Durham E-Theses

An investigation into the factors involved in hear death and thermal sensitivity in calliphora vicina R-D

Kashmeery, Amin M. S.

An Investigation into the Factors involved

in Heat Death and Thermal Sensitivity

in Calliphora vicina R-D.

(A Biochemical Study)

_by_

Amin M.S. Kashmeery, B.Sc.

Being a thesis submitted for examination for

the degree of Doctor of Philosophy of the

University of Durham, December, 1977.

Graduate Society,
Durham.

ACKNOWLEDGEMENT

not have been possible.

Finally, I pay tribute to my wife, and my parents, in Mecca, for their continual encouragement throughout this work. This thesis is dedicated to them.

ABSTRACT

The phenomenon of death at high temperatures in the blowfly Calliphora vicina has been studied, with particular emphasis on the respiratory activity of isolated flight muscle mitochondria (sarcosomes). These sarcosomes showed differential sensitivity towards several factors when different substrates are being repired. In the presence of α-glycerophosphate as substrate lower values for QO_2, RCl and ADP : 0 are obtained when the sarcosomes were isolated using the proteolytic enzyme 'nagarse' as compared with values obtained when the sarcosomes were prepared in the absence of 'nagarse'. It was also found that isolated sarcosomes respiring different substrates require different reaction conditions in order to show maximal respiratory performance.

Sarcosomes isolated from lethally heat treated flies showed impaired oxidative phosphorylation and loss of respiratory control when α-glycerophosphate is respired. However, in the presence of pyruvate and proline as substrates coupling is impaired but is still demonstrable.

Experiments were carried out to determine the effect of heat treatments when the heat dose is

split by an interval of recovery. It was found that the dose is accumulative unless the recovery period extends to 6 hours or more. Recovering of flies from a heat shock (L.D.$_0$), is temperature dependent with a high value for Q_{10}.

The effect of temperature on isolated sarcosomes indicated that the loss of respiratory control observed at the higher reaction temperatures might not be due to a general deterioration of the sarcosomal membranes, unless temperatures about 40°C or higher are used. Sarcosomes are more sensitive to heat in vitro than in vivo.

Sarcosomal α-glycerophosphate dehydrogenase has been studied following in vivo and in vitro heat treatment, and it was found to be relatively thermostable. Its specific activity and allosteric behaviour were not abolished by temperatures that are lethal to the whole animal.

Ultrastructural studies show that isolated sarcosomes exhibited changes in their morphology in different respiratory states. Work with sarcosomes from heat dead flies support the biochemical data, showing a differential thermosensitivity of sarcosomal respiration

with α-glycerophosphate as compared with pyruvate and proline respiration. Loss of the energized conformation seems to occur only in sarcosomes isolated from heat treated animals respiring on α-glycerophosphate.

No difference was seen in the appearance of the brain neurosecretory cells of control, lethally heated, or recovering flies.

It is concluded that α-glycerophosphate dehydrogenase may not be the temperature-sensitive site, and that thermal injury is a membrane-phenomenon.

The significance of the results is discussed, and suggestions are made for future work.

GLOSSARY

ADP	Adenosine - 5' - diphosphate.
ATP	Adenosine - 5' - triphosphate.
ATPase	Adenosine - 5' - triphosphatase (EC 3.6.1.3.).
BSA	Bovine Serum Albumin.
DNP	2:4 dinitrophenol.
EDTA	Ethylene diamine tetraacetic acid (sodium salt).
EGTA	Ethylene glycol-bis (β-amino-ethyl (ether) N N'-tetraacetic acid.
Km	Michaelis constant.
$L.D._{50}$	Experimental heat treatment causing 50% mortality in a sample of animals.
NAD^+	Nicotinamide-adenine-dinucleotide (oxidized form).
QO_2	Oxygen consumption expressed as ug atoms. Oxygen per hour per mg protein.
Tris	Tris (hydroxymethyl) aminomethane.
Vmax	Maximal reaction velocity.

TABLE OF CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

The underlying causes of heat death have been studied for a long time and have been followed in a wide variety of organisms, not only animals but also in microorganisms and plants. The literature has recently been reviewed, see Precht, Christophersen, Hensel & Larcher (1973). Much attention has been placed on ectothermal (poikilothermal) animals, particularly regarding the study of the phenomenon of resistance acclimation (Resistanzadoption, Precht, Christophersen & Hensel, 1955). It has for example been described in protozoans (Slater, 1954; Vogel, 1966), coelentrates (Mayer, 1914; Ushakov, Amosova, Chernokozheva, Dregolskaya, Pashkova & Skholl, 1977), flatworms (Ushakov & Bugayeva, 1975), annelids (Grant, 1955; Miles, 1963), nematodes (Wieser & Schiemer, 1977), Molluscs (Orr, 1955, Newell, Pye & Ahsanullah, 1971; Grainger, 1975), echinoderms (Orr, 1955; Ushakov et al, 1977). The work quoted is not meant to be exhaustive but simply to illustrate the variety of organisms studied. It is even more difficult to refer adequately to the literature on crustaceans, insects and fishes for the literature on these animal groups is quite vast; but those of particular interest to this study are mentioned. In crustaceans the detailed studies of Bowler, (1963,a,b),
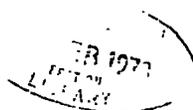
Gladwell, Bowler & Duncan (1976), McLeese (1956) are valuable. Similar studies on insects have been carried out by Davison (1969), Davison & Bowler, (1971), but the work of Dingley & Maynard Smith, (1968) as well as the earlier studies by Mellanby, (1954), Baldwin & House, (1954), on heat death in insects must be mentioned. Fewer studies have been carried out on arachnids, (Almquist, 1970). Studies on ectothermal vertebrates have been carried out by Brett (1944) and Cocking (1959) on fishes, by Volpe (1957), and Hutchison (1961), on amphibians.

As a consequence of the wide variety of these and other studies on heat death, much confusion occurs in the literature as to the causes of heat death, and the mechanisms of acquiring resistance acclimation, see Precht et al (1973) for the most recent review. The essence of the problem being that, as these phenomena are apparently universal throughout the animal (and plant) kingdom, fundamental mechanisms assumed to exist, which account not only for heat injury, but which are also modifiable by thermal acclimation. However, the literature has thrown up a number of theories, some mutually exclusive, as to the causes of heat death. Furthermore the state of literature concerning the mechanisms involved in resistance acclimation clearly shows how little is known of this phenomenon. Of the

many theories proposed to account for heat injury and heat death, it is possible to find contradictory or conflicting evidence for each of them and as a consequence the existence of a common cause(s) is in question. Winterstein (1905) proposed that heat death in many species was caused by tissue hypoxia, whilst this is supported by Weatherley's (1970) work in goldfish, Brett (1944), Fraenkel & Herford (1940) and Bowler (1963 a) showed heat death in the animals they worked with to be independent of the partial pressure of oxygen.

The increase in resistance to high temperature that occurs following a reduction in water content in resting stages of animals, as well of course in seeds and microorganisms, has led to the proposal that heat resistance of animals is caused by a change in the ratio of free to bound cell water, Hinton (1960), Fry (1958), Hutchison (1961). However, Mellanby (1958) clearly showed heat death points in meal worm larvae (Tenebrio molitor) to be unaffected by their degree of hydration. Suhrmann (1955) has also shown heat resistance of cellular activity in crucian carp (Carassius vulgaris) to be independent of the ratio of free to bound water.

A number of studies have also shown a breakdown of cellular permeability occurs during heat death,

resulting in "downhill" movements of Na^+ and K^+ between blood and tissue (Gladwell, Bowler & Duncan (1976), Grainger (1969), Ahearn (1970). However, Davison (1970) has shown this not to be the case during heat death in Calliphora erythrocephala.

In this search for a universal factor to account for heat death in such a variety of organisms, two theories are repeatedly returned to.

These are 1) that enzymes are "denatured" at high temperatures, and 2) that cellular lipids "melt" at high temperatures destroying cell structure.

The most prominant advocate of the enzyme "denaturation" theory is Ushakov (1964, 1966). He reports on the similarity in Arrhenius values (Ea) for cellular heat death and protein denaturation, and concluded that heat injury resulted from the latter effect.

It is, however, difficult to see how denaturation of enzymes, as originally meant, could account for the heat death of Aurelia at 14°C (Mayer, 1914) or for three species of Trematomus lower than 10°C (Somero & De Vries, 1967).

Indeed even Ushakov (1964) has remarked that most enzymes tested to that date have a greater thermostability than the cells or organisms from which they were extracted.

This led him to suggest that heat sensitivity of cells is determined by their least resistant proteins (Ushakov, 1964), however, the identity of these was obscure.

Read (1967) gave a clear lead in this field when he urged considerable caution in relating heat resistance of an organism to the thermostability of its proteins, unless it could be demonstrated that the temperatures at which protein function fails, closely coincides with those at which metabolism fails. In very few cases has this criterion been met. The best documented work has been in myosin ATP ase in lizards (Licht, 1964), on an amylase from a thermophilic bacterium (Manning & Campbell, 1961) and on the Mg^{2+} dependent ATP ase from crayfish muscle sarcolemma (Bowler & Duncan, 1967; Gladwell et al. 1976). More recently too the $Ca^{2+} Mg^{2+}$ ATP ase from crayfish muscle sarcoplasmic membranes has been shown to be heat sensitive at the same temperatures causing heat death (Cossins & Bowler, 1976).

More recent interpretations of the involvement of protein denaturation in thermal injury have turned to thermodynamic evidence, Rosenberg, Kemeny, Switzer & Hamilton (1971). They recalculated much data and found that the activation enthalpy (ΔH^*) and activation entropy (ΔS^*) for protein denaturation were related by the 'compensation' law:

$$\Delta S^* = a \Delta H^* + b$$

They described the values for the constants a & b as being very similar for a virus, yeasts, bacteria and <u>Drosophila</u> to the values obtained for protein denaturation. Banks, Demjanovic & Vernon (1972) have questioned the validity of the so-called "compensation" law, and furthermore when the data from a number of studies on multicellular organisms are so calculated, somewhat different values for a and b are obtained (Evans & Bowler, 1973). It is concluded that this thermodynamic approach is no more helpful in pinpointing proteins as the heat-sensitive cellular structures, than the more empirical approach.

Hochachka & Somero (1973) remark that proteins (enzymes) may become inactive at temperatures well below their denaturation temperature, as a result of temperature dependent changes in enzyme-substrate affinity. In allosteric enzymes this may also apply to a temperature dependent change in the sensitivity of the enzyme to its allosteric effector. In this way a loss of enzyme function could occur at temperatures well below those causing 'denaturation' in the protein coagulation sense. Thus heat death may result from temperature induced reversible inhibition of activity, which causes a cascade effect becoming more and more severe culminating in an enzyme or enzymes, metabolic chaos.

Alternatively, an irreversible change occurs in

tertiary or quaternary protein structure that is characteristic of coagulation.

For these reasons a detailed study of the effect of temperature on a mitochondrial enzyme, α-glycerophosphate dehydrogenase will be followed.

Attention was first drawn to the relationship between thermal sensitivity of an organism and the 'melting' profile of its lipids by Heilbrunn (1924). This theory of heat injury was extended by Belehrádek (1935, 1957) by the formalisation of the "lipoid liberation theory". This theory suggests that cellular heat injury is caused by the 'melting' of cellular lipids and that this has a deleterious effect on cell structure and as a consequence on cell function. In support of this, Belehrádek (1967), in a review, reported that fats and phospholipids are the only cell constituents whose molecular structure is dependent upon the temperature of formation.

The early work in this field did not distinguish between depot fat and membrane lipids, indeed it was conducted in ignorance of the function properties and cellular location of the lipid in question. The early work of Fraenkel & Hopf (1940) is a case in point, they reared two species Calliphora erythrocephala and Phormia terra-novae of blowfly at high and low temperatures. The lipids extracted from flies acclimated to lower temperatures were

more unsaturated than those isolated from flies reared at the higher temperature. The flies of both species when reared at the higher temperature were more heat resistant than flies of the same species reared at a lower temperature. However, the relationship between lipid saturation and heat death was not simple. Fraenkel & Hopf (1940) described that flies of the different species, when bred at the same temperature, although they had lipids of identical saturation, they had different resistances to high temperature exposure. House, Riordan & Barlow (1958) have, however, demonstrated a good correlation between the heat sensitivity of Pseudosarcophaga affinis larvae and the degree of saturation of their dietary lipids.

More recent work, specifically on membrane phospholipids, has shown a similar correlation between acclimation temperature and the degree of saturation of membrane lipids, Johnston & Roots (1964). These and other workers report that acclimation to low temperature leads to an increased incorporation of unsaturated fatty acids into membranes (Roots, 1968; Brañska & Wlodawer, 1969; Caldwell & Vernberg, 1970; Kemp & Smith 1970; Driedzic & Roots, 1975; Cossins, 1976).

The current interpretation of this phenomenon is that acclimation involves the ability to control the degree of membrane lipid unsaturation in order to maintain a specific degree of membrane 'fluidity'. Once again,

however, positive evidence relating cellular heat injury

with the degree of saturation of cellular (membrane)

lipids is missing; see Ushakov (1964; 1966).

What is quite evident, however, is that a number

of distinct studies show that membrane function is

impaired at temperatures that would be lethal to the

intact organism. Early work by Bowler (1963 b)

demonstrated a breakdown in cellular permeability to

cations occurred during heat death in the crayfish, a

study that was taken further by Bowler & Duncan (1967),

who related this to the inhibition of a muscle membrane

Mg^{2+} ATP ase.

Gladwell et al (1976) made a more detailed study.

They describe changes in the bioelectric properties of

the muscle membrane at high temperatures, and showed

too that the activity of the CNS of crayfish to be seriously

affected by the increase in haemolymph $[K^+]$ that occurred

during death, Bowler, Gladwell & Duncan (1973). The

following scheme, Fig. 1.1 was suggested by these workers

to account for heat death following impairment of membrane

function.

Similar, but less detailed studies in Arianta

(Grainger, 1969) in Centrioptera (Ahearn, 1970) in Helix

(Grainger, 1973 a) have all shown heat death in these

species to be accompanied by changes in the blood ionic

Fig. 1.1

Schematic representation of
the steps that lead to heat
death in the crayfish, as
proposed by Bowler et al. (1973)

ENVIRONMENT

Na⁺ | K⁺

GILLS

39·5 | 11·6

CNS

HAEMOLYMPH

MUSCLE

Na⁺ | K⁺

96·7 | 81·4

Na⁺ | K⁺

High

K⁺

1. Lethal high temperature inactivates sarcolemmal Mg²⁺-ATPase.

2. Sarcolemmal conductance (= permeability) increase.

2a. Muscle fibres depolarise.

3. Haemolymph Na⁺ and K⁺ change.

4. High haemolymph K⁺ causes increased CNS activity.

5. Progressive loss of co-ordination.

6. Death.
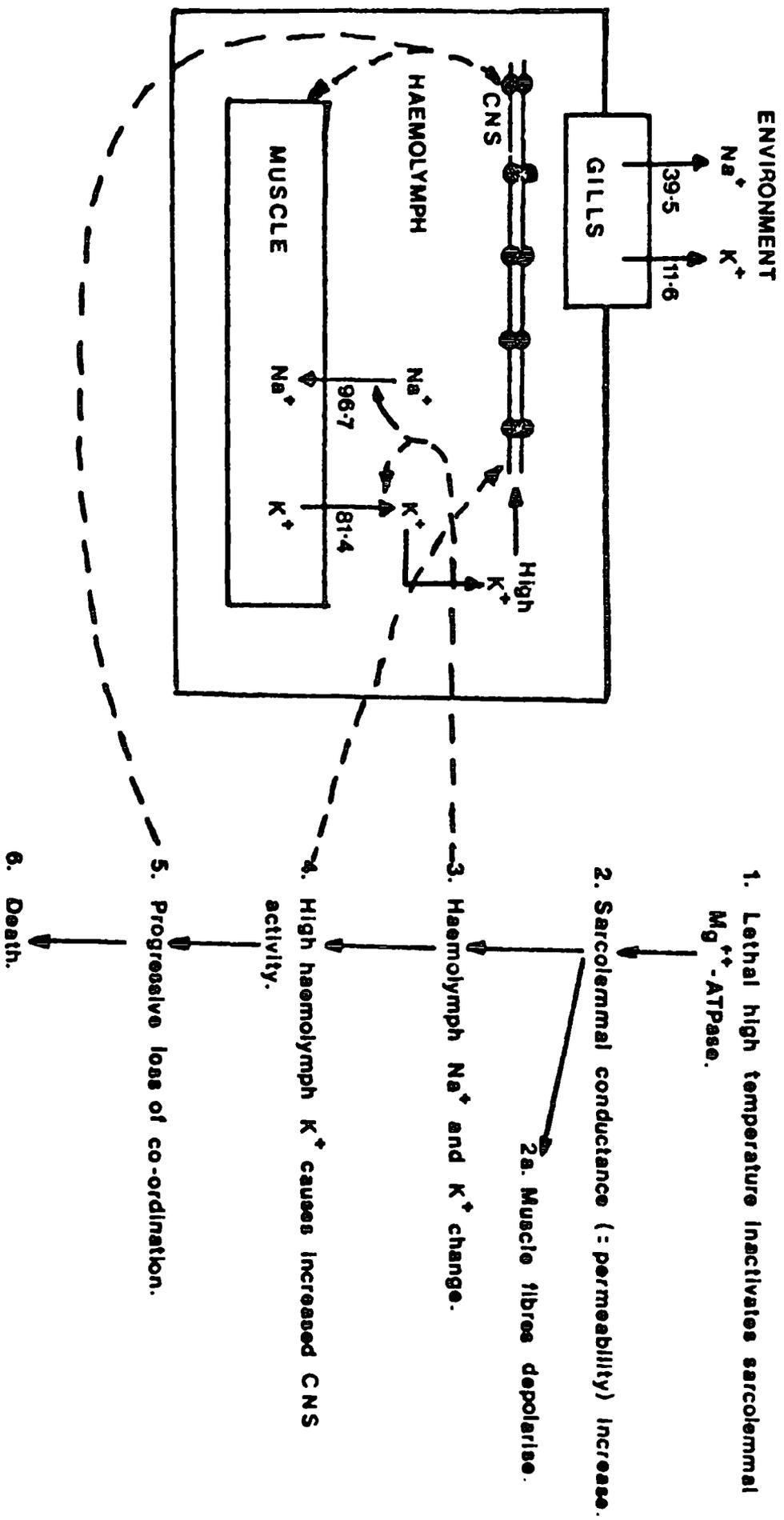
concentration.

Iandola & Ordal (1966) have earlier shown in the
bacterium (Staphylococcus aureus) that heat damage caused
a leakage of cellular contents across the cytoplasmic
membrane. Later, Bluhm & Ordal (1969) showed heat
treatment also caused a reduction in the activity of
S.aureus respiratory enzymes. Levy, Gollon & Elliot
(1969) noted, in an ultrastructural study of heat treated
Tetrahymena that structural changes occurred which they
interpreted as resulting from membrane damage. Ling
(1967) reported an increase in sarcolemma permeability
to sucrose in frog muscle at 35° - $40^\circ C$, temperatures
that are lethal to the frog. This would agree with Grainger's
work (1973 b) that resting potential of frog muscle membrane
fell during exposure to temperatures that are lethal.

Such work as this, together with studies on
Calliphora erythrocephala flight muscle sarcosomes by
Davison & Bowler (1971) to be mentioned below, led to the
suggestion that cellular heat injury, and by extrapolation
heat death, is a membrane-event, Bowler, Duncan,
Gladwell & Davison (1973). These workers proposed that
heat lesion was not necessarily related to either the
phospholipid or protein moiety but rather to the stability
of lipoprotein complexes, or of enzymes whose activity
is dependent upon the maintainance of membrane integrity.

In general it is shown that 'soluble' enzymes are thermally inactivated at temperatures well above those characteristic of heat death, see Brandt (1967), Read (1967), Ushakov (1967) and Cossins & Bowler (1976). Whereas a number of intrinsic membrane enzymes are heat inactivated at temperatures characteristic of heat death, see Cossins & Bowler (1976).

The work of Davison & Bowler (1971) deserves particular emphasis for it forms the particular basis for the proposed study. Davison & Bowler (1971) showed that sarcosomes have an equivalent thermal sensitivity to that of the whole animal.

Using α-glycerophosphate as a substrate, they found that sarcosomes, isolated immediately following sublethal or lethal heat treatment, were dramatically affected as compared with sarcosomes isolated from control untreated animals. Particularly after lethal heat treatments ($L.D._{50}$ and $L.D._{100}$) respiratory control was lost; phosphorylation was not measurable and oxidation was reduced to approximately two thirds of the value obtained from control untreated sarcosomes. Perhaps the most striking finding of this work was the fact, that after such treatments, respiration with pyruvate + malate and succinate were not so drastically affected, and partially coupled respiration with those substrates was observed

even after L.D.$_{100}$ treatment. Since α-glycerophosphate
is known to be one of the major substrates of flight muscle
sarcosomal respiration (Sacktor, 1958; Van den Bergh,
1962), a breakdown of the phosphorylation process coupled
with the oxidation of this substrate, which is itself
already reduced, could result in serious effects on the
animal as a whole.

Furthermore, Davison (1971b) demonstrated that
there is a correlation between the physiological lesions
observed with sarcosomes isolated from animals exposed
to lethal heat treatments and morphological impairment
of the sarcosomes, as revealed by electron microscopy.
This damage, consisted of the disruption of the lamellate
cristae and the appearance of a considerable amount of
electron dense granules which he attribu ted to the
precipitation of inorganic ions. These results led him
to suggest that sarcosomal sensitivity may be one of the
primary causes of heat death in the adult insect.

However, many questions arose again, if this is really
the case, which component of sarcosomal structure and
function is most sensitive to thermal stress. At the level
of the sarcosomal membranes, Davison (1970) concluded
that 'melting of phospholipids' is questionable for a
release of long chain fatty acids might then be expected which
would uncouple oxidative phosphorylation and also stimulate

oxygen consumption, this has not been conclusively
demonstrated. Alternatively denaturation of enzyme
molecules may account for the structural changes in
sarcosomes. This point can be tested by following the
recovery repair mechanism taking place after sublethal
heat treatment. It is assumed that such treatment would
cause either a reversible or an irreversible damage to
the mitochondrial enzymes. In the first case recovery
would gradually take place after the removal of the heat
stress; in the second case, recovery can only occur when
protein synthesis has replaced the damaged enzymes.
Reports by other workers, however, suggest that recovery
from sublethal heat treatment did take place in the absence
of protein synthesis (In Staphylococcus aureus see Bluhm
& Ordal (1969) or in Drosophila subobscura see Dingley
& Smith (1968)).

Despite the reasons, given by Davison (1970), that
led him to choose blowfly flight muscle sarcosomes as a
model for cellular heat injury, which included the point
that they can easily be isolated in an intact form, whose
functional efficiency can be readily measured, one must be
aware of a series of factors, on which the demonstration of
function relies, involved in dealing with isolated sarcosomes.

Many studies have been carried out on mitochondria

derived from different species and cell types (see Van den Bergh & Slater, 1962; Lardy & Ferguson, 1969; Lehninger, 1959; Chance & Hagihara, 1961; Hansford & Johnson. 1975). The diverse nature of the sources of mitochondria have led to some conflict in the interpretation of the optimal conditions necessary to demonstrate respiratory performance. In particular the isolation technique appears to be a point of some dispute.

A wide range of techniques is suggested regarding for instance breaking a tissue to free the mitochondria, homogenisation methods and periods, centrifugation times and speeds, resuspending the mitochondrial pellets after sedimentation and finally and most recently the storage of the preparation during the course of the experiment (Slack & Bursell, 1976a). There is also a lack of agreement on optimal assay conditions such as the reaction temperature, PH, the presence or absence of certain ions, the sequence of addition of reactants and the need for a primer for certain substrates.

The present study was begun with the intention of extending the work started by Davison (1970). It is the primary purpose of this thesis to examine the reported heat sensitivity of the flight muscle sarcosomes in more detail to determine if possible the primary lesion(s) in heat injury.

In the first instance, studies on recovery from

lethal and sublethal heat treatments were carried out.

Secondly, biochemical and ultrastructural work, on

the thermal sensitivity of isolated sarcosomes is

presented. Thirdly, the membrane - bound α;glycero-

phosphate dehydrogenase has been investigated as the

temperature-sensitive step in oxidative phosphorylation,

and therefore particular attention was focussed on it.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

The insects used in this study were male and female <u>Calliphora</u> <u>vicina</u> R-D. This stock was originally obtained from a mass-mated culture bred at the Pest Infestation Laboratory, Slough. It has been maintained in our laboratory for some eleven years.

1. Culture of stocks

The stock cultures used for breeding were kept in muslin covered cages, approximately 50cm x 35cm x 70 cm, with corrugated cardboard placed in the bottom to allow the flies to right themselves. These cages were kept at room temperature (24°C) and the stock cultures regularly replaced at two monthly intervals. The flies were fed sugar and water <u>ad libitum</u> and ox liver was provided regularly as a source of protein and to allow oviposition to occur.

Liver covered with eggs was removed from the stock cages and placed in a crystallizing dish (15cm diameter) for incubation. The liver was covered with a wet filter paper to provide a high relative humidity (R.H.), Davies (1949). The crystallizing dish was covered with muslin and transferred to an incubator maintained at $24 \pm 0.5°C$ to ensure larval emergence during the next 12 hours. Newly hatched larvae were placed on fresh liver in a crystallizing dish

filled with sawdust and the previous incubation process
was repeated. Larvae were fed for 4 - 5 days on liver,
which was replenished as required. Pupation took
place in the sawdust 8 - 9 days after oviposition. After
24 hours the puparia were collected and placed on fresh
dry sawdust in a 750 cm^3 conical flask, plugged with
cotton wool, and incubated at 24 $\pm$ 0.5C. The puparium
stage was 9 days long.

Method of exposure of flies to high temperatures

The animals were placed in a series of 7.5 cm x
2.5 cm glass vials, which were completely immersed in
a water bath at the required temperature. Racks were
constructed to hold 50 vials. Each vial was fitted to
a rubber bung, connected by glass and rubber tubing.
Air was circulated through the vials at 600 cm^3 per
minute and brought to a R.H. of 47.5 - 50.5 per cent
by bubbling through a saturated solution of Mg $(NO_3)_2$.
$6H_2O$ (Winston & Bates, 1960). The temperature of
the water bath was maintained at $\pm$ 0.05°C using a
500 watt immersion heater controlled by a Jumo
thermoregulator and sunvic relay (Type F103/4). The
water was continuously stirred. Air temperature in the
vials was also determined. In the temperature range
studied, 15 minutes was required for equilibration of
the air and water bath temperatures. This 15 minute
period was included in the 40 minute exposure. During

the remainder of the exposure the air temperature was
maintained at the temperature of the water bath
$\pm$ 0.05°C. (Davison 1970).

Isolation of Sarcosomes from flight muscle

Flight muscle sarcosomes were isolated after
the procedure used by Lewis & Slater (1954), Lewis &
Fowler (1960), Tribe (1967) and Davison (1970). 30 adults
were immobilised with either cardice or by placing them in
the deep-freeze for approximately 4 minutes, heads and
abdomens were quickly removed and the thoraces
transferred to a small glass cylinder, similar to the
one used by Greville, Munn & Smith (1965) and Davison
(1970). The cylinder was pre-cooled by placing it on
crushed ice. The thoraces were gently crushed in
2 cm^3 of ice-cold sucrose medium I for 2 minutes with
a cold, loosely fitting, flat-bottomed glass rod. Care
was taken not to rotate the rod or grind the pulp and
so damage the sarcosomes. The resulting pulp was
transferred with a further 1 cm^3 of sucrose medium I
to a cooled glass funnel lined with 4 layers of fairly
coarse muslin, previously boiled in distilled water and
soaked in ice cold sucrose medium I. The pulp was
forcibly squeezed through the muslin by hand and the
filtrate collected in a glass-centrifuge tube, also
surrounded with crushed ice. The filtrate was centrifuged
at 100xg for 4 minutes at 4°C in a Mistral 2 L

centrifuge (M.S.E.) to remove chitin and any flight muscle fibres, which had passed through the muslin. The supernatant was collected and centrifuged at 2,200xg for 10 minutes at 4°C in the same centrifuge and the resulting pellet suspended in 2 cm^3 of ice cold sucrose medium II. This suspension was recentrifuged at 2,200xg for 10 minutes and the final pellet of washed sarcosomes suspended in 0.75 cm^3 of ice cold KCl medium III. The average time for the preparation period was approximately 50 minutes.

3.   Isolation media used

Sucrose Medium I

                    0.32 M Sucrose

                        10 mM EDTA

                        2% BSA

                        10 mM tris/HCl Buffer at pH 7.3

Sucrose Medium II

                        As for Sucrose Medium I without
                                2% BSA

Medium III

                        0.15 M KCl

                        1 mM EDTA

                        10 MM tris/HCl Buffer at pH 7.3

4. Description of the oxygen electrode

Measurements of oxidative phosphorylation reported throughout this study were carried out using a Rank oxygen electrode (Rank Bros., Cambridge), which is of a similar design to the Clark oxygen electrode (Clark, 1956).

The electrode has a perspex reaction vessel surrounded by a water jacket and this unit screws onto a perspex base where a platinum cathode and silver anode are situated. When in use both are covered with a few drops of M KCl and this electrolyte is separated from the reaction vessel by a teflon membrane. The perspex screw cap fitted to the top of the reaction vessel has a small hole bored through the centre to allow additions to be made to the reaction media. The medium in the reaction vessel is continuously stirred using a small magnetic stirrer and "flea" (Rank Bros., Cambridge).

The principle of the oxygen electrode has been described by Davies & Brink (1942). When a polarising voltage of -0.6 volts is imposed across the two electrodes, the platinum negative to the silver, oxygen undergoes an electrolytic reduction

$$O_2 + 2\bar{e} + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + 2\bar{e} + 2H^+ \longrightarrow 2H_2O$$
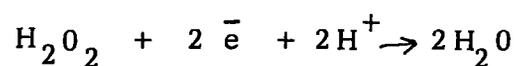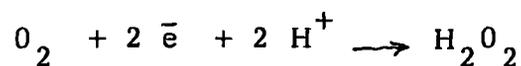
and the current flowing is directly proportional to the
oxygen content of the electrolyte. Chappell (1964)
has pointed out that the Clark electrode measures
activity and not the concentration of the oxygen present
in the reaction medium. Therefore in this study the
oxygen electrode has been calibrated by determining the
oxygen content of the reaction medium.

The current flowing through the electrode system
was passed through a helical potentiometer in series
with the platinum electrode and the voltage developed
across this resistance was fed into a Servoscribe recorder
(Goerz Electro) set at 2 mv sensitivity. The recorder
baseline was set by reducing the medium in the reaction
vessel of the oxygen electrode with sodium dithionite.
This effectively reduced the current across the electrodes
to zero. Reaction medium saturated with oxygen was
pipetted into the reaction vessel and the potential adjusted
with the helical potentiometer to give a full scale
deflection on the recorder.

5. <u>Measurement of oxidative phosphorylation</u>

Oxidative phosphorylation was measured at 24°C
(unless stated otherwise) using the oxygen electrode
described below. The reaction medium was similar to
that used by Davison (1970) unless otherwise stated.

Reaction medium:

50 mM KCl

30 mM phosphate buffer (Sörensen)
at pH 7.3

5 mM $MgCl_2 \cdot 6H_2O$

1 mM EDTA

20 mM Tris/HCl at pH 7.3

The medium was thoroughly equilibrated to 24°C for several hours to saturate it with atmospheric oxygen.

5 A. Polarographic measurement of oxygen consumption

3 cm^3 of the reaction medium were pipetted into the reaction chamber, the perspex screw cap was replaced such that all air bubbles were expelled. The surface of the reaction medium was allowed to rise about 5 mm up the central hole of the stopper to minimize the contact between the reaction medium and atmospheric oxygen. After about two minutes the recorder was adjusted using the helical potentiometer to give full scale deflection. Oxygen uptake from these 3 cm^3 of reaction medium was followed by determining the rate of change of deflection of the pen recorder after the following

additions :

> 50 ul of sarcosomal suspension.
>
> 50 ul of 2M DL - α Glycerophosphate
> solution made up in distilled
> water.
>
> 10 ul of 50 mM ADP solution made up in
> 30 mM phosphate buffer pH at
> 6.8 at 24°C.

These additions were made with microsyrénges
(Terumo Shandon, type MSG)

Care was taken not to allow the needle of the micro-

syrénge to reach the bottom of the reaction chamber and

so damage the teflon membrane.

Endogenous rates were neglected throughout this

study because they had low values which were insignificant.

The rate which followed the addition of substrate is

called 'substrate-rate' unless otherwise stated. The

rate which follows the addition of ADP is called 'ADP-

stimulatedrate' or state III (Chance & Williams, 1955b)

throughout this study.

5 B. QO_2 :

This is the sarcosomal oxygen consumption in

terms of µg AO per hour per mg protein. It can be

calculated from the following equation:

$$QO_2 = \frac{P \ x \ C \ x \ 60}{R - r \ X \ protein \ concentration}$$

Where:

P = rate of change of deflection (divisions/
minute)

C = initial oxygen content in reaction
medium saturated at a given temperature
(ug Atoms).

R = full scale deflection for the oxygen
saturated medium.

r = deflection obtained at anaerobiosis.

5 C. Respiratory Control Index : (RCl)

This is defined as the ratio of the respiratory
rate in the presence of added ADP to the rate following
its expenditure (Chance & Williams, 1956)   i.e.
$RCl = \dfrac{\text{state III}}{\text{state IV}}$. However, in some experimental
conditions (e.g. following heat treatment to the whole
animal) no respiratory "cut-off" occurred after ADP
addition, consequently it was not possible to measure
RCl according to the Chance & Williams definition.
RCl was therefore estimated throughout using the ratio
of the respiratory rate in the presence of added ADP
to the rate before its addition, that is the 'substrate-
rate'. (unless otherwise stated).

5 D. ADP :O ratio :

This is defined as the number of moles of ADP
esterified to ATP for each gram atom of oxygen
consumed.

ADP : O ratios have been calculated throughout this study using the method described by Chance & Williams (1955a). When a known amount of ADP is added to a mitochondrial suspension in the presence of substrate, the respiration rate increases and a quantity of oxygen is consumed. The uptake of oxygen can be measured from the trace by extrapolating the ADP-stimulated rate and the rate after its expenditure. The scale divisions between the point of the addition of ADP and the extrapolated point of its extinction, represent the amount of oxygen required to convert the added 0.5 μmole of ADP to ATP.

6.   Determination of Sarcosomal protein concentration :

For this purpose the Folin-phenol method of Lowry, Rosebrough, Farr & Randall (1951) was used with a slight modification. Bovine serum albumin fraction V was used as standard for constructing a calibration curve.

Reagents:-

A)   20% W/v   Sodium carbonate
      0.2% W/v Sodium potassium tartrate

B)   2%   W/v copper sulphate

C)   This was made up as follows:

5 volumes of (A) + o.5 volumes of (B) + 20 volumes distilled water.

D) This was made up by diluting 1 volume of
Folin-Ciocalteau phenol reagent with 10
volumes of distilled water.

Procedure :-

The sarcosomal suspension was diluted with
distilled water to give a protein concentration of less
than 400 µg/cm^3, which is the highest concentration of
the standards. These were prepared by making a serial
dilution from 400 µg BSA/cm^3 to give 300,200,150,
100,50 and 0 µg/cm^3. In some experiments, where,
due to dilution, the sarcosomal protein concentration
is expected to be less than 100 µg/ml, a further series
of standard solutions were made by serial dilution
from 100 µg BSA/cm^3 to give 75, 50, 25 and 0 µg/cm^3.
1 cm^3 of mixture C was added to 1 cm^3 of the protein
solution and allowed to stand for 15 minutes at room
temperature, then 1 cm^3 of reagent (D) was added and
allowed to stand for another 30 minutes. The solutions
were poured into a glass cuvette (1.0 cm light path)
and optical density was measured against water at 700 nm
in a Beckmann SP 1800 Ultraviolet Spectrophotometer.

Chemicals :

The chemical reagents used in the present study were AnalaR grade wherever possible, otherwise laboratory reagent grade was used.

Adenosine -5' - diphosphate (disodium salt), sodium pyruvate, proline, EGTA, bovine serum albumin (Fraction V) were purchased from Sigma Chemical Co.,Ltd.

DL α-glycerophosphate (sodium salt) was purchased from Koch-Light Laboratories Ltd., Colinbrook, Bucks, England.

ADP was stored in a frozen solution buffered at pH 6.8 at 24°C, for a maximal period of six weeks.

CHAPTER THREE

EFFECT OF ISOLATION PROCEDURE AND REACTION
CONDITIONS ON THE RESPIRATORY ACTIVITIES OF
ISOLATED SARCOSOMES

Introduction

The respiratory metabolism of insect flight muscle has been much studied. Watanabe & Williams (1951) clearly demonstrated that the respiratory organelles (Sarcosomes), isolated from flight muscle were homologous to mitochondria from other tissues. However, many of the earlier works on mitochondria from different sources gave rise to diverse results and interpretations. Chefurka (1965) suggested that the discrepancies in the results between the different researchers lay in differences in methodology between the various laboratories, not only in assay of the respiratory activity but in the method of preparation of the mitochondria. As a consequence, Chefurka (1965) ennumerated some of the parameters that may alter the stability of mitochondria and consequently their respiratory activity:

i) Composition of the isolation and reaction media.

ii) Extent of homogenization.

iii) Ageing of mitochondria.

iv) Other parameters such as age of insect, the order of addition of reactants, the concentration of mitochondrial suspension and perhaps even diurnal rhythm, may be important.

The most conventional technique for isolating sarcosomes is that originally reported by Watanabe & Williams (1951). It was developed on the grounds that mitochondria can be relatively easily released by rupturing the muscle fibres, and subsequently collected by filtration and centrifugation in an appropriate medium. This method has been used subsequently, with minor modifications, by many other workers, for example Lewis & Slater (1954), Sacktor & Cochran (1958), Estabrook & Sacktor (1958), Van den Bergh (1962), Tribe (1967), Davison & Bowler (1971), Bulos, Shukla & Sacktor (1972,1975).

Isolation methods employing the proteolytic enzyme nagarse have been widely used over the last ten years or so. It was first introduced by Chance & Hagihara (1961) in preparation of mitochondria from heart muscle tissue. Such methods are fully described by Chappell & Hansford (1972). The essence of this technique is the total digestion of the myofibrils, in an attempt to minimize the mechanical damage that might take place during isolation. This method has been shown to give a good yield of intact, non-

contaminated mitochondria (Chappell & Hansford, 1972).

Most recently, Slack & Bursell (1976 a) reported a new isolation method involving no proteolytic enzyme digestion, but a different isolation medium and milder resuspending methods than homogenization. They attributed the inconsistent results, reported by other workers, to the use of mitochondria which have been altered to an intermediate degree in structural integrity and functional ability, through inadequacy of the method of preparation.

Not only has there been a variety of isolation methods used to prepare sarcosomes, but different isolation media have also been used, for example, Sacktor & Childress (1967), Tribe & Ashhurst (1972), and Slack & Bursell (1976b), in the preparation of sarcosomes from blowfly flight muscle, have reported isolation media which differ in their tonicity osmolarity and the presence and absence of other constituents.

A variety of reaction media have also been used in assaying isolated insect flight muscle sarcosomes, e.g. the periodical cicada, Magicicada septendicem, (Hansford, 1971), the honey bee (Balboni, 1967),

Lucilia cuprina and Musca domestica (Birt, 1961), the

blowfly, Calliphora erythiocephala (Tribe & Ashhurst,

1972; Davison & Bowler, 1971), and many others.

The principal purpose of this study, as stated

earlier, is to determine possible lesions to sarcosomal

function caused by heat exposure, and consequently

to throw light on the mechanisms involved in cellular

heat injury. It has been reported earlier by several

workers that mitochondrial function is heat-sensitive,

particularly oxidative phosphorylation, (e.g.

Christiansen & Kvamme, 1969; Davison & Bowler, 1971;

Rochman, Lathe & Levell, 1967). The major part of

this thesis concerns therefore an investigation of the

effect of lethal heat doses to adults on sarcosomal

function. It was as a consequence considered necessary

to assess the effect of different isolation procedures

and assay conditions on sarcosomal function in control

sarcosomes.

α-glycerophosphate has been suggested as the

principal physiological substrate in dipteran flight

muscle mitochondria (Sacktor, 1964, 1970). On the

other hand, other workers (Gregg, Heisler &

Remmert, 1960- Van den Bergh, 1962) have suggested

that pyruvate plays a major role in supplying energy

for flight. No attempt was made in the present study
to use substrates of the citric acid-cycle intermed-
iates for they are usually partly oxidized by
sarcosomes because the sarcosomal membrane is
relatively impermeable to them (Van den Bergh &
Slater 1962); this may be due to lack of carrier
molecules (Hansford & Chappell, 1967).

In a preliminary work, Davison (1970) observed
that sarcosomes, isolated from the flight muscle of
lethally heat-treated blowflies, showed uncoupled,
markedly reduced respiration when α-glycerophosphate is
used as substrate. Whereas pyruvate showed some
measure of coupling, succinate showed tighter coupling
and higher oxidation rates than in control sarcosomes.

This differential thermal effect on the sarcosomal
respiratory systems leads to several speculations.
Amongst those is the possibility that in vivo heat
treatment might have rendered the sarcosomes susceptible
to isolation procedure thereafter. Thus it seems
reasonable to approach the problem from this angle in
the first instance.

The Chapter, therefore, will include some
detailed investigations into the effect of isolation
procedure and assay conditions on α-glycerophosphate,

as well as pyruvate-linked, respiration in sarcosomes isolated from control and heat treated blowflies.

Materials and Methods

The flies used in these experiments were control untreated, or $L.D_{50}$ treated 10-day old adults reared and maintained at $24°C$. Heat doses were given as described in Chapter 2.

1. Isolation procedures

A. Standard Method

This method has been fully described earlier in Chapter (2). It has been widely used by many other laboratories e.g. Lewis & Slater (1954), Sacktor & Cochran (1958), Van den Bergh (1962), Tribe (1967), Davison & Bowler (1971), Bulos, Shukla & Sacktor (1972, 1975). The basic method can be summarised as follows: the flight muscle fibres are ruptured, and subsequently the sarcosomes are collected, by filtration and subsequently isolated by centrifugation in an appropriate medium (Sucrose medium I).

B. Nagarse Isolation

The method used was similar to that described by Chance & Hagihara, (1961) and Chappell & Hansford

(1972), and was used as follows -

Isolation Medium

0.32 M sucrose

1 mM EGTA

5 mM Tris/HCl buffer at pH 7.7
at o°C

Isolation procedure:

Heads and abdomens were removed on a petri-dish.
The dish was kept cold by placing it on crushed ice,
whilst dissecting the flies. Thoraces were collected
in a small glass beaker placed also on crushed ice.
The flight muscle was expelled either by squeezing
the thorax, or by pressing all thoraces through one
layer of muslin, using a flat-footed pestle, on a glass
dish containing about 10 cm^3 of isolation medium. In
the first case, squeezing the thorax between fingers,
would yield an almost chitin-free preparation. The
second case, however, would yield a pure preparation,
if a low speed spin (100xg) is carried out for 4 minutes
to remove the chitin, before the next preparation step.

The expelled flight muscles were transferred
to an ice-cold 50 cm^3 glass beaker containing 20 cm^3
of cold isolation medium. Homogenization was performed
briefly and manually on ice, by passing the pestle of a

Dounce homogenizer twice through the medium. A fresh

solution of nagarse was prepared by dissolving 3.5 mg

in 5 cm^3 of the isolation medium. The ice-cold nagarse

solution was added into the homogenizer tube and two

further passes of the pestle were given. The prepar-

ation must stand on ice during the 5 minutes incubation

and in this time, one further homogenization was given.

The homogenate was then filtered through four layers

of muslin, which had been previously boiled in distilled

water for a few minutes, then soaked in ice-cold

isolation medium. Filtration was carried out via a cold

glass-funnel. The filtrate was divided into two equal

volumes in 50 cm^3 polyethylene centrifuge tubes and

centrifuged in an MSE high-speed 18 refrigerated

centrifuge for 2 minutes at 9000 x g . The supernatant

was discarded from both tubes and the pellets were

resuspended in 5 cm^3 each of fresh isolation medium.

Resuspending the pellets was carried out carefully

using the 'cold finger' technique, this being a glass test

tube filled with crushed ice. The cold bottom of this

tube was gently rubbed against the edge of the pellet

moving, in a circular manner, towards its centre. The

contents of the two tubes were added together, diluted

to 40 cm^3 and recentrifuged for 5 minutes at 9000 x g

in the same centrifuge. The supernatant was discarded and the pellet resuspended in 2 cm^3 of the isolation medium using the 'cold finger' method described above. Such a preparation yielded approximately 1 mg sarcosomal protein per 50 μl. During the experiment the sarcosomal preparation, as well as the substrates were placed on ice.

2. Reaction Conditions

The respiratory activity of sarcosomes isolated by the "standard" isolation method listed above was followed in four different reaction media -

Medium I

    50 mM KCl

     5 mM $MgCl_2 \cdot 6H_2O$

     1 mM EDTA

    20 mM tris/HCl buffer at pH 7.3 at 24°C

    30 mM Phosphate buffer at pH 7.3 at 24°C

Medium II

    50 mM KCl

     5 mM $MgCl_2 \cdot 6H_2O$

     1 mM EDTA

    20 mM tris/HCl buffer at pH 7.3 at 24°C

    30 mM Phosphate buffer at pH 7.3 at 24°C

    0.4 mg BSA/cm^3

Medium III

    154 mM KCl

    10 mM tris/HCl buffer at pH 7.3 at 24°C

    30 mM Phosphate buffer at pH 7.3 at 24°C

    0.4 mg BSA/cm^3

Medium IV

    154 mM KCl

    1 mM EDTA

    10 mM tris/HCl buffer at pH 7.3 at 24°C

    30 mM Phosphate buffer at pH 7.3 at 24°C

    0.4 mg BSA/cm^3

Measurement of oxidative phosphorylation

This was carried out as described in the General Materials and Methods, and the respiratory performance was expressed as RCl, ADP : O and QO_2. α-glycerosphosphate-dependent respiration was measured in 3 cm^3 of reaction medium I at 24°C. Pyruvate and proline-dependent respiration was measured in 2cm^3 of reaction medium III at 24°C. The RCl in the latter case was calculated as the ratio of respiration rate in the presence of ADP to that after the expenditure of added ADP (state III/state IV).

The substrate final concentrations were

33 mM, 2.5 mM and 2.5 mM for α-glycerophosphate, pyruvate and proline respectively.

Results

I. Effect of Isolation procedure on respiration of control sarcosomes

A α-glycerophosphate dependent respiration

As can be seen from Table (3.1) the isolation procedure had a profound effect on the sarcosomal respiratory behaviour when α-glycerophosphate is the substrate. The 'standard' method produced sarcosomes which gave QO_2 values in agreement with those of other workers in the literature (Tribe, 1967, Davison & Bowler, 1971; Childress & Sacktor 1967) who have worked on this or other blowfly species. The substrate rate oxidation was 34.42 μg A O.hr^{-1}. mg protein^{-1}, whilst this was stimulated to 84.57 μg A O.hr^{-1}. mg protein^{-1} by the addition of ADP. This resulted in obtaining good values for RCl, (mean = 2.48) and mean ADP :O of 1.79.

Nagarse prepared sarcosomes on the other hand had a significantly poorer respiratory performance throughout. Most dramatic was the low value for state III respiration, of only 37.87 μg A O.hr^{-1}. mg protein^{-1}. The resulting mean value for RCl was only 1.5 and the

calculated mean value for ADP : 0 was also significantly

lower at 1.37.

B Pyruvate and proline dependent respiration

As can be seen from Table (3.1), the use of

nagarse has very little effect, if any, on the pyruvate-

linked sarcosomal respiratory activity. The substrate

rate (state IV) in both bases was in agreement with that

reported by Tribe & Ashhurst (1972). The ADP-

stimulated rate (state III), also, was not affected by

the isolation technique and the results obtained were in

agreement with those obtained from housefly sarcosomes,

Musca domestica (Van den Bergh & Slater 1962) Phorm.ia

regina (Childress & Sacktor, 1966) and Calliphora

erythrocephela (Hansford 1972), see Table (3.1a).

Consequently the respiratory control index was

very little affected by the isolation procedures employed,

giving values of 8.27 $\pm$ 0.44 and 8.85 $\pm$ 1.81 for the

nagarse and standard isolation technique respectively.

These values are identical to those reported by other

workers (See Van den Bergh & Slater, 1962 and

Hansford & Chappell 1967).

Phosphorylation was not affected by either of the

isolation conditions applied as revealed by the high ADP : 0

ratios obtained.

Table 3.1a

Comparative state III respiration rates

and respiratory control indexes as

reported by different workers, using

Pyruvate + a primer as substrates.

Sp.	Van den Bergh and Slater (1962) _Musca domestica_	Childress and Sacktor (1966) _Phormia regina_	Tribe and Ashhurst (1972) _Calliphora_	Hansford (1972) _enythrocephala_	Present results (1977) _Calliphora vicina_
State III	57.77	59.28	27.14	39.30	43.81
RCI	8.9	6.4	4.4	–	8.27

II. Effect of isolation conditions on respiratory
     performance of sarcosomes isolated from
              Heat-treated blowflies

A) α-glycerophosphate-dependent respiration:

     The results of the effect of isolation technique

on sarcosomal respiratory performance following heat

treatment are also recorded in Table (3.1). As can be

seen from the Table, heat treatment impaired α-glycero-

phosphate-supported respiration, which showed a decline

of 30% and 46% in the substrate rates, 54% and 78% in

state III for sarcosomes isolated with and without

nagarse, respectively. In such sarcosomes ADP

stimulation was not demonstrable, hence an RCI value

of one was obtained, and ADP : 0 ratio was not

measurable.

B) Pyruvate-dependent respiration

     Sarcosomes isolated from L.D.$_{50}$ treated

blowflies showed a decline in state III respiration of

more than 60%, when pyruvate is used as substrate, where

as state IV (substrate rate) respiration increased by about

12 and 27 per cent in sarcosomes isolated with nagarse or

with the standard method, respectively. Nevertheless,

some coupling was still demonstrable, and ADP : 0 ratios

were measurable. This is in agreement with the prelim-

Table 3.1
_____

Effect of isolation procedure and heat treatment

on the sarcosomal respiratory performance in the

presence of 33 mM α-glycerophosphate, or 2.5 mM

pyruvate and 2.5 mM proline as substrates.

Reaction conditions:

Where α-glycerophosphate is the substrate, the

reaction medium was as follows:

> 50 mM KCl
>
> 30 mM Phosphate buffer at pH 7.3 at 24°C
>
> 5 mM Mg CL_2.$6H_2O$
>
> 1 mM EDTA
>
> 20 mM Tris/HCl buffer at pH 7.3 at 24°C

Where pyruvate and proline are the substrates, the

reaction medium was as follows:

> 154 mM KCL
>
> 30 mM Phosphate buffer at pH 7.3 at 24°C
>
> 10 mM Tris/HCl buffer at pH 7.3 at 24°C
>
> 0.4 mg BSA/cm^3

Respiration (QO_2) is shown as the mean values of

μg AO.hr^{-1}.mg prot.$^{-1}$ ($\pm$ s.e.)

n.m. = not measurable

Respiratory performance of sarcosomes isolated from the flight muscle of control and

LD$_{50}$-treated 10 day old blowflies using different isolation techniques

Substrate	Isolation Method	Treatment	QO$_2$		RCI	ADP : O ratio	n
			Substrate rate	ADP-rate			
α - GP	nagarse	Control	24.91±0.20	37.87±2.20	1.50±0.05	1.37±0.06	5
		LD$_{50}$	17.58±2.06	17.58±2.06	1	n.m.	3
	Standard	Control	34.42±0.87	84.57±2.60	2.48±0.1	1.79±0.03	12
		LD$_{50}$	18.43±0.4	18.43±0.4	1	n.m.	6
Pyr. + Prol.	nagarse	Control	5.30±0.18	43.81±2.11	8.27±0.44	2.87±0.04	6
		LD$_{50}$	5.97±0.14	15.33±0.60	2.57±0.10	2.49±0.08	6
	Standard	Control	5.53±0.12	48.95±3.00	8.85±1.81	2.89±0.06	8
		LD$_{50}$	7.02±0.15	17.43±0.26	2.48±0.05	2.23±0.08	6

inary results reported by Davison (1970.

III. Effect of the composition of the reaction medium on sarcosomal respiration

The data presented in Table (3.1) clearly shows that equally good results are obtained, for both α-glycerophosphate and pyruvate + proline supported respiration, in sarcosomes isolated following the "standard" method. This is not the case however for nagarse treated sarcosomes. Consequently, this study of reaction conditions on sarcosomal respiration has been followed using "standard" method produced sarcosomes only.

A. α-glycerophosphate-dependent respiration

As can be seen in Table (3.2), medium I supports sarcosomal respiration in a good functional state, with high QO_2 values, and with a mean RCI of 2.43. The ADP :O of 1.8 also implies a high level of coupling of oxidation and phosphorylation. The results obtained are entirely consistent with data produced by other workers in flight muscle sarcosomes, using similar reaction conditions.

As can be seen the inclusion of 0.4 mg/cm^3

Table 3.2
_____

Effect of composition of the reaction medium on the

respiratory activities of isolated blowfly flight

muscle sarcosomes.

Final concentration of the substrates used:

33 mM α-glycerophosphate, or 2.5 mM pyruvate +
2.5 mM proline

Reaction media:

I   II

50 mM KCl   Same as I but

 5 mM Mgcl$_2$. 6 H$_2$0   contains 0.4 mg

 1 mM EDTA   BSA per cm^3.

20 mM Tris/HCl buffer at pH 7.3
              at 24°C

30 mM Phosphate buffer at pH 7.3
              at 24°C

III   IV

154 mM KCl   Same as III but

 10 mM Tris/HCl buffer at pH 7.3   contains 1 mM EDTA
              at 24°C

 30 mM Phosphate buffer at pH 7.3
              at 24°C

0.4 mg BSA/cm^3

Sarcosomes in all cases were isolated using the "standard"

method described in Chapter (2). Respiration (QO$_2$) is

expressed as mean values of µg AO.hr^{-1}.mg prot^{-1}.($\pm$ s.e.)

Table 3.2

Various reaction media

Substrate		Med. I		Med. II		Med. III		Med. IV	
		α-GP	Pyr. + Prol.	α-GP	Pyr. + Prol.	α-GP	Pyr. + Prol.	α-GP	Pyr. + Prol.
QO_2	Substrate rate	36.01 ± 1.23	18.16 ± 1.29	37.10 ± 1.74	17.72 ± 3.16	38.91 ± 1.98	5.14 ± 0.60	12.55 ± 1.73	3.95 ± 0.57
	ADP-rate	88.02 ± 5.60	53.70 ± 3.80	82.70 ± 3.67	67.29 ± 5.98	58.48 ± 3.76	48.69 ± 5.86	21.60 ± 4.20	23.45 ± 2.25
RCI		2.43 ± 0.09	3.03 ± 0.16	2.27 ± 0.34	3.79 ± 0.13	1.50 ± 0.05	9.47 ± 0.52	1.71 ± 0.10	7.05 ± 1.00
ADP = O ratio		1.80 ± 0.03	2.82 ± 0.04	1.81 ± 0.04	2.66 ± 0.23	1.33 ± 0.04	2.90 ± 0.05	1.27 ± 0.09	2.88 ± 0.04
		7	6	5	5	7	6	3	5

of BSA in reaction medium I to give medium II, had no significant effect on any of the parameters measured, as compared to the values obtained in medium I $(p > 0.05)$.

Medium III however, which contained no Mg^{2+} nor EDTA, and had a raised K^+ concentration of 154 mM, was less good a medium for sarcosomal respiration with α-glycerophosphate. Whilst substrate rate oxidation was 38.91 μg A 0.hr^{-1}. mg prot.$^{-1}$, in contrast to 36.01 and 37.10 μg A o.hr^{-1}.mg prot.$^{-1}$ for media I and II, respectively. The state III respiration was however reduced to 58.48 μg A0.hr^{-1}. mg prot.$^{-1}$ from about 80 μg A0.hr^{-1}.mg prot.$^{-1}$ in media I and II. This of course resulted in a significantly poorer value for RCI of only 1.5 $(p < 0.01)$. The weaker coupling was also obvious from the mean values obtained for ADP :0 of only 1.33, which was substantially lower than the values found when media I and II were used $(p < 0.01)$.

Reaction medium IV is the same as medium III, except that it contains 1mM EDTA. This medium has even greater deleterious effect on α-glycerophosphate respiration. All the parameters measured, substrate rate $(p < 0.01)$, state III $(p < 0.01)$, RCI $(p < 0.01)$ and

ADP :O (p $<$ 0.01) were significantly lower than in either medium I or II. Mean oxidation rates, 12.55 (for substrate rate) and 21.6 (for state III) were also dramatically lower than when the sarcosomes were respiring in medium III.

B. Pyruvate and proline dependent respiration

It must be pointed out that for pyruvate and proline state Iv respiration is also referred to as 'substrate rate'.

As can be seen from Table (3.2), pyruvate and proline linked respiration was supported by medium I. However, the mean value obtained for RCl was only 3.03 $\overset{+}{-}$ 0.16. This was mainly owing to high state IV respiration, where a mean value of 18.16 μg AO.hr^{-1}. mg prot.$^{-1}$ was obtained. Nevertheless phosphorylation, as determined from ADP :O, was still relatively good, for a mean value of 2.82 $\overset{+}{-}$ 0.04 was obtained.

The enclusion of BSA in medium I, to give medium II, produced a significant improvement in the mean RCl to 3.79 (p $<$ 0.01). This occurred because higher values for state III respiration were obtained, 67.29 as compared with 53.70 μg AO.hr^{-1}.mg prot.$^{-1}$.

In this medium the mean ADP : 0 obtained was 2.66 $\pm$ 0.23 however but was not significantly different from the value calculated for medium I ($p > 0.05$).

Medium III appears to provide the best conditions of the four media for sarcosomes respiring pyruvate and proline. Whilst the QO_2 values for states III and IV are reduced as compared with media I and II, it is state IV respiration that is most affected. This results in significantly higher values for the RCl of 9.47 $\pm$ 0.52, (P = 0.001). Good values were also obtained for ADP : 0, and gave a mean of 2.9 $\pm$ 0.05.

The inclusion of EDTA in medium IV, further reduces sarcosomal QO_2, with state III being the more dramatically affected, mean values of 23.45 $\pm$ 2.25 and 48.69 $\pm$ 5.86 μg AO.hr^{-1}.mg prot.$^{-1}$ were obtained in media IV and III, respectively.

Consequently the mean RCl value obtained in medium IV was also lower, 7.05 $\pm$ 1 as compared with the highest value obtained in medium III of 9.47 $\pm$ 0.52, these were statistically different, ($p < 0.05$ but > 0.01).

Once again however, ADP :0 were less affected and a mean of 2.88 $\pm$ 0.04 was obtained, which was not different from the value determined in medium III ($p > 0.05$).

Discussion

I.   Effect of isolation conditions on sarcosomal
respiration

The results of measurements of respiratory

activity of flight muscle sarcosomes show that the

α-glycerophosphate-linked respiration is lower when

the sarcosomes were isolated using nagarse. Two

factors must, therefore, be taken into account. First,

the composition of the isolation medium, which is

different from that used in the standard method, in

that it contains Sucrose and EGTA instead of KCl and EDTA.

Van den Bergh & Slater (1962) found that housefly

sarcosomes isolated in KCl medium had higher respiratory

activities than those isolated in sucrose medium.

This has been recently confirmed by Slack (1975),

who critisized, however, the ability of KCl medium to

maintain high sarcosomal activity and to prevent loss

of endogenous material during storage. Stevenson (1968)

pointed out, that swelling was observed in E.M. in

mitochondria isolated in a KCl-medium, as compared

with a sucrose medium, if EDTA (1mM) was included.

This is in accord with the observation made by Azzone

& Azzi (1966), that the removal of Mg^{2+} ions from an

ionic K^+ medium promotes K^+ uptake into the mitochondria.

Nevertheless, many workers have reported results in

favour of ionic rather than a sucrose isolation medium.

Gamble & Gartid (1970) showed that the entrance of

sucrose into the matrix is balanced by loss of K^+

and inorganic phosphate. Van Dam & Tsou (1968)

proposed that a discharge of the K^+ gradient would lead

to inhibition of substrate translocation - the same was

suggested by Atsmon & Davis (1967) and Klein & Neff

(1960). Furthermore the results reported by Packer,

Pollak, Munn & Greville (1971) reveal the possibility

that high osmolarity of sucrose may have a directly

injurious effect on membrane sites through an initial

dehydration, resulting in swelling caused by alteration

in membrane configuration. The involvement of H^+

in the interaction between potassium and sucrose was

proposed by G ear & Lehninger (1968) that salt-free

mitochondria transferred to an ionic medium, will

exhibit a potassium in - hydrogen - out exchange, whereas

the exchange would occur in the opposite direction if

salt-containing mitochondria are transferred to a sucrose

medium and, consequently, the mitochondria would

experience both an anion efflux through loss of binding

sites, and uncoupling through proton uptake. The role

of Ca^{2+} in these processes will be dealt with separately

(see Chapter 6).

Secondly nagarse itself might alter some of the

properties of the sarcosomal membrane(s), since some

evidence already exists that nagarse may cause a

leakage of endogenous tricarboxylic acid cycle

intermediates and may even inactivate the inner

membrane carrier (Chappell & Hansford, 1972). In

addition, the possibility that mechanical damage occurs

during homogenization cannot be ru led out. Homogenization

is necessary, for without it nagarse action was found to

be unsatisfactorily slow, and oxidative levels of nagarse

prepared sarcosomes were slightly inhibited (Slack,1975).

Pyruvate-linked respiration, on the other hand,

was affected much less by the isolation technique. It

is possible that the composition of the reaction medium,

which was different from that used for α-glycerophosphate-

supported respiration (see later), may have restored

any loss of respiratory activity caused by the nagarse

isolation procedure. On the other hand, the possibility

that nagarse itself or, in combination with homogenization,

deleteriously alters sarcosomal membranes, does not

appear to have affected the pyruvate + proline respiration.

Recently, Bursell (1975) reported a new method

for isolating flight muscle sarcosomes with high

respiratory activity. This method involved the use of

the potassium salt of D-aspartate in the isolation medium to

replace KCl or sucrose as an osmoeffector. The reas-

oning behind the use of aspartate was to provide an isolation

medium of a more physiological nature. However, 20 mM

sucrose was included in a second medium, used for

washing the sarcosomes. Shortly afterwards, Slack &

Bursell (1976a), reported an isolation medium that

contained Ca^{2+} in addition to Mg^{2+}, the sarcosomes were

washed in a 40 mM sucrose medium, and finally resus-

pended (stored) in a 0.32 M sucrose solution, containing,

amongst other constituents, low concentration of Mg^{2+}.

More recently, Slack & Bursell (1977) reported work on

blowfly flight muscle mitochondria, which have been

isolated, stored and assayed in media containing 0.25 M

sucrose, or 0.16 M potassium aspartate. Why the

authors used the reagents they did, in different concent-

rations from time to time is not always made clear by

them.

One feature of their procedures is the large fall

in RCI values, during repeated assays, from the very

high initial values they obtained. This appears to be

caused by marked increases in state IV respiration with

each successive addition of ADP (Bursell & Slack 1976).

From the results presented in Table (3.1), it is

clear that pyruvate-linked oxidative phosphorylation is

only partially affected by thermally induced changes in

flight muscle sarcosomes.

It is well established that exposing an animal to lethal heat treatments causes a decline in oxygen consumption of the intact animal as well as its isolated tissues. This has been demonstrated in blowfly larvae (Fraenkel & Herford, 1940), Arianta arbustorum (Grainger, 1969).

Davison (1970) considered the decline in oxidative phosphorylation of sarcosomes isolated from heat treated blowflies to be too massive to be due to isolation procedure alone.

Moreover, it is of particular interest that some measure of coupling is still demonstrable in sarcosomes isolated from heat treated adults, when using substrates other than α-glycerophosphate. This phenomenon has been also observed by Davison (1970), who proposed some factors to be taken into account, such as altered permeability of sarcosomal membranes, disturbed divalent cations distribution, release of endogenous uncouplers, and/or thermal inactivation of respiratory enzyme(s).

Analysing these factors, it appears that some measure of altered membrane permeability has occurred. This has been shown electron microscopically as grossly swollen sarcosomes in the presence of α-glycerophosphate,

but not pyruvate (see Chapter 8). Perhaps the difference

in concentration between the two substrates may account

in part at least for the differences in the degree of

swelling (for detail see Chapter 8). Davison (1970)

interpreted increased succinate oxidation rates in

sarcosomes isolated from heat treated flies on the

grounds of altered membrane permeability, resulting

in more succinate penetrating the membrane, allowing its

oxidation by the citric acid cycle. The increased

pyruvate oxidation rate (state IV) observed in the present

study might also be accounted for in this way, although

Davison (1970) found that pyruvate oxidation prior to the

addition of ADP was slightly depressed. The difference

in pyruvate concentration (24 mM) and the nature of

the primer (8 mM malate), as compared with 2.5 mM

pyruvate and 2.5 mM proline used in the present study,

may account for the discrepancy recorded. However,

a similar decline in pyruvate oxidation in total absence

of ADP has also been observed in this study, which will

be discussed later (see Chapter 7).

On the other hand, altered membrane permeability

cannot explain depressed α-glycerophosphate oxidation by

sarcosomes isolated from heat treated flies, since this

substrate does not have to be translocated across the

inner membrane to be oxidised (Klingenberg & Buchholz, 1970). This has been fully discussed elsewhere in this study (see Chapter 6).

A similar differential sensitivity of these two systems (α-glycerophosphate and pyruvate) has been demonstrated by other workers. Van den Bergh & Slater (1962) have shown that pyruvate oxidation was not as drastically reduced by sonication as α-glycerophosphate oxidation in <u>Musca domestica</u> flight muscle sarcosomes. Furthermore, Tribe & Ashhurst (1972) reported uncoupling of sarcosomes associated with age, when α-glycerophosphate was used as substrate; this was less marked when pyruvate was the substrate.

The second possible factor in heat injury, suggested by Davison (1970), is the disturbance of divalent cations distribution. In this context Wehrle, Jurkowitz, Scott & Brierly, (1976) have recently reported that swollen beef heart mitochondria permit EDTA to enter the matrix, resulting in a 90 per cent reduction in mitochondrial Mg^{2+}, this, in turn, alters the membrane permeability to monovalent cations. Although Mg^{2+} is presently included in the reaction medium, it is not certain that it can gain access to the matrix to replace the loss in intramitochondrial Mg^{2+}. In this regard, Ca^{2+} should not interfere since its allosteric effect is not applicable at α-glycero-

phosphate concentrations as high as 33 mM, used in the present study, unless some thermally induced changes in the mitochondrion have altered this characteristic (see Chapter 6).

The third possible factor is the release of endogenous uncouplers. These are long chain fatty acids (Lewis & Fowler, 1960; Wojtczak & Wojtczak, 1960), which can be effectively removed by BSA (Sacktor et al. 1958, Wojtczak & Wojtczak, 1960). Davison(1970) tested the effect of BSA on sarcosomes isolated from heat treated flies in presence and absence of BSA, but there was no difference. However, throughout this study BSA was included either in the isolation or in the reaction medium (see Chapters 2 and 3). Nevertheless, it is quite possible that heat treatment may cause the release of an endogenous uncoupler of a different nature that is not removable by BSA, and specifically uncouples α-glycerophosphate-linked oxidative phosphorylation. Rogers & Higgins (1976), have recently reported that, in rat liver mitochondria, the action of an inhibitor depends upon the nature of the receptor site for inhibition, whether lipophilic or hydrophilic. Interestingly enough, they found not only that the differently linked dehydrogenases of the oxidative phosphorylation system respond differently to inhibition, but also that dehydrogenases

like succinate dehydrogenase and α-GP dehydrogenase,
which are both flavin-linked, are differently inhibited,
using the same inhibitor in both cases. It was, thus,
concluded that depression of respiratory control depends
upon the susceptible site, which may be different for
different respiratory substrates. The possibility that
respiratory enzyme(s) might have been thermally inact-
ivated, will be dealt with separately at the level of
α-glycerophosphate dehydrogenase (see Chapter 7).

II.   Effect of the reaction conditions

It must be pointed out that flight muscle sarcosomes,
employed in this part, have been isolated using the
standard method described in Chapter (2).

As mentioned earlier one of the parameters that
may alter the respiratory activity of the mitochondria is
the composition of the reaction medium (Chefurka, 1965).
The reaction media chosen have two constituents in common.
First, the presence of a phosphate buffer, whose
concentration is 30 mM, since there is some evidence
(Tulp, Stam & Van Dam, 1971) that low Pi concentration
(5 mM) would strongly inhibit a probable dicarboxylic
acid translocating system in the flight muscle sarcosomes.
Furthermore, it has been suggested that mitochondria in

the presence of divalent cations, but absence of phosphate,
were fragile (Brierley, 1963). On the other hand, Sacktor
& Hurlbut, (1966) reported that the optimal Pi
requirement appeared to be between 5 - 10 mM, more or
less at the physiological level of Pi in insect muscle.
The second common constituent in the four reaction media
used in this study is the tris/HCl buffer system at a
concentration of 20 mM. In spite of the wide controversy
about the side effects of tris, there is a general
agreement that its inhibitory effects are observed only
when high concentrations are applied (Sacktor & Childress
?(1967) pointed out that at concentrations above 20 mM
NAD-linked systems showed signs of inhibition.

There is an extensive literature on the requirement
for BSA in media to obtain high levels of respiratory
performance. In some cases the requirement for BSA
appears to be absolute (Sacktor, 1954; Sacktor, O'Neill
& Cochran, 1958, in Musca domestica; Wojtczak &
Wojtczak, 1960, in wax-moth larvae). On the other hand,
Gregg et al. (1960) in house fly, Newburgh, Potter
& Cheldelin, blowfly larvae (1960) and Van den Bergh &
Slater, housefly (1962) report high P : O ratios without
added serum albumin. The apparent protective effect of
serum albumin was attributed by Van den Bergh & Slater
(1962) to "its capacity to bind the fatty acids that form

the active component .... of the endogenous

uncoupling agent, enzymically ....." , The damaging

effect of free fatty acids on mitochondrial structure

has been recognised by many workers (Lehninger, 1962b;

Pressman & Lardy, 1956; and Borst, Loos, Christ &

Slater, 1962). This has been confirmed by other workers

(Kates, 1960 and Waite, Scherphof, Boshouwer & Van

Deenen, 1969), who postulated the inhibitory effect

of Ca^{2+} to be due to its catalytic action upon the fatty

acids releasing enzyme (phospholipase). Chefurka (1966)

suggested that the protective effect of serum albumin lies

in its capacity to trap the fatty acids but not to inhibit

the phospholipase activity.

 With the above discussion in mind it was thought

necessary to include BSA in medium II so that its effect

could be determined in comparison with sarcosomal

respiration in medium I. As can be seen from Table 3.2

sarcosomes used in this study did not apparently undergo

any release of fatty acids, since the addition of serum

albumin did not have any pronounced effect. This is

particularly the case for α-glycerophosphate supported

oxidation, however a significantly higher RCI was obtained

for pyruvate and proline supported oxidation, although

the mean ADP : 0 was lower in medium II as compared

to medium I. More significantly the respiratory

performance of sarcosomes with pyruvate + proline

as substrates, is very significantly poorer in media

I & II as compared to medium III. This argues that

it is a reaction medium constituent, other than BSA

which is either missing or when present has a

deleterious effect.

One significant different between media III

and I & II is the omission of Mg^{2+}, and a raised

K^+ concentration. Recently, Slack & Bursell (1976 b)

report that Mg^{2+} at concentrations above 10^{-5} M

increase state IV pyruvate respiration in blowfly

flight muscle sarcosomes, which, they consider, is

in part due to a stimulation of the sarcosomal ATPase.

Thus it may be that the relatively high state IV

observed with pyruvate-linked respiration in media

I and II, is attributable to the presence of Mg^{2+}.

This is reflected on partial uncoupling, resulting in

a fairly low RC1.

Earlier workers have also reported intact

muscle mitochondria possess a Mg^{2+} sensitive ATPase

(Azzone & Carafoli, 1960), however it is generally

believed it is latent unless the mitochondria are

damaged, Sugano & Nagai (1971), Waino (1970). Indeed,

uncoupling agents, such as DNP, are known to stimulate

this ATPase activity.

The removal of Mg^{2+} from media I and II has little effect on α-glycerophosphate supported substrate rate oxidation, but causes a significant fall in state III respiration with this substrate. It is unlikely that this is owing to any requirement for Mg^{2+} from the adenine nucleotide translocation system, for pyruvate respiration is apparently optimal in this medium III.

One must conclude, therefore, that whilst Mg^{2+} is a requirement for maximal sarcosomal respiration performance with α-glycerophosphate, this divalent cation is best excluded from reaction media when pyruvate and proline are being respired.

It is possible, that this differential effect results from an interaction of effects between the absence of divalent, and increased levels of monovalent cations.

Azzone & Azzi (1966) suggested that Mg^{2+} stabilizes the permeability of mitochondrial membranes to K^+, such that at low Mg^{2+}, potassium penetration would be increased. It is however difficult to see why any differential effect on the phosphorylating systems should occur, if K^+ entry is acting by interference with the proton gradient, and so affecting coupling (Mitchell & Moyle, 1969).

CHAPTER FOUR

RECOVERY OF THE BLOWFLY, CALLIPHORA

VICINA, FROM HEAT INJURY

Introduction :

The rationale behind the work dealt with in this Chapter concerns the reasonable assumption that the study of recovery from a sublethal (L.D.$_0$) or L.D.$_{50}$ exposure might give an insight to the nature of the damage caused by the heat treatment, and thus the cellular repair mechanisms. To this end two types of experiments were planned. 1. 'Split-dose' experiments and 2. the temperature dependency of recovery process.

Split-dose experiments have been widely used in the study of irradiation damage, in insects, as well as in representatives of other phyla, for a recent review of this work on insects see Rockstein & Miquel (1973). The essence of such experiments was to compare the damaging effect of a particular dose of X-irradiation given as a single dose or as two applications separated by a short time interval. Such work has provided valuable information on the permanent and temporary nature of irradiation damage, see Baxter & Blair (1969) and Webb, Hollingsworth, Mill & Davies (1976). Few

similar studies are reported in the field of heat injury.

The work of Kallman (1963) and Kvuur, Nelson, Frey & McGann (1972) on cells in culture is pertinent. These workers clearly showed that exposure of cell lines to sub-optimal temperatures causes sub-lethal injury that can accumulate with dose. Their data also revealed however such damage can be repaired.

On the other hand, Gerner & Schneider (1975), working with Hela cells interpreted their data as showing sub-lethal heat damage did not accumulate, this interpretation contrasts with that of Palzer & Heidelberger (1973) who concluded Hela cells were capable of repair of sublethal heat damage.

The second approach has been to study the effect of temperature on the rate of repair of a standard application of heat. Davison & Bowler (1971) in an earlier study on this stock of animals showed that repair of heat damage resulting from an $L.D._0$ exposure required 48 hours at $24°C$. It was not clear whether this process was temperature sensitive, and consequently whether the repair of heat damage required active cellular metabolism. Few similar studies have been carried out on organisms, and perhaps only the work on microorganisms is really pertinent to this problem. Christophersen (1973)

summarises this work in an extensive review. Sub-
lethal damage most commonly is seen as a delay in
multiplication (Postgate & Hunter, 1963) and recovery
is reported to require nutrient supplementation of the
growth medium (Clark & Ordal, 1969). Heinments (1960)
and Iandola & Ordal (1966) amongst others suggest
repair of sub lethal damage requires enzymatic activity.

Similar work on multicellular organisms has
been hindered by the lack of suitable criteria for
establishing that cellular structures have been damaged
as a result of heat treatment. Davison & Bowler (1971)
have clearly demonstrated a good correlation exists in
Calliphora erythrocephela between organism heat death
and damage to flight muscle sarcosomes. Indeed it
is the return of sarcosomal efficiency to the levels
obtaining in control flies that is used as the index of
repair in this study.

Materials and Methods

A. General methods

The flies used were reared and maintained
(24°C throughout) according to the method stated in
Chapter 2 of this thesis. The heating apparatus, and
application of heat treatment have also been described

in Chapter 2. During recovery the flies were housed
in small cages 22 cm x 22 cm x 22 cm constructed
from commercial biscuit tins with three sides removed.
Two sides were covered with polyglaze, and the third
had a muslin sleeve attached.

B. Split-dose experiments

960 adult male, 10 day old, blowflies were
used in this study. The heat dose given was 41°C for
40 min., this being slightly greater than the L.D.50
point for this aged fly reared and maintained at 24°C,
Davison (1969). The flies were used in groups of 80
individuals, ten of which received the heat treatment
as a single dose. The remainder were exposed for
20 min. at 41°C, followed by a period at 24°C, after
which they received a further 20 min exposure at
41°C. The time intervals between the split-doses were
10,20,40,80 min. and 4, 6 and 12 hours. The remaining
flies were randomly selected for these split-dose
periods. Following the completion of the heatings the
flies were returned to 24°C to recover.

Previous work by Davison & Bowler (1971) have
shown that those flies that will recover do so within
2 days at 24°C. Life table work on the same stock of

flies has shown few deaths occur naturally up to 20 days old, Tribe (1966). Thus the complication of natural death occurring during the experimental period can be ignored. The experiment was repeated on 12 separate occasions and the numbers dying at each trial were scored and pooled. The data were subjected to an analysis of variance and the results are shown in Table (4.1) and Figure (4.1).

C. Temperature - dependence of recovery

In these experiments some 270 10 day old flies were heated to an approximate L.D.$_0$ (40°C for 40 mins., Davison (1969). Thirty of these flies were taken immediately following heat treatment, and their flight muscle sarcosomes isolated as described in Chapter 2. The respiratory performance (QO_2, ADP : O ratio and RCl) was measured also as described fully in Chapter 2. This group was designated as the flies allowed no recovery in Table (4.2).

The remaining flies were placed at either 15° $\pm$ 0.5°C, 24° $\pm$ 0.5°C or 34° $\pm$ 0.5°C and allowed to recover from the heat treatment. The time dependency of recovery was followed at each temperature of

recovery by periodically taking batches of 30 flies, isolating the flight muscle sarcosomes and subsequently determining their respiratory performance. The recovery periods allowed were as follows:

> At 15° C, 2 days, 4 days and 6 days
>
> At 24°C, 1 day, 2 days.
>
> At 34°C, 8 hours, 16 hours and 24 hours.

Because the different maintenance temperatures might also cause acclimation changes in respiratory performance, control flies were placed at 15°, 24° or 34°C at the same time as the experimental flies. They were taken in batches of 30 animals at the same time intervals as the experimental group for determinations of sarcosomal respiratory performance.

This experiment was repeated on six separate occasions, the data were combined and the mean values of QO_2, RCI and ADP : 0 ratios are presented in Table (4.2). These values are plotted as a function of recovery periods at each temperature in Figs (4.2), (4.3) and (4.4).

Results

A) Split - dose experiments

Analysis of variance showed significant variation existed between the various treatments (P = 0.02 for 7 and 86 degrees of freedom). The means of numbers dying, at each treatment, can therefore be compared; these are shown in Fig. (4.1) in the form of a histogram. As can be clearly seen, the mean number dying as a result of a single application (control group) was 6.0 $\pm$ 0.62 (n = 12), and only when the interval between the split-doses was either 6 or 12 hours, was survival significantly greater than in the control group. For the 6 hour interval the mean number dying was 3.45 $\pm$ 0.72 (p = 0.0 2; n = 11) and for the 12 hour interval the mean number dying was 3.0 $\pm$ 0.63 (p = 0.01; n = 12). At the shorter time intervals then the injurious effect of heat is merely additive.

B) Effect of temperature on recovery

The results of the effect of temperature on recovery from heat treatment are shown in Table (4.2), and will be dealt with in two parts. The experimental group will be considered first. It must be mentioned that in all cases sarcosomal respiratory performance was measured at 24°C.

Table 4.1

Effect of giving an approximate

L.D.$_{50}$ heat treatment either as

a single dose (40 min at 41°C) or

as a split-dose of two separate

20 min exposures on survival

of 10 day old adult male blowflies.

Single dose		Split-dose of 20 min at 41° followed by the indicated period (in h) followed by a further 20 min period at 41°.													
		0.167		0.33		0.67		1.33		4		6		12	
D	A	D	A	D	A	D	A	D	A	D	A	D	A	D	A
72	48	81	39	61	59	66	44	69	51	59	61	38	72	33	77

D = Number dead after 2 days.

A = Number alive after 2 days.

Fig. 4.1

Mean number of blowflies dying as a result
of the state heat exposure. Single-dose
blowflies received a 40 min. exposure at
41°C; the split-dose blowflies received a
20 min exposure at 41°C, followed by the
stated period at 24°C before receiving the
final 20 min at 41°C. Flies were heated in
groups of ten for each treatment, and the
means were constructed from 11 or in some
cases 12 repeat experiments. The vertical
bars represent one standard error of the
mean.
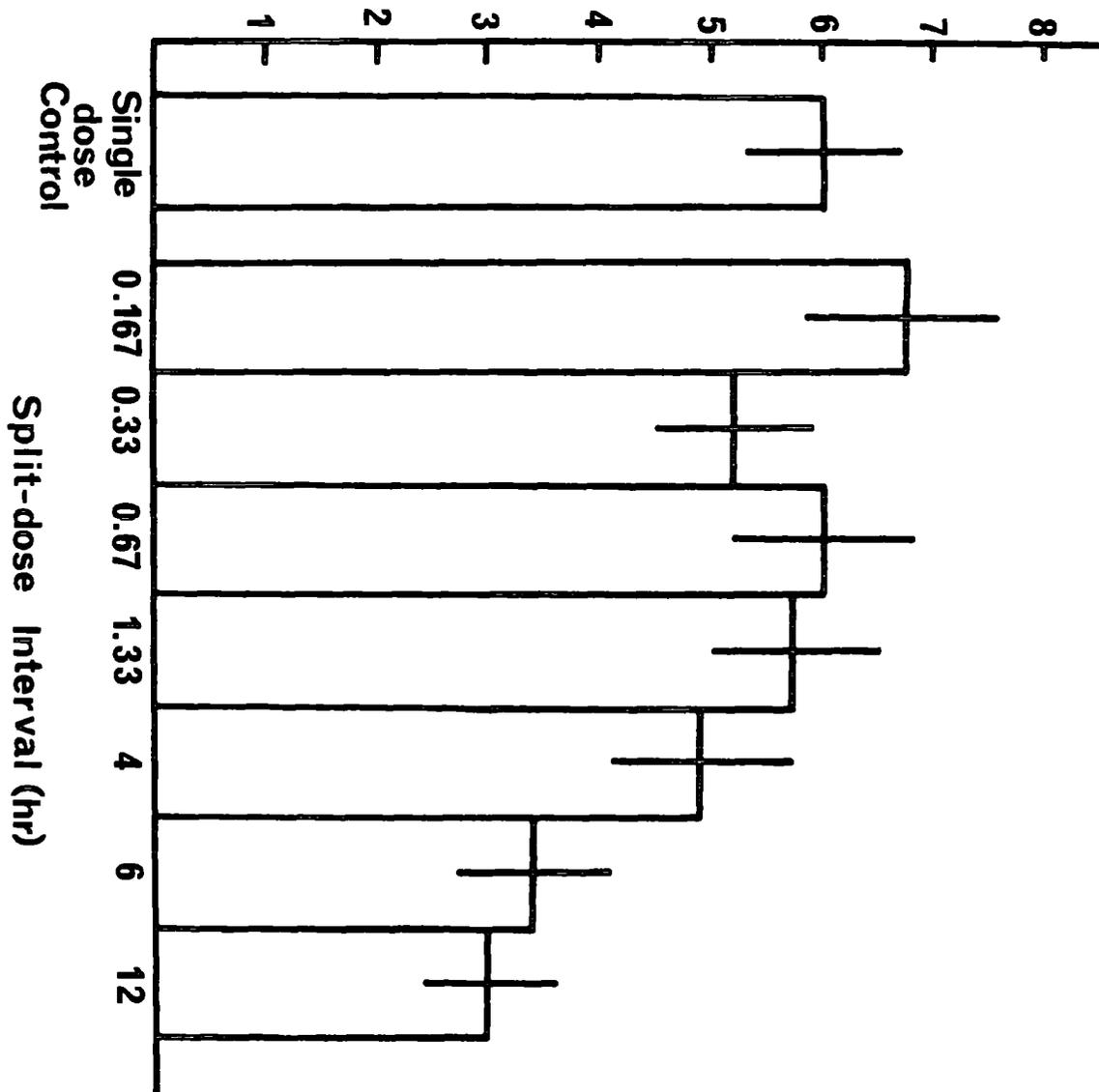
An L.D.$_0$ treatment to/the whole organism has a marked effect on sarcosomal respiratory performance. This can be seen most clearly immediately after the heating, and it is the ADP-stimulated oxygen consumption (state III) of sarcosomes that is most dramatically reduced (by about 40%) as compared with sarcosomes from control unheated flies. On the other hand substrate rates are not significantly different. This results in a significant reduction (p = 0.001) in RCl and ADP : O ratio.

15°C maintained flies

In the experimental group sarcosomes from flies taken 2 days after recovery at 15°C showed no recovery had occurred at all when compared with those taken immediately after heating. Only after a further 2 day recovery period was there any restoration of normal respiratory function in isolated sarcosomes. Indeed both state III, and substrate rate respiration in the sarcosomes from the experimental flies were the same as in the control flies, maintained at 15°C for 4 days. RCl and ADP : O ratios however were still significantly lower than in control flies (initial levels), although they were not different from those of control flies kept for 4 days at 15°C (p = 0.05). It was therefore decided

Table 4.2

Effect of temperature on the ability to recover
from 40°C : 40 min sublethal heat treatment

L.D.$_0$

Measurements of respiratory performance were

made of sarcosomes isolated from the flight muscle

of 10 day old adults, but allowed to recover for the time

shown at either 15°, 24° or 34°C.

Control flies were also placed at 15°, 24° or

34°C and were sampled after the same time intervals

as were the experimental flies.

The mean values shown were obtained from

6 separate preparations

Reaction conditions:-

    50 mM KCL
    30 mM Phosphate buffer at pH 7.3 at 24°C
     5 mM MgCl$_2$.6H$_2$)
     1 mM EDTA
    20 mM Tris/HCl buffer at pH 7.3 at 24°C

Sarcosomes in all cases were isolated using the

'standard' method described in Chapter (2).

QO$_2$ = µg Atoms of oxygen consumed per hour per

mg protein.   (µg AO.hr^{-1}.mg prot.$^{-1}$)

Table 4.2

Effect of temperature on recovery from sublethal heat treatment

Temperature	Recovery days	Experimentals QO$_2$ Subst. rate	Experimentals QO$_2$ ADP rate	Experimentals RCI	Experimentals ADP : 0	Controls QO$_2$ Subst. rate	Controls QO$_2$ ADP rate	Controls RCI	Controls ADP : 0
15°	0	30.04 ± 1.26	46.85 ± 1.81	1.57 ± 0.04	1.35 ± 0.02	32.83 ± 1.09	83.24 ± 2.56	2.54 ± 0.04	1.85 ± 0.04
	2	28.55 ± 0.99	45.84 ± 1.78	1.62 ± 0.04	1.39 ± 0.05	39.74 ± 2.12	84.30 ± 4.55	2.04 ± 0.11	1.72 ± 0.07
	4	31.93 ± 1.23	61.26 ± 3.15	1.92 ± 0.07	1.51 ± 0.07	31.07 ± 1.40	64.85 ± 2.61	2.11 ± 0.06	1.71 ± 0.07
	6	34.24 ± 0.9	68.59 ± 1.43	1.87 ± 0.07	1.65 ± 0.03	26.85 ± 1.37	60.86 ± 3.58	2.21 ± 0.07	1.66 ± 0.07
24°	1	28.80 ± 0.84	62.77 ± 2.43	2.11 ± 0.1	1.65 ± 0.03	34.96 ± 1.34	86.15 ± 4.08	2.46 ± 0.06	1.79 ± 0.03
	2	31.32 ± 1.22	72.88 ± 3.50	2.31 ± 0.06	1.80 ± 0.02	34.42 ± 0.87	84.57 ± 2.60	2.48 ± 0.096	1.79 ± 0.03
34°	0.33	41.98 ± 2.0	66.90 ± 6.0	1.57 ± 0.05	1.45 ± 0.04	34.56 ± 2.34	76.30 ± 5.42	2.19 ± 0.07	1.75 ± 0.05
	0.67	34.14 ± 2.49	64.82 ± 5.20	1.89 ± 0.07	1.67 ± 0.07	31.96 ± 1.12	71.78 ± 2.26	2.26 ± 0.11	1.67 ± 0.03
	1	29.95 ± 1.45	59.89 ± 3.1	2.04 ± 0.08	1.69 ± 0.02	27.55 ± 1.53	65.25 ± 4.6	2.32 ± 0.05	1.71 ± 0.02

Fig. 4.2

Recovery of the blowfly, at $15^{\circ}C$, from

sublethal heat treatment $(L.D._0)$, as

measured by the respiratory performance

of its isolated flight muscle sarcosomes.

	Control	experimental
Substrate-rate	●	O
ADP-stimulated rate	▽	▲
RCl	O	●
ADP : 0	△	▲

The standard error RCl and ADP : 0 falls

within the points.

Reaction and isolation conditions see Table (4.2).
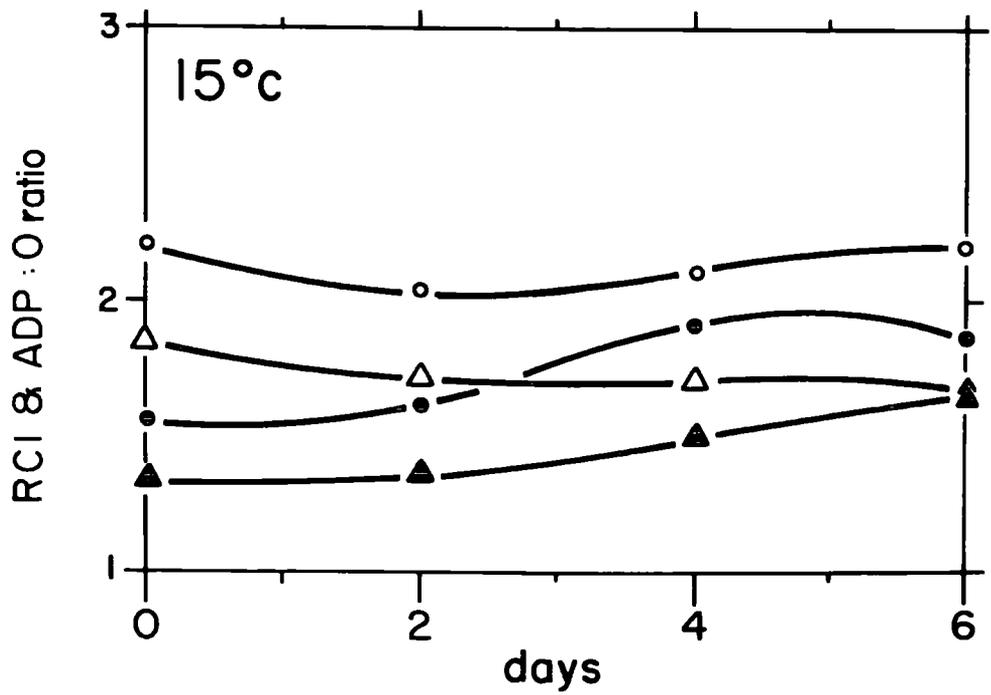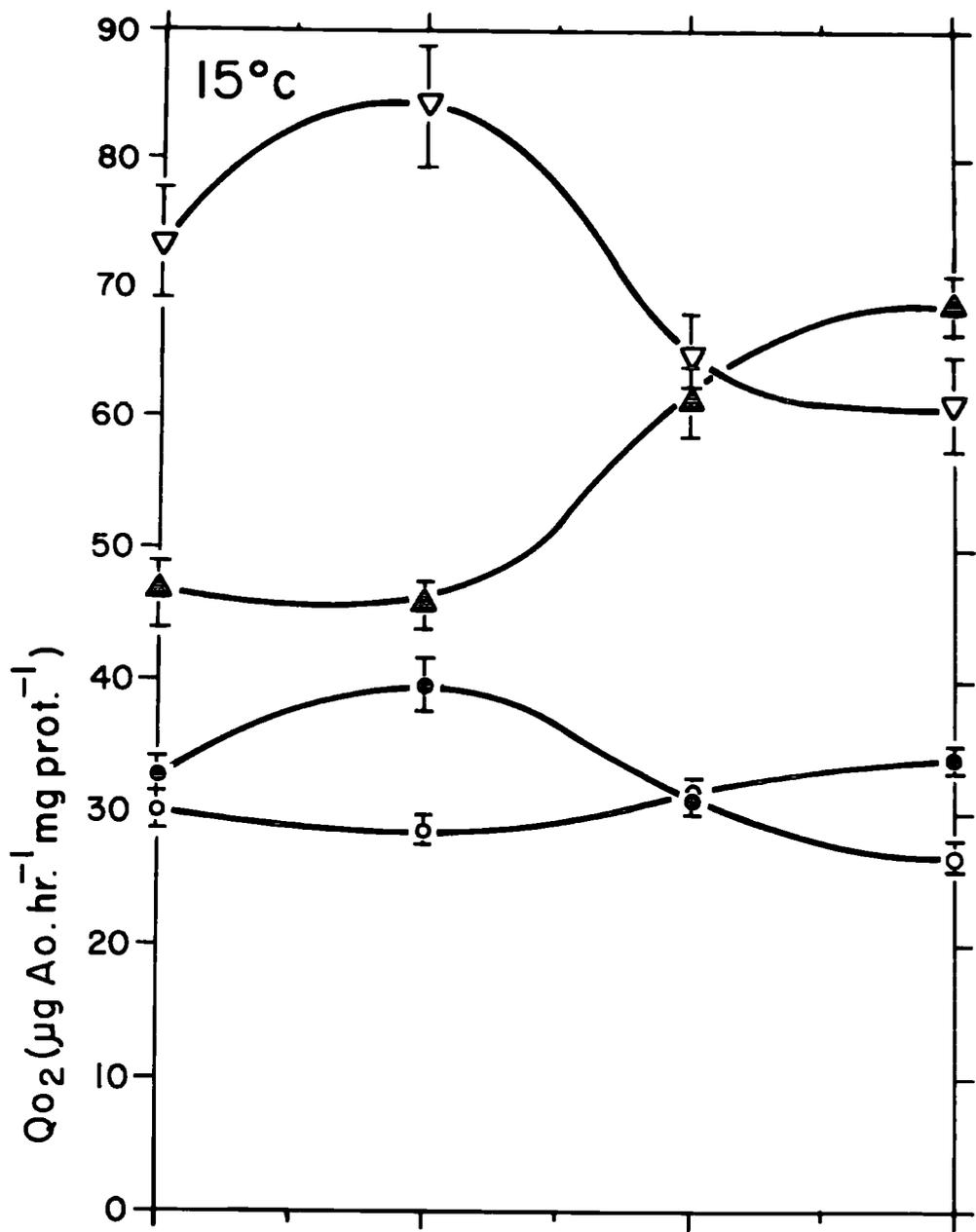
to leave the experimental flies for a further two days at 15°C to see if any further relative change of respiratory function took place. As can be seen, small, but statistically significant increases occurred in both substrate rate and state III respiration after 6 days recovery at 15°C. Furthermore, no differences occurred in either RCl or ADP : 0, these were still significantly below the initial value obtained for the control flies. Only in the case of RCl was the value lower in the sarcosomes from the experimental animals as compared with sarcosomes from control animals after 6 days at 15°C (p $<$ 0.05).

24°C maintained flies

In this group of flies, significant repair from the heat damage has occurred after 1 day recovery. This can be clearly seen in comparison with ADP stimulated oxygen consumption in the experimental groups, immediately after the heating. State III respiration has risen from 46.85 $\pm$ 1.81 to 62.77 $\pm$ 2.43 μg AO.hr^{-1}. mg prot.$^{-1}$. However, this is still significantly less than that of the control group kept at 24°C for 1 day (p $<$ 0.01). RCl (p$<$0.01), and more significantly ADP : 0 (p$<$0.001) are also less than in the control flies

Fig. 4.3

Recovery of the blowfly, at $24°C$, from sublethal heat treatment $(L.D._0)$, as measured by the respiratory performance of its isolated flight muscle sarcosomes

	Control	experimental
Substrate rate	●	○
ADP-stimulated rate	▽	▲
RC1	○	●
ADP : 0	△	▲

The standard error of RC1 and ADP : 0 falls within the points.

Reaction and isolation conditions see Table (4.2)

24°c

24°c

Qo_2 (μg $Ao.hr.^{-1}mg\,prot.^{-1}$)

RCI & ADP:O ratio

hours
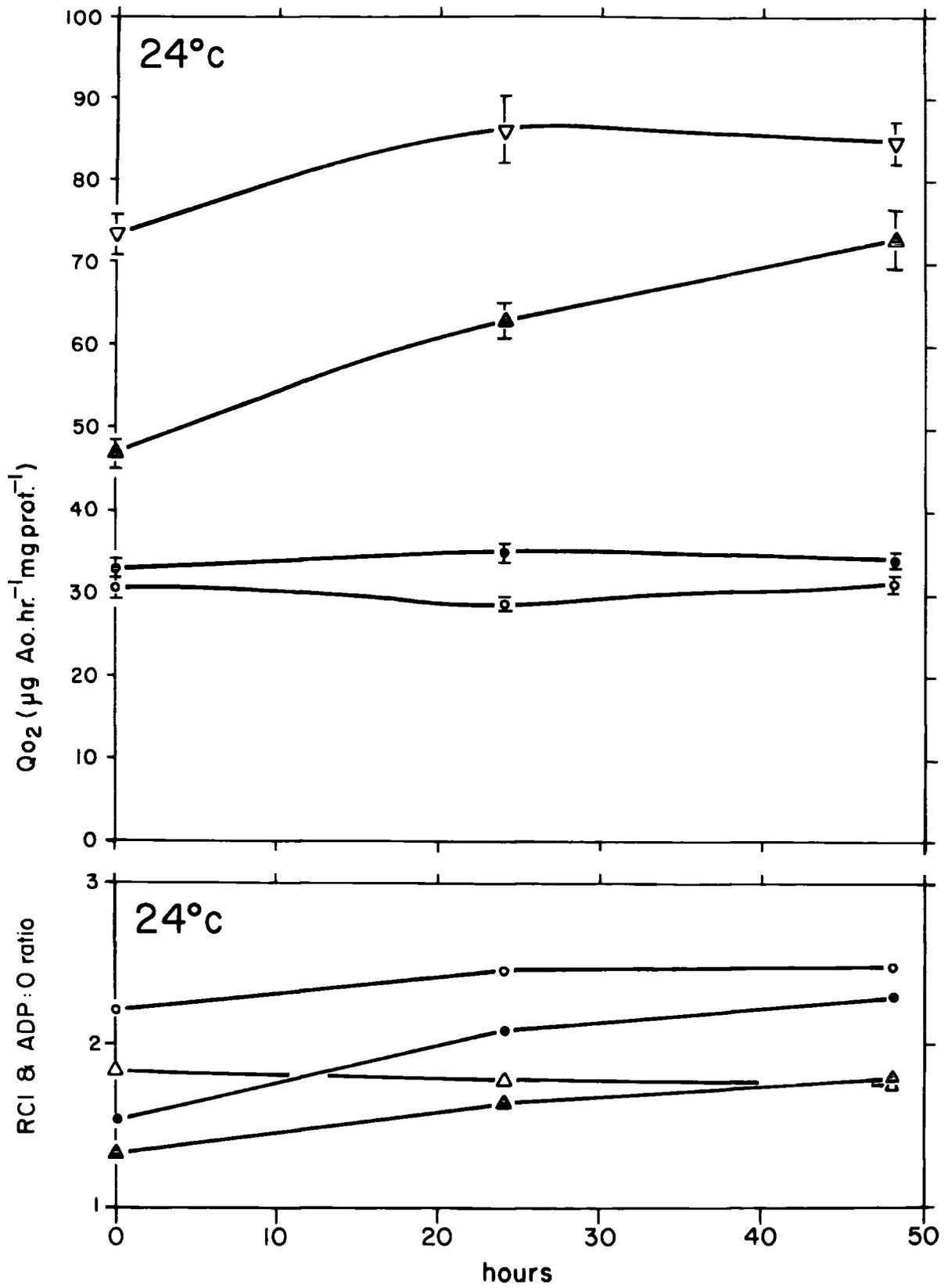
maintained for 1 day at 24°C.

However, after one further day recovery at 24°C sarcosomal RC1 and ADP : O ratios have improved in the experimental group. In both cases the values of 2.31 $\pm$ 0.06 and 1.80 $\pm$ 0.02 respectively are not significantly different from the controls maintained at 24°C, which were 2.48 $\pm$ 0.096 and 1.79 $\pm$ 0.03. State III respiration of the sarcosomes from the experimental group, 72.88 $\pm$ 3.5 μg AO.hr^{-1}. mg prot.$^{-1}$, is however significantly lower than that of the controls, 84.57 $\pm$ 2.6 μg AO.hr^{-1}. mg prot.$^{-1}$ (p $<$ 0.01).

34°C maintained flies

In the experimental group at this temperature, an 8 hour recovery period allows significant recovery to occur. It is marked however by a large rise in substrate rate respiration to 41.98 $\pm$ 2.0 μg AO hr^{-1}. mg prot.$^{-1}$, this is just significantly higher than in the sarcosomes from control flies after 8 hours at 34°C (p $<$ 0.05). Although sarcosomal state III respiration from the experimental flies is less than that from the control group, 66.90 $\pm$ 6 as compared with 76.30 $\pm$ 5.42 μg AO.hr^{-1}. mg prot.$^{-1}$, this is not statistically

Fig. 4.4

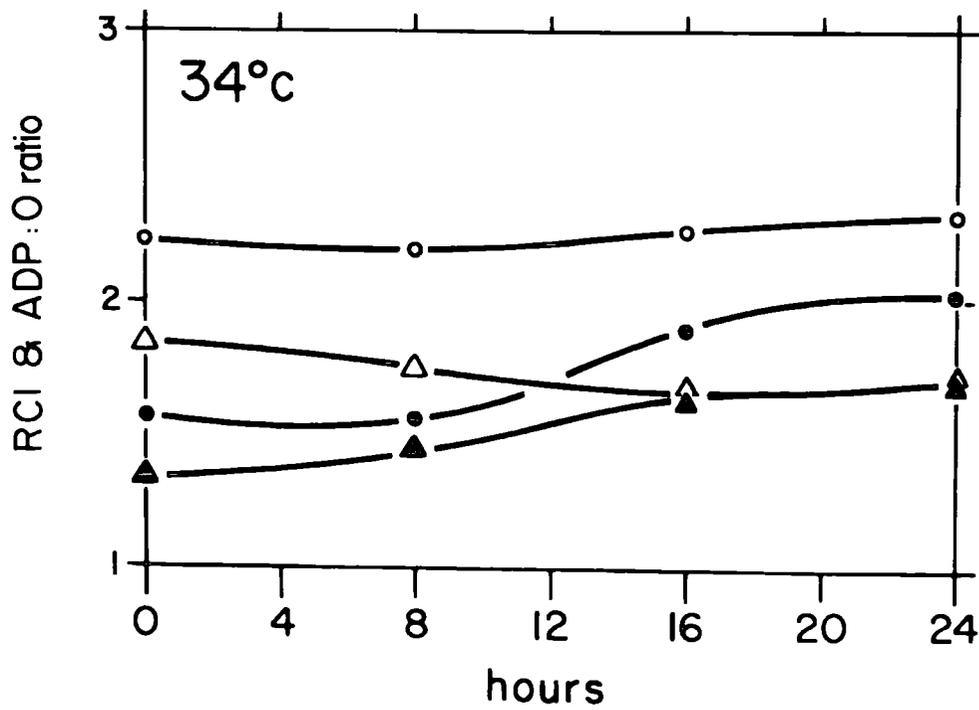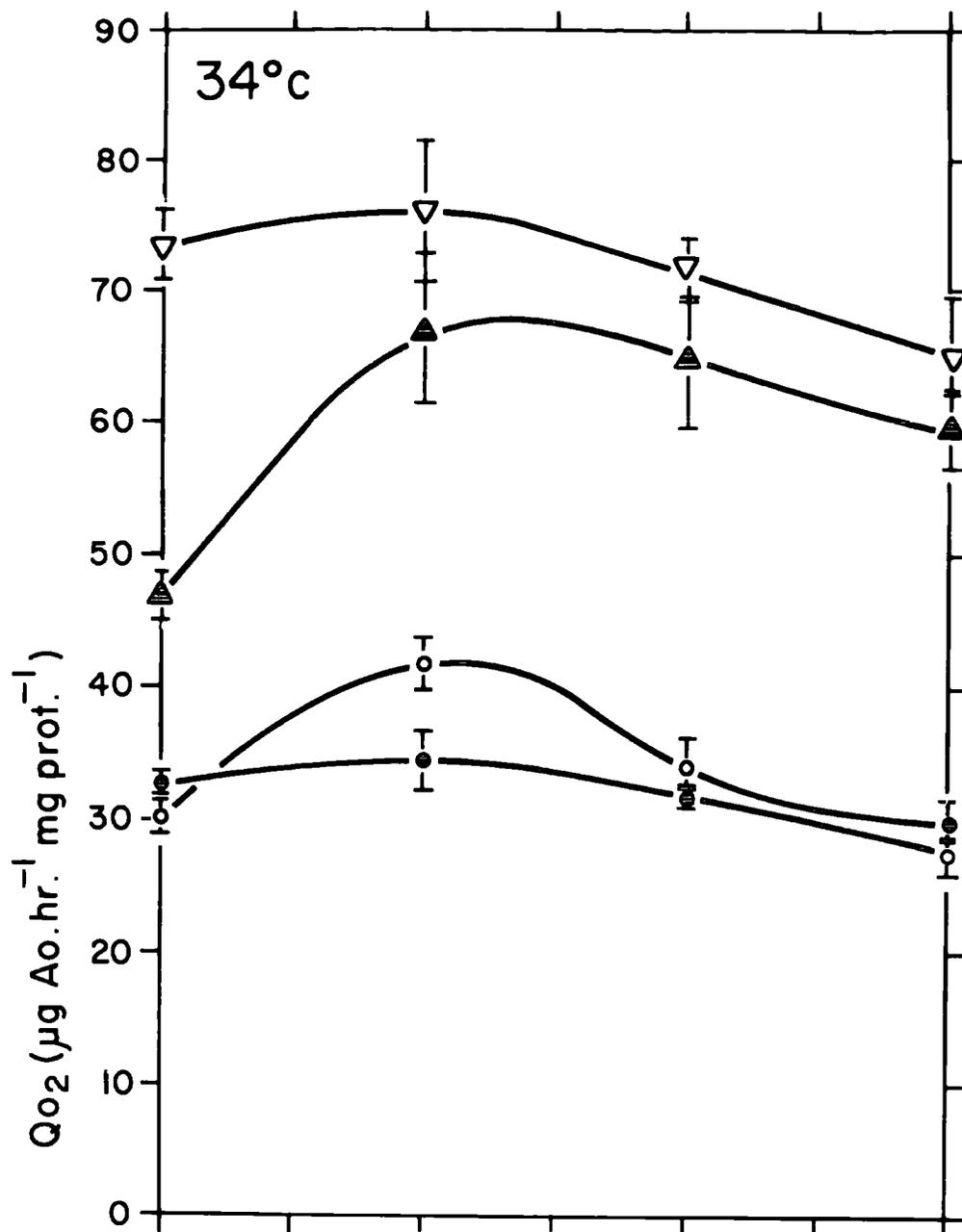Recovery of the blowfly, at $34°C$, from sublethal heat treatment $(L. D_{0})$ as measured by the respiratory performance of its isolated flight muscle sarcosomes.

	Control	experimental
Substrate-rate	⊖	○
ADP-stimulated rate	▽	▲
R Cl	○	●
ADP : 0	△	▲

The standard error of RCl and ADP : 0 falls within the point.

Reaction and isolation conditions see Table (4.2)

significant. RCl and ADP : 0 of the experiment group sarcosomes are highly significantly different from the control sarcosomes (p = 0.001 in both cases).

A further 8 hours recovery at 34°C brings a further marked improvement in the measured sarcosomal parameters. Substrate rate respiration falls to the same level as in the control group, whilst state III respiration shows only an insignificant fall over the value for the 8 hour recovery group. In this case also it is not significantly different from state III respiration of the control group sarcosomes, the values being 64.82 $\pm$ 5.2 and 71.78 $\pm$ 2.26 μg AO.hr^{-1}. mg prot.$^{-1}$ for experimental and control group sarcosomes, respectively. The consequence is an improvement in this 8 hour period in the RCl from 1.57 $\pm$ 0.05 to 1.89 $\pm$ 0.07, this latter value is still significantly less than that of the control flies after 16 hours at 34°C where the value is 2.26 $\pm$ 0.11 (p = 0.01). The ADP : 0 of the two groups are identical at 1.67. In experimental flies maintained for 1 day at 34°C only RCl is significantly different from the control group (p = 0.01), at 2.04 $\pm$ 0.08 it is less than that for control sarcosomes where a value of 2.52 $\pm$ 0.05 was found.

Effect of maintenance temperature on
sarcosomal performance in control flies

The initial values of respiratory function in

sarcosomes from control flies are of course obtained

at 24°C from flies maintained at 24°C. These values

are 32.83 $\overset{+}{-}$ 1.09 and 83.24 $\overset{+}{-}$ 2.56 µg AO.hr^{-1}.mg prot.$^{-1}$,

for substrate rate and state III respiration respectively.

Values of 2.54 $\overset{+}{-}$ 0.04 for RCl and 1.85 $\overset{+}{-}$ 0.04 for

ADP : 0 were obtained for these sarcosomes.

In flies kept on at 24°C no change was seen in

substrate rate or state III respiration, consequently

RCl remained fairly constant. ADP : 0 values at

1.79 $\overset{+}{-}$ 0.03 were not different from the initial values.

Transfer of control group flies to 15°C brought

a small rise in both substrate rate and state III

respiration of isolated sarcosomes measured at 24°C,

however only the substrate rates were different

(p = 0.05). ADP :0 (1.72 $\overset{+}{-}$ 0.07) was not significantly

different from the initial value. During the 6 day

period at 15°C both substrate rate and state III

respiration fell significantly to become 26.85 $\overset{+}{-}$ 1.37

and 60.86 $\overset{+}{-}$ 3.58 µg AO.hr^{-1}. mg prot.$^{-1}$ respectively.

In both cases these values are significantly less than

the initial values (p = 0.001) RCl also showed a

significant fall during the first 4 days at 15°C, but was
not significantly different from the initial value on
the sixth day at 15°C.

Substrate-rate respiration of sarcosomes,
isolated from control flies kept at 34°C for 24 hours,
showed a trend towards a significant decline from
34.56 ± 2.34 to 27.55 ± 1.53 µg AO.hr^{-1}. mg prot.$^{-1}$
($p < 0.01$). Such decline was less marked in state III
respiration of the same sarcosomes. Consequently,
a slight improvement was recorded for RCl values,
from 2.19 ± 0.07 to 2.32 ± 0.05. ADP : 0 ratio was
not affected during the 24 hour period at 34°C.

Discussion

As has been earlier pointed out by Bowler
(1963 a) a casual relationship obviously exists between
resistance acclimation and lethal death points of
ectothermal animals. Thus identifying the cause of
death at high lethal temperatures (finding the primary
lesion), could lead to an understanding of thermal
acclimation, for instance acclimation must modify the
factors associated with heat damage. With this point
in mind the work described on recovery from sublethal
heat treatments was carried out.

The split-dose experiments were attempted following the rationale of radiation work, much of which has been carried out on insects, see Rockstein & Miquel (1973). The radiation work has shown that two types of damage occur in insects. Acute damage, caused by an $L.D._{50}$ exposure is clearly repairable, for Baxter & Blair (1969) show even over-recovery may occur, such that higher doses are required to produce an $L.D._{.50}$ in Drosophila recovered from an earlier exposure, as compared with untreated controls. However, irradiation exposure, even at sublethal doses, is life shortening in insects. This point, together with the fact that repeated insults are additive in their effect, suggests this damage cannot be fully repaired. Acute radiation injury is thought to be cytoplasmic whereas the life-shortening injury is chromosomal (Baxter & Blair, 1969).

It has earlier been shown, in this stock of flies, that flies recovering from an acute heat stress ($L.D._{50}$) have a life expectancy just as long as unheated control flies of the same age, Davison (1970). In this respect then acute heat injury differs from irradiation injury, for heat damage once repaired, is not accumulative. This point has also been made for comparison of cold-injury and irradiation damage in cultured cell lines,

Kvuur et al., (1972).

These points support the hypothesis that heat damage is cytoplasmic rather than chromosomal, Bowler et al., (1973).

The clearest point emerging from the split-dose experiments is that the effects of the separate heat treatments are merely additive, unless separated by 6 hours or more. In the latter case sufficient repair occurs during the period at 24°C for the second dose to cause less mortality than the control dose applied at a single treatment.

No evidence is obtained which suggests that the first heat dose induces thermal resistance making the flies more thermo tolerant when exposed to the second dose. For in such a case one would expect the shorter period split-doses to be less effective than the longer period split-doses. Such induced thermotolerance was observed by Gerner & Schneider (1975) in Hela cells. Prior exposure to 44°C for 1 hour followed by a 2 hour period 37°C resulted in increased thermal tolerance when subsequently exposed to 44°C again. This increase in tolerance was time dependent at 37°C, reaching a plateau after about 2 hours. Gerner & Schneider however found the cells did not develop this thermotolerance if placed

at 0°C, a temperature which inhibits Hela cell metabolism.

It might also be concluded that the cellular repair mechanisms, whatever they are, are part of normal cellular processes, for if they were not, the split-dose heated flies might be expected to show a higher mortality than the control single treatment flies. The reasoning being that if the repair mechanism is only invoked after the damage caused by the first heating,

it would have been operant at the time of the second heat exposure in the split-dose flies. This being so it is reasonable to suppose it would then be liable to be damaged, thus further impairing repair and recovery. Such damage to any special repair mechanism might be supposed to increase mortality. It was clearly not so.

The work of Gerner & Schneider (1975) discussed above, as well as that by Webb, et al. (1976) showing that recovery from irradiation damage in Drosophila is also temperature dependent, suggests that the study of temperature dependence of repair of heat damage might be informative.

Some initial work on this problem was carried out by Davison (1970) and reported by Davison & Bowler (1971). These workers reported that the flight muscle sarcosome makes a good model for studying heat death, for they

showed that the functional efficiency of sarcosomes has the same thermal sensitivity to <u>in vivo</u> heatings as does the whole organism. That is, this system fulfills the criterion laid down by Read (1967), this being that the recorded deleterious change in sarcosomal activity occurred under the same conditions which caused organism heat death. Furthermore they report that recovery from sublethal (L.D $_0$) and lethal (L.D.$_{50}$) exposure of blowflies requires between 2 and 3 days at 24°C. At the end of this time the flies had a normal capacity for flight and isolated flight muscle sarcosomes had the same QO_2 values and RCl and ADP : 0 ratios as did sarcosomes from control unheated flies.

The results shown in Table (4.2) agree closely with those of Davison & Bowler (1971), in that exposure of the whole organism to an L.D.$_0$ treatment seriously impairs sarcosomal efficiency. The substrate rate oxygen consumption was little affected but the state III respiration of sarcosomes from the experimental group was only about 60% of the control values. RCl was consequently reduced from a value above 2 to less than 1.5; in both reports ADP : 0 ratios were also substantially lowered, but they were measurable.

During recovery at 24°C, see Table (4.2), the data presented here are very similar to that described

by Davison & Bowler (1971). The extent of recovery of normal sarcosomal function is a little more rapid in that in the present work, normal sarcosomal respiratory efficiency is restored after 2 days, as compared with data from control flies, except for state III respiration which is below the control sarcosomal level. Davison & Bowler (1971), on the other hand report rather less repair occurring after 1 day at $24°C$, and the various parameters of sarcosomal efficiency (RC1 and ADP :0) were still just below the control group values after 2 days.

In the present experiments it is necessary to erect a set of control flies, transferred to $15°$, $24°$ or $34°C$ at the same time as the experimental heated flies. These controls are essential because it is not certain whether changes in sarcosomal efficiency would occur as a result of transfer to a new temperature regime, or even as a result of age.

As can be seen from Table (4.2) recovery is markedly temperature sensitive, with a Q_{10} of about 3 over the $15°$ to $34°C$ temperature range, if the assumption is accepted that recovery is complete after 6 days at $15°C$ and 1 day at $34°C$. This marked temperature sensitivity infers that active cellular mechanisms are

involved in the repair process. Whether damaged cellular
components are degraded and replaced by newly
synthesized ones is not established by this work but is
clearly an important next step to take.

The effect of maintenance temperature on
sarcosomal efficiency in control flies is also quite
interesting, for this may reveal some acclimation
effect on sarcosomal performance.

As can be seen from Table (4.2), transfer
to 15°C does cause a substantial rise in substrate rate
QO_2 from 32.83 $\pm$ 1.09 to 39.74 $\pm$ 2.12 µg AO.hr^{-1}.
mg prot.$^{-1}$ after 2 days. This rate declines until after
6 days at 15°C it becomes 26.85 $\pm$ 1.37.

A similar story holds for sarcosomal state III
respiration too, which from the 15°C flies shows an
insignificant rise after 2 days, but then progressively
falls to be only 60.86 $\pm$ 3.58 µg A O.hr^{-1}.mg prot.$^{-1}$
after 6 days at 15°C. This value is significantly less
than that obtained for 24°C maintained flies (p = 0.001).
In the flies placed at 35°C sarcosomal state III
respiration declines from the initial (after 8 hours)
high value 76.30 $\pm$ 5.42 to be 65.25 $\pm$ 4.60 µg A O.hr^{-1}.
mg prot.$^{-1}$, however this decline is not significant,
but the value is significantly lower than that obtained

from sarcosomes of flies maintained at 24°C.

This situation is the converse of what is normally expected from a compensation standpoint. That is it is usual for low temperature acclimation to result in a higher activity than warm acclimation. This is seen in the partially acclimated animals at 34°C where substrate rate declines after 1 day to be 27.55 $\pm$ 1.53 μg A O.hr^{-1}. mg prot.$^{-1}$.

Values for RCl decline slightly on transfer to a new temperature. It is perhaps significant that these values gradually improve at 15°C to reach 2.21 $\pm$ 0.07 after 6 days, this may reflect compensatory changes occurring in sarcosomal respiration. Small but largely insignificant changes in ADP : 0 also occur on transfer and maintenance at 15° and 34°C as compared with 24°C

However these data must be considered in relation to the in vitro effects of temperature on sarcosomal performance as reported by Davison (1971 a). He described the QO_2 temperature curve as having 3 phases. The first phase over a low temperature had relatively little effect on QO_2. In this phase RCl and ADP :0 ratios were maximal and oxidative phosphorylation was tightly coupled. Above the plateau sarcosomal respiration was uncoupled and QO_2 again increased with temperature. This result

was found with 15°, 24° and 34° acclimated flies. The QO_2 temperature curves differed in the position of the plateau phase, which moved to a higher temperature range with higher temperature acclimation. Furthermore the 34°C acclimated flies showed the highest and the 15°C acclimated flies the lowest QO_2 in the plateau phase.

However, if this data and the present data are compared, the state III QO_2 of 34°C acclimated flies, measured at 24°C, is not at the plateau level, but is in the first phase (see Davison, 1971a) it is for this reason the respiration rate of the 34°C acclimated flies is lower than those at 24°C, see Table (4.2). As can also be seen from Davison's work, sarcosomes from 15°C acclimated flies have a lower potential for respiration as compared with sarcosomes from 24°C and more particularly from 34°C acclimated animals.

He concluded, this reflected an acclimation induced effect either on respiratory chain or coupling enzyme in oxidative phosphorylation.

CHAPTER FIVE

THE EFFECT OF INCUBATION TEMPERATURE, AND REACTION TEMPERATURE ON BLOWFLY FLIGHT MUSCLE SARCOSOMAL RESPIRATION

Introduction

A large literature exists demonstrating temperature-dependence of respiration in many insect species, indeed Keister & Buch, in their 1964 review, reflect that this is perhaps the most overdemonstrated phenomenon in insect physiology.

Work by Newell and his co-workers cast some doubts on the universality of the Krogh 'normal' curve (Krogh, 1914) interpretation for all ectothermal animals, Newell (1966; 1967). From work largely on intertidal animals, these workers proposed that whole organism respiration could be separated into 'active' and 'resting' phases, and only in the former phase was respiration temperature-dependent, Newell & Northcroft (1967). They described the 'resting' phase respiration as being temperature-independent over a wide temperature range.

Tribe & Bowler (1968) criticized Newell's interpretation of his data on the following grounds. First it ignored the vast literature which clearly demonstrated temperature dependent respiration, for

example, in insects (Keister & Buck; 1964), crustaceans
(Vernberg, 1959), annelids (Magnum & Sassaman, 1969),
Amphibians (Bishop & Gordon, 1967), reptiles
(Aleksiuk, 1971). Newell & Walkey, (1966) and later
Newell & Pye, (1971) supported their whole organism
work by work on mitochondria from a variety of
sources. Again Newell showed wide temperature-
independent plateaus of mitochondrial respiration.
This work has also been criticised by Tribe & Bowler,
(1968) and Davies & Tribe, (1969) for not only the choice
of inappropriate substrates but also the use of a-
typical isolation procedures and in no case was the
functional state of the isolated mitochondria determined.

Nevertheless, the ideas proposed in this
and subsequent papers, have greatly stimulated research
into the thermal respiratory physiology of ectothermal
animals. This point has been aptly stated by Wieser
(1973) in a recent review article.

> "Ectothermic animals (sometimes at least) are
> neither at the mercy of environmental temperature
> nor are they simple homeostatic systems. They
> may, in fact, be considered as "multistable"
> systems ...   in which in answer to a change
> in environmental temperature that state of the
> system is called into action which is best suited
> to the prevailing or anticipated temperature
> regime".

The dominant role of mitochondria in cellular respiration had led Newell and his co-workers to seek an explanation, at the biochemical level, of the temperature-independent metabolism recorded in the intact organism (Newell & Walkey, 1966, Newell & Pye, 1971). Such an explanation is necessary for a priori reasoning would suggest metabolic rate should follow the Arrhenius law.

However, work on mitochondria, from a variety of tissue and organism sources, shows respiration (Kemp, Groot, & Reitsma, 1969; Smith, 1973c and Lee & Grear, 1974) and adenine nucleotide translocation (Heldt & Klingenberg, 1968), to be very sensitive to temperature. This early work provided a basis for subsequent studies of the effect of temperature on mitochondria. Most notable is the work by Raison and his co-workers on the influence of temperature on respiratory enzyme systems (Raison, 1973). These investigations were carried out on mitochondria isolated from homeothermic and poikilothermic animals (Lyons & Raison, 1970a; Kumamoto, Raison & Lyons, 1971), as well as chilling resistant and chilling sensitive plants (Lyons & Raison, 1970 b); measuring state III and state IV of succinate oxidation. The arrhenius plots of his results showed a discontinuity in both

respiratory rates at about 25° and 12° in homeothermic animals and chilling sensitive plants respectively. This relationship was linear for poikilothermic animals and chilling resistant plants in both respiratory states (see Raison, Lyons, Mehlhorn & Keith, 1971, McMurchie, Raison & Cairncross, 1973). From spin-label studies Raison and his colleagues came to the conclusion that change in E_a, i.e. breaks in Arrhenius plots, can be considered a direct indication of a temperature-induced phase change in the membrane lipids (Raison, et al 1971).

Similar studies have been carried out by Smith on mitochondria isolated from fish tissues. He reports that both state III & IV respiration show a linear Arrhenius plot from mitochondria from a variety of fish. He too describes non-linear plots for mammalian mitochondria respiration (Smith, 1973 c).

Less attention has been paid to insect mitochondria, but the earlier work of Davison (1971 a), using the same stock of blowflies also shows a marked temperature sensitivity of sarcosomal respiration. Davison (1971 a) reported the existence of a plateau, where temperature has little effect on respiration, this was followed by a sharp increase with increasing temperature. Davies

& Tribe (1969), on the other hand, found that oxygen consumption of isolated blowfly flight muscle sarcosomes was temperature dependent and that there was no evidence of a plateau of temperature-independent respiration.

Much less attention has been focused on the effects of preincubation, at different temperatures, on subsequently determined respiratory performance.

The earlier work by Davison & Bowler, (1971) and similar data presented here (Chapter 3), shows that sarcosomal function is impaired following exposure of the fly to high lethal and sub-lethal temperatures. This begs the question of whether such temperature exposures act directly on the sarcosome or indirectly by a change in the cellular environment.

Little work on thermal preincubation of mitochondria has been carried out with the purpose of attempting to identify the lesions caused by heat damage. The fact that isolated mitochondria are susceptible to moderate temperatures is evident from the stress placed by many workers on maintaining mitochondria on ice during long assay periods, see remarks made by Slack, (1975). On the other hand, the preincubation of aliquots of mitochondria in the oxygen electrode reaction chamber, before the introduction of

substrates, has been used to obtain a better respiratory performance, Sacktor & Childress (1967), Hansford, (1975).

The following experiments are designed to compare the extent of heat damage caused following thermal preincubation in vitro with heat damage in vivo caused by lethal heat treatments. With this in mind flies acclimated at 15°, 24° and 34°C were used to determine whether changes occurred on sarcosomal thermal resistance, in parallel with changes in whole organism resistance occurring after such periods of acclimation.

Materials and Methods
Preparation of sarcosomes

Sarcosomes were isolated from 10 day old adult blowflies using the 'standard' procedure given in detail in Chapter (2).

Measurement of sarcosomal respiratory performance at different temperatures

Five oxygen electrodes were set up simultaneously at 5 temperatures in the range 5° to 37°C. The temperature of the water baths was maintained at the appropriate temperature to within $\pm$ 0.1°C, using

a 'Sunvic' relay and Jumo thermoregulator. Each

sarcosomal preparation was stored on ice prior to use,

and respiration was followed at a different temperature

in each oxygen electrode. Where possible the waterbath

temperatures were raised, and after a period of

stabilization, sarcosomal respiration was again followed

at the new temperature. In no case however were

sarcosomes used later than 60 mins. after extraction.

The data collected from work at thirteen different

temperatures is given in Table (5.1) and Fig. (5.1),

and were derived from five separate sarcosomal

preparations.

Oxygen consumption (QO_2), ADP : 0 and RCl

were determined using the "standard" reaction medium,

and following the same procedure as given in Chapter

(2). 33 mM α-glycerophosphate was used as substrate

and oxidation followed in the presence and absence of

0.5 μ mole ADP.

The oxygen tension of the reaction medium was

obtained from data presented by Davison, (1970).

Preincubation of sarcosomes:

The blowflies used in this series of experiments

were reared at 24°C and at eclosion were placed at

15°, 24° or 34°C to acclimate for 10 days. Sarcosomal

preparations were then made using the standard procedure as described in Chapter (2).

Preparations were divided into equal volumes, and one aliquot placed on ice and the other pipetted into a glass test tube (diameter 1.5 cm, length 10 cm), previously equilibrated at one of the following temperatures : 30°, 31°, 33°, 35°, 36° or 41°C. These water bath temperatures were maintained to within $\pm$ 0.1°C using a 'Sunvic' control and a Jumo thermo-regulator.

Sarcosomes from flies acclimated to 24°C were preincubated at 30°, 31°, 33°, 35°, 36° and 41° for varying periods of time, and in all cases but the last temperature the data shown in Tables (5.3 - 5.6) is from two separate preparations made for each temperature, except for preincubations at 31° and 33°, where each preparation was divided into 3 equal parts. Two of these parts were placed at the experimental temperature, and the third at 0°C to serve as a control for both experimental temperatures.

Sarcosomes from flies acclimated at 15° and 34°C were exposed to 36°C only. Four experimental and two control preparations were used for each acclimated group.

In all cases the effect of the preincubation

was determined by following respiratory rates at 24°C,

using 33 mM α-glycerophosphate as substrate in the

presence and absence of 0.5 μ mole ADP. 50 μl

samples of the preparation were withdrawn (after

shaking) for use in the oxygen electrode. Usually the

sampling alternated between the sarcosomes at the

experimental temperature and control sarcosomes held

on ice, in any event both the aliquots were sampled over

the same time period as is indicated in the results in

Tables (5.3 - 5.7).

Results:

A. Effect of reaction temperature on respiratory
performance

It must first be emphasized that the data presented

in this section are obtained from flies reared and

maintained throughout at 24°C. They are thus acclimated

at 24°C. Furthermore α-glycerophosphate was the only

substrate used in this series of experiments.

The complete data, showing the effect of reaction

temperature on QO_2 in state III and substrate rate

oxidation, RCl, and ADP : O, are given in Table (5.1).

The effect of reaction temperature on QO_2 is also shown

in the form of an Arrhenius plot in Fig. (5.1). For

Table 5.1

Effect of the reaction temperature on the respiratory performance of isolated blowfly flight muscle sarcosomes.

Sarcosomes were isolated using the "standard" method described in Chapter (2). Reaction conditions, see Table 4.2. The mean values shown were obtained from 5 separate preparations.

n. m = not measurable

-

Table 5.1

Effect of reaction temperature on isolated flight musdle sarcosomes

| Temp. °C | QO_2 (ug AO. hr^{-1}. mg prot.$^{-1}$) | | RCI | ADP : O |
	Substrate-rate	ADP-stimulated rate (State III)		
5	11.36 ± 1.30	11.36 ± 1.30	1.00	n.m.
8	15.18 ± 0.94	19.85 ± 1.30	1.31 ± 0.05	1.03 ± 0.04
10.5	22.86 ± 2.81	30.38 ± 2.93	1.33 ± 0.19	1.08 ± 0.12
13	23.61 ± 1.44	35.12 ± 2.69	1.49 ± 0.12	1.23 ± 0.08
15	23.62 ± 1.34	35.94 ± 1.25	1.54 ± 0.02	1.28 ± 0.04
18	23.73 ± 0.90	42.69 ± 2.78	1.82 ± 0.17	1.45 ± 0.10

21	26.49 ± 1.96	52.10 ± 2.45	1.97 ± 0.20	1.65 ± 0.06
24	27.84 ± 1.73	64.02 ± 5.07	2.30 ± 0.12	1.76 ± 0.03
27	36.13 ± 3.13	85.87 ± 4.58	2.46 ± 0.26	1.78 ± 0.05
29	45.51 ± 2.64	91.80 ± 7.50	2.07 ± 0.22	1.60 ± 0.12
31	48.19 ± 4.57	86.40 ± 7.79	1.89 ± 0.34	1.24 ± 0.04
34	63.63 ± 4.24	102.81 ± 13.27	1.61 ± 0.17	n.m.
37	77.30 ± 2.78	121.29 ± 7.81	1.56 ± 0.07	n.m.

Table 5.2

Changes in the Q_{10} values and
activation energy of isolated
blowfly flight muscle sarcosomes
respiring on α-glycerophophate.
Values are calculated from the
data presented in Table (5.1).

Table 5.2.

Temperature range °C	Respiration state	Q_{10}	Activation energy (kcal. mole^{-1})
5 - 10.5	- ADP	3.58	19.92
	+ ADP	5.93	27.76
10.5 - 24	- ADP	1.15	2.28
10.5 - 18	+ ADP	1.58	7.52
24 - 37	- ADP	2.22	14.00
18 - 27	+ ADP	2.16	13.27
27 - 37	+ ADP	1.41	4.67

convenience the effect of temperature on these various parameters will be discussed separately.

i) Substrate rate oxidation

It can be seen from Fig. (5.1), that the Arrhenius plot is non-linear and can be divided into three distinct phases, with 'breaks' at about $10°$ and $24°C$.

Phase 1 ($5°$ - $10°C$) The effect of temperature in this phase was characterised by a high value for E_a (19.92 Kcal. mole^{-1}), oxidation rates more than doubled between $5°$ and $10°C$ from 11.36 to 22.86 µg AO. hr^{-1}. mg prot.$^{-1}$. As can be seen from Table (5.2) this gave a high Q_{10} value in this temperature range of 3.58.

Phase 2 ($10°$ - $24°C$) This phase is characterised by the lowest E_a of only 2.28 kcl. mole^{-1}. Indeed very little rise in QO_2 is seen over this temperature range, it increased only by 5 ug AO from 22.86 to 27.84 µg AO. hr^{-1}. mg prot.$^{-1}$, a rise that is insignificant ($p > 0.05$) of 1.15. Thus in this temperature range substrate respiration is effectively temperature-independent.

Phase 3 This phase extended from $24°C$ to the upper

temperature studied, 37°C. The E_a for this temperature range is 14.00 kcal.mole^{-1}, and oxidation rate rises sharply from 27.84 to 77.3 µg AO.hr^{-1}. mg prot.$^{-1}$. Table (5.2) shows the calculated value for Q_{10} of 2.22, close to the predicted value from the 'Krogh' normal curve.

ii) <u>State III oxidation</u> <u>(ADP-stimulated respiration)</u>

The Arrhenius plot, in this case, can be divided into four distinct phases, with 'breaks' at about 10°, 18° and 27°C. (see Fig. 5.1).

<u>Phase 1</u>: (5° - 10°C) This phase, also, was characterised by a high value for E_a (27.76 kcal.mole^{-1}), oxidation rates nearly tripled between 5° and 10°C from 11.36 to 30.38 µg AO.hr^{-1}. mg prot.$^{-1}$ giving a very high Q_{10} value of 5.93.

<u>Phase 2</u>: (10° - 18°C) In this phase a low value for E_a (7.52 kcal.mole^{-1}) was observed. The increase in QO_2 over this temperature range was only by about 12 µg AO, from 30.38 to 42.69 µg AO.hr^{-1}. mg prot.$^{-1}$ The Q_{10} value calculated was 1.58.

<u>Phase 3</u>: (18° - 27°C) Over this temperature range oxidation

Fig. 5.1

An Arrhenius plot of the data presented
in Table (5.1).
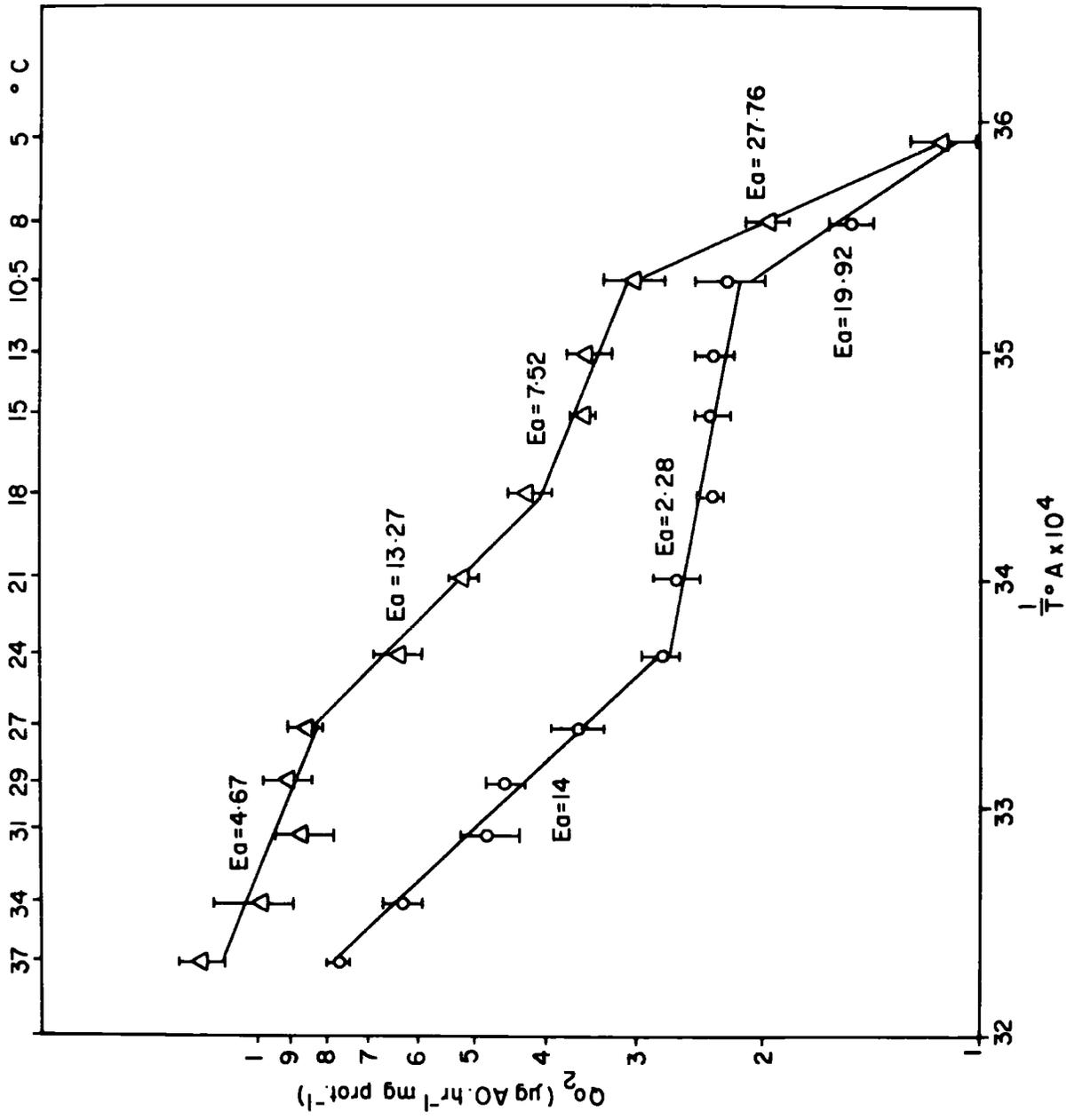
Ordinate   :   log scale representing oxidation

rates of α-glycerophosphate

expressed as ug AO.hr^{-1}.mg prot.$^{-1}$

Abscissa   :   Reciprocal of the absolute

temperature.

Values for Activation energy of the individual

phases, are given.   (See also Table 5.2)

Substrate rate respiration   O

State III respiration Δ

rates doubled from 42.69 to 85.87 μg AO.hr^{-1}.mg prot.$^{-1}$, and a relatively high E_a value was obtained (13.27 kcal.$mole^{-1}$), Q_{10} was 2.16.

Phase 4 (27° - 37°C) This phase was characterised by a low value for E_a (4.67 kcal.$mole^{-1}$). The increase in the oxidation rates was from 85.87 to 121.29 μg AO.hr^{-1}. mg prot.$^{-1}$, Q_{10} value of 1.41 was recorded.

Temperature dependence of RCl

Respiratory control is widely used as an index of the tightness of coupling of oxidative phosphorylation. As can be seen from Table (5.1) the highest values for RCl were obtained in the range 24 to 29°C and only here were values greater than 2 obtained. As can also be seen from Table (5.1), at 5°C the addition of ADP to the reaction medium failed to stimulate oxidation rates, and consequently an RCl of 1 was obtained. As reaction temperature was raised RCl increased up to 27°C where the highest value of 2.46 was obtained. Low values of RCl were obtained particularly at temperatures below 15°C, and the value of 1.82 for RCl obtained at 18°C is still significantly less than that obtained at 27°C ($p < 0.01$).

As the temperature was raised above 27°C, RCl progressively fell, and was significantly less than 2.46 at 34°C and 37°C when values of 1.61 and 1.56 were obtained respectively (p < 0.01) (see Fig. 5.1a)

ADP : 0

The theoretical maximum value for ADP : 0, when α-glycerophosphate is the substrate, is 2. As can be seen the highest values were obtained in the range 21° - 29°C when ADP : 0 were found greater than 1.6. These compare with the highest values given earlier in Chapter (3) (1.80), where the effect of reaction conditions on oxidative phosphorylation was reported. see Table (3.2).

It is not possible, using polarographic techniques to determine ADP : 0 when very low values for RCl are obtained. Consequently ADP : 0 were not measurable at 5°C or at 34° or 37°C. Indeed below 21°C the values calculated for ADP : 0 fell dramatically to be apparently about 1 at 10.5°C. (see Fig. 5.1a).

Fig. 5.1 a

Effect of the reaction temperature on

the respiratory performance of

isolated blowfly flight muscle sarcosomes.

(From data presented in Table 5.1)

Effect of reaction temp. on ADP:O ratio

Effect of reaction temp. on RCI
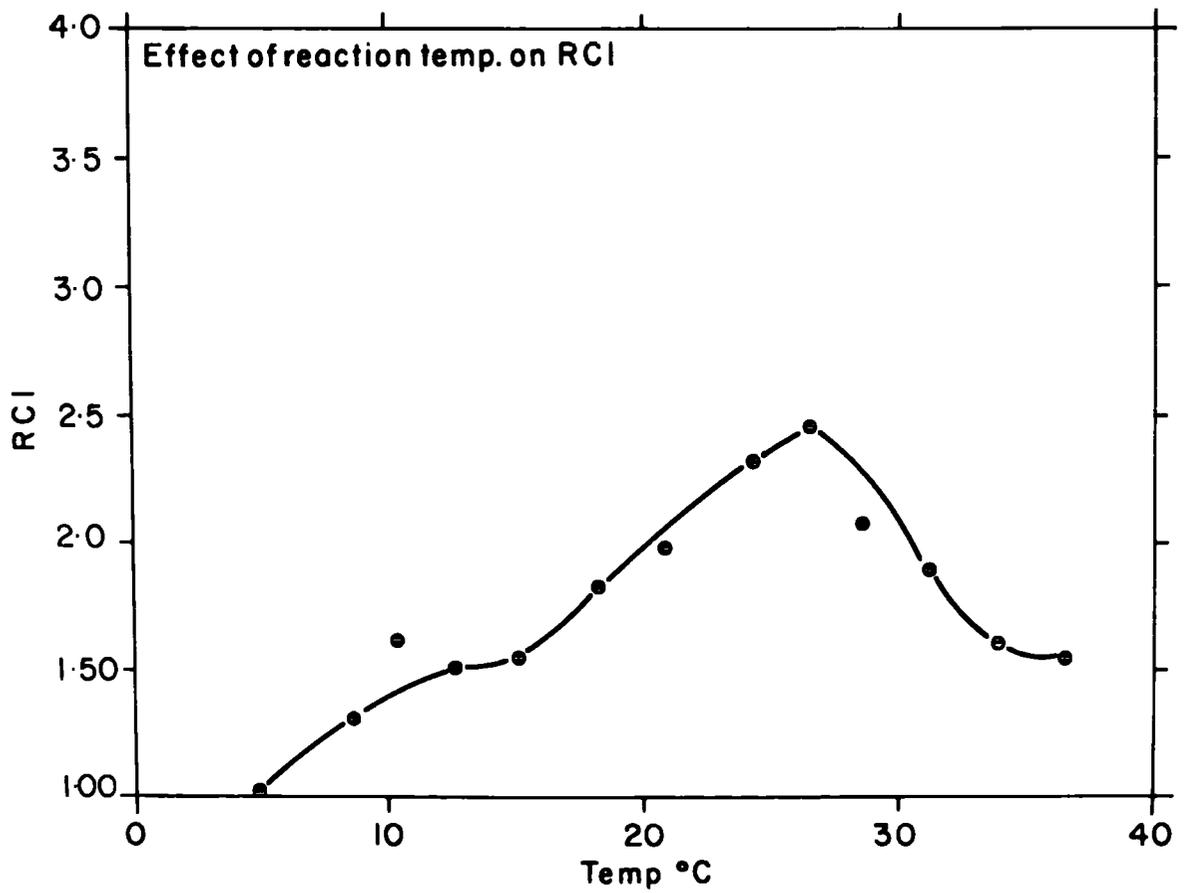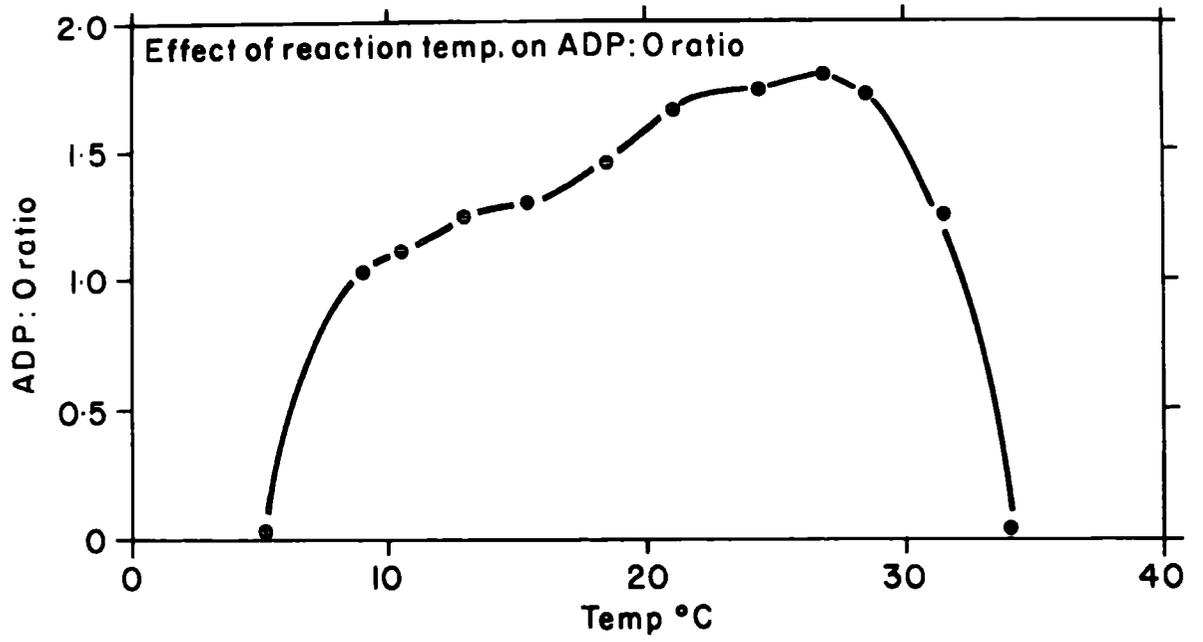
B)   The effect of thermal preincubation on
      sarcosomal respiratory performance

i.   Preincubation of Sarcosomes at 30°C

        As can be seen from Table (5.3) and Fig.(5.2),

substrate rate oxidation was little affected by

preincubation of sarcosomes at 30°C, as compared

with sarcosomes maintained at about 0 °c on ice. Only

the value obtained after 40 mins preincubation appeared

to be significantly less at 18.82 as compared with

22.46 μg AO.hr^{-1}. mg prot.$^{-1}$ in controls.

        Considering next ADP-stimulated respiration

(state III) there is no obvious trend in the effect of

preincubation at 0°C with the lowest value for QO_2

being obtained after 10 mins (42.4 μg AO.hr^{-1}.mg prot.$^{-1}$),

and the highest after 40 mins (47.98 μg AO.hr^{-1}

mg prot.$^{-1}$), however the value after 120 mins was still

45.96 μg AO.hr^{-1} mg prot.$^{-1}$, and it must be concluded

that ADP-stimulated rate oxidation is well maintained

during storage (preincubation) at 0°C. In the

experimental sarcosomes, preincubated at 30°C,

however state III rates were consistently, if not

significantly, higher than those obtained in the controls,

for up to 80 mins preincubation. A very significant

2 hrs. preincubation at 30°C, however, the value

being reduced to only 21.69 μg AO.hr^{-1}.mg prot.$^{-1}$,

Table 5.3
<hr>

Effect of preincubation at 30°C on the

subsequent respiratory performance of

isolated blowfly flight muscle sarcosomes.

(oxidative phosphorylation measured at 24°C).

Substrate : 33 mM α-glycerophosphate

Isolation : 'Standard' method (see Chapter 2)

Reaction conditions : See Table (4.2)

The mean values shown were obtained from

2 separate preparations. Range of values

is given in brackets.

QO_2 values are expressed as μg AO.hr^{-1}.mg prot.$^{-1}$.

Table 5.3

Incubation time (mins)	QO₂ Substrate-rate		QO₂ ADP-stimulated rate (State III)		RCI		ADP : O ratio	
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
5	18.38 (16.37–20.39)	18.74 (16.10–21.38)	46.94 (40.33–53.55)	46.35 (40.88–51.82)	2.55 (2.31–2.79)	2.47 (2.28–2.66)	1.87 (1.81–1.93)	1.97 (1.96–1.98)
10	19.51 (18.01–21.01)	19.72 (17.11–22.33)	49.59 (46.08–53.10)	42.40 (40.30–44.50)	2.53 (2.33–2.73)	2.15 (2.00–2.30)	1.91 (1.89–1.93)	1.83 (1.79–1.87)
20	19.86 (18.10–21.62)	20.95 (19.10–22.80)	48.09 (46.10–50.08)	44.89 (42.11–47.67)	2.42 (2.26–2.58)	2.14 (2.00–2.28)	1.95 (1.94–1.96)	1.86 (1.81–1.91)
40	18.82 (17.30–20.34)	22.46 (20.13–24.79)	48.09 (46.10–50.08)	47.98 (45.22–50.74)	2.56 (2.50–2.62)	2.14 (2.00–2.28)	1.89 (1.83–1.95)	1.77 (1.73–1.81)
60	23.00 (20.08–25.92)	22.46 (20.13–24.79)	51.23 (48.03–54.43)	46.96 (45.00–48.92)	2.23 (2.12–2.34)	2.10 (2.00–2.20)	1.73 (1.70–1.76)	1.67 (1.60–1.74)
80	23.00 (20.08–25.92)	21.35 (20.18–22.52)	48.09 (45.33–50.85)	45.99 (43.11–48.87)	2.09 (1.89–2.29)	2.15 (2.10–2.20)	1.63 (1.61–1.65)	1.67 (1.59–1.75)
120	19.72 (18.80–20.64)	21.35 (20.18–22.52)	21.69 (19.66–23.72)	45.96 (43.31–48.61)	1.10 (1.02–1.18)	2.15 (2.10–2.20)	n.m.	1.67 (1.60–1.74)

Fig. 5.2

Effect of preincubation at 30°C on the
respiratory performance of isolated
blowfly flight muscle sarcosomes.
Oxidative phosphorylation was
measured at 24°C.
(From data presented in Table 5.3)

	Control	experimental
Substrate - rate	O	●
ADP-stimulated rate	Δ	▲

30°C (24° acclimated)

QO2 (μg Ao. hr⁻¹. mg prot.⁻¹)

Time (mins)
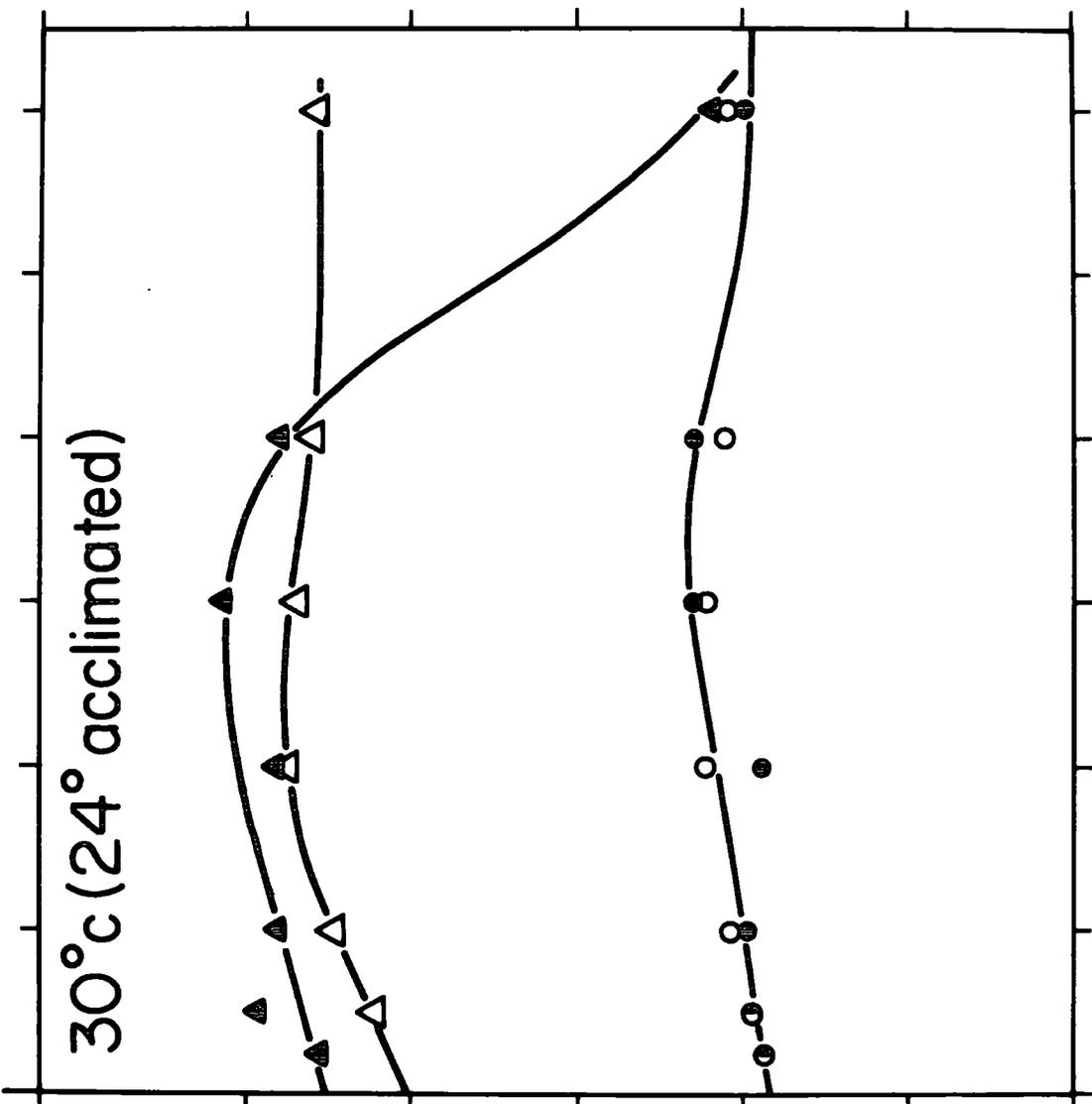
a reduction of more than 50% as compared with control
values.

In consequence, RCl showed differences between
control and experimental sarcosomes. Higher values
for RCl were obtained between 10 and 40 mins
preincubation for experimental as compared to control
sarcosomes, as can be seen from comparison of the
range of values obtained. Longer preincubation times
however, resulted in a fall in values of RCl, so that
after 2 hours preincubation the value was 1.10 for
experimental and 2.15 for control sarcosomal,
respectively.

ADP : 0 ratio obtained for control sarcosom̲e̲s
decreased over the first 60 mins from 1.97 to 1.67,
however this value was found to be consistent over the
following 60 mins. In experimental sarcosomes
ADP : 0 ratio did not show a major difference as
compared with the control values. Nevertheless the
highest value of 1.95 was obtained after 20 mins
preincubation at this temperature, and the lo̲w̲est value
of 1.63 after 80 mins. After 120 mins. however
ADP : 0 ratio was not measurable.

ii. Preincubation of Sarcosomes at 31°C

As can be seen preincubation at 31°C gave rise to an increase in substrate rate oxidation. This is particularly noticable for preincubations of 10 mins and longer, where the differences are clearly significant as the range of values obtained, at each sample point, do not overlap. During the 80 min duration of the experiment the oxidation rates rose in control sarcosomes from 33.55 to 38.68 μg AO.hr^{-1}. mg prot.$^{-1}$, a rise that is probably not significant. The rise in substrate rate in the experimental sarcosomes during the preincubation at 31°C is greater than in controls. However, this rise is not a progressive one for oxidation rates, after 60 or 80 mins, are no greater than after say 20 mins preincubation, see Table (5.4).

State III respiration in the control sample of sarcosomes shows a trend towards increased rates with longer times spent at 0°C. The highest rate was found after 45 mins and was 98.76 (95.51 - 102.01) μg AO.hr^{-1}. mg prot.$^{-1}$, subsequently the measured rate was about 89.5 μg AO.hr^{-1}. mg prot.$^{-1}$. In the sarcosomes held at 31°C however the highest state III respiration was found up to about 20 mins when values of about 110 μg AO.hr^{-1}. mg prot.$^{-1}$ were obtained. Subsequently

Table 5.4.

Effect of preincubation at 31°C on the
respiratory performance of isolated
blowfly flight muscle sarcosomes.
Oxidative phosphorylation was measured
at 24°C, under the same conditions
described in Table (5.3).

Means are derived from 2 separate
experiments.

Table 5.4

Incubation time (mins)	QO_2				RCI		ADP : O ratio	
	Substrate-rate		ADP-stimulated rate (State III)					
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
5	39.76 (33.99-45.53)	33.55 (30.21-36.89)	112.92 (99.38-126.46)	76.25 (73.50-79.00)	2.84 (2.72-2.96)	2.27 (2.19-2.35)	1.98 (1.97-1.99)	1.93 (1.90-1.96)
10	42.94 (38.11-47.77)	33.55 (30.21-36.89)	114.51 (101.02-128.00)	79.30 (78.61-79.99)	2.67 (2.55-2.79)	2.36 (2.28-2.44)	1.80 (1.76-1.84)	1.77 (1.73-1.81)
15	42.94 (38.11-47.77)	31.23 (30.81-31.65)	104.97 (98.11-111.83)	71.84 (70.62-73.06)	2.44 (2.34-2.57)	2.30 (2.28-2.32)	1.65 (1.60-1.70)	1.83 (1.78-1.88)
20	47.71 (40.81-54.61)	35.92 (32.32-39.52)	111.33 (105.31-117.35)	76.52 (74.03-79.01)	2.33 (2.15-2.58)	2.13 (2.10-2.16)	1.98 (1.97-1.99)	1.73 (1.70-1.76)
25	44.26 (40.11-48.41)	35.92 (32.32-39.52)	101.17 (97.33-115.01)	79.65 (78.11-81.19)	2.29 (2.16-2.42)	2.22 (2.12-2.32)	1.71 (1.68-1.74)	1.95 (1.93-1.97)
30	47.42 (43.22-51.62)	35.92 (32.32-39.52)	102.75 (99.50-116.00)	79.65 (78.11-81.19)	2.17 (2.00-2.34)	2.22 (2.12-2.32)	1.93 (1.89-1.97)	1.68 (1.66-1.70)

35	1.93 (1.91-1.95)	1.61 (1.58-1.64)	2.28 (2.22-2.34)	2.07 (1.89-2.25)	90.10 (86.10-94.10)	98.01 (96.01-100.01)	39.52 (38.12-40.92)	47.42 (43.22-51.62)
40	1.81 (1.78-1.84)	1.81 (1.77-1.85)	2.29 (2.26-2.32)	2.00 (1.81-2.19)	86.94 (82.81-91.01)	101.17 (97.33-15.01)	37.94 (37.00-38.88)	50.58 (47.99-53.17)
45	1.91 (1.89-1.93)	1.76 (1.74-1.78)	2.46 (2.38-2.54)	1.84 (1.62-2.06)	98.76 (95.51-102.01)	93.27 (90.81-95.73)	40.12 (38.06-42.18)	50.58 (47.99-53.17)
50	1.80 (1.78-1.82)	1.42 (1.37-1.47)	2.32 (2.28-2.36)	1.60 (1.43-1.77)	89.50 (86.78-92.22)	75.88 (73.34-78.42)	38.58 (36.73-40.43)	47.44 (44.23-50.65)
60	1.78 (1.71-1.85)	1.66 (1.46-1.86)	2.32 (2.28-2.36)	1.25 (1.14-1.36)	89.66 (86.30-93.02)	63.23 (60.12-66.34)	38.68 (36.12-41.24)	50.88 (47.50-54.26)
80	1.78 (1.71-1.85)	1.22 (1.01-1.43)	2.32 (2.28-2.36)	1.29 (1.11-1.47)	89.66 (86.30-93.02)	63.23 (60.12-66.34)	38.68 (36.12-41.24)	49.00 (46.31-51.69)

Fig. 5.3
_____

Effect of preincubation at 31°C on the

respiratory performance of isolated

blowfly flight muscle sarcosomes.

Oxidative phosphorylation was measured

at 24°C, (From data presented in

Table 5.4).

	Control	experimental
Substrate-rate	O	⊖
ADP-stimulated rate	Δ	▲
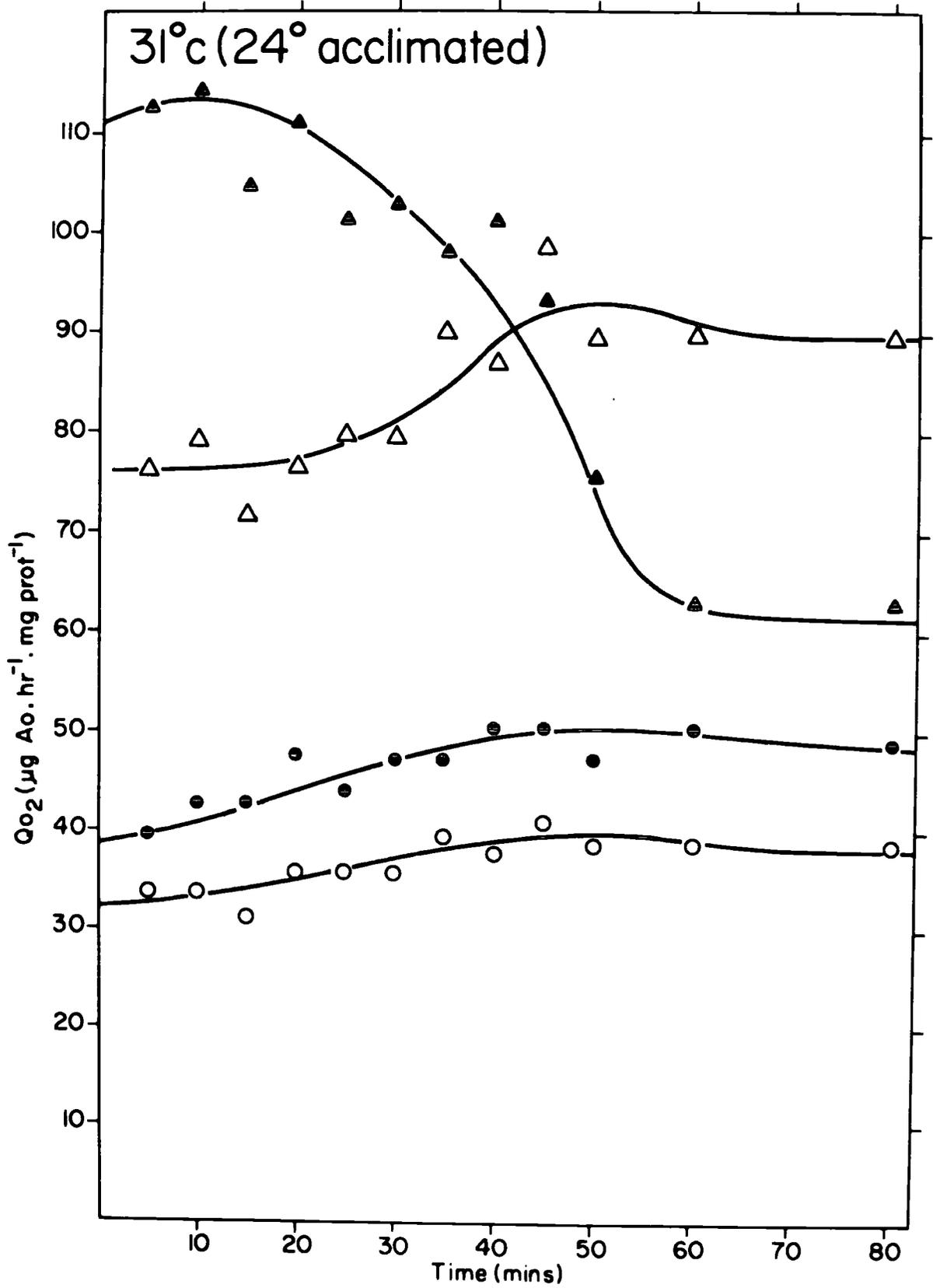
there was a decline in state III respiration and values

of only 63 µg AO.hr^{-1}.mg prot.$^{-1}$ were found after 60

and 80 mins at 31°C.

The measured values of RCl in the control group

are very similar throughout the 80 minute sampling

period, indeed the extreme values obtained are 2.1

and 2.54, the mean values vary only between 2.13 and

2.46. No obvious trend exists.

In the experimental sarcosomes, held at 31°C,

however the highest mean value for RCl, 2.48, is

obtained after 5 mins. Thereafter, RCl values fall

progressively to be only about 1.3 at the longest

preincubation times. It is clear however that the mean

RCl obtained for the experimental sracosomes is

significantly higher up to 10 mins preincubation, than

in the control sarcosomes.

No trend is apparent in the values obtained

for ADP : 0 in the control sarcosomes, and the mean

values obtained ranged between 1.68 (after 30 mins on

ice) to 1.95 (after 25 mins on ice).

In comparing the values obtained for ADP : 0

for the experimental sarcosomes, no trend is apparent,

and only after the longer exposure times, particularly

80 mins, did the value fall significantly to 1.22.

iii.   Preincubation of sarcosomes at 33°C

As can be seen from Table (5.5) and Fig. (5.4) preincubation at 33° caused an increase in substrate rate oxidation as compared with sarcosomes maintained on ice.   This increase was seen already after 5 min preincubation, where a value of 41.10 μgAO.hr^{-1}. mg prot.$^{-1}$ was obtained.   This value is probably significantly higher than that obtained from control sarcosomes, which was 33.55 μgAO.hr^{-1}.mg prot.$^{-1}$, as the two ranges of values obtained are non-overlapping. The rise in substrate rate oxidation of sarcosomes preincubated at 33°C is greater than in control sarcosomes, and is a progressive one reaching 51.21 μgAO.hr^{-1}. mg prot.$^{-1}$, with the exception of the value obtained after 40 min. preincubation, see Table (5.5).

ADP-stimulated respiration behaved very similar to that described for 31° preincubation. QO_2 values were higher for the experimental as compared with the control sarcosomes up to about 35 mins preincubation. With the values for the experimental sarcosomes being about 100 μg AO.hr^{-1}.mg prot.$^{-1}$, in contrast to values of 70 - 80 μg AO.hr^{-1}.mg prot.$^{-1}$, for control sarcosomes. Thereafter the QO_2 of experimental sarcosomes fell to be less than that of controls, which increased, during the next 45 mins at 33°C.

Table 5.5

Effect of preincubation at $33^{\circ}C$ on the
respiratory performance of isolated
blowfly flight muscle sarcosomes.
Oxidative phosphorylation was measured
at $24^{\circ}C$, under the same conditions
described in Table (5.3).

The mean values shown were derived from
2 separate experiments.

Table 5.5

Incubation time (mins)	QO$_2$				RCI		ADP : O ratio	
	Substrate-rate		ADP-stimulated rate (State III)					
	Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
5	41.10 (38.11-44.09)	33.55 (32.00-35.10)	105.91 (100.31-111.51)	76.25 (74.40-78.10)	2.58 (2.44-2.72)	2.27 (2.21-2.33)	1.93 (1.91-1.95)	1.93 (1.90-1.96)
10	42.68 (40.77-44.59)	33.55 (32.00-35.10)	104.33 (101.34-107.32)	79.30 (76.33-82.27)	2.44 (2.38-2.50)	2.36 (2.30-2.42)	1.66 (1.60-1.72)	1.77 (1.73-1.81)
15	44.26 (41.31-47.21)	31.23 (30.89-31.57)	107.49 (105.91-109.07)	71.84 (70.38-73.30)	2.43 (2.35-2.51)	2.30 (2.28-2.32)	1.86 (1.78-1.94)	1.83 (1.79-1.87)
20	44.26 (41.16-47.36)	35.92 (34.01-38.83)	104.33 (100.32-108.34)	76.52 (72.81-80.23)	2.36 (2.30-2.42)	2.13 (2.11-2.15)	1.66 (1.60-1.72)	1.73 (1.68-1.78)
25	48.01 (46.08-49.94)	35.92 (33.81-38.03)	104.02 (100.11-107.93)	79.65 (78.13-81.17)	2.17 (2.00-2.34)	2.22 (2.20-2.24)	1.73 (1.68-1.78)	1.95 (1.93-1.97)
30	46.41 (45.00-47.82)	35.92 (32.31-39.53)	104.02 (99.22-108.82)	79.65 (78.13-81.17)	2.24 (2.11-2.37)	2.22 (2.20-2.24)	1.55 (1.53-1.57)	1.68 (1.66-1.70)

35	47.42 (46.11-48.73)	39.52 (35.99-43.05)	101.17 (97.38-104.96)	90.10 (86.88-93.32)	2.13 (2.00-2.26)	2.28 (2.24-2.32)	1.81 (1.79-1.83)	1.93 (1.91-1.95)
40	44.26 (42.01-46.51)	37.94 (35.88-40.00)	99.59 (95.18-104.00)	86.94 (82.33-91.55)	2.25 (2.00-2.50)	2.29 (2.26-2.32)	1.49 (1.41-1.57)	1.81 (1.78-1.84)
45	49.92 (47.88-51.96)	40.12 (38.12-42.12)	91.78 (88.08-95.48)	98.76 (97.52-100.00)	1.84 (1.80-1.88)	2.46 (2.41-2.51)	1.72 (1.66-1.78)	1.91 (1.89-1.93)
50	51.53 (49.33-53.73)	38.58 (35.79-41.37)	90.17 (86.18-94.16)	89.50 (87.81-91.19)	1.75 (1.70-1.80)	2.32 (2.30-2.34)	1.58 (1.55-1.61)	1.80 (1.78-1.82)
60	52.81 (50.18-55.44)	38.68 (36.10-41.26)	84.81 (80.11-89.51)	89.66 (87.88-91.44)	1.66 (1.60-1.72)	2.32 (2.30-2.34)	1.59 (1.55-1.63)	1.78 (1.71-1.85)
80	51.21 (50.11-52.31)	38.68 (36.10-41.26)	75.21 (71.44-78.98)	89.66 (87.88-91.44)	1.47 (1.35-1.59)	2.32 (2.30-2.34)	1.34 (1.30-1.38)	1.78 (1.71-1.85)

Fig. 5.4

Effect of preincubation at $33\,^{\circ}$C on the

respiratory performance of isolated

blowfly flight muscle sarcosomes .

Oxidative phosphorylation was measured

at $24\,^{\circ}$C, (From data presented in

Table 5.5)

	Control	experimental
Substrate-rate	O	●
ADP-stimulated rate	△	▲

33°c (24° acclimated)
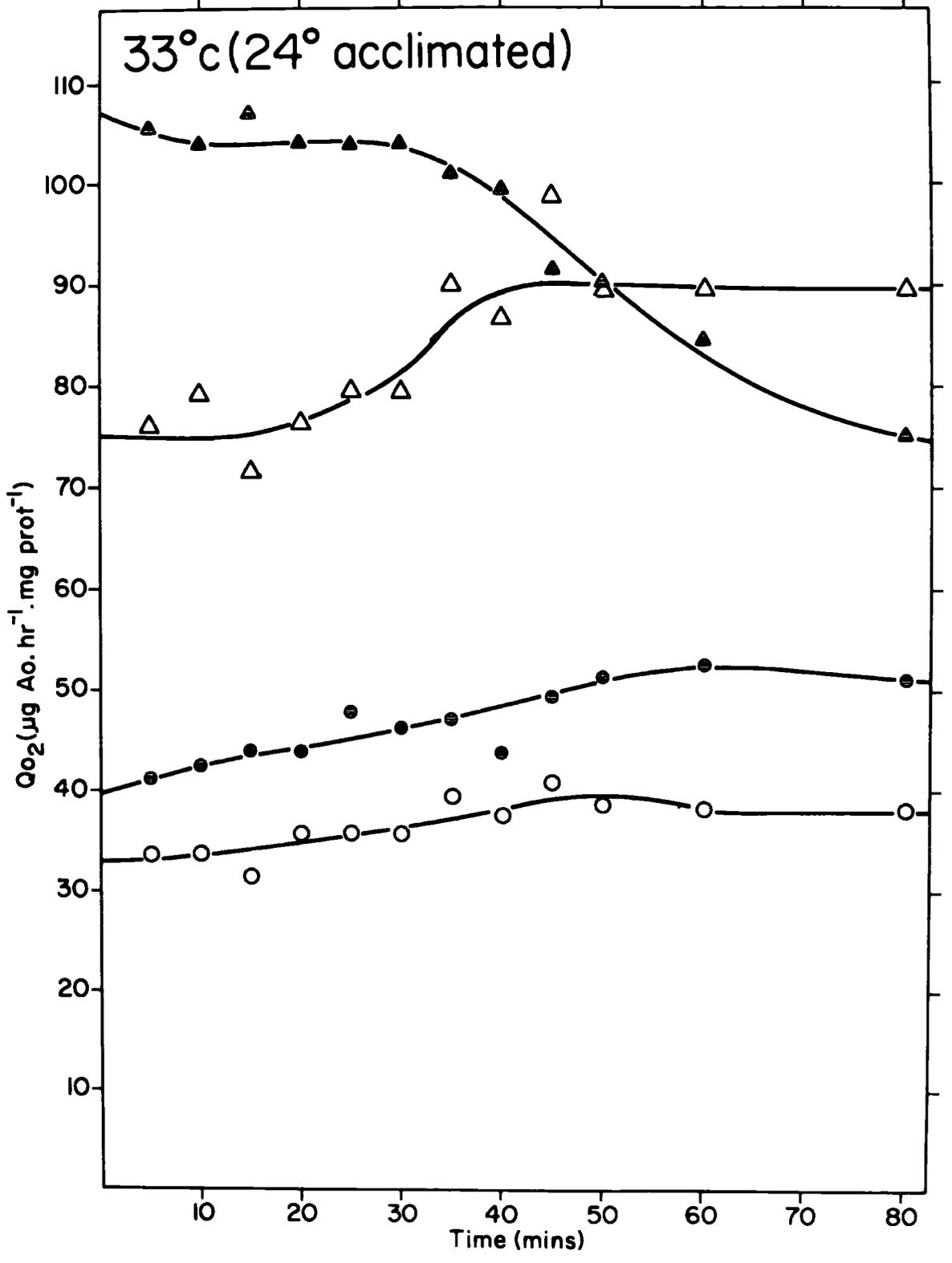
The measured values of RCl in the experimental sarcosomes, held at 33°C, were higher than those of the control sarcosomes up to 20 mins, and the highest value (2.58) was found after 5 mins preincubation. Subsequently there was a progressive fall in RCl values, which reached 1.47 after 80 mins.

The effect of preincubation of sarcosomes at 33°C on ADP : O ratio is similar to that observed at 31°C. No trend is apparent; and only after 80 mins a significant fall to 1.34 was observed.

iv.   Preincubation of sarcosomes at 35°C

As can be seen from Table (5.6), preincubation at 35°C had no consistent nor dramatic effect on substrate rate oxidation. Indeed the values obtained for the experimental sarcosomes are probably not different from the control sarcosomes held on ice.

State III respiration in sarcosomes held at 35°C did not show a trend towards increased rates as seen with previously described temperatures (31° & 33°C). Instead a progressive fall in state III respiration was observed, the values obtained of preincubation times of 30 mins and later, are probably significantly lower than the control values for the ranges of values obtained are non overlapping. After 60 mins state III respiration was

Table 5.6

_____

Effect of preincubation at 35°, 36° & 41°C,

on the respiratory performance of isolated

blowfly flight muscle sarcosomes. Oxidative

phosphorylation was measured at 24°C, under

the same conditions described in Table (5.3).

The mean values were obtained from two

separate preparations, except for 41°C, where

a single preparation has been used.

Table 5.6

Incubation temp °C	Incubation time (mins)	QO₂				RCI		ADP : O ratio	
		Substrate-rate		ADP-stimulated rate (State III)					
		Exp.	Control	Exp.	Control	Exp.	Control	Exp.	Control
35°	5	29.01 (26.33-31.69)	26.77 (25.58-27.96)	62.53 (55.82-69.40)	62.53 (59.35-65.71)	2.16 (2.12-2.19)	2.34 (2.32-2.35)	1.88 (1.83-1.93)	1.76 (1.69-1.83)
	15	31.13 (28.90-33.36)	26.60 (25.58-27.62)	58.40 (49.42-68.39)	64.06 (59.09-69.33)	1.88 (1.71-2.05)	2.41 (2.31-2.51)	1.89 (1.83-1.95)	1.83 (1.76-1.90)
	20	28.77 (27.66-29.88)	31.00 (29.25-32.75)	57.05 (54.21-59.76)	64.53 (58.50-70.09)	1.98 (1.96-2.00)	2.08 (2.02-2.14)	1.88 (1.79-1.97)	1.71 (1.59-1.83)
	30	29.01 (27.23-30.78)	31.25 (28.68-33.82)	53.60 (48.74-58.79)	67.01 (60.23-73.73)	1.85 (1.79-1.91)	2.14 (2.10-2.18)	1.81 (1.79-1.83)	1.88 (1.86-1.90)
	35	29.18 (26.96-31.40)	26.60 (25.34-27.86)	44.71 (40.44-48.98)	66.04 (61.58-70.49)	1.53 (1.50-1.56)	2.48 (2.43-2.53)	1.78 (1.74-1.82)	1.83 (1.78-1.88)
	45	29.65 (29.09-30.21)	31.00 (28.82-33.18)	43.05 (41.02-45.01)	71.60 (57.93-85.27)	1.45 (1.41-1.49)	2.29 (2.01-2.57)	1.66 (1.60-1.72)	1.70 (1.58-1.82)

|									
36°	60	31.65 (28.28-35.02)	31.25 (28.68-33.82)	38.43 (29.69-47.98)	71.47 (63.96-79.48)	1.21 (1.05-1.37)	2.29 (2.23-2.35)	1.47 (1.32-1.62)	1.61 (1.55-1.67)
	10	31.51 (26.72-36.50)	26.69 (26.23-27.14)	69.77 (62.53-77.01)	63.30 (59.02-67.58)	2.21 (2.34-2.11)	2.37 (2.25-2.49)	1.84 (1.75-1.93)	1.85 (1.79-1.91)
	20	30.45 (28.82-32.08)	29.01 (27.88-30.14)	56.05 (55.05-57.05)	64.53 (60.93-68.13)	1.84 (1.91-1.77)	2.22 (2.19-2.26)	1.61 (1.57-1.65)	1.71 (1.64-1.79)
	25	29.01 (26.33-31.70)	29.01 (27.88-30.14)	51.22 (44.44-58.00)	64.53 (60.93-68.13)	1.77 (1.70-1.83)	2.22 (2.19-2.26)	1.66 (1.57-1.76)	1.89 (1.84-1.95)
	40	30.44 (28.31-32.55)	31.00 (28.10-33.90)	35.79 (32.56-39.02)	66.12 (59.01-73.22)	1.18 (1.15.1.20)	2.13 (2.10-2.16)	1.41 (1.35-1.47)	1.72 (1.55-1.88)
41°	25	31.05	27.39	81.00	71.74	2.61	2.62	1.42	1.62
	7	31.36	35.92	34.69	93.97	1.11	2.62	n.m.	1.80

Fig. 5.5

Effect of preincubation at $35\,^{\circ}C$ on

the respiratory performance of

isolated blowfly flight muscle

sarcosomes. Oxidative phos-

phorylation was measured at

$24\,^{\circ}C$.

(From date presented in Table 5.6)

	Control	experimental
Substrate-rate	O	⊖
ADP-stimulated rate	▲	△
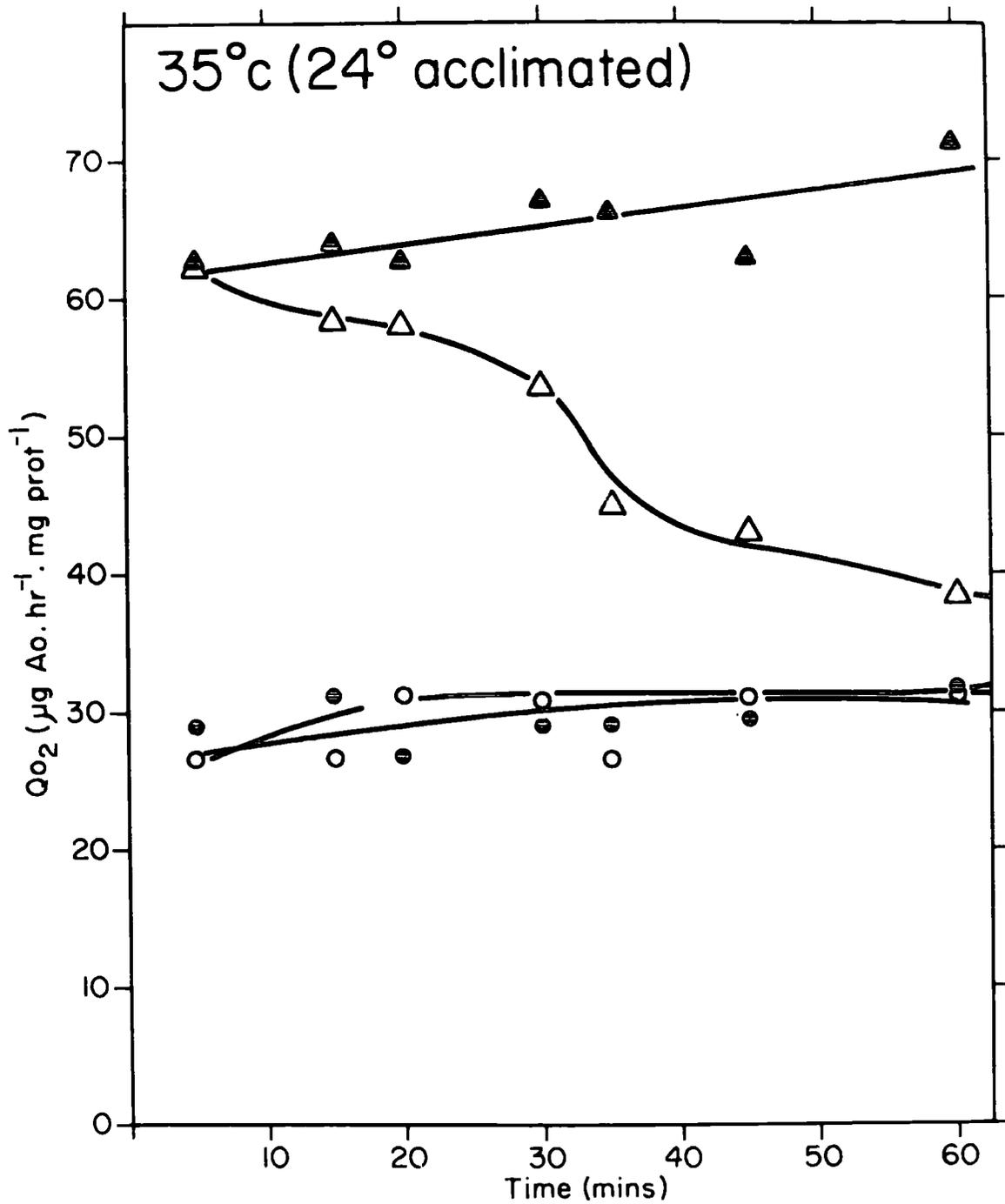
only 38.43 μg. AO. hr^{-1}. mg prot. $^{-1}$ in sarcosomes held at 35°C, which is approximately half the corresponding control value.

In sarcosomes kept on ice during the 60 mins duration of the experiment state III respiration showed a small increase from 62.53 to 71.47 μg AO. hr^{-1}. mg prot. $^{-1}$ see Table (5.6) and Fig. (5.5).

The measured mean values of RCl throughout the experiment were less in experimental sarcosomes than those obtained in control sarcosomes. The highest value (2.16) was obtained after 5 mins. preincubation but, with longer times at 35°, RCl showed a progressive decrease, and was only 1.21 after 60 mins. The mean RCl values of control sarcosomes ranged between 2.14 and 2.48 during the 60 min period on ice.

No trend is apparent in the ADP :0 ratios in experimental sarcosomes up to 45 mins preincubation at 35°C, where values ranging between 1.66 and 1.89 were obtained. These values are almost idential to those obtained in control sarcosomes. After 60 mins preincubation at 35°C ADP : 0 ratio declined to 1.47.

v. Preincubation of Sarcosomes at 36°C

As can be seen from Table (5.6) preincubation at 36°C has little effect on substrate rate QO_2 values,

indeed these data for the experimental sarcosomes are probably not significantly different from those of control sarcosomes on ice.

State III respiration in control sarcosomes was not significantly affected by 40 mins exposure at $0°C$. In the sarcosomes held at $36°C$ however the highest value $(69.77 \text{ }\mu g AO.hr^{-1}. \text{ mg prot.}^{-1})$ was obtained after 10 mins preincubation. Subsequently, there was a decline in state III respiration, and a value of only $35.79 \text{ }\mu g \text{ } AO.hr^{-1}.\text{mg prot.}^{-1}$ was found after 40 mins. (see Fig. 5.6).

The mean values of RCl in the control group are ranging between 2.13 and 2.37. In the experimental sarcosomes, held at $36°C$ however mean RCl values were throughout the experiment lower than the corresponding control values. The highest value (2.21) was obtained after 10 mins preincubation at $36°C$, which showed a progressive decline and reached 1.18 after 40 mins. The mean values for ADP : 0 were not significantly affected by 40 mins exposure at $0°C$. In contrast, the mean values for ADP : 0 for experimental sarcosomes at $36°C$ showed a significant decline, falling from 1.84 (1.75 - 1.98) after 10 mins to 1.41 (1.35 - 1.47) after 40 mins.

Fig. 5.6

Effect of preincubation at 36° and 41°C on the respiratory performance of isolated blowfly flight muscle sarcosomes. Oxidative phosphorylation was measured at 24°C. (From data presented in Table 5.6)

	Control	experimental
Substrate-rate	O	Δ
ADP-stimulated rate	●	▲
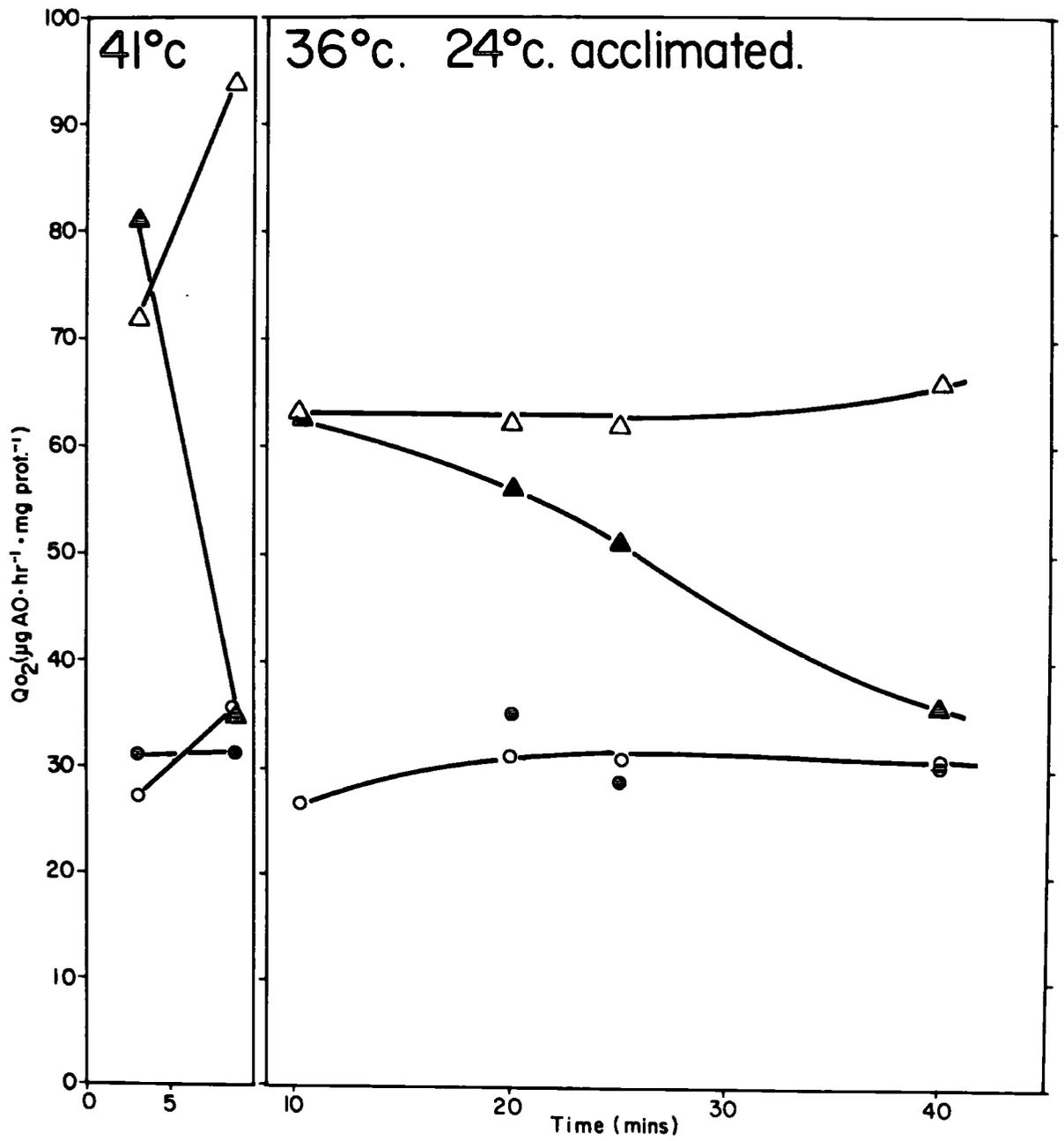
vi. Preincubation of sarcosomes at 41°C

Preincubation times at this temperature were short, only 2.5 or 7 mins, and had no significant effect on substrate rate oxidation. Although the duration of the experiment was relatively short, a dramatic fall in state III respiration was observed from 81.00 to 34.69 µg AO.hr^{-1}.mg prot. $^{-1}$ within 7 mins. As a result RCI fell from 2.61 to 1.11 and ADP : 0 ratio was not measurable after this time (see Table 5.6 and Fig. 5.6)

C) Effect of acclimation of animals to 15° or 34°C
    on response of sarcosomes to preincubation at 36°C

i. 15°C acclimated flies

Sarcosomes from 15° acclimated flies had a substrate rate oxidation unaffected by a 30 min exposure at 0°C. Exposure at 36°C did cause a fall in mean QO_2 from 38.54 to 32.9 µg AO.hr^{-1}.mg prot. $^{-1}$, but it is unlikely this is a significant decline in QO_2.

ADP-stimulated respiration was also unaffected in the control sarcosomes during a 30 min period at 0°C. In contrast, the experimental sarcosomes were markedly affected by exposure at 36°C. This is particularly evident after 20 mins exposure where the

mean QO_2 had fallen to be only 50% of the value for control sarcosomes. This decline continued and became only one third of that for control sarcosomes. (see Table 5.7 and Fig. 5.7).

This is reflected in the differences in RC1 obtained for control and experimental sarcosomes. For the former mean values of about 2.4 were obtained throughout the period of the experiment; whereas for the experimental sarcosomes RC1 values declined with time at $36°C$, being 2.03, 1.24 and 1 after 10, 20 and 30 mins exposure, respectively.

In the values obtained for ADP : O in control sarcosomes no trend is apparent; the values obtained were 1.84, 1.79 and 1.81 after 10, 20, and 30 mins on ice, respectively.

In the experimental sarcosomes ADP : O showed a fall with exposure time. The mean value obtained after 10 mins exposure (1.82) is not different from that obtained from control sarcosomes on ice. However, after 20 mins exposure the mean value obtained was only 1.58 and a further 10 min exposure resulted in a complete loss of demonstrable coupling.

ii.   34°C acclimated flies

As can be seen from Table (5.7) preincubation at 36°C gave rise to a small decrease in the QO_2 values of substrate rate oxidation, from 27.28 after 10 mins to 21.90 µg AO.hr^{-1}.mg prot.$^{-1}$ after 30 mins. However, these differences are not significant as the range of the values obtained at each sample point is overlapping.

Substrate rate oxidation in control sarcosomes increased from 26.91 to 30.10 µg AO.hr^{-1}.mg prot.$^{-1}$ during the 30 mins duration of the experiment, but again these values are not significantly different from each other.

State III respiration declined from 52.30 to only 21.90 µgAO . hr^{-1}.mg prot.$^{-1}$ in sarcosomes preincubated at 36°C. As can be clearly seen the latter value is identical to that obtained for substrate rate oxidation after the same period of time (30 mins), resulting, consequently in an RCI of only 1.

No trend is apparent in the values obtained for state III respiration in control sarcosomes. These values were similar throughout the experiment (see Fig. 5.7).

Table 5.7.

Effect of preincubation at $36°C$ on the
respiratory performance of sarcosomes
isolated from the flight muscle of blowflies
acclimated to $15°$ & $34°C$. Oxidative
phosphorylation was measured at $24°C$,
under the same conditions described in
Table (5.3).

The mean values are obtained from 4 and
2 separate preparations for experimentals
and controls, respectively.

Table 5.7

Acclim. temp. °C	Incub time (mins)	QO₂ Substrate-rate Exp.	QO₂ Substrate-rate Control	QO₂ ADP-stimulated rate (State III) Exp.	QO₂ ADP-stimulated rate (State III) Control	RCI Exp.	RCI Control	ADP : O ratio Exp.	ADP : O ratio Control
15°	10	38.54 (25.87-51.22)	38.81 (36.33-41.29)	78.09 (56.05-100.12)	94.11 (89.88-98.34)	2.03 (1.95-2.47)	2.42 (2.38-2.47)	1.82 (1.73-1.91)	1.84 (1.82-1.86)
	20	36.21 (31.04-47.09)	38.88 (37.00-40.76)	44.94 (37.62-58.86)	95.31 (94.33-96.29)	1.24 (1.16-1.30)	2.45 (2.36-2.55)	1.58 (1.51-1.79)	1.79 (1.75-1.83)
	30	32.90 (26.19-40.03)	40.12 (38.13-42.11)	32.90 (26.19-40.03)	96.00 (94.89-98.11)	1.00	2.39 (2.33-2.49)	n.m.	1.81 (1.77-1.85)
34°	10	27.28 (23.57-30.61)	26.91 (22.88-30.94)	52.30 (42.13-63.57)	75.16 (63.38-86.94)	1.91 (1.58-2.08)	2.79 (2.77-2.81)	1.70 (1.59-1.87)	1.77 (1.75-1.79)
	20	26.07 (19.96-28.90)	27.32 (25.13-29.51)	40.32 (33.26-45.76)	75.45 (68.86-82.04)	1.57 (1.20-1.89)	2.76 (2.74-2.78)	1.54 (1.37-1.73)	1.83 (1.79-1.87)
	30	21.90 (20.71-24.02)	30.10 (28.15-32.15)	21.90 (20.71-24.02)	76.00 (70.66-81.34)	1.00	2.52 (2.51-2.53)	n.m.	1.79 (1.75-1.83)

Fig. 5.7
_____

Effect of preincubation at $36°C$ on the

respiratory performance of sarcosomes

isolated from the flight muscle of

blowflies acclimated previously at

$15°$ or $34°C$ for 10 days.

(From data presented in Table 5.7)

Oxidative phosphorylation was measured

at $24°C$ under the conditions described in

Table (4.2) and was expressed as $\mu gAO.hr^{-1}$.

mg prot.$^{-1}$. Points represent mean values

of 4 separate preparations.

Data at 0time is the initial control values

	$15°C$-acclimated	$34°C$-acclimated
Substrate rate	O	⊕
ADP-stimulated rate	Δ	▲
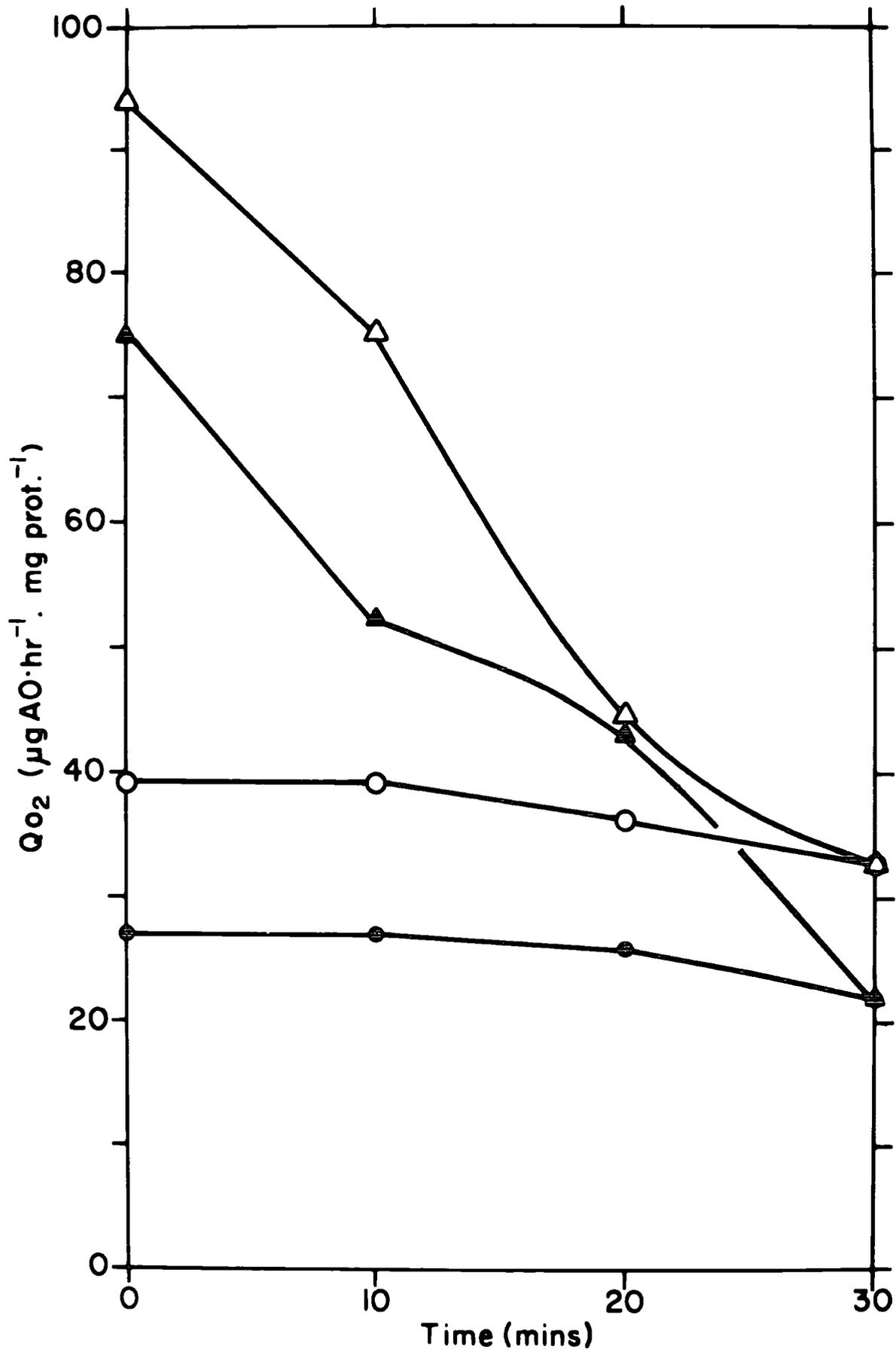
RCl values for control sarcosomes were found ranging between 2.52 and 2.79. The highest value was obtained after 10 mins period on ice, and the lowest after 30 mins. In the experimental sarcosomes, however, a 10 minute preincubation period resulted in RCl value of 1.91, which is significantly lower than the corresponding control value. After 20 mins the mean RCl was found to be only 1.57; however the range of the values is overlapping with that obtained after 10 mins preincubation, but after 30 mins RCl value of 1 was obtained.

No trend was obvious in ADP : O values for control sarcosomes. Throughout the experiment these values were similar, ranging between 1.77 and 1.83. In experimental sarcosomes, a fall in mean ADP :O values was observed from 1.70 to 1.54 after 20 minute preincubation at $36°C$. However, this difference is probably not a significant one. After 30 mins however, ADP : O was not measurable owing to the loss of respiratory control.

Discussion

The effect of reaction temperature on mitochondrial performance has been followed by many workers. Frequently, such studies have concerned determination of oxidation rates, presented as Arrhenius curves, e.g. Lyons & Raison (1970 a & b) Smith, (1973 c). However, attention has been drawn by Heldt & Klingenberg (1968) to low temperature sensitivity of mitochondrial adenine nucleotide translocase. Kemp, et al., (1969) concluded that it was indeed this translocation process that was rate limiting in oxidative phosphorylation in rat liver mitochondria in the temperature range 0 - 23°C.

The most interesting feature of Arrhenius plots of oxidation rates of mitochondria is that, in most cases, breaks, or discontinuities (as they are usually called) occur. These breaks yield distinct phases over different temperature ranges and consequently distinct activation energies, since these are calculated from the slopes of the different phases.

However, some differences of opinion are held concerning the causes of, and therefore interpretation of, "breaks" in Arrhenius curves. Raison (1973), and Kumamoto et al., (1971) clearly stated the currently

held views on the nature of "breaks" for membrane-bound enzymes.

In an authorative text, Dixon and Webb (1958) objected to the interpretation made by Sizer and Josephson (1942), that Arrhenius plots for the activity of crystalline enzymes, such as invertase, lipase, Trypsin and pepsin, show a discontinuity at $0°C$ owing to a phase change in the water which forms one of the reactants. The objection was on the grounds that a change in activation energy at $0°C$ was also observed with pancreatic lipase in a reaction mixture containing 36.5% glycerol. Drost-Hansen, (1972), on the other hand, attributed many of the thermal anomalies in enzyme kinetics to the alteration in the physical properties of vicinal water. It was the similarity between the temperature at which changes in activation energy are observed and the temperature at which changes are observed in the structure of water adjacent to an interface, that led him to this postulation. He stressed that these thermal anomalies occur at or near the same temperature for all systems regardless of the chemical nature of the substrates involved. The temperatures mentioned are $17°$, $28°$, $43°$ and $60°C$. He emphasized that changes in vicinal water must be superimposed upon the changes in the biological macro-

molecules, and these will undoubtedly be influenced by environmental changes. Raison and his co-workers believe that there is no doubt that "breaks" or true discontinuities in Arrhenius plots do exist. This conclusion is supported by the observation of large gaps between two non-intersecting straight lines at the transition temperature. Therefore, Kumamoto, et al., (1971) adopted, for the interpretation of these breaks, a theory which employs a phase change. They stated that this theory "can adequately accommodate all the data for temperature "breaks" in Arrhenius plots". Furthermore, they took it that this concept should cover a large number of systems, such as whole organisms, subcellular organelles and membranes, and others.

The two plateaus, observed in the substrate rate and state III respiration, at temperatures above the break at $10.5°$ (see Fig. 5.1) in part confirm the results reported by Davison (1971 a). In his R/T curves, Davison observed a plateau at the level of substrate respiration, which started at $24°$ and extended to $34°$. He did not observe the first sharp break, for his experimental temperature only ranged between $14°$ and $39°C$.

For α-glycerophosphate oxidation (substrate rate) the plateau extends to about $24°C$ before a second discontinuity occurs.

State III respiration gives a plot of a different nature; here the plateau extends only to about $18°C$, above which the slope of the curve increases (to 13.27 k cal. mole^{-1} from 7.52 k cal. mole^{-1}) to $27°C$ where a third discontinuity occurs. The divergence of the curves for substrate rate and state III oxidation over this temperature range results in increasing values being obtained for RCI as reaction temperature increases.

The increase in slope following a "break" in an Arrhenius plot is an interesting feature of these curves and requires an explanation; for it is more usual for the Ea of the slopes of the lines to be smaller as the progression occurs from lower to higher temperatures. Davison (1971 a), who also found this phenomenon, attributed it to high temperature uncoupling of oxidative phosphorylation. This may also be the case in the present study, for RCI values are seen to fall progressively as the temperature rises above $27°C$. At $34°$ and $37°C$ it was no longer possible to determine ADP : 0.

The data of Newell & Walkey, (1966) and
Newell & Northcroft, (1967), which show mitochondria
from a variety of sources, having a low, and
essentially temperature-independent respiration,
but which suddenly show a dramatic increase at a
high temperature, can probably be best explained in
the same way. For in certain cases at least, substrates
were provided which the mitochondria were not
normally permeable to. At high reaction temperatures,
membrane damage presumably occurred which allowed
the substrate to penetrate.

Few other reports exist showing this
phenomenon and it is most likely to be because the
studies have not continued to the higher reaction
temperatures, where mitochondrial membranes might
be damaged. Lee & Gear, (1974) in a comprehensive
study, carried out their studies on rat liver
mitochondria only to $36°C$, whereas Smith, (1973 c)
and Kemp et. al., (1969) confined themselves to an
even lower temperature range.

The cause or causes of the breaks in the
Arrhenius plots can only be a matter of speculation.
In simpler systems, non-linear plots have been
obtained for a wide variety of membrane-bound
enzymes. Most notable are the $Na^{+} - K^{+}$—ATPase

(Gruener & Avi-Dor, 1966 ; Bowler & Duncan, 1968;

Taniguchi & Iida, 1972; Tanaka & Teruya, 1973) and

Ca^{2+} - Mg^{2+}- ATPase (Charnock & Frankel, 1971;

Deamer, 1973).

A great deal of evidence is now available for

the dependence of membrane-bound enzymes on the

lipid component of the membrane for activity.

Fleischer, Brierley, Klouwen & Slautterback (1962)

have demonstrated such requirement in enzymes of

the electron transport system. Furthermore, many

such enzymes were found to show a partial or obligate

requirement for unsaturated fatty acids (Rothfield &

Finkelstein, 1968; Sekuzu, Jurtshuk & Green, 1963;

Cunningham & Hager, 1971). Proudlock, *et al.* in their experiments

on diet regulated yeasts, showed similar results on their

mitochondria (Proudlock, Haslam & Linnane, 1969).

Since such lipids are expected to change their

state with changing temperatures, one would expect this

to be reflected on the activity of membrane-bound

enzymes. Indeed, there is growing evidence for this

phenomenon. Several workers have established a close

correlation between the transition temperature of

enzyme activity and the transition temperature of the

molecular mobility parameter of a hydrophobic probe

(Raison, et al., 1971; Grisham & Barnett, 1973;

Rottem, Cirillo, DeKruyff, Shinitzky & Razin, 1973).

The effect of temperature on respiration of

mammalian and avian mitochondria usually gives rise

to non-linear Arrhenius plots, with one or more

discontinuities. Smith (1973 c) Lyons & Raison, (1970a),

Lenaz, Sechi, Parenti-Castelli, Landi & Bertoli (1972),

Roberts, Arine, Rochelle & Chaffee (1972), Kemp

et al., (1969), Lee & Grear, (1974). However, similar

systems from ectothermal animals have been reported

to yield linear Arrhenius plots, Smith, (1973 c) and

Lyons & Raison, (1970 a) for fish; but Pye (1973) and

Davison, (1971 a) report non-linear plots for frog and

insect mitochondria, respectively.

The interpretation offered by other workers of

the effect of temperature on their mitochondrial systems

are of interest. Smith, (1973 c), points out clearly

that Arrhenius plots of respiration using succinate and

glutamate are similar, and also that switching from

state III (adenine nucleotide translocase limited) to DNP

uncoupled respiration does not affect the plot. He

considers this may indicate that the effect of temperature

on the enzymes is not crucial. Raison et al., (1971)

reached much the same conclusion. Lee & Gear, (1974) also concluded that the discontinuities are caused by some process common to the entire respiratory chain, for they are common to both oxidation and phosphorylation, indeed the intact energy-transducing membrane is necessary. They describe some enzymes, malate dehydrogenase (matrix) and rotenone-insensitive NADH cyt.c reductase (outer membrane)gave linear Arrhenius plots.

Whether 'breaks are caused as a result of phase changes occurring in mitochondrial lipids, or a moiety of lipids, is problematical. Intrinsic membrane enzymes do usually give non-linear plots, but Lenaz et al., (1972) suggests caution be applied in interpretation, as they found the 'break' temperature was different for different enzymes' systems from beef heart mitochondria, and cytochrome oxidase showed no 'break' at all.

If 'breaks' are simply related to the fatty acid composition of phospholipids then one might expect more uniformity of response to temperature by these several enzyme systems. This is also true for blowfly sarcosomes as reported here, for the curves for state III and substrate rate oxidation are different. Lee &

G ear, (1974) also found divers effects of temperature on a variety of mitochondrial functions.

Another interesting feature of studies on the effect of temperature on membrane-bound enzymes is the fact that the intactness of the membrane is required for 'breaks' in the Arrhenius plots to be shown at any temperature. This has been demonstrated in sonicated rat liver mitochondria by Lee & G ear (1974). A similar effect was found in the presence of detergents or phospholipases (Taniguchi & Iida, 1972; Raison, Lyons & Thomson, 1971; Charnock, Cook & Casey, 1971).

As can be seen, the results reported in the literature on this subject are rather contradictory; and the discrepancies render an interpretation of the observations made in this study, as well as others, to be not an easy one. Many questions remain to be answered, whether it is the adenine nucleotides translocation, despite the almost similar effect observed on the substrate respiration in the absence of exogenous ADP; or   a temperature-induced phase transition in the lipids of the mitochondrial membrane(s); although the very recent work reported by Feo,Ca n uto, Garcear, Avogadro, Villa & Celasco (1976) showed that a transition started at -17° and centred around -6° for total lipid extracted from rat liver mitochondria;

another transition started at about +55°C and centred

around + 70°C for whole liver mitochondria, whereas

breaks in Arrhenius plots were recorded at about

27°, 20° and 24° for succinate-cyt.c reductase,

NADH-cyt.c reductase and ATPase for the same

mitochondria, respectively.

However, it can be safely concluded, that

temperature dependent changes are taking place in the

mitochondrial membrane(s), since sonication (Lee &

G ear, 1974) or the application of a detergent (Raison,

1973) abolish the discontinuity in Arrhenius plots.

However, no attempt was made in the present study to

investigate such effect on insect flight muscle

sarcosomes. Furthermore, blowfly flight muscle

sarcosomes appear to be able to illustrate a consid-

erable amount of adaptability to thermal stress over

a relatively wide range of temperature in both

substrate, as well as state III respiration, in order

to maintain the best possible respiratory control, which

is a sensitive parameter in measuring sarcosomal

respiratory performance. Phosphorylation, however,

has proved to be more sensitive to extreme temperatures.

Nevertheless, drawing a distinction between

temperature-induced changes in insect flight muscle

sarcosomes and mitochondria from other sources is
a subject of a considerable interest and therefore
demands further investigations.

The elevated substrate rate oxidation which
occurred at reaction temperatures above about $27°C$
(see Fig. 5.1), may simply be the normal Q_{10} response
to temperature rise. However, it could also occur
through a raised temperature 'deterioration' of the
sarcosomes resulting in a partial uncoupling of
oxidative phosphorylation. It is of course likely that
the phenomenon is caused by an interaction of the two
temperature induced events. The preincubation
experiments were carried out in part to elucidate this
phenomenon.

With the point in mind that high experimental
temperatures might cause mitochondrial membranes
to deteriorate, it is interesting that even for mammalian
mitochondria $25°C$ is frequently chosen as the reaction
temperature rather than $37°C$; for example Bellamy,
(1962); G ear & Lehninger, (1968); Butler & Judah, (1970).

In blowfly flight muscle sarcosomes, if this is
true then high reaction temperatures in vitro more
seriously affect the coupling process than the respiratory

chain enzymes. The very high rates of oxidation may reflect temperature induced increase in permeability to substrates.

Preincubating isolated mitochondria at a given temperature prior to initiating respiration at the same temperature has been reported on several occasions. Sacktor & Childress (1967) pointed out that rate of pyruvate oxidation decreased as elapsed time between the addition of mitochondria and of substrate increased; this loss, however, could be prevented by including BSA and ATP in the reaction medium. Hansford, (1972), investigating the effect of phosphate concentration on the rate of oxygen uptake by blowfly mitochondria as function of temperature, preincubated sarcosomes at temperatures as high as 30.5° for up to 8 minutes, more recently still, Hansford (1975), in a study of the interaction between uncoupler and ATP hydrolysis, preincubated blowfly sarcosomes at 22°C for up to 10 minutes. Whether such treatment would not cause membrane deterioration, is not made clear.

With this in mind the effect of preincubation at temperatures sub-lethal to the intact organism was determined on isolated sarcosomal performance.

The data presented in Tables (5.3) to (5.7)
require some detailed discussion. First, when
considering QO_2 values, it can be seen that QO_2 of
control sarcosomes varies between experiments, it is
for this reason that each preincubation at 30° or
higher must be accompanied by preincubation of an
aliquot of the same preparation on ice.

One interesting feature is that substrate rate
oxidation QO_2 values are little affected by preincubation
at 0°C (controls) even for long periods. Preincubation
at 30°C even for 120 mins also has no uncoupling
or inhibiting effect on α-glycerophosphate oxidation.
At higher preincubation temperatures however substrate
rate QO_2 were found to be slightly stimulated by
preincubation, this was found after some 30 mins at 31°C,
but in as soon as 5 mins at 33°C. At 35°C, preincub-
ation for 60 mins had neither stimulatory nor inhibitory
effect on QO_2. Shorter preincubation times were used at
both 36°C and 41°C and in both cases preincubation was
without an effect on QO_2 for substrate rate oxidation.

The situation regarding state III respiration
is somewhat different. Preincubation at 30°C caused
a slight but probably insignificant stimulation of
state III respiration, but after 2 hrs. ADP addition
failed to stimulate respiration. In consequence the

highest RCl values were found in the experimental sarcosomes (except at 80 and 120 mins). Much the same pattern was found for state III QO_2 values of sarcosomes preincubated at 31°C and 33°C, with preincubation causing a marked stimulation for periods of time up to 45 mins. Once again much longer exposure times were deleterious and a marked reduction in QO_2 was found. Once again this results in the experimental preincubated sarcosomes giving higher values for RCl than the cold kept controls.

At 35°C however, preincubation failed to stimulate state III respiration, indeed it was inhibitory at all exposure times longer than 5 mins. In no case was a higher RCl obtained for preincubated as compared with control sarcosomes. This was distinctly the pattern for 36° and 41°C preincubated sarcosomes also where in the latter little respiratory control could be demonstrated after 7 mins preincubation.

It would seem that the changes occurring in RCl values may be the most sensitive indicator of thermal damage. It is also quite clear that sarcosomes are much more temperature sensitive to in vitro than in vivo heating. Davison & Bowler (1971) report, from the same stock of flies that temperatures as high as

40°C for 40 mins are necessary <u>in vivo</u> to cause damage
to sarcosomal function. Furthermore, as has been shown
in this thesis, it is possible to maintain adult flies
at 34°C for long periods without any obvious damage to
their flight apparatus. Sarcosomes subsequently isolated
also have a normal respiratory function, see Table 5.7
and Chapter (4).

It seems reasonable to suggest that the enzymes
of the oxidation pathway are relatively unaffected by
exposure to temperatures between 30° and 41°C, one
can say this for even after 40 mins preincubation at
36° substrate rate oxidation showed no inhibiton, this
is also true following 7 mins preincubation at 41°C. In
both cases however, these preincubation times seriously
inhibit state III respiration. As substrate rate oxidation
was not seen to increase markedly, following
preincubation, even at longer exposure times at 30°,
31° and 33°, it would suggest that oxidative phosphory-
lation is not uncoupled under these conditions. Rather
it would seem that phosphorylation is inhibited, and so
results in bringing state III respiration to substrate rate
levels. With this in mind, it would have been useful to
determine, whether this inhibited state III respiration
in preincubated sarcosomes could be stimulated by
uncouplers such as DNP. It seems that the very long

times required at 30° and 31°C to bring about damage,
may be more 'ageing' rather than a thermal phenomenon.

A similar work, on spinach chloroplasts, has
been recently published by Santarius, (1975). It was
found that heat treatment caused inactivation of
photophosphorylation, the coupling factor (CF1) more
heat stable than photophosphorylation, and that heat
causes irreversible changes in the permeability of
chloroplast membranes.

One feature of thermal sensitivity is that in
ectothermal animals it should be modifiable by changes
in the thermal history of the animal, Read, (1967),
Bowler et al (1973). With this in mind, some blowflies
were placed at 15°C and 34°C to acclimate prior to the
isolation of their sarcosomes, which were then
preincubated at 36°, after which their respiratory
performance determined at 24°C. These can also be
compared with sarcosomes so treated from flies
maintained at 24°C, data previously discussed and given
in Table (5.6).

In sarcosomes from all three groups of
acclimated flies, preincubation either at 0°(controls)
or at 36°C caused no marked effects on substrate rate

oxidation. Once again state III respiration is more
dramatically affected, being progressively inhibited
as preincubation time increases. No obvious acclim-
ation effect can be seen. In the extreme acclimation
states (15° and 34°C) respiratory control is lost
after 30 mins exposure. The 15°C acclimated group
may be the more sensitive, for RCI shows the most
rapid decline and is but 1.24 after 20 mins preincub-
ation compared with 1.57 for sarcosomes from 34°
acclimated flies. The 24°C acclimated flies, however,
have sarcosomes which appear the more resistant and
have an RCI that is still 1.84 after 20 mins.

The apparent thermostability of the substrate
rate respiration, observed throughout the preincub-
ation experiments described above, suggests that the
enzymes participating in this pathway are not likely
to be susceptible to in vitro heat application. Indeed,
α-glycerophosphate dehydrogenase has been shown
elsewhere in this thesis (see Chapter 7), to be resistant
to temperatures as high as 50°C, when assayed under
conditions excluding the respiratory chain from the process
of electron transfer. This finding, together with those
reported in this Chapter, strongly suggest that the
oxidative enzyme system as a whole, in blowfly flight

muscle   sarcosomes, is not sensitive to heat exposure

under the conditions described above.

CHAPTER SIX

EFFECT OF CALCIUM ON SARCOSOMAL

α-GLYCEROPHOSPHATE OXIDATION

Introduction:

Davison, (1970) described a differential thermal sensitivity of blowfly flight muscle sarcosomal oxidative phosphorylation when α-glycerophosphate was used as a substrate as compared with pyruvate and malate. In Chapter (3) this preliminary finding by Davison, (1970) has been studied further. The data presented there clearly point to the possibility that the initial enzyme in α-glcerophosphate oxidation as being the thermal sensitive focus.

α-glycerophosphate dehydrogenase is thought to be located on the outer surface of the inner membrane (Donnellan, Barker, Wood & Beechey, 1970; Klingenberg & Buchholz, 1970) although recently Slack & Bursell (1977) have questioned this interpretation. It has been shown by Hansford & Chappell (1967) that α-glycerphosphate dehydrogenase is an allosteric enzyme, maximally stimulated by $10^{-5} \text{gm} - \text{ion.}^{-1} \text{Ca.}^{2+}$. This finding clearly explaining the inhibitory effect of EDTA (Estabrook & Sacktor, 1958) and EGTA (Hansford & Chappell, 1967) on α-glycerophosphate oxidation by flight muscle sarcosomes.

Klingenberg & Buchholz (1970) and Hansford (1971) have also shown a similar Ca^{2+} activation for α-glycerophosphate oxidation in sarcosomes from locust and the periodical cicada, <u>Magicicada</u> <u>septendicem</u> flight muscle, respectively. It is significant that the enzyme is activated maximally by concentrations of free calcium that are close to those estimated to activate myofibrillar ATPase (Sacktor, 1970). The level of extra mitochondrial free calcium in resting muscle is apparently so low that α-glycerophosphate dehydrogenase would not be allosterically activated (Hansford & Chappell, 1967) and would be relatively inactive at the estimated cellular concentration of α-glycerophosphate, 2 mM (Sacktor & Wormser-Shavit, 1966). Thus the allosteric activation by raised calcium facilitates the rapid increase in sarcosomal function that follows the demand for ATP by muscle fibres in contraction (for a recent review article see Sacktor, 1974).

With this regard it is also pertinent that Smith, (1966) reports insect flight muscle possesses a scant sarcoplasmic reticulum. This raised a question as to the identity of the organelles undertaking the function of Ca^{2+} sequestration. However, Carafoli, Hansford, Sacktor & Lehninger, (1971) have studied the interaction

of Ca^{2+} with blowfly flight muscle sarcosomes, and

found that Ca^{2+} is accumulated by respiring blowfly

sarcosomes with a low efficiency. The authors

concluded that such accumulation occurs through

slow, concentration-dependent, physical diffusion

through the membrane, and is not mediated by the

action of a specific carrier.

Dawson, Dunnett & Selwyn (1971), and Hansford

(1971; 1972) have worked on the mechanism of the

stimulation of respiration by exogenous Ca^{2+} Slack &

Bursell (1977) have also suggested Ca^{2+} might be

involved in the stimulation of substrate transport into

the sarcosomes of blowflies.

To return to the particular stimulation of

α-glycerophosphate dehydrogenase, it would seem

possible that if in vivo heating does affect this enzyme

then its allosteric behaviour might reveal this thermal

sensitivity as suggested by Hochachka & Somero (1973).

This is because allosterism requires a high level of

structural order in the enzyme molecule, and heat is a

well documented perturbator of protein tertiary

structure (Brandt 1967). If such thermally induced

perturbations occur to the α-glycerophosphate

dehydrogenase, at heat death temperatures the property

of allosteric stimulation by calcium may be lost before

catalytic function (Stadtman 1966). In this way it may explain why substrate oxidation of α-glycerophosphate is reduced in sarcosomes from $L.D_{50}$ treated flies whilst pyruvate oxidation (state IV) by the same sarcosomes is, if anything, increased.

Materials and methods:

Sarcosomes were isolated from either control or $L.D_{50}$ heat treated blowflies (10 day old) as described in Chapter 3. α-glycerophosphate dehydrogenase activity was determined as the rate of oxygen consumption by sarcosomes in presence of α-glycerophosphate, by conventional polarographic methods as described earlier in Chapter 2.

Enzyme activity was followed in the conditions below:

1. Calcium free medium:

    100 mM KCl
    10 mM Tris HCl buffer pH 7.1 at 24°C.
    1 mM EGTA

2. Calcium containing medium:

    100 mM KCl
    10 mM Tris HCl buffer pH 7.1 at 24°C
    10^{-5} gm-ion.1^{-1} ca^{2+}

This low level of calcium was maintained by using a Ca^{2+} - EGTA buffer system. Equal volumes of 1 mM EGTA and 1 mM $CaCl_2$ were mixed. The method used to calculate the level of free calcium is that suggested by Portzehl, Caldwell & Ruegg (1964).

Both reaction media were fully equilibrated with air at 24°C for several hours. $2 cm^3$ of medium was pipetted into the reaction chamber of the oxygen electrode. This was followed by 3 µl of an alcoholic solution of carbonyl-cyanide-p-trifluoro methoxy phenylhydrazone (FCCP) to give a final concentration of 0.5 µM.

50 µl of sarcosomal suspension was then added and this was followed by 50 µl of α-glycerophosphate solution which started the reaction.

The substrate concentrations (final) used were as follows:
1, 2, 4, 5, 6, 8, 10, 15 20, 25, 30, 35, 40 and 50 mM. All additions to the reaction chamber were made using terumo microsyringes.

Any one set of substrate concentrations were followed on the same sample of sarcosomes, working from the higher to the lower substrate concentrations.

The experiment was repeated five times for control and three times for sarcosomes from $L.D_{50}$ treated flies. The data shown in Table (6.1) shows the mean values obtained for QO_2 as well as the range of values at different concentrations of α-glycerophosphate. The mean values are also plotted versus substrate concentration in Fig. (6.1).

Protein assay was carried out as described in Chapter (2). All chemicals were purchased from Sigma Chemical Co., Ltd., except FCCP which was purchased from Boehringer Chemicals.

Results

The allosteric effect of including 10^{-5} gm-ion.1^{-1} Ca^{2+} in the reaction medium is clearly shown in Fig. (6.1) and in more detail in Table (6.1) In Fig. (6.1) these data from sarcosomes from both control and $L.D_{50}$ treated flies are shown as a plot of V against S.

Sarcosomes from control flies

Taking the reaction in the absence of Ca^{2+} first, it can be seen that the rate-substrate concentration curve has a sigmoidal shape. At substrate concentrations below 10 mM α-glycerophosphate low rates of oxygen consumption were recorded. Whilst the highest rates,

(about 40 μg AO.hr^{-1}.mg prot.$^{-1}$) were found only when α-glycerophosphate concentrations were higher than 30 mM. The estimated value for app. $Km(S_{0.5})$ is 14 mM.

In constrast, in the presence of free calcium rapid oxidation rates were observed even at 1mM α-glycerophosphate, $QO_2 = 13.45$ μg AO.$\overset{.}{hr}^{-1}$ mg prot.$^{-1}$ Values as high as this were observed in the absence of free calcium only at much higher substrate concentrations. Values for QO_2 approximating to Vimax for the enzyme were obtained at substrate concentrations of 10 mM. The form of the curve is a classical rectangular hyperbola of Michaelis-Menten kinetics. The app. Km for the enzyme in the presence of 10^{-5} mg-ion.l^{-1} free calcium falls to about 2 mM α-glycerophosphate.

Sarcosomes from heat treated flies

As can be seen from Fig. (6.1) the most dramatic effect of the in vivo heating on the α-glycerophosphate dehydrogenase activity is to simply depress the activity without destroying the ability to respond to the allosteric effector, Ca^{2+}.

The substrate concentration - velocity curve

in the absence of free Ca^{2+} is still sigmoidal in shape.
As in the control flies QO_2 values are very low below
5 mM α-glycerophosphate, the values obtained are only
about 30% of those obtained for control sarcosomal
enzyme activity in this concentration range, similar
to the effect earlier reported in Table (3.1). QO_2
values approaching Vmax did not appear to have been
reached even at 50 mM α-glycerophosphate, this is
in contrast with control sarcosomal enzyme kinetics.
The value for the QO_2 was only 18.68 μgAO.hr^{-1}.
mg prot.$^{-1}$, whereas the value for QO_2 was only
18.68 μg AO.hr^{-1}. mg prot.$^{-1}$, whereas the value for
QO_2 at Vmax in presence of Ca^{2+} was 21.37 μg AO.hr^{-1}.
mg prot.$^{-1}$. These values are only about half the rates
for Vmax obtained for the enzyme from control
sarcosomes.

It is significant however that in the sarcosomes
from the heated flies the addition of 10^{-5}gm-ion.1^{-1}
Ca^{2+} converts the substrate concentration - velocity
curve from a sigmoidal to a rectangular hyperbolic
form. However the app. Km in presence of Ca^{2+} is
7 mM α-glycerophosphate, this being much lower than
the value calculated in absence of Ca^{2+}, which was 20 mM.

Table 6.1

Saturation kinetics of blowfly flight

muscle sarcosomal α-glycerophosphate

dehydrogenase from control untreated,

and L.D.$_{50}$ treated adults, in the

presence and absence of 10^{-5} g-ion.l^{-1}

free Ca^{2+}.

o.5 μM FCCP was present in all cases

Isolation : Nagarse method (see Chapter 3)

Reaction conditions   : 100 mM KCl

                            10 mM Tris/HCl buffer
                            at pH 7.1 at 24 $^{\circ}$C.

Reaction temperature   : 24 $^{\circ}$C.

Table 6.1

$$QO_2$$
$$(mg\ AO.\ hr^{-1}.\ mg\ prot.^{-1})$$

Substrate Concentration (mM)	CONTROL − Calcium			CONTROL + Calcium			LD$_{50}$ − Calcium			LD$_{50}$ + Calcium		
	$\bar{x}$	Range	n	$\bar{x}$	Range	n	$\bar{x}$	Range	n	$\bar{x}$	Range	n
1	-	-		13.45	(5.13-21.01)	4	-	-		-	-	
2	-	-		23.79	(7.58-33.23)	4	-	-		4.18	(4.14-4.22)	3
4	-	-		29.30	(14.49-38.07)	4	-	-		6.80	(5.93-7.66)	3
5	5.59	(2.08-10.18)	5	-	-		1.68	(1.38-1.98)	3	-	-	
6	-	-		32.91	(20.10-45.71)	2	-	-		8.79	(7.93-9.65)	3
8	-	-		36.38	(28.16-44.59)	2	-	-		11.17	(9.88-12.46)	2

10	11.77	(6.25–16.66)	5	40.96	(25.00–60.13)	4	4.70	(4.13–5.60)	3	11.69	(9.93–13.44)	3
15	21.19	(10.83–31.59)	5	–	–		8.04	(7.75–8.32)	3	15.25	(13.81–16.69)	2
20	28.90	(16.19–44.76)	5	41.07	(26.11–60.00)	4	10.14	(9.28–10.99)	3	16.68	(13.82–19.53)	3
25	31.85	(21.55–46.30)	4		–		–	–		17.91	(15.00–21.62)	3
30	38.90	(24.14–53.90)	3	–	–		13.86	(12.10–15.83)	3	19.20	(15.79–22.06)	3
35	39.24	(26.73–48.76)	4	–	–		–	–		–	–	
40	39.62	(29.31–44.83)	3	–	–		16.86	(16.35–17.37)	3	21.37	(20.37–22.21)	3
50	41.98	(32.76–49.01)	3	43.87	(26.50–60.00)	3	18.68	(18.40–18.96)	3			

Fig 6.1

Saturation kinetics of α-glycerophosphate

dehydrogenase. (See Table 6.1).

Ordinate : Oxidation rate (QO_2) expressed

as ug $AO.hr^{-1}$ mg prot.$^{-1}$

Abscissa : α-glycerophosphate concentration in mM.

$\triangle$ Control, in the absence of Ca^{2+}.

$\bigcirc$ Control, in the presence of 10^{-5} g-ion.l^{-1}
free Ca^{2+}.

$\triangledown$ $L.D._{50}$ treated, in the absence of Ca^{2+}.

$\square$ $L.D._{50}$ treated in presence of 10^{-5} g-ion.l^{-1}
free Ca^{2+}.

Arrows on the abscissa represent the app. Km value

for each graph with the corresponding symbol.
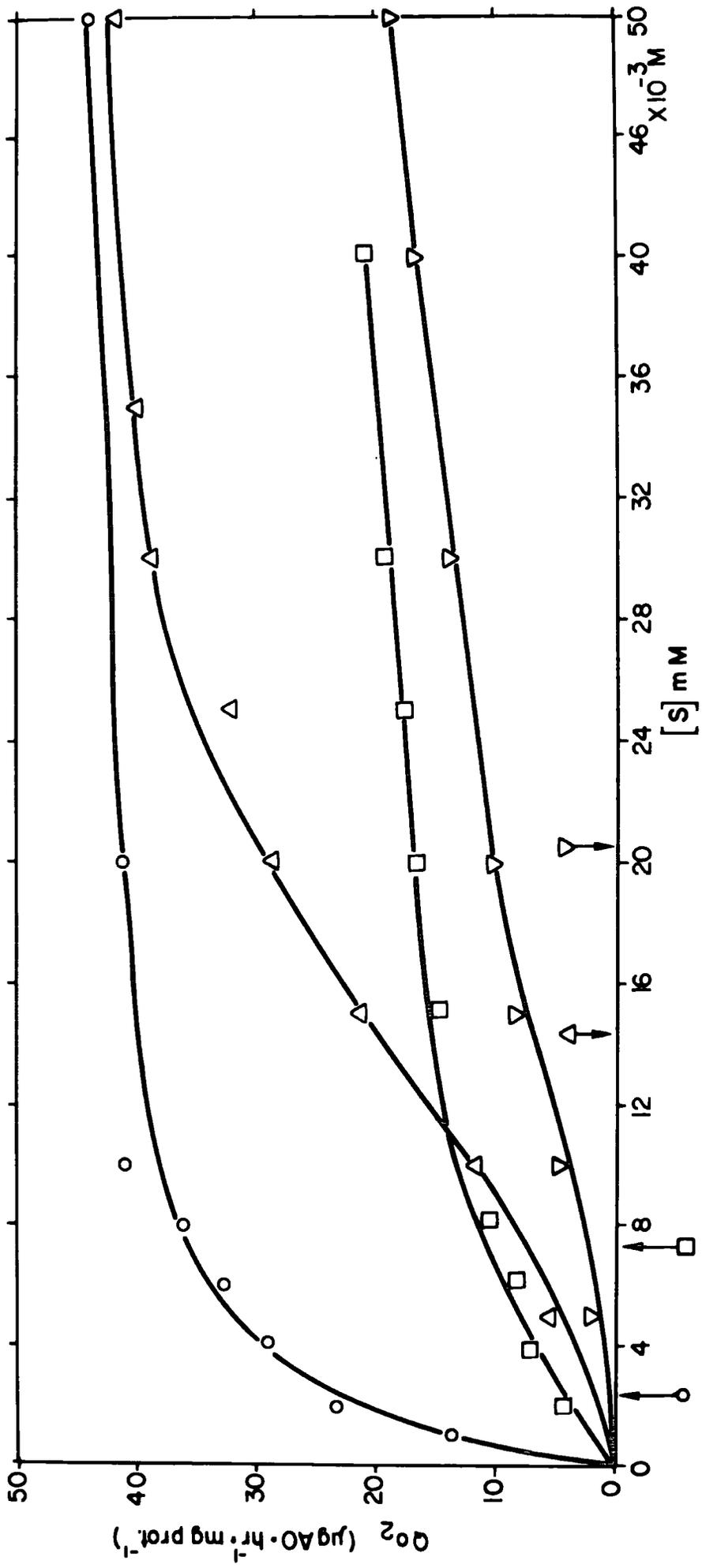
Discussion

The data presented in Table (6.1) and Fig.(6.1) are consistent with those obtained by Hansford & Chappell, (1967) on flight muscle sarcosomes from $\underline{Calliphora}$ $\underline{vomitora}$. They described Ca^{2+} (10^{-5} gm-ion.1^{-1}) reduced the app. Km for substrate from about 10 to 2 mM. In the present work this reduction is from 14 to 2 mM.

Hansford & Chappell, (1967) excluded the possibility that this Ca^{2+} activation of α-glycerophosphate oxidation was merely on the respiratory chain and energy conserving systems because Ca^{2+} was without effect on any aspect of pyruvate oxidation. They also demonstrated, by comparing results with sonicated and intact sarcosomes, that this effect was not mediated through a stimulation of penetration of the sarcosomal membrane of either substrate or product. This strongly suggests that the α-glycerphosphate dehydrogenase is directly allosterically stimulated by calcium.

However, this leads to the question of the localization of the dehydrogenase. A variety of workers (Klingenberg & Buchholz, 1970; Balboni,1972) have studied this problem,and the generally held view

is that this enzyme is located on the outer surface of the inner mitochondrial membrane, which means that α-glycerophosphate is oxidized without having to be translocated across the inner membrane.

This also argues against the Ca^{2+} stimulation of α-glycerophosphate oxidation being mediated by an effect on permeation of the sarcosomal membrane. Recently, however, a contrary view has been proposed by Slack & Bursell (1977) from work on the blowfly, Sarcophaga modosa. They claimed that α-GP dehydrogenase is located on the inner, rather than the outer surface of the inner membrane. This conclusion has been based on the observation that NADH oxidation, which is mediated by an intramitochondrially situated enzyme, is parallel to α-glycerophosphate oxidation, when the mitochondria are subjected to increasing intensities of sonication. They questioned the previously reported view that α-glycerophosphate dehydrogenase is located on the outer surface of the inner membrane, which has been supported by experiments involving the reduction of the non-penetrant ferricyanide to ferro-cyanide on addition of α-glycerophosphate in antimycin A treated mitochondria. Their criticism was based on the argument that high concentrations of α-glycerophosphate

might alter the permeability of the inner membrane, thereby enabling the normally non-penetrant ferricyanide to penetrate the membrane and so reversing the inhibition, being capable of denaturing antimycin A (Walter & Lardy, 1964). Slack & Bursell (1977) concluded, therefore, that the membrane transport of α-glycerophosphate is dependent on membrane translocation.

Ca^{2+} also acts as an allosteric inhibitor of flight muscle sarcosomal isocitrate dehydrogenase (Vaughan & Newsholme, 1970), an enzyme located intramitochondrially, according to Carafoli, et al. (1971). In insects the modulation of those two enzymes must occur simultaneously on the initiation of flight (Carafoli et al., 1971).

Furthermore, the possibility that the high concentrations of α-glycerophosphate used, might affect the structure of the mitochondrial membrane, has been suggested by Wojtczak, Vlodower & Zborowski, (1963). This may well have been the case in the rat liver mitochondria used by these workers for α-glycerophosphate is not the normal substrate for these mitochondria. Still the concentration of α-glycerophosphate used in the present study, and by other workers, on flight muscle sarcosomes are frequently much higher than those experienced

physiologically (Sacktor & Wormser-Shavit 1966).

However, Klingenberg & Buchholz (1970) reported that

α-glycerophosphate concentrations as high as 50 mM,

hardly induced swelling in flight mitochondria, this

they took to be indicative of an unaltered membrane

permeability. Thus there seems to be some

uncertainty of the exact mechanism of Ca^{2+} stimulation

of α-glycerophosphate oxidation. The thorough work

by Hansford & Chappell (1967) is compelling,

particularly as the allosteric behaviour of the enzyme

was observed in both intact and sonicated sarcosomes.

Ca^{2+} interaction only being lost when sarcosomal

membranes were treated with triton.

A variety of enzymes have been shown to be

desensitized to their allosteric effectors, before loss

of catalytic activity, when exposed to such agents as

mercuric salts, freezing digestion with proteases etc.

Aspartate transcarbomylase lost sensitivity

to cytidine triphosphate inhibition following exposure

to mercuric salts, or urea (Gerhart & Pardee, 1962).

In case of trehalose synthetase also the loss of

susceptibility to allosteric control following treatment

with mercuric salts or exposure to high ionic strength,

is accompanied by a conversion from sigmoidal to

Michaelis-Menten type kinetics (Murphy & Wyatt, 1965).

In a number of cases the exposure of the enzyme to heat also destroys its allosteric properties. This has been shown in phosphoribosyl-ATP pyrophosphatase extracted from Salmonella typhimurium (Martin, 1963), aspartate transcarbamylase from Escherichia coli (Gerhart & Pardee, 1962), and acetohydroxy acid synthetase in Salmonella typhimurium (Bauerle, Freundlich, Stormer & Umbarger, 1964). However in these cases the heat exposure used has been very unphysiological, that is temperatures of about $50°C$ or more.

In this respect, very few, if any, reports occur in which the sensitivity of the allosteric properties of an enzyme have been studied following the exposure to in vivo temperatures that are just lethal to the organism.

In the present study, both in the presence and absence of calcium, lethal heat treatment causes an increase in the calculated app. Km for α-glycerophosphate dehydrogenase. But unlike some instances discussed earlier there is no change in the saturation kinetics of the enzyme. In sarcosomes from both heated and control flies, sigmoidal kinetics are obtained in absence, and Michaelis-Menten kinetics in the presence of calcium.

However in the absence of calcium the app. Km for α-glycerophosphate rises by 6 mM for the enzyme from heated flies. A similar rise occurs in the app. Km in the presence of calcium,when the value rises from 2 to 7 mM when the flies are heated.

It is not possible to estimate whether the effectiveness of calcium as a stimulator of α-glycerophosphate dehydrogenase is altered following the in vivo heat exposure. However, it is noticeable that the amount the app. Km is reduced in control and heat exposed enzyme by the presence of calcium is about 12 mM in both cases.

It is clear however that catalytic efficiency is seriously affected for, under conditions that give Vmax, enzyme activity is reduced by about 50% following in vivo heatings.

As Lardy, Lee & Takemori (1960) report that α-glycerophosphate dehydrogenase is the rate limiting enzyme in α-glycerosphosphate oxidation, it is tempting to assume the inactivation in oxidation described above results from heat damage to α-glycerophosphate dehydrogenase. This assumption is supported by the data presented in Table (3.1) Chapter(3). Here an L.D.$_{50}$ heat treatment causes loss of respiratory

control and inhibition of substrate rate (and state III)
respiration by up to 50% when α-glycerophosphate is
the substrate. However, when pyruvate and proline
are being respired state IV respiration is increased
in sarcosomes from heated flies as compared with
control flies This implies that the part of the respir-
atory chain, common to both substrates, (i.e. past
coenzyme Q-cytochrome b) is not damaged by the
heating.

At this point, therefore, it seems reasonable
to suggest the mitochondrial α-glycerophosphate
dehydrogenase is one thermal sensitive structure, in
that its catalytic efficiency is restricted following heat
applied in vivo. With this in mind further work using
dye reduction to assay α-glycerophosphate dehydrogenase
will be carried out. Using this technique the need to
use the whole respiratory chain in the assay is avoided.
This work is presented in Chapter (7), and further explores
the possibility of α-glycerophosphate dehydrogenase
being inactivated by heating in vivo and in vitro.

CHAPTER SEVEN

THE ACTIVITY OF AND THERMOSENSITIVITY OF SARCOSOMAL α-GLYCEROPHOSPHATE DEHYRODGENASE

Introduction:

The results presented in Chapter (3)

together with those reported by Davison (1970) strongly

indicate that the failure of α-glycerophophase-

supported oxidative phosphorylation mechanism,

exhibited by sarcosomes isolated from heat-treated

animals, cannot be at the level of the respiratory

chain. This comment is justified by the observation

that sarcosomes from heat treated animals carry out

oxidative phosporylation when pyruvate and proline

are used as substrates, see Chapter (3). This is

particularly puzzling because the conventional view

is that electrons, irrespective of the substrate

source, are fed into a common electron transfer

pathway (Lehninger, 1964; Pullman & Schatz,1967;

Lardy & Ferguson, 1969).

However the results mentioned above, and

presented in detail in Chapter (3), confirm the

preliminary observations made by Davison (1970).

Implicit in Davison's (1970) interpretation

of his results is that oxidative phosphorylation

associated with α-glycerophosphate may be carried out

by a respiratory assembly, different to those associated

with other substrates.   This is perhaps supported

by the recent report by Rogers & Higgins (1976) that,

in rat liver mitochondria, the degree of inhibition of

respiration by tetraalkylammonium bromides, depends

upon the respiratory substrates used.

Davison (1970) has also mentioned the possibility

of thermal inactivation of the respiratory enzymes

associated with α-glycerophosphate-dependent respiration.

In this regard, it is noteworthy to remember that the

results presented in Chapter (4) on recovery from

sublethal heat treatment, suggest that biosynthetic

processes may be involved in the repair mechanism

following heat damage.

Similar thermal inactivation has been reported

for several respiratory enzymes of <u>Staphylococcus</u>

<u>aureus</u> (Bluhm & Ordal, 1969), and for some liver

mitochondrial enzymes from a respresentative series of

vertebrate animals (Smith, 1973 a, b).

At this stage it seems quite reasonable to expect

that heat injury might be at the level of α-glycerophosphate

dehydrogenase (EC.1.1.99.5); the first enzyme in α-glycerophos-

phate respiratory pathway.   Therefore, this Chapter will

represent a detailed investigation into the nature of this enzyme, its activity and thermostability. The results will be discussed in the light of other relevant parts of this study, together with the results reported by Davison (1970) and Davison & Bowler (1971).

Materials and Methods:

A) Measurement of sarcosomal α-glycerophosphate dehydrogenase activity

Sarcosomal α-glycerophosphate dehydrogenase activity was measured using the method described by Lee & Lardy (1965).

The essence of the technique is based on the reduction of the tetrazolium salt (INT) by electrons transferred directly from the dehydrogenase via phenazine methosulphate (PMS), resulting in the formation of insoluble formazan. The optical density of a solution of this formazan in toluene is proportional to the enzyme activity.

Sarcosomes were isolated from the flight muscle of 10 blowflies, aged 1, 10 or 20 days, using the standard isolation technique (see Chapter 2). However, the final pellet was resuspended in 8 cm^3 of the resuspension medium, Chapter 2 giving a

preparation with sarcosomal protein concentration of
less than 0.05 mg/50ul Preparations with protein
concentrations higher than that, were found to give
non-linear activity with time, under the present assay
conditions.

Reaction medium:

66 mM α-glycerophosphate

2 mM Potassium cyanide

33 mM Phosphate buffer at pH 7.3 at 24°C.

Artificial electron acceptor:

0.044% INT (2-p-iodophenyl-3-p-nitro-
phenyl-5-phenylmonotetrazolium
chloride)

0.09% PMS (phenazine methosulphate)

made up separately in 33 mM phosphate
buffer at pH 7.3 at 24°C.

Assay Procedure:

1)   Five glass test tubes were wrapped in aluminium
        foil and held in a water bath maintained at 24°C.

2)   Into each tube 0.5 cm^3 of the reaction medium
        was introduced and allowed to equilibrate for
        5 minutes.

3)   This was followed by an addition of 50 μl of the
        mitochondrial preparation and allowed to stand

for a further 5 minutes.

4) The reaction was initiated by the addition of 0.5 cm^3 of a mixture of equal volumes of the artificial electron acceptors,

5) The reaction was terminated by the addition of 0.5 cm^3 of 10% trichloroacetic acid (TCA) after 0, 2, 4, 6 or 8 minutes.

6) The tubes were then taken out of the water bath and 4 cm^3 of toluene were added into each to solubilize the formazan formed after the reduction of the INT.

7) Complete solubilization was achieved by mixing thoroughly on a whirli-mixer for a few seconds.

8) The tubes were then centifuged in a Gallenkamp Junior centrifuge for 2 minutes at 1000 xg.

9) The upper layer, which consisted of toluene and solubilized formazan, was carefully decanted and the intensity of the colour was read at 500 mu in a Baush & Lamb spectronic 20.

10) The same steps were repeated using sarcosomes isolated from $L.D._{50}$ treated 10-day old flies.

Extra care was taken to avoid exposing the tubes to direct sunlight, or any bright light, at all stages of the experiment since the PMS-INT solution was extremely sensitive to light.

The heat treatments given to the experimental flies of different ages were as follows:

1 day old adults 97 minutes at 41°C.

10 day old adults 37.22 minutes at 41°C.

20 day old adults 39.75 minutes at 41°C.

These heat doses were used as they correspond to the L.D.$_{50}$ treatment for each age (Davison, 1969).

B) <u>Effect of thermal preincubation in vitro of sarcosomes on α-glycerophosphate dehydrogenase activity</u>

Sarcosomes were isolated from the flight muscle of ten 10-day old blowflies, using the "standard" isolation procedure (see Chapter 2).

Glass test tubes were equilibrated for 20 minutes in a water bath at one of the following temperatues: 40°, 42°, 43°, 50° and 55°C.

The temperature of each bath was maintained at $\pm$ 0.1°C with a thermoregulator and 'Sunvic' control. A 50 μl aliquot of the sarcosomal preparation, containing

approximately 0.05 mg protein, was introduced into

each tubes and then preincubated for the following

period of time:

5, 10, 15, 20, 30, 40, 50, 60, 80, 100 and 120 mins.

After the preincubation period, the tubes were then

transferred to a 24°C water bath and allowed to

equilibrate for 5 mins. Thermal inactivation of the

enzyme was followed, using the method of dye

reduction described above (see Section A).

C) Effect of freezing and thawing

As for experiments investigating this effect

on isolated blowfly flight muscle sarcosomes, oxidation

rates of α-glycerophosphate or pyruvate + proline were

measured polarographically in a reaction medium that

contained:

154 mM Kcl

10 mM tris/Hcl buffer at pH 7.3 at 24°C.

33 mM phosphate buffer at pH 7.3 at 24°C.

0.4 mg BSA/cm^3

The assay was carried out at 24°C, and

sarcosomes were isolated in all cases using the nagarse

isolation technique (see Chapter 3).

Freezing

This treatment was given to the isolated

sarcosomes by placing the preparation for 12 hours in

a deep freeze at $-20°C$.

The preparation was allowed to stand at room

temperature for about 15 mins. to thaw. Sarcosomes

from both control and L.D.$_{50}$ treated flies were

subjected to this procedure.

Results

A) α-glycerophosphate dehydrogenase activity:

The data given in Table (7.1), represent values

for α-glycerophosphate dehydrogenase activity of

sarcosomes isolated from the flight muscle of control

and heat-treated adult blowflies of different ages. It

can be clearly seen that low enzyme activity is a charac-

teristic of one day old flies. This activity however,

increased over twofold by the age of 10 days, and remained

at that higher level in sarcosomes isolated from 20 day

old flies.

However, in sarcosomes isolated from adult flies,

which have been given the L.D.$_{50}$ heat treatment corres-

ponding to their age, a decline of 19.2, 17.8 and 16.5 per

cent was recorded in sarcosomal enzyme activity of 1, 10

Table 7.1

α-glycerophosphate dehydrogenase activity of

sarcosomes isolated from the flight muscle of

1, 10 or 20 day old blowflies, of either control

(unheated) flies or flies given a heat treatment

equivalent to an L.D.$_{50}$ dose. α-glycerophosphate

dehydrogenase activity was determined under the

following conditions:

Reaction medium

66 mM α-glyercophosphate

2 mM Potassium cyanide

33 mM Phosphate buffer at pH 7.3 at 24°C

Artificial electron acceptor:

0.044%   INT

0.09%   PMS

made up separately in 33 mM phosphate buffer at

pH 7.3 at 24°C.

Enzyme activity is expressed as ΔOD_{500} min^{-1}.mg prot.$^{-1}$.

Mean values from 10 separate extractions $\pm$ 1 standard

error are given.

Table 7.1

Age and α-GP dehydrogenase thermostability

Age (days)	Treatment	Activity (ΔOD/min/mg prot.)		% Inactivation	n
		Experiment	Control		
1	41°/97 mins	1.05 ± 0.19	1.3 ± 0.166	19.2	10
10	41°/37.22 mins	2.35 ± 0.17	2.86 ± 0.23	17.8	10
20	41°/39.75 mins	2.48 ± 0.28	2.97 ± 0.27	16.5	10

and 20 day old flies, respectively. However, the enzyme
activity levels were not statistically significantly
different from those obtained from control sarcosomes
(p $>$ 0.05).

B) Table (7.2) and Fig. (7.1) present α-glycero-
phosphate dehydrogenase thermal inactivation, when
isolated sarcosomes have been preincubated at the given
temperatures for the indicated periods of time, before
being assayed at 24°C. Two control values are given for
each temperature, representing enzyme activity at the
beginning and the end of the experiment. Values for
enzyme activity after the various treatments, are
expressed in terms of percentage of enzyme activity
in control sarcosomes recorded at the beginning of the
experiment. As can be seen, temperatures as high as
40°C required up to 60 minutes preincubation period
to bring about 50% inactivation to α-glycerophosphate
dehydrogenase. Indeed after 2 hours preincubation at
this temperature, enzyme activity was still measurable.
A similar effect was recorded when the temperature
was raised to 42°C. At 43°C, however, a 10% inactivation
was observed after only 10 minutes of preincubation; this
percentage increased gradually with time, and reached
15, 25 and about 30 per cent after 20, 30 and 40 minutes

Table 7.2.

_____

Effect of thermal preincubation in vitro
on the activity of α-glycerophosphate
dehydrogenase of sarcosomes isolated from
the flight muscle of 10-day old adult
blowflies.

Measurements of the enzyme activity was
carried out under the same conditions
described in Table (7.1).

Enzyme activity is expressed as

$\triangle OD_{500} \cdot min^{-1} \cdot mg\ prot.^{-1}$

Table 7.2

Effect of thermal preincubation in vitro on sarcosomal

α-glycerophosphate dehydrogenase activity

Treatment °C	Control		5		10		15		20		30	
	Initial	Final	Activity	% of Cont.	Activity	% of Cont.	Activity	% of Cont.	Activity	% of Cont.	Activity	% of Cont.
40	3.09	2.48			3.09	100	3.09	100	2.88	84.79	2.78	89.97
42	3.09	2.74			2.97	90.02	3.08	99.98				
43	3.30	2.94							2.82	85.41	2.47	74.81
50	2.26	2.22							1.73	76.60		
55 no Ca²⁺	2.61	2.51							0.98	37.55		
55 + Ca²⁺	2.47	2.45	2.07	83.83	1.44	58.31	1.28	51.71	1.06	42.91	0.71	28.60

INCUBATION TIME (Mins)

| Treatment °C	Control		40		50		60		120	
	Initial	Final	Activity	% of Cont.	Activity	% of Cont.	Activity	% of Cont.	Activity	% of Cont.
40	3.09	2.48					1.5	48.54	1.25	40.50
42	3.09	2.74	2.20	71.19					1.43	46.45
43	3.30	2.94	2.40	72.27						
50	2.26	2.22	1.50	66.37			1.19	52.54		
55 no Ca²⁺	2.61	2.51	0.55	21.03			0.47	18.12		
55 +Ca²⁺										

Fig. 7.1

Effect of preincubation in vitro on
the activity of α-glycerophosphate
dehydrogenase of sarcosomes isolated
from the flight muscle of 10-day old
adult blowflies.

Residual activity of sarcosomal glycero-
phosphate dehydrogenase activity
(Δ O.D.$_{500}$ min^{-1} mg prot.$^{-1}$) after
preincubation of sarcosomes for varying
periods of time at $40°$, $42°$, $43°$, $50°$
and $55°C$. At the latter temperature
preincubation was carried out in presence
and absence of 10^{-5} g-ion l^{-1} of free Ca^{2+}.
Activity was determined at $24°C$ under the
conditions described in Table (7.1).

The points on the ordinate represent the
activity level prior to preincubation.
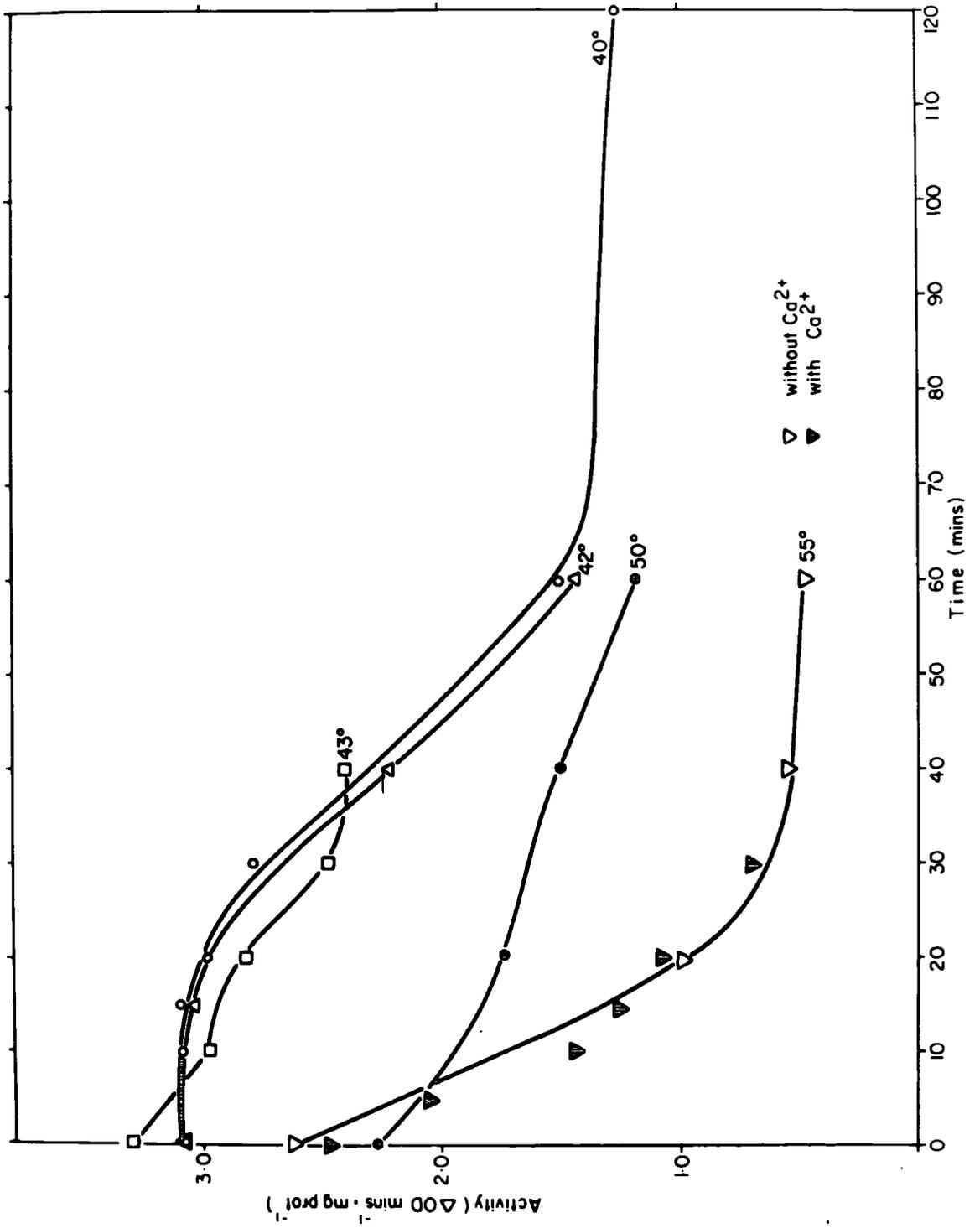
preincubation respectively. When the temperature was raised to 50°C, about 50% inactivation required, also, a long period of preincubation, approximating 60 minutes, to occur. Nevertheless, 55°C was adequately high to cause 50% inactivation within 20 minutes of preincubation, and after 40 and 60 minutes, enzyme activity of 0.55 and 0.47 units was recorded, which was only 21 and 18 per cent of the control initial value, respectively. The same effect of this temperature was observed on enzyme activity, when the reaction medium included 10^{-5} g-ion/l of free Ca^{2+} (see Chapter 6).

C) Effect of freezing and thawing on sarcosomes

The data recorded in Table (7.3) show that the oxidation rates of pyruvate + proline and α-glycerophosphate by untreated nagarse-isolated blowfly flight muscle sarcosomes, were 4.34 $\pm$ 0.33 and 28.05 $\pm$ 1.70 μg AO.hr^{-1}.mg prot.$^{-1}$, respectively. When such a preparation was frozen overnight at -20°C and thawed the following morning these rates of oxidation declined considerably, giving a value of only 1.15 $\pm$ 0.14 for pyruvate and proline, a decline of about 73 per cent. α-glycerophosphate gave, after such treatment, respiratory rates of 18.74 $\pm$ 2.83, showing a fall of

Table 7.3

The effect of freezing and thawing of sarcosomes, isolated by 'nagarse' method, from control and $L.D._{50}$ treated flies ($41°$ for 40 mins) on oxidation rates in presence of 33 mM α-glycerophosphate or 2.5 mM pyruvate + 2.5 mM proline.

Oxidation rates are shown as mean values $\pm$ s.e. as μg AO.hr^{-1}. mg prot.$^{-1}$. The figure in brackets is the number of preparations used. Activity was determined at $24°C$ under the following conditions:

154 mM KCl, 10 mM Tris/HCl buffer at pH 7.3 at $24°C$, 30 mM phosphate buffer at pH 7.3 at $24°C$, o.4 mg BSA/cm^3.

Oxidation rates of α - glycerophosphate or pyruvate + proline by nagarse

isolated sarcosomes after various treatments

	Untreated		Frozen and thawed (in vitro)		L.D.$_{50}$ (in vivo)		L.D.$_{50}$ (in vivo), frozen and thawed (in vitro)	
	α- GP	Pyr. + Prol.	α- GP	Pyr. + Prol.	α- GP	Pyr. + Prol.	α- GP	Pyr. + Prol.
Oxidation rate ± S.E.	28.05 ± 1.70 (5)	4.34 ± 0.33 (6)	18.74 ± 2.83 (6)	1.15 ± 0.14 (5)	16.81 ± 1.77 (4)	3.24 ± 0.16 (6)	11.83 ± 1.46 (6)	1.73 ± 0.13 (6)
% Of Control			66.81	26.50	59.93	74.65	42.18	39.86

approximately 33 per cent. On the other hand,
sarcosomes isolated from L.D.$_{50}$ treated, 10 day
old adult flies, using the nagarse isolation procedure
described, showed a decline of only 25 per cent when
using pyruvate and proline as substrate, whereas
α-glycerophosphate oxidation rate declined by about
40 per cent. When a preparation, isolated from
L.D.$_{50}$ treated flies, was frozen and thawed in the
same manner described earlier, a fall of approximately
60 per cent and 58 per cent was recorded for pyruvate +
proline and α-glycerophosphate as substrates res-
pectively.

Discussion:

Investigations, involving a possible thermal
inactivation at the level of α-glycerophosphate
dehydrogenase, have been carried out. It was found,
that the insignificant reduction in the enzyme activity
is not compatible with the depression of α-glycero-
phosphate oxidation observed with sarcosomes isolated
from heat treated flies. Furthermore, α-glycerophos-
phate dehydrogenase proved to be relatively thermo-
stable, for temperatures as high as 50°C produced only
25 per cent inactivation after a preincubation period of
20 minutes. At 55°C about 15 minutes were required to

produce 50 per cent inactivation (see Fig. 7.1) This
is not surprising, for enzymes are generally known
to be thermostable, and many are inactivated at
temperatures only above about 55 to 60°C (Lehninger,
1975). Moreover, the temperature tolerable limits
are, in general, narrowest for intact animals, somewhat
wider at the level of tissues and cells, and even much
wider when inactivation is measured on isolated
enzymes (Prosser, 1973).

α-glycerophosphate dehydrogenase manifests
a variety of responses to temperature in different
animals. It has been found sensitive to temperature
changes in the screwworm fly (Bush & Neck, 1976),
but thermostable in microorganisms, Bacillus
stearothermophilus (Amelunxen, Sauvan & Mira, 1974).
In the crayfish Cambarus bartoni, cold acclimation
increased the enzyme activity in the abdominal muscle,
but decreased it in the hepatopancreas of the same animal
(Popham & Dandy 1976).

α-glycerophosphate dehydrogenase, is known to
contain iron as a metal component, which is essential
for its catalytic activity (Lehninger, 1975). The
possibility that in vivo heat treatment might have liberated
this metal component, is ruled out by the fact that the

enzyme has tolerated in vitro preincubation temperature
as high as 50°C. However, Yakovleva & Grubnitskii,
(1970) have described the evaluation of enzyme thermo-
stability by determining its catalytic activity as being
not always sufficient. This view was based on the
observation that bull-liver glutamate dehydrogenase
manifests different thermostability in its catalytic
and allosteric properties when forward and reverse
reactions occur; the enzyme is less thermostable in the
direction of the faster reaction. Furthermore, it has
been demonstrated by Tung (1961) that in α-glycero-
phosphate dehydrogenase of brain mitochondria the
degree of inhibition by cholate depends upon the nature
of the electron acceptor; it is drastic when the acceptor
is atmospheric oxygen, whereas low concentrations of
cholate accelerate the reaction when estimated by
methyleneblue. When phenazine methosulfate is used
as electron acceptor, increasing the amount of cholate
decreases the oxidation rate to a level less than that
observed with methyleneblue.

It is possible that in vivo heat treatment may
well interrupt the action of the glycerol 1-phosphate
cycle (Bücher cycle). This is because the cytoplasmic
α-glycerophosphate dehydrogenase has been reported

to be relatively thermolabile (Young & Pace, 1958).
Furthermore, the nonprotein component of this enzyme
has been found removable by several ways including heat
treatment (Van Eys, Nuenke & Patterson, 1959). However,
this should not interfere with the function of isolated
sarcosomes where "Bücher cycle" is already interrupted,
and the action is one-sided, incomplete.

There is now accumulating evidence that the
activity of membrane-bound enzymes is dependent on the
structure of the membrane. Fleischer et al., (1962)
investigating the role of phospholipids in electron
transfer, found that the lipid component of the membrane
is required by the enzymes of the electron transfer system
to show their activity. In addition, Cunningham & Hager
(1971), reported an allosteric effect of phospholipid on
crystalline pyruvate oxidase from Escherichia coli. In
beef heart mitochondria, hydroxybutyric apodehydrogenase
was found to require lipid for activity (Sekuzu et al.,
1963). Yeast mitochondria clearly demonstrated the effect
of membrane fatty acid composition on their function,
by showing high P/O ratios and respiratory control in
yeasts given a diet, which contained high levels of
unsaturated fatty acids, and vice versa (Proudlock et al., 1969).

In the present study <u>in vivo</u> heat treatment might have partially delipidated the sarcosomes, and in doing so altering specific activities of membrane-bound enzymes, including α-glycerophosphate dehydrogenase. This, however, would raise the question as to why this inactivation was not observed when the enzyme was assayed with dye reduction. The stimulatory effect of KCN on glycerophosphate dehydrogenase, reported by Emmelot & Bos (1962), may account in part for this discrepancy.

The results presented in Table (7.3) provide some information about the nature of two different mitochondrial systems under study serving oxidative phosphorylation. The objective of these experiments was a comparative study on these two systems as a function of <u>in vivo</u> heat treatment, and <u>in vitro</u> freezing and thawing of isolated sarcosomes in total absence of ADP. Neither α-glycerophosphate nor pyruvate oxidation showed signs of uncoupling following freezing and thawing of isolated sarcosomes, suggesting that freezing directly inactivates the enzymes associated with both systems. Similar reduction in activity has been reported by Fishbein & Griffin (1976), measuring succinate state IV respiration by isolated frozen and thawed mouse

liver mitochondria, when liquid nitrogen was used as freezing agent. They attributed this to damage to the electron transfer chain.

Whatever the damage is, there appears to be a quantitative correlation between α-glycerophosphate oxidation rate in L.D.$_{50}$ treated, and frozen-thawed sarcosomes. The reduction is approximately of the same magnitude, suggesting that the damage, caused by heat treatment, might be structural. Furthermore, the drastic fall in pyruvate oxidation, caused by freezing and thawing, indicates that the damage is not likely to be merely at the level of the respiratory chain, since the latter is common for both systems (Lehninger, 1964). This conclusion can, also, be valid for L.D.$_{50}$ heat treatment, to which the two systems responded differently, showing a reduction of about 40 and 25 per cent for α-glycerophosphate and pyruvate respectively.

However, when both treatments are applied on the same preparation the effect is different, α-glycerophosphate oxidation appears to have suffered an additive effect of both treatments, resulting in a further reduction of up to about 60 per cent, whereas heat treatment prior to freezing and thawing appears to be beneficial for pyruvate oxidation. This protecting effect of in vivo heat treatment against in vitro freezing may be due to the release of a

constituent, which acted as a specific cryoprotectant for pyruvate oxidation. Fishbein & Griffin (1976) reported that sucrose is a very powerful cryoprotectant for succinate cytochrome C reductase, however, it protected the mitochondrial structure only moderately, and oxidative phosphorylation inefficiently.

CHAPTER EIGHT

ELECTRON MICROSCOPIC APPEARANCE OF SARCOSOMES, ISOLATED FROM CONTROL AND HEAT-TREATED BLOWFLIES, DURING RESPIRATION IN DIFFERENT RESPIRATORY STATES

Introduction:

Insect flight muscle sarcosomes are large relative to mitochondria from other sources. Smith for example, who was one of the early workers in this field, described sarcosomal appearance in the dragon fly Aeshna sp. Smith (1961 b) and the blowfly Calliphora erythrocephala Smith (1963) flight muscle. Davison, (1971 b) and Tribe & Ashhurst, (1972) have also used sarcosomes from this species in their work. Flight muscle sarcosomes from the honey bee, Apis mellifera (Herold, 1965) and tettigoniid, Homorocoryphus nitidulus vicinus (Anstee, 1971) have also been similarly described. In most of these reports, sarcosomes are reported to have a dimension of about 3 um long. The most striking feature, in the ultrastructure of blowfly sarcosomes, is the cristae. These were described by Smith, (1963) as being "unusually highly oriented, forming sub parallel arrays of about 30 cristae per micron, whose successive fenestrations are precisely aligned one behind the next."

Such sarcosomes are, therefore, expected to be very vulnerable and fragile to isolation procedures. Thus, an ultrastructural study of isolated sarcosomes will be helpful in interpreting their functional state, following heating in vivo. It is pertinent that sarcosomal function is known to be dependent upon their structural integrity (Lehninger, 1964).

The reversible ultrastructural changes, which occur as a result of changes in the energy state of sarcosomes is another aspect, which is difficult to follow in situ. These conformational changes have been studied by fixation of isolated mitochondria, respiring in a known energy state. Following fixation the mitochondria are prepared using routine techniques for electron microscopy. The original observations of this kind were made by Hackenbrock, (1966), using mouse liver mitochondria, confirming earlier suggestions made by Lehninger (1959, 1962 b). Similar observations have been reported from Green's laboratory (see Penniston, Harris, Asai & Green, 1968) and G oyer & Krall (1969) in other mammalian mitochondria.

In mouse liver mitochondria, the main forms described by Hackenbrock, (1966) are :

i)   Orthodox conformation which is characterized by a

large matrix volume resulting in decreased intracristal

space and outer compartement volume. This form was

exhibited by mitochondria incubated for 15 minutes in

respiratory states I and IV respiration.

ii) Condensed conformation, which is characterized

by a decreased matrical volume resulting in a large

outer compart ment and intracristal space. This

conformation is displayed, with various degrees of

intensity, in states II, III and after short(1.5 mins)

incubation in states I and IV respiration.

On the other hand, in beef heart mitochondria

the main conformational changes described by Green

and his co-workers (see Green, Asai, Harris &

Penniston, 1968) are:

i) Non-energized configuation, which is characterized

by thin, electron-dense pairs of membranes tightly

compressed together. Such configuration is displayed

in the presence of respiratory inhibitors (antimycin,

or rutamycin).

ii) Energized configuration, which is hallmarked by

pairs of darkly stained parallel membranes separated

by a less electron-dense region. This configuration

took place in the presence of oxidizable substrate.

iii) Energized-twisted configuration, whose diagnostic

Fig. 8.1

Schematic represation, showing the
main configurational changes at
different energy states as described
by other workers

A) Mouse liver mitochondria

   OR = orthodox, CON = Condensed

   (Hackenbrock, 1966)

B) Beef heart mitochondria

   1) Non-energized

   2) Energized

   3) Energized-twisted

   (Green et al., 1968)

A

STATE I   STATE II   STATE III   STATE IV
1·5 mins 15 mins 1·5 mins 15mins 1·5 mins 1·5 mins 15 mins
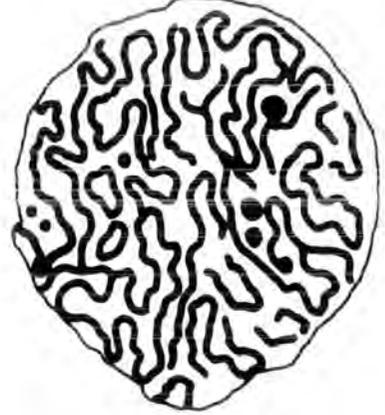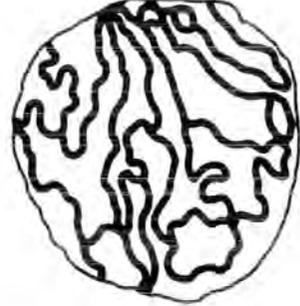
OR

CON

B

features are, the highly twisted character of the cristal membranes, and the absence of an electron-transparent region between them. This configuration is displayed by mitochondria incubated in the presence of oxidizable substrate and inorganic phosphate. The energized-twisted configuration can be discharged, and transformed into non energized, by using uncouplers, ADP or Ca^{2+}. (see Fig. 8.1)

Such conformational changes have, also been reported for Calliphora erythrocephala (Tribe & Ashhurst, 1972), Phormia regina, and Musca domestica (Smith, Smith & Yunis, 1970).

On the other hand, Butler & Judah, (1970) have urged caution concerning such work and suggested that the differences in conformation reported might be artifacts of fixation procedures.

To help interpret the biochemical work reported elsewhere in this study, which shows that in vivo heating disrupts sarcosomal function, it has been deemed important to confirm whether sarcosomes from heated flies undergo the conformational changes in structure, reported by Hackenbrock, (1966).

In fact there is little published work in this field. However, Davison (1971b) has shown dramatic changes in the morphology of the flight muscle sarcosomes

occur in situ, after severe heat treatments, given to the intact animal.

In the present study ultrastructural changes of isolated sarcosomes will be investigated, as well as, the effect of heat treatment on their morphology, together with the response of configurational changes, mentioned above, in presence of α-glycerophosphate, and pyruvate as substrates. It must be re-emphasized that α-glycerophosphate-supported system failed to show state III respiration, when sarcosomes were isolated from lethally heat treated flies, whereas state III respiration was observable in the same sarcosomes, but with pyruvate as substrate. (see Chapter 3).

The observations will be discussed in the light of those previously reported, with particular emphasis on Davison's (1971 b) observations on the one hand, and the results presented in Chapter (3) on the other.

Materials and methods:

Sarcosomes were extracted from 10-day old flies using the 'standard' isolation procedure described in Chapter 2.

Oxygen consumption was measured polarographically at 24°C.

However, for reasons given later, the reaction medium contained :

154 mM KCL

1 mM EGTA

30 mM Phosphate buffer at pH 7.3 at 24°C

10 mM Tris/HCl buffer at pH 7.3 at 24°C

0.4 mg BSA/ml

Fixative and embedding medium:

A) Glutaraldehyde fixative:

2% Glutaraldehyde

2% Acrolein

0.32 M Sucrose

50 mM Cacodylate buffer at pH 7.4 at 24°C

B) Osmium fixative

2% Osmium tetroxide

0.32 M Sucrose

50 mM cacodylate buffer at pH 7.4 on ice

Embedding medium : (Epoxy resin)

|	
Epon 812	47 g
DDSA	21 g
MNA	32 g
DMP-30	1.4 cm^3

Fixation and embedding procedure:

1)   Two cm^3 reaction medium, containing sarcosomes at the desired energy state (see Fig. 8.2), were taken rapidly out of the reaction chamber of the oxygen electrode with a 5 cm^3 pipette, mixed with an equal volume of the glutaraldehyde fixative A in 15 cm^3 polythene centrifuge tubes and allowed to stand at room temperature for 15 minutes.

2)   The tubes were centrifuged at 3600 r.p.m (2200xg) for 10 minutes in a refrigerated 2L MSE centrifuge.

3)   Pellets were resuspended in 2 cm^3 of the osmium fixative B and allowed to stand for 60 minutes on ice.

4)   The tubes were recentrifuged at 3600 r.p.m. (2200xg) in the same centrifuge for a further 10 minutes.

5)   Pellets were dehydrated in a series of ethanol concentrations (50% - 70% - 95% and absolute ethanol) for 10 minutes each. This was followed by 2 x 15 minutes of propylene oxide, left overnight in a 50/50 mixture of propylene oxide and epon embedding medium (see above).

Fig. 8.2

Schematic representation of an

oxygen electrode trace, showing

the points at which fixation of the

respiring sarcosomes was

achieved.

X

0.5 µmole
ADP

B

αGP

C

D

Y

A

B

0.5 µmole
ADP

αGP

C

Z

A

B

0.5 µmole
ADP

Pyr. + Prol.

C

D
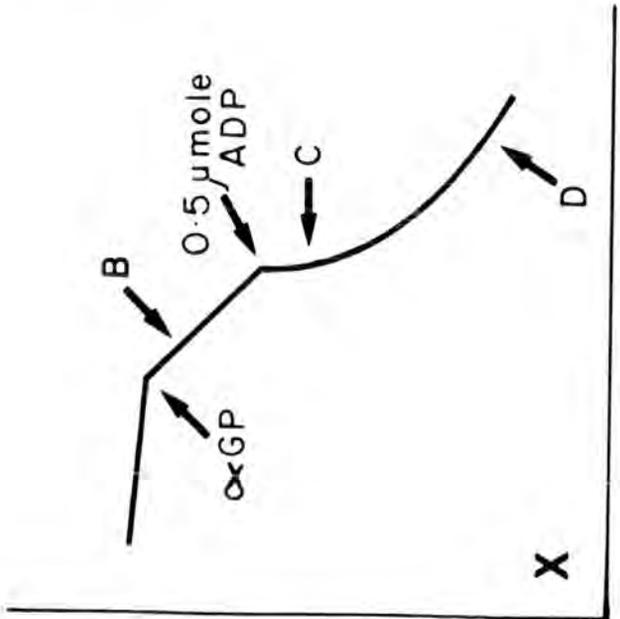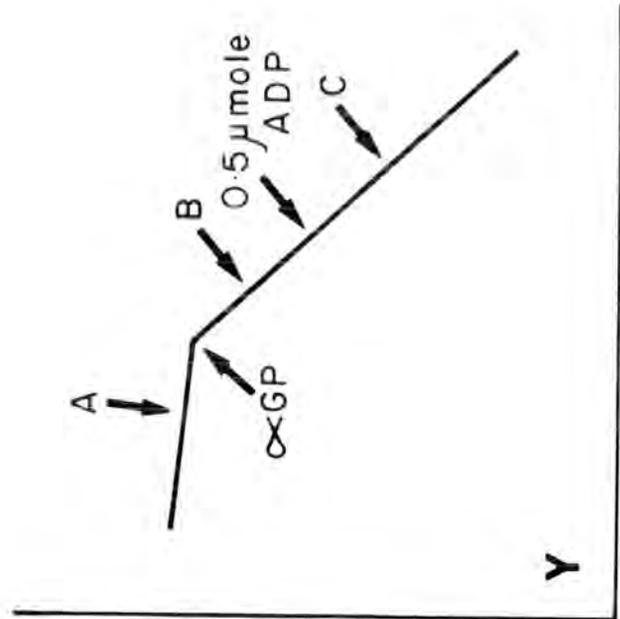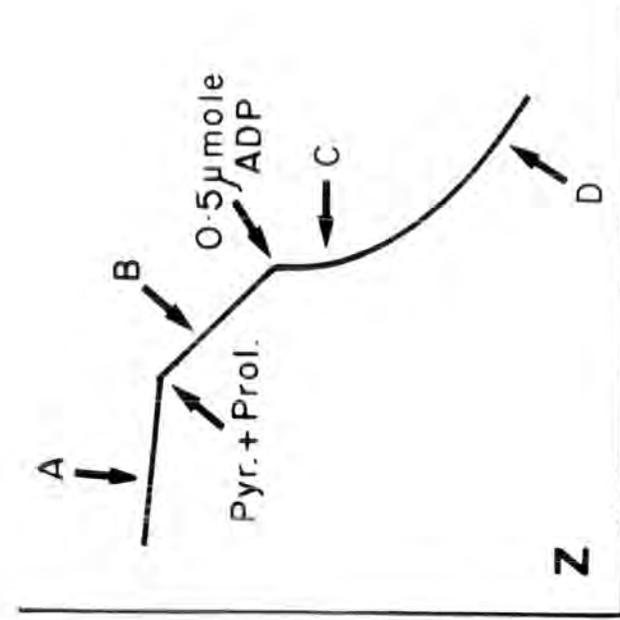
6)   Pellets were infiltrated for 6 hours in absolute
epon, then transferred to foil dishes containing absolute
epon, which was polymerized at 60° for 48 hours.

7)   Ultra-thin sections were cut with glass knives
using either a Reichert Omv3 or LKB ultramicrotome.

8)   The sections were mounted on uncoated grids,
stained with uranylacetate (Mercer & Birbeck 1966),
followed by lead citrate and examined with an AEI EM 801
electron microscope operated at 80 KV.

9)   The entire experiment was repeated twice using
control sarcosomes, and sarcosomes isolated from $L.D._{50}$
treated 10-day old flies.

Observations

A) Control unheated sarcosomes

        33 mM α-glycerophosphate was the only substrate
used. Sarcosomes were removed from the reaction
chamber at the points shown in Fig. 8.2 (X) B,C & D.

Substrate rate respiration

        Fig. 8.3A shows the appearance of the sarcosomes

in "substrate rate" respiration. The vast majority of

the sarcosomes appeared intact, but a few are fractured.

Some sarcosomes also appear vacuolated in that the

cristae do not entirely fill the intramitochondrial space.

In these, and all the other electron micrographs

of sarcosomes it is difficult to determine the extent of

the matrix and intracristal spaces. This is because

of the very close packing of the cristal membranes

in these sarcosomes. This makes the identification of

the various conformational states, described by

Hackenbrock (1966) and reported earlier in the Introduction,

very difficult to interpret in sarcosomes.

However, no sarcosomes in what could be described

as a "discharged" or "non-energized" form were seen

in substrate respiration.

State III Respiration

Sarcosomes removed from the reaction chamber

in state III respiration (Fig. 8.3B) were of mixed

appearance. Again very few seemed damaged or fractured

in any way. However, many sarcosomes were in the

"discharged" form, as described by Tribe & Ashhurst

(1972), that is having a condensed matrix leading to spaces

developing intracristally, see Fig. 8.3 B. Although the

Fig. 8.3
___

Three electron micrographs showing sarcosomes

respiring at different energy states, isolated from

the flight muscle of control (unheated) 10-day old

adult blowflies.

Substrate   : 33 mM α-glycerophosphate

Isolation   : "Standard" method (see Chapter 2)

Reaction   : 154 mM KCl, 1 mM EGTA,
      medium
                     30 mM Phosphate buffer at pH 7.3
                                               at 24°,
                     10 mM Tris/ HCl buffer at pH 7.3
                                               at 24°.

Fixative   :1) 2% Glutaraldehyde

                     2% Acrolein

                   2) 2% Osmium tetroxide.

A)   Sarcosomes fixed at the point B shown in

     Fig. 8.2 (X), in "substrate rate" respiration. The

     majority are intact, some are fractured or vacuolated.

     No "discharged form is apparent, all seem to be

     "energized".

B)   Sarcosomes fixed at the point C shown in Fig. 8.2(X)

     in state III respiration. A mixed population of

     "energized" and "discharged" sarcosomes can be

     seen.

C)   Sarcosomes fixed at the point D shown in Fig. 8.2(X)

     in state IV respiration. A mixed population can be

A

E

im
om→

B

D

C

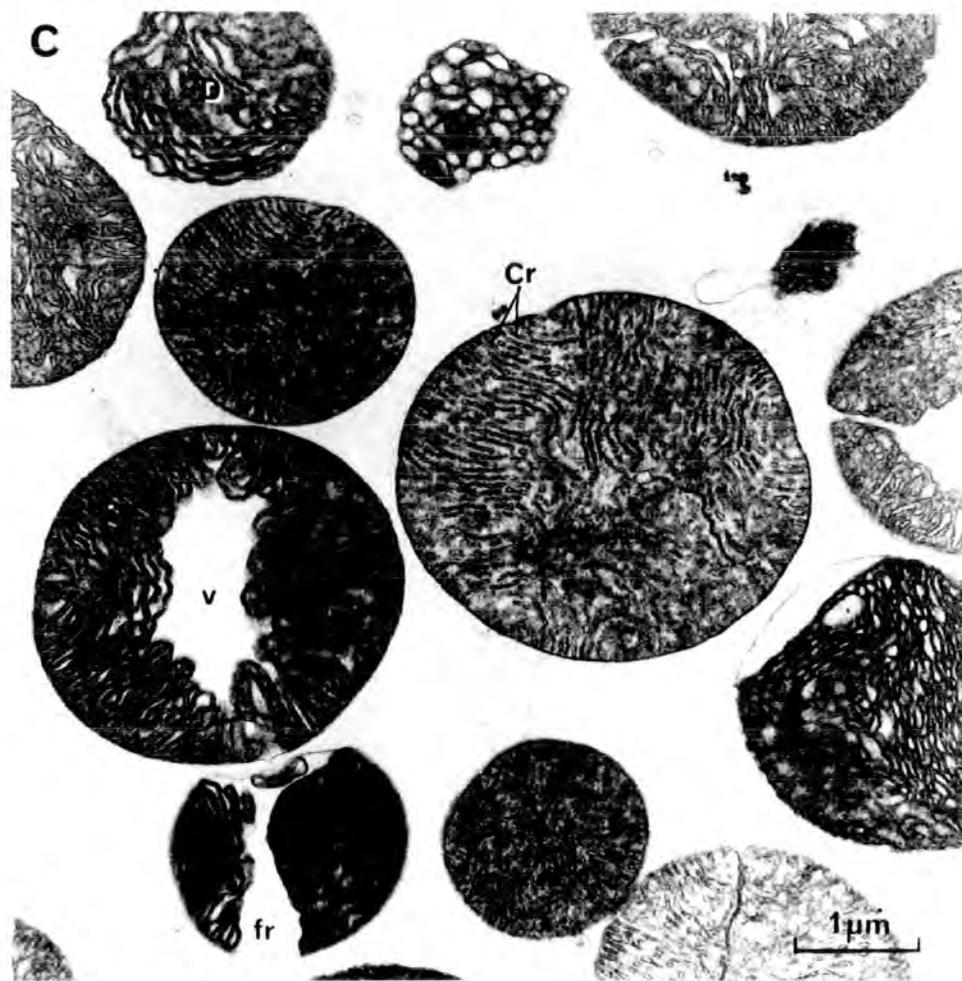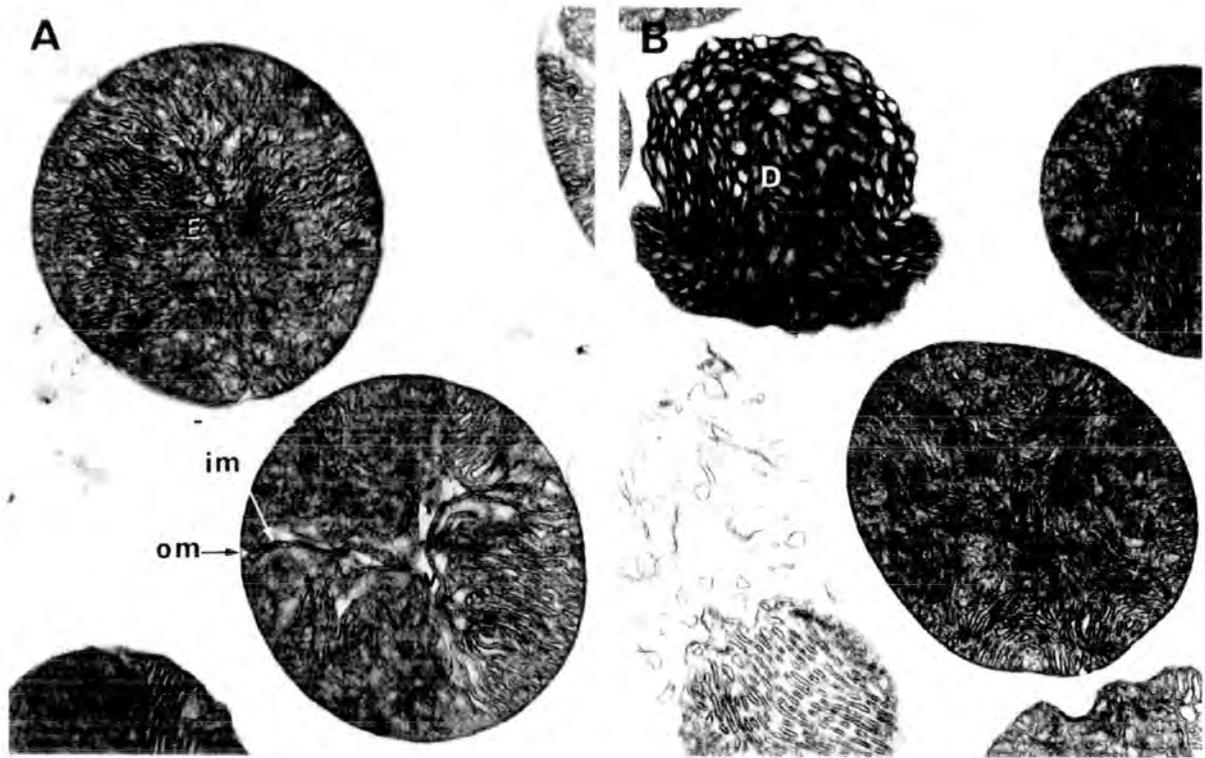D

Cr

v

fr

1μm

Fig. 8.3 (cont.)

seen. Most of the sarcosomes are "energized",

some are "discharged". Fractured and

vacuolated sarcosomes are also evident.

E   =   Energized

D   =   Discharged

fr =   fractured

V   =   vacuolated

Om =   Outer membrane

im =   Inner membrane

C   =   Cristae

All plates are the same magnification as indicated

in C.

sarcosomes showing this "discharged" form do so to greater or lesser extents. Some sarcosomes however remain in the "energized" form.

State IV Respiration

Fig. 8.3 c shows the appearance of sarcosomes taken in state IV respiration. Once more the vast majority are intact. As can be seen, mixed populations exist with both "energized" and "discharged" forms evident. It is not possible to distinguish with certainty the appearance of the sarcosomes in states III and IV on this evidence.

B) Sarcosomes from flies given L.D.$_{50}$ heat treatment

i) α-glycerophosphate-supported respiration

The sarcosomes were taken from the reaction chamber at the points shown in Fig. 8.2 (Y) A - C.

Fig. 8.4A shows, in low magnification, the appearance of the sarcosomes immediately after addition to the reaction medium, but before the addition of substrate. As can be seen, several are ruptured, in some they are C-shaped. There is also little evidence of swelling of sarcosomes. As can be seen at higher

Fig. 8.4

Electron micrographs showing sarcosomes

isolated from the flight muscle of 10-day

old adult blowflies given an L.D.$_{50}$ heat

treatment. Fixation was achieved after

addition of sarcosomes to the reaction medium,

at the point A shown in Fig. 8.2 (Y).

For isolation, and reaction and fixation

conditions see Fig. (8.3).

A)   low magnification showing intact, ruptured

      and C-shaped sarcosomes.

B)   higher magnification showing many sarcosomes
&
C)   in the "non-energized" form. Note the dense

      granules formed in the sarcosomes.

NE   =   Non-energized.

 g   =   granules.

A. fr, D, v, 2 µm

B. NE, 1 µm

C. g, 1 µm
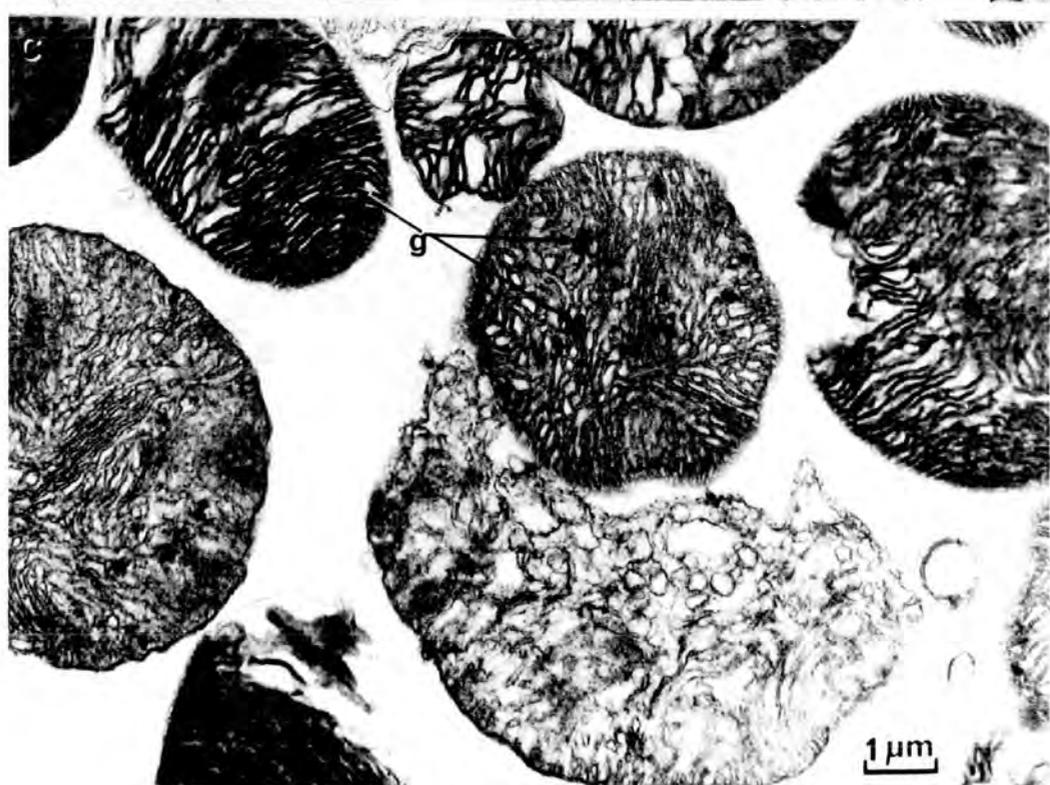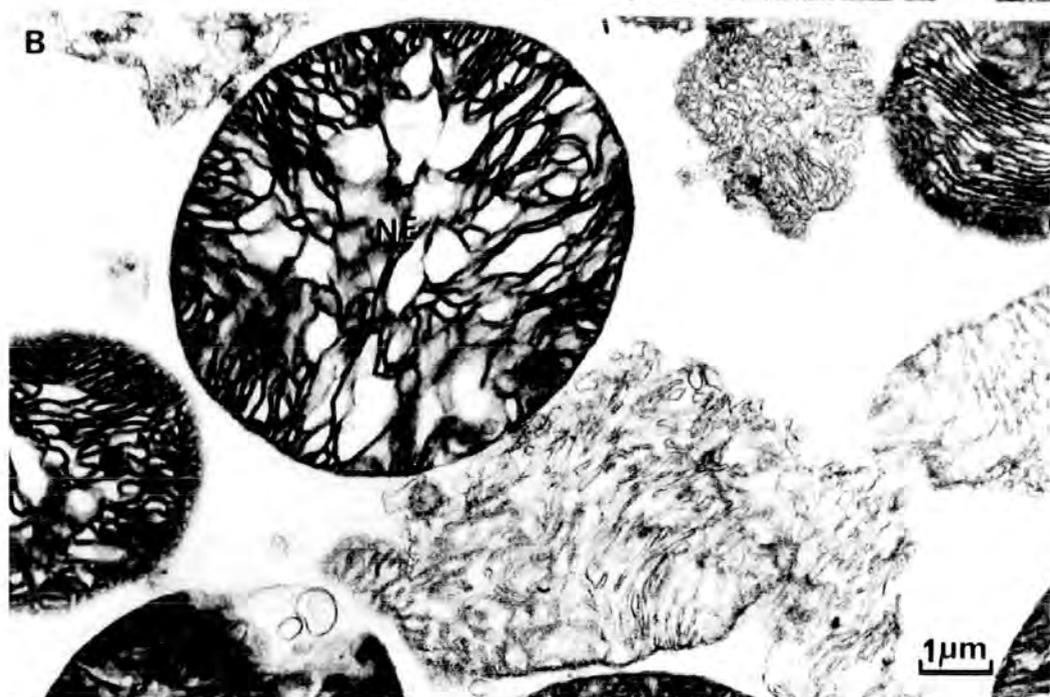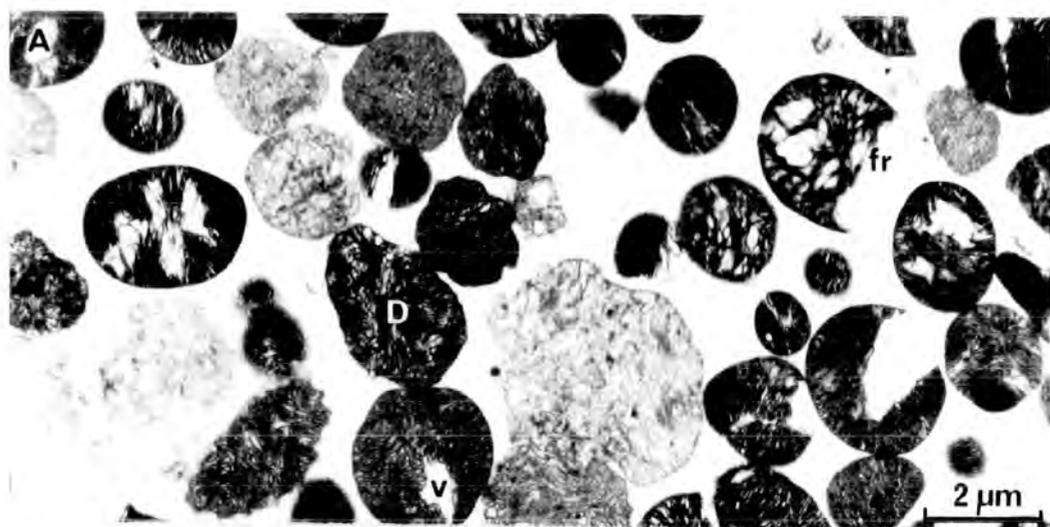
magnification (Fig. 8.4 B & C) many of the sarcosomes
are not in the "energized" form rather they are closer
in appearance to the "non-energized" configuration
.described for beef heart mitochondria respiring in the
presence of antimycin or rutamycin, Green et al.,(1968).
In some sarcosomes there are also dense granules formed,
these were not evident in any control sarcosomes.

Substrate-rate respiration

The appearance of the sarcosomes is shown in
Fig. 8.5A. Many now appear grossly swollen, some even
to the point of rupture. Those sarcosomes that are not
yet swollen are apparently in the "discharged" configuration.
Fig. 8.5B also shows, at higher magnification, grossly
swollen sarcosomes. Note that dense granules occur
in both swollen and unswollen sarcosomes.

State III respiration

The most significant difference in the appearance
of the micrographs of the pellets of sarcosomes, after
the addition of ADP, is the high background of sarcosomal
fragments, see Fig. 8.6A. Grossly swollen sarcosomes
are evident, and the normal sarcosomes appear to be in
the "discharged" configuration.

Fig. 8.6B shows at higher magnification a sarcosome
in the final stages of swelling, as well as many cristal

Fig. 8.5

Electron micrographs showing sarcosomes isolated from the flight muscle of 10-day old adult blowflies, given an $L.D._{50}$ heat treatment.

Fixation was achieved in presence of 33 mM α-glycerophosphate, at the point B shown in Fig. 8.2 (Y).

For isolation, reaction and fixation conditions see Fig. (8.3)

A) Low magnification, showing intact, swollen and ruptured sarcosomes. Many of the intact sarcosomes are apparently in the "discharged" form. Dense granules are also evident.

B) Higher magnification showing grossly swollen sarcosomes.

S = swollen.

sf = sarcosomal fragments.

A

g

st

D

2 µm

B

S

1 µm

Fig. 8.6

Electron micrographs showing sarcosomes isolated from the flight muscle of 10-day old adult blowflies, given an L.D $_{50}$ heat treatment.

Fixation was achieved in presence of 33 mM α-glycerophosphate and 1 umole ADP, at the point C shown in Fig. 8.2 (Y).

For isolation, reaction and fixation conditions, see Fig. (8.3).

A) Low magnification showing "discharged", swollen and fragmented sarcosomes.

B) Higher magnification, showing many cristal fragments, grossly swollen sarcosomes and intact sarcosomes apparently displaying the "discharged" form. The dense granules are also evident.

C. fr = Cristal fragments.

A

B

S

g

C fr

2 µm

1 µm
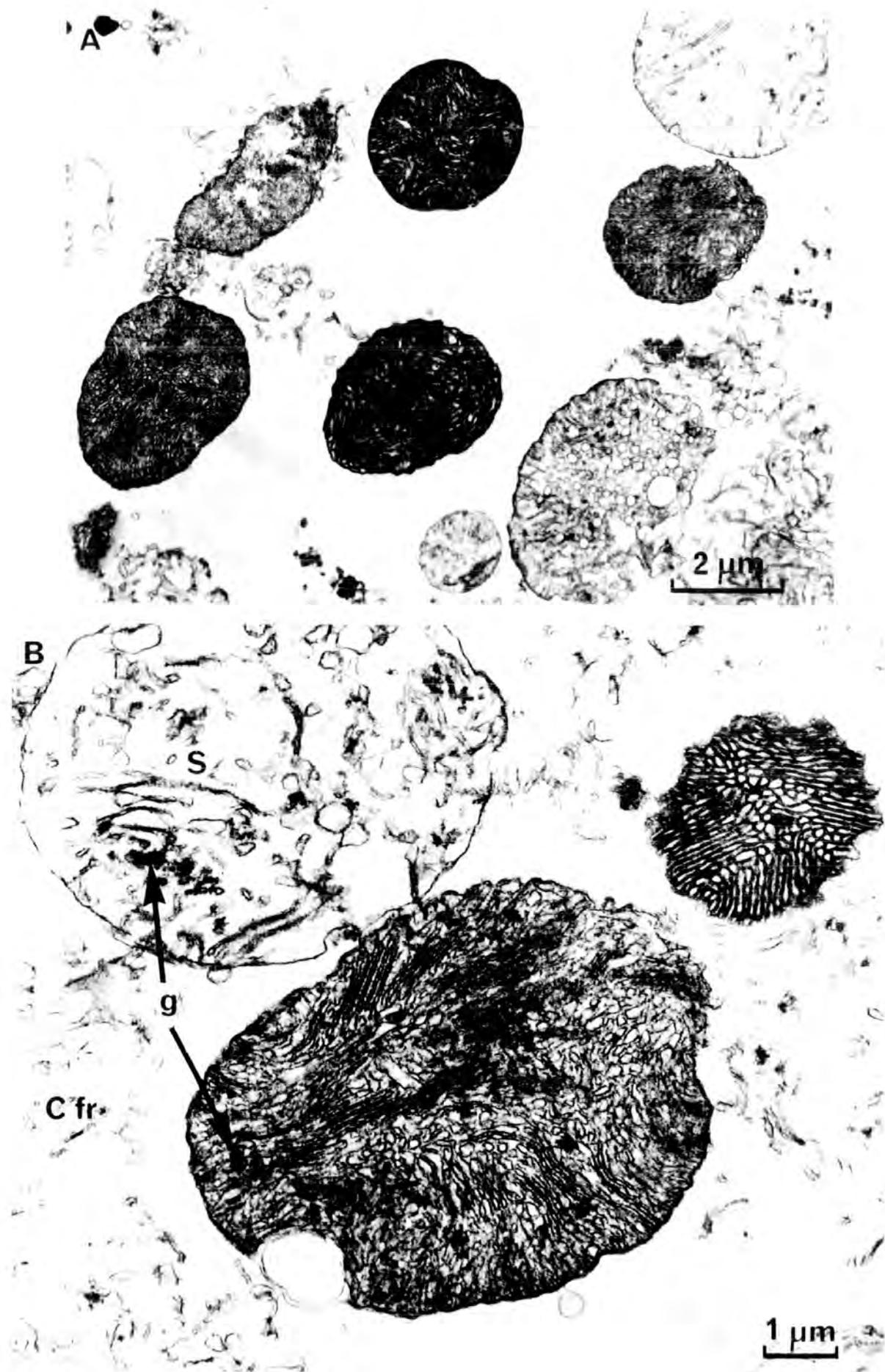
fragments from broken sarcosome. Note also the normal sarcosomes, again which seem to display the "discharged" configuration. Swollen, unswollen and cristal fragments all possess dense granular accretions.

Note that as respiratory control is lost in sarcosomes from heated flies, when respiring on α-glycerophosphate, no attempt has been made to determine the appearance of the pellets from sarcosomes in state IV respiration.

ii)   Pyruvate + proline-dependent respiration

The sarcosomes were removed from the reaction chamber at the points shown in Fig. (Z) A-D.

Substrate rate respiration

Fig. 8.7A shows the appearance of the sarcosomal pellet taken from the reaction chamber when respiring pyruate + proline. There is a mixed population. A proportion show signs of swelling, but many others are normal, of the latter group, sarcosomes displaying the "discharged" and the energized configuration can be seen. Note also the incidence of dense granules in the cristae. Fig. (8.7 B & C) show in detail both swollen and normal sarcosomes from the same pellets.
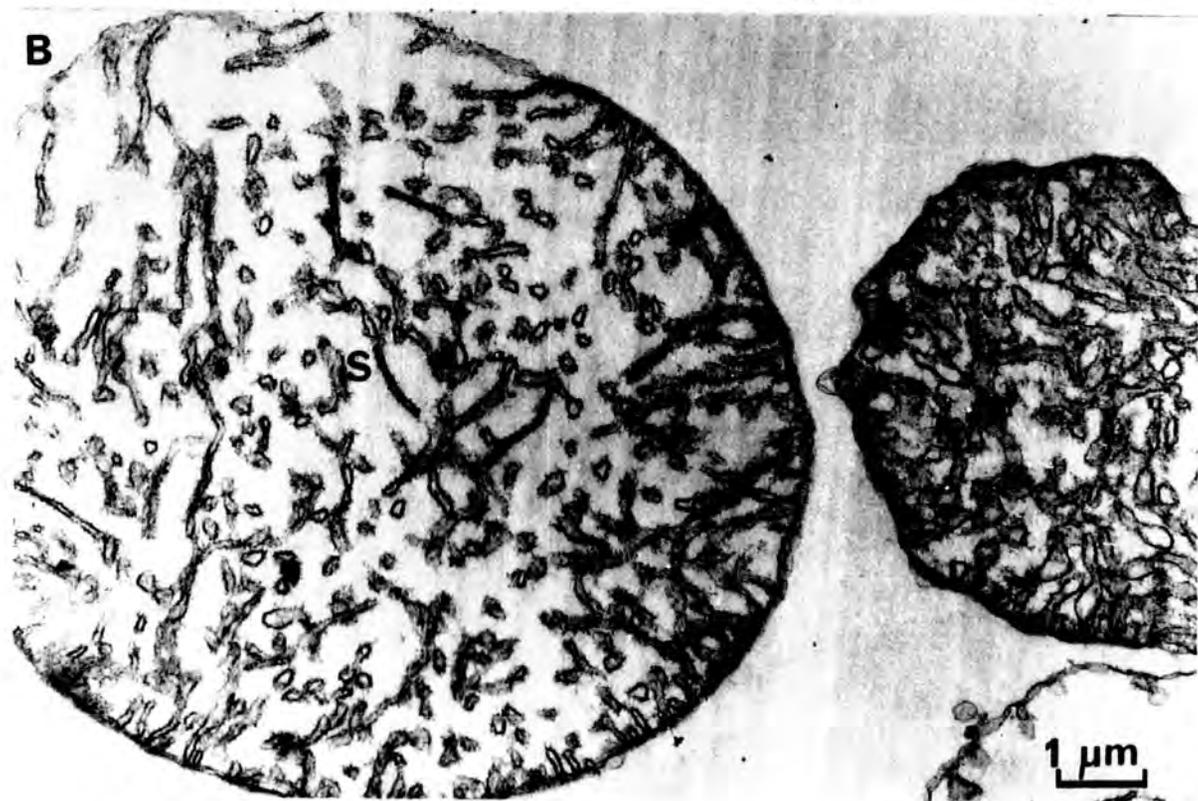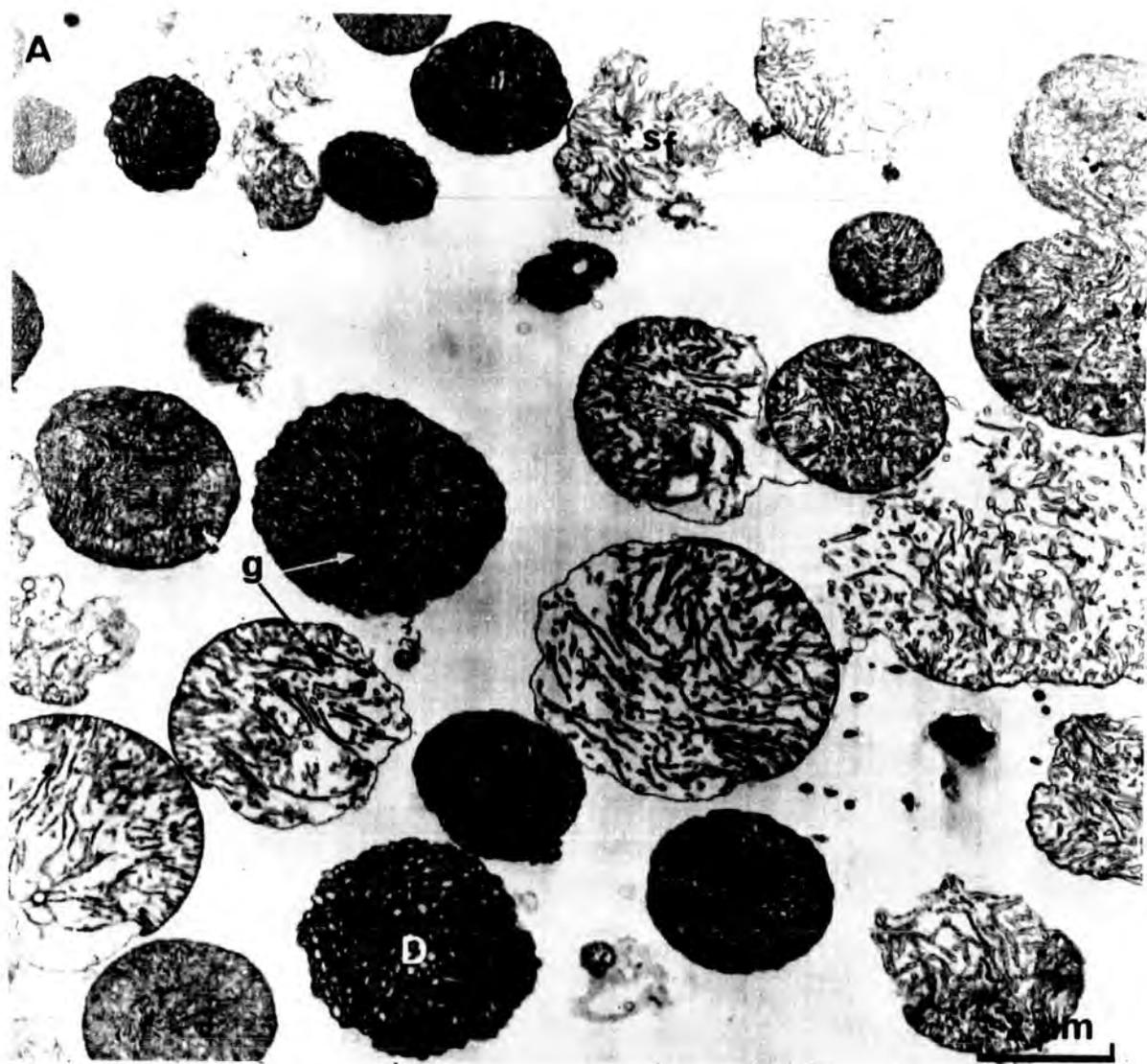
Fig. 8.7

Electron micrographs showing sarcosomes
isolated from the flight muscle of 10-day old
adult blowflies, given an L.D.$_{50}$ heat
treatment.

Fixation was achieved in presence of 2.5 mM
pyruvate plus 2.5 mM proline, at point B shown
in Fig. 8.2 (Z).

For isolation, reaction and fixation conditions
see Fig. (8.3).

A) Low magnification showing a proportion of the
    sarcosomes with signs of swelling, the others
    are normal and appear to be displaying the
    "discharged" and "energized" forms.

B) Higher magnification of the same pellet
&
C) described above.

    The dense granules are also observable.

A

v

fr

S

2 μm

B

g

E

1 μm

C

S

1 μm
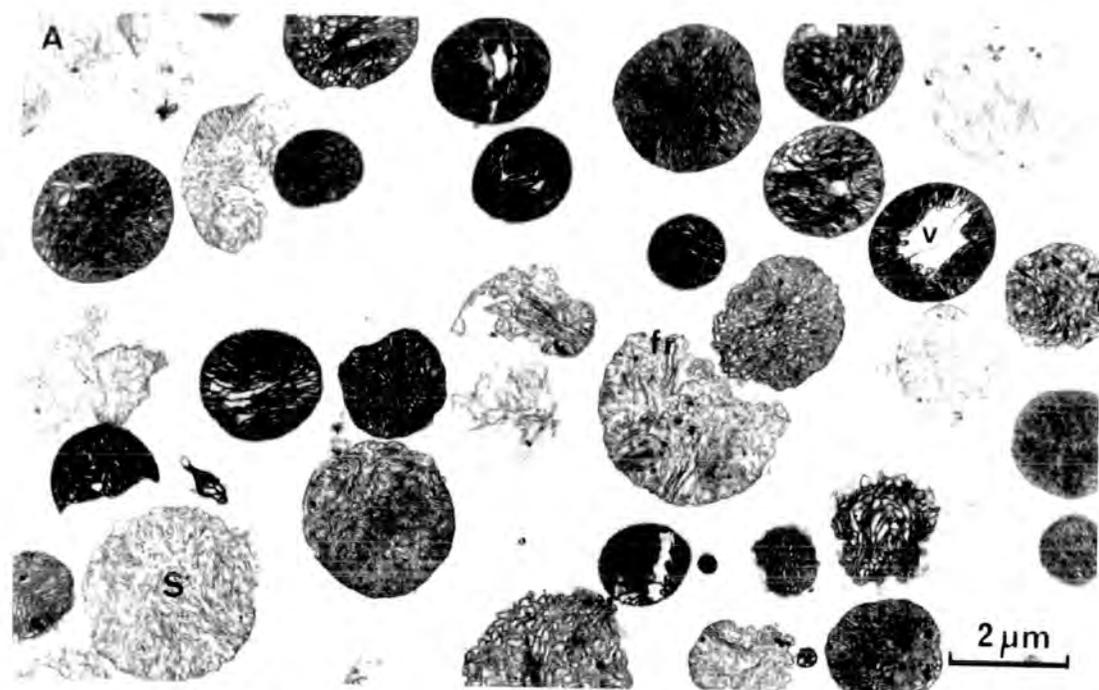
State III respiration

Again the general appearance of the electron micrograph seen at low magnification, is of a mixed population of sarcosomes, see Fig. 8.8A. There is perhaps more cristal debris than is evident in Fig. 8.7 but there does not seem to be a greater proportion of sarcosomes that are swollen.

The normal looking sarcosomes almost all seem to be in the "discharged" configuration. Dense granular accretions are again in evidence, see Figs. 8.8 B & C.

State IV respiration

Fig. 8.9A shows at low power the appearance of the sarcosomes in state IV respiration. More sarcosomes appear to be at an advanced state of swelling, and this probably accounts for the large amount of sarcosomal debris in the background. Sarcosomes in both "energized" and "discharged" forms are present, however, higher magnification views can be seen in Figs. 8.9 B & C.

Discussion:

Although some configurational changes have been observed in the present study, they are in no way identical with those reported for mouse liver (Hackenbrock,1966) or
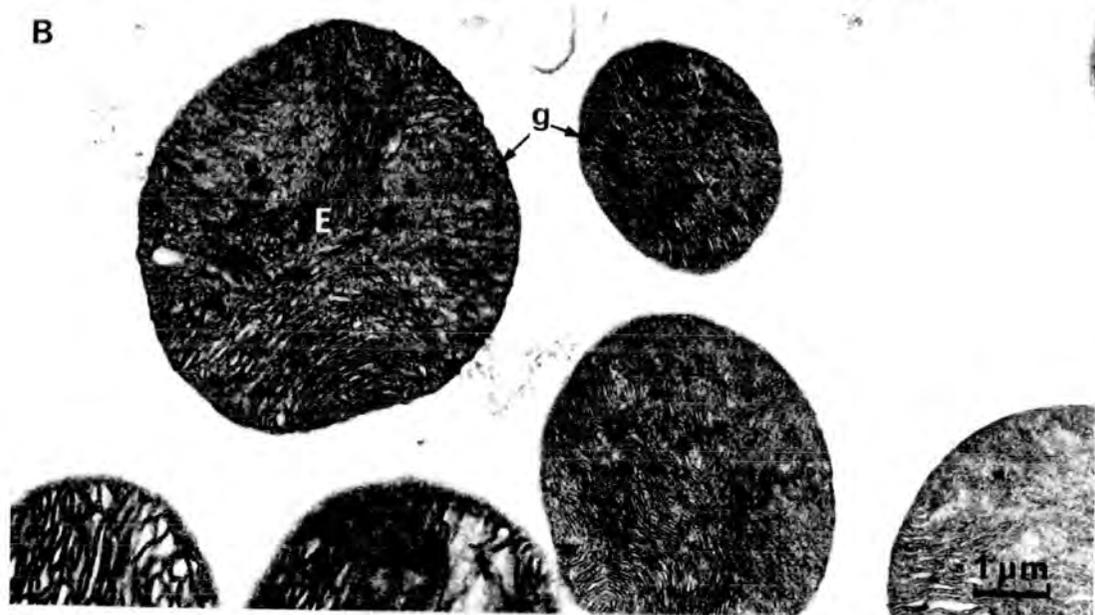
Fig. 8.8

Electron micrographs showing sarcosomes isolated from the flight muscle of 10-day old adult blowflies, given an $L.D._{50}$ heat treatment.

Fixation was achieved in presence of 2.5 mM pyruvate plus 2.5 mM proline, and 1 uMole ADP, at the point C shown in Fig. 8.2 (Z).

For isolation, reaction and fixation conditions see Fig. (8.3).

A) Low magnification showing a mixed population of sarcosomes. The majority are intact, few are swollen or fragmented.

B) & C) Higher magnification showing almost all the sarcosomes seen to be in the "discharged" form.

Dense granular accretions are in evidence.
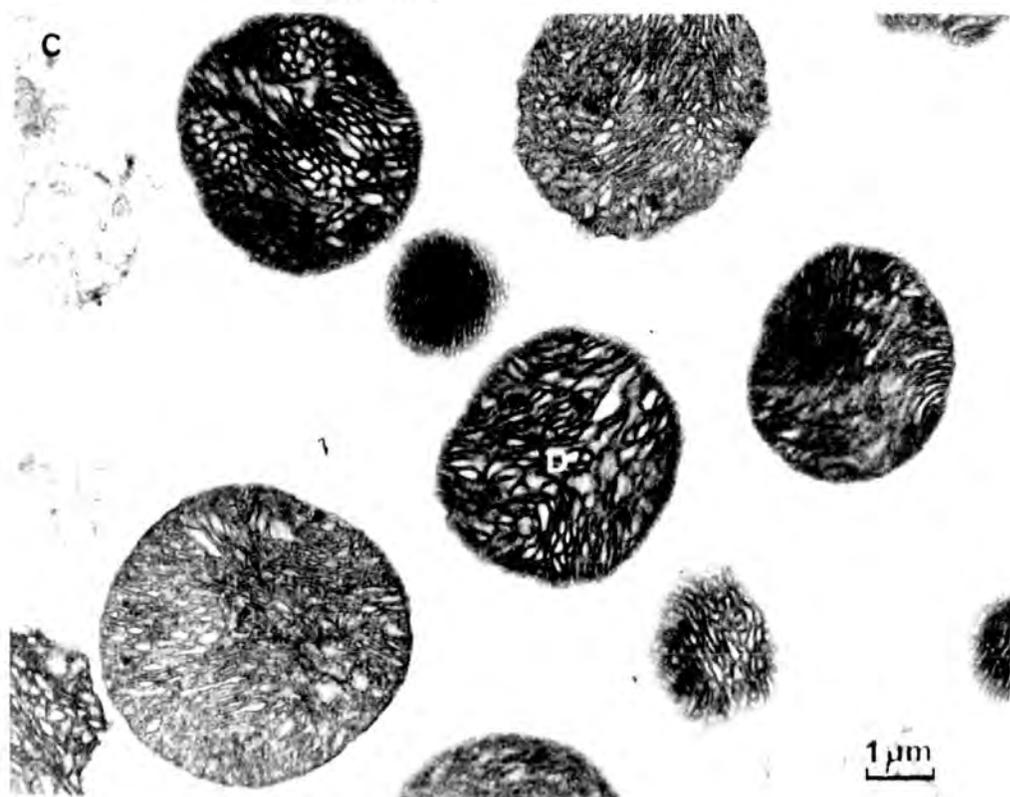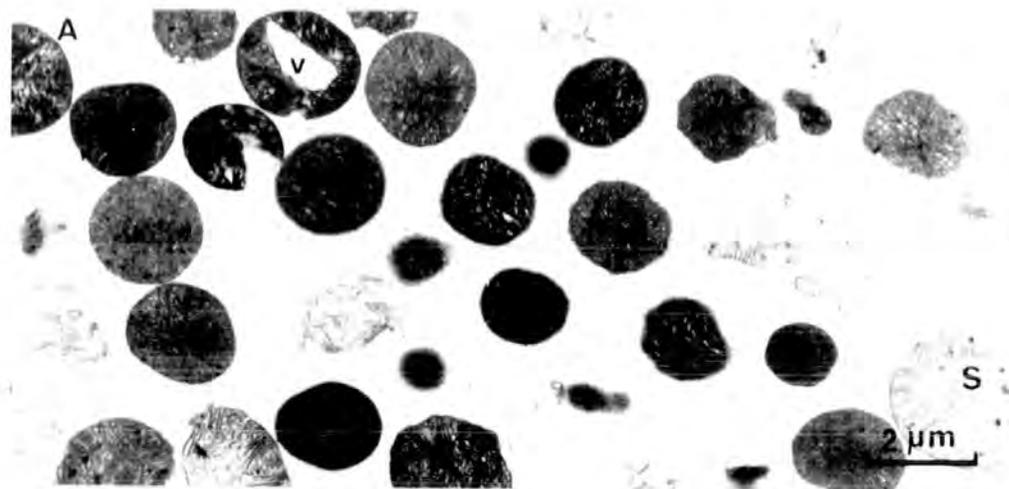
Fig. 8.9

Electron micrographs showing sarcosomes isolated

from the flight muscle of 10-day old adult

blowflies, given an L.D.$_{50}$ heat treatment.

Fixation was achieved in presence of 2.5 mM

pyruvate plus 2.5 mM proline, and 1 u mole ADP

(state IV), at the point D shown in Fig. 8.2 (Z).

For isolation, reaction and fixation conditions

see Fig. (8.3).

A)   Low magnification showing sarcosomal debris

     in the background, some sarcosomes are at

     an advanced state of swelling. The majority

     of the sarcosomes are intact and seen to

     display both "energized" and "discharged"

     forms.

B)   Higher magnification of the sarcosomes described

     above.

     Dense granules can also be seen.

A

C fr

D

2 µm

B

E

C

D

D

1 µm
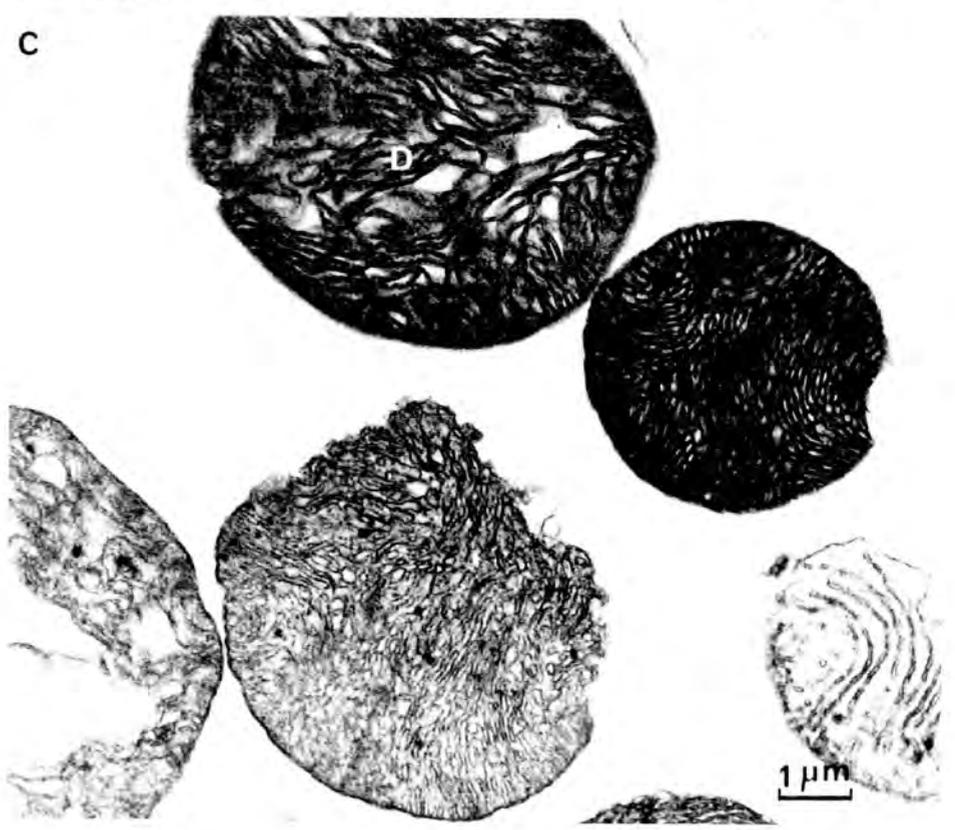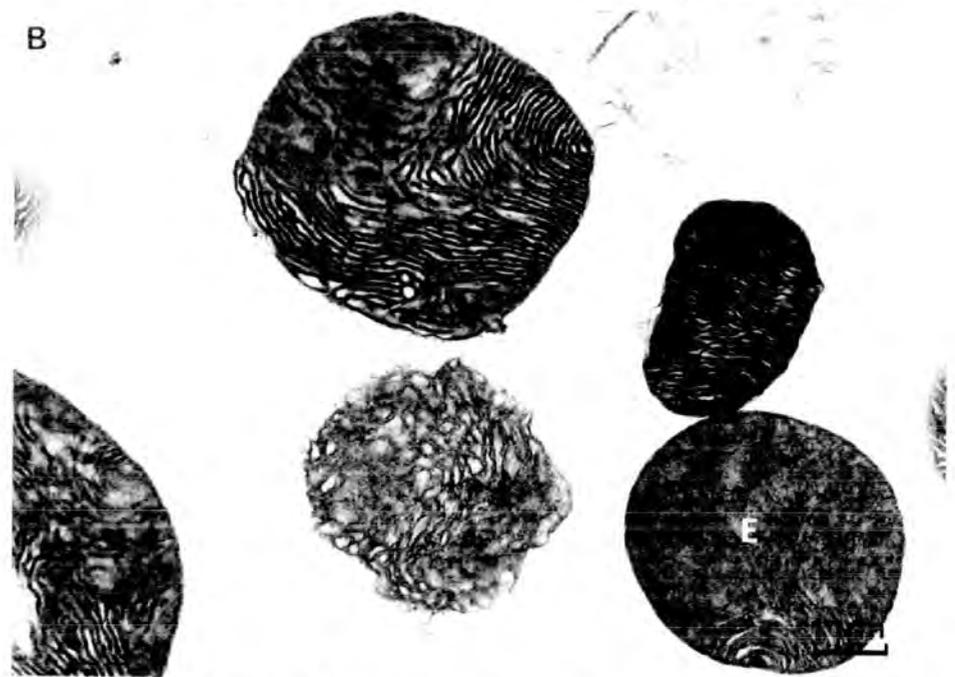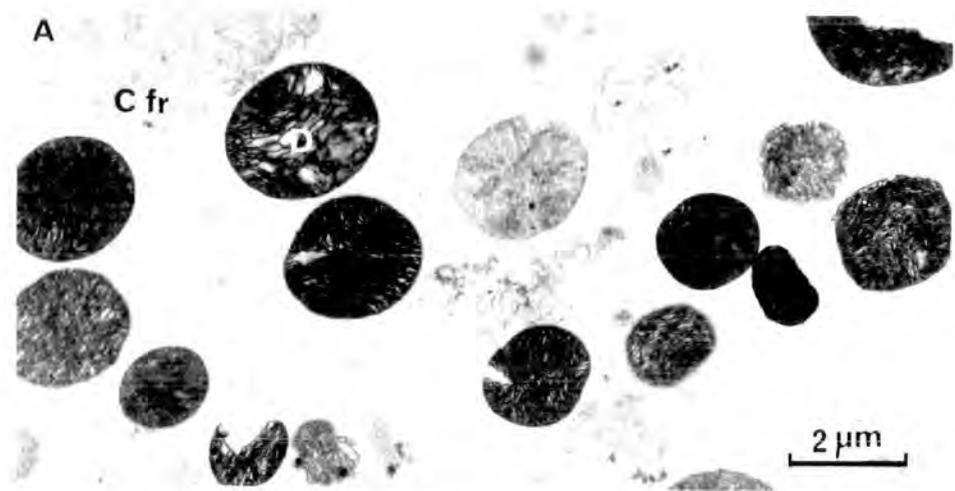
beef heart mitochondria (Green et al., 1968). There are major differences in many respects; for example a highly condensed form of mouse liver mitochondrial inner components and an expanded outer compartment are not clearly recognizable in insect sarcosomes. The characteristic features of the cristae of beef heart mitochondria displayed in the energized-twisted configuration were not as diagnostically observed in the present study. The interpretation of these differences may be based on the dis-similarity between mammalian and insect mitochondrial structure. In the latter case sarcosomes manifest an extreme close-packing of cristae, Calliphora (present study; Smith, 1963; Davison 1971b), (for a variety of insects see Smith, 1961a, 1961b, 1962), which may be up to 30 cristae per micron. For this reason it is often difficult to identify the matrix from the intracristal spaces. Green & Hatefi, (1961) concluded that the greater the number of cristae per unit area, the greater the oxidative function of the mitochondria. Furthermore, they attributed the relatively low density of cristae contained in liver mitochondria not only to their low rate of activity, but also to their involvement in auxiliary functions other than the primary mitochondrial function.

Isolated blowfly flight muscle sarcosomes with more or less similar morphology to those described above have been previously reported (Tribe & Ashhurst, 1972).

The criticisms of Butler & Judah (1970) must be mentioned. They have questioned such functionally induced morphological changes in isolated mitochondria and presented evidence after detailed investigations that these changes might be artifacts. Their argument was based on the observations that various fixatives produce various morphological patterns; the same effect was seen to be brought about when various osmoeffectors were used. Furthermore, the depth of sectioning was also recorded to be a significant factor; although this has, already, been admitted by Hackenbrock, (1966), who pointed out that such a risk has been avoided by using quick-sampling and micropellet procedures. Bronk & Jasper, (1970) have also questioned that mitochondrial condensation is obligatory for oxidative phosphorylation.

Sjöstrand's view was also cautious in this context. On several occasions, he and his co-workers pointed out that conventionally used fixatives and dehydrants are responsible for modifications observed in the structure of mitochondrial membranes by protein

denaturation (Sjöstrand & Barajas, 1968; Sjöstrand & Kretzer, 1975). Furthermore, Sjöstrand & Bernhard, (1976) have shown that there is a correlation between the appearance of intracristal space and protein denaturation; that is when no such space was observed in preparations where care was taken to reduce the risks for denaturation of mitochondrial membrane proteins. It was obvious from their results that osmium tetroxide can produce artifactual intracristal spaces.

Most recently, Sjöstrand (1977) questioned measurements of mitochondrial membrane thickness in that, alterations might have taken place caused by nonreversible plastic flow of the embedding material and the membrane material during sectioning.

Muscatello, Guarriera-Bobyleva & Buffa, (1972), carried out detailed investigations on configurational changes caused by changes in respiratory states in unfixed, negatively stained rat liver mitochondria. They were able to demonstrate many relevant features of configurational changes, which were in accord with observations made on similar material using different techniques reported by other workers; for example, Hackenbrock, (1966), Harris, Penniston, Asai & Green, (1968), Packer & Tappel, (1960) and Penniston et al.,(1968).

However, some discrepancies have been recorded as to

the length of the time elapsing during transition from

one respiratory state to the other. Moreover,

statistically significant variations were found in

the extent of metabolically linked structural changes

when different substrates were used. In their

conclusion Muscatello et al., (1972) did not support the

hypothesis proposed by Hackenbrock (1966) and Green &

Harris, (1969), that reversible changes in configuration

may form a basis for the mechanism of energy

transformations in mitochondria and that a high-energy

form of the electron transport membrane may serve

to produce ATP-synthesis.

In the present study, the reaction medium that

incubated sarcosomes to be processed for electron

microscopical studies did not include Mg^{2+}. That was

done to avoid the possibility of a Mg^{2+} induced discharge

of a probable energized-twisted configuration, and to

ensure a low state IV pyruvate-supported respiration.

Ca^{2+} has been shown to discharge energized-twisted

configuration in beef heart mitochondria (Green et al.,

1968).

The contradictory state of the literature on the

interpretation of such data, and particularly on the

relationship between the structural appearance and functional state of isolated sarcosomes, makes this present work difficult to interpret. The problem is exacerbated by the dissimilarity in appearance of blowfly flight muscle sarcosomes and those used by Hackenbrock, (1966) and Green ét al., (1968) to describe the different energized states of mitochondria.

As has been pointed out in the Results section, no clear structural changes were seen in sarcosomes in states III and IV, as might be predicted from the work of Green et al., (1968). It is clear, however, that in 'substrate rate' respiration, prior to the addition of ADP, there was no visual evidence for sarcosomes in the "discharged" form. This was not true of sarcosomes in state III respiration where sarcosomes of various forms could be identified. It was particularly evident that relative changes had occurred involving the matrix and intracristal spaces. It was not possible to distinguish between sarcosomes in state III and IV respiration, the same type of mixed populations existed.

What is very clear however is that sarcosomes from flies receiving an L.D.$_{50}$ treatment display a different appearance from those from control flies. Sarcosomes taken after suspension in the reaction medium,

but before substrate addition, appear to be in the
"non-energized" configuration as described by Green
et al., (1968) for beef heart mitochondria in the presence
of inhibitors such as antimycin.

Also in contrast to sarcosomes from control
flies, obvious signs of swelling can be seen in some
sarcosomes. This swelling seems to be either progressive
with time, or is induced by the addition of substrate
to the reaction medium. This is because many sarcosomes
from heated flies, taken when respiring α-glycerophosphate,
show gross swelling, some are even at the point of
rupture. The swelling clearly continues after the addition
of ADP and from the high levels of sarcosomal fragments,
seen in the background, many sarcosomes have clearly
burst by this time.

The only "normal" looking sarcosomes present,
seem to be in the "discharged" form. This is in marked
contrast to control sarcosomes in state III respiration
where both the "discharged" and "energized" forms exist
in the same pellet, see Fig. 8.3.

Thus quite marked differences exist between the
control and experimental sarcosomes, the latter from
heat-treated flies, are characterized by the high degree
of swelling and rupture, and the apparent absence of the

energized state. One further point, also noted by
Davison (1971b) using sarcosomes in situ, is the appear-
ance of many dense granules associated with cristal
membrane in the experimental sarcosomes.

As has been shown in Chapter (3), respiratory
control in sarcosomes from L.D.$_{50}$ heated flies cannot be
demonstrated when α-glycerophosphate as the substrate,
but is present when pyruvate and proline are being
respired. This point makes it necessary to determine
whether the same morphological effect can be seen in
sarcosomes, from L.D.$_{50}$ treated animals, respiring
pyruvate and proline, as has just been discussed for
α-glycerophosphate-dependent respiration.

From comparisons of the appearance of sarcosomes
in Figs. 8.7 - 8.9 with the corresponding figures obtained
during α-glycerophosphate dependent respiration, the
following observations can be made.

First swelling occurs in a proportion of sarcosomes,
and in state III and IV respiration, the obvious quantities
of sarcosomal debris, reflect the breaking up of swollen
sarcosomes. However the swelling process does not seem
to procede as rapidly in pyruvate and proline respiration,
or not to such a great extent.

The 'normal' unswollen sarcosomes in 'substrate

rate" respiration are in a mixed population, with both 'energized' and 'discharged' configurations being displayed. This is again in contrast to the appearance in substrate rate respiration by α-glycerophosphate where all appear to be in the discharged form. After the addition of ADP (state III respiration) all sarcosomes appear to take up the "discharged" configuration, whereas in state IV respiration mixed populations are seen again. Thus, it does seem that even in sarcosomes from L.D.$_{50}$ treated flies, if pyruvate and proline are the substrates, then the sarcosomes can display conformational changes. This is in marked contrast to the situation in the same sarcosomes respiring α-glycerophosphate.

A point of similarity throughout is the appearance of the dense granules in sarcosomes from heated flies. These seem to be accretions on cristal membranes, and even can be seen in the fragments of ruptured sarcosomes.

Similar work, investigating ultrastructural changes in heat damaged sarcosomes, but in situ has been carried out by Davison, (1971b), who predicted that his observations may be quite different to those in heat damaged isolated sarcosomes. His prediction was

based on a report set forth by Weinbach, Garbus & Sheffield, (1967) that the injection of pentachlorophenol into rats caused no morphological changes in their liver mitochondria in situ, but when isolated they displayed distorted morphology and reduced respiratory performance. Such changes were identical to those observed when the drug was applied in vitro. However, a comparison between in situ and in vitro studies of sarcosomes of heat treated blowflies may reveal some information about the nature of the damage. Most obvious is the susceptibility of the sarcosomes to isolation, although this might not be the primary lesion in the failure of oxidative phosphorylation, since submitochondrial fractions are capable of carrying out this function. (Lehninger, 1975).

As has been emphasized, swelling is a dominant feature of sarcosomes from heated flies. A series of articles concerned with the mechanism of swelling in beef heart mitochondria has been published by Green's laboratory. Green and his co-workers concluded that there are two types of swelling (Asai, Blondin, Vail & Green, 1969); (i) an energized swelling, which takes place by electron transfer or ATP-hydrolysis, and (ii) a pseudoenergized swelling, which takes place not under energized conditions, but in the presence of appropriate

salts. In this context, it is noteworthy that pseudo-energized swelling manifested configurational changes in beef heart mitochondrial inner membrane, similar to those observed at different energy states. This raises questions as to whether the saltsdirectly induce that effect or do they effectively energize the membrane? It was found that the entire swelling process is a concerted phenomenon (Blondin, Vail & Green,1969; Blondin & Green, 1969).

In this regard, the work carried out in Lehninger's laboratory is of great interest. A series of articles has been published investigating the swelling process in isolated rat liver mitochondria (Lehninger & Schneider, 1959; Lehninger, Ray & Schneider, 1959; Lehninger & Gotterer, 1960; Lehninger, 1962a; Neubert & Lehninger, 1962). It was found that certain physiologically occurring substances (e.g. inorganic phosphate, thyroxine) drive such mitochondria to undergo swelling. Such swelling can be reversed by ATP in the presence of Mg^{2+} and BSA (Lehninger, 1959). However, ATP failed to reverse mitochondrial swelling induced by glutathione if the mitochondria employed are present in relatively dilute suspensions. This observation led Lehninger and his co-workers to the finding that glutathione causes leakage into the medium and dilution of a critical factor, which was designated as "contraction factor", whose presence

at a certain effective concentration is required in
extrusion of water from mitochondria. Interestingly
enough this factor was found to be heat-labile. The
subject has been reviewed by Lehninger (see
Lehninger, 1962 b).

However, these observations would not have
been possible to make on sarcosomes fixed in situ.
Nevertheless, there is one major common feature
of heat effect, that is the existence of electron dense
granules. Davison (1971 b) suggested two alternative
answers to the nature of these granules, i) they were
a precipitation of inorganic ions, and ii) they may be
parts of the inner mitochondrial membrane which have
become rolled in ball-shaped masses.

Ashhurst, (1967), observing similar dense
deposits on electron micrographs of osmium fixed
flight muscle sarcosomes of giant water-bugs in situ,
suggested that their preferential occurrence over the
matrix may be indicative of some substance in discrete
areas readily stainable with lead. In the blowfly,
however, such phenomenon was not clearly observable
in untreated sarcosomes, and the occurrence of such
substance in experimental sarcosomes suggests that there
are certain thermally induced alterations in the

stainability of some mitochondrial components. This

may present a basis for further investigations.

Furthermore, the fact that such opaque granules were

not observable when L.D.$_{50}$ treated sarcosomes fixed,

stained and sectioned in situ (Davison 1970) suggests

that isolation of sarcosomes, following L.D.$_{50}$

treatment given to the intact animal, might enhance

precipitation of such deposits. Alternatively, the

swelling that isolated, heat treated sarcosomes

undergo makes these granules more easily observable.

In spite of all the uncertainties over interpretation of

such work, morphological differences exist between

control and heat-treated sarcosomes. Most

interesting is the different appearance of heat-treated

sarcosomes respiring α-glycerophosphate and pyruvate,

for this correlates closely with other biochemical

differences reported in Chapter 3.

CHAPTER NINE

TEMPERATURE STRESS AND BRAIN NEUROSECRETORY CELLS IN THE BLOWFLY CALLIPHORA VICINA

Introduction

It is long understood that hormones play an important integrative role in many aspects of organism function, this is true not only in vertebrates but also in invertebrates. The literature on arthropods is particularly well documented.

What is less well appreciated is the cellular action of many hormones, and whilst the cyclic AMP mediated action of many mammalian hormones has been recently demonstrated, the site of action of insect hormones, for example, remains to be demonstrated conclusively.

Hormones have also been shown to have metabolic effects in insects. Clarke and his co-workers claim that brain neurosecretions stimulate protein synthesis, Clarke & Langley (1963), Clarke & Gillott(1967).

Novak (1966) has suggested brain neurosecretions are the source of "activation" hormone, a hormone with a variety of direct and indrect effects on insect metabolism. Thomsen, (1952) describes various roles for this hormone, including its involvement as a

trophic factor in corpora allata function, in <u>Calliphora</u>

<u>erythrocephala</u>.   Thomsen & Möller (1952) also believe

it stimulates intestinal proteinase activity in the

blowfly.

Both diuretic and antidiuretic hormones have

also been claimed exist in a variety of insects. The

most convincing demonstration is of course in

<u>Rhodnius</u> by Maddrell (1966), but Highnam, Hill &

Gingell (1965) made claims that such hormones exist

in <u>Schistocerca</u>.

The 'stress' response in mammals, results

in a release of pituitary gland adrenocorticotrophic

hormone which activates the adrenal cortex to release

glucocorticoid hormones. The existence of an analogous

stress response has not been demonstrated in insects,

but it is well documented that quite dramatic changes

in the neurosecretory material in some brain cells

take place, and temperature change is one factor that

can apparently induce these changes , Clarke (1966);

Peacock, Anstee & Bowler (1976); Ivanovic, Jankovic-

Hiadni & Milanovic (1975).

The early work of Thomsen (1949) showing that

respiration in the blowfly (<u>Calliphora</u> <u>erythrocephala</u>)

is impaired following allatectomy, points towards insect

hormone(s) having an analogous effect on mitochondria as has been shown for thyroid hormones in mammals (Hulbert, Augee & Raison, 1976; Tata, Ernster, Lindberg, Arrhenius, Pederson & Hedman, 1963; Bronk, 1966; Lee & Lardy, 1965).

Indeed Keeley, (1970) working on Blaberus discoidalis fat body mitochondria, and Slama, (1964) working on Pyrrhocoris apterus, both report that cardiatectomized-allatectomized insects have mitochondria with lower respiratory performance as compared to controls. Thus evidence is accumulating for endocrine control of mitochondrial respiration, see also Clarke & Baldin, (1960) and Ralph & Matta, (1965). The subject has been recently reviewed by Steele, (1976).

Taking these various points into account, together with the heat-induced injury to sarcosomes, it seemed necessary to determine whether the heat damage was secondarily caused via heat-induced alterations in neurosecretions in the insect.

Consequently the histological appearance of the brain neurosecretory cells was studied in control and heat-treated flies.

Materials and Methods:

Control and heat-treated 10-day old adult male
flies were fixed in Bouin's fluid after removing abdomens,
wings and legs. Embedding was carried out in paraffin
wax. Serial horizontal sections (8 µm) were cut through
head and thorax using a Cambridge Rocking Microtome,
then mounted on glass slides, to which they were attached
with egg albumen.

For staining the neuroendocrine system the
aldehyde fuchsin technique, described by Ewen (1962),
was employed. Aldehyde fuchsin was supplied by
High Wycombe, Bucks., England. A stock solution of
0.75 g of aldehyde fuchsin in 100 cm^3 of 70 per cent
ethanol was made up.

Staining procedure:

1) Paraffin wax was removed from the sections with
xylene and the sections were then hydrated via graded
dilutions of ethanol.

2) The sections were oxidized for one minute in
acid permanganate, which was made up as follows:

$$1 \text{ volume } 2.5\% \text{ KMnO}_4$$
$$1 \text{ volume } 5\% \text{ H}_2\text{SO}_4$$
$$6 \text{ volumes Dist. water}$$

3) The sections were rinsed in distilled water.

4) Decolorization was performed in 2.5% sodium bisulphite.

5) The sections were passed through rinses of distilled water, 30% and 70% ethanol to aldehyde fuchsin and stained for two minutes.

6) The sections were washed in 95 per cent ethanol and differentiated for half a minute in acid-alcohol

$$\text{Absolute alcohol} \quad 100 \text{ cm}^3$$
$$\text{Concentrated HCl} \quad 0.5 \text{ cm}^3$$

7) Rinses of 70 per cent and 30 per cent ethanol and distilled water were carried out.

8) The following composition of phosphotungstic-phosphomolybdic acid was used to mordant the sections for 10 minutes:

Phosphotungstic acid	4.0	g
Phosphomolybdic acid	1.0	g
Distilled water	100	cm^3

9) After rinsing in distilled water, the sections

were counterstained for $1\frac{1}{2}$ hours in the following dye:

Distilled water	100 cm^3
Light green SF yellowish	0.4 g
Orange G	1.0 g
Chromotrope 2R	0.5 g
Glacial acetic acid	1.0 cm^3

10)   The slides were then well rinsed in 0.2% acetic acid in 95% ethanol, dehydrated rapidly through absolute ethanol, cleared in xylol and finally mounted in canada balsam.

The heat treatments given to the animals were either $L.D._0$ or $L.D._{50}$ (see Chapter 2). Flies allowed to recover, were fed on water and sugar _ad libidum,_ and recovery temperature was maintained at $24°C \pm 0.5$. Housing the flies for recovery was as described in Chapter 4.

Observations:

Figure (9.1A) shows a representative example of the 4 flies fixed and sectioned as untreated controls. Figure (9.1B) represents an example of the 3 flies subjected to $L.D._{50}$ heat treatments prior to fixation and sectioning. An example of the 2 flies subjected to an $L.D._0$ heat treatment is shown in Fig. (9.1C).

Fig. 9.1

Horizontal sections through the brain of 10-day old adult male blowflies after the following treatments:

A) Control untreated, representative of 4 samples.

B) $L.D._{50}$ treated, representative of 3 samples.

C) $L.D._0$ treated, representative of 2 samples.

D) 18 hr recovery from $L.D._0$ treatment, representative of 3 samples.

E) 48 hr recovery from $L.D._0$ treatment, representative of 2 samples.

Neurosecretory cells (NC) are shown having the same appearance in all groups. They are large and filled with neurosecretory material; in some cases transport nuclei can also be seen (white arrow). The location of the neurosecretory cells is in all cases near the median furrow of the pars intercerebralis (PI).

Methods : see text.

All plates are the same magnification as indicated in E.
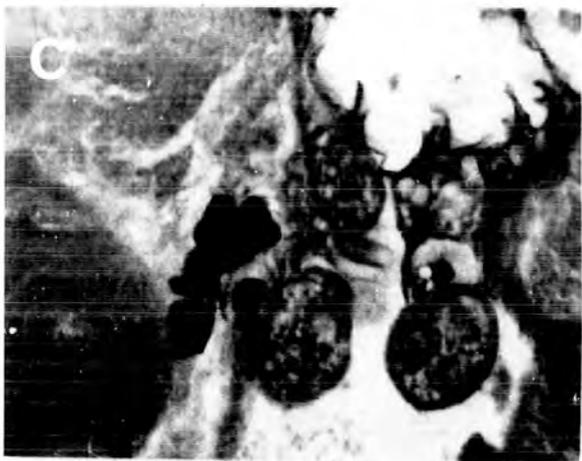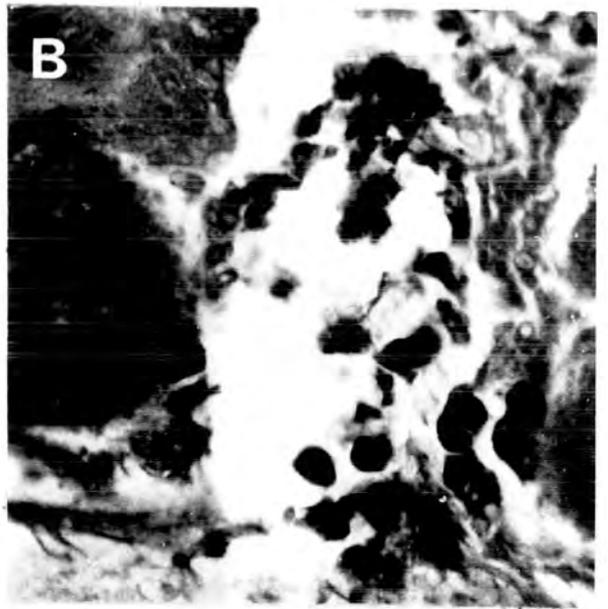
Figures (9.1D & E) show representative examples of the

3 and 2 flies allowed to recover at 24°C from an L.D.$_0$

heat treatment, for 18 and 48 hours, respectively.

Examination showed in all cases the neurosecret-

ory cells had the same appearance.

As can be seen from Fig. (9.1A - E), the

neurosecretory cells are conspicuous, and neurosecretory

material is shown as a dark stain filling their cytoplasm.

The cells are in all cases located near the median

furrow of the pars intercerebralis. In some of the cells

large, transparent nuclei are also observable.

Discussion

Under the conditions described in the present

study no major difference in neurosecretory activity has

been observed between differently heat treated, recovering

or control flies. This suggests that neurosecretory cells

in the brain of the blowfly are not sensitive to heat

treatment given to the intact animals in the way described.

The aim of the present histological investigations

was to study the effects of heat damage, and the nature

of repair mechanism during recovery from sublethal

heat treatment. The suggestion made by Davison (1970)

that protein synthesis might be involved in the repair
mechanism of heat damage, could be studied further
on the basis of one of the following approaches. First,
heat treated flies are allowed to recover following the
application of one of the drugs which are known to
inhibit protein synthesis such as ethidium bromide
(Zylber, Vesco, & Penman, 1969). If such flies show
recovery judged by their appearance and their flying
ability, or measured by respiratory performance of
sarcosomes isolated from their flight muscle, then heat
injury is not likely to have been caused by an irreversible
denaturation of proteins, for this would require new
proteins to be resynthetized, in the blowfly <u>Calliphora</u>
<u>erythrocephala</u>. The process of protein synthesis has
been suggested to be affected by the hormonal control of
the intestinal proteinase activity (Thomsen and Moller,
1959).

This technique, however, has not been employed
in the present study, since there exists considerable
uncertainty about what effects such drugs might have on
living organisms, other than inhibition of protein
synthesis. Indeed, there is evidence that ethidium
bromide inhibits oxidative phosphorylation in rat liver
mitochondria respiring on either glutamate or succinate
(Higgins <u>et al</u>, 1975). The nature of this inhibition is

not fully understood; nor is it well known yet whether

mitochondria obtained from other sources would exhibit

similar symptoms in response to the action of ethidium

bromide. It is, also, not known whether other protein

synthesis inhibitors are capable of producing a similar

diminishing effect on state III respiration.

Furthermore, such agents have been reported

to alter the rate of degradation of some enzymes. This

subject has been reviewed by Schimke & Doyle (1970).

Dingley & Smith (1968) used cycloheximide to

inhibit protein synthesis in <u>Drosophila</u>, recovering from

sublethal heat treatments. The observation that damaged

Drosophila did recover, led the authors to conclude

that protein denaturation is not involved in thermal

injury. However, it has been recently claimed by

Milner, (1976) that cycloheximide selectively inhibits

cytoplasmic protein synthesis.

In the present study it is reasoned that if heat

damage causes irreversible inactivation of proteins then

in recovering flies these proteins will need to be replaced.

As has been mentioned earlier many aspects of insect

physiology are under hormonal control, for example the

production of intestinal proteinases (Thomsen & Moller,

1959). Those authors suggest cellular protein synthesis

might also be indirectly affected by the dependence of
the supply of aminoacids by the action of the proteinase.
The source of this hormone (and others) is the brain
neurosecretory cells, Novak (1966). It is also of
interest that several workers have shown that
temperature changes are one of the factors which do
cause changes in the appearance (and by extrapolation
function too) of these neurosecretory cells (Clarke,
1966; Peacock et al. 1976).

As no difference was found, in the appearance
of the brain neurosecretory cells, between control,
heat treated and recovering flies, it is difficult to make
any statements whether protein synthesis is involved in
the repair mechanism of heat damage, and therefore
impairment of α-glycerophosphate-dependent respiration
in sarcosomes isolated from heat-treated blowflies may
or may not be a result of damaged respiratory enzymes.

However, the work is not complete, and further
investigations are required, to find out how lethal
heat treatments, given to the intact insect, affect
other parts of the endocrine system, particularly
corpora cardiaca and corpus allatum.

CHAPTER TEN

GENERAL DISCUSSION

The principal aims of this thesis are to understand better the mechanisms involved in thermal injury at the cellular level. The work owes much to an earlier study on the same stock of insects (<u>Calliphora</u> <u>erythrocephala</u>) by Davison (1970). In the latter work, Davison clearly showed that giving flies heat treatments equivalent to L.D.$_{50}$ impaired flight muscle sarcosomal function. The sensitivity of these sarcosomes, to <u>in vivo</u> heat treatment, so closely mirrored the sensitivity of the whole animal that subsequently Davison & Bowler (1971) were able to propose that these sarcosomes would make a good model system for studying heat death. The authors recognized that the damage they reported to have occurred in sarcosomes from heat treated flies, may have been, at least in part, because those sarcosomes were made more sensitive than control sarcosomes to the isolation procedures.

With this point in mind the early part of this thesis concerned the use of two different isolation procedures, the 'standard' procedure and one using the proteolytic enzyme 'nagarse'.

One other problem that arose was the choice of the appropriate raction medium to determine sarcosomal respiratory performance. A variety of recommended

media are published for sarcosomes from several

insect species, but only infrequently are the reasons for

the choice of a particular set of conditions given, Birt,

(1961); Balboni, (1967); Hansford, (1971); Tribe &

Ashhurst, (1972); Bursell & Slack, (1976). It was

necessary therefore to make a preliminary study to assess

the composition of reaction media that were suitable for

use with both isolation techniques and different substrates.

The essence of the 'nagarse' technique is to

minimize the mechanical damage that might occur to the

sarcosomes during homogenization, and so, sarcosomes

are released following enzymatic digestion of the muscle

fibres. This technique however was found to affect

α-glycerophosphate supported respiration in such a way

that a dramatic fall in the QO_2 values and RCl were

recorded as compared with values obtained when sarcosomes

are isolated using the conventional method described in

Chapter 2. Pyruvate-supported respiration, on the other

hand, was not so affected when different isolation methods

are applied.

It was therefore decided that, during the course

of the present study, in those experiments where

α-glycerophosphate was the substrate, flight muscle

sarcosomes would be isolated without the use of

nagarse, except in experiments where only substrate-rate

oxidations were measured, e.g. the allosteric effect

of Ca^{2+} (Chapter 6), and effect of freezing and thawing (Chapter 7).

On the other hand, no difference was observed in respiratory activities of flight muscle sarcosomes isolated in presence or absence of the proteolytic enzyme nagarse, when pyruvate and proline are used as substrate. It was concluded that these results strongly suggest that the proteolytic enzyme might have altered the structure of the sarcosomal membrane, and in doing so affecting the activity of membrane-bound enzymes, such as α-glycerophosphate dehydrogenase (EC 1.1.99.5).

The principal point about the composition of the reaction media seemed to rest on whether Mg^{2+} was present or not. When pyruvate and proline were the respiratory substrates (Table 3.2, media I and II) the presence of Mg^{2+} depressed RCI, this seems to be largely owing to an increase in state IV respiration. The reason for this is in some dispute and it has been proposed to be because of an increased Mg^{2+}-stimulated ATPase activity.

In fact there is a dispute about Mg^{2+} and whether it stimulates ATP-ase activity in intact (Azzone and Carafoli, 1960) or only in aged and damaged mitochondria

(Sugano and Nagai 1971; Waino 1970).

Consequently, it was decided to omit Mg^{2+} from reaction medium, where pyruvate and proline are substrates.

Magnesium ions are however a requirement in α-glycerophosphate driven respiration by the same sarcosomes, see Table 3.2, and so it is necessary to include them in the reaction medium when α-glycerophosphate is the substrate for oxidative phosphorylation.

Throughout this study, substrates were injected into the reaction chamber immediately following the injection of the sarcosomes, this avoided the problems of preincubation of sarcosomes prior to the initiation of respiration.

Having established these points, the other parts of the work were concerned with thermal sensitivity and heat death in the blowfly, <u>Calliphora vicina</u>. This problem has been dealt with, on the same insect, by Davison (1970), who suggested that a breakdown of oxidative phosphorylation might be a main factor in heat death in this species. The consequence would be a loss of ATP synthesizing capacity and may lead to the inactivation of ATP dependent pathways. Heat-shocked ($L.D._0$) animals cannot fly nor right themselves and so

co-ordinated movement is definitely impaired in heat-damaged flies, they do gradually regain their flight ability, and Davison & Bowler (1971) have shown this closely parallels the recovery of normal sarcosomal function as witnessed by high values for RCl and normal values of ADP:0. Earlier Naguib & Christophersen (1965) reported in yeast that cellular ATP levels fall at high temperature. Bluhm & Ordal (1969) also report reversible inactivation of several respiratory enzymes directly after sublethal heat treatment of <u>Staphylococcus aureus</u>. This, together with the work by Santarius (1975) on heat damage to photophosphorylation in chloroplasts, leads to the suggestion that ATP generating systems may well be very temperature sensitive structures.

Davison's work (Davison & Bowler, 1971) did not give any indication of which part of the complex functions of the sarcosome might be most seriously affected. Several different approaches have been made to shed light on this aspect of the problem.

Chapter 4 represents an approach to this question based on the grounds of recovery from sublethal heat treatment. It is argued that if the repair process can be followed and studied, this may shed light on the type of damage caused. It was found that recovery from $L.D._0$ treatment is very dependent on the temperature at which

the animals are allowed to recover.

Recovery of 'normal' sarcosomal function had a high Q_{10} (about 3), this implies the involvement of active metabolic processes in repair of sarcosomal heat-damage. The nature of the damage being repaired, and the repair-mechanism is not evident, but probably involves re-synthesis. This is in contradiction to the work of Dingley & Smith (1968) - they report :

i) Cycloheximide effectively reduces protein sythesis in Drosphila, ii) sublethally heat treated Drosphila recovered in spite of an application of cycloheximide, and iii) the rate of protein synthesis was not compatible with the recovery state. The authors concluded that undoubtedly protein synthesis is not involved in recovery from thermal injury. However, cycloheximide is claimed to selectively inhibit cytoplasmic protein synthesis (Milner, 1976), therefore mitochondria driven protein synthesis may have aided recovery without being affected by cycloheximide. It would be interesting to find out how recovery is affected, in Dingley & Smith's (1968) work, following the application of chloramphenicol, since this is claimed to specifically inhibit mitochondria driven protein synthesis (for review see Milner, 1976).

The use of inhibitors of protein synthesis was

not applied in the present study because these agents
when injected in intact animals to inhibit protein
synthesis of a specifically suspected enzyme, may have
side effects on other parts of the organism. Indeed
there is evidence that these agents not only inhibit
protein synthesis but also, amongst other effects, alter
the rate of degradation of some enzymes (for review
see Schimke and Doyle (1970). Furthermore, Higgins
and his co-workers reported that ethidium bromide
(an inhibitor of protein synthesis) inhibits oxidative
phosphorylation of rat liver mitochondria (Higgins
et al., 1975).

The split-dose experiments, also reported in
Chapter 4, show clearly that the effect of two doses
is merely additive, unless they are separated by periods
of 6 hours or more. In the latter case significant
recovery has taken place and the effect of the split-
dose heat treatment is less than when given as a single
dose. This is in accord with Dingley & Smith's (1968)
work on Drosophila, they described recovery from a
25 mins exposure to 35°C to be almost complete after
2 hours at 20°C. The rate of recovery, in their work,
is measured by the survival time of Drosophila exposed
to a second treatment at 35°.

It would appear that the repair processes are part of normal on-going cellular metabolism, for it is reasoned, if they were only invoked following injury, the repair mechanism(s) itself would have become susceptible to damage during the second heat application in the split-dose. This would have resulted in increased mortality, which did not occur. It is also of interest to compare heat injury with radiation injury caused in split-doses. In a review article, Rockstein & Miquel (1973) report that irradiation in insects is life shortening, and that some proportionality occurs between dose and life shortening effects. This implies that this type of injury is non-repairable and is suggested to be chromosomal in nature, Baxter & Blair (1969). Davison (1970) has shown however that flies recovered from an L.D.$_{50}$ heat-treatment has the same life-expectancy as control unheated flies of the same age. Acute heat-injury is therefore different from life-shortening irradiation damage in insects, and may well be more similar to repairable acute radiation damage which is thought to be principally cytoplasmic in nature, Baxter & Blair,(1969).

One Chapter in the present study has been concerned with the effect of in vivo heat treatment on the neuro-secretion in the blowfly, Calliphora vicina. The idea was

stimulated by numerous reports presenting strong evidence

that there exists controlling effect of hormones on

respiratory enzymes. (Thomsen, 1949, 1952; Keeley,

1970; Lee & Lardy, 1965). Also, the fact that neurosecretion

exerts a controlling factor on protein synthesis (Thomsen

& Moller, 1959) made such investigations seem useful in

providing some information leading to a better under-

standing of the nature of heat injury and repair mechanism.

However, the observations made on neurosecretory cells

of the brain showed no significant difference between

control and lethally heat treated flies. No difference was

observable either in brain neurosecretory cells of control

flies or sublethally heat treated flies, nor in the latter

after 18 hr. or 48 hr. recovery at 24°C.

The effect of temperature on sarcosomal function

has been considered under several approaches, but

basically concerns _in vivo_ and _in vitro_ effects and the

comparison of the effects of moderately high temperature

with those of more 'normal' temperature _in vitro_.

The effect of reaction temperature on state III

and substrate rate oxidation using α-glycerophosphate as

a substrate will be discussed first. As can be seen in

Fig. 5.1 when oxidation rates are plotted, as a function

of $1/\circ A$, a non-linear plot is obtained which can be

divided into four phases with breaks at about $10°$, $18°$ and $27°C$. A Q_{10} value of 5.93 was obtained at the lower temperature range ($5°$ - $10°C$), 1.58 for the second phase ($10°C$ - $18°C$), 2.16 for the third ($18°$ - $27°C$) and 1.41 at the highest temperature range ($27°$ - $37°C$). The substrate rate oxidation, on the other hand, yielded a non-linear plot with only three phases, showing breaks at about $10°$ and $24°C$. The Q_{10} values obtained were 3.58, 1.15 and 2.22 for the first, second and third phase, respectively.

In the search for an explanation for these breaks, several suggestions have been made by different workers. In the early work by Heldt & Klingenberg, (1968) attention has been drawn to low temperature sensitivity of mitochondrial adenine nucleotide translocase. Kemp et al., (1969) have shown that this translocation process is rate limiting in oxidative phosphorylation in rat liver mitochondria. Drost-Hansen, (1972) suggested that changes in the physical properties of vicinal water must be superimposed upon the changes in the biological macro-molecules. Raison and his co-workers are the most prominent advocates for 'lipid phase change' theory (Kumamoto et al., 1971). The Arrhenius plot for substrate rate oxidation is in close agreement with that reported

by Pye (1973) for potato mitochondria using different substrates. The author drew attention to the observation that temperature-independent plateaus may occur in the presence of high concentrations of substrates.

The effect of temperature on mitochondria has received much stimulus in recent times because many workers have attempted to explain the temperature-independent plateaus observed in organism respiration, (Newell, 1967; Newell & Walker, 1966; Pye, 1973; Wieser, 1973) in terms of effect of temperature on mitochondrial function. The conflict in many of these studies has been bedevilled by a lack of rigour in demonstrating mitochondria isolated from an organism, in good functional states, a point made by Tribe & Bowler (1968) in criticism of work by Newell & Walkey (1966).

A further point that future work of this kind ought to consider is that the pH of the reaction medium will change with reaction temperature. Often no account has been taken of this in constructing media for mitochondria temperature-studies, and some of the phenomena described may be, at least in part, pH effects. In the present work no attempt was made to correct media

pH for temperature changes, the reaction media were always made up at 24 °C, the temperature of acclimation of the flies. It was considered that this situation might more closely reflect events intracellularly. Howell, Rahn, Goodfellow & Herreid (1977) have reported that haemolymph pH decreases as body temperature increases in a variety of ectotherms, and further Malan, Wilson & Reeves (1977) report a good correlation between the temperature- dependence of blood pH and intracellular pH in ectothermal vertebrates. Thus it seems likely that intracellular pH, in vivo, will be expected to change with temperature, at least in the same direction as predicted from the various studies of Reeves and his co-workers (Reeves, Howell & Rahn, 1977). A point more notice should be taken of in such studies.

In the present work, the 'breaks' seen in the Arrhenius plot (Fig. 5.1) confirm earlier reports by many other workers mentioned above, particularly the temperature-independent plateaus observed for both substrate-rate and state III respiration after the first sharp break at about 10 °C. Similar plateaus have been described by Davison (1971 a) for blowfly flight muscle sarcosomes. What is evident is that the efficiency of sarcosomal function is maximised over a wide temperature range, irrespective of 'breaks' and 'plateaus'.

As was described by Davison (1971a) it seems that in vitro heat is more damaging to sarcosomes than in vivo heat, for as can be seen from Table 5.1, RCI values fall rapidly at temperatures higher than 27°C, and Davison found in his experiments that respiration was uncoupled at 34°C (Davison, 1971 a). A temperature he reports,(and has been shown in this study),/that insects can be reared at. Davison also described an acclimation effect in that at 34°C, RCI and ADP :0 values for sarcosomes from 34°C acclimated flies indicated that these sarcosomes were more tightly coupled than those from either 24° or 15°C acclimatized flies.

In taking this problem of in vitro sensitivity to heat a little further, a series of preincubation experiments were conducted in which sarcosomes were preincubated at temperatures between 30° and 41°C for varying periods, and their functional efficiency was subsequently determined at 24°C. Few similar studies exist in the literature to give comparison, those in which mitochondria have been preincubated, the preincubation was to serve a purpose other than to study their thermal sensitivity (Sacktor & Childress, 1967; Hansford, 1975).

The results of these experiments confirm the greater thermal sensitivity in vitro, for temperature as 36°C was adequately high to bring about a rapid decline in state III respiration, resulting in low RCI values.

Substrate rate respiration, on the other hand, was not affected by preincubation, and slight but insignificant fluctuations of QO_2 values were obtained. In groups previously acclimated to 15° and 34°C, preincubation of isolated sarcosomes resulted in similar effects as described above, and here again 36° caused loss of repiratory control, where values approximating one were recorded within 30 mins. ADP : 0 values were not measurable after such treatments. Previous acclimation to high (34°C) or low (15°C) temperatures did not result in major differences in the tolerance of the sarcosomes to exposure to 36°C.

A comparison of the effect of preincubation at moderate temperatures (Tables 5.5 and 5.6) effect of those temperatures as a reaction condition on sarcosomal function leads to the following conclusion. The high oxidation rates seen at say 34°C (reaction temperature) can not be owing to an uncoupling effect of heat, nor from release of endogenous uncouplers, because prior exposure during preincubation (up to 60 mins) causes no major changes in oxidation rates as compared with control preincubated sarcosomes. The thermal preincubation therefore seemed to cause no deleterious effects on sarcosomes unless it was for a long period or at

temperatures over about 40°C.

Perhaps the most interesting and significant part
of this work has been concerned with a comparative study
between α-glycerophosphate and pyruvate + proline-
supported respiration in sarcosomes isolated from the
flight muscle of 10-day old adult blowflies which have
been exposed to an L.D.$_{50}$ heat treatment (40 mins
at 41°C). The results revealed that α-glycerophosphate-
linked respiration is more sensitive to this heat
treatment than pyruvate-linked respiration. In the first
case substrate rate respiration was reduced by about
30 per cent, and furthermore no stimulation was observed
upon addition of ADP. This was not the case when pyruvate
was used as substrate. Indeed some measure of coupling
was still demonstrable, and state IV respiration was
higher than in control experiments. These results are in
accord with preliminary work reported by Davison (1970)
on the same stock of flies. An atte mpt was, therefore,
made to investigate this phenomenon, considering the
possibility that heat treatment given to the intact animal
may have rendered the sarcosomes susceptible to isolation
procedure thereafter, and perhaps differentially affected.

It is reasonable to assume that this sarcosomal
damage is not occurring at the level of the respiratory

chain. This is because the commonly held view is that electrons from all substrates share a common fate once they enter the respiratory chain. Thus if an enzyme in the chain was damaged this ought to be reflected in inhibition of both α-glycerophosphate and pyruvate oxidation, this is clearly not the case. Davison (1970) who first reported this phenomenon suggested that the coupling factors (enzymes) might be the temperature sensitive structures, but this would imply that different substrates have different respiratory pathways, a point not usually held; but recently some indirect evidence has been produced that this might be the case in rat liver mitochondria (Rogers & Higgins, 1976).

It is however necessary to consider other reactions in α-glycerophosphate oxidation not common to pyruvate oxidation. The most obvious is that by α-glycerophosphate dehydrogenase. This enzyme is known to be allosterically modulated by low concentrations of free calcium (10^{-5} gm-ion/l) (Hansford & Chappell, 1967). The allosteric property of some enzymes has been previously shown to be very sensitive to heat treatment and other insults; for example aspartate transcarbamylase from Escherichia coli (Gerhart & Pardee, 1962) and acetohydroxy acid synthetase in Salmonella typhimurium (Bauerle et al. , 1964). Consequently it is pertinent to enquire

whether sarcosomal α-glycerophosphate dehydrogenase has its allosteric function destroyed during in vivo lethal heat treatments. The data, given in Table 6.1 and Fig. 6.1 clearly shows that the sarcosomal α-glycerphosphate dehydrogenase is still allosterically stimulated by low levels of Ca^{2+} even after the flies received an L.D.$_{50}$ heat treatment. As can be seen the presence of Ca^{2+} reduces the app Km for α-glycerophosphate from about 14 to 2 mM in controls as compared with 20 mM to 7 mM in heat treated sarcosomes. The app Km is slightly higher in the latter group, but what is also evident is the point that Vmax is again much lower in the experimental heat treated sarcosomes, this supports the data presented in Table 3.2. Thus the inhibition of α-glycerophosphate oxidation in sarcosomes from heated flies cannot be attributed to a loss of responsiveness to the allosteric stimulator Ca^{2+}.

The data in the above experiments was collected polarographically, and so the entire respiratory chain is needed, however in the next series of experiments α-glycerophosphate dehydrogenase activity was directly measured. In these experiments electrons were transferred directly from the dehydrogenase via phenazine methosulphate to INT, the artificial electron acceptor

which is reduced , and an insoluble product, formazan

is produced. The amount of formazan formed is

proportional to the activity of the enzyme.

This technique, with slight modifications, has

been widely used to assay enzyme activity. For example

Lee and Lardy (1965) used PMS and INT to estimate the

activity of mitochondrial as well as cytoplasmic

α-glycerophosphate dehydrogenase from various tissues

of rat. A similar method has been used by Vaughan &

Newsholme (1969), Walker & Birt (1969) for α-glycero-

phosphate dehydrogenase , Kun & Abood (1949), Glock

& Jensen (1953) and Nachlas Margulies & Seligman (1960)

for succinic dehydrogenase. However, Walker & Birt

(1969) suggested that a high concentration of INT might

damage the mitochondrial membranes. This suggestion

was based on the impression of very low tonicity of the

assay system they used, and on the incorrect interpretation

of the failure of Ca^{2+} to relieve the inhibition caused

by EGTA to α-glycerophosphate dehydrogenase (high

Ca^{2+} concentration).

In the present experiments no significant difference

in α-glycerophosphate dehydrogenase activity levels was

demonstrable between sarcosomes from control and $L.D._{50}$

treated flies, see Table 7.1. At first sight this result

was not expected, particularly as the activity of the same

enzyme when measured by the consumption of oxygen was significantly less in sarcosomes isolated from heat treated flies. In making that observation it is of course assumed that it is the α-glycerophosphate dehydrogenase that is rate limiting in α-glycerophosphate oxidation as has been shown by Lardy et al., (1960).

These results prompted the study of the effects of in vitro heating on sarcosomal α-glycerophosphate dehydrogenase. The sarcosomes were preincubated for various times at temperatures between 40° and 55°C, and their residual enzyme activity was monitored at 24°C after this thermal preincubation. As can be seen (Table 7.2) the enzyme tolerated preincubation temperatures higher than 40°C for long periods of time, and it was not until the temperature was raised to 55°C that 50% inactivation was observed within 20 mins. This is in accord with the results described earlier (see Table 5.6) where the activity of this enzyme was measured by oxygen consumption (substrate-rate oxidation), and it has been shown that temperatures as high as 41°C could not produce a pronounced effect of the enzyme activity after 7 mins preincubation. One must conclude, therefore, that α-glycerophosphate dehydrogenase is a thermostable structure, and that the reduction in its activity in sarcosomes isolated from lethally heat treated flies, as

measured by oxygen consumption, might not be due to thermal inactivation occurring to this enzyme.

A point that deserves to be raised, is whether the enzyme (α-glycerophosphate dehydrogenase) inactivated by in vivo heatings, and if so why is it not as sensitive to heat when the sarcosomes are preincubated in vitro?

It could possibly be that the experiments in which the artificial electron acceptors are used, do not accurately reflect the true level of enzyme activity owing to the very high affinity of PMS for electrons. Nachlas et al., (1960) showed that PMS effectively competes with molecular oxygen to bind electrons and the competition is so successful that there was no need for anaerobiosis to be induced. Such a high electron affinity might mask the reduced enzyme activity following in vivo heating.

If α-glycerophosphate dehydrogenase is unaffected by in vivo heating, as it seems to be to in vitro heating, then is the heat lesion elsewhere in the respiratory chain?

If this is the case, why then is the respiratory chain still functioning normally when pyruvate and proline are used? This presents a paradoxical situation for as has been mentioned earlier it is commonly held that electrons, irrespective of substrate source, all follow the common pathway of respiratory chain enzymes to the final

acceptor, oxygen. It may be this is not the case and different substrates use separate assemblies of respiratory enzymes, this is however not proven by this data. An alternative explanation would involve thermal inactivation of one or more of the reactions between α-glycerophosphate dehydrogenase and the first enzyme, in the common respiratory chain, Coenzyme Q. This is most definitely a worthwhile possibility to explore in any further studies. It may well be possible to dissect respiratory chain function with the use of specific inhibitors, such as antimycin A and rotenone, in sarcosomes from heated and non-heated flies.

The problem first mentioned by Davison & Bowler (1971) of the nature of the primary lesion in heat injury remains. The foregoing discussion does not take account of the fact that state III respiration is more dramatically impaired in sarcosomes from heat treated flies than is substrate rate oxidation, see Table 3.2. This point is also true in sarcosomes preincubated at temperatures above 35°C, see Table 5.6, as well as in the experiments carried out to construct the Arrhenius plots, see Fig.5.1. There too state III oxidation was more seriously affected by raising temperatures above 27°C than was substrate rate, as witnessed by the falling RCl values shown in Table 5.1.

The point Davison & Bowler (1971) make is that the coupling factors may be the most temperature sensitive component of oxidative phosphorylation. This conclusion was reached because <u>in vivo</u> heat did not appear to uncouple oxidative phosphorylation, for state IV (substrate rate) oxidation rates were reduced rather than elevated; at the same time state III respiration could not be demonstrated with RCI of about 1 being found.

Once again the fact that pyruvate and proline driven respiration is differentially affected by <u>in vivo</u> heating causes a problem of interpretation. This is because it is believed, as has been said earlier, that electrons from substrates are processed in a common oxidative phosphorylation pathway. So if coupling factors are heat-damaged for α-glycerophosphate oxidation, pyruvate and proline oxidation ought to be similarly damaged. Whilst the ADP : 0 and RCI in sarcosomes from heated flies are lower than in control flies, when pyruvate and proline are respired, coupling can still be demonstrated. It may be that one should think in terms of different substrates being processed through separate respiratory assemblies, but direct evidence is lacking.

It is interesting, however, that Santarius (1975) has shown clearly that photophosphorylation in chloroplasts

is temperature sensitive, and he describes non-cyclic photophosphorylation as being more sensitive than cyclic photophosphorylation. The light requiring thiol-activated membrane ATPase was heat sensitive in a manner parallel to cyclic photophosphorylation.

The data presented in Table 7.3 is informative, for it allows a comparison to be made between α-glycerophosphate and pyruvate + proline oxidation systems as a function of in vivo heat treatment, and in vitro freezing and thawing of isolated sarcosomes. Both systems were inactivated following freezing and thawing, a treatment which destroys the structure of the sarcosome. It is also interesting that there is a quantitative correlation in the degree of reduction between α-glycerophosphate oxidation in in vivo heated, and in vitro frozen and thawed sarcosomes, suggesting that the damage caused by heat treatment might be structural. This was not the case for pyruvate and proline system, the fall in oxidation rate following freezing and thawing was drastic, (about 70%), whereas heat treatment resulted in only 25% inactivation of this system.

The results presented in Chapter (8) are also very pertinent to this discussion. The EM appearance of the mitochondria was difficult to interpret in the manner used for liver mitochondria by Hackenbrock (1966) or heart mitochondria by Green et al., (1968) see Fig. 8.2,

and as a consequence a tentative interpretation is made.
It is clear that the appearance of the sarcosomes does
change somewhat with respiratory state. What is most
pertinent to this discussion is the appearance of the
sarcosomes from heat treated animals, which show gross
morphological abnormalities, particularly when respiring
α-glycerophosphate. In that case swelling became
progressive, see Figs. 8.5, 8.6 and many sarcosomes
were broken.

In marked contrast when pyruvate and proline
were used as substrates much less swelling was evident
and sarcosomes in different morphological forms were
also evident in the different energy states, see Figs.
8.7 - 9. This does again provide concrete evidence
that the pyruvate and proline driven oxidative phosphory-
lation is less damaged by the in vivo heating as compared
with the α-glycerophosphate system.

Could in vivo inactivation of sarcosomal function
kill the organism? This would seem unlikely unless the
thermal injury seen in flight muscle sarcosomes reflects
a similar state in other cells. Indeed, this would seem to be
an important next step to take. In vitro work on isolated
malpighian tubules might be used to provide a clue to
the generality of heat damage.

The results, presented in this study, support Davison's (1970) work, and amply show that sarcosomal function is thermosensitive over the same temperatures that kill the organism, and so fulfills one of Read's (1967) conditions. Further this work supports the general hypothesis laid down by Bowler et al., (1973) that all heat injury is a membrane phenomenon.

With this theory of heat injury in mind the possible mechanisms of thermal perturbation of membrane function must now be considered. The facility with which ecto-thermal animals and microorganisms change the fatty acid composition of their membrane phospholipids may well provide a guide. In general, acclimation to a low temperature results in an increased incorporation of longer chain more unsaturated fatty acids, this has been demonstrated in Crayfish (Cossins, 1976), goldfish (Driedzic & Roots, 1975; Kemp & Smith, 1970; Anderson, 1970). The adaptive properties of such changes have been described in terms of 'capacity' adaptation, the maintenance of an acceptable state of fluidity of the 'liquid-crystalline' membrane as proposed by Singer & Nicolson, (1972).

Compensatory effects have not been conclusively demonstrated in ectothermal animals, but good evidence does exist that adaptation of membrane properties and

composition (fluidity) do co incide in prokaryotes (Haest, De Gier & Van Deenen, 1969; Esser & Souza, 1974) and in protozoans (Cullen, Phillips & Shipley, 1971; Nazaira, Iida, Fukushima, Ohki & Ohnishi, 1974), grown at different temperatures. This points to the importance of a control mechanism which regulate the degree of membrane fluidity and which is sensitive to environmental temperature. Less importance has been placed on changes in membrane unsaturation influencing the thermal stability of membranes and so being involved in 'resistance' adaptation. Membranes from cold acclimated animals may show a more rapid breakdown of functional integrity at high temperatures as compared to the more saturated membranes from warm acclimated animals.

However, the correlation between lipid saturation and lethal temperatures has not been conclusively demonstrated. House et al., (1958) did find a correlation in Pseudosarcophaga affinis larvae, Fraenkel & Hopf (1940) and Ushakov (1964; 1966) in Calliphora have shown altered lipid composition does not necessarily affect thermostability. Once again the data from bacteria is less equivocal. Esser & Souza (1974) have provided

evidence that maximal growth in <u>B. stearothermophilus</u> is determined by the physical properties of the cell membrane, which is grossly influenced by its lipid composition. This is supported by evidence that a thermosensitive strain of this bacterium has a decreased ability to alter its membrane lipids in response to a temperature change. In a detailed study of crayfish muscle, Cossins (1976) showed that acclimation to lowered temperatures caused an increase in the proportion of mono and polyunsaturated fatty acids, mainly in the acidic phospholipids. However, the same worker was unable to show that this affected the thermostability of the sarcosomal $Mg^{2+} + Ca^{2+} + ATPase$ (Cossins & Bowler, 1976). At the same time however the sarcosomal enzyme was sensitive to temperature in the same temperature range as heat death occurred, whereas the glycolytic soluble enzyme pyruvate kinase was considerably more heat stable (Cossins & Bowler, 1976). It may well be that changes in the phospholipid composition of bulk-lipid bilayer are not the most significant in controlling membrane integrity at different temperatures for the protein content of membranes must also be considered. The presence of a distinct lipid halo, forming a micro-environment, for the protein (see Stier & Sackmann, 1973) may be more significant in determining the thermostability of a protein, rather than any bulk changes to membrane

phospholipids. This might only be true ofcourse should the halo lipids be chemically different from the bulk lipid. However, Raison and his co-workers claim that the 'breaks' seen in Arrhenius plots of mitochondrial oxidation reflect phase changes in membrane (bulk) lipids, (Kumamoto et al., 1971). This suggests that enzyme function (if not thermostability) is conditioned by the characteristics of the bulk phospholipids.

If the essential thermostability of sarcosomal function is determined, at least in part, by the nature of the mitochondrial phospholipids, then three patterns ought to be demonstrable. These are: 1) Changes in phospholipid composition of sarcosomes with adult age, for Davison (1970) showed that both organismal and sarcosomal thermosensitivity changed dramatically during the first 10 days of age. 2) The time course of those changes in thermosensitivity are dependent upon maintenance temperature and so must be any changes in phospholipid composition. 3) Davison (1970) also showed organism heat death to be modified by acclimation; it ought therefore to be possible to establish temperature induced changes in sarcosomal phospholipid composition as the sarcosome provides a good model for organism heat death (Davison & Bowler, 1971).

Whilst this study has gone further than that of Davison (1970) in attempting to pinpoint a primary lesion in heat injury in the blowfly, much work still remains. In this discussion possible future avenues of approach have been mentioned which might provide more central answers to the questions of what factors are involved in heat death, cellular heat injury and resistance acclimation. It can be concluded that the work presented here in no way contradicts the general hypothesis set out by Bowler et al., (1973) that cellular heat injury (and therefore heat death) may well be related to the stability of lipoprotein complexes.

B I B L I O G R A P H Y

AHEARN, G.A. (1970) Changes in the haemolymph properties accompanying heat death in the desert tenebrionid beetle <u>Centrioptera municata</u>. <u>Comp. Biochem. Physiol</u>. <u>33</u>: 847-857.

ALEKSIUK, M. (1971) An isoenzyme basis for instantaneous cold compensation in reptiles: Lactate dehydrogenase kinetics in <u>Tamnophis sirtalis</u>. <u>Comp. Biochem. Physiol</u>., <u>40</u>: 671-681.

ALMQUIST, A. (1970) Thermal tolerance and preferences of some dune-living spiders. <u>OIKOS</u>, <u>21</u>: 230-236.

AMELUNXEN, R.E., SAUVAN, R.L. & MIRA, O.J. (1974) Thermostable glycerol-3-phosphate dehydrogenase from <u>Bacillus stearothermophilus</u>. <u>Physiol. Chem. Phys</u>. 6; 515-526.

ANDERSON, T.R. (1970) Temperature adaptation and the phospholipids of membranes in goldfish (Carassius auratus). <u>Comp. Biochem. Physiol</u>, 33: 663-687.

ANSTEE, J.H. (1971) An ultrastructual study of tettigoniid muscle in relation to function. <u>J. Insect. Physiol</u>., <u>17</u>: 1983-1994.

ASAI, J., BLONDIN, G.A., VAIL, W.J. & GREEN, D.E. (1969) The mechanism of mitochondrial swelling IV. Configurational changes during swelling of beef heart mitochondria. <u>Arch. Biochem. Biophys</u>., <u>132</u>: 524-544.

ASHHURST, D.E. (1967) The fibrillar flight muscles of giant water-bugs: An electron-microscope study. <u>J. Cell Sci</u>., 2: 435-444.

ATSMON, A & DAVIS, R.P. (1967) Mitochondrial respiration under conditions of varying osmolarity. Biochem. Biophys. Acta, 131: 221-233.

AZZONE, G.F. & AZZI, A. (1966) In "regulation of metabolic processes in mitochondria" (Tager, J.M., Papa, S. Quagliariello, E. and Slater, E.C., eds.). Elsevier, Amsterdam. B.B.A. Lib., Vol. 7, pp. 332-345.

AZZONE, G.F. & CARAFOLI, E. (1960) Biochemical properties of skeletal muscle mitochondria. I. Oxidative phosphorylation. Exptl. Cell Res., 21: 447-455.

BALBONI, E.R. (1967) The respiratory metabolism of insect flight muscle during adult maturation. J. Insect Physiol, 13: 1849-1856.

BALBONI, E.R. (1972) Control of glycerol-phosphate oxidation by H^+ and Ca^{2+}. J. Insect Physiol, 18: 355-358.

*see p. 265

BANKS, B.E.C., DAMJANOVIC, V. & VERNON, C.A. (1972) The so-called thermodynamic compensation law and thermal death. Nature (Lond.), 240: 147-148.

BAUERLE, R.H., FREUNDLICH, M., STORMER, F.C. & UMBARGER, H.E. (1964) Control of isoleucine, valine and leucine biosynthesis. II. End product inhibition by value of acetohydroxy acid synthetase in Salmonella typhimurium. Biochim. Biophys. Acta, 92: 142-149.

BAXTER, R.C. & BLAIR, H.A. (1969) Recovery and over-recovery from acute radiation injury as a function of age in Drosophila. Rad. Res., 39: 345-360.

BELLAMY, D. (1962) The endogenous citric acid-cycle intermediates and amino acids of mitochondria. Biochem. J. 82: 218-224.

BELEHRADEK, J. (1935) Temperature and living matter. Protoplasm Monographien, 8: 50-54.

BELEHRADEK, J. (1957) Physiological aspects of heat and cold. A. Rev. Physiol., 19: 59-82.

BELEHRADEK, J. (1967) Intermolecular aspects of the structural stability of protoplasm at the temperature extremes. In: The Cell and Environment Temperature (ed. A.S. Troshin). Pergamon.

BIRT, L.M. (1961) Flight muscle mitochondria of Lucilia cuprina and Musca domestica. Biochem. J., 80: 623-631.

BISHOP, L.G. & GORDON, M.S. (1967) Thermal adaptation of metabolism in anuran amphibians. In "Molecular mechanisms of temperature adaptation (ed. C.L. Prosser), AAAS Publ. 84.

BLONDIN, G.A. & GREEN, D.E. (1969) Mechanism of mitochondrial swelling. III. Two forms of energized swelling. Arch. Biochem. Biophys., 132: 509-523.

BLONDIN, G., VAIL, W.J. & GREEN, D.E. (1969) The mechanism of mitochondrial swelling. II. Pseudoenergized swelling in the presence of alkali metal salts. Arch. Biochem. Biophys., 129: 158-172.

BLUHM, L. & ORDAL, Z.J. (1969) Effect of sublethal heat on the metabolic activity of Staphylococcus aureus. J. Bact., 97: 140-150.

BORST, P., LOOS, J.A., CHRIST, E.J. & SLATER, E.C. (1962) Uncoupling activity of long-chain fatty acids. Biochim. Biophys. Acta, 62: 509-518.

BOWLER, K. (1963a) A study of the factors involved in acclimatization to temperature and death at high temperatures in <u>Astacus pallipes</u>. I experiments on intact animals. <u>J. Cell. Comp. Physio.1.</u>, <u>62</u>: 119-132.

BOWLER, K. (1963b) A study of the factors involved in acclimatization to temperature and death at high temperatures in <u>Astacus pallipes</u>. II experiments at the tissue level. <u>J. Cell. Comp. Physio.1.</u>, <u>62</u>: 133-146

BOWLER, K. & DUNCAN, C.J. (1967) Evidence implicating a membrane ATPase in the control of passive permeability of excitable cells. <u>J. Cell. Physio.1.</u>, <u>70</u>: 121-1 5.

BOWLER, K. & DUNCAN, C.J. (1968) The effect of temperature on the Mg^{2+}- dependent and $Na^{+}- K^{+}$- ATPases of rat brain microsomal preparation. <u>Comp. Biochem. Physoil.</u>, <u>24</u>: 1043-

BOWLER, K., DUNCAN, C.J., GLADWELL, R.T. & DAVISON, T.F.(1973) CELLULAR HEAT INJURY, <u>Comp. Biochem. Physoil.</u>, <u>45A</u>; pp. 441-450.

BOWLER, K. GLADWELL, R.T. & DUNCAN, C.J. (1973) Acclimatization to temperature and death at high temperatures in the Crayfish <u>Austropotemobius pallipes</u>. Freshwater Crayfish, Ed. S. <u>Abrahamsson (Lond.)</u> <u>1973</u>, 121-131.

BRANDT, J.F. (1967) Heat effects on proteins and enzymes. In: <u>Thermobiology</u> (ed. A.H. Rose). <u>Academic Press, London.</u>

BRANSKA, J. & WLODAWER, P. (1969) Influence of temperature on the composition of fatty acids and on lipogenesis in frog tissues. <u>Comp. Biochem. Physio.l.</u>, <u>28</u>: 553-570.

BRETT, J.R. (1944) Some lethal temperature relations of Algonquin Park fishes. <u>Pub. Ontario Fish Res. Lab.</u>, No. 63: 5-49.

BRIERLEY, C.P. (1963) In "<u>Energy-linked functions of mitochondria</u>". (Ed. B. Chance; <u>Acad. Press; London, and New York</u>), p.237.

BRONK, J.R. (1966) Thyroid hormone: effects on electrons transport. <u>Science, 153</u>: 638-639.

BRONK, J.R. & JASPER, D.K. (1970) The relationship between mitochondrial structual changes and respiration rate in intact mucosal cell. <u>Biochem. J.</u>, <u>116</u>; 33 p.

BULOS, B., SHUKLA, S. & SACKTOR, B. (1972) Bioenergetic properties of mitochondria from flight muscle of aging blowflies. <u>Arch. Biochem. Biophys.</u>, <u>149</u>: 461-469.

BULOS, B.A., SHUKLA, S.P. & SACKTOR, B. (1975) Bioenergetics of mitochondria from flight muscles of aging blowflies: Partial reactions of oxidation and phosphorylation. <u>Arch. Biochem. Biophys.</u>, <u>166</u>: 639-644.

BURSELL, E. (1975) Substrates of oxidative metabolism in dipteran flight muscle. <u>Comp. Biochem. Physio.l.</u>, <u>52B</u>: 235-238.

BURSELL, E. & SLACK, E. (1976) Oxidation of proline by sarcosomes of the tsetse fly, <u>Glossina morsitans</u>. <u>Insect Biochem.</u>, <u>6</u>: 159-167.

BUSH, G.L. & NECK, R.W. (1976) Ecological genetics of the screw worm fly, Cochliamyia nominivorax and its bearing on the quality control of mass-reared insects. Environ. entomol. 5: 821-826.

BUTLER, W.H. & JUDAH, J.D. (1970) Preparation of isolated rat liver mito-chondria for electron microscopy. J. Cell. Biol., 44: 278-289.

CALDWELL, R.S. & VERNBERG, F.J. (1970) The influence of acclimation temperature on the lipid composition of fish gill mitochondria. Comp. Biochem. Physiol., 34: 179-191.

CARAFOLI, E., HANSFORD, R.G., SACKTOR, B. & LEHNINGER, A.L. (1971) Interaction of Ca^{2+} with blowfly flight muscle mitochondria. J. Biol. Chem. 246: 964-972.

CHANCE, B. & HAGIHARA, B. (1961) Symp. Intracellular respiration: Phos-phorylating non-phosphorylating oxidation reactions. Proc. 5th int. Congr. Biochem., Moscow, 5, 3, (Slater, E.C. Ed., Pergamon Press, London, 1963)

CHANCE, B. & WILLIAMS, G.R. (1955a) Respiratory enzymes in oxidative phos-phorylation. I. Kinetics of oxygen util-ization. J. Biol. Chem. 217: 383-393.

CHANCE, B., & WILLIAMS, G.R. (1955b) Respiratory enzymes in oxidative phosphorylation. III. The steady state. J. Biol. Chem., 217: 409-427.

CHANCE, B. & WILLIAMS, G.R. (1956) The repiratory chain and oxidative phosphorylation. Adv. Enzymol., 17: 65-134.

CHAPPELL, J.B. (1964) The oxidation of citrate, isocitrate and cis-aconitate by isolated mitochondria. Biochem J, $\underline{90}$: 225-237.

CHAPPELL, J.B. & HANSFORD, R.G. (1972) In "Subcellular components Preparation and Fractionation". 2nd ed. edited by G.D. Birnie, Butterworths, University Park Press, Baltimore, and Butterworth & Co. Ltd., London.

CHARNOCK, J.S., COOK, D.A. & CASEY, R. (1971) The role of cations and other factors on the apparent energy of activation of $(Na^+ + K^+)$ - ATPase. Arch. Biochem. Biophys., $\underline{147}$: 323-329.

CHARNOCK, J.S. & FRANKEL, D. (1971) Ca^{2+} uptake and energy utilization by sarcoplasmic reticulum. Abstracts 2nd International Congress of Muscle Diseases, Perth, Abs. $\underline{45}$.

CHEFURKA, W. (1965) Some comparative aspects of the metabolism of carbohydrates in insects. Ann. Rev. Entomology $\underline{10}$: 345-382.

CHEFURKA, W. (1966) Oxidative phosphorylation in in vitro aged mitochondria. I. Factors controlling the loss of the dinitrophenol-stimulated adenosine triphosphatase activity and respiratory control in mouse liver mito-chondria. Biochemistry, $\underline{5}$; 3887-3903.

CHILDRESS, C.C. & SACKTOR, B. (1966) Pyruvate oxidation and the permeability of mitochondria from blowfly flight muscle. Science, $\underline{154}$: 268-270.

CHRISTIANSEN, E.N. & Effects of thermal treatment on mito-
KVAMME, E. (1969)
chondria of brain, liver and ascites

cells. Acta physiol. scand., 76: 472-484.

CHRISTOPHERSEN, J. (1973) Basic aspects of temperature

action on microorganisms. In "Temperature

and Life" (ed. Precht, Christophersen,

Hensel and Larcher). Springer-Verlag

Berlin.

CLARKE, K.U. (1966) Histological changes in the endocrine

system of Locusta migratoria L. associated

with the growth of the adult under

different temperature regimes. J. Insect

Physiol., 12: 163-170.

CLARKE,K.U. & The effect of insect hormones and of 2:4
BALDWIN, R.W. (1960)
dinitrophenal on the mitochondria. of

Locusta migratoria. J. Insect Physiol.,

5: 37-46.

CLARKE, K.U. & Studies on the effects of the removal of
GILLOTT, C. (1967)
the frontal ganglion in Locusta migra-

toria L. II. Ribonucleic acid synthesis.

J. exp. Biol., 46: 27-34.

CLARKE, K.U. & Studies on the initiation of growth and
LANGLEY, P.A. (1963a)
moulting in Locusta migratoria migratorioides

R. and F. I. The time and nature of the

initiating stimulus. J. Insect Physiol.,

9: 287-292.

CLARKE, K.U. & II The role of the stomatogastric nervous
LANGLEY, P.A. (1963b)
system. J. Insect Physiol. 9: 363-373.

CLARK, L.C. Jun. (1956) Monitor and control of blood and

tissue oxygen tension. Trans. Ann. Soc.

artif. internal organs, 2: 41-65.

CLARK, C.W. & ORDAL, Z.J. (1969) Thermal injury recovery of _Salmonella typhimurium_ and its effect on enumeration procedures. J.Appl. Microbiol., 18: 332-336.

COCKING, A.W. (1959) The effect of high temperatures on the roach (Rutilus rutilus). I. The effects of constant high temperatures. J. exp. Biol., 36: 203-216.

COSSINS, A.R. (1976) Changes in muscle lipid composition and resistance adaptation to temperature in the freshwater crayfish, _Austropotambius pallipes_. Lipids II, 307-316.

COSSINS, A.R. & BOWLER, K. (1976) Resistance adaptation of the freshwater crayfish and thermal inactivation of membrane-bound enzymes. J. Comp. Physiol., 111: 15-24.

CULLEN, J., PHILLIPS, M.C. & SHIPLEY, G.G. (1971) The effect of temperature on the composition and physical properties of the lipids of Pseudomonas fluorescens. Biochem. J., 125: 733-742.

CUNNINGHAM, C.C. & HAGER, L.P. (1971) Crystalline pyruvate oxidase from Escherichia coli III. Phospholipid as an allosteric effector for the enzyme. J. Biol. Chem., 246: 1583-1589.

DAVIES, P.W. & BRINK, F. JUN. (1942) Microelectrodes for measuring local oxygen tension in animal tissues. Rev. Scient. Instum., 13: 524-533.

DAVIES, L. (1949) The temperature and humidity relations of various stages in the life history of some calliphorine flies. Ph.D. Thesis, University of Durham.

DAVIES, P. & TRIBE, M.A. (1969) Temperature dependence of metabolic rate in animals. Nature, 224: 723-724.

DAVISON, T.F. (1969) Changes in temperature tolerance during the life cycle of Calliphora erythrocephala. J. Insect Physiol., 15: 977-988.

DAVISON, T.F. (1970) An investigation into the factors involved in acclimatization to temperature and death at high temperature in Calliphora erythrocephala (Meig). Ph.D. Thesis, University of Durham.

DAVISON, T.F. (1971a) The effect of temperature on oxidative phosphorylation in isolated flight muscle sarcosomes. Comp. Biochem. Physiol., 38B: 21-34.

DAVISON, T.F. (1971b) Ultrastructural changes in sarcosomes after exposure of adult Calliphora erythrocephala to lethal and sublethal high temperatures. J. Cell. Physiol., 78: 49-57.

DAVISON, T.F. & BOWLER, K. (1971) Changes in the functional efficiency of flight muscle sarcosomes during heat death of adult Calliphora erythrocephala J. Cell. Physiol., 78: 37-48.

DAWSON, A.P., DUNNETT, S.J.& SELWYN, M.J. (1971) Calcium uptake by mitochondria from locust flight muscle. Europ. J. Biochem., 21: 42-47.

DEAMER, D.W. (1973) Isolation and characterization of a lysolecithin-adenosine triphosphatase complex from lobster muscle microsomes. J. Biol. Chem., 248: 5477-5485.

DINGLEY, F. & SMITH, J.M. . (1968) Temperature acclimatization in the absence of protein synthesis in Drosophila subobscura. J. Insect Physiol., 14: 1185-1194.

DONNELLAN, J.F., BARKER, M.D., WOOD, J. & BEECHEY, R.B. (1970) Specificity and locale of the L-3-glycero-phosphate flavoprotein oxidoreductase of mitochondria isolated from insect flight muscle. Biochem. J. 120: 467-478.

DRIEDZIC, W.M. & ROOTS, B.I. (1975) Glycerophosphatide content and com-position of trout (Salvelinus fontinalism) brain. J. Thermal Biol., 1: 7-10.

DROST-HANSEN, W. (1972) Chemistry of the cell interface. Part (B) H.D. Brown (ed.) Academic press Inc., New York, p. 170.

EMMELOT, P. & BOS, C.J. (1962) A stimulatory effect of KCN on glycerol phosphate dehydrogenase of normal and neoplastic tissues. Biochim., Biophys. Acta. 59: 495-497.

ESSER, A.F. & SOUZA, K.A. (1974) Correlation between thermal death and membrane fluidity in Bacillus stearo-thermophilus. Proc. Nat. Acad. Sci., U.S.A., 71: 4111-4115.

ESTABROOK, R.W. & SACKTOR, B. (1958) α-Glycerophosphate oxidase of flight muscle. mitochondria. J. Biol. Chem. 233: 1014-1019.

EVANS, P.R. & BOWLER, K. (1973) Thermal death and the denaturation of proteins. Subcell. Biochem., 3: 91-93.

EWEN, A.B. (1962) An improved Aldehyde Fuchsin staining technique for Neuro-secretory products in insects. Trans. of Amer. Microsc. Soc. Vol. LXXXI (1): 94-96.

FISHBEIN, W.N. & GRIFFIN, J.L. (1976) Studies on the mechanism of freezing damage to mouse liver. IV. Effects of ultrarapid freezing on structure and function of isolated mitochondria. Cryobiology, 13; 542-556.

FEO, F., CANUTO, R.A., GARCEA, R., AVOGADRO, A., VILLA, M. & CELASCO, M. (1976) Lipid phase transition and breaks in the arrhenius plots of membrane-bound enzymes in mitochondria from normal rat liver and hepatoma AH-130. FEBS Letters, Vol. 72, No. 2, 262-266.

FLEISCHER, S., BRIERLEY, G., KLOUWEN, H. & SLAUTTERBACK (1962) Studies of the electron transfer system XLVII. The role of phospholipids in electron transfer. J. Biol. Chem. 237: 3264-3272.

FRAENKEL, G.S. & HERFORD, G.V.B. (1940) The physiological action of abnormally high temperatures on poikilotherm animals, II. The respiration at high sublethal and lethal temperatures. J. exp. Biol. 17: 386-395.

FRAENKEL, G. & HOPF, H.S. (1940) The physiological action of abnormally high temperatures on poikilothermic animals I Temperature adaptation and the degree of saturation of the phosphatides. Biochem. J. 34: 1085-1092.

FRY, F.E.J. (1958) Temperature compensation. Ann. rev. Physiol. 20: 207-224.

GAMBLE R.L. & GARTID, K.D. (1970) The permeability of the mitochondrial inner membrane to sucrose. Biochim. Biophys. Acta, 211: 223-232.

GEAR, A. &   Rapid, respiration-indepandent binding
LEHNINGER, A.L. (1968)
                   of alkali metal cationa by rat liver

                   mitochondria. J. Biol. Chem. 243: 3953-

                   3962.

GERHART, J.C. &   The enzymology of control by feedback
PARDEE, A.B. (1962)
                   inibition. J. Biol. Chem., 237: 891-896.

GERNER, E.W. &   Induced thermal resistance in Hela
SCHNEIDER, M.J. (1975)
                   cells. Nature, 256: 500-502.

GLADWELL, R.T.,   Heat death in the crayfish Austropotamobius
BOWLER, K. &
DUNCAN, C.J. (1976)pallipes - ion movements and their effects

                   on excitable tissues during heat death.

                   J. Thermal Biol., 1: 79-94.

GLOCK, E. &   The colorimetric determination of plant
JENSEN, C.O. (1953)
                   succinic dehydrogenase. J. Biol. Chem.,

                   201: 271-278.

GOYER, R.A. &   Ultrastructual transformation in mito-
KRALL, R. (1969)
                   chondria isolated from kidneys of normal and

                   lead intoxicated rats. J. Cell. Biol.,

                   41: 393-400.

GREEN, D.E.,   Conformational Basis of energy transform-
ASAI, J.,
HARRIS, R.A. &   ation in membrane systems. III Configur-
PENNISTON, J.T. (1968)
                   ational changes in the mitochondrial inner

                   membrane induced by changes in functional

                   states. Arch. Biochem. Biophys., 125:

                   684-705.

GREEN, D.E. &   The mitochondrion and biochemical machines.
HATEFI, Y. (1961)
                   Science, 133: 13-19.

GRAINGER, J.N.R. (1969) Heat death in Ariauta arbustorum.

                   Comp. Biochem. Physiol., 29: 665-670.

GRAINGER, J.N.R. (1973a) A study of heat death in nerve-muscle preparations of Rana temporaria L. Proc. Roy. Irish Acad. 73B: 283-290.

GRAINGER, J.N.R. (1975) Mechanism of death at high temperatures in Helix and Patella. J. Thermal Biology, 1: 11-13.

GRANT, W.C. Jun. (1955) Temperature relationships in the megascolecid earthworm, Pheretima hupeiensis. Ecology, 26: 412-417.

GREGG, C.T., HEISLER, C.R. & REMMERT, L.F. (1960) Oxidative phosphorylation and respiratory control in housefly mitochondria. Biochim. Biophys. Acta, 45: 561-570.

GREVILLE, G.D., MUNN, E.A. & SMITH, D.S. (1965) Observations on the fragmentation of isolated flight muscle mitochondria from Calliphora erythrocephala (Diptera). Proc. R. Soc. B., 161: 403-420.

GRISHAM, C.M. & BARNETT, R.E. (1973) The role of lipid-phase transitions in the regulation of the (sodium + potassium) adenosine triphosphatase. Biochemistry, 12: 2635- 2640.

GRUENER, N. & AVI-DOR, Y. (1966) Temperature dependence of activation and inhibition of rat-brain adenosine triphosphatase activated by sodium and potassium ions. Biochem. J., 100: 762-767.

HACKENBROCK, C.R. (1966) Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J. Cell Biol., 30: 269-297.

HAEST, C.W.M., DEIGIER, J. & VAN DEENEN, L.L.M. (1969) Changes in the chemical and the barrier properties of the membrane lipids of E. Coli by variation of the temperature of growth. Chem. Phys. Lipids, 3: 413-417.

HANSFORD, R.G. (1971) Some properties of mitochondria isolated from the flight muscle of the periodical cicada, Magicicada septendicem Biochem. J., 121: 771-780.

HANSFORD, R.G.(1972) Some properties of pyruvate and 2-oxoglutarate oxidation by blowfly flight muscle mitochondria. Biochem. J., 127: 271-283.

HANSFORD, R.G. (1975) The control of tricarboxylate-cycle oxidations in blowfly flight muscle. The oxidized and reduced nicotinamide-adenine dinucleotide content of flight muscle and isolated mitochondria, the adenosine triphosphate and adenosine diphosphate content of mitochondria, and the energy status of the mitochondria during controlled respiration. Biochem. J., 146: 537-547.

HANSFORD, R.G. & CHAPELL, J.B. (1967) The effect of Ca^{2+} on the oxidation of glyceral phosphate by blowfly flight muscle mitochondria. Biochem. Biophys. Res. Commun., 27: 686-692.

HANSFORD, R.G. & JOHNSON R.N. (1975) The steady state concentrations of coenzyme A-SH and coenzyme A thioester, citrate and isocitrate during tricarboxylate cycle oxidations in rabbit heart mitochondria. J. Chem. 250: 8361-8375.

HARRIS, R.A.,
PENNISTON, J.T.,
ASAI, J. &
GREEN, D.E. (1968)
The conformational basis of energy conservation in membrane systems, II. Correlation between conformational changes and functional states. Proc. Natl. Acad. Sci. U.S.A., 59: 830-837.

HEILBRUNN, L.V. (1924) The celloid chemistry of protoplasm. Am. J. Physiol., 69: 190-199.

HEINMENTS, F. (1960) An analysis of the concept of cellular injury and death. Int. J. Radiation Biol., 2: 341-352.

HELDT, H.W. &
KLINGENBERG, M. (1968)
Differences between the reactivity of endogenous and exogenous adenine nucleotides in mitochondria as studied at low temperature. European J. Biochem., 4: 1-8.

HEROLD, R.C. (1965) Development and ultrastructural changes of sarcosomes during honey bee flight muscle development. Devl. Biol., 12: 269-286.

HIGGINS, E.S.,
DUNLAYVEY, B.L.,
FRIEND, W.H. &
ROGERS, K.S. (1975)
Ethidium bromide inhibits mitochondrial phosphorylating oxidation. P. Soc. Exp. M., 149: 1055-1058.

HIGHNAM, K.C.,
HILL, L &
GINGELL, D.J. (1965)
Neurosecretion and water balance in Schistocerca. J. Zool., 147: 201-215.

HINTON, H.E. (1960) Cryptobiosis in the larva of Polypedilum vanderplanki hint. (chironomidae). J. Insect Physiol., 5: 286-300.

HOCHACHKA, P.W. &
SOMERO, G.N. (1973)
Strategies of Biochemical Adaptation. W.B. Sauders Co. Philadelphia, London, Toronto, p. 358.

HOUSE, H.L.,
RIORDAN, D.F. &
BARLOW, J.S. (1958)
Effects of thermal conditioning and of degree of saturation of dietary lipids on resistance of an insect to a high temperature. Can. J. Zool., 36: 629-632.

HOWELL, B.J.,
RAHN, H.,
GOODFELLOW, D. &
HERREID, C. (1973)
Acid-base regulation and temperature in selected invertebrates as a function of temperature. Am. Zool., 13: 557-563.

HULBERT, A.J.,
AUGEE, M.L. &
RAISON, J.K. (1976)
The influence of thyroid hormones on the structure and function of mitochondrial membranes. Biochem. Biophys. Acta, 455: 597-601.

HUTCHINSON, V.F.. (1961) Critical thermal maxima in salamanders Physiol. Zool., 34: 92-125.

IANDOLA, J.J. &
ORDAL, Z.J. (1966)
Repair of thermal injury in Staphylococcus aureus. J. Bact. 91: 134-142.

IVANOVIC, J.P.,
JANKOVIC-HLADNI, M.I.,
MILANOVIC, M.P. (1975)
Possible role of neurosecretory cells: type A in response of Morimus funereus larvae and the effect of temperature. J. Thermal Biol., 1: 53-57.

JOHNSTON, P.V., &
ROOTS, B.I. (1964)
Brain lipid fatty acids and temperature acclimation. Comp. Biochem. Physiol., 11: 303-309.

KALLMAN, R.I. (1963) Recovery from radiation injury: A proposed mechanism. Nature, 197: 557-560.

KATES, M. (1960) in "Lipid metabolism", (Ed. K. Block, Wiley Press, N.Y.), p. 188.

KEELEY, L.L. (1970) Insect fat body mitochondria: Endocrine and age effects on respiratory and electron transport activities. Life Science, 9: 1003-1011.

KEISTER, M. & BUCK, J. (1964)

Respiration: Some exogenous and endo-
genous effects on rate of respiration.
In " The Physiology of Insecta" Academic
Press, N.Y., Lond.

KEMP, A.J., GROOT, G.S.P. & REITSMA, H.J. (1969)

Oxidative phosphorylation as a function
of temperature. Biochem. Biophys. Acta,
180: 28-34.

KEMP, P. & SMITH, M.W. (1970)

Effect of temperature acclimatization on
the fatty acid composition of goldfish
intestinal lipids. Biochem. J. 117: 9-15.

KLEIN, R.L. NEFF, R.S. (1960)

Osmotic properties of mitochondria
isolated from Acauthamoeba Sp. Exptl. Cell
Res., 19: 133-155.

KLINGENBERG, M. & BUCHHOLZ, M. (1970)

Localisation of the glycerol-phosphate
dehydrogenase in the outer phase of the
mitochondrial inner membrane. Europ.
J. Biochem., 13: 247-252.

KROGH, A. (1914)

The quantitative relation between temp-
erature and standard metabolism in animals.
Intern. Z. Physik - Chem. Biol., 1:
491-508 .

KUMAMOTO, J., RAISON, J.K. & LYONS, J.M. (1971)

Temperature "Breaks" in Arrhenius plots:
A thermodynamic consequence of a phase
changes. J. Theoret. Biol., 31: 47-51.

KUN, E. & ABOOD, L.G. (1949)

Colorimetric estimation of succinic
dehydrogenase by triphenyltetrazolium
chloride. Science, 109: 144-146.

KVUUR, J., NELSON, R.J., FREY, H.E. & McGANN, L.E. (1972)

Recovery from sublethal cold-induced damage
in mammalian cells. Exptl. Cell Res. 74:
514-518.

LARDY, H.A. & FERGUSON, S.M. (1969)   Oxidative phosphorylation in mito-chondria.   <u>A. Rev. Biochem.</u> <u>38</u>: 991-1034.

LARDY, H.A., LEE, Y.P. & TAKEMORI, A. (1960)   Enzyme responses to thyroid hormones.   <u>Ann. N.Y. Acad. Sci.</u>, <u>86</u>: 506-511.

LEE, Y.P. & LARDY, H.A. (1965)   Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat.   <u>J. Biol. Chem.</u>, <u>240</u>: 1427-1436.

LEE, M.P. & GEAR, A.R.L. (1974)   The effect of temperature on mitochon-drial membrane - linked reaction.   <u>J. Biol. Chem.</u>, <u>249</u>: 7541-7549.

LEHNINGER, A.L. (1959) Reversal of Thyroxine-induced swelling of rat liver mitochondria by adenosine Triphosphate.   <u>J. Biol. Chem.</u>, <u>234</u>: 2187-2195.

LEHNINGER, A.L. (1962a) A heat-labile factor required in extrusion of water from mitochondria. <u>J. Biol. Chem.</u>, <u>273</u>: 946-951.

LEHNINGER, A.L. (1962b) Water uptake and extrusion by mito-chondria in relation to oxidative phos-phorylation.   <u>Physiol. Rev.</u>, <u>42</u>: 467-577.

LEHNINGER, A.L. (1964) The mitochondrion.   <u>Benjamin Inc. Press New York, U.S.A.</u>

LEHNINGER, A.L. (1975) Biochemistry 2nd ed.   <u>Worth Publ., Inc. N.Y.</u>, pp. 1104.

LEHNINGER, A.L. & GOTTERER, G.S. (1960)   A soluble protein required in mito-chondrial contraction; " leakage" of active factors from mitochondria.   <u>J. Biol. Chem.</u>, <u>235</u>: PC 8

LEHNINGER, A.L.,   The swelling of rat liver mitochondria
RAY, B.L. &
SCHNEIDER, M.J. (1959) by thyroxine and its reversal. J.

Biophys. Biochem. Cytol., 5: 97-108.

LEHNINGER, A.L. &   Mitochondrial swelling induced by
SCHNEIDER, M.J. (1959)
glutathione. J. Biophys. Biochem.,

Cytol., 5: 109-116.

LENAZ, G.,   Activation energies of different mito-
SECHI, A.M.,
PARENTI-CASTELLI, G.,   chondrial enzymes; breaks in Arrhenius
& BERTOLI, E. (1972)
plots of membrane-bound enzymes occur at

different temperatures. Biochem. Bio-

phys. Res. Commun., 49: 536-542.

LEVY, M.R.,   Effects of Hyperthermia on Tetrahymena.
GOLLON, C.E. &
ELLIOT, A.M. (1969) I. Localization of acid hydrolases and

changes in cell ultrastructure. Expl.

Cell. Res., 55: 295-305.

LEWIS, S.E. &   Nature and origin of an inhibitor of
FOWLER, K.S. (1960)
oxidative phosphorylation present in

freshly isolated sarcosomes from blow-

fly theraces. Biochem. Biophys. Acta.,

38: 564-565.

LEWIS, S.E. &   Oxidative phosphorylation in insect
SLATER, E.C. (1954)
sarcosomes. Biochem. J., 58: 207-217.

LICHT, P. (1964)   The temperature dependence of myosin

adenosine triphosphatase and alkaline

phosphatase in lizards. Comp. Biochem.

Physiol., 12: 331-340.

LING, G.N. (1967)   Effects of temperature on the state of

water in the living cell. In: Thermo-

biology (ed. A.H. Rose), 5-24. Acad.

Press, London.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1957) Protein measurement with Folin-phenol reagent. J. Biol. Chem., 193: 265-275.

LYONS, J.M. & RAISON, J.K. (1970a) A temperature-induced transition in mitochondrial oxidation; contrasts between cold and warm-blooded animals. Comp. Biochem. Physiol., 37: 405-411.

LYONS, J.M. & RAISON, J.K. (1970b) Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol., 45: 386-389.

MADDRELL, S.H.P. (1970) Diuretic hormone in Rhodnius and its neurohaemal release. Symp. Roy. ent. Soc. Lond., 5: 101-116.

MAGNUM, C.F. & SASSAMAN, C. (1969) Temperature sensitivity of active and resting metabolism in a polychaetous annelid. Comp. Biochem. Physiol., 30:111-116.

MALAN, A., WILSON, T.L. & REEVES, R.B. (1976) Intracellular pH in cold-blooded vertebrates as a function of body temperature. Respir. Physiol., 28: 29-47.

MANNING, G.B. & CAMPBELL, L.L. (1961) Thermostable α- amylase of Bacillus stearothermophilus. J. Biol. Chem., 236: 2952-2957.

MARTIN, R.G. (1963) The first enzyme in histidine biosynthesis : The nature of feedback inhibition by histidine. J. Biol. Chem., 238: 257-268.

MAYER, A.G. (1914) The effects of temperature on tropical

marine animals. <u>Pap. Tortugas Lab. 6</u>,

1-24.

MELLANBY, K. (1954) Acclimatization and the thermal death

point in insects. <u>Nature (Lond.) 173</u>:

582-583. <u>Entomol. Exp. Appl., 1</u>: 153-160.

MERCER, E.H. &   Electron microscopy: A hand book for
BIRBECK, M.S.C. (1966)

biologists 2nd Ed. <u>Blackwell; Oxford</u>.

MILES, H.B. (1963)   Heat-death temperature in <u>Allolobophora</u>

<u>terrestris</u> (Sav.), forma longa (Ude) and

<u>Eisenia foetida</u> (Sav.). <u>Nature, 199</u>: 826.

MILNER, J. (1976)   The functional development of mammalian

mitochondria. <u>Biol. Rev., 51</u>: 181-209.

MITCHELL, P. &   Translocation of some anions, cations and
MOYLE, J. (1969)

acids in rat liver mitochondria. <u>Eur.</u>

<u>J. Biochem., 9</u>: 149-155.

MURPHY, T.A. &   The enzymes of glycogen and trehalose
WYATT, G.R. (1965)

synthesis in silk moth fat body. <u>J</u>.

<u>Biol. Chem., 240</u>: 1500-1508.

MUSCATELLO, A.,   Configurational changes in isolated rat
GUARRIERA-BOBYLEVA, V.,
& BUFFA, P. (1972)   liver mitochondria as revealed by negative

staining. II. Modifications caused by

changes in respiratory states. <u>J</u>.

<u>Ultrastr. Res., 40</u>: 235-260.

McLEESE, D.W. (1956) Effect of temperature, salinity and

oxygen on the survival of the American

lobster. <u>J. Fish. Res. Board Can. 13</u>:

247-272.

McMURCHIE, E.J.
RAISON, J.K. &
CAIRNCROSS, K.D.
(1973)
Temperature-induced phase changes in membranes of heart: a contrast between the thermal response of poikilotherms and homeotherms. Comp. Biochem. Physiol., 44B: 1017-1026.

NACHLAS, M.M.,
MARGULIES, S.I. &
SELIGMAN, A.M. (1960)
A colorimetric method for the estimation of succinic dehydrogenase activity. J. Biol. Chem., 235: 249-503.

NAGUIB, M. &
CHRISTOPHERSEN, J.
(1965)
Über die Beteiligung von ATP bei der Regelung temperaturbedingter Leistungs-änderungen von Hefezellen. Naturwissenschaften, 52: 193-194.

NAZAWA, Y., IIDA, H.,
FUKUSHIMA, H.,
OHKI, K. &
OHNISHI, . (1974)
Studies on Tetrahymena membranes: Temperature-induced alterations in fatty acid composition of various membrane factions in Tetrahymena pyriformis and its effect on membrane fluidity as inferred by spin-label steady. Biochim. Biophys. Acta, 367: 134-147.

NEUBERT, D. &
LEHNINGER, A.L. (1962)
The effect of thiols and disulfides on water uptake and extrusion by rat liver mitochondria. J. Biol. Chem., 237: 952-958.

NEWBURGH, R.W.,
POTTER, L.N. &
CHELDELIN, V.H. (1960)
Oxidative phosphorylation in Phormia regina larvae. J. Insect Physiol., 4: 348-349.

NEWELL, R.C. (1966)
Effect of temperature on the metabolism of poikilotherms. Nature, 212: 426-428.

NEWELL, R.C. (1967) Oxidative activity of poikilotherm mitochondria as a function of temperature. _J. Zool._, _151_: 299-311.

NEWELL, R.C. & NORTHCROFT, H.R. (1967) A re-interpretation of the effect of temperature on the metabolism of certain marine invertebrates. _J. Zool._, _151_: 277-298.

NEWELL, R.C. & PYE, V.I. (1971) Quantitative aspects of the relationship between metabolism and temperature in the winkle _Littorina littorea_ (L). _Comp. Biochem. Physiol._ _38B_: 635-650.

NEWELL, R.C., PYE, V.I. & AHSANULLAH, M. (1971) The effect of thermal acclimation on the heat tolerance of the intertidal prosorbranchs _Littorina littorea_ (L) and _Monodonta lineata_ (Da Costa). _J. Exp. Biol._, _54_: 525-533.

NEWELL, R.C. & WALKEY, M. (1966) Oxidative activity of mammalian liver mitochondria as a function of temperature. _Nature (Lond.)_, _212_: 428-429.

NOVAK, V.J.A. (1966) In "Insect hormones". _Methuen & Co._ Ltd., London, p. 478.

ORR, P.R. (1955) Heat death, I. Time-temperature relationships in marine animals. _Physiol. Zool._, _28_: 290-294.

PACKER, L., POLLAK, J.K., MUNN, E.A. & GREVILLE, G.D. (1971) Effect of high sucrose concentrations on mitochondria: Analysis of mitochondrial populations by density-gradient centrifugation after fixation with glutaraldehyde. _J. Bioenerg._ _2_: 205-316.

PACKER, L. &
TAPPEL, A.L. (1960)
Light scattering changes linked to oxidative phosphorylation in mitochondrial membrane fragments. J. Biol. Chem., 235: 525-530.

PALZER, R.J. &
HEIDELBERGER, C. (1973)
Studies on the quantitative biology of hyperthermic killing of Hela cells. Cancer Res. 33: 415-421.

PEACOCK, A.J.,
ANSTEE, J.H. &
BOWLER, K. (1977)
The effect of temperature on development and the histology of the neuroendocrine system of the tettigniid, Jamaicana flava (Caudell). J. Thermal Biol., 2: 35-40.

PENNISTON, J.T.,
HARRIS, R.A.,
ASAI, J. &
GREEN, D.E. (1968)
The conformational basis of energy transformations in membrane systems, I. Conformational changes in mitochondria. Proc. Nat. Acad. Sci. U.S.A., 59: 624-631.

POPHAM, J.D. &
DANDY, J.W.T. (1976)
The specific activities of lactic dehydrogenase and α - GP dehydrogenase in acclimated crayfish, Cambarus bartoni. Comp. Biochem. Physiol. B, 53: 97-99.

PORTZEHL, H.,
CALDWELL, P.C. &
RUEGG, J.C. (1964)
The dependence of contraction and relaxation of muscle fibres from the crab Maia squenado on the internal concentration of free calcium ions. Biochim. Biophys. Acta, 79: 581-591.

POSTGATE, J.R. &
HUNTER, J.R. (1963)
The survival of starved bacteria. J. Appl. Bacteriol., 26: 295-306.

PRECHT, H.,
CHRISTOPHERSEN, J. &
HENSEL, H. (1955)
Temperatur und Leben. Springer-Verlag, Berlin.

PRESSMAN, B.C. & LARDY, H.A. (1956) Effect of surface active agents on the latent ATPase of mitochondria. Biochim. Biophys. Acta, 21: 458-466.

PROSSER, C.L. (1973) In "Comparative animal physiology", 3rd Ed. Saunders Co. Philadelphia/London/ Toronto. p. 368

PROUDLOCK, J.W., HASLAM, J.M. & LINNANE, A.W. (1969) Specific effect of unsaturated fatty acid depletion on mitochondrial oxidative phosphorylation in Sacchoromyces cerevisiae. Biochem. Biophys. Res. Commun. 37: 847-852.

PULLMAN, M.E. & SCHATZ, G. (1967) Mitochondrial oxidations and energy coupling. A. Rev. Biochem., 36: 539-610.

PYE, V. (1973) Acute temperature change and the oxidation rates of ectotherm mitochondria. In "Effects of temperature on ectothermic organisms". Ed. by W. Wieser. Springer-Verlag Berlin, Heidelberg, N.Y.

RAISON, J.K. (1973) The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. Bioenergetics 4: 285-309.

RAISON, J.K., LYONS, J.M., MEHLHORN, R.J. & KEITH, A.D. (1971) Temperature-induced phase chages in mitochondrial membranes detected by spin labelling. J. Biol. Chem. 246: 4036-4040.

RAISON, J.K., LYONS, J.M. & THOMSON, W.W. (1971) The influence of membranes on the temperature-induced changes in the kinetics of some respiratory enzymes of mitochondria. Arch. Biochem. Biophys. 142: 83-90.

RALPH, C.L. & MATTA, R.J. (1965) Evidence for hormonal effects on metabolism of cockroaches from studies of tissue homogenates. J. Insect Physiol., 11: 983-991.

READ, K.R.H. (1967) Thermostability of proteins in poikilotherms. In: Molecular Mechanisms of Temperature Adaptation (ed. C.L. Prosser). Amer. Assoc. Adv. Sci., Washington D.C.

REEVES, R.B., HOWELL, B. & RAHN, H. (1977) Protons. Proteins. Temperature. Publications in acid-base physiology. State University of New York at Buffalo.

REYNOLDS, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol., 17: 208-213.

ROBERTS, J.C., ARINE, R.M., ROCHELLE, R.H. & CHAFFEE, R.R.J. (1972) The effects of temperature on oxidative phosphorylation of liver mitochondria from hamster, rat and squirrel monkey. Comp. Biochem. Physiol., 41B: 127-135

ROCHMAN, H., LATHE, G.H. & LEVELL, M.J. (1967) The effect of temperature and anoxid of kidney on the subsequent oxidative phosphorylation of mitochondria. Biochem. J., 102: 48-52.

ROCKSTEIN, M. & MIQUEL, J. (1973) Aging in insects. In "Physiology of the Insecta" Vol. 1, pp.371-478. Acad. Press, N.Y.

ROGERS, K.S. & HIGGINS, E.S. (1976) Respiratory control depression by tetraalkylammonium bromides in rat liver mitochondria. Chem-biol. Interact. 12: 71-79.

ROOTS, B.I. (1968) Phospholipids of goldfish (Carassius auratus L.) brain: The influence of environmental temperature. Comp. Biochem. Physiol. 25: 457-466.

ROSENBERG, B., KEMENY, G., SWITZER, R.C. & HAMILTON, T.C. (1971) Quantitative evidence for protein denaturation as the cause of thermal death. Nature (London) 232: 471-473.

ROTHFIELD, L. & FINKELSTEIN, A. (1968) Membrane biochemistry. Ann. Rev. Biochem., 37: 463-496.

ROTTEM, S., CIRILLO, V.P., DE KRUYFF, B., SHINITZKY, M. & RAZIN, S. (1973) Cholesterol in mycoplasma membranes: correlation of enzymic and transport activities with physical state of lipids in membranes of Mycoplasma mycoides var. capri adapted to grow with low cholesterol concentrations. Biochem. Biophys. Acta 323: 509-519.

SACKTOR, B. (1954) Investigations on the mitochondria of the Housefly, Musca domestica L. J. General Physiol., 37: 343-359.

SACKTOR, B. (1958) A biochemical basis of flight muscle activity. 4th Int. Conference of Biochemistry (Vienna) Pergamon Press, Oxford.

SACKTOR, B. (1965) Energetics and respiratory metabolism of muscular contraction. In "The Physiology of Insecta" (M. Rockstein, ed.), Vol. 2, pp. 483-580. Acad. Press, N.Y.

SACKTOR, B. (1970) Regulation of intermediary metabolism, with special reference to the control mechanisms in insect flight muscle. Advances in Insect Physiology, Vol. 7, 267-347.

SACKTOR, B. (1974) Biological oxidations and energetics in insect mitochondria. In "The Physiology of Insecta" (ed. M. Rockstein). Acad. Press, N.Y., London, pp. 271-353.

SACKTOR, B. & CHILDRESS, C.C. (1967) Metabolism of proline in insect flight muscle and its significance in stimulating the oxidation of pyruvate. Arch. Biochem. Biophys., 120: 583-588.

SACKTOR, B. & COCHRAN, D.G. (1958) The repiratory metabolism of insect flight muscle. I. Manometric st..udies of oxidation and concomitant phosphorylation with sarcosomes. Arch-Biochem. Biophys. 74: 266-276.

SACKTOR, B. & HURLBUT, E.C. (1966) Regulation of metabolism in working muscle in vivo. II. Concentrations of adenine nucleotides, arginine phosphate, and inorganic phosphate in insect flight muscle during flight. J. Biol. Chem., 241: 632-634.

SACKTOR, B., O'NEILL, J.J. & COCHRAN, D.G. (1958) The requirement of serum albumin in oxidative phosphorylation of flight muscle mitochondria. J. Biol. Chem., 233: 1233-1235.

SACKTOR, B. & WORMSER-SHAVIT, E. (1966) Regulation of metabolism in working muscle in vivo. I. Concentrations of some glycolytic, tricarboxylic acid cycle, and amino acid intermediates in insect flight muscle during flight. J. Biol. Chem., 241: 624-631.

SANTARIUS, K.A. (1975) Sites of heat sensitivity in chloroplasts and differential inactivation of cyclic and noncyclic photophosphorylation by heating. J. Thermal Biol., 1: 101-107.

SCHIMKE, R.T. & DOYLE, D. (1970) Control of enzyme levels in animal tissues. Ann. Rev. Biochem., 39: 929-976.

SEKUZU, I., JURTSHUK, D. & GREEN, D.E. (1963) Studies on the electron transfer system L Isolation and characterization of the D-(-)-B- hydroxybutyric apodehydrogenase from beef heat mitochondria. J. Biol. Chem., 238: 975-982.

SINGER, S.J. & NICHOLSON, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science (Wash. D.C.), 175: 720.

SIZER, I.W. & JOSEPHSON, E.S. (1942) Kinetics as a function of temperature of lipase, trypsin, and invertase activity from -70° to 50°C, (-94 to 122°F). Food Res. 7: 201-209.

SJOSTRAND, F.S. (1977) The arrangement of mitochondrial membranes and a new structural feature of the inner mitochondrial membranes. J. Ultrastr. Res., 59: 292-319.

SJOSTRAND, F.S. & BARAJAS, L. (1968) Effect of modifications in conformation of protein molecules on structure of mitochondrial membranes. J. Ultrastr. Res., 25: 121-155.

SJOSTRAND, F.S. & BERNHARD, W. (1976) The structure of mitochondrial membranes in frozen sections. J. Ultrastruct. Res., 56: 233-246.

SJOSTRAND, F.S. &   A new freeze-drying technique applied
KRETZER, F. (1975)   to the analysis of the molecular struc-
                        ture of mitochondrial and chloroplast
                        membranes. J. Ultrastruct. Res., 53:
                        1-28.

SLACK, E.N. (1975)   Mitochondria from insect flight muscle.
                        Ph.D. Thesis, University of London.

SLACK, E.N. &
BURSELL, E. (1976a)   The isolation of mitochondria from
                        dipteran flight muscle. Biochim. Bio-
                        phys. Acta, 449: 491-499.

SLACK, E. &
BURSELL, E. (1976b)   Oxidation of pyruvate by mitochondria
                        isolated from the flight muscles of
                        blowflies. Insect Biochem., 6: 637-647.

SLACK, E. &   Oxidation of α - glycerophosphate by
BURSELL, E. (1977)   mitochondria isolated from flight muscle
                        of the blowfly. Insect Biochem., 7:
                        159-167.

SLATER, J.V. (1954)   Temperature tolerance in Tetrahymena.
                        Am. Naturalist, 88: 168-171.

SLAMA, K. (1964)   Hormonal control of respiratory
                        metabolism during growth, reproduction
                        and diapause in female adults of Pyrrhocoris
                        apterus L. (Hemiptera). J. Insect Physiol.,
                        10: 283-303.

SMITH, C.L. (1973a)   Thermostability of some mitochondrial
                        enzymes of lower vertebrates - I. General
                        survey. Comp. Biochem., Physiol., 44B:
                        779-788.

SMITH, C.L. (1973b) Thermostability of some mitochondrial enzymes of lower vertebrates - II. Fresh-water teleosts. <u>Comp. Biochem. Physiol.</u>, <u>44B</u>: 789-801.

SMITH, C.L. (1973c) The temperature dependence of oxidative phosphorylation and of the activity of various enzymes systems in liver mitochondria from cold- and warm- blooded animals. <u>Comp. Biochem. Physiol.</u>, <u>46B</u>: 4 445-461.

SMITH, D.S. (1961a) The structure of insect fibrillar flight muscle. A study made with special reference to the membrane systems of the fiber. <u>J. Biophysic and Biochem. Cytol</u>, <u>Vol. 10</u>, No. 4, Part 2.

SMITH, D.S. (1961b) The organization of the flight muscle in a dragonfly, <u>Aeshna sp</u>. (Odonata). <u>J. Biophys. and Biochem. Cytol.</u>, <u>11</u>: 119-145.

SMITH, D.S. (1962) Cytological studies on some insect muscles. With special reference to the sarcoplasmic reticulum. In: International symposium on progress in muscle research. I. Skeletal muscle. <u>Rev. Canad. Biol.</u>, <u>21</u>: 279-301.

SMITH, D.S. (1963) The structure of flight muscle sarcosomes in the blowfly, <u>Calliphora erythrocephala</u>. (Diptera). <u>J. Cell Biol.</u>, <u>19</u>: 115-138

SMITH, D.S. (1966) In "Progress in biophysics and molecular biology". (Butler, J.A. & Huxley, H.E. eds.) <u>Pergamon Press, Oxford</u>, p. 107.

SMITH, U.,
SMITH, D.S. &
YUNIS, A.A. (1970)
Chloramphenicol-related changes in mitochondrial ultrastructure. J. Cell Sci., 7: 501-521.

SOMERO, G.N. &
DE VRIES, A.L. (1967)
q
Temperature tolerance of some Antarctic fishes. Science, 156: 257-258.

STADTMAN, E.R. (1966) Allosteric regulation of enzyme activity. Advan. in Enzymology, 28: 41-154.

STEELE, J.E. (1976) Hormonal control of metabolism in insects. In:"Advances in Insect Physiology" ed. Treherne, J.E., Besridge, M.J. & Wigglesworth, V.B. Acad. Press, London, N.Y., S. Fr.

STEVENSON, E. (1968) Carbohydrate metabolism in the flight muscle of the southern armyworm moth. Prodenia eridania. J. Insect Physiol., 14: 179-198.

STIER, A. &
SACKMANN, E. (1973)
Spin labels as enzyme substrates. Heterogeneous lipid distribution in liver microsomal membranes. Biochem. Biophys. Acta, 311: 400-408.

SUGANO, T &
NAGAI, O. (1971)
Effect of magnesium ion on brain mitochondrial respiration. I. Activation of brain mitochondrial phosphotransferases by magnesium ion. J. Biochemistry, 70: 417-427.

SUHRMANN, R. (1955) Weitere Versuche über die Temperaturadaptation der Karauschen. (Carassius vulgaris Nils). Biol. Zbl., 74: 432-448.

TANAKA, R. &
TERUYA, A. (1973)
Lipid dependence of activity-temperature relationship of (Na^+-K^+)- activated ATPase. Biochim. Biophys. Acta, 323: 584-591.

TANIGUCHI, K. & IIDA, S. (1972)   The effect of phospholipids on the apparent activation energy of (Na^+-K^+)-ATPase. Biochem. Acta, <u>274</u>: 536-541.

TATA, J.R., ERNSTER, L., LINDBERG, O., ARRHENIUS, E., PEDERSON, S. & HEDMAN, R. (1963)   The action of thyroid hormones at the cell level. Biochem. J., <u>86</u>: 408-428.

THOMSEN, E. (1949)   Influence of corpus allatum on the oxygen consumption of adult <u>Calliphora erythrocephala</u>, Meig. <u>J. exp. Biol.</u>, <u>26</u>: 137-149.

THOMSEN, E. (1952)   Functional significance of the neuro-secretory brain cells and the corpus cardiaeum in the female blowfly, <u>Calliphora erythrocephala</u> Meig. <u>J. exp. Biol.</u>, <u>29</u>: 137-172.

THOMSEN, E. & MOLLER, I. (1959)   Neurosecretion and intestinal proteinase activity in an insect, <u>Calliphora erythrocephala</u>. <u>Nature, London, 183</u>: 1401-1402.

TRIBE, M.A. (1966)   The effect of diet on longevity in <u>Calliphora erythrocephala</u> Meig. <u>Exp. Geront.</u>, <u>1</u>: 269-284.

TRIBE, M.A. (1967)   Changes taking place in the respiratory efficiency of isolated flight muscle sarcosomes associated with the age of the blowfly. <u>Comp. Biochem. Physiol.</u>, <u>23</u>: 607-620.

TRIBE, M.A. & BOWLER, K. (1968)   Temperature dependence of "standard metabolic rate" in a poikilotherm. <u>Comp. Biochem. Physiol.</u>, <u>25</u>: 427-436.

TRIBE, M.A. &   Biochemical and structural variations in
ASHHURST, D.E. (1972)
                  the flight muscle mitochondria of ageing

                  blowflies. <u>Calliphora erythrocephala</u>.

                  <u>J. Cell. Sci.</u>, <u>10</u>: 443-469.

TULP, A.,   Permeability of housefly mitochondria
STAM, H., &
VAN DAM, K. (1971) towards dicarboxylate ions. <u>Biochem</u>.

                  <u>Biophys. Acta</u>, <u>234</u>: 301-305.

TUNG, C.C. (1961) Study on the mitochondrial L - -

                  glycerophosphate dehydrogenase of brain

                  IV. Effect of cholate on the electron

                  transferring system of the mitochondrial

                  L- - glycerophosphate dehydrogenase.

                  <u>Jour. Formosan Med. Assoc.</u>, <u>60</u>: 43-48.

USHAKOV, B.P. (1964) Thermostability of cells and proteins

                  of poikilotherms and its significance in

                  speciation. <u>Physiol. Rev.</u> <u>44</u>: 518-560.

USHAKOV, B.P. (1966) The problem of associated changes in

                  protein thermostability during the

                  process of speciation. <u>Helgoländer</u>

                  <u>Wiss. Meeresunters</u>, <u>14</u>: 466-481.

USHAKOV, B.P.,   The environmental temperature and
AMOSOVA, I.S.,
CHERNOKOZHEVA, I.S., physiological polymorphism of populations
DREGOLSKAYA, I.N.,
PASHKOVA, I.M. &   - III. Heat acclimation and the popula-
SKHOLL, E.D. (1977)
                  tion response to selection caused by

                  heating. <u>J. Thermal Biol.</u> <u>2</u>: 17-22.

USHAKOV, B.P. &   The effect of heat acclimation on variab-
BUGAYEVA, E.A. (1975)
                  ility and survival of a population at

                  elevated temperature. <u>J. Thermal Biol.</u>,

                  <u>1</u>: 1-6.

VAN DAM, K. & TSOU, C.S. (1968)   Accumulation of substrates by mitochondria. _Biochem. Biophys. Acta,_ 162: 301-309.

VAN DEN BERGH, S.G. (1962) Respiration and energy production in the flight muscle of the housefly _Musca domestica_. _Ph.D. Thesis, University of Amsterdam._

VAN DEN BERGH, S.G. & SLATER, E.C. (1962)   The respiratory activity and permeability of housefly sarcosomes. _Biochem. J.,_ 82: 362-371.

VAN EYS, J., NUENKE, B.J. & PATTERSON, M.K. (1959)   The nonprotein component of α - glycerophosphate dehydrogenase. Physical and chemical properties of the crystalline rabbit muscle enzyme. _J. Biol. Chem.,_ 234: 2308-2313.

VAUGHAN, H. & NEWSHOLME, E.A. (1969)   The effect of Ca^{2+} and ADP on the activity of NAD-linked isocitrate dehydrogenase of muscle. _Fed. Eur. Biochem. Soc. Lett._ 5: 124-126.

VAUGHAN, H. & NEWSHOLME, E.A. (1970)   Effects of calcium ion and electron-acceptor concentration on the activity of mitochondrial glycerol-1-phosphate dehydrogenase from insect flight muscle. _Biochem. J.,_ 116: 31pp.

VERNBERG, F.J. (1959) Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus _Uca._ III. The influence of temperature acclimation on oxygen consumption of whole organisms. _Biol. Bull._ 117: 582-593.

VOGEL, W. (1966)   Uber die Hitze - und Kälteresistenz

von Zoothamnium hiketes Precht (Ciliata,

Peritr.). Z. Wiss. Zool., 173: 344-378.

VOLPE, E.P. (1957)   Temperature tolerance and rates of

development of different species and

races of Bufo. Physiol. Zool., 30:

164-176

WAINO, W.W. (1970)   In "The mammalian mitochondrial

respiratory chain". (ed. Acad. Press

N.Y.), p.253.

WAITE, M.,   Differentiation of phospholipases A in
SCHERPHOF, G.L.,
BOSHOUWER, F.M.G. & mitochondria and lysosomes of rat liver.
VAN DEENEN, L.L.M.
(1969)   J. Lipid Res., 10: 411-420.

WALKER, A.C. &   Development of respiratory activity and
BIRT, L.M. (1969)
                     oxidative phosphorylation in flight muscle

mitochondria of the blowfly, Lucilia cuprina.

J. Insect Physiol., 15: 305-317.

WALTER, P. &   Effect of antimycin A on oxidative
LARDY, H.A. (1964)
                     phosphorylation with ferricyanide as

electron acceptor. Biochemistry 3:

305-317.

WATANABE, M.I. &   Mitochondria in the flight muscles of
WILLIAMS, C.M. (1951)
                     insects. 1. Chemical composition and

enzymatic content. J. gen. Physiol.,

34: 675-689.

WEATHERLEY, A.H. (1970) Effect of superabundant oxygen on

thermal tolerance of goldfish. Biol.

Bull., 139: 229-238.

WEBB, J.V.,   Temperature dependence of repair of
HOLLINGSWORTH, M.J.,
MILL, A.J. &   sublethal damage in Drosophila. Rad.
DAVIES, R.W. (1976)
                     Res., 65: 187-190.

WEHRLE, J.P., Mg^{2+} and the permeability of heart
JURKOWITZ, M.,
SCOTT, K.M. & mitochondria to monovalent cations.
BRIERLY, G.P. (1976)
Archives of Biochem & Biophys., 174:

312-323.

WEINBACH, E.C., Morphology of mitochondria in the
GARBUS, J. &
SHEFFIELD, H.G. (1967) coupled, uncoupled and recoupled

states. Exptl. Cell. Res., 46: 129-143.

WIESER, W. (1973) In "Effects of temperature ectothermic

organisms" ed. by W. Wieser. Springer-

Verlag Berlin, Heidelberg. N.Y.

WIESER, W., & The ecophysiology of some marine
SCHIEMER, F. (1977)
nematodes from Bermuda: Seasonal aspects.

J. exp. Mar. Biol. Ecol., 26: 97-106.

WINSTON, P.W. & Saturated solutions for control of
BATES, D.H. (1960)
humidity in biological research. Ecology,

41: 232-246.

WINTERSTEIN, H. (1905) Warnelahmung und Narkose. Z. allg.

Physiol., 5: 322-350.

WOJTCZAK, L., Adenasine triphosphate-induced contrac-
VLODOWER, P. &
ZBOROWSKI, J. (1963) tion of rat liver mitochondria and syn-

thesis of mitochondrial phospholipids.

Biochim. Biophys. Acta, 70: 290-305.

WOJTCZAK, L. & Uncoupling of oxidative phosphorylation
WOJTCZAK, A.B. (1960)
and inhibition of ATP-Pi exchange by a

substance from insect mitochondria. Bio-

chim. Biophys. Acta, 39: 277-286.

YAKOVLEVA, V.I. & Effect of heating on the catalytic and
GUBNITSKII, L.S. (1970)
allasteric properties of bull-liver

glutamate dehydrogenase. DOKL AKAD

NAUK SSSR, 190: 231-234.

YOUNG, H.L. &
PACE, N. (1958)
Some physical and chemical properties of crystalline   - glycerophosphate dehydrogenase. <u>Arch. Biochem. Biophys.</u>, <u>75</u>: 125-141.

ZYLBER, E.,
VESCO, C. &
PENMAN, S. (1969)
Selective inhibition of the synthesis of mitochondria-associated RNA by ethidium bromide. <u>J. Mol. Biol.</u>, <u>44</u>: 195-203.

Additional References

BALDWIN, W.F. &
HOUSE, H.L. (1954)
Studies on effects of thermal conditioning in two species of sawfly larvae. <u>Canad. J. Zool., 32</u>: 9-15.

DIXON, M. &
WEBB, E.C. (1958)
"<u>Enzymes</u>", Longmans, Green and Co., London, p.150.

MELLANBY, K. (1958)
Water content and insect metabolism. <u>Nature, 181</u>: 1403.

PRECHT, H.,
CHRISTOPHERSEN, J.,
HENSEL, H. &
LARCHER, W. (1973)
"<u>Temperature and Life</u>". Springer-Verlag, Berlin.