TRANSPORT AND METABOLISM OF PROLINE AND TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY ISOLATED FLIGHT MUSCLE MITOCHONDRIA OF THE TSETSE FLY, GLOSSINA MORSITANS

By

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D E C L A R A T I O N

I, ELIUD NYAGA MWANIKI NJAGI, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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ABREVIATIONS

ADP	Adenosine-5 -diphosphate
ATP	Adenosine-5 -triphosphate
BSA	Bovine Serum Albumin
CCCP	Carbonylcyanide-p-trichloromethoxyphenyl-hydrazone
CoA	Coenzyme A
CO_2	Carbon dioxide
$CuSO_4$	Copper sulphate
DNP	2,4-dinitrophenol
Ea	Activation Energy
EC	Enzyme Commission
EDTA	Ethylene diaminetetra acetic acid
EGTA	Ethylene glyco bis - (aminoethylether) tetra acetic acid
g	Gravitational force or gram
H^+	Hydrogen ion
^3H_2O or T_2O	Tritiated water
INT	2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride
K^+	Potassium ion
KCN	Potassium cyanide
α-kg	α-ketoglutarate
KH_2PO_4	Potassium dihydrogen phosphate
Km	Michaelis constant
LDH	Lactate dehydrogenase
Na^+	Sodium ion

NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
NEM	N-ethylmaleimide
NH_3	Ammonia
NH_4^+	Ammonium ion
O	Oxygen atom
OH	Hydroxide ion
Pi	Inorganic phosphate
POPOP	1,4-bis 2-(5-phenyloxazolyl) benzene
PPO	2.5-Diphenyloxazole
Rb^+	Rubiduim ion
r.p.m	Revolutions per min
TMPD	Tetramethyl-p-phenylene diamine
Tris	Tris (hydroxy methyl) aminoethane
V_{max}	Maximum velocity
V/V	Volume per volume

UNIT ABBREVIATIONS

Cal	Calorie
cm	Centimetre
Ci	Curie
J	Joule
KCal	Kilocalorie
M	Molar concentration

mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mmol	Millimole
nm	Nanometer
sec	Second
µCi	Microcurie
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromole

SUMMARY

TRANSPORT AND METABOLISM OF PROLINE AND TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY ISOLATED FLIGHT MUSCLE MITOCHONDRIA OF THE TSETSE FLY, GLOSSINA MORSITANS

It has been demonstrated that in tsetse flies and some species of Beetles, energy for flight metabolism is derived from the partial oxidation of proline to alanine. The rates of proline oxidation have been shown to be very high during the first few seconds of flight. Such rates would require a fast proline uptake system by the mitochondria. Other studies have shown that alanine arising from the oxidation of proline in the flight muscle is transported to the fatbody where it is used in the resynthesis of the latter in these insects. The proline so resynthesized in the fatbody is transported through the haemolymph into the flight muscle where it is used to provide energy for flight metabolism. This would imply that proline from the haemolymph must gain its way into the flight muscle mitochondria where its oxidation takes place.

This study investigates the mechanism by which proline is transported into the tsetse fly flight muscle mitochondria and further elucidates the pathway through which this amino acid is metabolished in

this insect. It has earlier been shown that this pathway involves the use of a section of the tricarboxylic acid cycle between ketoglutarate and malate. Further work is also carried out to study how these tricarboxylic acid cycle intermediates between ketoglutarate and malate are transported and metabolised in this insect.

The transport of L-proline was studied by measuring the rate of mitochondrial swelling when incubated with isoosmatic solutions of proline. Swelling was determined as a function of optical density at 520 nm. The results showed that proline was transported into the mitochondria as seen from the decrease in optical density. Other amino acids run parallel with proline, namely, L-alanine, L-hydroxy proline and L-valine also caused swelling of mitochondria suggesting that they were slowly transported into this organelle. In all cases, mitochondrial swelling was inhibited by N-Ethylmaleimide and greatest inhibition was observed with proline. This latter observation suggest that proline could have a carrier.

The uptake of proline was further investigated using ^{14}C-L-Proline. Plots of proline concentration versus uptake showed saturation similar to that of Michaelis-Menten kinetics. Double reciprocal plots gave an apparent km of uptake of 85 μM and a V_{max} of

0.962 nmoles/min/mg protein. The uptake of proline was again inhibited by N-Ethylmaleimide and stimulated by neutral amino acids L-valine, L-alanine, L-methionine, L-phenylalanine, L-tryptophan and L-hydroxyproline. Inhibition by N-Ethylmaleimide support and confirm results obtained from mitochondrial swelling studies. These results suggest the uptake of proline may be carrier mediated and the stimulation by the above amino acids suggest that the uptake of this amino acid may be regulated by these neutral amino acids.

The effect of pH and temperature on the uptake of proline was investigated in the pH range 5 to 9 and temperature range of $0^{o}C$ to $30^{o}C$. The maximum uptake rate of proline was found to be at pH 7.0. Arrhenius plots of log (rate of uptake) versus absolute temperature gave an activation energy of 11 Kcal/mol. These results are consistent with the view that the process under study is the membrane transport of proline rather than the binding of proline to fixed sites in the tsetse fly flight muscle mitochondria.

To determine if proline uptake is up or down a concentration gradient, the matrix proline concentration was calculated at a fixed external proline concentration of 0.4 mM and was found to be 1.8 mM. This indicates that proline uptake is up a concentration gradient and must involve the use of energy. Proline uptake was inhibited by the uncoupler,

carbonylcyanide-p-trichloromethoxyphenyl hydrazone suggesting that energy in the form of pH gradient and/or membrane potential generated during electron transport could also drive proline inside the mitochondria.

In an earlier postulated pathway, proline was suggested to be first oxidized to glutamate which transaminates with pyruvate to give alanine and ∝-ketoglutarate. The latter has been suggested to be oxidized through the normal tricarboxylic acid cycle via succinate, fumarate, malate and finally to pyruvate which is used in the transamination reaction. To confirm this pathway the oxidative capacity of tsetse fly flight muscle mitochondria on intermediates of proline oxidation namely glutamate, ∝-ketoglutarate, succinate, fumarate and malate were investigated in parallel with proline. Results showed that tsetse mitochondria only respired appreciably with proline and succinate as substrates. Other substrates, glutamate, ∝-ketoglutarate, fumarate and malate showed no detectable oxygen consumption with these mitochondria.

To find if the limitation of oxidation of these substrates by tsetse fly flight muscle mitochondria was due to permeability barrier, detergents triton X-100 or Digitonin were used to permeabilise the mitochondrial membranes. Results showed that the proline

oxidation rates were decreased in the triton X-100 treated mitochondria. Digitonin treatment did not affect the rate of proline oxidation. In the two detergent treatments, the oxidation rates of glutamate, ketoglutarate, fumarate and malate were not detectable. These results suggest that if the above substrate have to be metabolised through the proline oxidation pathway postulated earlier (see Bursell 1977) there must be a permeability barrier which cannot be removed by these treatments.

The factors affecting the oxidation rates of proline in tsetse fly flight muscle mitochondria were investigated by following the effect of increasing concentration of proline on its oxidation rates. Saturation kinetics similar to those of Michaelis-Menten for enzyme systems were obtained. Double reciprocal plots gave an apparent Km of oxygen uptake of 6.06 mM and a V_{max} of 300 nmoles of oxygen atoms/min/mg protein. Similar work repeated at increasing concentration of ADP on the state-III rate of proline oxidation by tsetse mitochondria showed saturation kinetics similar to those of Michaelis-Menten. Double reciprocal plots of the above relationship gave an apparent Km of oxidation of 192 µm and a V_{max} of 235 nmoles oxygen atoms/min/mg protein. These results suggest that low concentration of proline are required to saturate the proline oxidation system of the

tsetse fly flight muscle mitochondria and the low Km value for ADP for this system suggest that ADP controls the rate of proline oxidation at the level of the electron transport chain but not at the level of the dehydrogenase.

Further work carried out to investigate the effect of temperature and pH on the proline oxidizing system of the tsetse mitochondria showed that this system is both temperature and pH dependent. Arrhenius plots of log (rate of proline oxidation) versus the absolute temperature gave an activation energy of 4,803 Ca/mol. The bell-shaped curve of pH versus the rate of oxidation of proline gave a maximum rate of pH of about 7.0. Further work showed that pH affected the V_{max} but not the Km of the proline oxidizing system.

Studies carried out with electron transport chain inhibitors showed that rotenone, antimycin A and cyanide completely inhibited proline oxidation and this oxidizing system is sensitive to oligomycin. The inhibition by oligomycin was relieved by the uncoupler 2, 4-dinitrophenol. Rotenone inhibition was relieved by succinate and ascorbate/tetramethyl-p-phenylenediamine. These results confirm that proline oxidation is controlled at the level of the electron transport chain and further indicates that the reducing

equivalents from the proline oxidation are channelled through the whole span of the chain. These results are further confirmed by the observation that the ADP/O ratio ranged between 2.7 to 2.9. They also indicate that the proline oxidation pathway goes through succinate as a metabolic intermediate since it is completely inhibited by malonate.

Respiration with succinate was carried out in the presence of respiratory chain inhibitors. Rotenone did not inhibit succinate oxidation. Antimycin A and cyanide completely inhibited succinate oxidation and the inhibition by antimycin A was relieved by Ascorbate/TMPD. Succinate oxidation was insensitive to oligomycin and the uncoupler 2, 4-dinitrophenol. It was also not dependent on the presence of ADP. These results indicate that the reducing equivalent from succinate oxidation are channelled through the normal electron transport chain between ubiquinone and cytochrome oxidase (aa_3). The insensitivity to ADP, oligomycin and 2, 4-dinitrophenol would suggest that either these mitochondriai preparation contains partly damaged mitochondria or succinate alters the properties of the mitochondria and uncouples them in which case once uncoupled, they should be uncoupled on any substrate or there might be a carrier for succinate which transports it into the matrix for oxidation.

Respiration with succinate by tsetse mitochondria were inbibited by malonate and ATP. Other tricarboxylic acid cycle intermediates namely ketoglutarate and malate tested decreased the rate of succinate oxidation. These results taken together suggest that succinate dehydrogenase and since the above substrates are not transported by these mitochondria, they could be interferrign with the affinity of the succinate carrier possibly by competing with the latter for the binding sites.

The products of oxidation of the tricarboxylic acid cycle intermediates ketoglutarate, succinate fumarate and malate were studied in the absence of glutamate. The results showed that pyruvate production was only detectable as the end product with succinate. In the other intermediates, there was no detectable pyruvate production. This indicated that succinate oxidation goes through a section of the postulated proline oxidation pathway.

In this study, the rate of proline oxidation by tsetse fly flight muscle mitochondria was shwon to range from 130 to 300 nmoles oxygen atoms/min/mag protein. It was shown by Olembo (1977) that in proline oxidation pathway, ketoglutarate dehydrogenase has the lowest enzyme activity of 20 nmoles/min/mg protein

It would therefore appear that may be the oxidation rate of proline through the whole pathway to alanine would be limited by its transport.

CHAPTER 1

INTRODUCTION

1.1 THE PROLINE-ALANINE OXIDATION PATHWAY IN INSECTS

Most insects have been found to utilize either lipids, carbohydrates or both as an energy source for flight. Amino acids were previously considered to be quantitatively unimportant as a direct source of energy (Wigglesworth, 1949; Hicks and Kerly, 1960, William and Krebs, 1961). Recently, Bursell (1960, 1963, 1966) found high concentrations of proline in the Haemolymph and thorax of the tsetse fly. This was confirmed in other insects (Wyatt, 1961; Sacktor 1965).

Bursell (1963), working on the flight muscle of the tsetse fly, Glossina morsitans showed that during flight there was a rapid disappearance of proline followed by a stoichiometric rise in alanine concentrations during the first two minutes. Glutamate showed a transient increase after the first minute of flight, while ∝-ketoglutarate increased ten-fold after two minutes. These observations suggested that glutamate might constitute an intermediary in the conversion of proline to ∝-ketoglutarate. ∝-ketoglutarate would then be metabolised through the normal tricarboxylic acid cycle to oxaloacetate, and the latter would there-

fore be decarboxylated to pyruvate, alanine thus appearing as the end product of proline oxidation (Figure 1).

In latter experiments, Bursell (1965, 1966, 1976) using ^{14}C-labelled amino acids confirmed the presence of this proline-alanine pathway. Since then, various workers have obtained evidence for the presence of the same pathway in other species of Glossina.

The key enzymes of proline metabolism such as proline dehydrogenase, and alanine-aminotransferase have been noted to be particularly active in the flight muscle of Glossina, in keeping with their postulated role (Crabtree and Newsholme 1970, 1975; Bursell 1975a). More recently, NAD-linked malic enzyme has also been found to be particularly active in the flight muscle of Glossina (Hoek et al, 1976; Norden and Matanganyidze 1977, 1979) suggesting its participation in the decarboxylation of malate. Earlier investigation had shown that oxaloacetate could be decarboxylated to pyruvate by an oxaloacetate decarboxylase (Bursell, 1965). However, due to its cytosolic location, and its acidic pH optimum (5.9), it is unlikely that it functions physiologically in the mitochondrial pathway of proline oxidation. A more likely route for pyruvate production from malate is directly via NAD-linked malic enzyme. This enzyme is very active

in the flight muscle of proline metabolising Glossina than in carbohydrate metabolising Sarcophaga tibialis (Konji et al 1984). Moreover, its mitochondria location, its kinetics and pH optimum (7.8) would fit it for this function (Hoek et al 1976; Norden and Matanganyidze, 1977).

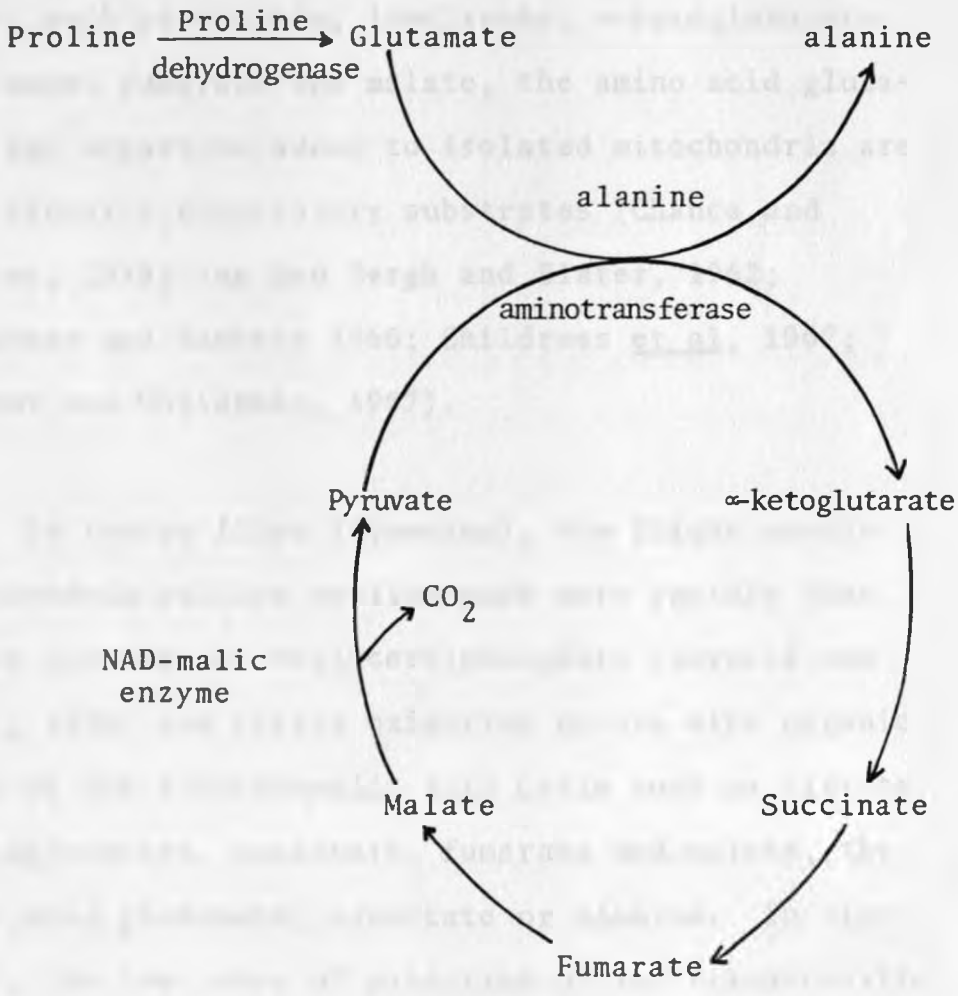
Bursell and Slack (1976) using ^{14}C-labelled proline, were able to follow the pathway of proline oxidation in Glossina. They found that glutamate was the first intermediate to be labelled, followed by succinate, then pyruvate, while alanine and carbon dioxide appeared as the the end products.

A similar pathway for partial oxidation of proline has been implicated in other proline metabolising insects, the beetles, Leptinotarsa decemlineata and Heliocopris dilloni (Hansford and Johnson, 1975; De Kort et al 1972; Khan and De Kort 1978; Mordue and De Kort 1978, Pearson et al 1979; Weeda et al 1980a). Hence a proline dependent energy metabolism appears not to be confined to the tsetse fly.

Proline is believed to be resynthesized from alanine in the fat body, by a system in which alanine appears to be converted to pyruvate by transamination with ketoglutarate, Carboxylated to oxaloacetate and then condensed with acetyl CoA, derived from fat

breakdown to form citrate which then forms ∝-ketoglutarate through the tricarboxylic acid cycle steps. Glutamate could be formed from this ∝-ketoglutarate by transamination and be sequently reduced to form proline (McCabe and Bursell 1975; Konji et al 1984).

Figure 1: Proline Oxidation Pathway



1.2 LIMITATION OF APPARENT POTENTIAL SUBSTRATES FOR RESPIRATION IN INSECTS.

Certain dipteran mitochondria have been shown to oxidize pyruvate and α-glycerolphosphate rapidly (Van den Bergh, 1964; Childress and Sacktor, 1966; Bursell and Slack, 1976), but respire relatively slowly with proline as the only substrate (Bursell, 1975). In contrast intermediates of the tricarboxylic acid cycle, such as citrate, isocitrate, α-ketoglutarate, succinate, fumarate and malate, the amino acid glutamate and aspartate added to isolated mitochondria are not effective respiratory substrates (Chance and Sacktor, 1958; Van den Bergh and Slater, 1962; Childress and Sacktor 1966; Childress et al, 1967; Sacktor and Childress, 1967).

In tsetse flies (Glossina), the flight muscle mitochondria oxidize proline much more rapidly than either pyruvate or α-glycerolphosphate (Bursell and Slack, 1976) and little oxidation occurs with organic acids of the tricarboxylic acid cycle such as citrate, α-ketoglutarate, succinate, fumarate and malate, the amino acid glutamate, aspartate or alanine. In blowflies, the low rates of oxidation of the tricarboxylic acid cycle intermediates may be accounted for by the fact that intact flight muscle mitochondria are not readily permeable to these compounds (Van den Bergh

and Slater, 1962) as demonstrated by the stimulation of respiration on these substrates in mitochondria whose membranes have been disrupted.

Van den Berg (1967) using the ammonium swelling technique demonstrated that the citric acid cycle intermediates (succinate, malate, citrate and isocitrate) do not permeate housefly or locust mitochondria. Both the ammonium salts of succinate and malate were inactive in the presence and absence of 5 mM phosphate in this test with mitochondria from the blowfly Sarcophaga barbat (Donnellan et al 1970). In an apparent contradiction, Tulp and Van Don (1969) have claimed that succinate can exchange for malate in housefly mitochondria. However, the carrier is saturated by low concentrations of phosphate, so that in the presence of phosphate little succinate can enter. In the absence of phosphate or in the presence of phosphate and mersalyl, an inhibitor of phosphate transport in insect mitochondria (Carafoli et al, 1971), succinate readily enters and is rapidly oxidized.

Pearson et al (1979) working with Beetle mitochondria demonstrated appreciable oxygen uptake rates with proline, glycerolphosphate and succinate as substrates. Respiration in the presence of 10 mM proline was consistently two to four times higher than 20 mM glycerol-3-phosphate and three to five times

higher than with 10 mM succinate.

Hansford (1971) demonstrated that mitochondria from the flight muscle of the periodical cicada, magicicada septendecim differ from preparation of other insects in that they are permeable to glutamate and ketoglutarate but not readily permeable to other members of the Krebs cycle. He proposed that specific membrane carriers are genetically determined and function related to the physiology of the tissue. In other words, the absence of dicarboxylate and tricarboxylate anion carriers in flight muscle of most insects is an adaptation to prevent the efflux of these intermediates from the mitochondrion rather than simply to inhibit their entry. The extent to which the impermeability of isolated mitochondria to various substrates affects the rates of oxidation has not been studied in Glossina.

1.3 MITOCHONDRIAL ANION TRANSPORT

1.3.1 Requirement for Mitochondrial Transport systems

The functions of mitochondria in cellular metabolism require that a large number of compounds be transported across the mitochondrial membrane (Slater et al, 1969; Tager et al, 1969). At neutral pH, most of these compounds are charged, the majority being anions (Slater et al, 1969; Tager et al, 1969).

The mitochondrion is a cellular organelle whose primary function is to generate energy as ATP, for use by the remainder of the cell. This organelle is surrounded by two membranes (Sjostrand, 1953) forming an intermembrane and matrix compartment. The outer membrane seems to be permeable to molecules having a molecular weight of 10,000 or less (Ernster and Kuylenstierna, 1969; Garland et al, 1969) so that the intermediate space contains metabolites at concentrations equivalent to those found in the cytosol. On the other hand, the inner membrane is characterized by a general impermeability to most substrates with the exception of uncharged molecules with a molecular weight not larger than 100-150 (e.g. CO_2 and NH_3) (Klingenberg 1963; Chappell, 1968, 1969). The impermeability of the membrane results primarily from the inability of hydrophilic ions to penetrate its hydrophobic lipid bilayer. Thus specific permeases must exist to facilitate the movement of anions across the inner membrane. Many of these anions accumulate in the matrix and a mitochondria/cytosolic gradient results.

The transport of certain mitochondrial metabolites is required since enzymes for some metabolic pathways are distributed in between the mitochondrial matrix and the cytosolic compartment. Thus the products of the mitochondrial enzyme reactions must be

transported to the cytosol to be used as substrates. In addition, the matrix compartment contains enzymes of the tricarboxylic acid cycle, while enzymes for fatty acid β-oxidation electron transport and ATP synthesis are associated with the inner membrane. Therefore substrates for most of these reactions must also be able to penetrate the inner membrane.

1.3.2 Methods for Determining Mitochondrial Permeability.

Several methods are available for the study of substrate anion transport across the mitochondrial membrane (Klingenberg, 1970). The permeability of the mitochondrial membrane may be assessed by (a) Measuring the rate of intramitochondrial reactions with exogenously added substrates. This method is useful for anions whose metabolism is NAD^+-linked. This is done by incubating mitochondria in a medium containing an uncoupler and rotenone in a fluorimeter. The uncoupler fully oxidizes matrix NADH and the rotenone prevents further metabolism of NADH via the electron transport chain. The addition of the anion causes reduction in the matrix NAD^+ if it penetrates. This reduction is monitored in a fluorimeter at 480 nm.

A second method consists of measurement of the swelling that occurs when mitochondria are suspended

in an isoosmotic solution of the permeant anion in the presence of a permeant cation such as NH_4^+ or K^+ in the presence of valinomycin. Swelling occurs because NH_3 which crosses the membrane by diffusion down a concentration gradient associates with protons leaving an excess of hydroxyl ions in the matrix which exchange with permeant anions. This causes an increase in the number of ions and therefore the osmotic pressure in the matrix, since water has been replaced with OH^- or an anion and NH_4^+. Thus mitochondria swell due to osmosis. This swelling causes an increase in light scattering or decrease in optical density of 520 nm. Ammonium salts can be replaced by K^+ salts but since K^+ cannot cross the membrane, valinomycin has to be added to facilitate K^+ -entry. In this method, swelling will only occur if the anion exchanges for hydroxyl ions (e.g. phosphate). Otherwise there will be no increase in the number of ions in the matrix since hydroxyl ions will not be removed from the matrix and dissociation of water will not take place. An anion/anion exchange will only occur if it is coupled to an exchange with hydroxyl ions (e.g. dicarboxylate and tricarboxylate transporter). The use of this method in mitochondrial studies was introduced by Chappell et al (1966, 1967, 1968) and has since been widely employed.

The transport of anions across the mitochondrial

membrane may also be studied directly by measuring their uptake, their efflux or both. The method compares the amount of the radio labelled anion into mitochondria with that of tritiated water and ^{14}C-labelled sucrose. T_2O can penetrate into the whole mitochondria and ^{14}C-labelled sucrose can only penetrate upto, but not into the matrix. The metabolism of the anion under investigation has to be inhibited in order to investigate only its permeability properties. This is achieved by using rotenone and/or antimycin A. The mitochondria are incubated in media containing radio label and the reaction is stopped by centrifugation. From the radioactivity in the pellet fraction one can determine whether the anion under investigation has penetrated into the matrix or not. This method employs very small quantities of material.

1.3.3 Mitochondria Anion Translocators

Two basic types of translocators have been described for the transport of metabolites across the inner mitochondrial membrane (Fig. 2).

Electrogenic exchange is characterized by the net movement of charge during the transport process. Thus the translocator catalyzes the exchange of two anions which bear different charges (e.g. Adenine

FIGURE 2: Mitochondrial Anion Translocation

EXTERNAL VOLUME	INNER MITOCHONDRIA MEMBRANE	MATRIX VOLUME	TRANSLOCATOR	TYPE	INHIBITORS
ADP^{3-}		ATP^{4-}	Adenine Nucleotide	Electrogenic	Atractloside, coatractyloside, Bongkrekic acid
Glutamate H^{+}		Aspartate	Glutamate - Aspartate	Electrogenic	Glisoxepide
Phosphate		OH^{-}	Phosphate	Electro neutral Proton compensated	N-ethyl maleimide mersalyl
Glutamate		OH^{-}	Glutamate	-----	N-ethylmaleimide
Pyruvate		OH^{-}	Pyruvate	------	∝-cyano (4.OH) cinnamate
$Malate^{2-}$		citrate $^{3-}_{+}$ H^{+}	Tricarboxylate	------	
$Malate^{2-}$		Phosphate^{2-}	Dicarboxylate	Electro neutral	n-Butyl malonate 2-Phenyl succinate
Malate^{2-} or Malonate^{2-}		∝-kg^{2-}		-	Aspartate
Glutamine		Glutamate			

nucleotide and Glutamate-aspartate carriers).

Electroneutral exchange on the other hand is characterized by the absence of net electron movement For the dicarboxylate and ∝-ketoglutarate carriers, The requirement is satisfied by the exchange of similarly charged anions, whereas the glutamate, phosphate, monocarboxylate (pyruvate) and tricarboxylate carriers maintain electroneutrality by proton compensation.

1.4 DRIVING FORCES OF MITOCHONDRIAL ANION TRANSPORT.

In addition to being impermeable to anions, the inner mitochondrial membrane also has a low permeability to protons. As a result, the membrane has a very low electrical conductivity (Greville, 1969). According to Mitchell's (1961, 1966a, 1966b, 1969) chemiosmotic hypothesis, however, the redox components of the mitochondrial electron transport chain permit the extrusion of protons from the mitochondrial matrix which do not readily leak back in due to the low membrane permeability. The proton translocation can occur because the components of the electron transport chain span the membrane in a series of three loops, each related to an energy conservation site for ATP synthesis. Thus at the inner surface of the mitochondrial membrane a carrier is reduced by acceptance of protons while at the outer surface of the membrane

another component ejects protons into the intermembrane space. The close proximity of these components in the membrane, as well as their characteristic midpoint potentials, permit their continuous oxidation and reduction when an electron source (e.g. NADH, succinate, or ascorbate plus tetramethylphenylenediamine) is provided. The resulting redox interactions cause reduction of oxygen to water and translocation of protons.

Mitchell's (1969; 1966b) initial hypothesis proposed that $2H^+$ were ejected from the mitochondria per each electron pair which passed through one of the three energy conservation sites. Thus a $2H^+$/ATP (Mitchell and Moyle, 1967) stoichiometry was postulated. Although Skulachev (1971) obtained a similar value, other researchers (Azzone and Massau, 1973; Papa et al, 1974) have criticized these results. Brand et al (1976) demonstrated a H^+/energy conservation site ratio of at least 3.0, while values as high as 4.0 were possible.

Mitochondrial respiration, therefore, acts as a proton pump which due to H^+ efflux, produces both a pH gradient and an electrical potential gradient. The protonmotive force is defined as $\Delta p = \Delta\psi - 2.3\ RT/F\ \Delta pH$ milli volts (1) where $\Delta\psi$ is the electrical potential gradient (membrane potential$_{in}$ - membrane potential$_{out}$)

and ΔpH is the proton gradient (pH_{in} - pH_{out}). This model, therefore, provides two driving forces for anion transport; a ΔpH component for electroneutral exchanges and a $\Delta\psi$ component for electrogenic exchanges.

For exchanges such as the glutamate-hydroxyl, phosphate-hydroxyl, pyruvate-hydroxyl and citrate-malate of rat liver mitochondria, which are electroneutral due to proton compensation, the ability of the pH gradient to act as a driving force is clear. Under conditions, however, where net efflux of protons occur (e.g. glutamate efflux in rat liver mitochondria) the metabolite concentration may act as a driving force. This can occur because of the relationship of anion distribution to the pH gradient (Palmieri, 1970) where

$$\log(A_{in}^{n-} / A_{out}^{n-}) = n\Delta pH \quad (2)$$

since the ΔpH is positive, a metabolite distributed according to this equation will be concentrated in the mitochondrial matrix.

The effect of ΔpH on the electroneutral H^+ compensated exchanges has been analyzed by numerous investigators by artificially collapsing the pH gradient. For this purpose, uncoupler (proton ionophores), such as carbonyl cyanide-p-trichloro-methoxy phenyl-hydrazone (CCCP) may be used since they facilitate proton translocation across the membrane (Mitchell

and Moyle, 1965; Mitchell, 1966a). It can be predicted, therefore that the addition of uncouplers to mitochondria should enhance efflux of metabolites such as glutamate, pyruvate and citrate whose transport are coupled to proton efflux. Thus it has been shown that the efflux of phosphate^{1-} (Papa et al 1970a) glutamate (Brouwer et al, 1973; Harris et al, 1973; Meijer et al, 1972), pyruvate (Brouwer et al, 1973) and citrate exchange for external malate (Papa et al, 1971; Robinson et al, 1971a) was enhanced by collapse of the ΔpH. In addition increase of the external pH has also been found to lower the ΔpH (Palmieri et al, 1970, Papa and Paradies, 1974) and thus as such should increase efflux or decrease uptake of these anions. In agreement with this idea, investigators have found that uptake of glutamate (Bradford and McGivan, 1973; Palmieri et al, 1971), phosphate (McGivan and Klingenberg, 1971) and pyruvate (Papa and Paradies, 1974) is decreased at higher external pH, which was also found to stimulate citrate efflux in exchange for malate (Robinson et al, 1971a).

Exchanges which are electroneutral but not H^+-compensated can also be driven by the ΔpH through coupling to the H^+-compensated translocators. Thus the $malate_{out}$-$phosphate_{in}$ exchange can be coupled to the $phosphate_{out}$- $hydroxyl_{in}$ exchange. The monovalent phosphate anion is deprotonated in the matrix for

exchange with the divalent malate anion. In agreement with this theory, McGivan and Klingenberg (1971) showed that a higher external pH (lower ΔpH) decreases malate uptake, the effect being brought about by a decreased uptake of phosphate^{1-} on the phosphate-hydroxyl exchange. In addition, collapse of the pH gradient enhanced uptake of phosphate in exchange for malate (Papa et al, 1971) while inhibiting the reverse process (Papa et al, 1970a). Again these effects are indirectly brought about by coupling to the phosphate-hydroxyl carrier.

The α-ketoglutarate$_{out}$-malate$_{in}$ exchange is also coupled to proton translocation through the dicarboxylate and phosphate-hydroxyl carriers. Thus phosphate^{1-} uptake permits influx of malate^{2-} which in turn can be translocated to extramitochondrial space in exchange for α-ketoglutarate^{2-}. The reverse exchange (malate$_{out}$-α-ketoglutarate$_{in}$) is also coupled to H^+ movement but through coupling to the citrate$_{out}$-malate$_{in}$ exchange.

Evidence for the relationship of the ΔpH to anion distribution was first demonstrated by Palmieri et al (1970) who showed that a plot of log (anion gradient) versus the ΔpH gave a slope which was theoritically equal to the ion charge according to equation (2). Acetate and phosphate^{-1} yielded slopes of

0.8 and 0.6 respectively, while malate and phosphate^{2-} gave slopes of 1.5 and 1.6. A similar plot by Papa and Paradies (1974) for pyruvate distribution produced a value of 0.8. Although these values were all lower than theoretical, the deviation probably results from a limited capacity of the mitochondria for anion accumulation when excess anion is provided in the medium (Palmeiri et al, 1970). Additional evidence for the relationship was obtained by a plot of the log (citrate gradient) versus log (malate gradient) the slope of which should equal the citrate charge/ malate charge (1.5). In experiments published by Robinson et al (1971) slopes of 1.4 to 1.8 were obtained lending credence to the relationship in equation (2).

Electrogenic transport, unlike electroneutral transport is driven by the mitochondrial electrical potential gradient, which provides the primary contribution to the total proton motive force (Greville, 1969). With isolated mitochondria, the $\Delta\psi$ can be estimated by the use of valinomycin, a K^+ (and Rb^+) ionophore, which facilitates the transport of K^+ across the membrane. Thus estimation of K^+ (or Rb^+) distribution will give the membrane potential ($\Delta\psi$) according to the equation

$$\Delta\psi = -2.3\ RT/F\ (\log K_{in}/K_{out})\ \text{Milli volts} \quad (3)$$

(Padan and Rottenberg, 1973), while Δp is calculated from equation (1).

In rat liver mitochondria, incubated with succinate, state 4 and state 3 respiration gave a $\Delta\psi$ of 125 and 124 mV, respectively, with a total protonmotive force of 148 and 143 mM (Padan and Rottenberg, 1973). Mitchell and Moyle (1969) on the other hand, incubated the mitochondria with β-hydroxybutyrate and measured a Δp of 225 mV in both state 4 and state 3 with the $\Delta\psi$ being 169 and 145 mV, respectively. Although Nicholls (1974) measured a similar Δp under state 4 conditions with rat liver mitochondria (Δp = 228 mV, $\Delta\psi$ = 150 mV), the Δp decreased during state 3 respiration to 170 mV, but returned to the original state 4 values when state 3 respiration ceased. In the presence of rotenone plus uncoupler the protonmotive force was only -0.6 mV with the $\Delta\psi$ = -086 mV and the ΔpH = +85 mV (Nicholls, 1974). Under similar conditions Mitchell and Moyle (1969) measured a $\Delta\psi$ = -33 mV and a ΔpH = +33 mV. Nicholls (1974) estimated a Δp = 220 mV as a result of ATP hydrolysis (Lardy et al, 1958), the Δp slowly decayed with a $t_{\frac{1}{2}}$ = 140 seconds, which agreed well with that measured for proton equilibration across the inner mitochondrial membrane (Mitchell and Moyle, 1974).

Since the membrane potential is more negative at

the inner side of the inner mitochondrial membrane, the $\Delta\psi$ can act to drive the adenine nucleotide and glutamate-aspartate translocators. Because the exchange of ATP^{4-}_{out} for ADP^{3-}_{in} must go against the membrane potential, the observed asymmetry of the carrier results. Since this asymmetry is abolished by deenergization of the mitochondria, it must result from the electrogenicity of the carrier (Pfaff and Klingenberg, 1968). Although the uptake of aspartate in exchange for glutamate is greater when the mitochondria are deenergized by uncoupling (Bremer and Davis, 1975) the carrier still maintains some asymmetric characteristics (Tischler, 1977). Studies by LaNoue et al (1974a) demonstrated that aspartate transport is facilitated by the $\Delta\psi$, this conclusion being reflected in the apparent first order rate constant which decreased from 125 $minute^{-1}$ under conditions of a maximal $\Delta\psi$ to 8 $minute^{-1}$ at the lowest $\Delta\psi$.

1.5 THEORIES FOR AND AGAINST THE EXISTENCE OF CARRIER SYSTEMS FOR NEUTRAL AMINO ACIDS IN BIOLOGI-MEMBRANES

In rat liver mitochondria, carriers have been postulated for glycine (Galfinkel, 1963) and for neutral amino acids (Gamble and Lehninger, 1973). Garfinkel (1963) postulated a glycine carrier on the basis of experiments in which the equilibrium optical

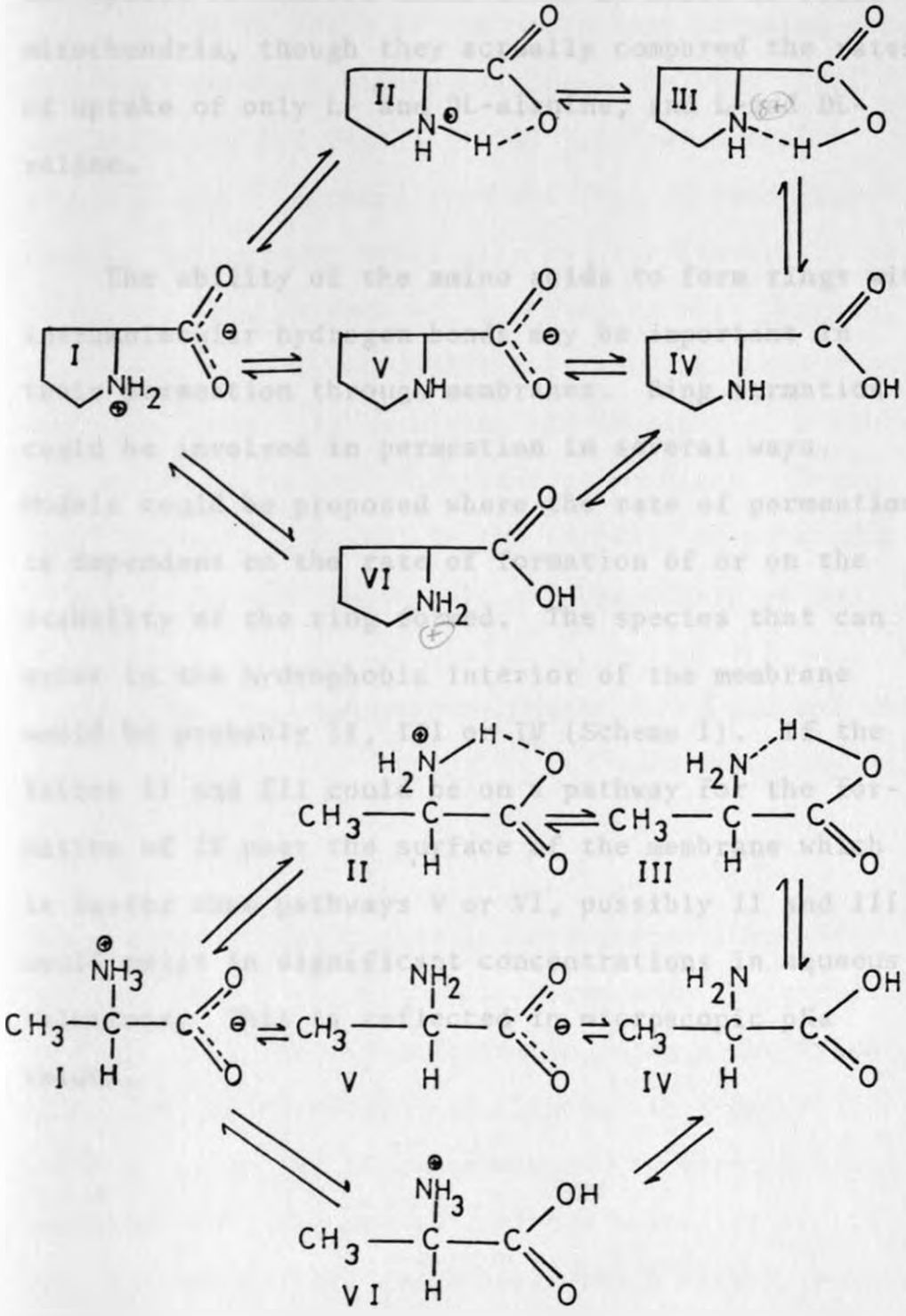
density of mitochondria suspended in glycine solutions was measured. But since mitochondria will swell in solutions of permeant solutes until membrane damage occurs and allows efflux of internal osmotically active species this data had no meaning, for if there is no additional impermeant species outside, the permeant species cannot provide the permeant osmotic support needed for the equilibrium.

Gamble and Lehninger (1973) proposed a general carrier for neutral amino acids from the finding that rat liver mitochondria swell in glycine, alanine, proline, valine and citrulline but rat heart mitochondria did not swell in citrulline. The mechanism of citrulline permeation is not defined (Chappell et al, 1972). Furthermore, it is not possible to accept that the amino acids are able to cross the inner mitochondrial membrane by simple diffussion at the observed rates since on the basis of macroscopic dissociation constants, the calculated fraction unionized is similar to that for the dicarboxylic acids which are generally impermeant in the absence of a specific carrier (Chappell and Haarhoff, 1966).

Several lines of evidence support the possibility that there are no specific transport mechanisms for neutral amino acids. Brand and Chappell (1974) found that the rates of swelling of both brain and liver

Scheme 1:

Possible forms of L-Proline and L-alanine. II and III could be either tautomers or canonical forms of a resonance hybrid. Ring form(s) are referred to as II and III in the text.



mitochondria were proportional to the concentration of GABA in the suspending medium and no evidence was obtained for saturation kinetics. Halling et al (1973) found that there was no stereospecificity for the uptake of neutral amino acids by brain or liver mitochondria, though they actually compared the rates of uptake of only L- and DL-alanine, and L-and DL-valine.

The ability of the amino acids to form rings with intramolecular hydrogen bonds may be important in their permeation through membranes. Ring formation could be involved in permeation in several ways. Models could be proposed where the rate of permeation is dependent on the rate of formation of or on the stability of the ring formed. The species that can exist in the hydrophobic interior of the membrane would be probably II, III or IV (Scheme 1). If the latter II and III could be on a pathway for the formation of IV near the surface of the membrane which is faster than pathways V or VI, possibly II and III could exist in significant concentrations in aqueous solutions. This is reflected in microscopic pKa values.

1.6 AMINO ACID TRANSPORT

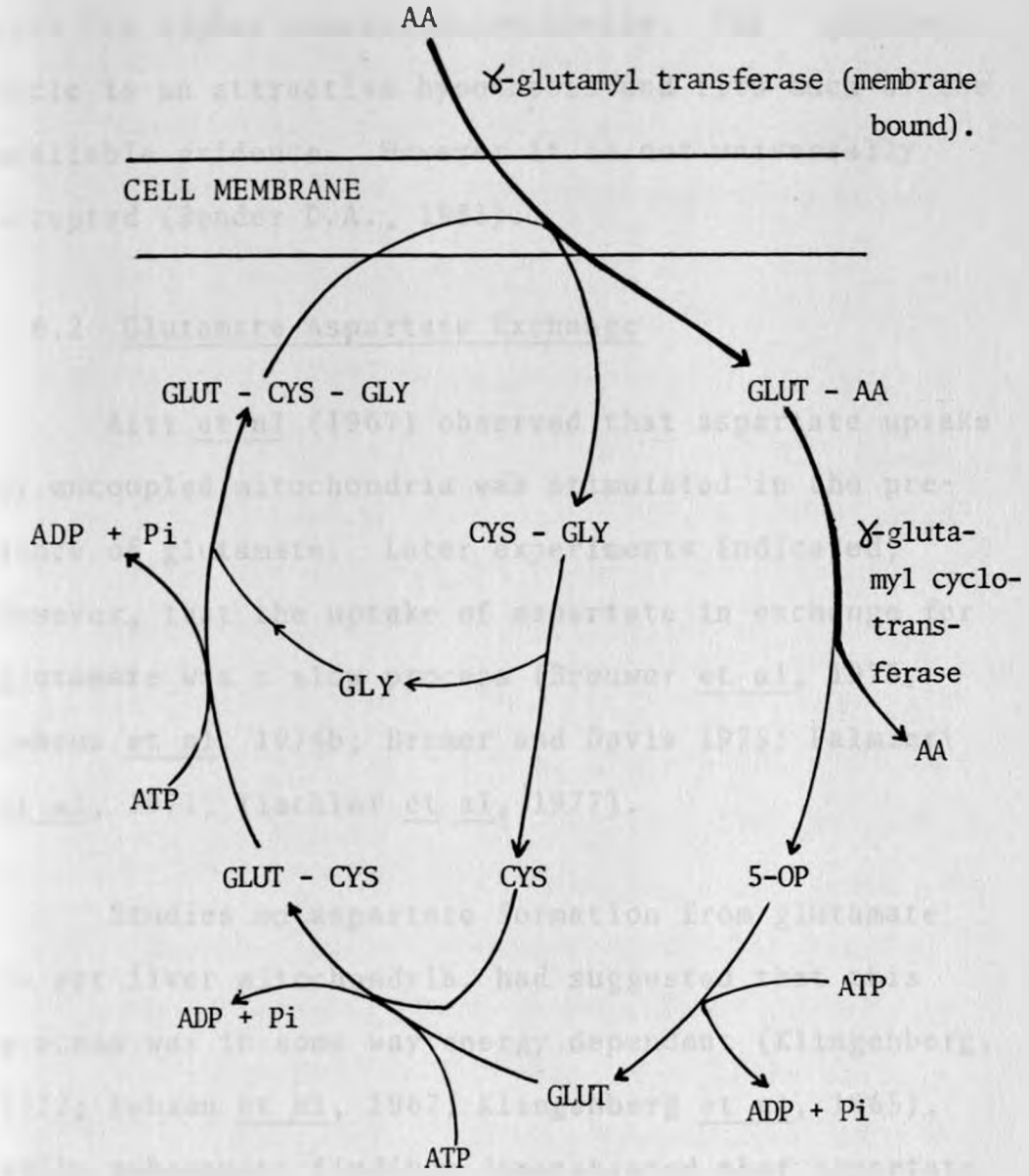
1.6.1 The γ-Glutamyl Cycle For The Transport of Amino Acids Across Cell Membranes

Little is known of the enzymology of amino acid transport across membrane barriers. Meister (1981 and Rosenberg and Tanaka (1978) proposed a cyclic formation of γ-glutamyl peptides (Fig 3) from glutathione, followed by hydrolysis and resynthesis of glutathione. Six enzymes are involved in this pathway, the central one being γ-glutamyltransferase which is membrane bound. It catalyses the transfer of a glutamyl residue from the tripeptide glutathione (γ-glutamyl cysteinylglycine) to the incoming amino acid to form a γ-glutamyl-amino acid and cysteinylglycine:

amino acid + γ-glutamylcysteinylglycine ——> γ-glutamyl-amino acid+cysteinylglycine.

The γ-glutamyl-amino acid is transported across the membrane and in the cytosolic compartment is cleaved by γ-glutamyl-cyclotransferase to release the amino acid. The other product of cleavage is 5-oxoproline, which is converted to glutamate and in turn back to glutathione in the cytosol. Three molecules of ATP are utilized in this resynthesis which serves to keep

FIGURE 3: The γ-Glutamyl Cycle



Each reaction is catalysed by a separate enzyme.

Abreviations: AA, amino acid being transported;
GLUT, glutamate; GLY, glycine;
CYS, cystein; 5-OP, 5-oxoproline.

the γ-glutamyltransferase reaction out of equilibrium so that the amino acid is 'pulled' into the cell despite its higher concentration inside. The -glutamyl cycle is an attractive hypothesis and fits much of the available evidence. However it is not universally accepted (Bender D.A., 1983).

1.6.2 Glutamate-Aspartate Exchange

Azzi et al (1967) observed that aspartate uptake by uncoupled mitochondria was stimulated in the presence of glutamate. Later experiments indicated, however, that the uptake of aspartate in exchange for glutamate was a slow process (Brouwer et al, 1973; LaNoue et al, 1974b; Bremer and Davis 1975; Palmieri et al, 1971; Tischler et al, 1977).

Studies on aspartate formation from glutamate in rat liver mitochondria, had suggested that this process was in some way energy dependent (Klingenberg, 1972; Dehaan et al, 1967; Klingenberg et al, 1965), while subsequent findings demonstrated that aspartate efflux was an energy dependent step (LaNoue and Williams, 1971; LaNoue et al, 1973; LaNoue et al, 1974a). Thus aspartate efflux was inhibited in uncoupled rat heart mitochondria (LaNoue and Williams, 1971) while ATP or substrate level phosphorylation provided energy for aspartate efflux (LaNoue et al, 1975a). Palmieri

et al (1971) showed that aspartate uptake was decreased during mitochondrial respiration or ATP hydrolysis. In agreement with this observation, Bremer and Davis (1975) later showed that mitochondria de-energization by addition of uncoupler stimulated aspartate uptake.

Although both glutamate and aspartate are monovalent anions at physiological pH, the glutamate-aspartate translocator is electrogenic (LaNone et al, 1974b; Tischler et al, 1977). Studies by LaNone et al (1974b) demonstrated that the exchange of extramitochondrial glutamate for matrix aspartate was accompanied by the uptake of protons. Whereas the glutamate-aspartate stoichiometry was 1:1, the proton; aspartate ratio was (0.66-0.81):1 which suggested that the exchange was at least 66% electrogenic. Experiments by Tischler et al (1977) show however that the exchange is 100% electrogenic. Previous studies by Brouwer et al (1973) on the aspartate-glutamate exchange in submitochondrial particles had also demonstrated the 1:1 (aspartate:glutamate) stoichiometry. Because these particles are inverted relative to intact mitochondria, aspartate uptake would be favoured over its efflux.

Kinetic studies of the glutamate-aspartate exchange gave an external glutamate Km = 7.0 mM, internal aspartate Km = 3.7 mM and V_{max} = 23.1 nmoles/min/mg protein at 10°C (Viale et al, 1977; Tischler et al,

1977). LaNoue et al (1974a) had previously calculated apparent first order rate constants for aspartate efflux in both rat liver and heart mitochondria, under different energy states. In state IV, where ADP availability is limited and respiration is slow, the constants measured were 14.0 and 21.1 min^{-1} in heart and liver respectively. Under state III conditions, which is characterized by maximal rates of respiration, the respective values were 151 and 104 min^{-1}. When the mitochondria were de-energized by addition of uncoupler, the constants decreased to 1.3 min^{-1} in heart and 7.7 min^{-1} in liver mitochondria. Thus the energy dependence of the translocation is clearly reflected in these measurements.

1.6.3 Glutamin Transporter

A Glutamin-glutamate exchange had been demonstrated in pig kidney mitochondria (Crompton and Chappell, 1973; Adam and Simposon, 1974). A glutamine translocation was demonstrated by Goldstein (1975) in rat renal mitochondria. The glutamine transport was stimulated by energy and inhibited by structural analogues, mersayl and p-chloromercuribenzoate (Goldstein 1975). Though a carrier mediated transport of glutamine in rat liver mitochondria has not been demonstrated, Karl and Brosnan (1973) have suggested that glutamine must be transported in rat liver mitochondria

since it is intramitochondrially deaminated. Otherwise it had been generally been accepted that glutamine crosses the mitochondrial membrane by free diffusion like other neutral amino acids (Garfinkel, 1963; Jones and Jones, 1970, King and Diwan, 1973; Halling et al, 1973).

1.6.4 Leucine Transporter

A leucine transporter apparently exists in rat liver mitochondria as evidenced by the transport saturation kinetics and inhibition of both leucine uptake and efflux by isoleucine, methionine, valine and L-cycloleucine. The uptake was stimulated by ATP and inorganic phosphate and was inhibited by N-ethylmaleimide (Buchanan et al, 1969).

1.6.5 Proline Transporter

Proline like the other neutral amino acids has been generally considered to enter mitochondria by free diffusion (Garfinkel, 1963; Jones and Jones, 1970; King and Diwan, 1973; Halling et al, 1973). However, Meyer (1977) obtained some evidence in support of a specific carrier for proline transport in rat liver mitochondria. He noted that:

a) the transport system was found to be stere-

ospecific for L-protein.

(b) Proline was accumulated against a concentration gradient.

(c) Energization of the mitochondria stimulated while de-energization inhibited proline transport.

(d) Mersalyl and p-chloromercuribenzoate inhibited proline transport.

(e) Proline transport showed saturation kinetics with an apparent Km = 7 mM. The energy of activation was 9 Kcal/mol (Q_{10} = 1.7). The uptake of proline showed first order kinetics with a time constant of 0.69 min^{-1}. Proline transport showed no variation with pH in the range 6.0.0. The rate of proline oxidation was approximately equal to the rate of proline transport suggesting that proline transport is rate limiting in proline oxidation in the rat liver mitochondria.

In Leishmania tropica, Law and Mukkada (1979) showed that proline transport is through an active uptake system that has saturation kinetics, temperature dependence, a requirement for metabolic energy and transport against a concentration gradient. The uptake system has a relatively broad specificity; it

is completely inhibited by D-proline as well as by alanine, methionine, valine, azetidine-2-carboxylate, thioproline, 3-4-dehydroproline, hydroxyproline and α-aminoisobutyric acid.

1.7 AIMS OF THE STUDY

It has been demonstrated that in tsetse flies (Glossina)(Bursell 1963 1966 1967; Bursell and Slack 1976; Bursell et al 1974)and some species of Beetles (Crabtree and Newsholme 1970; DeKort et al 1973; Hansford and Johnson 1975; Mordue and De Kort 1978), energy for flight metabolism is derived from the partial oxidation of proline to alanine. The rates of proline oxidation have been shown to be very high during the first few seconds of flight (Bursell 1963; Hargrove 1976; Olembo and Pearson 1982). Such rates would require a fast proline uptake system by the mitochondria. Other studies (Bursell 1963, 1966, 1967; Hoek et al 1976) have shown that alanine arising from the oxidation of proline in the flight muscle is transported to the fat body where it is used in the resynthesis of the latter (Bursell 1977; McCabe and Bursell 1975; Weeda et al 1980b; Konji et al 1984). The proline so resynthesized in the fat body is transported through the Haemolymph into the flight muscle where it is used to provide energy for flight metabolism. This would imply that proline from the Haemo-

lymph must gain its way into the flight muscle mitochondria where its oxidation takes place.

The transport of proline into isolated rat liver mitochondria occurs against a concentration gradient, and is thought to involve a carrier system (Meyer 1977). In Glossina, the rapid utilization of proline at initiation of flight would require a well controlled uptake system which hitherto is uninvestigated. This study investigates the mechanism by which proline is transported into the tsetse fly flight muscle mitochondria and further elucidates the pathway through which this amino acid is metabolished in this insect. It has earlier been postulated (Bursell 1963, 1966, 1967; Hoek et al 1976) that this pathway involves the use of a section of the tricarboxylic acid cycle between α-ketoglutarate and malate (Norden and Matanganyidze 1977; Olembo 1980).

Pearson et al (1979) observed that Beetle flight muscle mitochondria respired appreciably with succinate as a substrate. In tsetse flight muscle mitochondria similar observations were expected and this was actually observed and an explanation had to be offered. This study was also set out to explain why these mitochondria respired appreciably with succinate.

CHAPTER 2

MATERIALS AND METHODS

2.1 INSECTS AND RATS

Mature male tsetse flies Glossina morsitans were kindly supplied by the International Centre for Insect Physiology and Ecology (ICIPE), Nairobi, the Walter Reed Project (Kabete), Nairobi and the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi. They were fed on rabbit blood for the first two days by tying the upper part of rabbit's ear to the meshed side of the cage and then maintained by feeding every other day. They were starved for 24 hrs before dissection.

Male sprague-Dawley rats 3-4 months old and weighing 250-300 g were obtained from the International Laboratory for Research on Animal Disease. The animals were housed, given rat pellets and water ad libitum.

2.2 MITOCHONDRIAL PREPARATION

2.2.1 TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Before the removal of thoraces, insects were first immobilized in the cold room at $4^{o}C$ for about

30 minutes. Tsetse fly muscle mitochondria were prepared by the method of Bursell (1974). Thoraces were obtained from sixty immobilized mature male tsetse flies by pulling off heads and legs and cutting off the abdomen and the anterior wall of the thorax. These were then transfered to ice-cold isolation medium containing 250 mM sucrose, 5 mM potassium phosphat buffer pH 7.4, 0.5% BSA (essentially fatty acid free and 1 mM EDTA. The medium was homogenized in a glass homogenizer with four-up and down strokes of a hand driven teflon pestle and the tissue suspension was then filtered through muslin cloth to remove the muscle fibres. The filtrate was spun at 3020 g at 4°C for 7 minutes in a refrigerated centrifuge to bring down the mitochondrial pellet. The mitochondrial pellet was washed once with fresh isolation medium and then resuspended in 1.5. ml of medium to give a final protein concentration of 20 mg/ml. The mitochondrial suspension was then stored in ice ready for use.

2.2.2. Rat Liver Mitochondria

Rat liver mitochondria were prepared as described by Hogeboom (1965) with the following modifications. Rats fasted overnight, were sacrificed by decapitation and allowed to bleed into the sink for one to two minutes. The liver was removed, the adhering fat

trimmed and immediately placed in a beaker of ice-cold 0.25 M sucrose solution to remove the adhering blood. The washing was repeated twice and the liver chopped up with scissors into small pieces in the cold room at 4°C. The scissor-minced slices were homogenized by three up and down strokes in ice-cold 0.25 M sucrose solution by means of a Potter-Elvehjem type of homogenizer at "25" r.p.m. consisting of a glass grinding vessel and a motor-driven pestle with a teflon grinding head. Approximately three parts of 0.25 M sucrose per one part of liver tissue (25%) are used.

The 25% homogenate was centrifuged for 10 minutes at 755 g with the MSE High speed 18 centrifuge. The supernatant fraction was pipetted off with the exception of some liquid above the sediment containing poorly sedimented material. This step removes most of the unbroken cells, nuclei, erythrocytes and debris.

The supernatant fraction was centrifuged for 10 minutes at 5090 g. The resulting supernatant liquid was decanted and discarded and the sediment was suspended in an amount of 0.25 M sucrose solution corresponding to half the volume of the preceeding fraction. The suspension was again centrifuged for 15 minutes at 5090 g. The sediment after suspension in a small volume of 0.25 M sucrose solution to a

protein concentration of approximately 15 mg/ml was used for experiments. This always fell within 2.5 ml to 3.0 ml.

2.3 PROTEIN DETERMINATION

Protein was determined spectrophotometrically by the procedure of Lowry et al (1951) using Bovine serum Albumin as a standard. The standard curve was constructed using a serum albumin concentration ranging from 10-200 µg in 0.6 ml volume. To each protein sample 3.0 ml of solution A (prepared freshly by mixing 0.5 ml 1% $CuSO_4$, 0.5 ml of 2% Na-K-tartarate with 49 ml 2% Na_2CO_3 in 0.1 M NaOH) was added. The solution was allowed to stand for 10 minutes. 0.3 ml solution B (Folin phenol reagents diluted 1:4 with water) was then added and thoroughly mixed. The solutions were stored in the dark for 30 minutes and the optical density was then read at 750 nm in a 'spectronic 20'. Blanks were prepared using 0.6 ml of distilled water, run through the same process.

Sample aliquots (0.1 ml in 0.6 ml) were also run through the same procedure and the protein concentrations read against the standard curve.

Samples for protein determination were prepared as follows: Aliquots of 0.1 ml of mitochondrial sus-

pension were withdrawn and transferred to 0.1 ml of 10% trichloroacetic acid and centrifuged at 3000 g for 5 minutes. The protein precipitate was washed with 1.5 ml Ethanol-acetone mixture (1:1 V/V) and finally with 1.5 ml of Ether to remove the adhering lipids. The washed precipitate was dissolved in 0.5 ml of 7% sodium hydroxide. The hydroxide extract was then diluted to 10.0 ml with water and 0.1 ml aliquots withdrawn from it and used for protein determination.

2.4 OSMOTIC SWELLING EXPERIMENTS

The mitochondrial swelling studies were conducted using the general method of Chappell and Crofts (1966). Mitochondrial volume changes were measured by recording the changes in the absorbance of mitochondrial suspension at 520 nm and 25^{o}C in isoosmotic solutions of amino acids, supplemented with 20 mM Tris buffer, pH 7.5, containing 0.5 mM EGTA to suppress changes due to movement of endogenous Ca^{2+} and mg^{2+}, and rotenone (1 µm) to suppress endogenous respiration.

Swelling due to the entry of solute and accompanying water results in an increase in the light transmitted and a decrease in absorbance, recorded on strip charts with a Perkin-Elmer 550 S UV/VIS spectrophotometer.

2.5 UPTAKE EXPERIMENTS

Uptake of ^{14}C-proline was initiated by adding mitochondria (1 mg) to 1 ml of incubation medium in a 25 ml flask. The incubation medium contained 250 mM sucrose, 10 mM KH_2PO_4 and 1 µM Rotenone at pH 7.0 and 22°C. After time t, 0.4 ml aliquots were withdrawn from the incubation medium and transferred into 0.5 ml Eppendorf tubes. The mitochondria were separated from the medium by rapid centrifugation of 8,000 g for one minute with an Eppendorf 32-microcentrifuge. The supernatant fluid was discarded and the tightly parked pellet was quickly washed with 0.4 ml ice-cold incubation medium and the pellet dissolved in 0.3 ml of 4% sodium deoxycholate solution. The radioactivity of the redissolved pellet was measured in an intertechnique SL30 scintillation counter following the addition of 0.3 ml aliquots to 10 ml of the scintillation medium. The scintillation medium contained 0.9 g 2, 5-diphenyloxazole (PPO), 6.0 g 1,4- bis [2-(5-phenyloxazohyl)] benzene (POPOP) in one litre of ethanol-toluene mixture 3:7, V/V).

Corrections for prolone present in the sucrose space were made as follows: In a parallel series of tubes ^{14}C-sucrose or $^{3}H_2O$ was added instead of ^{14}C-proline and the radioactivity of the pellets was measured as described for proline.

The count rate of 0.3 ml aliquots of the pellet dissolved in the sodium deoxycholate without having been incubated with the radioactive anion was determined to act as a blank (X).

The count rate of 0.3 ml aliquots of the pellet dissolved in the sodium deoxycholate which had been incubated with the radioactive anion(s) was also determined (Y).

The count rate of 0.3 ml aliquots of the incubation medium with 0.4 mM anion with a known amount of radioactivity was determined (W).

From these three values, then the amount of anion taken up could be calculated from the following formula:

$$\text{anion taken up} = \frac{(Y-X)}{(W-X)} \times C \text{ (nano moles/litre)}$$

where

C = was the concentration of anion whose count rate was (W-x) and (Y-x) was the count rate of the anion whose concentration was to be determined.

2.6 DETERMINATION OF MITOCHONDRIA SPACE

Mitochondria (2 mg) were suspended in 10 ml centrifuge tubes containing 3 ml of a medium of 250 mM sucrose, 10 mM KH_2PO_4, 0.5 m EGTA and 1 µM rotenone at pH 7.0 and 24°C. ^{14}C-sucrose was added to 0.2 µCi/ml radioactivity concentration and tritiated water to 5 µCi/ml. After the incubation time of 5 minutes at 25°C, the tubes were centrifuged at 6,000 g for 5 minutes at 25°C and thepellet washed with fresh incubation medium. The supernatant and pellet were both treated with 5% (w/v) perchloric acid. Protein precipitates were removed by centrifugation and the potassium perchlorate that precipitated when the extracts were adjusted to pH 6.8 were also removed by centrifugation. The volumes of both extracts were recorded and the contents of tritium and ^{14}C-determined in an intertechnique SL 30 scintillation counter following the addition of 0.3 ml aliquots to 10 ml scintillation medium.

The space penetrated by each compound was calculated using the formular:

$$\text{Permeable space} = \frac{B \times Vi \times Vp}{A \times Vs}$$

where

A = concentration of supernatant extract

B = Concentration in pellet extract

Vi = Initial incubation volume

Vs = Volume of supernatant extract

Vp = Volume of pellet extract

From the radioactivity of the extract containing ^{14}C-sucrose, we could determine the extra matrix space (permeable to sucrose) and from the extract containing $^{3}H_2O$ the total water space (permeable to $^{3}H_2O$).

2.7 RESPIRATION EXPERIMENTS

Measurement of oxygen uptake by either isolated tsetse fly flight muscle mitochondria or rat liver mitochondria were made polarographically at 25°C, using a Clark oxygen electrode (yellow springs instruments) in an assay medium containing 250 mM sucrose, 1 mM EDTA, 10 mM Potassium phosphate buffer, pH 7.4, 10 mM proline, 10 mM succinate or 10 mM Glutamate and approximately one mg of mitochondrial protein in a total volume of 1.5 ml.

During the course of respiration, small aliquots of ADP were added by means of a long needle Hamilton microsyringe in order to establish several cycles of state - IV (no ADP)/state - III (in presence of ADP)/ state - IV respiration. The ADP/O ratio (i.e. the ratio of the moles of ADP used up to atoms of oxygen consumed) and the respiratory control ratio (RCR)

which is the ratio of state - III over state - IV rates of oxygen uptake, were calculated according to Estabrook (1967).

2.8 ENYZME ASSAYS

2.8.1 Glutamate Dehydrogenase Activity (E.C.1.4.1.2)

The enzyme was assayed according to Schmidt (1974) in 50 mM Triethanolamine hydrochloride buffer, pH 8.0, 2.5 mM EDTA, 0.2 mM NADH, 100 mM ammonium acetate, 10 µg/ml LDH and 7 mM α-ketoglutarate. Oxidation of NADH was followed in cuvettes of 1 cm light path unless otherwise stated using an unicam 1800 spectrophotometer at 30°C, following the change in optical density at 340 nm. Molar extinction coefficient at 340 nm was taken as 6.22×10^3 1 mol^{-1} cm^{-1}.

2.8.2 Proline Dehydrogenase Activity

This enzyme was assayed essentially as described by Crabtree and Newsholme (1970) based on the reduction of 2-p-iodophenyl-3-p-nitrophenyl-5-phenylmonotetrazolium chloride (INT). The assay medium contained 50 mM sodium phosphate buffer, pH 7.5, 1 mM Potassium cyanide, 100 mM L-proline, 1.7 mM acetaldehyde, 0.2 mg yeast alcohol dehydrogenase and 2000 µg mitochondrial

protein (prepared in 250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.4 and 0.1 mM EGTA). The reaction was initiated by the addition of 100 µl phenazine methosulphate (10 mg/ml) followed by 200 µl of saturated INT (5 mg/ml). The final volume of the incubation medium was 1.5 ml. An incubation period of 5 min at 25°C (room temperature) was allowed before 1.5 ml of 10% (w/v) trichloroacetic acid was added to the medium to terminate the reaction. Ethyl acetate (4.0 ml) was added to each tube, the content shaken vigorously for 30 seconds and the tubes let to stand until the ethylacetate layer cleared. This layer was carefully removed with a pasteur pipette and the absorption measured at 490 nm against ethylacetate. Control samples were treated in a similar manner except for the omission of proline from the medium. The extinction coefficient of reduced INT in ethylacetate was taken to be 20.1×10^3 l mol^{-1} cm^{-1}.

2.8.3 Succinate Dehydrogenase Activity (E.C.1.3.99.1)

The enzyme was assayed essentially as described by Pennington (1961). The assay was based on the reduction of 2-p- Iodophenyl-3-p-nitrophenyl-5-phenyl-monotetrazolium chloride (INT). The assay medium contained 50 mM potassium phosphate buffer, pH 7.4, 50 mM sodium succinate, 25 mM sucrose and 1000-2000 µg mitochondrial protein. The reaction was initiated by

addition of 100 µl phenazine methosulphate (10 mg/ml) followed by 200 µl of saturated INT (10 mg/ml). The final volume of incubation medium was 1.0 ml. After an incubation period of 5 min at 25°C, 1.0 ml of 10% Trichloroacetic acid was added to the medium to terminate the reaction. Ethyl acetate (4.0 ml) was added to each tube, the contents shaken vigorously for 30 seconds and the tubes left to stand until the ethyl acetate layer cleared. This layer was carefully removed with a pasteur pipette and the absorption measured at 490 nm against ethylacetate. Control samples were treated in a similar manner except for the omission of succinate from the medium. Molar extinction coefficient for the reduction of INT in ethylacetate was taken to be 20.1×10^3 1 mol^{-1} cm^{-1}.

2.9 ASSAY OF PYRUVATE

When the end products of succinate oxidation were to be determined, incubations were carried out in a Dubnoff metabolic shaking incubator in a 25 ml Erlenmeyer flasks at 25°C with a 1 ml final volume in order to allow proper aeration. Metabolism was stopped by deproteinization with ice-cold perchloric acid to a final concentration of 5% (w/v). The deproteinized samples were neutralized with 6 m KOH. Aliquots of the protein free neutral extracts were used for metabolic determination. Pyruvate was deter-

mined at 25°C on a Perkin Elmer 550 S UV/VIS spectrophotometer coupled to a Perkin Elmer 561 recorder using cuvettes of 1 cm light path.

An extinction coefficient of 6.22×10^{-6} per mole of NADH used was used in all the determinations at 340 nm. The concentration of pyruvate in each sample assayed was estimated using the following formula:

$$C = \frac{\Delta E}{6.22} \times \frac{V}{v}$$

where C = concentration of pyruvate in µmoles/ml of sample

ΔE = change in absorbance during the assay due to the presence of volume v of sample in a cuvette containing final volume V of assay mixture.

Pyruvate was assayed immediately after neutralization of the deproteinized extract in a reaction mixture containing 300 mM Triethanolamine buffer, pH 7.6, 3 mM EDTA and 0.1 mM NADH in a 1 cm cuvette. Deproteinized samples ranging from 0.4 ml to 0.6 ml were added to the reaction mixture. The reaction mixture was preincubated until a stable absorbance was obtained (Eo). This procedure allowed the reaction mixture to acquire a uniform temperature. After the pre-incubation period, the reaction was started by

the addition of 5.5 I.U. of lactate dehydrogenase and allowed to go to completion and the absorbance (E_1) read. The ΔE pyruvate at 340 nm was determined and used to estimate the concentration of pyruvate in each sample assayed.

2.10 TRITON X-100/DIGITONIN TREATMENT

Triton X-100 or Digitonin was added to 1-2 mg of mitochondrial protein in a medium containing 250 mM sucrose, 5 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA and 0.5% BSA. After an incubation period of 10 minutes at 0-4^{o}C, the medium was centrifuged at 3020 g for 7 minutes at 4^{o}C. The pellet was discarded and the supernatant used for assay of enzymes.

CHAPTER 3

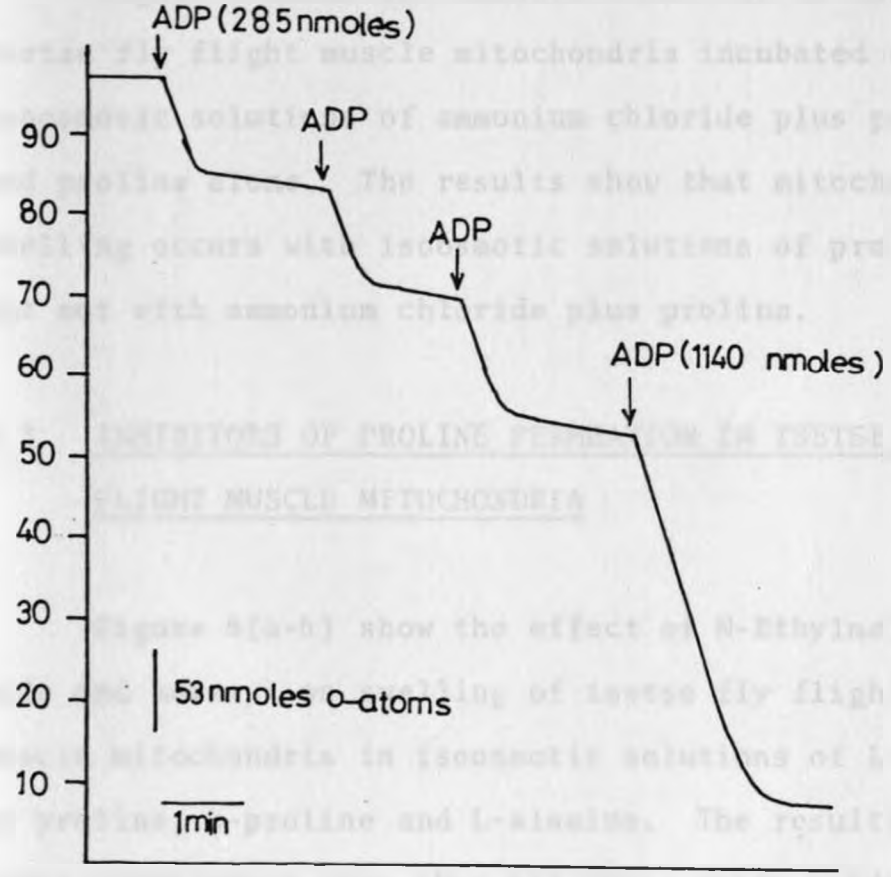
PROLINE TRANSPORT IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

3.1 RESPIRATORY CONTROL RATIO AND ADP/O RATIO

Mitochondria integrity was assessed by the determination of the ADP/O ratio, defined as the ratio of the µmoles of ADP added to ng-atoms of oxygen consumed. In addition respiratory control ratio (RCR) was determined. This is defined as the ratio of the rate of oxygen uptake in the presence of ADP to the rate of oxygen uptake in the absence of ADP normally known as state -III and state -IV respectively (Chance and Williams 1955a,b). This is shown in figure 4 where the rate of oxygen uptake by isolated tsetse fly flight muscle mitochondria incubated with proline as substrate was monitored with the oxygen electrode. It is seen that there is a slow rate of oxygen uptake in coupled tsetse fly flight muscle mitochondria incubated with proline until ADP is added, then oxygen is taken up rapidly until all the ADP is phosphorylated at which point the oxygen uptake rate slows down again. Several cycles of state -III and state -IV respiratory rates could be established by adding similar small aliquots of ADP. These experiments showed that the mitochondria were coupled since the ADP/O

FIGURE 4: Oxygen consumption by tsetse fly flight muscle mitochondria in the presence of proline. Mitochondria (1 mg) were added to 1.5 ml of the following medium: 250 mM sucrose, 10 mM phosphate buffer, pH 7.4, 1 mM EDTA and 10 mM L-proline. The reaction was started by the addition of 285 nmoles of ADP. Rates of oxygen consumption are expressed in nmoles oxygen atoms/min/mg protein. The experiment was carried out at 25°C.

FIG. 4


ADP(285nmoles)
ADP
ADP
ADP(1140 nmoles)
90
80
70
60
50
40
30
20
10
53nmoles o-atoms
1min

ratio ranged from 2.7 to 2.9 and the respiratory control ratio was always over 20.

3.2 PROLINE PERMEATION IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Figure 5 shows the osmotic behaviour of isolated tsetse fly flight muscle mitochondria incubated with isoosmotic solutions of ammonium chloride plus proline and proline alone. The results show that mitochondrial swelling occurs with isoosmotic solutions of proline but not with ammonium chloride plus proline.

3.3 INHIBITORS OF PROLINE PERMEATION IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Figure 6(a-h) show the effect of N-Ethylmaleimide and mersayl on swelling of tsetse fly flight muscle mitochondria in isoosmotic solutions of L-hydroxy proline, L-proline and L-alanine. The results from these experiments show that all three amino acids caused mitochondrial swelling as measured as a function of optical density and all are inhibited by N-ethylmaleimide but not by mersayl. The greatest inhibition of mitochondrial swelling by N-ethylmaleimide was observed with L-proline followed by L-alanine.

FIGURE 5: OSMOTIC BEHAVIOUR OF ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA INCUBATED WITH ISOOSMOTIC SOLUTIONS OF L-PROLINE AND AMMONIUM PROLINE

Mitochondria (1 mg) were suspended in 3.0 ml of a medium containing 20 mM Tris, 0.5 mM EDTA, 1 µM rotenone, 250 mM proline or 250 mM NH_4 proline, at pH 7.5 and 25°C. OD was measured at 520 nm.

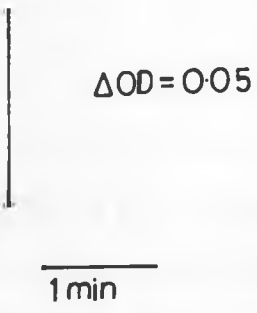
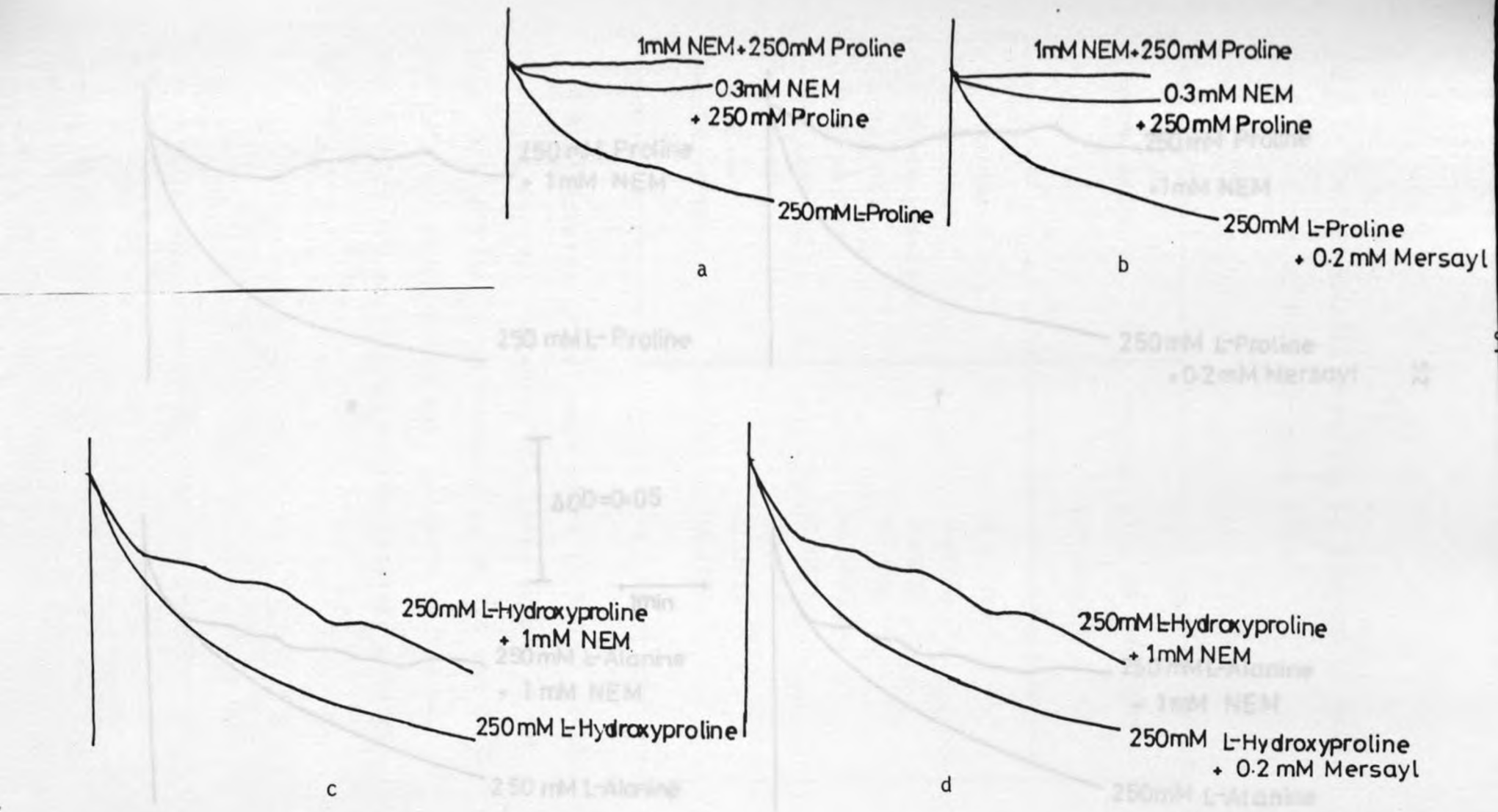


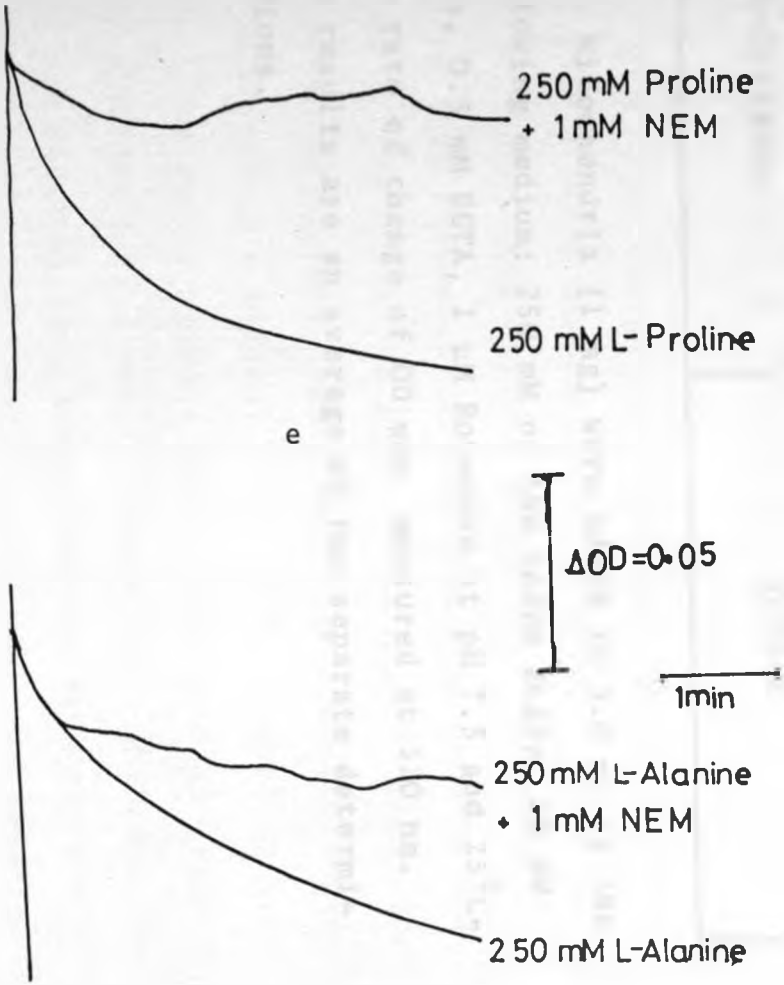
FIG. 5

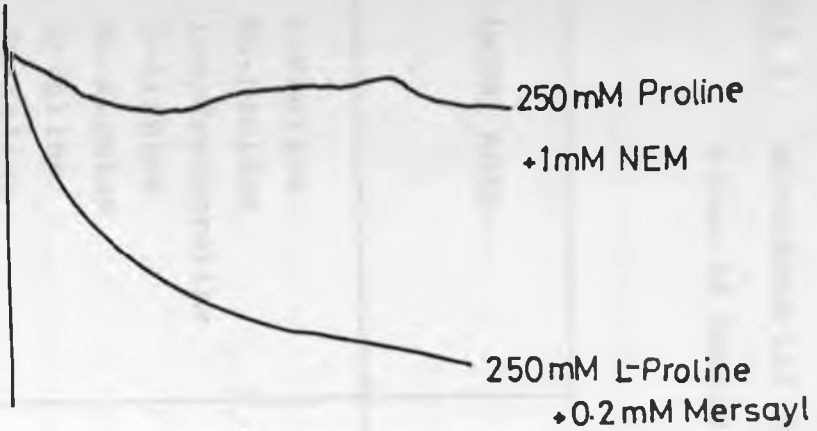
NH_4^+ Proline $\simeq$ NH_4^+Cl + Proline

FIGURE 6:(a-h) EFFECT OF N-ETHYLMALEIMIDE AND MERSAYL ON SWELLING OF TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA IN ISOOSMOTIC SOLUTIONS OF L-PROLINE, L-HYDROXYPROLINE AND L-ALANINE.

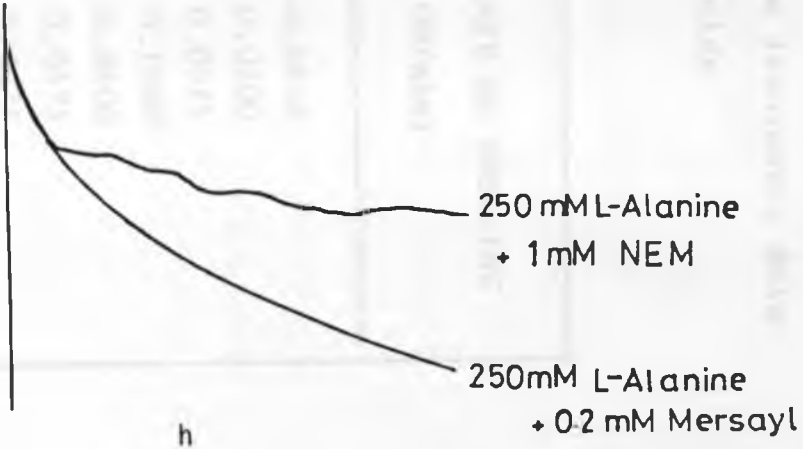
Mitochondria (1 mg) were added to 3.0 ml of the following medium: 250 mM L-proline or L-hydroxyproline or L-alanine, 20 mM Tris, 0.5 mM EGTA, 1 μM rotenone at pH 7.5 and 25^{o}C. OD was measured at 520 nm. Inhibitors were present in the medium before the addition of mitochondria.


1mM NEM+250mM Proline
0·3mM NEM
+ 250mM Proline
250mM L-Proline
a
1mM NEM+250mM Proline
0·3mM NEM
+250mM Proline
250mM L-Proline
+ 0·2 mM Mersayl
b
250mM L-Hydroxyproline
+ 1mM NEM
250mM L-Hydroxyproline
c
250mM L-Hydroxyproline
+ 1mM NEM
250mM L-Hydroxyproline
+ 0·2 mM Mersayl
d


250 mM Proline
+ 1mM NEM
250 mM L-Proline
e
ΔOD=0·05
1min
250 mM L-Alanine
+ 1 mM NEM
250 mM L-Alanine
g


250 mM Proline
+1mM NEM
250 mM L-Proline
+0.2 mM Mersayl

f


250 mM L-Alanine
+ 1mM NEM
250mM L-Alanine
+ 0.2 mM Mersayl

h

TABLE I: Mitochondrial Swelling in Isoosmotic Solutions of Neutral Amino Acids

AMINO ACID	INITIAL RATE OF SWELLING (OD/min)
L-Proline	0.0950
DL-Proline	0.0300
L-Hydroxyproline	0.0625
L-Alanine	0.1000
DL-Alanine	0.0400
L-Valine	0.0575
DL-Valine	0.0275
Glycine	0.0625
L-Cysteine	0.0400

Mitochondria (1 mg) were added to 3.0 ml of the following medium: 250 mM of the amino acids, 20 mM Tris, 0.5 mM EGTA, 1 µM Rotenone at pH 7.5 and 25°C. The rate of change of OD was measured at 520 nm. The results are an average of two separate determinations.

3.4 STEREOSPECIFICITY OF AMINO ACID PERMEATION IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Table 1 shows the mitochondrial swelling in isoosmotic solutions of neutral amino acids L-proline, L-hydroxy proline, L-alanine, DL-alanine, DL-proline, L-valine, DL-valine, Glycine and L-cysteine. The initial rates of mitochondrial swelling in the presence of L- and DL-alanine, L- and DL-proline, L- and DL-valine shows that the permeability of these mitochondria is stereospecific for these compounds since the initial rates as well as the extent of swelling are over two times higher with L- than with the DL-isomers.

3.5 TIME COURSE OF ^{14}C-PROLINE UPTAKE

The time course of ^{14}C-proline uptake is shown in figure 7 where tsetse fly flight muscle mitochondria were incubated with a fixed amount of ^{14}C-proline at varying time intervals ranging from 0 seconds to 180 seconds. The uptake is shown to be almost linear for the first 30 seconds, after which it gradually decreases approaching a maximal level of 0.8-1 mmol/mg protein. The uptake shows a first order kinetics with a rate constant of 1.2 sec^{-1} (Figure 7b). The reaction does not start at zero time since the reaction was stopped not by an inhibitor but by rapid centrifugation. The determination of the matrix volume as

FIG. 7

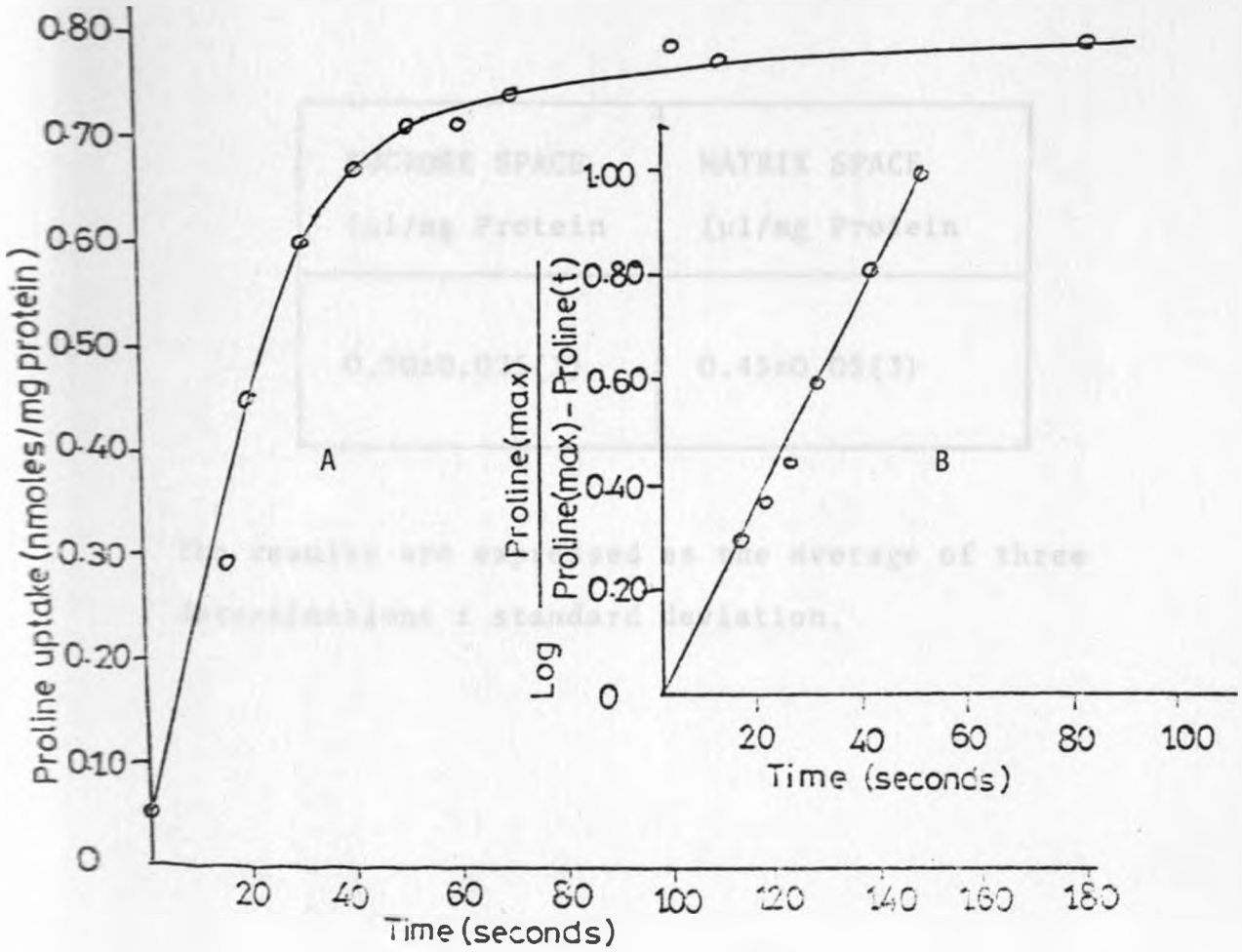

Proline uptake (nmoles/mg protein)
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0
20
40
60
80
100
120
140
160
180
Time (seconds)
A
Log Proline(max) / Proline(max) - Proline(t)
1·00
0·80
0·60
0·40
0·20
0
20
40
60
80
100
Time (seconds)
B

FIGURE 7A: TIME COURSE OF 1-^{14}C-PROLINE UPTAKE BY ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA.

Mitochondria (1 mg) were added to 1.0 ml of medium containing 250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, Rotenone (1 μM) and 0.4 mM L-Proline (0.2 μCi) at 25°C. The results are an average of two separate determinations.

FIGURE 7B: Shows a kinetic plot of figure 7A. It shows a plot of

$$\log_{10}\left[\frac{\text{Proline}_{(max)}}{\text{Proline}_{(max)} - \text{Proline}_{(t)}}\right] \text{ versus time}$$

For experimental details see legend for figure 7A.

TABLE II: Accessible Space in Packed Tsetse Fly Flight Muscle Mitochondria Determined By The Method of Meyer and Vignais (1973)

SUCROSE SPACE (µl/mg Protein	MATRIX SPACE (µl/mg Protein
0.70±0.025(3)	0.45±0.05(3)

The results are expressed as the average of three determinations ± standard deviation.

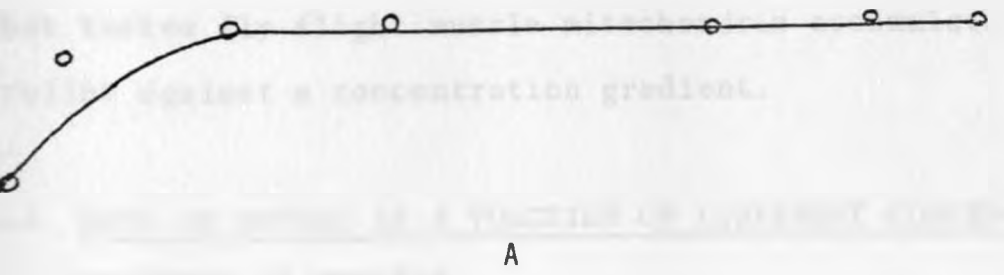
FIGURE 8A: CONCENTRATION DEPENDENCE OF PROLINE UPTAKE IN ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Mitochondria (1 mg) were added to 1.0 ml of medium containing 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 1 μM Rotenone, 0.05 - 1.0 mM L-Proline (0.2 μCi) at 25°C. Uptake was stopped after one minute. The plotted values are an average of two separate determinations.

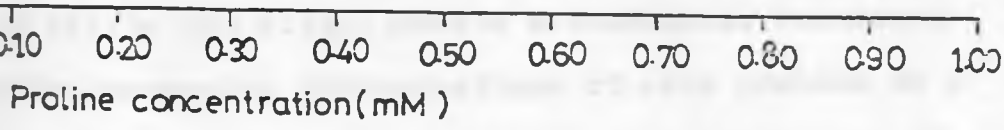
FIGURE 8B: LINEWEAVER-BURK PLOT OF L-^{14}C-PROLINE PENETRATION IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

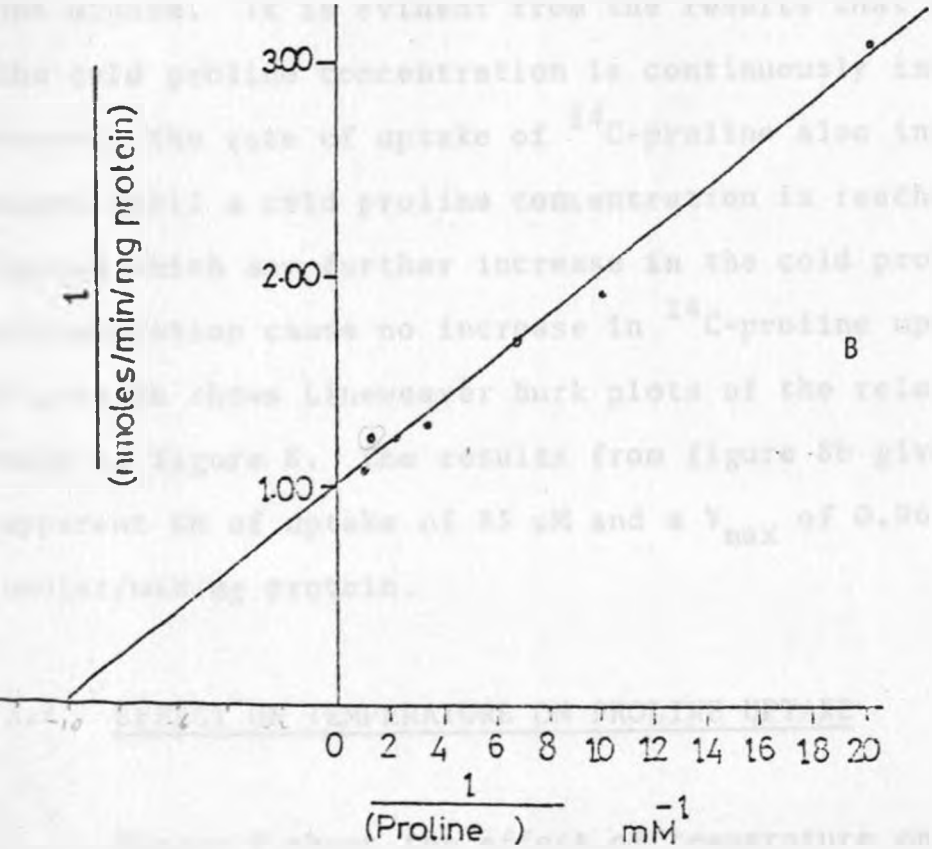
For experimental details see the legend of figure 8A.

8



A


0·10
0·20
0·30
0·40
0·50
0·60
0·70
0·80
0·90
1·00
Proline concentration (mM)


3·00
2·00
1·00
1
(nmoles/min/mg protein)
B
0
2
4
6
8
10
12
14
16
18
20
1
(Proline)
mM⁻¹

0.4 - 0.5 µl/mg of mitochondrial protein (Table II) would imply a matrix proline concentration of 1.8 mM at 30 seconds while the external medium proline concentration is 0.4 mM. This again, figure 7 shows that tsetse fly flight muscle mitochondria accumulate proline against a concentration gradient.

3.6 RATE OF UPTAKE AS A FUNCTION OF DIFFERENT CONCENTRATIONS OF PROLINE

Figure 8 shows the rate of uptake of ^{14}C-proline by tsetse fly flight muscle mitochondria incubated with increasing concentrations of cold proline at a fixed hot proline concentration at a fixed time of one minute. It is evident from the results that as the cold proline concentration is continuously increased, the rate of uptake of ^{14}C-proline also increases until a cold proline concentration is reached beyond which any further increase in the cold proline concentration cause no increase in ^{14}C-proline uptake. Figure 8b shows Lineweaver Burk plots of the relationship of figure 8. The results from figure 8b give an apparent Km of uptake of 85 µM and a V_{max} of 0.962 nmoles/min/mg protein.

3.7 EFFECT OF TEMPERATURE ON PROLINE UPTAKE

Figure 9 shows the effect of temperature on the

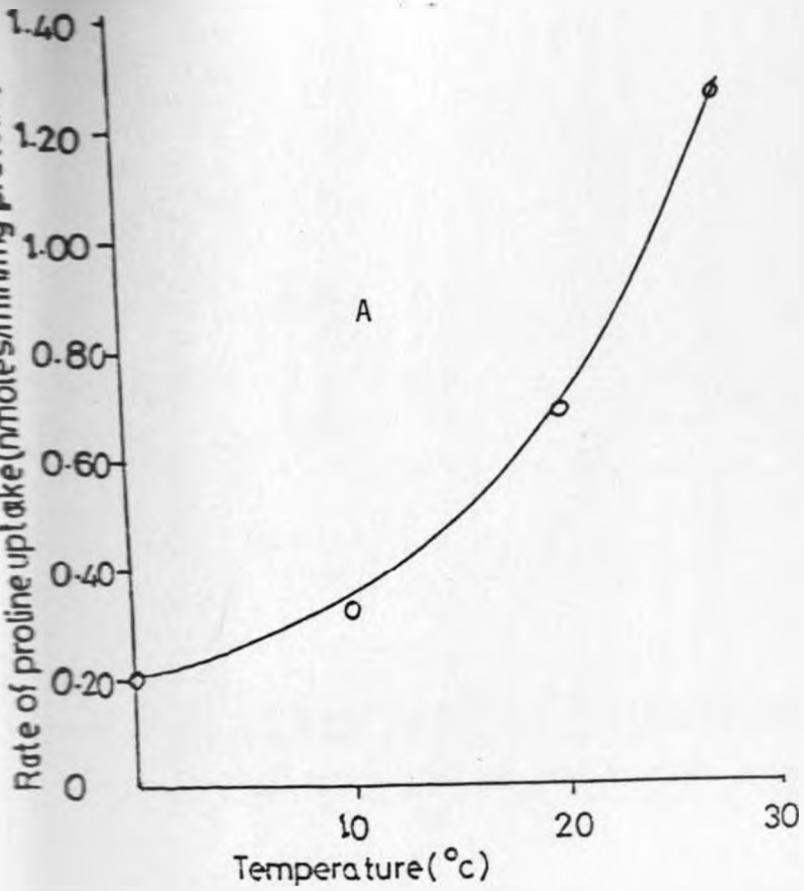
FIGURE 9A: TEMPERATURE DEPENDENCE ON L-^{14}C-PROLINE UPTAKE BY TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA IN VITRO

Mitochondria (1 mg) were added to 1.0 ml of medium containing 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, 1 µM Rotenone, 0.4 mM L-Proline (0.2 µCi) at temperatures ranging from 0°C to 30°C. Plotted points are and average of two determinations.

FIGURE 9B: ARRHENIUS PLOT OF THE RELATIONSHIP BETWEEN L-^{14}C-PROLINE UPTAKE VERSUS ABSOLUTE TEMPERATURE.

Conditions same as those of figure and data plotted is that of figure

FIG. 9


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
Rate of proline uptake(nmoles/min/mg protein)
A
10
20
30
Temperature(°c)

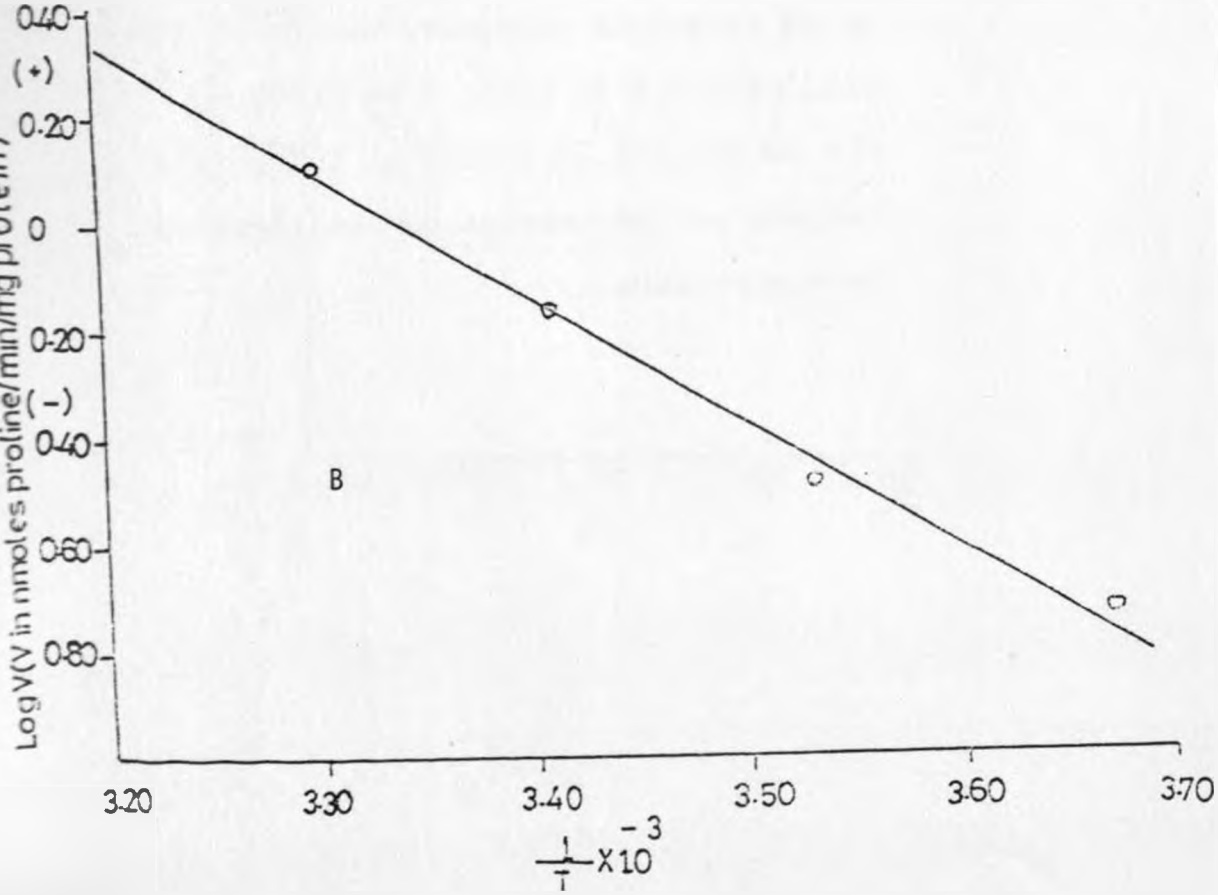
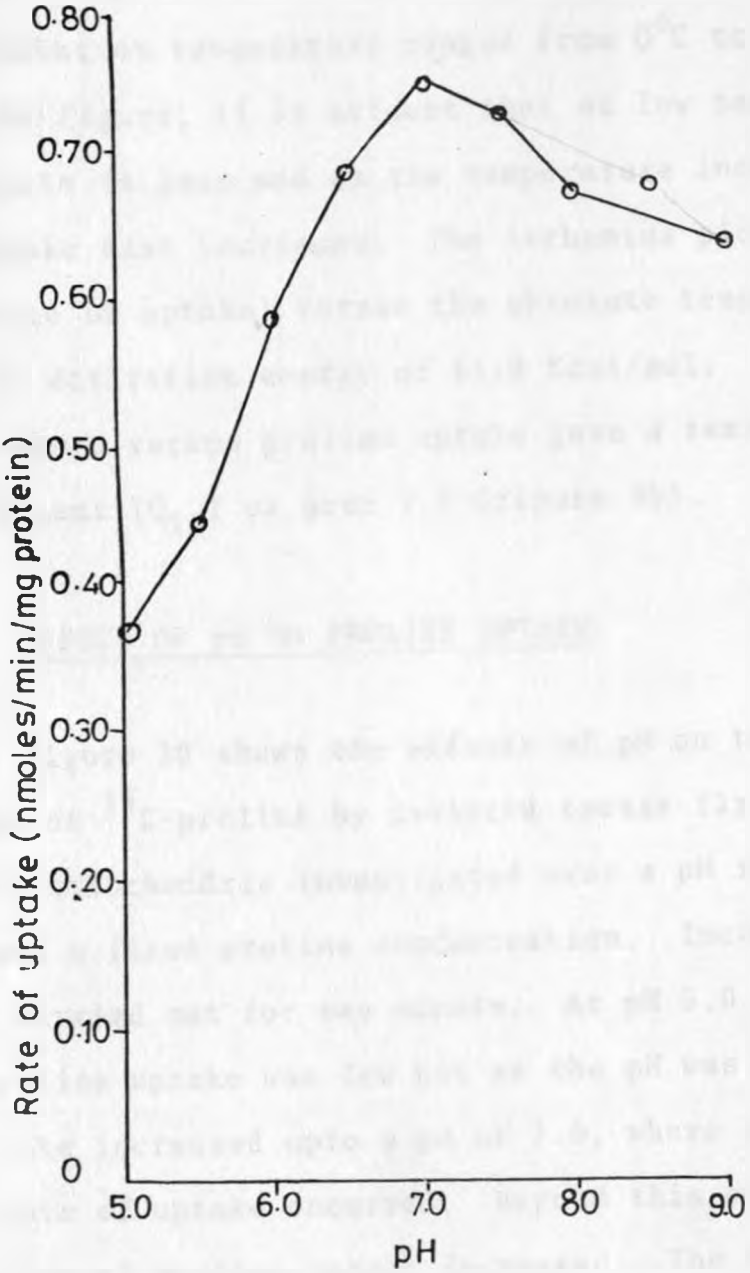

0.40
(+)
0.20
0
0.20
(-)
0.40
0.60
0.80
Log V(V in nmoles proline/min/mg protein)
B
3.20
3.30
3.40
3.50
3.60
3.70
$\frac{1}{T} \times 10^{-3}$

FIGURE 10: EFFECT OF pH ON L-^{14}C-PROLINE UPTAKE IN TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

Mitochondria (1 mg) were added to 1.0 ml of medium containing 250 mM Sucrose, 10 mM Potassium phosphate buffer at varying pH's 0.5 mM EGTA, 1 µM Rotenone, 0.4 mM Proline (0.2 µCi) at 25^{o}C. The results are an average of two separate determinations.

FIG. 10


0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0
Rate of uptake (nmoles/min/mg protein)
5.0
6·0
7·0
8·0
9.0
pH

uptake of ^{14}C-proline by isolated tsetse fly flight muscle mitochondria incubated with a fixed concentration of ^{14}C-proline and a fixed time of one minute. The incubation temperature ranged from 0^{o}C to 30^{o}C. From the figure, it is evident that at low temperatures, the uptake is less and as the temperature increases the uptake also increases. The Arrhenius plots of log (rate of uptake) versus the absolute temperature gave an activation energy of 11.0 Kcal/mol. The temperature versus proline uptake gave a temperature coefficient (Q_{10}) of over 2.0 (figure 9b).

3.8 EFFECT OF pH ON PROLINE UPTAKE

Figure 10 shows the effects of pH on the rate of uptake of ^{14}C-proline by isolated tsetse fly flight muscle mitochondria investigated over a pH range of 5-9 and a fixed proline concentration. Incubations were carried out for one minute. At pH 5.0, the rate of proline uptake was low but as the pH was increased the rate increased upto a pH of 7.0, where the maximum rate of uptake occurred. Beyond this optimum pH, the rate of proline uptake decreased. The pH-uptake profile gives a bell-shaped curve.

3.9 ENERGY DEPENDENCE OF ^{14}C-PROLINE UPTAKE

The energy dependence of ^{14}C-proline uptake by

TABLE III: Energy Dependence of L-^{14}C-Proline uptake By Isolated Tsetse Fly Flight Muscle Mitochondria.

ADDITIONS	INITIAL RATE OF PROLINE UP TAKE (nmoles/min/mg)
0.4 mM Proline	0.66 ± 0.02(3)
" + Succinate	1.41 ± 0.015(3)
" + Succinate + Antimycin A	1.18 ± 0.016(3)
" + Antimycin A + Ascorbate + TMPD	0.83 ° 0.025(3)
" + Antimycin A + Ascorbate + TMPD + KCN	0.73 ± 0.04(3)
" + CCCP	0.56 ± 0.012(3)
" + Oligomycin (30 µg)	0.86 ± 0.015(3)

Mitochondria (1 mg) were added to 1.0 ml of the following medium: 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, 1 µM Rotenon, and 0.4 mM Proline (0.2 µCi) at 25°C. Substrate or inhibitors were present before the addition of mitochondria at the following concentration: 5 mM Succinate, 10 µM Antimycin A, 5 mM Ascorbate, 100 µM TMPD, 2 µM CCCP and 1 mM KCN. Results are expressed as mean ± standard deviation of three determinations.

tsetse fly flight muscle mitochondria is shown in table III. When mitochondria are energized by the addition of the respiratory substrate, succinate whose reducing equivalent after oxidation enter the respiratory chain at ubiquinone Q, the rate of proline uptake increases. When mitochondria are energized with succinate, the addition of the respiratory chain inhibitor antimycin A, which blocks electron transport between cytochrome b and cytochrome C, is seen to decrease the rate of proline uptake. In the presence of antimycin A, the addition of the artificial respiratory chain substrate, Ascorbate/TMPD whose reducing equivalents after oxidation, enter the respiratory chain at the level of cytochrome C, decreased the uptake of proline. Addition of cyanide into the antimycin A, ascorbate/TMPD containing medium further decreased the rate of uptake of proline. Cyanide is known to inhibit the cytochrome oxidase (aa_3) complex. The inclusion of the uncoupler, carbonylcyanide-p-trichloromethoxyphenylhydrazone which disconnects the coupling between ATP synthesis and electron transport into the proline uptake medium decreased the uptake of proline more drastically than the respiratory chain inhibitors. Oligomycin does not affect the initial rate of proline uptake.

TABLE IV: Effect of Proline Analogs and Other Neutral Amino Acids on Proline Uptake

COMPOUND ADDED	RATE OF UPTAKE (nmoles/min/mg)	% STIMULATION OF INITIAL RATE
0.4 mM Proline	0.88 ± 0.02(3)	-
Proline + L-Hydroxy Proline (1 mM)	1.51(2)	72
" + Thioproline (1 mM)	1.39(2)	58
" 3,4-Dehydro-proline (1 mM)	1.45(2)	65
" L-Alanine (1 mM)	1.41(2)	60
" L-Valine (1 mM)	1.70(2)	93
" L-Methionine (1 mM)	2.13(2)	142
" L-Phenylalanine (1 mM)	1.70(2)	93
" L-Tryptophan (1 mM)	1.40(2)	59

Mitochondria (1 mg) were added to 1.0 ml of the following medium: 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, 1 µM Rotenone, 0.4 mM Proline (0.2 µCi) and 1 mM of the amino acids/proline analogs at 25°C. The results shown are an average of number of determinations given in brackets.

TABLE V: The effect of ADP and ATP on Proline Uptake

ADDITIONS	RATE OF UPTAKE (nmoles/min/mg Protein)
Proline	0.78(2)
Proline + 1 mM ADP	0.62(2)
Proline + 5 mM ADP	0.63(2)
Proline + 1 mM ATP	0.75(2)
Proline + 5 mM ATP	0.72(2)

Mitochondria (1 mg) were added to 1.0 ml of the following medium: 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, 1 µM Rotenone and 0.4 mM Proline (0.2 µCi) at 25°C. Nucleotides were present before the addition of the mitochondria at the concentrations indicated. Results are expressed as an average of two separate determinations.

3.10 SPECIFICITY OF THE PROLINE TRANSPORT SYSTEM OF TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

The effect of other L-amino acids and/or proline analogs on the proline transport system of the tsetse fly flight muscle mitochondria was studied by incubating these mitochondria with the amino acids and/or analogs of proline before the addition ^{14}C-proline. Results shown in table IV indicate that other amino acids and/or proline analogs tend to increase the initial rate uptake of ^{14}C-proline.

3.11 EFFECT OF ADENINE NUCLEOTIDES ON PROLINE UPTAKE

The proline transport system of the tsetse fly flight muscle mitochondria was studied in the presence of two concentrations of ADP and ATP as shown in table V. ADP at both 1 and 5 mM concentrations was shown to decrease the initial rate of proline uptake by 21%. ATP at both 1 and 5 mM concentrations did not affect the initial rate of proline uptake.

3.12 EFFECT OF THIOL BLOCKING AGENTS ON PROLINE UPTAKE

The effect of thio-blocking agents, N-Ethylmaleimide and mersalyl on the uptake of proline by tsetse fly flight muscle mitochondria was studied by incubating these mitochondria with the inhibitors

TABLE VI: Effect of Thiol Blocking Agents on Proline Uptake

ADDITIONS	INITIAL RATE OF UPTAKE (nmoles/min/mg Protein)
NONE	0.86 ± 0.03(3)
NEM (0.2 mM)	0.60 ± 0.05(3)
NEM (0.4 mM)	0.40 ± 0.015(3)
MERSALYL (0.2 mM)	0.85 ± 0.01(3)

Mitochondria (1 mg) were added to 1.0 ml of the following medium: 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.0, 0.5 mM EGTA, 1 μM Rotenone and 0.4 mM Proline (0.2 μCi) at 25°C. Inhibitors were present before mitochondrial addition at the concentrations indicated. Results are expressed as mean ± standard deviation of 3 separate determinations.

before the addition of ^{14}C-proline. From the results of table VI, N-Ethylmaleimide at concentrations of 0.2 and 0.4 mM inhibited the uptake of proline by tsetse fly flight muscle mitochondria by 30% and 53% respectively. Thiol blocking agent mersalyl had no effect on the uptake of proline by these mitochondria.

CHAPTER 4

OXIDATION OF PROLINE AND INTERMEDIATES OF ITS OXIDATION BY ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

4.1 SUBSTRATE OXIDATION

State-III respiration of tsetse fly flight muscle mitochondria incubated with the substrates, proline, glutamate α-ketoglutarate, fumarate, malate and succinate was studied (Table VII). The rate of substrate oxidation was measured as a function of oxygen consumption. The results show that proline and succinate were the only substrates which could be oxidized by these mitochondria. The other substrates, namely glutamate, α-ketoglutarate, fumarate and malate did not give detectable rates of oxygen cunsumption and therefore appeared not to have been oxidized. The rate of proline oxidation measured as a function of oxygen consumption was higher than that of succinate oxidation.

4.2 EFFECT OF DETERGENTS ON SUBSTRATE OXIDATION

Having observed that only proline and succinate showed appreciable oxygen uptake rates (Table VII) but not the other substrates (glutamate, α-ketoglutarate, fumarate and malate) it was necessary to find out

TABLE VII: State III Respiration of Tsetse Fly Flight Muscle Mitochondria Incubated with various Substrates.

SUBSTRATE	RATE OF OXIDATION (nmoles of O_2 atoms/min/ mg Protein)
Proline	188 ± 11.8
Glutamate (10 mM)	N.D
α-ketoglutarate (10 mM)	N.D
Fumarate (10 mM)	N.D
DL-malate (10 mM)	N.D
Succinate (10 mM)	153 ± 6.4

The incubation medium contains 250 mM sucrose, 10 mM KH_2PO_4, 0.1 mM EGATA, substrates at the concentrations shown, 1 mg of mitochondria protein and 1 mM ADP in a total volume of 1.7 ml and pH 7.4. Reaction was carried out at 25°C. The vaues given are an average of three separate determinations.

N.D. = Not detectable.

whether the low rates of oxidation of these substrates was due to insufficient permeability of mitochondria towards them. This could only be done by increasing mitochondrial permeability towards these substrates. In this study the detergents, Triton X-100 and Digitonin were used for this purpose.

4.2.1 Effect of Triton X-100

The effect of Triton X-100 on the oxidation of proline, glutamate, ∝-ketoglutarate, fumarate and malate by tsetse fly flight muscle mitochondria was investigated (Table VIII). Results showed that triton X-100 did not stimulate the oxidation of glutamate, ∝-ketoglutarate, fumarate or malate. The rate of oxidation of proline was decreased by the presence of triton X-100 at a concentration of 170 µg/mg protein in the incubation medium. Further work was carried out to find the effect of increasing concentration of triton X-100 on the rate of proline oxidation and the results showed that as the detergent concentration was increased the rate of oxidation of this substrate decreased until a detergent concentration was reached which totally inhibited oxidation (Table IX).

TABLE VIII: Effect of Triton X-100 on Oxygen Uptake by Isolated Tsetse Fly Flight Muscle Mitochondria Using Various Substrates

SUBSTRATES	RATES OF OXYGEN UPTAKE (nmoles O_2 atoms/min/mg protein)	
	WITHOUT TRITON X-100	WITH TRITON X-100 (170 µg)
L-Proline (10 mM)	196 ± 17(3)	76 ± 13(3)
∝-ketoglutarate (10 mM)	N.D	N.D
L-Glutamate (10 mM)	N.D	N.D
DL-Malate (10 mM)	N.D	N.D
Fumarate (10 mM)	N.D	N.D

Results are an average of three separate determinations. Experimental legends were same as those used in table VII except that 170 µg triton X-100/mg protein were present before the reaction was started with 1 mM ADP.

TABLE IX: Effect of Triton X-100 on Oxygen Uptake by Tsetse Fly Flight Muscle Mitochondria using Proline

TRITON X-100 µg/mg Protein	RATE OF OXYGEN UPTAKE (nmoles O_2 atoms/min/mg Protein
0	208
42.8	108
127.5	86
170	76
225	64
295.5	62
340	60
382.0	0
425.0	0

Experimental details were same as those of table VII except that varing amounts of triton X-100 were added before the reaction was started with 1 mM ADP.

4.2.2 Effect of Digitonin

The effect of digitonin on oxidation of proline, glutamate, α-ketoglutarate, fumarate and malate by tsetse fly flight muscle mitochondria was also studied (Table X). The results showed that presence of digitonin at a concentration of 170 µg/mg protein in the incubation medium did not stimulate the oxidation of glutamate, α-ketoglutarate, fumarate or malate. However, it is observed that the same detergent concentration decreased the oxidation of proline significantly.

The effect of increasing concentration of digitonin on the oxidation rate of proline was studied (Table XI). Several cycles of state-III/state-IV respiration were established by adding small aliquots of ADP into the suspension medium which contained the tsetse mitochondria at increasing detergent concentration. These results showed that during proline oxidation, the state-IV respiratory rates were increased, while the state-III respiratory rates were decreased by the presence of this detergent. These two opposing effects would account for the decrease in the respiratory control ratio observed in this experiment.

Further experiments with increasing concentra-

TABLE X: Effect of Digitonin Treatment on Oxygen Consumption of Isolated Tsetse Fly Flight Muscle Mitochondria Incubated with various Substrates

SUBSTRATES	RATES OF OXYGEN UPTAKE (nmoles atoms /min/mg)	
	WITHOUT DIGITONIN	WITH 170 µg DIGITONIN
Proline (10 mM)	244.5 ± 32(3)	145 ± 4
Glutamate (10 mM)	N.D	N.D
∝-ketoglutarate (10 mM)	N.D	N.D
Fumarate (10 mM)	N.D	N.D
Malate (10 mM)	N.D	N.D

The reaction medium contained 250 mM Sucrose, 10 mM Potassium phosphate buffer, pH 7.4, 10 mM substrate, 1 mg of mitochondrial protein, 1 mM ADP and 0.5 mM EGTA at 25°C. Rates of oxygen consumption were measured as nmoles of oxygen atoms/min/mg of mitochondrial protein. The valuesgiven are an average of three separate determinations. Rates of oxygen uptake indicated are same in the presence of both NAD^+ (0.1 mM) and CoA (10 µg/ml).

TABLE XI: The effect of increasing concentration of Digitonin on the respiratory control ratio, State III and state IV respiratory rates on tsetse fly flight muscle mitochondria incubated with 10 mM proline.

Digitonin Conc. (µg/mg Protein)	State III rate of Oxygen uptake	State IV rates of oxygen uptake	RCR
0	417	20	20.85
34	329	23	14.30
68	329	33	9.97
102	326	26	12.54
136	284	48	5.92
170	317	75	4.23
204	366	100	3.66
238	287	107	2.68
272	236	110	2.15
306	236	129	1.83
340	224	143	1.57
374	180	127	1.42

The reaction medium contained 250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.4, 0.5 mM EGTA, 10 mM substrate, 1 mg of mitochondrial protein, and 1 mM ADP in a total volume of 1.7 ml at 25^{o}C. Rates of oxygen consumption are measured as nmoles oxygen atoms/min/mg mitochondrial protein. Each reading is an average of two observations.

FIGURE 11: Latency of Glutamate dehydrogenase from the tsetse fly flight muscle mitochondria. One mg of mitochondrial protein were incubated in a medium containing 250 mM sucrose, 5 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.5% BSA and varying amounts of triton X-100 or digitonin at 0 -4^{o}C for 10 minutes. After the incubation period is over, the medium is centrifuged at 3020 g for 7 minutes at 4^{o}C and the pellet discarded. Glutamate dehydrogenase activity was then assayed in the supernatant using the method given in section 2.8.1. The results are an average of two separate determinations. The plotted points are within 20% of the mean.

FIG. 11

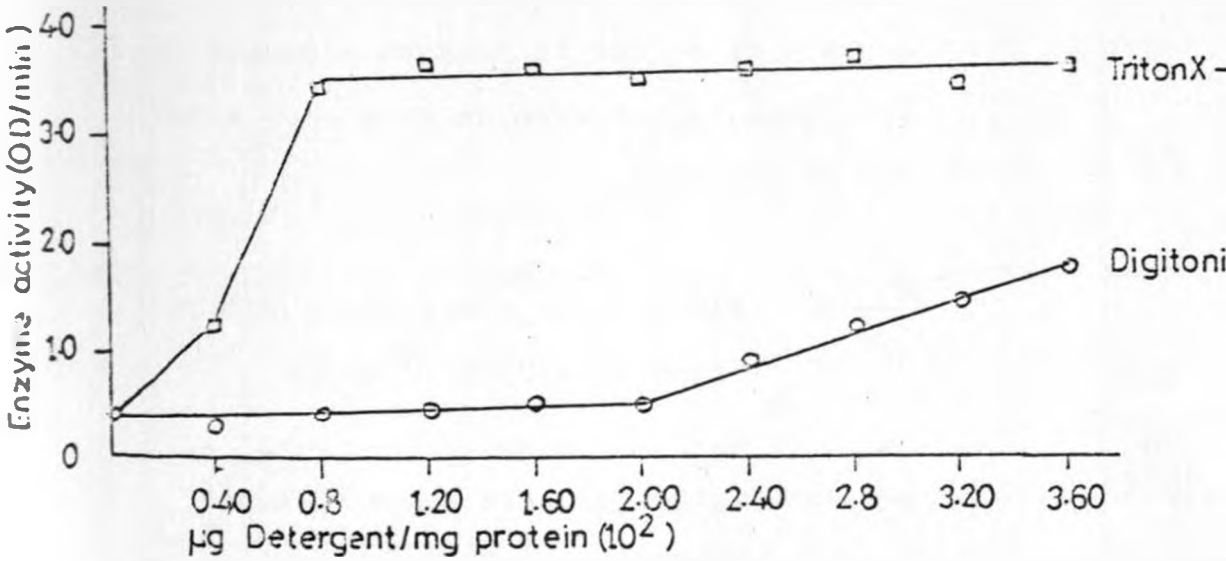
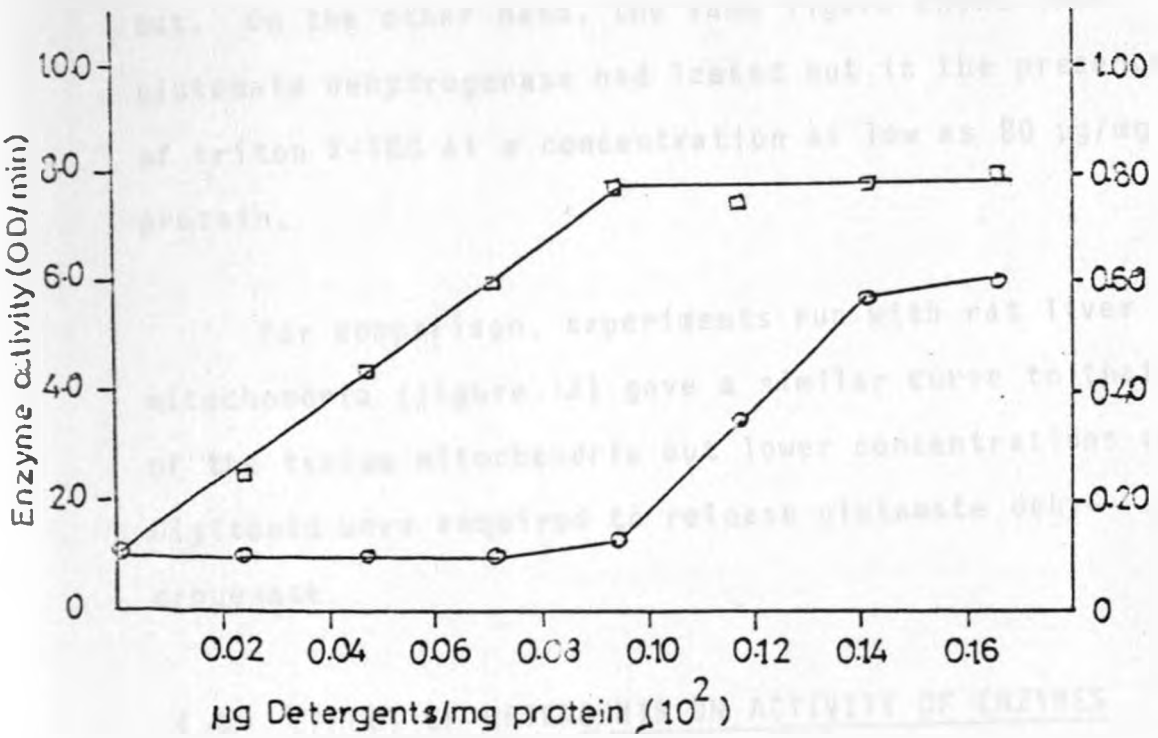

Enzyme activity(OD/min)
40
30
20
10
0
TritonX
Digitoni
0.40
0.8
1.20
1.60
2.00
2.40
2.8
3.20
3.60
µg Detergent/mg protein (10²)

FIGURE 12: Latency of glutamate dehydrogenase from male rat liver mitochondria. The experimental conditions were same as those found in the legend of figure 11. No correction was made of the initial activity encountered at zero detergent concentration. This activity was assumed to be due to damaged mitochondria. The given values were in each case with 20% of the mean.

□——□ Triton X-100 curve whose OD/min ranges from 0.00 to 10.00

o——o Digitonin curve whose OD/min ranges from 0.00 to 10.00.

FIG. 12


Enzyme activity (OD/min)
10.0
8.0
6.0
4.0
2.0
0
1.00
0.80
0.60
0.40
0.20
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
µg Detergents/mg protein (10²)

tion of digitonin on the tsetse fly flight muscle mitochondria were carried out to investigate whether or not the matrix enzyme, glutamate dehydrogenase had leaked out at 170 µg/mg of mitochondrial protein concentration of either triton X-100 or Digitonin. The results in figure 11 show that the inhibitory effect of Digitonin on proline oxidation occurred even before the matrix enzyme glutamate dehydrogenase had leaked out. On the other hand, the same figure shows that glutamate dehydrogenase had leaked out in the presence of triton X-100 at a concentration as low as 80 µg/mg protein.

For comparison, experiments run with rat liver mitochondria (figure 12) gave a similar curve to that of the tsetse mitochondria but lower concentrations of Digitonin were required to release glutamate dehydrogenase.

4.3 EFFECT OF DETERGENTS ON ACTIVITY OF ENZYMES

Since the decrease in proline oxidation rate in the presence of detergents could have been brought about by the action of the detergents on the activity of the mitochondrial membrane enzymes, the effect of increasing concentration of the two detergents on the activity of membrane enzymes, proline dehydrogenase and succinate dehydrogenase were studied. Comparable experiments were carried out with rat liver mitocho-

FIGURE 13: Effect of triton X-100 and digitonin on proline dehydrogenase activity of the tsetse fly flight muscle mitochondria. The experimental conditions are the same as those given in section 2.8.2 except that increasing concentrations of triton X-100 and digitonin are present before the reaction is started. The plotted points are an average of two separate determinations.

□———□ Digitonin

o———o Triton X-100

FIG. 13

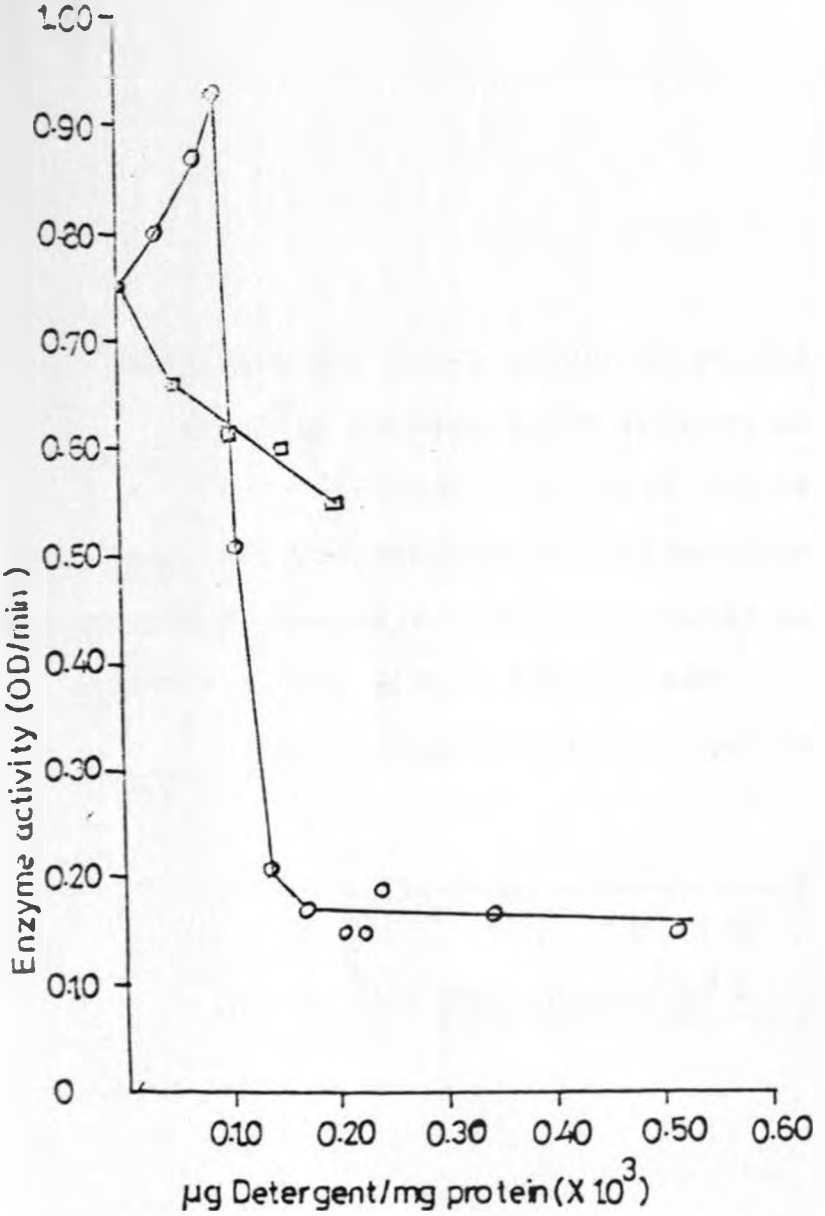

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
Enzyme activity (OD/min)
0.10
0.20
0.30
0.40
0.50
0.60
µg Detergent/mg protein (X 10^3)

FIGURE 14: Effect of triton X-100 and digitonin on proline dehydrogenase activity of rat liver mitochondria. The experimental conditions are the same as those given in the legend of figure 13. The plotted points are an average of two separate determinations.

o———o Triton X-100

□———□ Digitonin

FIG. 14

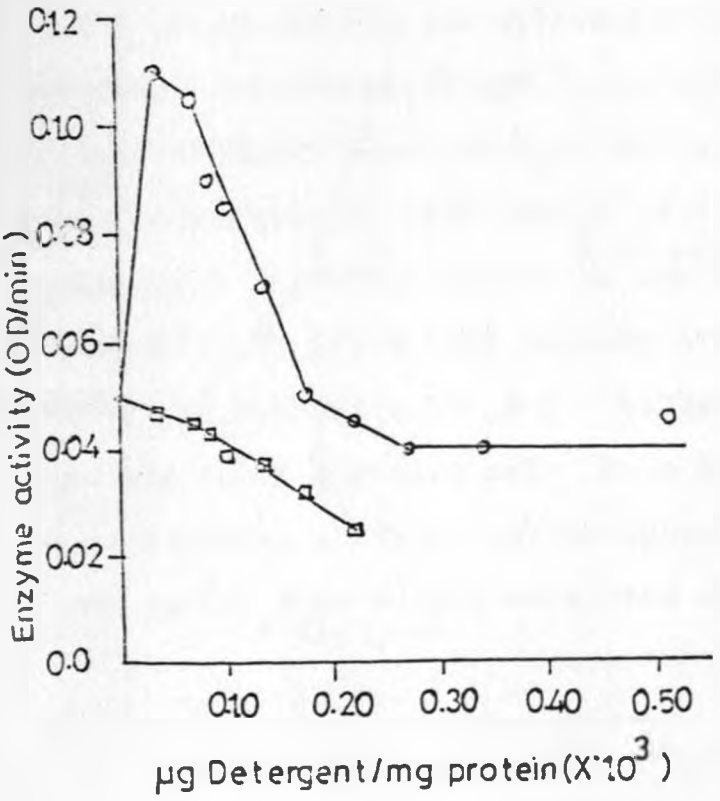
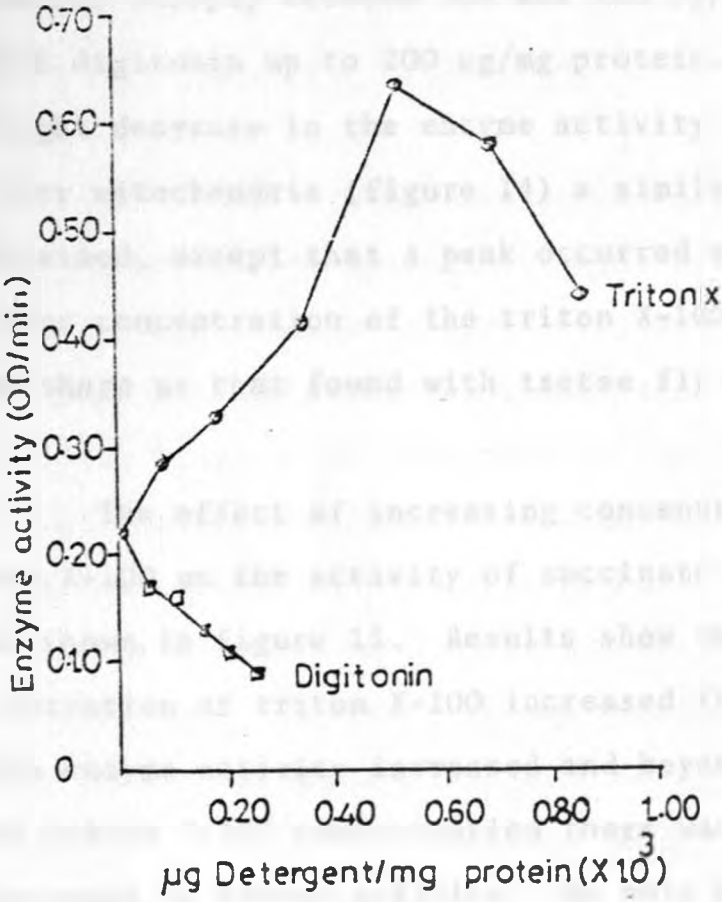

0.12
0.10
0.08
0.06
0.04
0.02
0.0
Enzyme activity (OD/min)
0.10
0.20
0.30
0.40
0.50
µg Detergent/mg protein(X 10^3)

FIGURE 15: Effect of triton X-100 and Digitonin on succinate dehydrogenase activity of the tsetse fly flight muscle mitochondria. The experimental conditions are the same as those found in section 2.8.3 except that increasing concentrations of triton X-100 and digitonin were present before the reaction was started. 1 mg of mitochondrial protein was used. The points plotted are an average of two separate determinations. The indicated points were within 20% of the mean.

FIG. 15


0·70
0·60
0·50
0·40
0·30
0·20
0·10
0
Enzyme activity (OD/min)
Triton x-100
Digitonin
0.20
0.40
0.60
0.80
1.00
µg Detergent/mg protein (X 10³)

ndria. The results of figure 13 show that with triton X-100 at a concentration of 170 µg/mg of mitochondrial protein, proline dehydrogenase was inhibited. Otherwise, the curve shows that as the triton X-100 concentration increases from 0-100 µg/mg of mitochondrial protein, the enzyme activity first increases but decreases sharply between 100 and 150 µg/mg protein. With digitonin up to 200 µg/mg protein, there was a slight decrease in the enzyme activity. With rat liver mitochondria (figure 14) a similar curve was obtained, except that a peak occurred at a somewhat lower concentration of the triton X-100 which was not as sharp as that found with tsetse fly mitochondria.

The effect of increasing concentrations of triton X-100 on the activity of succinate dehydrogenase is shown in figure 15. Results show that as the concentration of triton X-100 increased from 0-500 µg/mg the enzyme activity increased and beyond this value of triton X-100 concentration there was an apparent decrease in enzyme activity. We note here that triton X-100 concentration of 170 µg/mg protein is now stimulating. When the effect of increasing concentration of Digitonin on the activity of succinate dehydrogenase was studied, a decrease in enzyme activity as the detergent concentration increased was observed (figure 15). These results may indicate that the inhibitory effect produced by incubating tsetse mito-

chondria with proline in the presence of these detergents could be exerted at the level of membrane enzymes.

4.4 FACTORS AFFECTING THE OXIDATION OF PROLINE BY TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

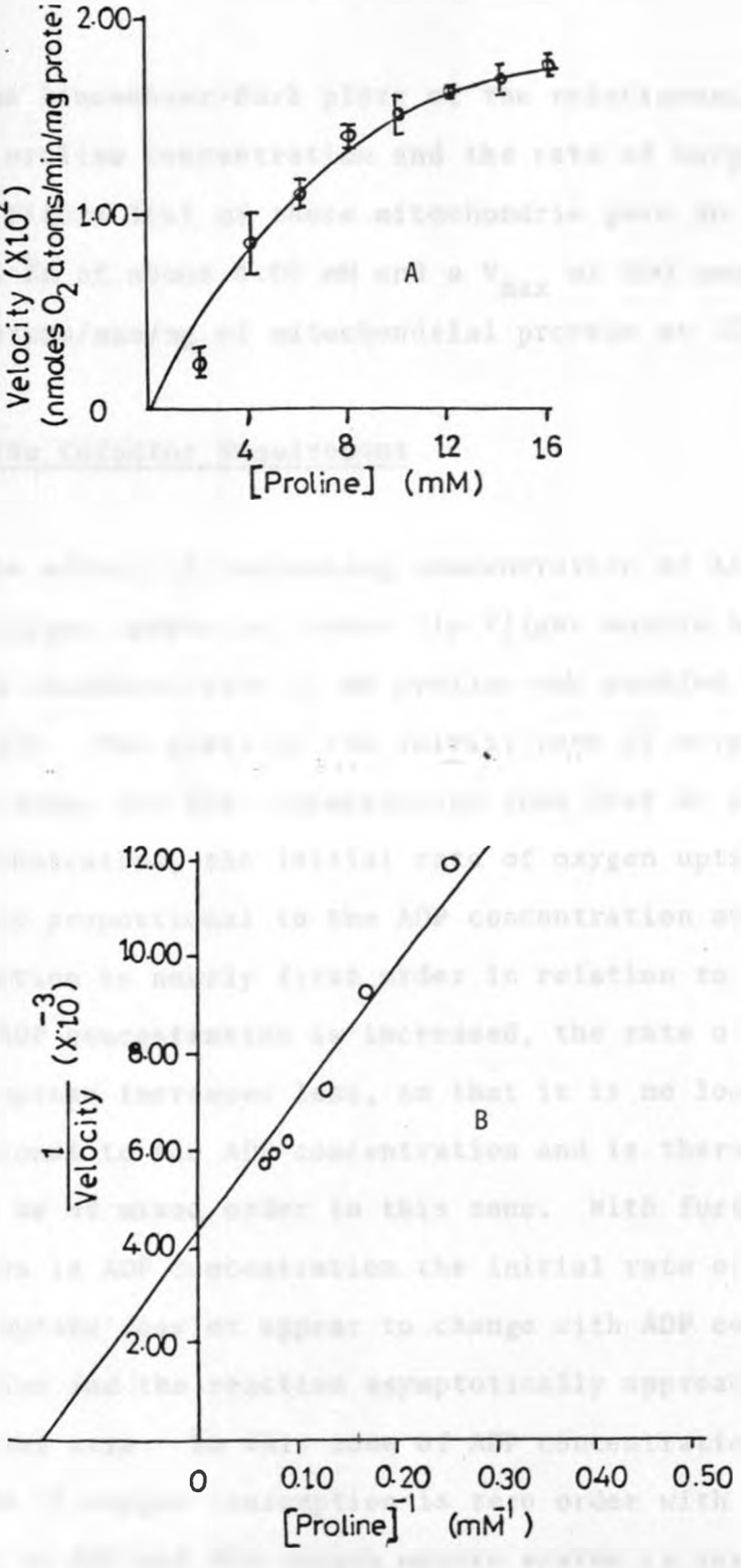
4.4.1 The Substrate Requirement

The effect of increasing concentration of proline on the oxygen uptake by tsetse fly muscle mitochondria was studied (figure 16). From the plots of the initial rate of oxygen uptake versus the substrate concentration, it is seen that at low concentrations of substrates, the initial rate of oxygen uptake is proportional to the substrate concentration and the reaction is approximately first order with respect to the substrate. However, as the substrate concentration is increased, the initial rate of oxygen uptake increases less so that it is no longer proportional to the substrate concentration. In this zone the reaction is mixed order. With a further increase in the substrate concentration, the rate of oxygen consumption becomes essentially independent of substrate concentration and asymptotically approaches a constant rate. In this range of substrate concentration the rate of oxygen uptake is essentially zero order with respect to the substrate and the oxygen uptake

FIGURE 16: THE EFFECT OF PROLINE CONCENTRATION ON OXYGEN UPTAKE BY ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

The reaction cell contains the incubation medium (250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.4, 0.1 mM EGTA). 1 mg of mitochondrial protein and 0-16 mM proline in a total volume of 1.7 ml. The reaction was started with 1 mM ADP. These experiments were conducted at 25^{o}C.

FIG. 16


Velocity (X10^2)
(nmoles O_2 atoms/min/mg protein)
2.00
1.00
0
A
4
8
12
16
[Proline] (mM)
$\frac{1}{\text{Velocity}}$ (X10^{-3})
12.00
10.00
8.00
6.00
4.00
2.00
B
0
0.10
0.20
0.30
0.40
0.50
[Proline]$^{-1}$ (mM^{-1})

system is said to be saturated with substrate.

The Lineweaver-Burk plots of the relationship between proline concentration and the rate of oxygen uptake (figure 16b) of these mitochondria gave an apparent Km of about 6.00 mM and a V_{max} of 300 nmoles oxygen atoms/min/mg of mitochondrial protein at 25°C.

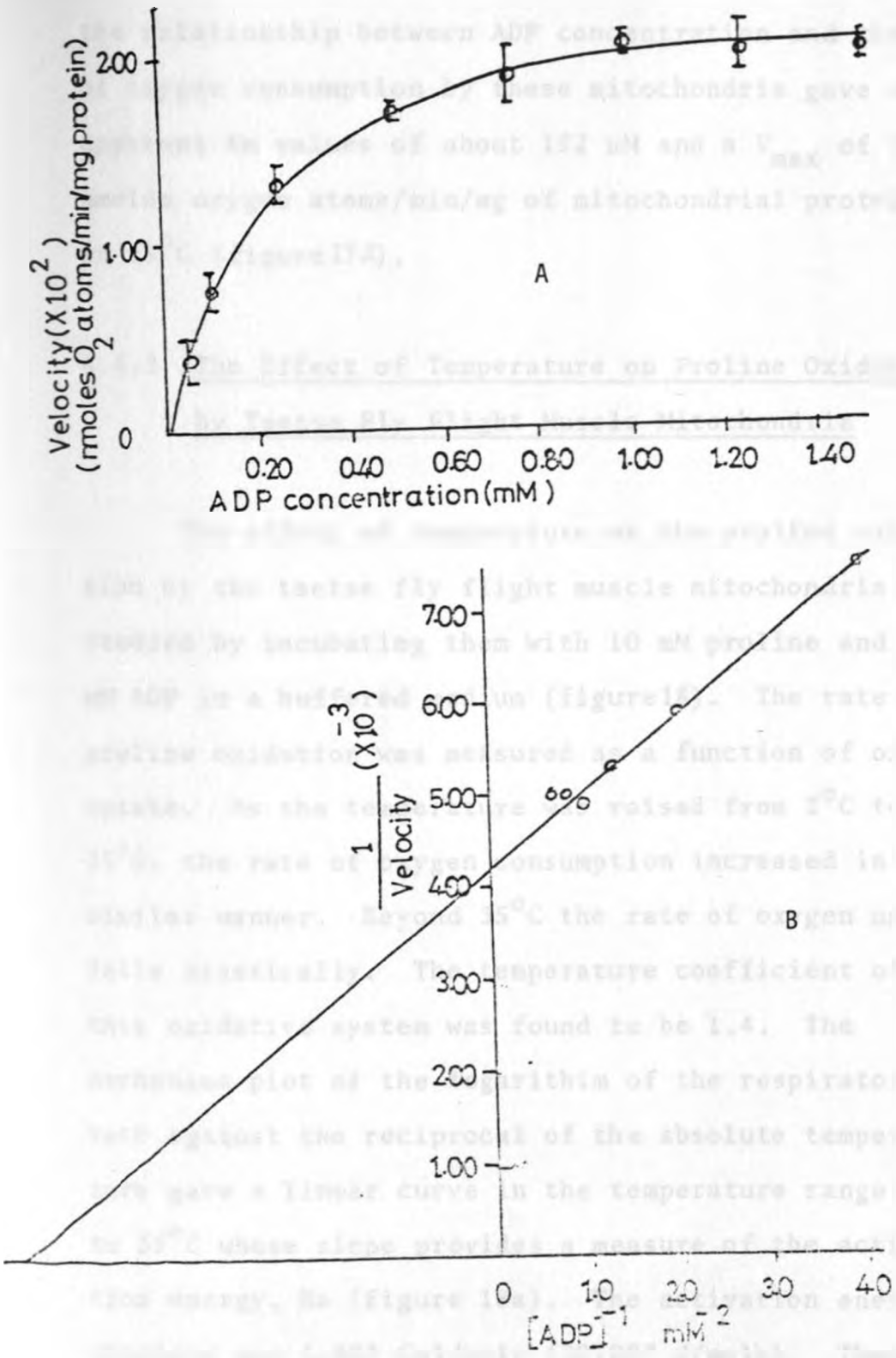
4.4.2 The Cofactor Requirement

The effect of increasing concentration of ADP on the oxygen uptake by tsetse fly flight muscle mitochondria incubated with 10 mM proline was studied (figure17). The plots of the initial rate of oxygen uptake versus the ADP concentration show that at low ADP concentration, the initial rate of oxygen uptake is nearly proportional to the ADP concentration and the reaction is nearly first order in relation to ADP. As the ADP concentration is increased, the rate of oxygen uptake increases less, so that it is no longer proportional to the ADP concentration and is therefore said to be of mixed order in this zone. With further increases in ADP concentration the initial rate of oxygen uptake does'nt appear to change with ADP concentration and the reaction asymptotically approaches a constant rate. In this zone of ADP concentration the rate of oxygen consumption is zero order with respect to ADP and the oxygen uptake system is said

FIGURE 17: THE EFFECT OF ADP CONCENTRATION ON THE RATE OF OXYGEN UPTAKE BY TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA INCUBATED WITH PROLINE.

The reaction cell contains the incubation medium (250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.4, 0.1 mM EGTA), 10 mM Proline and 1 mg mitochondrial protein in a total volume of 1.7 ml. The reactions were started by adding varying amounts of ADP. The experiments were conducted at 25^{o}C.

FIG. 17


Velocity (X10^{2}) (nmoles O_2 atoms/min/mg protein)
2.00
1.00
0
A
0.20
0.40
0.60
0.80
1.00
1.20
1.40
ADP concentration (mM)
$\frac{1}{\text{Velocity}}$ (X10^{-3})
7.00
6.00
5.00
4.00
3.00
2.00
1.00
B
0
1.0
2.0
3.0
4.0
$[ADP]^{-1}$ mM^{-2}

to be saturated with ADP. Double reciprocal plots of the relationship between ADP concentration and the rate of oxygen consumption by these mitochondria gave an apparent Km values of about 192 μM and a V_{max} of 235 nmoles oxygen atoms/min/mg of mitochondrial protein at 25°C (figure 17A).

4.4.3 The Effect of Temperature on Proline Oxidation by Tsetse Fly Flight Muscle Mitochondria

The effect of temperature on the proline oxidation by the tsetse fly flight muscle mitochondria was studied by incubating them with 10 mM proline and 1 mM ADP in a buffered medium (figure18). The rate of proline oxidation was measured as a function of oxygen uptake. As the temperature was raised from 2°C to 35°C, the rate of oxygen consumption increased in a similar manner. Beyond 35°C the rate of oxygen uptake falls drastically. The temperature coefficient of this oxidative system was found to be 1.4. The Arrhenius plot of the logarithim of the respiratory rate against the reciprocal of the absolute temperature gave a linear curve in the temperature range 2° to 35°C whose slope provides a measure of the activation energy, Ea (figure 18a). The activation energy obtained was 4,803 Cal/mole (20,097 J/mole). These results indicate that temperature changes could regulate the rate of metabolism of various substrates.

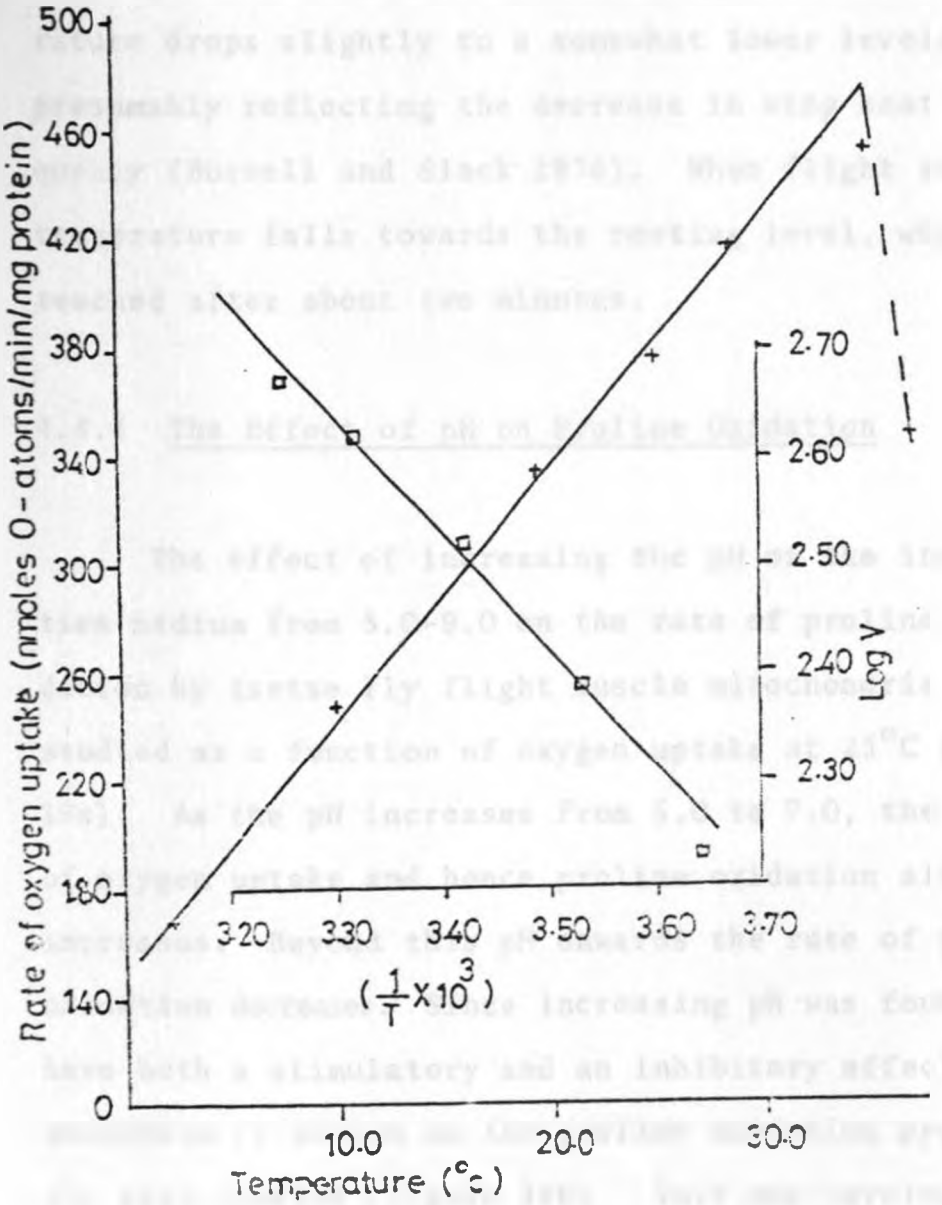
FIGURE 18: THE EFFECT OF TEMPERATURE ON THE STATE-III OXIDATION OF PROLINE BY MITOCHONDRIA ISOLATED FROM THE FLIGHT MUSCLE OF TSETSE FLIES

The reaction cell contains the incubation medium (260 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4, 0.1 mM EGTA), 1 mg mitochondrial protein and 10 mM proline and in a total volume of 1.7 ml. The reactions were started with 1 mM ADP and the experiments are conducted at the temperatures shown

-x--x- (A) TEMPERATURE VERSUS RATE OF OXYGEN UPTAKE CURVE.

-□--□- (B) LOG RATE OF OXYGEN UPTAKE VERSUS 1/ABSOLUTE TEMPERATURE CURVE.

FIG. 18


Rate of oxygen uptake (nmoles O-atoms/min/mg protein)
500
460
420
380
340
300
260
220
180
140
0
10.0
20.0
30.0
Temperature (°c)
2.70
2.60
2.50
2.40
2.30
Log V
3.20
3.30
3.40
3.50
3.60
3.70
(1/T X10^3)

This has been shown to be so with tsetse flies since during rest the thoracic temperature is about 25.2°C and at the onset of flight, this temperature rises by 2.3 degrees after a minute following which the temperature drops slightly to a somewhat lower levels, presumably reflecting the decrease in wing beat frequency (Bursell and Slack 1976). When flight stops the temperature falls towards the resting level, which is reached after about two minutes.

4.4.4 The Effect of pH on Proline Oxidation

The effect of increasing the pH of the incubation medium from 5.0-9.0 on the rate of proline oxidation by tsetse fly flight muscle mitochondria was studied as a function of oxygen uptake at 25°C (figure 19a). As the pH increases from 5.0 to 7.0, the rate of oxygen uptake and hence proline oxidation also increases. Beyond this pH onwards the rate of proline oxidation decreases. Since increasing pH was found to have both a stimulatory and an inhibitory effect its mechanism of action on the proline oxidation system was also studied (figure 19b). This was carried out by incubating tsetse fly flight muscle mitochondria with varying proline concentrations and at each proline concentration, the effect of increasing the pH of the incubation medium from 5.0 to 9.0 was investigated. Plots of substrate concentration versus rate

FIGURE 19A: THE EFFECT OF pH ON PROLINE OXIDATION BY ISOLATED TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA.

The reaction cell contains the incubation medium (250 mM sucrose, 5 mM phosphate buffer at varying pH's 0.1 mM EGTA), 1 mg of mitochondrial protein and 10 mM L-proline in a total volume of 1.7 ml. The reactions were started with 1 mM ADP. The experiments were conducted at $25^{o}C$.

FIG. 19A

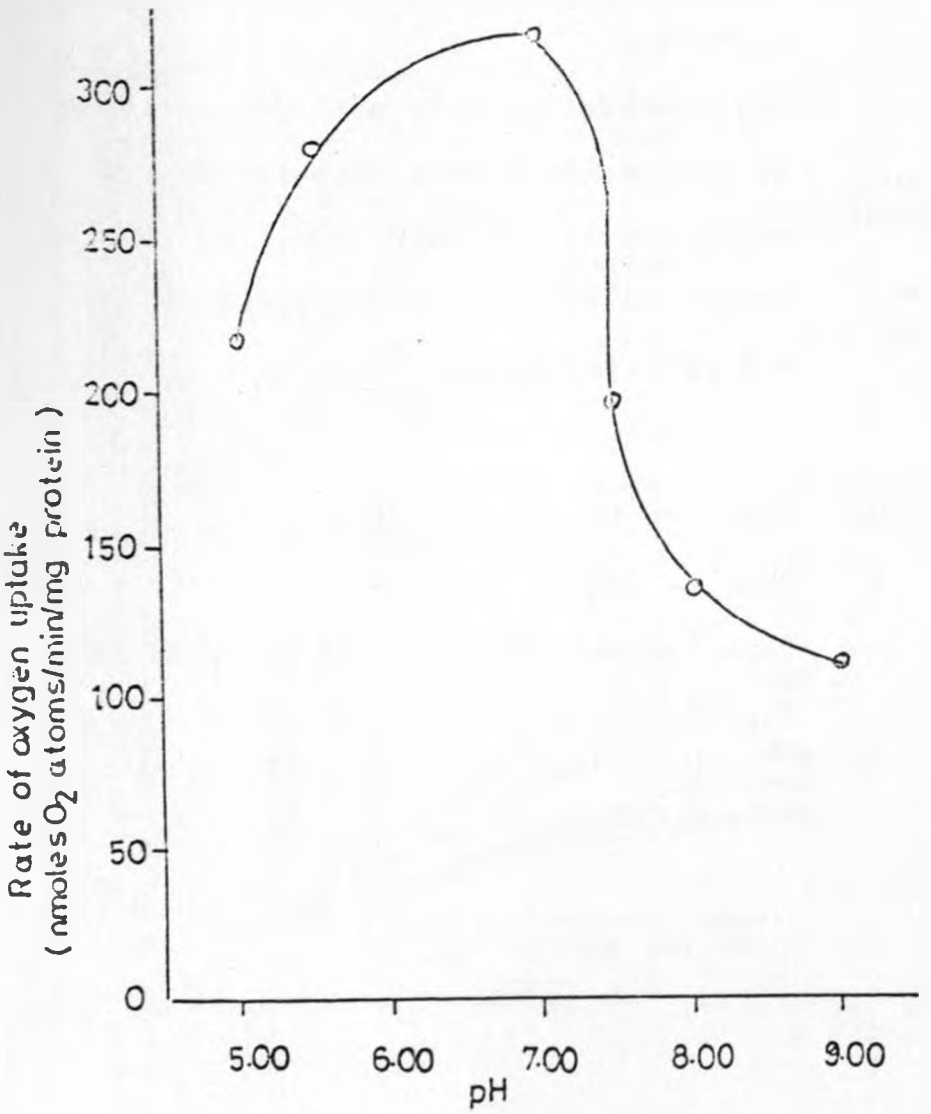
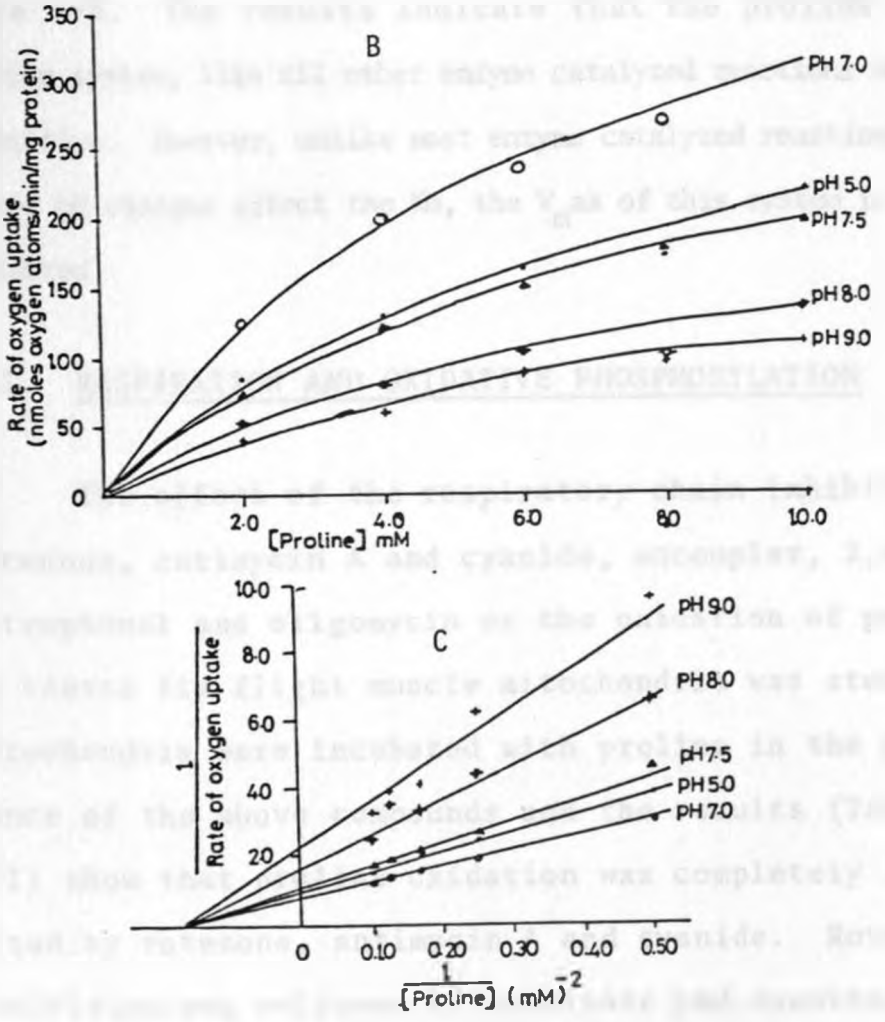

Rate of oxygen uptake
(nmoles O_2 atoms/min/mg protein)
300
250
200
150
100
50
0
5.00
6.00
7.00
8.00
9.00
pH

FIGURE 19B: Mechanism of action of pH on proline oxidation.
Experimental details are same as those of Figure 19a except that in this, at each proline concentration, the rate of oxygen uptake was investigated at all the pH's indicated.

FIGURE 19C: Shows the Lineweaver-Burk plots of the data of figure 19b.
Experimental details same as those of figure 19b.

FIG. 19


B
Rate of oxygen uptake (nmoles oxygen atoms/min/mg protein)
350
300
250
200
150
100
50
0
2.0
4.0
6.0
8.0
10.0
[Proline] mM
pH 7.0
pH 5.0
pH 7.5
pH 8.0
pH 9.0
C
1/Rate of oxygen uptake
10.0
8.0
6.0
4.0
2.0
0
0.10
0.20
0.30
0.40
0.50
1/[Proline] (mM)⁻²
pH 9.0
pH 8.0
pH 7.5
pH 5.0
pH 7.0

of oxygen uptake plotted and double reciprocal plots of the above relationship (figure 19C) showed that only the V_{max} of the proline oxidative system of the tsetse fly flight muscle mitochondria was affected but not the apparent Km. This was also shown in figure 19B. The results indicate that the proline oxidative system, like all other enzyme catalyzed reactions is pH sensitive. However, unlike most enzyme catalyzed reactions, where pH changes affect the Km, the V_max of this system is affected.

4.5 RESPIRATION AND OXIDATIVE PHOSPHORYLATION

The effect of the respiratory chain inhibitors, rotenone, antimycin A and cyanide, uncoupler, 2,4-dinitrophenol and oligomycin on the oxidation of proline by tsetse fly flight muscle mitochondria was studied. Mitochondria were incubated with proline in the presence of the above compounds and the results (Table III) show that proline oxidation was completely inhibited by rotenone, antimycin A and cyanide. Rotenone inhibition was relieved by succinate and ascorbate/tetramethyl-p-phenylenediamine. Antimycin A inhibition was relieved by ascorbate/tetramethyl-p-phenylenediamine.

Oligomycin was also shown to completely inhibit proline oxidation although its inhibitory effect was removed by the uncoupler 2,4-dinitrophenol. Succinate

TABLE XII: Shows the effects of respiratory chain inhibitors/effectors on proline oxidation by isolated tsetse fly flight muscle mitochondria.

The reaction medium contained 250 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mg mitochondrial protein, 10 mM proline and 5 Tris or succinate in a total volume of 1.7 ml. Inhibitors/effectors are present at the concentrations shown. The reactions were started with 0.1 mM ADP and the experiments are conducted at 25^{o}C. Rates are expressed as nmoles of oxygen atoms/min/mg protein. The values given are an average of two separate determinations.

TABLE XII: The effects of Respiratory Chain Inhibitors/Effectors on Proline Oxidation by Tsetse Fly Flight Muscle Mitochondria

SUBSTRATES INHIBITORS/EFFECTORS	RATE OF OXIDATION
Proline (10mM)	145
Proline (10mM) + KCN (1mM)	N.D
Proline (10mM) + 6µg antimycin A	N.D
Proline (10mM) + 2µg rotenone	N.D
Proline (10mM)	127
Proline (10mM) + DNP (12µM)	163
Proline (10mM)	136
Proline (10mM) + 6µg oligomycin	N.D
Proline (10mM) + 6µg oligomycin + DNP (12µM)	186
Proline (10mM) + 6µg oligomycin + 5mM succinate	204
Proline (10mM)	154
Proline (10mM) + 2µg rotenone	N.D
Proline (10mM) + 2µg rotenone + 5mM succinate	200
Proline (10mM) + 5mM succinate	204
Proline (10mM)	154
Proline (10mM) + 6µg antimycin A	N.D
Proline (10mM) + 6µg antimycin A + succinate (5mM)	N.D
Proline (10mM) + 6µg antimycin A + 5mM ascorbate/TMPD	270
Proline (10mM) + 5mM ascorbate/TMPD	266
Proline (10mM) + KCN (1mM) + 5mM ascorbate/TMPD	N.D
Proline (10mM) + malate (1mM)	N.D

TABLE XIII: Oxidation of Succinate by Isolated Tsetse Fly Flight Muscle Mitochondria

SUBSTRATE/INHIBITOR	RATE OF OXYGEN UPTAKE (nmoles o-atoms/min/mg Protein)
Tris-succinate (5 mM)	144
Sodium-succinate (5 mM)	140
Tris-succinate(5 mM)+Rotenone	142
Sodium-succinate (5 mM) + Rotenone	145
Tris-succinate (5 mM) + antimycin A	0
Sodium-succinate (5 mM) + antimycin A	0
Tris-succinate (5 mM) + KCN	0
Sodium-succinate(5mM) + KCN	0
Tris-succinate (5 mM) + Oligomycin	143
Sodium-succinate (5 mM) + Oligomycin	142

Reaction medium contained 250 mM sucrose, 10 mM Potassium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mg mitochondrial Protein, 5 mM Tris-or sodium succinate, 6µg of each inhibitor in a total volume 1.7 ml. Incubations were carried out at 25°C. Results are an average of two separate determinations.

also relieved the inhibitory effect of oligomycin. The inhibition by antimycin A though complete occurred gradually but not immediately after the addition of the inhibitor. Malonate was also shown to inhibit the proline oxidative system of the tsetse fly.

4.6 SUCCINATE OXIDATION BY TSETSE MITOCHONDRIA

4.6.1 The Effect of Succinate Concentration on the rate of Oxygen Uptake by Tsetse Fly Flight Muscle Mitochondria

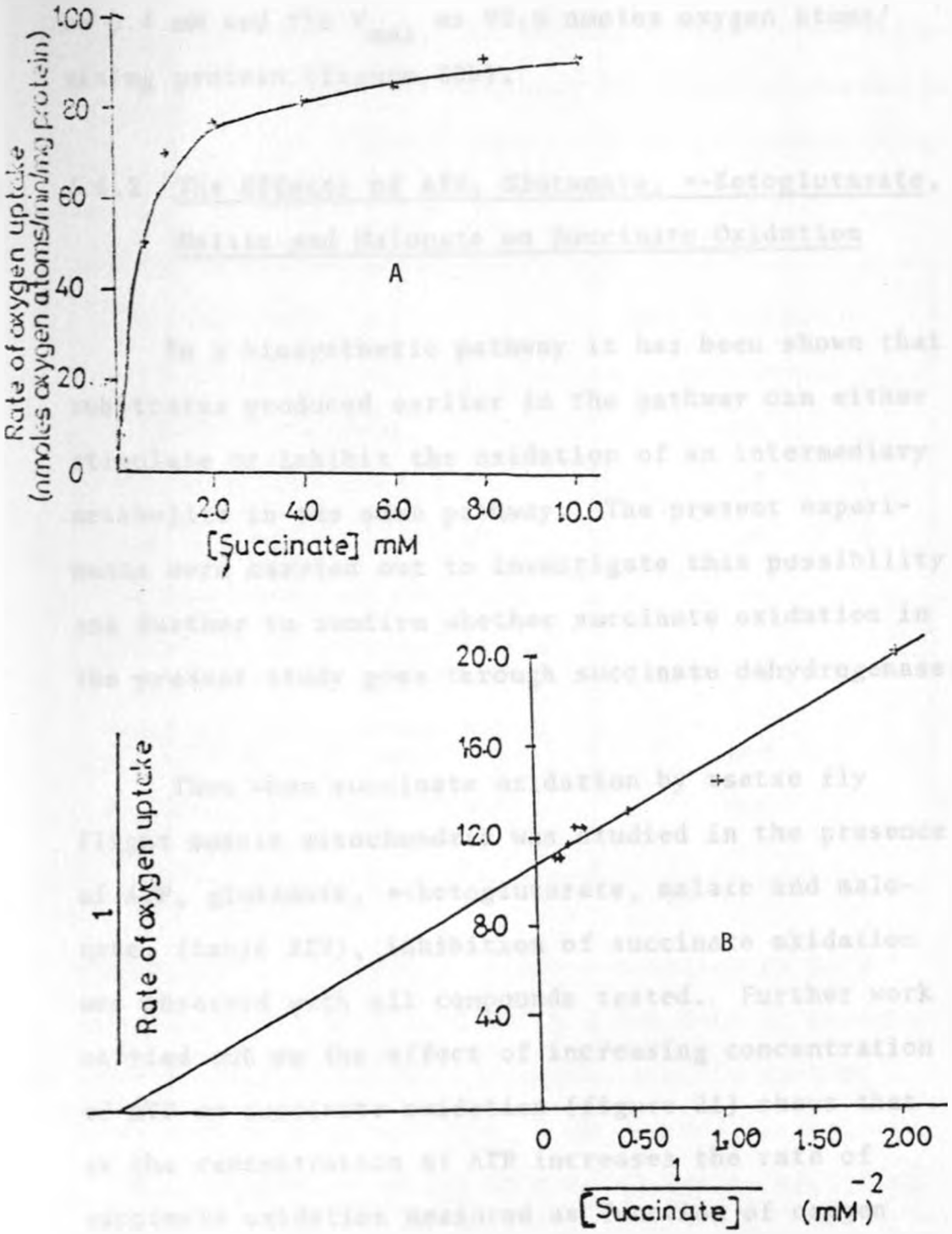
The effect of succinate concentration on the rate of oxygen uptake by tsetse fly flight muscle mitochondria was investigated in the presence and absence of 1 mM ADP at 25^{o}C. The results (Figure 20) show that ADP does not affect the rate of oxidation of succinate since the presence and absence of ADP does not alter the saturation curve of succinate oxidation. Figure 20 also shows that increasing succinate concentration also increased the rate of oxygen uptake until a succinate concentration was reached beyond which the rate of oxygen uptake was not altered by any increment in succinate concentration.

The Lineweaver-Burk plot of the relationship between succinate concentration versus the rate of

FIGURE 20: Shows the effect of succinate concentration on the rate of oxygen uptake by tsetse fly flight muscle mitochondria.

The reaction medium contained 250 mM sucrose, 1 mM EDTA, 5 mM potassium phosphate buffer, pH 7.4, 1 mg mitochondrial protein, and 5 mM succinate in a total volume of 1.7 ml. The system consumed oxygen in the same rate with and without 1 mM ADP. The reaction was conducted at 25^{o}C.

FIG. 20


Rate of oxygen uptake
(nmoles oxygen atoms/min/mg protein)
100
80
60
40
20
0
A
2.0
4.0
6.0
8.0
10.0
[Succinate] mM
20.0
16.0
12.0
8.0
4.0
B
1
Rate of oxygen uptake
0
0.50
1.00
1.50
2.00
1
[Succinate]
-2
(mM)

oxygen uptake gave a linear curve from which the apparent Km for succinate oxidation was calculated to be 0.4 mM and the V_{max} as 92.6 nmoles oxygen atoms/ min/mg protein (figure 20b).

4.6.2 The Effects of ATP, Glutamate, α-Ketoglutarate, Malate and Malonate on Succinate Oxidation

In a biosynthetic pathway it has been shown that substrates produced earlier in the pathway can either stimulate or inhibit the oxidation of an intermediary metabolite in the same pathway. The present experiments were carried out to investigate this possibility and further to confirm whether succinate oxidation in the present study goes through succinate dehydrogenase.

Thus when succinate oxidation by tsetse fly flight muscle mitochondria was studied in the presence of ATP, glutamate, α-ketoglutarate, malate and malonate, (table XIV), inhibition of succinate oxidation was observed with all compounds tested. Further work carried out on the effect of increasing concentration of ATP on succinate oxidation (figure 21) shows that as the concentration of ATP increases the rate of succinate oxidation measured as function of oxygen uptake decreases. Results indicate that succinate oxidation goes through succinate dehydrogenase.

TABLE XIV: Effect of ATP, Malate, Malonate ∝-ketoglutarate and Glutamate on Succinate Oxidation by Isolated Tsetse Fly Flight Muscle Mitochondria

ADDITIONS	RATE OF OXIDATION
Succinate (5 mM) alone	100
Succinate(5 mM) + ATP(5 mM)	19
Succinate(5 mM) + ATP (10 mM)	0
Succinate(5 mM) + Malonate(1 mM)	0
Succinate(5 mM) + ∝-ketoglutarate (5mM)	73.2
Succinate(5 mM) + ∝-ketoglutarate (5 mM) + Glutamate (5 mM)	59.8
Succinate(5 mM) + Malate (5mM)	75.6

Reaction cell contains: incubation medium (0.25 M sucrose, 0.001 M EDTA, 0.005 M K_2HPO_4) 50 µl mitochondrial suspension 5 mM succinate, 5 or 10 mM ATP, 5 mM malate, 1 mM malonate, 5 mM ∝-ketoglutrate, and 5 mM glutamate at pH 7.4 in a total volume of 1.7 ml. Incubations were carried out at 25°C. Rates have been expressed as a percentage of the rate of succinate. Results are an average of two separate determinations.

FIGURE 21: THE EFFECTS OF INCREASING CONCENTRATIONS OF ATP ON SUCCINATE OXIDATION

Reaction cell contains the reaction medium which consists of 250 mM sucrose, 10 mM phosphate buffer, pH 7.4, 0.5 mM EGTA, 1mg mitochondrial protein, 5 mM succinate and increasing ATP concentrations in a total volume of 1.5 ml. Incubation were carried out at 25^{o}C. Rates of oxygen consumption are expressed as nmoles of oxygen atoms/min/mg protein. Results are an average of two separate determinations. Indicated results were within 10% from the mean.

FIG. 21

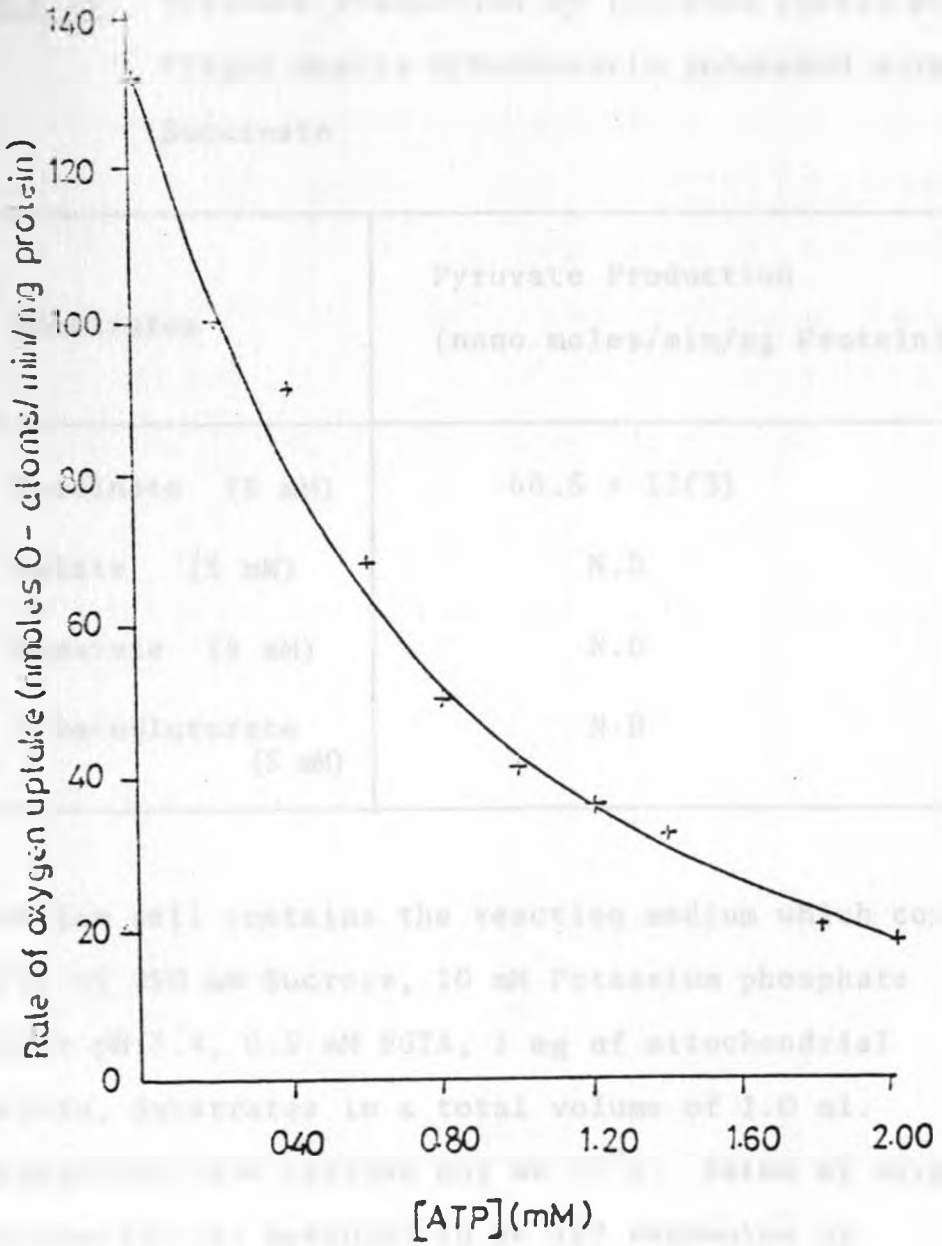

Rate of oxygen uptake (nmoles O- atoms/min/mg protein)
140
120
100
80
60
40
20
0
0.40
0.80
1.20
1.60
2.00
[ATP] (mM)

TABLE XV: Pyruvate Production by Isolated Tsetse Fly Flight Muscle Mitochondria incubated with Succinate

Substrates	Pyruvate Production (nano moles/min/mg Protein)
Succinate (5 mM)	66.5 ± 12(3)
Malate (5 mM)	N.D
Fumarate (5 mM)	N.D
α-ketoglutarate (5 mM)	N.D

Reaction cell contains the reaction medium which consists of 250 mM Sucrose, 10 mM Potassium phosphate buffer pH 7.4, 0.5 mM EGTA, 2 mg of mitochondrial protein, Substrates in a total volume of 1.0 ml. Incubations were carried out at $37^{o}C$. Rates of oxygen consumption was measured to be 157 nanomoles of oxygen atoms/min/mg of mitochondrial Protein.

4.6.3 Pyruvate Production During Succinate Oxidation

In an attempt to determine qualitatively the end product of substrate oxidation, tsetse fly flight muscle mitochondria were incubated with ∝-ketoglutarate, succinate, fumarate and malate in the absence of glutamate and the amount of pyruvate produced assayed. Table XV shows that only succinate produced pyruvate. The other substrates, namely, ∝-ketoglutarate, fumarate and malate produced no detectable quantities of pyruvate within the 15 minutes of incubation.

CHAPTER 5

DISCUSSION

5.1 PERMEABILITY OF TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA TO NEUTRAL AMINO ACIDS

Since proline and the other neutral amino acids namely, alanine, valine, hydroxyproline and glycine have pK values of about 2 and 10 and therefore have an isoelectric pH of about 6, they are largely present as either a zwitterion or a form related to it by internal proton movement at pH of about 7.0. Therefore, the net movement of these amino acids into the matrix compartment of respiration inhibited tsetse fly flight muscle mitochondria suspended in isoosmotic solutions of ammonium salts of amino acids and of amino acids themselves was examined. The requirements for osmotic swelling due to permeation of these amino acids was also examined.

The mitochondrial swelling observed (sections 3.2; 3.3) when tsetse fly flight muscle mitochondria were incubated in isoosmotic solutions of amino acids and not the ammonium salts of the amino acids indicates that these mitochondria are permeable to these amino acids (Halling et al 1973; Meyer, 1977) and that the movement of these amino acids into the matrix is

not linked to any other species. The lack of swelling observed when these mitochondria are incubated with isoosmotic solutions of ammonium salts of amino acids may be due to a reaction of ammonia with the carrier protein to form a complex with no transporting ability. The observation that the uncoupler, CCCP inhibited mitochondrial swelling with isoosmotic solutions of proline indicated that this swelling is not dependent on the presence of agents that allow the dissipation of a membrane potential and that the permeant species must have no net charge (Mitchell and Moyle, 1965; Mitchell, 1966a; Papa et al, (1970a; Papa et al, 1971).

5.2 STEREOSPECIFICITY AND THE PROBABLE LOCATION OF -SH GROUPS OF PROLINE CARRIER

The uptake of the amino acids, proline, alanine and valine by tsetse fly flight muscle mitochondria is stereospecific (Table 1). Meyer (1977) also showed a similar stereospecificity with proline uptake by rat liver mitochondria. On the other hand, Halling (1973) had found no stereospecificity for the uptake of alanine and valine by brain or rat liver mitochondria and concluded that neutral amino acids cross the mitochondrial membrane by free diffussion via ring configuration. The present results with tsetse fly flight muscle mitochondria show

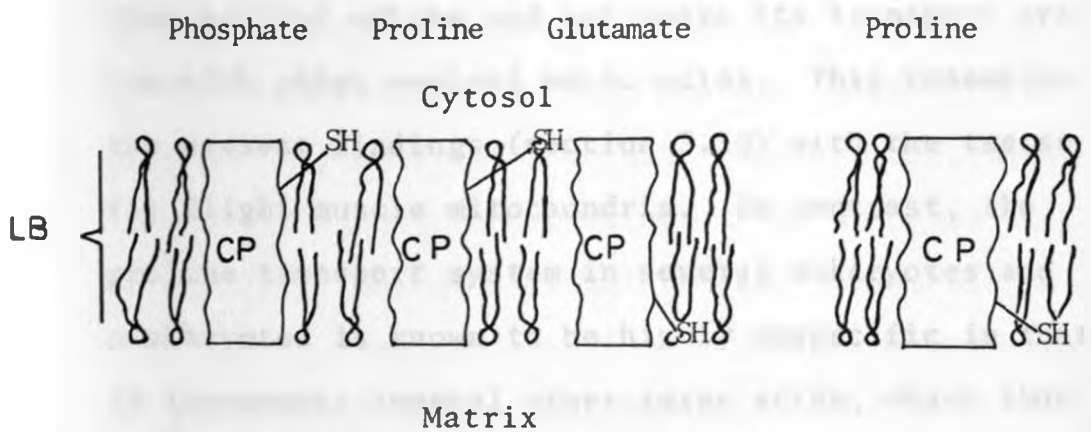
markedly that the uptake of several neutral amino acids is stereospecific since the initial rates as well as the extent of swelling are over two times higher with L- than with the DL-isomer which immediately precludes a free diffussion mechanism. The uptake of glutamate and aspartate by rat liver mitochondria is also stereospecific (King and Diwan, 1972) and the permeation of these amino acids into the mitochondrial matrix is mediated by specific carriers (Azzi et al, 1967; Meijer et al, 1972; Meyer and Vignais, 1973; La None et al, 1974).

The observation that the penetrant thiol blocking reagent N-ethylmaleimide inhibited the uptake of proline more than that of the other neutral amino acids (section 3.3) suggests that proline does not share its transport system with other neutral amino acids. Further work in the present study showed that mersalyl could not inhibit this transport process by reacting with sulfhydryl groups of extramitochondrial glutathione of carrier proteins (Meyer, 1977). These results combined together would suggest that the sulfhydryl groups involved in proline transport of the tsetse fly flight muscle mitochondria might be located in a hydrophobic region or on the matrix side of the membrane (figure 22). This resembles the location of the sulfhydryl groups involved in the glutamate-hydroxyl carrier of the rat liver

FIGURE 22: Probable position of thiols involved in the transport of glutamate, phosphate and proline in the rat liver mitochondria and proline in the tsetse fly flight muscle mitochondria as deduced from Meyer and Vignais (1973) Guerin et al (1970), Meyer (1977) and the results of present investigations (section 3.3).

(a) RAT LIVER MITOCHONDRIA

(b) TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA



LB Lipid Bilayer of the inner mitochondrial membrane

CP Carrier Protein of the inner mitochondrial membrane

mitochondria which could only be reached by lipophilic reagents able to penetrate in the membrane and thus located in a hydrophobic region or on the matrix side of the membrane (Meyer, 1977). In contrast, the sulfhydryl groups involved in the transport of proline and phosphate in the rat liver mitochondria can only be effectively inhibited by mersalyl but not N-ethylmaleimide and are therefore presumed to be located on the outside of the mitochondrial membrane in a hydrophilic environment (Meyer and Vignais 1973; Guerin et al, 1970, Meyer, 1977).

5.3 SPECIFICITY OF THE PROLINE CARRIER

In rat liver mitochondria, Meyer (1977) showed that proline uptake did not share its transport system with other neutral amino acids. This resembles the present findings (section 3.10) with the tsetse fly flight muscle mitochondria. In contrast, the proline transport system in several eukaryotes and prokaryotes is known to be highly unspecific in that it transports several other amino acids, which thus shows inhibitory effect on its transport (Law and Mukkada, 1979; Eavenson and Christensen 1967; Hampton, 1970; Christensen and Oxender, 1963; Schwencke and Schwencke, 1969; Christensen and Thomas, 1971). In some prokaryotes, proline transport system are far more specific and are inhibited by only a few proline

analogs (Hirata et al, 1971; Kay and Gronlund, 1969; Tristam and Neale, 1968).

The stimulatory effect of other neutral amino acids and/or proline analogs on the uptake of proline by tsetse fly flight muscle mitochondria (section 3.10) are not easy to explain though it can be argued that either these amino acids/proline analogs are necessary for proline transport and they combine with the transport system to form a ternary complex with the metabolite that is transported across the membrane, probably not affecting the affinity of the transport system for proline (Goldner et al, 1969). Alternatively these compounds are transported in first and latter exchange with proline as it enters (Feiguson and Williams, 1966; Chappell and Haarhoff, 1967). This requirement for maximum uptake rates are not only restricted to the uptake of proline by tsetse fly flight muscle mitochondria since De Kort et al (1973) showed an absolute requirement of proline for the maximum uptake of ^{14}C-Leucine into isolated colorado beetle mitochondria.

The observation that the other amino acids caused mitochondrial swelling (section 3.3) and their uptake was inhibited by N-ethylmaleimide, while they were not oxidized suggests that these amino acids are not involved in energy generation. However, such

compounds could be used for other energy consuming activities like protein synthesis (De Kort et al, 1973; Buchanan et al, 1969).

5.4 ENERGETICS OF THE PROLINE CARRIER

From the time-course of proline uptake (Fig.7), Proline was shown to accumulate against a concentration gradient in tsetse fly flight muscle mitochondria. A similar result was obtained with proline uptake in rat liver mitochondria (Meyer, 1977) and Leishmania tropica (Law and Mukkada, 1979). In contrast, the transport of proline in Plasmodium Lophurae (Sherman and Tanigoshi, 1974) and Fasciola hepatica (Isseroff and Read, 1969) is by simple diffusion. From the observations that proline was transported up a concentration gradient, it was deduced that proline transport was energy dependent. This was also indicated by the observation that de-energization of the tsetse fly flight muscle mitochondria by the addition of the uncoupler, carbonylcyanide-p-trichloromethoxylphenylhydrazone decreased the initial rate of proline uptake. This energy was shown to be provided by the respiratory chain activity, since the respiratory substrate, succinate increased the initial rate of proline uptake when included in the incubation medium. Studies with microbial systems have demonstrated that proline transport is dependent

upon substrate oxidation (Klein and Boyer, 1972; Berger, 1973).

The observation that the uptake of proline is an active transport suggests that this amino acid exists transiently in a different form in passing through the membrane separating the medium and matrix amino acid levels. In order for the dissociation of such a complex to give a high level of amino acid inside the matrix than outside, energy must be provided. Two possible ways are suggested in which this could occur: (a) A compound of high energy may be split in the formation of the carrier-amino acid complex, which may then inturn be of sufficiently high energy to release the amino acid at an elevated concentration or (b) A structural change of the amino acid complex may be produced within the matrix to increase its energy content so that the amino acid is dissociated from it at a higher concentration. However, the nature of the high energy intermediate remains to be established. The reduction of the initial rate of uptake of proline by the respiratory chain inhibitors antimycin A and cyanide indicates a clear requirement of metabolic energy for the concentrative uptake of proline. Respiratory chain inhibitors do not allow the generation of a pH gradient due to electron influx and thus no proton motive force is set up across the mitochondrial membrane.

Proline transport was shown not to be inhibited by oligomycin suggesting that ATP itself was not the energy transfer intermediate in this system. This was further indicated by the observation that proline uptake was not affected by the presence of ATP in the incubation medium. ADP showed an inhibitory effect on proline uptake by tsetse fly flight muscle mitochondria. ATP can be used as a source of energy under conditions of anaerobiosis (Klein and Boyer, 1972). From these results one can conclude that a phosphorylated intermediate is not a requirement for the transport of proline in the tsetse fly flight muscle mitochondria. These results were similar to those obtained for proline transport (Meyer, 1977) and glutamate transport (Meyer, 1975) in rat liver mitochondria which have been shown not to be inhibited by oligomycin, and therefore do not use ATP as an energy transfer intermediate.

An alternative energy source for the active transport of proline is the membrane potential, in which case proline would enter the mitochondria accompanied by a cation crossing the membrane downward from the membrane potential (positive outside). In S. cerevisae (Scaston et al, 1973) and Halobacterium halobium (Hubbard et al, 1976) a proline-proton electrogenic symport was evidenced. In the tsetse fly flight muscle mitochondria, the proton movement

may not be a candidate for such a role as shown from the swelling experiments though the striking decrease in uptake of proline in the presence of the uncoupler, CCCP and the pH dependence of proline uptake would have suggested so. This resembles the transport of proline in rat liver mitochondria (Meyer, 1977) which has been shown not to be pH dependent and proton linked but differs from the glutamate-transport in rat liver whose uptake is a strongly pH dependent and proton-substrate cotransport (Meyer and Vignais, 1973; Tischler, 1977). However, an active transport can be driven by a membrane potential without involving proton transfers as for example in the system proposed for lysine transport in S. aureus (Niven and Hamilton, 1974).

Proline transport by tsetse fly flight muscle mitochondria (section 3.7, fig. 9) was shown to be temperature dependent. At low temperatures ($0^{o}C$), the uptake rate was low and at higher temperatures ($20^{o}C$) the uptake rate was high. These results are consistent with the view that the process under study is the membrane transport of proline rather than the binding of proline to fixed sites in the particles (Martin and Smith, 1972). The temperature coefficient of about 2 obtained for this transport system was of the order expected for carrier systems. The Arrhenius plot of this uptake system was linear

with an activation energy of 11.0 Kcal/mole. Most carrier systems have an activation energy of between 15 and 30 Kcal/mole (Palmeiri et al, 1972a, 1972b; Meyer and Vignais 1973; Halestrap, 1975) although the proline uptake system of the rat liver mitochondria has been shown to be 9 Kcal/mole (Meyer, 1977).

5.5 KINETICS OF THE PROLINE CARRIER

Proline transport by tsetse fly flight muscle mitochondria (section 3.6, Fig.8) was shown to obey Michaelis-Menten kinetics with saturation of the uptake system by proline. The apparent Km for the uptake of proline was quite low (85 μM). This suggested that the system involves a specific site to which the substrate must bind reversibly in order to be transported across the membrane. It also suggests that the rate of binding of the substrate on one side of the membrane or its release on the other side can set up a limit to the transport, just as the rate of formation or breakdown of an enzyme-substrate complex can limit the rate of an enzymatic reaction. Other carrier mediated transport systems like the phosphate-hydroxyl exchange (Coty and Pedersen, 1974), the dicarboxylate-dicarboxylate exchange (Gimpel et al, 1973; Kunz et al, 1969; Bohnensack and Kunz, 1971; Robinson and Chappell 1967a), the α-ketoglutarate-malate exchange (Palmieri et al, 1972a; Papa et al,

1969c), the tricarboxylate translocator (Robinson et al, 1971a; Palmieri et al 1972b, the Glutamate-hydroxyl exchange (Harris et al, 1973; Bradford and McGivan, 1973; Meyer and Vignais, 1973; Mandella, 1976; Mandella et al, 1977), the proline translocator (Meyer, 1977) etc. have been shown to exhibit saturation of the transport system.

5.6 PERMEATION OF SUBSTRATES TO TSETSE MITOCHONDRIA

Bursell (1963) suggested that in active tsetse flight muscle proline was first oxidized to glutamate which transaminates with pyruvate to give alanine and be oxidized through the normal tricarboxylic acid cycle via succinate, fumarate, malate and finally to the pyruvate involved in the transamination reaction. In an attempt to confirm this pathway, the oxidative capacity of tsetse fly flight muscle mitochondria on intermediates of proline oxidation, namely glutamate, α-ketoglutarate, succinate, fumarate and malate, were investigated in parallel with proline. The observation that these mitochondria respired appreciably with both proline and succinate, but not with the other substrates, suggested a lack of permeability towards these intermediates (Hansford and Johnson, 1975; Van den bergh and Slater, 1962). These findings support those of other investigators (Bursell

1963, 1966, 1975; Bursell and Slack, 1976) who showed that proline was the only substrate of the many tested which supported respiration appreciably with these mitochondria in the presence of ADP and phosphate (state-III of Chance and Williams, 1955a). The observation in the present study that these mitochondria also respired appreciably with succinate suggests that this substrate may be used by this insect to provide metabolic energy. This was not clearly defined in the results of Bursell and Slack (1976) who obtained a low oxidation rate with this substrate, but would appear to be in line with the findings of D'Costa et al (1973) who showed the carbohydrate reserves might serve as a source of flight energy in flight muscle of the tsetse fly.

The observation that the rate of proline oxidation is greater than that of succinate could be explained by suggesting that the mitochondrial preparation used contained partly damaged mitochondria. This possibility might be possible since if the respiratory rate observed with succinate as a substrate is normal, then the amount of oxygen used per min per mg protein would be higher than that by proline (Chance and Williams, 1955a). This would be so since succinate appears to donate its electrons into the electron transport chain at a lower level than proline.

The results could also suggest that there might exist a carrier system for these substrates in tsetse mitochondria. For proline, this has been shown to be so in the present work. The possibility of a carrier system for succinate in this study agrees with the findings of Tulp and Van Dam (1969) who while working with houseflies demonstrated that a carrier system exists for succinate in these insects. However, Hansford (1971) indicated the existence of carrier systems for glutamate and ∝-ketoglutarate in the flight muscle mitochondria of the periodical cicada, Magicicada septendecim but no carriers exist for other members of the tricarboxylic acid cycle. He proposed that specific membrane carriers are genetically determined and functionally related to the physiology of the tissue. One may postulate that the absence of dicarboxylate and tricarboxylate anion carriers in flight muscle of most insects is an adaptation to prevent the efflux of these intermediates from the mitochondrion, rather than simply to inhibit their entry. In the flight muscle, tricarboxylic acid cycle intermediates are needed almost exclussively for respiratory function, as substrates or as precursors of oxaloacetate for catabolism of pyruvate, and not for their participation in various biosynthetic processes outside the mitochondrion, as found in tissues such as mammalian liver Sacktor 1975).

5.7 INFLUENCE OF DETERGENTS ON MITOCHONDRIA PERMEABILITY.

Biological membranes contain a continuous non-polar hydrocarbon core contributed by phospholipid tailes. The hydrophilic heads of the phospholipid are exposed to the aqueous phase.

Within the membrane, there are proteins and other lipids such as cholesterol and ergosterol among others The organization of the membrane makes the biological membranes intrinsically impermeable to polar molecules. Usually permeation of polar molecules through biological membranes is effected via natural specific carrier proteins distributed within the membranes/ The permeability of cell membranes to polar molecules can be artificially increased by altering the arrangement of the membrane components or selectively removing some of these components.

When cell membranes are treated with digitonin, an insoluble complex of digitonin and membrane cholesterol is formed. ("Bentley and Drivers, 1960"). This results in the formaition of pores, thus abolishing the selective permeability of the membrane.

In this investigation, digitonin and triton X-100 treatment have been used in an attempt to make the inner mitochondrial membrane permeable to glutamate, α-ketoglutarate, fumarate and malate. The permeability of these compounds would be evidenced by an increase in the rate of respiration. In this case, this was not so. The lack of stimulation for these substrates suggests that if the above substrates have to be metabolished through the proline oxidation pathway postulated earlier (Bursell 1963) there must be a permeability barrier which cannot be removed by these detergents. This appears to be the only deduction since respiration experiments performed with the same substrates in the presence of NAD^+ and CoA did not show any increase in the rate of respiration. The decrease in the proline oxidation rate caused by both these detergents suggests that either they interfered with the membrane integrity while the proline oxidation requires intact mitochondria or the detergents have an inhibitory effect on the enzymes catalyzing proline oxidation (Wadkins and Lehninger, 1963).

Further work on proline oxidation in the presence of digitonin proposes that this detergent inhibits proline oxidation by tsetse fly flight muscle mitochondria by uncoupling them since as the concentration of the detergent increases, the rate

of proline oxidation at state-III decreases and that at state-IV increases. This is further confirmed by the increase in the glutamate dehydrogenase activity as the detergent concentration increases. Such observations have also been noted by Wadkins and Lehninger (1963) while working with rat liver mitochondria. This brought about a decrease in the respiratory control ratio. The respiratory control ratio is a measure of mitochondrial intactness. It is infinity for coupled mitochondria and one for uncoupled ones.

5.8 EFFECT OF DETERGENTS ON MEMBRANE AND MATRIX ENZYMES OF TSETSE MITOCHONDRIA

To locate the site of inhibition of the detergents, the effects of these detergents on two membrane enzymes, (proline dehydrogenase and succinate dehydrogenase) and one matrix enzyme (Glutamate dehydrogenase) were studied. Digitonin was shown to inhibit both the membrane enzymes but had no effect on the matrix enzyme, glutamate dehydrogenase even at a concentration of 170 µg/mg protein. Triton X-100 though had an inhibiting effect at a concentration of 170 µg/mg protein, showed both the stimulatory and the inhibitory pattern as the detergent concentration is increased. Solubilisation by Triton X-100 treatment may not only unmask latent enzyme

activity but may also disrupt any ordered arrangement between the individual component enzymes of the complex. The stimulatory phase can be explained by assuming that low concentrations of triton X-100 expose the intact enzymes to substrates and cofactors, thus causing the enzyme activities to increase. As the concentration of the detergent increases, the enzymes become dissociated and the ordered arrangement of the enzymes within the membrane is thereby disorganized. Substrates and cofactors then have to diffuse to the appropriate enzymes now free in solution before they can be acted upon (Oduro et al, 1980). Thus the site of inhibition of these detergents on proline oxidation is at membrane enzyme level.

The observation that a higher concentration of triton X-100 is required to start inhibiting succinate dehydrogenase than proline dehydrogenase suggests that the two enzymes operate in different lipid environments. The higher the lipid content involved in enzyme activity the faster the inactivation by the detergent.

5.9 CONTROL OF PROLINE OXIDATION

The proline oxidizing system of the tsetse mitochondria showed saturation kinetics with an apparent Km of about 6.00 mM. This was similar to

that obtained by Hansford and Johnson (1975) working with Japanese Beetle Popillia japonica flight muscle mitochondria respiring with proline. Bursell and Slack (1976) found a value which was half this value.

The high respiratory control ratio, the full uncoupling attained with 2.4-dinitrophenol and the low requirement for ADP in tsetse mitochondria used in this study indicates that the oxidation of proline is tightly coupled to phosphorylation. This is further confirmed by the observation that state-III oxidation of proline is completely oligomycin sensitive (Slater, 1967; Hansford and Johnson, 1975).

The higher Km value for ADP in this study compared to that of Bursell and Slack (1976) would be accounted for by the differences in experimental techniques or the degree of intactness of the mitochondrias preparations used. Bursell and Slack (1976) argued that the requirement of ADP increases as the degree of mitochondrial damage increases. The Km value for ADP in this study is lower than those reported for insect mitochondria where ADP takes part in controlling the activity of the various dehydrogenases (Hansford and Sacktor, 1970; Hansford (1970). Thus ADP exerts its stimulatory effect at the level of the electron transport chain but not at the level of the dehydrogenase.

5.10 OXIDATION OF PROLINE

The inhibition of proline oxidation by the respiratory chain inhibitors, rotenone, antimycin A and cyanide indicates that the reducing equivalents from proline oxidation are channelled through the whole span of the chain. The observed ADP/O ratio of about three implies that proline donates its electrons to the electron transport chain through NADH and that the first enzyme in proline breakdown/catabolism requires NAD^+ as the first cofactor but not FAD^+. These findings are similar to those of Bursell and Slack (1976) who found a P/O ratio close to 3. The observation that proline oxidation was inhibited by malonate suggested that proline oxidation involves succinate as an intermediate. The oxidation of succinate requires succinate dehydrogenase which is inhibited by malonate.

The observation that succinate and ascorbate/TMPD relieved inhibition by rotenone and antimycin A respectively and that oligomycin does not block succinate oxidation suggests that either these substrates alter the properties of the mitochondria and uncouples them or these mitochondrial preparations contain partly damaged mitochondria or these mitochondria respire with these substrates for energy metabolism. Chance and Williams (1955a) suggests that if these

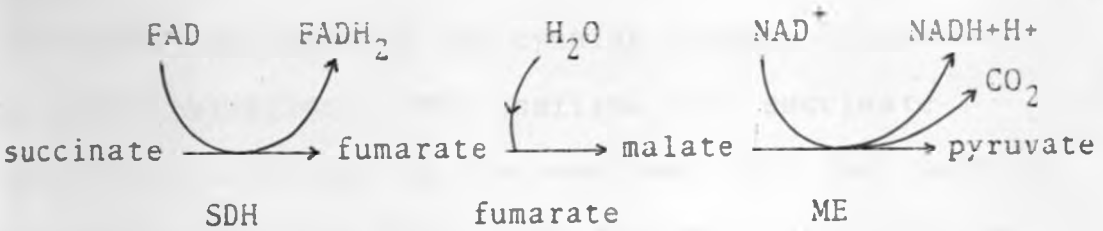
mitochondria actually respired with these substrates, then the rates of oxidation measured as a function of oxygen uptake would increase in the order proline--> succinate--->ascorbate/TMPD. Otherwise the expected ratios of the rates of oxygen uptake when these mitochondria are respiring with proline, succinate and ascorbate/TMPD would be 1:2:3 if the same rate of ATP synthesis is to be attained respectively. From the results of this study, oxygen consumption rates increase in the order proline, succinate and ascorbate/TMPD, but the ratios are not as expected. They are actually 1:1.3:1.7. suggesting that the last two respiratory substrates uncoupled the mitochondria.

5.11 SUCCINATE OXIDATION

Sacktor (1965) showed that Blow fly mitochondria respired with pyruvate and α-glycerolphosphate quite rapidly. In contrast, the respiratory rates with proline and the tricarboxylic acid cycle intermediates were very low. Bursell and Slack (1976) showed that tsetse fly flight muscle mitochondria, respired very rapidly with proline as substrate and little oxidation occurred with the tricarboxylic acid cycle intermediates and the amino acid glutamate, aspartate and alanine. Pearson et al (1979) showed that beetle flight muscle mitochondria respire appreciably with succinate as the substrate though

these investigators attributed this to mitochondrial disruption during the isolation procedures. Bursell and Slack (1976) also proposes that the intermediates of succinate oxidation are fumarate, malate and finally pyruvate.

In this study, succinate oxidation by tsetse fly flight muscle mitochondria showed saturation with the substrate suggests that the reactions involved in its oxidation are very likely to be enzymatic. The observation that these mitochondria produced pyruvate when incubated with succinate in the absence of glutamate confirmed that succinate oxidation is enzyme catalyzed. If glutamate was present in the incubation medium, the product would have been alanine. This is because alanine aminotransferase would have catalyzed the transamination reaction between pyruvate and glutamate. This enzyme has been shown by investigators to be present in this insect at high concentrations (Pearson et al, 1979; Konji et al, 1984; Crabtree and Newsholme, 1970). This data leads to the deduction that succinate is oxidized to pyruvate via fumarate and malate as intermediates.



SDH succinate dehydrogenase ME = NAD^+-linked malic enzyme

The observation that malonate inhibit succinate oxidation suggests that the oxidation of this substrate in these insects is catalyzed by succinate dehydrogenase. Malonate, a substrate analog of succinate can not be catabolised by this enzyme. This molecule is a competitive inhibitor of succinate. The presence of this enzyme in tsetse flies have been demonstrated by several investigators (Norden and Paterson, 1969; Norden and Matanganyidze, 1979) and in the present study. These results confirm the finding of earlier investigators (Bursell, 1963; 1966, 1967; Bursell et al 1976) who showed that the catabolic products of proline is alanine and that prolone oxidation makes use of a section of the tricarboxylic acid cycle from ketoglutarate to malate.

Experiments conducted with succinate by incubating it with the respiratory chain inhibitors, rotenone, antimycin A and cyanide showed that only rotenone did not inhibit succinate oxidation. Other

inhibitors, antimycin A and cyanide totally inhibited succinate oxidation. This confirms that succinate donates its electrons in the usual way into the electron transport chain from FADH to ubiquinone Q and that the terminal electron acceptor for these electrons is cytochrome oxidase (aa_3). Thus the reducing equivalents from the oxidation of succinate are channelled through the normal electron transport chain between ubiquinon and cytochrome aa_3 (cu).

The observation that succinate oxidation is ADP insensitive suggests that though these mitochondrial preparation appear to be intact with respect to proline they either contain partly damaged mitochondria or succinate alters the properties of the mitochondria and uncouples them in which case once uncoupled, they should then be uncoupled on any substrate. This might be so since small amounts of succinate added before proline, eliminates the state IV/III rates of oxygen uptake. However, Hansford and Sacktor (1971) showed that mitochondrial preparations containing intact and some damaged mitochondria both oxidized α-glycerolphosphate rapidly, although oxidation with damaged mitochondria were without control and were not sensitive to ADP and Pi. On the other hand, partially disrupted mitochondria having a lesion on the Krebs cycle, such as loss of NAD, CoA, OAA or one of the soluble enzymes components did not oxidize pyruvate. These invetigations

also showed that the presence of partially damaged mitochondria did not alter the respiratory control ratio for pyruvate except that they contributed much to the ATPase activity. Thus the above results can be explained likewise.

That, the mitochondrial preparation used in this study could have contained partially damaged mitochondria was confirmed by the observations that succinate oxidation was insensitive to oligomycin and was not stimulated by the uncoupler, 2, 4-dinitrophenol, which relieves respiratory inhibiton by oligomycin by discharging some high energy intermediate which has no phosphate. Oligomycin inhibits tightly coupled respiration (Wadkins and Lehninger, 1963). The observation that with various different mitochondrial preparations, the rates of proline oxidation was sometimes higher and at other times lower than those of succinate oxidation could indicate possibilities in the degree of mitochondrial disruption.

5.12 WHAT IS THE RATE LIMITING STEP IN PROLINE OXIDATION BY TSETSE FLY FLIGHT MUSCLE MITOCHONDRIA

The work carried out in the present study shows that proline transport in the flight muscle mitochondria of the tsetse fly is carrier mediated. The

transport systems of metabolites across the mitochondrial membranes have regulatory roles in cell metabolism (Meijer and Van Dam, 1974). Such a role is possible for proline transport in these insects since the rate of proline oxidation is over 80 times greater than proline transport (Bursell and Slack, 1976). Thus the rate of proline oxidation through the whole pathway to alanine would be limited by its transport.

The low rate of proline transport compared to its oxidation suggests that flight in tsetse flies would become limited by lack of this fuel. This is probably associated with the characteristic short interrupted flight behaviour of this insect, with periods of rest being necessary to allow proline level to reach sufficient concentration to support renewed activity. It is likely that the transport of proline into the matrix of tsetse flies is under neuro-endocrinal control as it is in other insects (Bailey, 1975) in which case proline transport during flight might be increased to levels at which it could make an effective contribution to the demands of oxidative metabolism.

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