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**Elthon, Thomas E.**

**BIOENERGETICS OF PROLINE OXIDATION AND RELATED PROCESSES  
IN PLANT MITOCHONDRIA**

*Iowa State University*

**PH.D. 1983**

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**Bioenergetics of proline oxidation and related processes  
in plant mitochondria**

by

**Thomas E. Elthon**

**A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of the  
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## ABBREVIATIONS

DH	- dehydrogenase
P5C	- $\Delta^1$ -pyrroline-5-carboxylic acid
GSA	- glutamic- $\gamma$ -semialdehyde
$\alpha$ -KG	- $\alpha$ -ketoglutaric acid
FCCP	- <i>p</i> -trifluoromethoxycarbonylcyanide phenylhydrazone
SHAM	- salicylhydroxamic acid
RCR	- respiratory control ratio
DCIP	- 2,6-dichlorophenolindophenol
<i>o</i> AB	- <i>o</i> -aminobenzaldehyde
EGTA	- ethyleneglycol-bis( $\beta$ -aminoethyl ether) N,N'- tetraacetic acid
OAA	- oxaloacetic acid
FAD	- flavin adenine dinucleotide
FMN	- flavin mononucleotide
PMS	- phenazine methosulfate
MTT	- Mes, Tes, Tricine
sec	- second(s)
min	- minute
h	- hour
cyt	- cytochrome
NAD	- nicotinamide adenine dinucleotide
NADH	- nicotinamide adenine dinucleotide (reduced)

ADP	- adenosine 5'-diphosphate
ATP	- adenosine 5'-triphosphate
BSA	- bovine serum albumin
TCA	- trichloroacetic acid
(A)ABS-	absorbance
%T	- percent transmittance
mV	- millivolt
A/D	- analog/digital
HGR	- high resolution graphics
Pi	- inorganic phosphate
g	- times gravity
PVP	- polyvinyl-pyrrolidone
nm	- nanometers
SRM	- standard reaction medium
mM	- millimolar
$\mu$ mol	- micromoles
wt	- weight
dpm	- decays per minute
T4C	- L-thiazolidine-4-carboxylic acid
PM	- 2-pyrrolidine methanol
A2C	- L-azetidine-2-carboxylic acid
D-pro	- D-proline
L-pip	- L-pipecolic acid
4-OH	- 4-hydroxy-L-proline
3,4-dehydro	- 3,4-dehydro-DL-proline

## PLANT MITOCHONDRIAL RESEARCH - SOME THOUGHTS

As an introduction to my dissertation, I have decided to briefly review the field of plant mitochondrial research, and to discuss my views on some of the more recent developments. Unlike many areas of botany, plant mitochondriology is a relatively new field in that it had its beginnings in the early 1950s (Lance 1978). It was during this time that mitochondria were first isolated. Before their isolation, mitochondria were solely the subject of microscopists, and their structure and function were somewhat of a mystery.

With the isolation of mitochondria, it became possible to study their physiology *in vitro*. Of the techniques available to investigators, Warburg manometry was most widely used since it was a direct measure of the physiological response - oxygen uptake. However, use of manometry restricted the experiments performed, since it required large quantities of mitochondria and long response times. In addition, the Warburg apparatus was simply technically difficult to use.

In 1961, introduction of the oxygen electrode allowed for oxygen uptake to be measured relatively easily with small quantities of mitochondria. Thus, progress on mitochondrial research increased dramatically until the

1970s. Essentially all of the work prior to 1961 has been reevaluated using the oxygen electrode and is thus primarily of developmental or historical interest. Since the early seventies, there has been a lull in plant mitochondrial research because of stimulated interest in other areas of plant physiology. To make matters even more depressing, the percentage of plant physiological research devoted to plant mitochondria is still decreasing, and it appears that the plant mitochondriologist may be becoming extinct.

The influence of animal mitochondrial research. Before looking at the conceptual development that has led to our current understanding of mitochondrial structure and function, it is necessary to explain the situation that plant mitochondrial research has been in since its inception. Unfortunately (or fortunately), plant mitochondrial research has consistently been overshadowed by research on animal mitochondria. Thus, much of what we know (or assume to be true) about plant mitochondria has been derived from research on animals. We have benefited from this association since we have gained insight into areas yet untouched by our research interests or capabilities. However, after years of such an association, it has become difficult to differentiate between what has been assumed to be true, and that which has actually been proven.

The first paper included within this dissertation consists of a chemiosmotic model for plant mitochondria. We have developed this model out of necessity, to help explain our experimental results, and those of others. Since it is presented as a model for mitochondrial function, it relies heavily on animal mitochondrial research. Even though it will be years before many of the concepts presented are evaluated with plant mitochondria, we felt that it was better to have a working model for experimental design and evaluation, than to have nothing at all.

Conceptual developments. I thought it would be interesting to pull out some of the greatest contributions to our understanding of mitochondrial function, and discuss them briefly. In the period up to 1961, the overall function of the respiratory chain was elucidated. As reviewed in Peter Mitchell's Nobel Prize lecture (Mitchell 1979), the basic concept of the respiratory chain was developed in the laboratories of Keilin, Warburg, and Wieland. That concept was that the respiratory chain is a "water-insoluble complex of redox carriers operating serially between the reducing substrates or coenzymes and molecular oxygen."

The respiratory chain was originally thought to be involved only in these redox reactions, but it was soon discovered that it was also coupled to the phosphorylation

of ADP to ATP. It was assumed that this phosphorylation occurred through a mechanism similar to that of substrate-level phosphorylation. The search then began, to find chemical intermediates such as those formed during phosphoryl-transfer by the glycolytic enzymes 3-phosphoglycerate dehydrogenase and phosphoglycerate kinase. The search continued into the seventies with no energy-rich intermediates being found.

During this time, several facts became apparent that linked the process of phosphorylation with the necessity for an intact membrane system. In contrast, and quite remarkably, it was found that the respiratory chain alone did not show a similar dependency. Thus, the coupling of phosphorylation to the respiratory chain occurred via some mechanism that was dependent upon intact membrane structure.

In the early sixties, a hypothesis consistent with the above observations, the chemiosmotic hypothesis, was formulated by Peter Mitchell. The essence of this hypothesis was that during electron flow through the respiratory chain, protons are translocated across the membrane resulting in the formation of an electrochemical gradient. This gradient was proposed to be the source of energy used to drive phosphorylation and other energy dependent membrane processes. The chemiosmotic hypothesis

has since been proven to be conceptually correct and has recently been acclaimed the chemiosmotic theory.

Thus, I feel that there have been 3 fundamental concepts that have provided a basis for all mitochondrial research. The first, is that the respiratory chain is a series of membrane bound redox carriers that are involved in the oxidation of soluble substrates and the reduction of molecular oxygen. The second, is that the energy released during this process is linked to the formation of ATP. And the third, is the development of the chemiosmotic hypothesis.

## PROGRESS WITH PLANT MITOCHONDRIA

Although it is beyond reason to review all the higher plant mitochondrial research in detail, I would like to give some impression of the progress that has been made in a few areas. This will not only complement the model we are presenting, but will also lead into a discussion of our research on mitochondrial proline oxidation.

Classical electron transport. The plant electron transport chain has been extensively investigated as to its redox properties (Storey 1980). Through these investigations, most of the components present have been identified. In addition, the electron transport chain components have been ordered as to sequence based on their redox potentials. Thus, an approach very similar to that used with animal mitochondria has been followed.

Few attempts have been made to solubilize the plant electron transport chain. Thus at present, there are no established methods for its fractionation and reconstitution. This is a severe limitation, since it is through the use of solubilized electron transport chain complexes, that the nature of the animal electron transport chain is being elucidated. With the isolation of these complexes from plant mitochondria, it will be possible to determine the components present within each complex and to

evaluate their physiological roles.

Alternative electron transport. One of the most obvious differences between plant and animal mitochondria, is the presence of an alternative (cyanide-resistant) terminal oxidase in plant mitochondria. This complex has been isolated in Bonner's laboratory (Bonner and Rich 1978), but has not yet been sufficiently characterized. This alternative pathway of electron transport branches from the classical electron transport chain via ubiquinone. It is operative, primarily under conditions where the cytochrome portion of the classical electron transport chain is limited (Lambers 1982). Nearly 50% of the higher plant mitochondrial research in the past few years has been devoted to the investigation of this pathway. Yet its structure and *in vivo* functioning are still relatively obscure.

Formation and utilization of the electrochemical gradient. There are only a few papers in the plant mitochondrial literature that deal with this subject (Moore 1978, Moore and Bonner 1981, Moore and Bonner 1982, Moore *et al.* 1978, Moreau and Romani 1982). From this research, it is evident that the plant electron transport chain generates an electrochemical gradient that is subsequently used to drive other membrane dependent processes (such as

ATP synthesis and metabolite transport). These papers evaluate the fluctuations that occur in the gradient during state 3 to state 4 transitions and during alternative oxidase activity. They have proven that the chemiosmotic theory is directly applicable to plant mitochondria, and have provided a basis for further investigation of plant mitochondrial bioenergetics.

Metabolite transport systems. Transport of the following substrates has been investigated with plant mitochondria: pyruvate, lactate, succinate, malate,  $\alpha$ -ketoglutarate, citrate, proline, glutamate, and glycine. The exchange of ATP for ADP, and the transport of various ions ( $\text{P}_i$ ,  $\text{Mg}^{++}$ ,  $\text{K}^+$ , and  $\text{Ca}^{++}$ ), has also been studied. A general scheme for metabolite transport is presented in the chemiosmotic model presented in this dissertation.

Substrate oxidation systems. The investigation of substrate oxidation systems represents the largest volume of plant mitochondrial literature. The oxidation of the normal *in vivo* substrates NADH, glutamate, proline, glycine, pyruvate, succinate, and malate has been investigated. In addition, the oxidation of formate (Oliver 1981) and of Krebs cycle intermediates has also been worked on somewhat. Of the above substrates, malate and NADH oxidation have received the most attention.

There are two different enzymes capable of oxidizing

malate in mitochondria. Malate dehydrogenase is a well-characterized matrix enzyme that yields oxaloacetate and reduced NAD as its products. Malic enzyme is the second enzyme capable of oxidizing malate, and releases pyruvate,  $\text{CO}_2$ , and possibly reduced NAD as products (Moreau and Romani 1982). The submitochondrial location and exact nature of malic enzyme still remains a controversy. Malic enzyme may be involved in providing pyruvate to run the Krebs cycle during the oxidation of substrates other than pyruvate. It is also involved in carbon assimilation during photosynthesis by some  $\text{C}_4$  plants (Gardestrom and Edwards 1983).

The only other active controversy in this area, is over the nature of NADH oxidation. Traditionally, the plant electron transport chain is thought to contain 2 NADH dehydrogenases. One of these is associated with the oxidation of exogenous NADH, while the other oxidizes NADH produced within the matrix. Recently, it has been suggested that a third NADH dehydrogenase is present that also oxidizes endogenous NADH. This NADH dehydrogenase has been proposed to be associated specifically with the alternative pathway (Moller and Palmer 1982, Palmer and Moller 1982). At present, there is some support for this notion (Moreau and Romani 1982, Walker *et al.* 1982), but also

considerable criticism (Wiskich and Day 1982).

Amino acid metabolism. The role of mitochondria in amino acid metabolism is becoming more and more evident. Glycine produced during photorespiration is known to enter the mitochondria, where it is readily converted to serine (Walker *et al.* 1982). This conversion results in the release of CO<sub>2</sub> and in the release of considerable NH<sub>4</sub><sup>+</sup> into the matrix. The fate of this ammonium is at present uncertain, but it may be incorporated into various amino acids within the matrix. Mitochondria are also intimately involved in fatty acid metabolism (Mettler and Beevers 1980). Fatty acid metabolism (to sucrose) in plants occurs through the concerted action of glyoxysomes, mitochondria, and a number of cytoplasmic enzymes. During this process, mitochondria are involved in several amino acid shuttle systems with the glyoxysome, with the most prevalent being the malate-aspartate and the  $\alpha$ -ketoglutarate-glutamate shuttles. Thus *in vivo*, the mitochondria represent an active site of amino acid metabolism.

As will be discussed in detail, in the second paper of this dissertation, the conversion of proline to glutamate and of ornithine to glutamate also occur within mitochondria. In addition, we have presented some evidence indicating that the oxidation of arginine to ornithine is mitochondrial.

## CONCLUDING REMARKS

The main body of this dissertation consists of 3 papers. The first is our chemiosmotic model for plant mitochondria. This paper serves as an excellent literature review for the mitochondrial aspects of our research. In addition, many of the concepts presented are novel. This is the first time such a model has been written (even for animal mitochondria). Our second paper represents a good portion of my research. In this paper, we further characterize the enzyme system involved in proline oxidation, and summarize the current status of research in that area. Our third paper concerns the inhibition of proline oxidation with L-thiazolidine-4-carboxylic acid. The results have allowed us to reaffirm previous findings on the role of proline oxidation during wa'er stress. In addition, this inhibitor will be useful in mitochondrial transport studies. The experiments with barley leaves in this last paper were contributed by Cecil Stewart.

**A CHEMIOSMOTIC MODEL FOR PLANT MITOCHONDRIA**

**Thomas E. Elthon and Cecil R. Stewart**

**Botany Department**

**Iowa State University**

**Ames, IA 50011**

## ABSTRACT

A model is presented which provides a balanced application of chemiosmosis to plant mitochondria. Electron transport carriers are sequenced to achieve proton translocation across the inner membrane. The processes of proton translocation, utilization and release are integrated to yield a description of the effective proton gradient available for metabolite transport and ATP synthesis.

## INTRODUCTION

General acceptance of the chemiosmotic theory has led to a proliferation of research ideas and applications. This theory combines knowledge of membrane structure and function with enzymology to explain membrane associated processes such as electron transport, ATP synthesis, and metabolite transport. Chemiosmotic models for membrane transport systems and animal mitochondria (Hinkle and McCarty 1978, Papa 1982) have provided a framework for experimental design and evaluation. The current lack of such a model for plant mitochondria has hindered interpretation of results from a chemiosmotic view.

Several excellent reviews that deal with plant mitochondrial structure and function have been published and include a review by Palmer (1976) on the organization and regulation of electron transport, a review by Wiskich (1977) on mitochondrial metabolite transport, and a review on cyanide-resistant respiration by Solomos (1977). In addition, there are several chapters in the series *The Biochemistry of Plants* including one by Hanson and Day (1980) on the occurrence, structure, and function of mitochondria; one by Day, Arron, and Laties (1980) on the alternative pathway; and one by Storey (1980) on the electron transport chain. We have integrated data from

these reviews and from the animal mitochondrial literature to develop a physiological-level chemiosmotic model for plant mitochondria. To achieve a concise, easy to follow presentation, the material from these reviews is not referenced. Therefore, the resulting model needs to be used in conjunction with these reviews and was not intended to be a separate entity.

Our interest in this model stems from the design of experiments to determine the effects of environmental stresses on mitochondrial function. It has provided a basis for understanding net energy conservation in mitochondria, which results from the integrated processes of metabolite transport, substrate oxidation, and electron transport coupled to ATP synthesis.

Some of the specific questions addressed during formulation of the model were: What are the theoretical ADP:O ratios of various mitochondrial substrates? To what degree does protonmotive metabolite transport affect ADP:O ratios? What is the meaning of respiratory control and of respiratory control ratios chemiosmotically? How do the alternative pathway and the oxidation of exogenous NADH fit into the chemiosmotic model?

General mitochondrial structure. Mitochondria are fairly complex organelles composed of four distinct regions,

each with its own characteristic complement of enzymes. Two separate membranes delineate these regions, an outer membrane, an intermembrane space including the intracristal regions, and an inner membrane which encloses the matrix. Proteins associated with either membrane can occur in a variety of positions within the membrane. Peripheral or extrinsic proteins associate primarily with the inner or outer surfaces since they are predominantly hydrophilic. Other proteins are primarily hydrophobic and are deeply embedded, sometimes extending completely through the membrane. These are integral or intrinsic proteins. Lipids, as well as the proteins within membranes, are also differentially arranged. Some membrane proteins are thought to be functional only when in association with specific membrane lipids. Other lipids may be involved only as structural components of the membrane.

The inner mitochondrial membrane is highly convoluted and surrounds a protein-rich matrix that contains a number of enzymes including those of the Krebs cycle. The invaginations of the inner membrane (cristae) greatly increase its surface area. The inner mitochondrial membrane contains a number of protein complexes including those of the electron transport chain, ATPases, and transporters. This membrane is very fluid, with this fluidity being essential for electron transport. The inner membrane is

only permeable to some small neutral molecules ( $MW < 150$ ), including  $H_2O$  and the gases  $O_2$  and  $CO_2$ . Substrates or other molecules that pass through the inner membrane, generally go through proteinaceous carriers. It is relatively impermeable to large molecules, ions, or protons, and therefore can support the development of an osmotic pressure gradient.

The outer mitochondrial membrane is thought to have only a few enzymes associated with it and contains a NADH DH (dehydrogenase) system. The function of this DH system is not clearly understood. In contrast to the inner mitochondrial membrane, the outer membrane is a fairly rigid membrane and will not support an osmotic pressure gradient since it is very permeable to medium and low molecular weight compounds ( $MW < 4000$ ).

The space between the inner and outer membranes is also thought to contain relatively few enzymes and may contain soluble cyt c. The intermembrane space continues into the cristae, with these intracristal regions comprising the greater part of the intermembrane space.

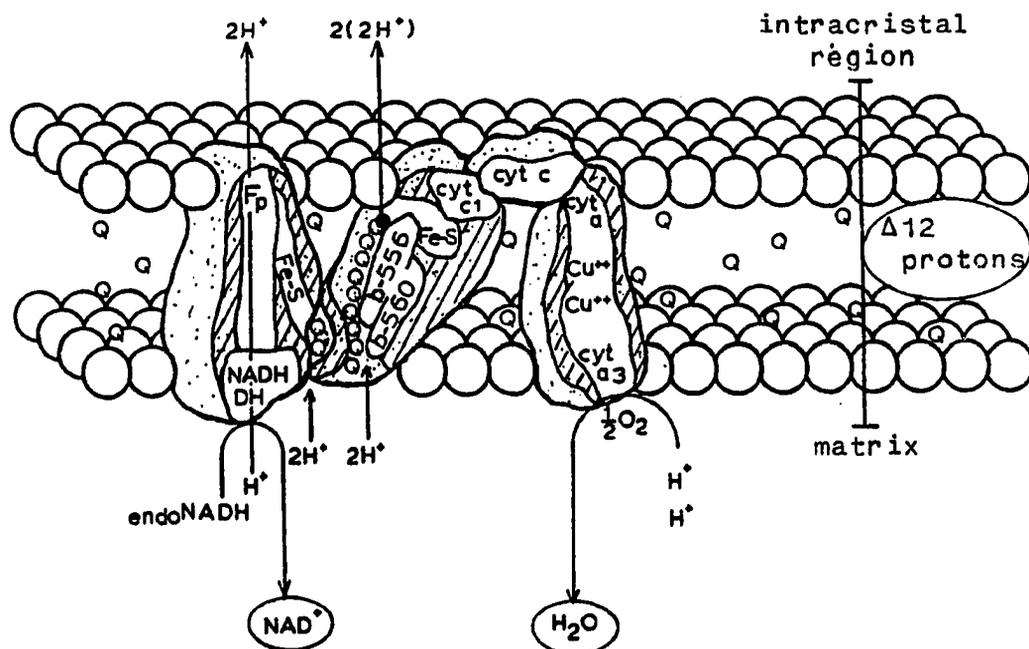
The essential features of the chemiosmotic theory are based upon the structural arrangement of specific proteins and lipids within membranes so that reactions occur vectorally (i.e., in a specific direction). As a result,

ions or molecules are transported across the insulating membrane, establishing a gradient. This established gradient is a source of potential energy that can be used to drive other membrane localized reactions (Mitchell 1979).

In mitochondria, the electron transport chain consists of a series of electron carriers that carry electrons from various substrates to oxygen. Because of their nature, some of these must also carry protons. The topographical arrangement of these two types of carriers within the inner membrane results in the vectorial transport of protons out of the matrix into the intermembrane region during electron flow. Since the inner membrane is relatively impermeable to protons, a gradient is established. This proton gradient is then used to drive oxidative phosphorylation and a variety of substrate and ion uptake mechanisms.

## A CHEMIOSMOTIC MODEL FOR PLANT MITOCHONDRIA

The oxidation of endogenous NADH. One of the primary functions of mitochondria, is to generate ATP for use within the cytoplasm. This is usually achieved through the oxidation of pyruvate (to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ ) via the Krebs cycle and the electron transport chain. Energy from the Krebs cycle is transferred to the electron transport chain in the form of NADH and succinate. NADH produced by the Krebs cycle enzymes (and other enzymes within the matrix), diffuses in the matrix until it binds with a membrane bound endogenous NADH DH complex (Figure 1). This complex corresponds to complex I in animal mitochondria (Hatefi *et al.* 1979) and contains an NADH DH (exposed to the matrix), a flavoprotein ( $\text{Fp}_{1a}$ ), and a series of iron-sulfur proteins (probably 3). This complex is also thought to contain other dehydrogenases. Soluble NADH is oxidized by the DH, with 2 electrons and 1 proton passed on to the flavoprotein. Since flavoproteins require 2 electrons and 2 protons for reduction, a second proton is picked up from the matrix. This second proton was originally released into the matrix during NAD reduction, and thus comes from the substrate. The flavoprotein is positioned so that it spans the membrane and has an exposed surface within the intracrystal region.



**Figure 1. Oxidation of Endogenous NADH**

In this figure and in those that follow, we have used large circles (outlining the membrane) to represent the hydrophilic regions of the membrane, with the area between being hydrophobic. Diagonal lines were used to indicate cross-sections of various protein complexes. Overall products of a reaction sequence are given in ovals.

The electrons are then passed on through a series of iron-sulfur proteins. Since iron-sulfur proteins only carry electrons (necessarily one at a time), 2 protons are released into the intracristal region. This transition from a carrier reduced by 2 electrons to a carrier reduced by 1, is facilitated by the ability of flavoproteins to release one electron at a time (forming a semiquinone intermediate). The iron-sulfur proteins are spatially positioned so that they traverse the membrane, transferring their electrons to ubiquinone on the matrix side of the inner membrane. The reduction of ubiquinone requires 2 electrons and protons, thus 2 protons are picked up from the mitochondrial matrix. This transition from a 1 electron to a 2 electron process is possible because ubiquinone also forms a semiquinone intermediate. The endogenous NADH DH complex can be inhibited by rotenone, amytal, or piericidin A.

Ubiquinone is hydrophobic (thus soluble within the lipid portion of the membrane) and serves to transfer electrons from the endogenous NADH DH complex (and protons obtained from the matrix) to the cyt  $bc_1$  complex (complex III in animal mitochondria (Hatefi *et al.* 1979)). Ubiquinone is represented by "Q" in the hydrophobic region of the membrane, but the concentration would necessarily be higher than illustrated. This transfer of electrons via ubiquinone most likely occurs through association of the

endogenous NADH DH complex with the cyt  $bc_1$  complex (Heron *et al.* 1978). In this association, a few of the soluble ubiquinone molecules are trapped between the complexes and are used in the transfer. This association between complexes is thought to form, dissociate, and re-form readily; potentially with a different portion of the soluble ubiquinone pool being trapped each time.

The cyt  $bc_1$  complex is thought to contain an iron-sulfur protein, 2 molecules of cyt b (b-556 and b-560), one molecule of cyt  $c_1$  (c-552), and a layer of functionally integrated ubiquinone. The ubiquinone molecules associated with this complex appear to be bound to the complex (Yu and Yu 1981) and do not participate in connecting the endogenous NADH DH complex with the cyt  $bc_1$  complex. The 2 electrons and 2 protons carried by the lipid soluble ubiquinone pool are transferred to the bound ubiquinone pool. These electrons and protons are then transferred within the bound pool of ubiquinone to the intracrystal side of the inner membrane. Ubiquinone passes an electron to each the iron-sulfur protein and cyt b-556, releasing 2 protons into the intracrystal region (Trumpower 1981a). Cytochromes are similar to iron-sulfur proteins in that they carry only one electron at a time and do not carry protons.

The reduction of the Fe-S protein and cyt b-556 could

occur through two possible mechanisms, the first being through interaction at the junction of the Fe-S protein and cyt b. Upon oxidation of ubiquinone, one electron could go to the iron-sulfur protein and the other to cyt b. This possibility would allow for an efficient transition from ubiquinone that carries two electrons, to the Fe-S protein-cytochrome system that carries only one electron per component at a time. The second possible mechanism is similar to the above, except that the oxidation of ubiquinone would occur in two steps. First, the Fe-S protein would be reduced, leaving a semiquinone that would then reduce cyt b-556.

At this point in the sequence, it is thought that one electron is already present within the second cyt b molecule (b-560). The two cyt b molecules are positioned so that together they traverse the membrane. Thus upon reduction of cyt b-556, the 2 electrons within the cyt b molecules are used to reduce a molecule of bound ubiquinone at the inner surface of the membrane (with 2 protons being picked up from the matrix). This reduction of ubiquinone most likely occurs via cyt b-560, and would occur through a 2 step reaction (i.e., 1 electron being passed to ubiquinone at a time). The 2 electrons and protons then pass to the outer surface of the membrane within the bound ubiquinone pool. The Fe-S protein and cyt b-556 are reduced, with 2 more

protons released into the intracrystal region. Thus, the system is rejuvenated (1 electron within the b-cytochromes) for acceptance of 2 more electrons and protons from the endogenous NADH DH complex. Therefore, it can be seen that the b-cytochromes do not participate in direct electron transport to oxygen, but are involved in this "quinone-cycle" (Mitchell 1979, Papa 1982, Trumpower 1981b). For each pair of electrons passing through the cyt  $bc_1$  complex, a total of 4 protons are translocated into the intracrystal region. The cyt  $bc_1$  complex has been isolated from plant mitochondria by Ducet and Diano (1978). Antimycin A inhibits the cyt  $bc_1$  complex presumably by interaction with the cyt b-ubiquinone portion of the complex.

Each time the Fe-S protein is reduced the electron is passed to cyt  $c_1$  (c-552) and then to cyt c (c-550) which is loosely associated with the outer surface of the inner membrane. Cyt c then passes the electron to the cytochrome oxidase complex (cyt  $aa_3$  or complex IV in animal mitochondria (Hatefi *et al.* 1979)). Thus, cyt c serves to connect the cyt  $bc_1$  complex with the cyt  $aa_3$  complex. Bomhoff and Spencer (1977) and Maeshima and Asahi (1978) have succeeded in isolating the cyt  $aa_3$  complex from plant mitochondria.

The cyt  $aa_3$  complex contains a molecule of cyt a, 2

copper atoms, and a molecule of cyt  $a_3$ . These components are thought to accept electrons in that order with cyt  $a_3$  eventually reducing oxygen to form water (Azzi 1980). The reduction of molecular oxygen by this complex is a four electron process that consumes four protons from the matrix, thus contributing to the establishment of a proton gradient. The mechanism of this reduction process is not well-understood. Inhibitors of this complex include cyanide, azide, carbon monoxide, and sulfide.

Components of the established proton gradient. The translocation of protons by the electron transport chain results in the formation of a pH gradient across the inner membrane. In addition to a pH gradient, an electrical gradient also forms because of the separation of positive charges (protons into the intracristal region) from negative charges (hydroxyls remaining within the matrix).

Because of the electrical and pH potentials across the membrane, protons will flow back into the matrix if provided with an appropriate carrier. This movement of protons back into the matrix can be coupled to ATP synthesis and a number of substrate and ion transport mechanisms. In addition, the charge separation across the membrane can be used to drive transport mechanisms. The term protonmotive force represents the sum of these two potential energy sources, that being an electrochemical gradient.

This relationship can be expressed by the following equation (Mitchell 1979):

$$\Delta p = \Delta Y - Z \Delta pH$$

where  $\Delta p = \Delta \mu_{H^+}$  = protonmotive force

$\Delta Y$  = the electrical potential

$-Z \Delta pH$  = the pH potential

and  $Z = 2.3RT/F$  = about 60 at 25 C

Because of convention for membrane potentials (outside - inside), the  $\Delta pH$  value becomes negative. Thus, a negative sign is included in the equation to allow summation of the two potentials.

So what are the contributions of the electrical and pH potentials to the total protonmotive force? In plant mitochondria, they both have values of near 100 mV (Moore and Bonner 1981). Thus, the total protonmotive force across the inner mitochondrial membrane is near 200 mV. This force corresponds to a pH gradient of around 1.7 pH units. It must be kept in mind, that this is an average potential for the inner membrane.

As a result of the electrical gradient across the membrane, protons tend to associate with its outer surface. Thus, it has been postulated that the protons are not released into the bulk intracrystal solution. Instead, it is envisioned that the protons flow very near the surface of

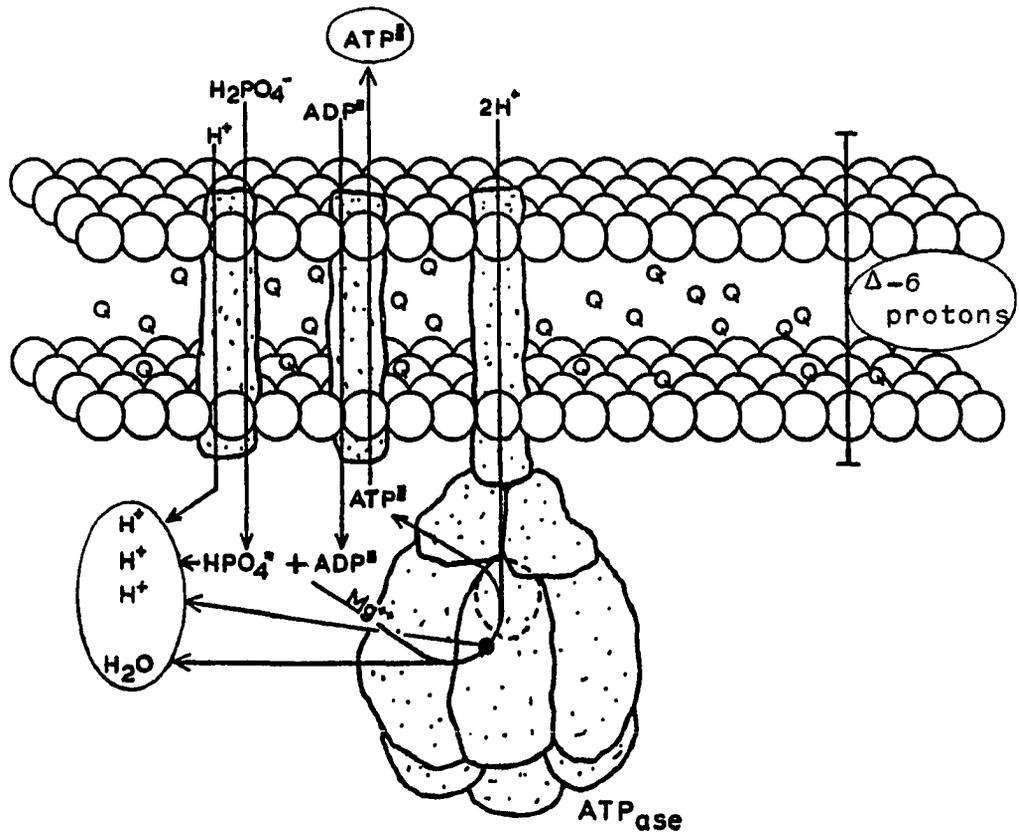
the membrane or perhaps at the membrane-solution interface (Kell 1979). This flow would occur from regions of high concentration (sites of translocation) to regions of low concentration (sites of utilization) (de Kouchkovsky and Haraux 1981).

When considering utilization of the established electrochemical gradient, it is difficult to separate the electrical and pH components. However, for electron transport to continue, it is evident that the pH potential must be fully utilized or the matrix would become depleted of protons. Therefore, various transport mechanisms would necessarily be involved in keeping the electrical potential in balance as the proton potential is utilized.

Coupling of protonmotive force to ATP synthesis. As shown in Figure 1, the oxidation of endogenous NADH is thought to result in the translocation of 6 protons into the intracrystal region. Four of these protons were removed from the matrix solution. The remaining 2 were provided by substrate dependent NAD reduction, and the resulting NADH oxidation. A transmembrane potential of 10 protons (4 removed from the matrix, with 6 ending up in the intracrystal region) is created. In addition, 2 protons are consumed within the matrix during the reduction of oxygen establishing a net potential of 12 protons across the membrane. The positive charge on  $\text{NAD}^+$  does not contribute

to the electrical gradient because its charge balances that of  $\text{NADH} + \text{H}^+$ . The established proton gradient serves as an energy source which can then be used to drive reactions that occur via specific proteins within the membrane.

One such protein complex is the ATPase complex (Figure 2). This complex (complex V in animal mitochondria (Hatefi *et al.* 1979)) consists of two general regions, a hydrophobic proton channel embedded within the membrane ( $F_0$ ) and a large hydrophilic head ( $F_1$ ). A pair of protons passes through the hydrophobic channel (down the potential gradient) and drives either the synthesis of ATP or its release within the hydrophilic head region (Kagawa *et al.* 1979). There is some evidence in the literature that 3 protons may be required for this process, however we will assume that 2 are sufficient (Duszyński *et al.* 1981, Moore and Bonner 1981, Papa 1982). The 2 protons may function by removing an oxygen from  $\text{Pi}^{-2}$  ( $\text{HPO}_4^{-2}$ ) by formation of water. The intermediate formed is proposed to combine with ADP producing ATP. During this process, the proton associated with  $\text{HPO}_4^{-2}$  would be released into the matrix. Alternatively, ATP may be synthesized independent of proton flow, with the proton gradient essential for the release of synthesized ATP from the complex. The ATPase complex can be inhibited by oligomycin and DCCD ( $\text{N,N}'$ -



**Figure 2.** ATP Synthesis and Related Transport Processes

dicyclohexylcarbodiimide), both of which affect proton translocation by the  $F_o$  portion of the complex.

To synthesize ATP, transport of  $Pi^{-1}$  ( $H_2PO_4^{-1}$ ) and ADP into the matrix is also necessary.  $ADP^{-3}$  uptake occurs in exchange for  $ATP^{-4}$ . The uptake of  $Pi^{-1}$  is thought to be driven by the simultaneous uptake of a proton (resulting in neutral uptake), utilizing a transmembrane potential of 2 protons. Once inside the matrix, the phosphate dissociates because of the more basic conditions, releasing a proton (Fox 1982) to further diminish the transmembrane potential by 1 proton. Thus, phosphate uptake requires a membrane potential equivalent to 3 protons. It is the  $Pi^{-2}$  form of phosphate that is used in ATP synthesis. During ATP synthesis, the proton in  $HPO_4^{-2}$  is released, reducing the membrane potential by 1 proton. ATP synthesis is dependent upon 2 protons passing through the ATPase for each ATP produced. Thus, a transmembrane potential of 6 protons is needed for the synthesis of 1 molecule of ATP. Therefore as presented, the oxidation of endogenous NADH results in the synthesis of 2 ATPs. The 6 protons removed from the matrix during electron transport, have been returned during ATP synthesis. Thus, the pH potential has been fully utilized. The electrical gradient has also been exhausted.

In the above scheme, substrate transport into the

matrix was not considered. What is the demand of various substrate transport mechanisms on the protonmotive force? Since the uptake of substrate occurs through a number of mechanisms, the demand on the proton gradient varies (Figure 3). The uptake of monocarboxylic acids is thought to occur along with the simultaneous uptake of a proton, utilizing a transmembrane potential of 2 protons. Uptake of dicarboxylic acids usually occurs in exchange for  $\text{Pi}^{-2}$  and tricarboxylic acids are transported simultaneously with a proton in exchange for dicarboxylic acids. The uptake of  $\text{Pi}$  utilizes a transmembrane potential of 3 protons, with di- and tricarboxylic acid transport requiring an additional 1 and 3 protons respectively. Since the total oxidation of a substrate often results in the formation of several molecules of NADH, this high cost of transport is minimized.

The oxidation of succinate. As previously discussed, succinate is a Krebs cycle intermediate that is oxidized (to fumarate) by the electron transport chain. This oxidation occurs through the action of the succinate DH complex (complex II in animal mitochondria (Hatefi *et al.* 1979)). This complex (Figure 4) is associated with the inner surface of the membrane and contains a dehydrogenase, a flavoprotein ( $\text{Fp}_{\text{ha}}$ ), a b-cytochrome (b-565), and a series of iron-sulfur proteins (probably 3). During succinate oxidation, the DH

passes 2 electrons and 2 protons to the flavoprotein. In contrast to the endogenous NADH DH complex, the flavoprotein in the succinate DH complex does not traverse the membrane. Thus upon transfer of the electrons to cyt b-565 (one at a time), the 2 protons are released into the matrix. Therefore, the succinate DH complex is not protonmotive.

The 2 electrons then pass through the series of iron-sulfur proteins, reducing soluble ubiquinone that is trapped through association of the succinate DH complex with the cyt  $bc_1$  complex. This association is thought to be similar to that which is formed between the endogenous NADH DH complex and the cyt  $bc_1$  complex, except that it may involve more protein-protein interactions. The 2 electrons and 2 protons (picked up from the matrix) are utilized as previously discussed, resulting in a net transport of 4 protons across the membrane (2 derived from succinate) and a transmembrane potential of 6 protons. Two protons are also removed from the matrix during reduction of oxygen via cyt  $aa_3$ . Thus, a total transmembrane potential of 8 protons is established during succinate oxidation (not considering substrate transport). This potential would be sufficient to synthesize 1.33 ATP molecules. Inhibition of the succinate DH complex is usually achieved through addition of malonate, which is a competitive inhibitor.

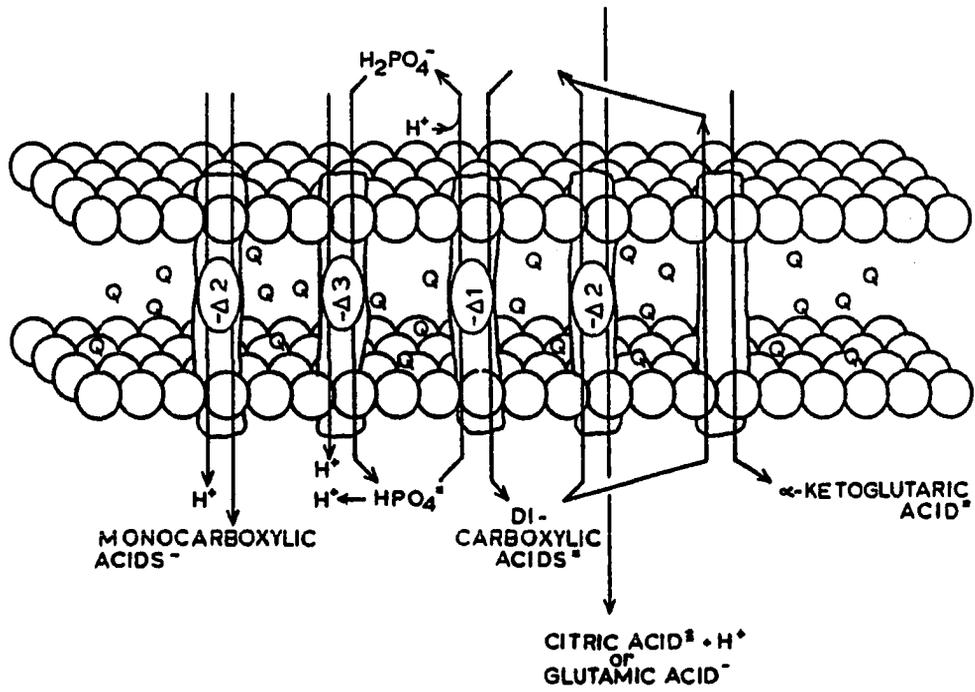


Figure 3. A Generalized Scheme for Substrate Transport

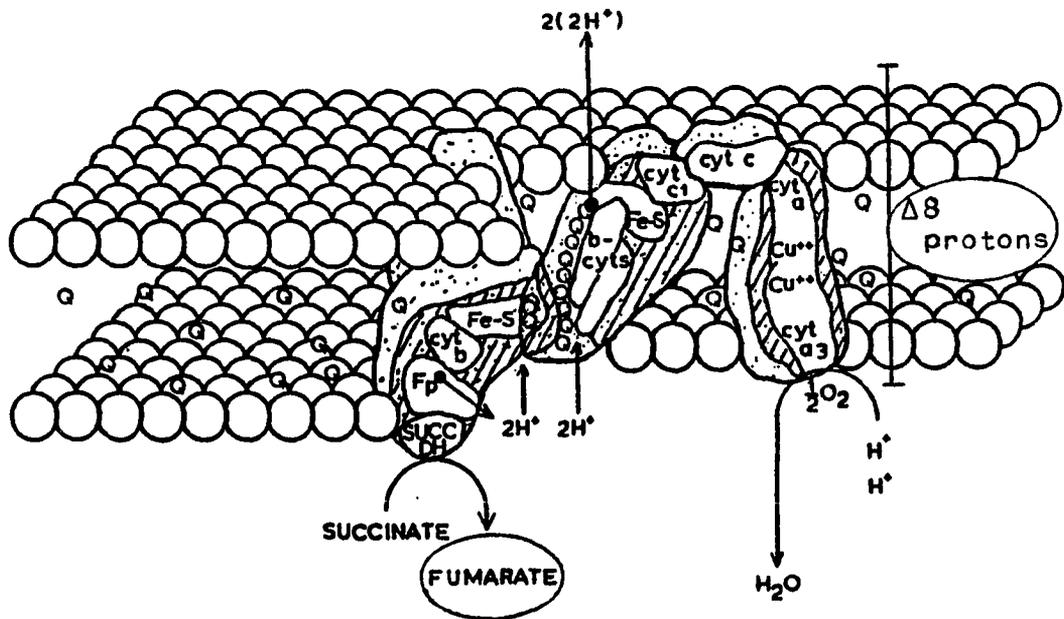


Figure 4. Oxidation of Succinate

The oxidation of exogenous NADH. There are two dehydrogenase complexes involved in the oxidation of exogenous NADH. This NADH would be derived from either the cytoplasm or from the intermembrane region of the mitochondria. One complex is associated with the outer membrane of the mitochondria, while the other is associated with the inner membrane. The inner membrane exogenous NADH DH complex contains a dehydrogenase, a flavoprotein ( $Fp_{vha}$ ), and perhaps a b-cytochrome (b-554) (Figure 5). This complex is positioned with its DH facing the intracrystal region, and its flavoprotein traversing the membrane. The flavoprotein probably passes its electrons and protons to the bound ubiquinone pool of the cyt  $bc_1$  complex near the inner surface of the inner membrane. The 2 electrons and 2 protons are utilized as previously discussed, resulting in 4 protons being translocated across the inner membrane. Two of these protons are from the matrix and one was from NADH. The fourth proton was initially removed from the intracrystal region (via the exogenous NADH DH) during the oxidation of NADH. This proton does not contribute to the proton gradient because the reduction of NAD occurred in the cytoplasm and only NADH would be expected to diffuse into the intracrystal region. Therefore, a transmembrane potential of 5 protons is established.

As with succinate oxidation, two protons are utilized

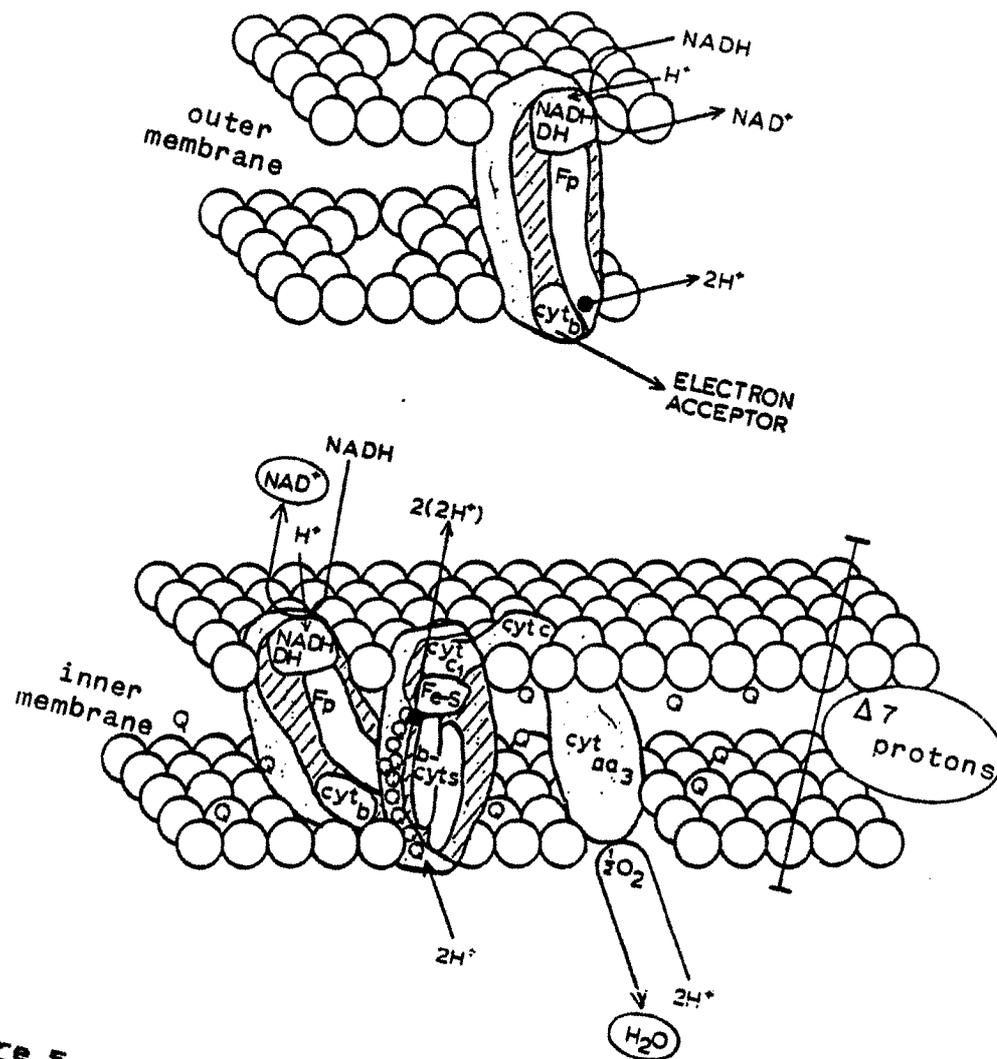


Figure 5. Oxidation of Exogenous NADH

within the matrix during the formation of water. Thus, a transmembrane potential of 7 protons is established, which is capable of driving the synthesis of about 1.17 ATP molecules (no substrate transport involved). If a b-cytochrome is associated with this complex, it may be residual and thus would not participate in normal electron flow. NADH oxidation by this complex is sensitive to antimycin and inhibitors of the  $\text{cyt aa}_3$  complex.

With exogenous NADH, further complications arise due to the presence of the outer membrane NADH DH complexes. Each of these complexes is thought to contain a dehydrogenase, a flavoprotein, and a b-cytochrome (b-555) (Figure 5). The function of this complex is not understood, although it has been proposed that it may transfer reducing equivalents to the inner membrane respiratory chain via soluble cyt c. It has also been suggested that the inner membrane exogenous NADH DH complex could have arisen through a modification of the outer membrane NADH DH and its insertion into the inner membrane. We have proposed the presence of a residual b-cytochrome in the inner membrane exogenous NADH DH complex to be consistent with this possibility. The inner membrane exogenous NADH DH complex is not present in animal mitochondria.

The alternative or cyanide-resistant pathway. While investigating the effect of various inhibitors on substrate

dependent oxygen uptake, it was observed that some tissues exhibited or could develop considerable insensitivity to cyanide and antimycin. Most of these tissues were found to exhibit insensitivity readily with substrates that yielded endogenous NADH or with succinate, while the oxidation of exogenous NADH was only insensitive in thermogenic tissues.

The cyanide resistant pathway (Figure 6) is responsible for this oxidation, and is positioned within the inner membrane. It contains at least a flavoprotein ( $Fp_{ma}$ ), a component (perhaps a nonheme iron protein) that is sensitive to hydroxamic acids such as SHAM (salicylhydroxamic acid), and a terminal oxidase. Under conditions where the cytochrome portion of the primary respiratory chain is not functioning to capacity, electrons and protons from the various DH complexes can be utilized by the alternative pathway via soluble ubiquinone (Ladies 1982). The reduced ubiquinone passes its electrons and protons to the flavoprotein of the alternative oxidase. This flavoprotein does not traverse the membrane, and thus the protons are released into the matrix when the electrons are passed on to the oxidase (i.e., the complex is not protonmotive). The electrons eventually reduce oxygen, consuming 2 protons from the matrix to form water.

With endogenous NADH as substrate, a transmembrane

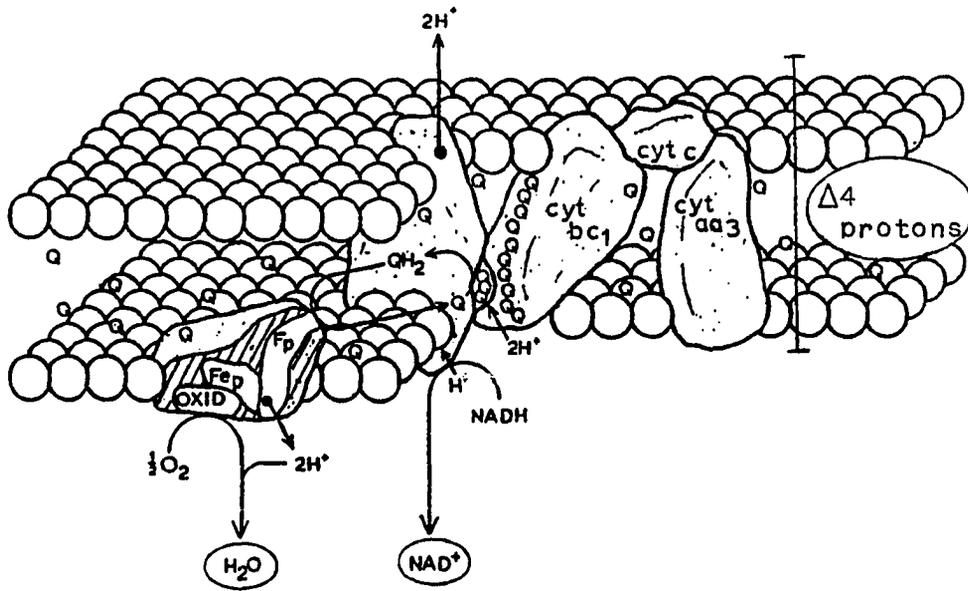


Figure 6. The Alternative Pathway

potential of 4 protons is achieved. Two protons derived from NADH are translocated into the intracrystal region, and 2 protons are consumed in the formation of water via the alternative oxidase. This is sufficient to drive the synthesis of 0.67 ATP molecules (not considering transport). In the case of succinate (or exogenous NADH in some tissues) as substrate, the 2 protons used to reduce oxygen would be provided by the substrate. Thus, no membrane potential would be generated (Moore *et al.* 1978).

The alternative pathway is not always operational in mitochondria whose cytochrome chain (the cyt  $bc_1$  and  $aa_3$  complexes) is fully functional. In these mitochondria, a developmental time is required before activity of the alternative pathway is observed. During this developmental period, the association of the endogenous NADH DH complex with the cyt  $bc_1$  complex, and that of the succinate DH complex with the cyt  $bc_1$  complex, may be weakened. This "loosening" of the membrane would cause less efficient trapping of ubiquinone between the complexes, and result in reduced ubiquinone escaping into the soluble ubiquinone pool where it can interact with the alternative pathway.

With the exogenous NADH DH complex, stronger protein-protein interactions may need to be weakened before it could interact with soluble ubiquinone. Therefore, in only a few exceptional tissues, such as thermogenic tissues, has this

complex been found to feed electrons and protons to the alternative pathway. In addition to the proposed weakening of associations between complexes, developmental time may also be necessary for *de novo* synthesis of a limiting component of this pathway or perhaps to achieve increased membrane fluidity. The alternative oxidase complex is not present in animal mitochondria. In addition to SHAM,  $\alpha$ -CLAM ( $\alpha$ -chlorobenzhydroxamic acid) and disulfiram are effective inhibitors. This pathway functions in the dramatic production of heat in thermogenic tissues and is also involved in the climacteric rise in respiration during the ripening of some fruits.

Regulation of the electron transport chain. As presented in the model, upon addition of substrate a proton gradient is thought to be established through proton translocation into the intracrystal region. Substrate oxidation becomes limited as the proton gradient increases to a certain potential. There are at least 2 possible mechanisms through which this influence of the proton gradient may be exerted upon electron flow through the respiratory chain.

With continued substrate oxidation, the proton concentration within the matrix may become limiting because of proton translocation. Both ubiquinone and oxygen must be

reduced at the matrix side of the membrane for electron flow to continue. Thus, the rate of substrate oxidation may be limited to the rate of proton influx back into the matrix.

Control of substrate oxidation (by the proton gradient) may also be exerted on the intracrystal side of the membrane. As the proton concentration increases within the intracrystal region, proton release may be inhibited. This inhibition would occur not only as a result of the pH (proton concentration), but also because of charge since the intracrystal region would be becoming increasingly more positive. This influence would be exerted at all points of proton release exposed to the intracrystal region.

When the proton gradient potential reaches a steady state, the rate of substrate oxidation will be constant. All factors that influence the transmembrane potential will contribute to this steady state. These factors include proton translocation into the intracrystal region, proton utilization, proton flux back into the matrix, and charge separation across the membrane.

When substrate oxidation is being limited by such a steady state condition (a state 4 rate), addition of ADP in the presence of Pi and Mg<sup>++</sup> results in a stimulation of the oxidation rate (a state 3 rate). This stimulation occurs because of increased utilization of the proton potential via ATPase activity. When all of the ADP has been

phosphorylated to form ATP, the substrate oxidation rate is reduced to its previous level. The respiratory control ratio is a ratio of the state 3 to the state 4 rate, and has been used as an indicator of the integrity of the inner mitochondrial membrane. Respiratory control ratios vary depending upon the substrate utilized.

In mitochondria with active alternative oxidase activity, a large portion of the oxygen uptake during the state 4 rate is thought to occur through this pathway. Under these conditions (state 4), the cyt pathway is limited and reduced ubiquinone may be released from associations between complexes (before it can be oxidized) into the soluble ubiquinone pool. It thus becomes available for oxidation via the alternative oxidase complex. During state 3, this process would be reduced because of the increased flow of electrons through the cyt pathway.

The phenomenon of substrate oxidation being stimulated by ADP is referred to as the coupling of phosphorylation to substrate oxidation. Uncouplers are often used in mitochondrial research and function by carrying protons (down the potential gradient) from the intracrystal region back into the matrix. Thus, uncouplers deplete part of the proton gradient and allow for more rapid substrate oxidation. However, if a substrate requires active uptake

into the matrix, then addition of excess uncoupler can result in diminished substrate uptake which reduces the oxidation rate. Common uncouplers used include FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), and DNP (2,4-dinitrophenol).

The coupling of phosphorylation to respiration results in a system that is regulated by ADP concentration *in vivo*. When the energy demand within the cell is low, ADP becomes scarce. This scarcity causes a shutdown of ATP synthesis which results in a relatively high proton gradient being maintained. This gradient in turn slows substrate oxidation which in itself regulates Krebs cycle activity. Alternatively, the electron transport chain can be regulated by substrate availability.

Rationalization of measurable ADP:O ratios. An ADP:O ratio represents the number of molecules of ADP phosphorylated per pair of electrons passing through the respiratory chain to form water (utilizing  $1/2 O_2$ ). ADP:O ratios are measured by adding a small quantity of ADP (initiating a state 3 rate) and measuring the amount of oxygen consumed during its phosphorylation to ATP. When all of the ADP has been phosphorylated, substrate oxidation returns to a state 4 rate. Measured ADP:O ratios for substrates such as malate and pyruvate that involve NADH

oxidation via the endogenous NADH DH are normally in the range of 2.0 to 2.3. For the oxidation of succinate and exogenous NADH, the ADP:O ratios are usually between 1.2 to 1.5. Like respiratory control ratios, ADP:O ratios have been used as indicators of mitochondrial integrity.

We will first consider the oxidation of exogenous NADH via the inner membrane exogenous NADH DH, since it is simpler in that substrate transport is not involved. The outer membrane NADH DH system does not contribute significantly to NADH oxidation in isolated mitochondria unless an electron acceptor is added. Using the model, the oxidation of exogenous NADH can drive the synthesis of 1.17 ATPs. This value is reasonably consistent with measurable ADP:O ratios, although a little low. Succinate oxidation yields a potential for the synthesis of 1.33 ATPs. This figure does not consider substrate transport and thus measurable ADP:O ratios of 1.2 to 1.5 are reasonably consistent but also a little low. The oxidation of endogenous NADH establishes a proton potential capable of driving the synthesis of 2.0 ATP molecules. This value is lower than the usually measured ADP:O ratios of 2.0 to 2.3. In addition, a portion of the proton potential would be needed for substrate uptake and thus would not be available for ATP synthesis, further lowering the measured ADP:O

ratio.

Thus, the measured ADP:O ratios, are generally higher than anticipated in the model. This discrepancy can be explained in at least two ways. The media in which ADP:O ratios are measured, always contain Pi which is necessary not only for phosphorylation, but also for substrate uptake into the matrix. As a result, prior to addition of ADP, the mitochondria may have become loaded with Pi using the electrochemical gradient created during state 4 respiration. Thus, the measured ADP:O ratios may not reflect the need for Pi uptake. In addition, it can be seen that substrate loading may also occur to some extent. Thus, measured ADP:O ratios probably do not reflect the theoretical transport requirement. Artificially high measured ADP:O ratios would result (Hinkle and Yu 1979, Moore and Bonner 1981).

Another possible explanation for the discrepancy is that additional proton translocation may be occurring. Depending upon the technique, between 6 and 12 protons can be measured as being translocated for each pair of electrons flowing through the respiratory chain. The model we have presented is consistent with that of Mitchell (1979) and can only account for 6 protons. If additional protons are translocated, it is probable that their transport is not linked directly to electron flow. For example, there is considerable evidence in the literature that the cyt aa<sub>3</sub>

complex is protonmotive when incorporated into membrane vesicles. This proton translocation occurs (in the opposite direction of electron flow) within a protein subunit of the complex. This illustrates that proton translocation may occur in addition to that directly associated with electron flow (Casey *et al.* 1981, Papa 1982).

This matter of redox potentials. The electron carriers within the respiratory chain are involved in cyclic reduction/oxidation reactions. Electron flow has classically been described as electrons passing from one carrier to the next based upon their redox potentials, with electrons flowing from components with more negative redox potentials to those with more positive potentials. Between some of the carriers in the electron transport chain, large differences in redox potentials are found. It was assumed that ATP synthesis was driven by the energy released between these carriers.

The chemiosmotic theory dictates that electron flow is not responsible for ATP production directly, but rather that oxidative phosphorylation occurs via the proton gradient. Thus, the classical description of ATP production occurring at 3 sites along the respiratory chain, which was based on sufficiently large redox potential differences between carriers, is not appropriate. Although there still is a

tendency to infer that proton translocation should occur at these same sites, this apparently does not occur. Electron flow through the electron transport chain is more dependent upon the overall redox potential difference between substrate and oxygen, than it is upon such differences between individual carriers. Thus, it is believed that proton translocation can theoretically occur anywhere in the electron transport chain, with the energy being provided by this overall redox potential difference (Fox 1982).

Although the analogy is not entirely correct, it is useful to think of the electron transport chain as a siphon. The potential between the two ends determines the amount of energy that can be obtained from the system. As with a siphon, the energy can be used to perform work (proton translocation) at any point along the respiratory chain. Thus, the energy for proton translocation is derived from the overall redox potential difference, with proton translocation itself resulting from the spatial arrangement of carriers within the membrane.

The overall redox potential difference with NADH as substrate is around 1.9 volts per pair of electrons flowing to oxygen. This represents the total amount of energy available from the system. The amount of energy required to transfer a proton from the matrix into the intracristal region varies depending upon the protonmotive force present.

If the protonmotive force across the membrane was near 200 mV, then it would take that much electrical energy to transfer a proton across the membrane. Thus, under these conditions, there would be sufficient energy within the system to transport 9 protons.

The energy that can be derived from a translocated proton would also be equal to the protonmotive force present. So how much energy does it take to synthesize an ATP molecule, and how many protons would be required to provide energy for that process? The amount of energy required to synthesize ATP (including all transport processes) has been determined to be near 660 mV for plant mitochondria (Moore and Bonner 1981). Thus, it would take 3 protons under a transmembrane potential of 220 mV to drive this process. This is consistent with the use of a transmembrane proton potential of 6 protons as presented in the model.

## CONCLUSIONS

What has been presented is an explanation as to how plant mitochondria may function chemiosmotically. As with any model, its purpose is to provide the best explanation possible with our current understanding. It will be useful for experimental design and evaluation, in addition to providing a basis of understanding for researchers not actively involved in this area.

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PROLINE OXIDATION IN CORN MITOCHONDRIA:  
INVOLVEMENT OF NAD, RELATIONSHIP TO ORNITHINE METABOLISM,  
AND SIDEDNESS ON THE INNER MEMBRANE

Thomas E. Elthon and Cecil R. Stewart  
Botany Department  
Iowa State University  
Ames, IA 50011

## ABSTRACT

Proline dependent oxygen uptake in corn mitochondria (*Zea mays* L. B73 x Mo17 or Mo17 x B73) occurs through a proline dehydrogenase (pH optimum around 7.2) bound to the matrix side of the inner mitochondrial membrane. Sidedness was established by determining the sensitivity of substrate dependent ferricyanide reduction to antimycin and FCCP (*p*-trifluoromethoxycarbonyl-cyanide phenylhydrazone). Proline dehydrogenase activity did not involve NAD reduction, and thus electrons and protons from proline enter the respiratory chain directly. P5C ( $\Delta^1$ -pyrroline-5-carboxylate) derived from proline was oxidized by a P5C dehydrogenase (pH optimum approximately 6.4). This enzyme was found to be similar to proline dehydrogenase in that it was bound to the matrix side of the inner membrane and fed electrons and protons directly into the respiratory chain.

Ornithine dependent oxygen uptake was measurable in corn mitochondria and resulted from an ornithine transaminase coupled with a P5C dehydrogenase. These enzymes existed as a complex bound to the matrix side of the inner membrane. P5C formed by ornithine transaminase was utilized directly by the associated P5C dehydrogenase and was not released into solution. Activity of this dehydrogenase involved the reduction of NAD.

## INTRODUCTION

The accumulation of proline in several species of plants is a well-established response to stress. Proline accumulation results primarily from stimulated synthesis and the concomitant inhibition of proline oxidation (Stewart *et al.* 1977). The oxidation of proline occurs within the mitochondria and is more sensitive to inhibition by water stress than is the oxidation of other mitochondrial substrates (Sells and Koeppel 1981). Inhibition of proline oxidation by stress is necessary to cause proline to accumulate, although it alone does not account for observed rates of accumulation. Accumulated proline may serve as a neutral osmoticum and as a reserve of nitrogen. Upon relief of stress, accumulated proline is rapidly oxidized, suggesting a role as an energy reserve (Stewart 1972, Stewart *et al.* 1977).

The enzymes involved with proline oxidation recently have been partially characterized as to their submitochondrial location and electron transport characteristics (Boggess *et al.* 1978, Boggess *et al.* 1975, Elthon and Stewart 1981, Huang and Cavalieri 1979, Stewart and Lai 1974). The first enzyme in this process is proline DH, which catalyzes the conversion of proline to

P5C. This enzyme activity had previously been referred to as proline oxidase activity; however, proline DH is more appropriate (see Conclusions).

The P5C thus formed is oxidized to glutamate by a P5C DH, with this reaction possibly involving the formation of the intermediate GSA. In the present study, we provide evidence for two distinct mitochondrial enzymes capable of oxidizing P5C. One of these P5C DH activities can only be measured in disrupted mitochondria and is involved in the oxidation of ornithine. Previous papers have shown that mitochondria contain ornithine transaminase activity (Bone 1959, Mazelis and Fowden 1969, Taylor and Stewart 1981). In this paper, we have further characterized the enzyme systems involved with the oxidation of proline and ornithine.

## MATERIALS AND METHODS

Corn seedlings (*Zea mays* L. B73 x Mo17 or Mo17 x B73) and mung bean seedlings (*Vigna radiata* L.) were grown in the dark at  $30 \pm 2$  C in moist vermiculite. Mitochondria were isolated from shoots of 3- to 4-day-old seedlings according to Day and Hanson (1977). Protein was estimated by the method of Lowry *et al.* (1951) using BSA (fraction V) as the standard. Assays were conducted at  $26 \pm 2$  C unless otherwise indicated, and were initiated upon addition of substrate. FCCP (3.0 mM), antimycin A (0.15 mM), rotenone (3.0 mM), and SHAM (300 mM) were solubilized in 80% ethanol.

Oxygen uptake. Oxygen utilization was measured at  $26 \pm 1$  C in 3.0 to 3.2 ml of medium using a Clark O<sub>2</sub> electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, OH). ADP:O ratios, RCR, and O<sub>2</sub> content of air-saturated water were determined according to Estabrook (1967).

Enzyme assays. Substrate dependent NAD reduction or NADH oxidation was followed by measuring changes in *A* at 340 nm. Ferricyanide reduction was measured by following decreasing *A* at 420 nm. The reduction of DCIP was followed as decreasing *A* at 600 nm. The following mM extinction coefficients were used:  $E_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  for NADH,  $E_{420} = 1.03 \text{ mM}^{-1} \text{ cm}^{-1}$  for ferricyanide,  $E_{600} = 21.0 \text{ mM}^{-1} \text{ cm}^{-1}$  for DCIP, and  $E_{260} = 15.4 \text{ mM}^{-1} \text{ cm}^{-1}$  for ADP (Elthon and

Stewart 1981).

Preparation and assay of P5C. P5C was synthesized according to the procedure of Williams and Frank, with the P5C concentration determined using ninhydrin (Williams and Frank 1975). It was concentrated by evaporation to dryness under an air stream (to remove the HCl) and redissolved in water. Purity of P5C preparations was evaluated by using partially purified P5C reductase from mung bean hypocotyls (no measurable P5C reductase was present in corn seedlings). Approximately 90% correlation between NADH oxidation and P5C utilization was found. This result corresponds closely with results of Williams and Frank, and indicates a purity of at least 90% after concentration. However, when using relatively high concentrations of P5C (around 10 mM), a contaminant with a maximum concentration of 1 mM is possible. Therefore, it was essential to correlate observed activity with P5C utilization.

P5C utilization was measured by using the ninhydrin technique of Chinard for proline and ornithine determination (Chinard 1952). P5C exhibits an absorption spectrum similar to proline in this assay, while glutamate does not react under the same conditions.

P5C formation was followed by reacting it with 20 mM oAB in 5% TCA/ethanol (w/v). One ml of the oAB solution was

added to 2 ml samples, followed by centrifugation at 30,000g for 10 min. Absorbance at 443 nm of the clear supernatant was determined after 40 min total incubation. The oAB reagent was prepared immediately before use (Williams and Frank 1975).

Isolation of P5C reductase from mung bean hypocotyls.  
Mung bean hypocotyls (50 g) were homogenized with a mortar and pestle in 100 ml of 0.4 M sucrose, 50 mM  $\text{KH}_2\text{PO}_4$ , and 5 mM EGTA (pH 7.6). All operations were conducted at 4 C. The resulting slurry was squeezed through 4 layers of cheesecloth and centrifuged (all centrifugations were at 30,000g for 10 min). The supernatant was fractionated by using ammonium sulfate, with the 40 to 50% pellet containing P5C reductase activity. This pellet was resuspended in 5 ml of 10 mM Tes (pH 7.2) and dialyzed against the same solution for 1 hr. The suspension was then centrifuged, with the supernatant adjusted to 10% ammonium sulfate (to help stabilize the enzyme) and stored at -20 C. This procedure results in a 12-fold purification and a preparation that exhibits no interfering NADH oxidation. The enzyme preparation is relatively stable since 50% of the activity was still present after 8 months storage.

## RESULTS AND DISCUSSION

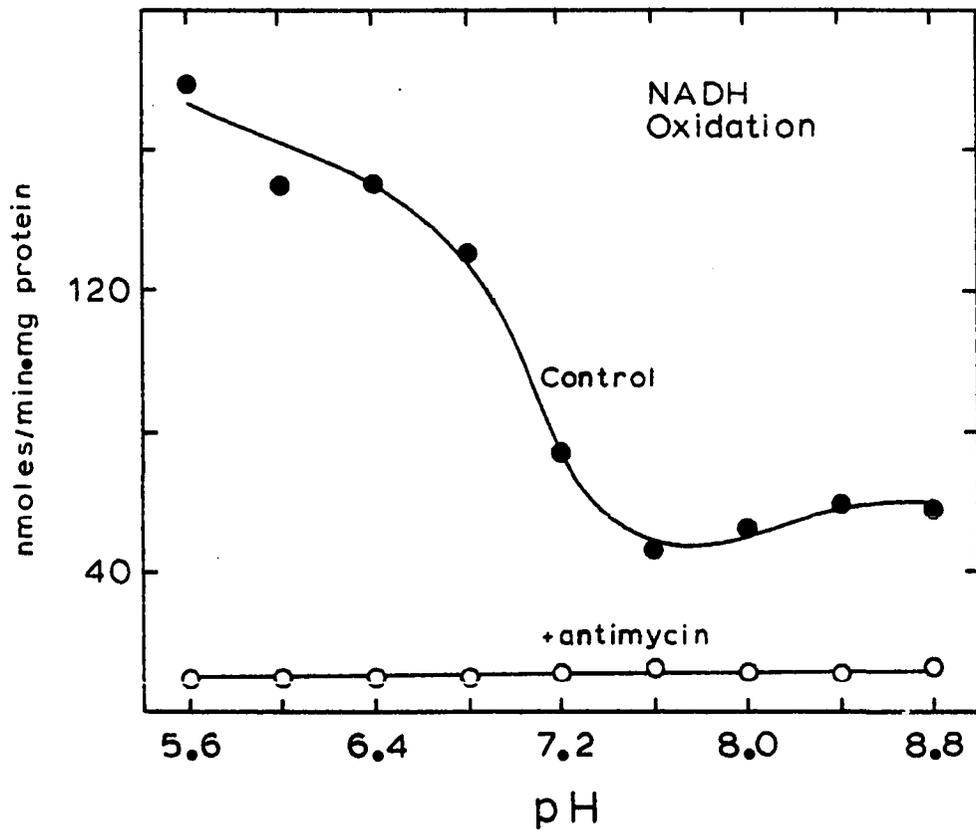
Involvement of NAD in proline and P5C oxidation. To determine if NAD reduction is involved in the activities of proline DH and P5C DH, we suspended mitochondria in a medium of low osmolarity (10 mM each Mes, Tes, and Tricine), which allowed sufficient permeability of substrates into the mitochondrial matrix. In comparison to intact mitochondria (suspended in media with 250 mM sucrose), mitochondria suspended in media without sucrose are referred to as swollen mitochondria. In swollen mitochondria, the outer membrane has ruptured, as indicated by a 6-fold increase in succinate dependent cyt c reduction over that found in intact mitochondria. In contrast, the inner membrane does not rupture since malate DH activity does not increase in the 30,000g supernatant of suspended swollen mitochondria as compared to intact (Elthon and Stewart 1981).

Measurement of NAD reduction within swollen mitochondria, required inhibition of as much of the NADH DH activity as possible. This inhibition was achieved through the addition of 0.5  $\mu$ M antimycin A, which left a residual rate of NADH oxidation of around 10 nmol/min/mg protein over a broad pH range (Figure 1). Higher concentrations of antimycin (1  $\mu$ M) or inclusion of other inhibitors (10  $\mu$ M

**Figure 1. Inhibition of NADH Oxidation by 0.5  $\mu$ M  
Antimycin in Swollen Mitochondria**

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Assays were conducted in 3.0 ml of 30 mM MTT and were initiated upon addition of 100  $\mu$ M NADH. Mitochondrial protein averaged 0.64 mg/assay. Data represent the mean of three separate experiments.



rotenone, 1 mM  $\text{NaN}_3$ , or 1 mM SHAM) resulted in little additional inhibition. This residual NADH oxidation is sometimes attributable to contaminating endoplasmic reticulum in the mitochondrial preparation.

To ensure that we could follow NAD reduction within the matrix of swollen mitochondria, we measured the activities of malate and glutamate DH. Malate dependent NAD reduction was measurable (32.2 nmol/min/mg protein with 10 mM malate) and required the presence of a small amount of glutamate (0.5 mM), presumably for the removal of OAA by transamination. Glutamate dependent NAD reduction was also measurable (7.45 nmol/min/mg protein with 10 mM L-Glu). The rates of NAD reduction obtained underestimate the actual rates by the amount of residual NADH oxidation (Figure 1). We also followed NADH oxidation by these enzymes and found activity with both malate DH (7320 nmol/min/mg protein with 250  $\mu\text{M}$  OAA) and glutamate DH (19.9 nmol/min/mg protein with 10 mM  $\alpha$ -KG and 5 mM  $\text{NH}_4\text{Cl}$ ). NADH oxidation by a particular enzyme was measured as an increase in oxidation over the residual rate, and thus represents the actual rate. Therefore, by using swollen mitochondria, it was possible to measure both NAD reduction and NADH oxidation by enzymes within the matrix.

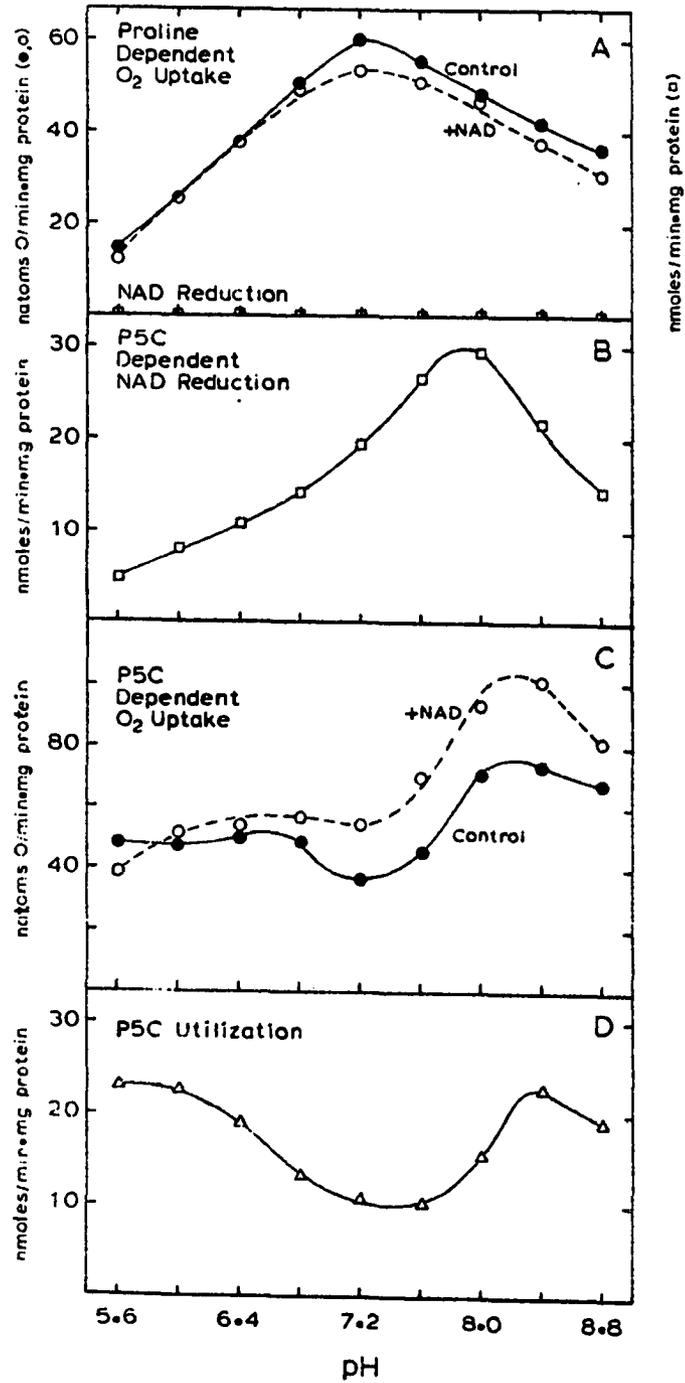
If NAD reduction is involved in the activity of proline

substrate dependent oxygen uptake and NAD reduction. We followed these activities over a broad pH range in swollen mitochondria (Figure 2). Proline dependent  $O_2$  uptake (Figure 2A) was found to exhibit a pH optimum of 7.2 as previously reported for intact mitochondria (Elthon and Stewart 1981). This activity was not stimulated by NAD, and NAD reduction could not be measured under the same conditions. In addition, no PSC dependent NADH oxidation (reversal of proline dependent NAD reduction) could be measured. This result clearly establishes that proline DH activity does not involve the reduction of NAD, and thus electrons and protons are fed directly into the respiratory chain. Indications that proline DH is flavin-linked are consistent with this result (Huang and Cavalieri 1979). In corn mitochondria, we could find no stimulation of  $O_2$  uptake upon addition of 0.5 mM FAD, although we did observe some stimulation (26%) with 0.5 mM FMN. Proline DH activity measured as DCIP reduction in swollen mitochondria (60  $\mu$ M DCIP with 0.5  $\mu$ M antimycin) also exhibited a pH optimum of 7.2 in either the presence or absence of 1 mM PMS. The rates in the presence of PMS were decreased considerably. Proline dependent  $O_2$  uptake was stimulated by  $MgCl_2$  (40% with 5 mM) as previously reported (Huang and Cavalieri 1979).

Figure 2. Effects of pH on the Oxidation of Proline and P5C in Swollen Mitochondria

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NAD reduction was assayed in 3.0 ml of 30 mM MTT containing 2 mM NAD, 0.5  $\mu$ M antimycin, and either 10 mM L-proline or 6.5 mM DL-P5C. Oxygen uptake was followed in 3.0 ml of 30 mM MTT containing 10 mM L-proline or 13 mM DL-P5C.  $O_2$  uptake was followed in the presence and absence of 2 mM NAD. For the measurement of P5C utilization, mitochondria were incubated at  $25 \pm 1$  C for 30 min in 2.5 ml of 30 mM MTT containing 1.44 mM DL-P5C (neutralized HCl solution). The reaction was terminated by addition of 2.5 ml of glacial acetic acid. P5C content was determined by using ninhydrin (Chinard 1952). Mitochondrial protein averaged 1.38 mg/assay for A, 0.67 mg/assay for B, 1.36 mg/assay for C, and 1.44 mg/assay for D. All data represent the mean of three separate experiments.



When P5C dependent NAD reduction was measured (Figure 2B), an optimum of around pH 8 was observed. This is similar to that previously reported for crude mitochondrial isolations from several tissues (Stewart and Lai 1974) and to that obtained from detergent solubilized mitochondria (Boggess *et al.* 1975), although the rates are considerably higher than those previously reported. However, it is markedly different from the optimum of pH 6.1 for P5C dependent  $O_2$  uptake in intact mitochondria (Elthon and Stewart 1981). Therefore, P5C dependent  $O_2$  uptake was measured in swollen mitochondria (Figure 2C). Two optima were observed, one near pH 6.4 that was not stimulated by NAD, and another around pH 8 that was. When P5C utilization was measured (Figure 2D), two corresponding activities were obtained. Therefore, under these conditions, two enzymes are involved in the oxidation of P5C. P5C dependent  $O_2$  uptake at pH 6.4 was not stimulated by 0.5 mM FMN, and was inhibited somewhat (27%) by 0.5 mM FAD. The pH 6.4 activity was stimulated 17% by 5 mM  $MgCl_2$ . P5C and  $MgCl_2$  (as low as 1 mM) were found to react above pH 7.2, with visible precipitation occurring around pH 8. This reaction explains  $MgCl_2$  inhibition of P5C DH observed by Boggess *et al.* (1975).

Reversal of P5C dependent NAD reduction (glutamate dependent NADH oxidation) was not measurable over the pH

range. The inability to measure such activity suggests that the pH 8-P5C DH may not be significantly reversible. However, this could occur as a result of an equilibrium greatly toward glutamate formation (analogous to malate DH). Thus, reversibility possibly could be measured, if the P5C is removed as it is formed. All attempts to demonstrate that this occurs have failed. These include trying to trap the P5C with 1 mM oAB, and attempts to remove the P5C using partially purified P5C reductase from mung bean hypocotyls. Reversibility using added P5C reductase was tried with intact mitochondria and with mitochondria solubilized in 0.030% Triton X-100 (Elthon and Stewart 1981). In each, considerable P5C DH activity (measured as NAD reduction in the mitochondria) and considerable P5C reductase activity (P5C dependent NADH oxidation) were present in the reaction medium. No reversibility of P5C DH could be measured (either as glutamate dependent NADH oxidation or  $^{14}\text{C}$ -proline production from  $^{14}\text{C}$ -glutamate), indicating that the failure to measure reversibility of P5C DH under these conditions is not merely a result of a simple equilibrium reaction. It was also possible that additional cofactors may be necessary for reversal. Inclusion of  $\text{MgCl}_2$  and ATP (1 mM each) was tried since these are known to be involved in glutamate to P5C conversion during proline synthesis (Morris *et al.*

1969), however no reversibility was observed.

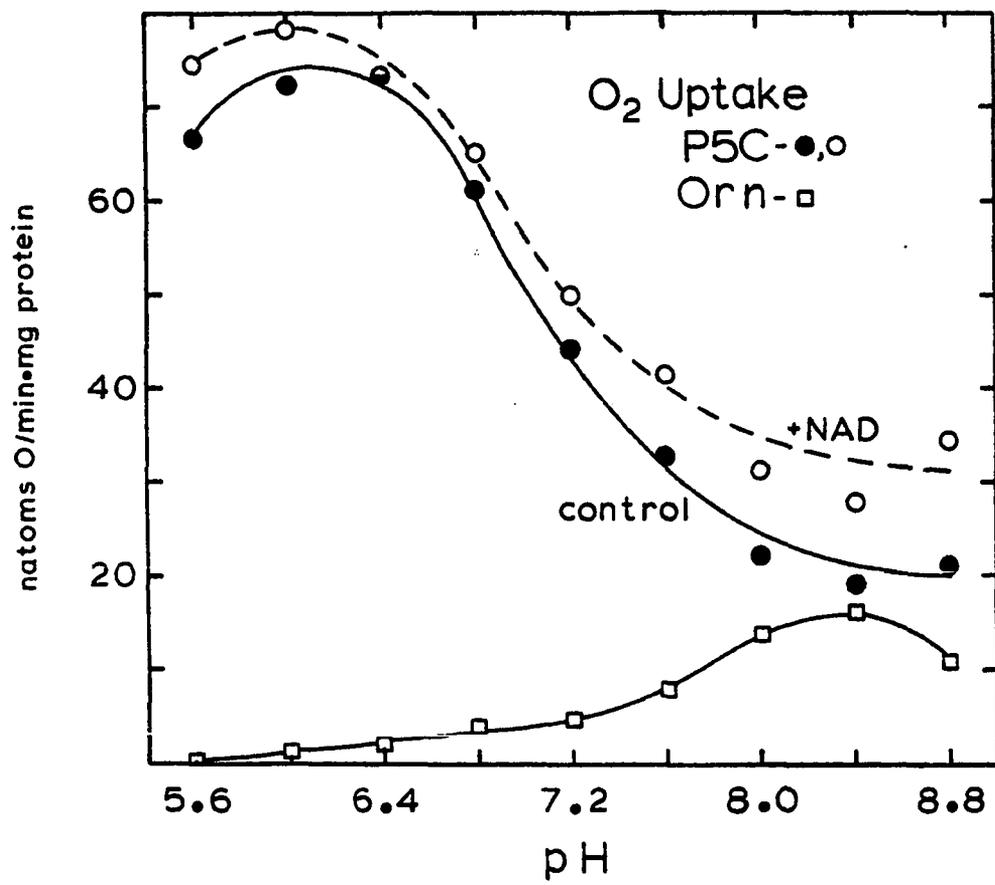
Ornithine dependent  $O_2$  uptake and P5C DH activities.  
P5C dependent NAD reduction cannot be measured in intact mitochondria (Bogges *et al.*, 1975, Elthon and Stewart 1981). Consistent with this, the associated P5C dependent  $O_2$  uptake activity (optimum near pH 8) is not measurable (Figure 3). A possible explanation of this, is that P5C, NAD, or both, are not reaching the enzyme active site in intact mitochondria around pH 8. Upon swelling of the mitochondria, this inaccessibility is relieved. Inaccessibility could result from P5C not being able to penetrate the inner membrane (assuming the active site is exposed to the matrix), or from this enzyme being part of an enzyme complex. Since P5C dependent  $O_2$  uptake can be measured due to the other P5C DH (compare Figures 2C and 3), P5C evidently can penetrate the membrane (refer to the section on sidedness).

If the pH 8 P5C-DH is part of an enzyme complex, this complex would be involved in the degradation of proline or ornithine, since these two processes are the only such degradative processes known to involve P5C as an intermediate. Since ornithine transaminase activity is known to be present within the mitochondria (Bone 1959, Mazelis and Fowden 1969, Taylor and Stewart 1981), we attempted to measure ornithine dependent  $O_2$  uptake in intact

**Figure 3. Effects of pH on the Oxidation of PSC and Ornithine in Intact Mitochondria**

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Oxygen uptake was followed in 3.0 ml of 250 mM sucrose, 5 mM  $\text{KH}_2\text{PO}_4$ , and 30 mM MTT containing 10 mM DL-PSC (or 10 mM L-ornithine in the presence of 10 mM  $\alpha$ -KG). PSC dependent  $\text{O}_2$  uptake was measured as an increase in the rate above that with  $\alpha$ -KG. Mitochondrial protein averaged 1.55 mg/assay. Data represent the mean of three separate experiments.



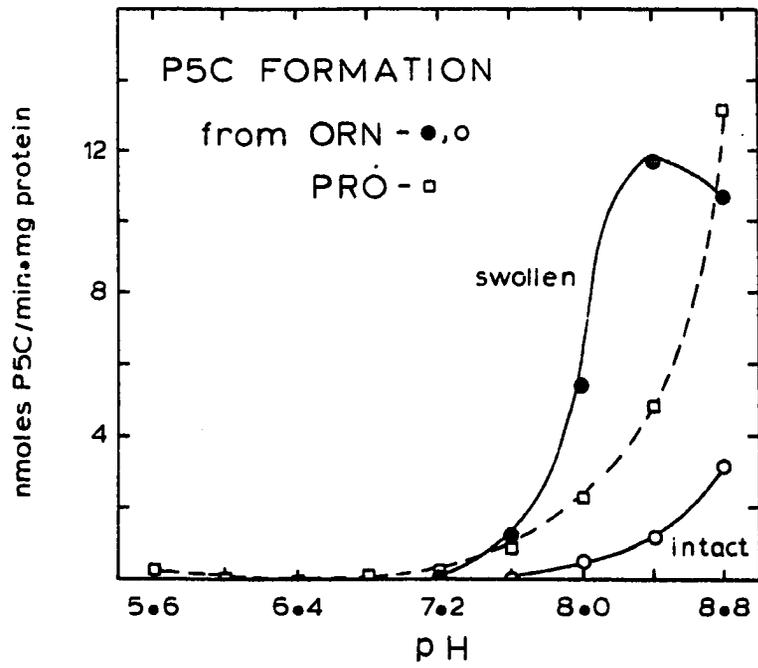
mitochondria. Activity was present and had a pH optimum of around 8.4 (Figure 3). The presence of  $\alpha$ -KG was essential for measurement of ornithine dependent  $O_2$  uptake, presumably as an amino acceptor for ornithine transaminase activity. Ornithine dependent  $O_2$  uptake decreases upon mitochondrial swelling (by 60%), correlating with the appearance of measurable P5C dependent NAD reduction (and the associated P5C dependent  $O_2$  uptake). These results suggest that these enzymes are complexed.

Further evidence to support the association of ornithine transaminase with the pH 8-P5C DH was obtained by measuring both proline and ornithine dependent P5C production (Figure 4). Proline dependent P5C formation in intact mitochondria was measurable only above pH 7.6. Thus, P5C derived from proline is utilized by the pH 6.4-P5C DH, while the pH 8-P5C DH activity (only measurable in swollen mitochondria) is not involved. This pH response explains the stoichiometric production of P5C from proline at pH 8.5, as measured by Huang and Cavalieri (1979). In addition, those experiments were conducted in the presence of  $MgCl_2$  which effectively removes P5C from solution at that pH. Earlier attempts to demonstrate reasonable P5C production from proline failed since the mitochondria were incubated at pH 7.6 (Boggess *et al.* 1978).

Figure 4. P5C Formation from Proline and Ornithine

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Assays were conducted in 2.0 ml of 30 mM MTT and 5 mM  $\text{KH}_2\text{PO}_4$  ( $\pm$  250 mM sucrose). Ornithine dependent P5C formation was followed by using 10 mM L-ornithine and 10 mM  $\alpha$ -KG in the presence of 0.5  $\mu\text{M}$  antimycin. Proline dependent P5C formation was determined in the presence of sucrose, using 10 mM L-proline. Samples were incubated with substrate for 30 min at  $26 \pm 1$  C. The reaction was terminated by addition of the oAB solution. Mitochondrial protein averaged 0.42 mg/assay. Data represent the mean of three experiments.



When P5C production from ornithine was followed with intact mitochondria (in the presence of antimycin), very little P5C formation was observed. However, considerable P5C formation occurred when this same activity was followed in swollen mitochondria. This activity (ornithine transaminase) had a pH optimum of 8.4. Thus, P5C derived from ornithine is normally not released into solution *in vivo*, but is utilized directly by the pH 8-P5C DH. Consistent with this, P5C in solution does not have access to the active site of this enzyme in intact mitochondria. Upon swelling the mitochondria and disrupting the complex, P5C derived from ornithine is released into solution (thus measurable), and P5C in solution gains access to the active site of this enzyme, resulting in measurable P5C dependent NAD reduction and the associated P5C dependent O<sub>2</sub> uptake. Thus, it is clear that ornithine transaminase and the pH 8-P5C DH are complexed. This P5C DH is associated with the inner mitochondrial membrane (Elthon and Stewart 1981).

Ornithine dependent O<sub>2</sub> uptake in corn mitochondria has a very low specific activity. This activity is not significantly stimulated by exogenous NAD or higher concentrations of ornithine (in intact or swollen mitochondria). In addition, ornithine dependent NAD and ferricyanide reduction cannot be measured. Thus, to characterize ornithine dependent O<sub>2</sub> uptake further, a tissue

with higher activity must be used. Arginine dependent  $O_2$  uptake (10 mM L-arginine and 10 mM  $\alpha$ -KG) was also measurable in intact mitochondria with a rate of around 8 natoms  $O_2$ /min/mg protein at pH 8. This activity was measured as an increase in the rate above that with  $\alpha$ -KG.

Inhibitor sensitivities and measurable ADP:O ratios.

The oxidation of proline is known to be sensitive to inhibition by rotenone, antimycin, and azide (Elthon and Stewart 1981). Thus, electrons and protons from proline enter the respiratory chain before the rotenone sensitive iron-sulfur proteins. Further, proline oxidation has a measurable ADP:O ratio similar to that of malate + pyruvate (Boggess *et al.* 1978, Stewart and Elthon 1981). The ADP:O ratio and inhibitor sensitivities previously published for P5C oxidation are similar to those for proline oxidation and are for the pH 6.4-P5C DH activity (Elthon and Stewart 1981). Since the pH 8-P5C DH activity is not measurable in intact mitochondria, ADP:O ratios cannot be determined. With ornithine, ADP:O ratios could not be accurately determined because of low rates of ornithine dependent  $O_2$  uptake and interference from  $\alpha$ -KG (which itself exhibits an ADP:O ratio).

Inhibitor sensitivities of ornithine oxidation could not be determined due to low specific activity in corn

mitochondria. When substrate oxidation occurs at a rate considerably below the potential capacity of the electron transport chain, inhibitor studies are not relevant because electron flow occurs primarily through the remaining uninhibited capacity of the system. A similar response can be obtained with substrates that are oxidized with a high specific activity if the rates are determined under adverse conditions such as insufficient cofactor availability or measurement at pHs considerably different than the optimum.

P5C dependent  $O_2$  uptake (near pH 8 in swollen mitochondria) results primarily from NADH oxidation since glutamate oxidation is too slow under similar conditions. Thus, a comparison was made between the inhibitor sensitivities of exogenously added NADH and the oxidation of P5C (Table I). As can be seen, the oxidation of P5C was very sensitive to rotenone and SHAM in comparison with the oxidation of exogenously added NADH. Thus, electrons and protons from NADH produced by this P5C DH enter the respiratory chain through the endogenous NADH DH. In corn mitochondria, we consistently find that the oxidation of substrates having rotenone sensitivity show similar sensitivity to SHAM. This includes the oxidation of malate, proline, and P5C (pH 6.4).

Sidedness on the inner mitochondrial membrane. Since proline DH and the associated pH 6.4-P5C DH feed electrons

Table I. Comparison of NADH and P5C Dependent O<sub>2</sub> Uptake in Swollen Mitochondria at pH 8

Assays were conducted in 3.0 ml of 5 mM KH<sub>2</sub>PO<sub>4</sub> and 30 mM MTT (pH 8). Mitochondrial protein averaged 1.47 mg/assay. Data represent the mean of three experiments.

	Average	10 μM	0.5 μM	1 mM	1 mM
Substrate	Control	Rate-	Anti-	NaN <sub>3</sub>	SHAM
	Rate	none	mycin		
	atoms O/min/mg protein				% inhibition
10 mM DL-P5C	109	76	75	19	78
0.5 mM NADH	239	4	85	33	+22

and protons directly into the respiratory chain, we wanted to determine their sidedness on the inner mitochondrial membrane. To do this, we followed the sensitivity of substrate dependent ferricyanide reduction to antimycin inhibition. Ferricyanide is an electron acceptor that cannot permeate the inner mitochondrial membrane. Thus, it can only accept electrons from enzymes exposed to the intermembrane region or from cyt c which is located within the outer phase of the inner membrane (Douce *et al.*, 1973). Electrons that would normally flow to  $O_2$  were shunted to ferricyanide by blocking cytochrome oxidase with 1 mM  $NaN_3$  (P5C interferes with KCN inhibition (Elthon and Stewart 1981)). With enzymes exposed to the intermembrane region, ferricyanide reduction would be relatively insensitive to antimycin inhibition since these enzymes can reduce ferricyanide directly. Antimycin inhibits electron flow through the respiratory chain before cyt c reduction. However, with enzymes exposed to the matrix, such as succinate and malate DH, ferricyanide reduction is sensitive to antimycin inhibition since this reduction can occur only by electron flow through cyt c.

Therefore, the sidedness of an enzyme can be indicated by the amount of substrate dependent ferricyanide reduction that is sensitive to antimycin inhibition (Table II). As with the oxidation of malate or succinate, we found that

Table II. Sidedness of Various Enzyme Active Sites on the Inner Mitochondrial Membrane

Assays were conducted in 3.0 ml of 30 mM MTT (pH 8) containing 250 mM sucrose, 5 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{NaN}_3$ , and 0.7 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ . Mitochondrial protein averaged 0.60 mg/assay. The antimycin and FCCP data are the means of three separate experiments.

<u>Ferricyanide Reduction</u>			
	Average	0.5 $\mu\text{M}$	10 $\mu\text{M}$
Substrate	Control	antimycin	FCCP
	Rate	sensitive	sensitive
nmol/min/mg protein			
10 mM Malate +			
0.5 mM Glu	133	81.0	80.7
10 mM Succinate	249	189	159
10 mM L-Proline	159	151	80.9
10 mM DL-P5C	350	122	107
(pH 6.4)			

proline and P5C dependent ferricyanide reduction showed considerable sensitivity to antimycin. This indicates that the active sites of proline DH and the pH 6.4-P5C DH are exposed to the matrix. If percent inhibition is compared between substrates, P5C dependent ferricyanide reduction appears to be relatively insensitive to antimycin. This does not result from less inhibition by antimycin, but from a high control rate.

The use of this technique assumes that the enzyme is capable of reacting directly with ferricyanide and that antimycin only inhibits electron transport. This assumption is usually verified by disrupting the mitochondria to relieve the antimycin inhibition. We found that swelling the mitochondria (in the absence of antimycin) increased the rate of malate oxidation, but decreased the rates for the other substrates in Table II. When following the relief of antimycin inhibition by swelling, we observed increased rates of antimycin insensitive oxidation for all the substrates. However, since it was not possible to distinguish between rate changes resulting from swelling and those due to relief of antimycin inhibition, these data were not included. Instead, an independent technique using uncouplers was used to help establish the sidedness of these enzymes.

If a substrate is oxidized on the matrix side of the

inner membrane, and if transport of that substrate requires the energy of a proton gradient, the substrate oxidation will be sensitive to FCCP (or other uncouplers). However, if the substrate is oxidized on the outer surface of the inner membrane (or if a proton gradient is not required for uptake), the addition of FCCP will have little effect (Wiskich 1977). As presented in Table II, the results with FCCP correlate with those obtained using antimycin. Both proline and P5C dependent ferricyanide reduction showed considerable sensitivity to FCCP. The percent inhibition with P5C again was influenced by the high control rate, and not by lack of inhibition by FCCP.

Ferricyanide reduction was not measurable in intact corn mitochondria with ornithine or P5C (pH 8) as substrates. Thus, these techniques could not be used to determine the sidedness of these enzymes.

## CONCLUSIONS

The enzyme previously referred to as proline oxidase is bound to the inner mitochondrial membrane and is exposed to the matrix. Electrons and protons from proline enter the respiratory chain before the rotenone sensitive iron-sulfur proteins, with proline dependent  $O_2$  uptake thus having an ADP:O ratio similar to that of malate + pyruvate. Proline dependent  $O_2$  uptake has a pH optimum of 7.2. Activity does not involve the reduction of soluble NAD, and therefore electrons and protons enter the respiratory chain directly, possibly through a flavoprotein. It is clear that proline dependent  $O_2$  uptake occurs through a proline dehydrogenase functionally linked to the respiratory chain, and not through a proline oxidase. Therefore, this activity should be referred to as proline dehydrogenase activity (Enzyme Nomenclature 1979).

P5C derived from proline oxidation is oxidized via a P5C DH that has a pH optimum of 6.4. This P5C DH feeds electrons and protons directly into the respiratory chain and thus is bound to the inner mitochondrial membrane. Its active site is exposed to the matrix. This activity has an ADP:O ratio and inhibitor sensitivities similar to those of proline oxidation.

Ornithine dependent  $O_2$  uptake is measurable in corn

mitochondria and occurs through an enzyme complex consisting of ornithine transaminase (pH optimum 8.4) and the pH 8-P5C DH. This complex is facing the matrix because NADH produced by this P5C DH is preferentially oxidized by the endogenous NADH DH.

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INHIBITION OF PROLINE OXIDATION IN BARLEY BY THE PROLINE  
ANALOG L-THIAZOLIDINE-4-CARBOXYLIC ACID

Thomas E. Elthon and Cecil R. Stewart  
Botany Department  
Iowa State University  
Ames, IA 50011

## ABSTRACT

The effect of various proline analogs on proline oxidation in mitochondria isolated from etiolated barley (*Hordeum vulgare*) shoots was investigated. Of the analogs tested, only T4C (L-thiazolidine-4-carboxylic acid) was an effective inhibitor. T4C (1 mM) inhibited proline (10 mM) dependent  $O_2$  uptake an average of 67%. T4C was also oxidized to some degree (12.9 natoms O/min/mg protein). The effect of T4C on the oxidation of other mitochondrial substrates was also tested. T4C inhibited P5C ( $\Delta^1$ -pyrrolidine-5-carboxylic acid) dependent oxygen uptake slightly (13%), the oxidation of malate + pyruvate even less (6%), and stimulated the oxidation of succinate (+11%), exogenous NADH (+19%), and citrate (+20%). Thus, inhibition by T4C in mitochondria is relatively specific to proline oxidation.

The effect of T4C on proline metabolism in detached green barley leaves was also investigated. T4C inhibited proline oxidation in turgid leaves, increasing the proline content of these leaves slightly. In wilted leaves (that are synthesizing proline rapidly), T4C inhibited proline synthesis, which resulted in a decrease in the proline content of the leaves. T4C had no influence on the incorporation of proline into protein.

## INTRODUCTION

The oxidation of proline to glutamate occurs within mitochondria (Boggess *et al.* 1978, Elthon and Stewart 1981, 1982), and is believed to provide energy and carbon skeletons for a number of processes. During water stress, inhibition of proline oxidation and stimulation of proline synthesis cause proline to accumulate (Boggess *et al.* 1976b, Stewart *et al.* 1977). Accumulated proline may lend increased survivability to stressed plants since it is a cellular osmolyte that may alleviate the effect of the stress (Munns *et al.* 1979, Paleg *et al.* 1981) and because it may serve as an energy supply for use after the stress is relieved (Stewart 1972). Proline may also provide energy for other processes such as pollen germination (Pálfi and Pálfi 1982).

The enzymes that oxidize proline (proline and P5C dehydrogenases) are bound to the matrix side of the inner mitochondrial membrane where they are associated with the first portion of the electron transport chain (Elthon and Stewart 1981, 1982). Therefore, proline must penetrate the inner mitochondrial membrane for oxidation to occur. The fact that proline uptake into the matrix is stereospecific (Cavalieri and Huang 1980), reversibly sensitive to sulfhydryl reagents (Cavalieri and Huang 1980), and

sensitive to uncouplers (Elthon and Stewart 1982) suggests that proline uptake may be a carrier-mediated energy-dependent process.

The oxidation of proline is more sensitive to water stress than is the oxidation of other mitochondrial substrates (Sells and Koeppel 1981). Since proline oxidation can be envisioned as being regulated at either the transport or dehydrogenase level, a proline oxidation inhibitor would be very useful in investigating the integration and regulation of these two mitochondrial processes. Such an inhibitor would also be useful in further investigating the relationships that lead to proline accumulation during environmental stress. An inhibitor of proline oxidation would also be useful for assessing the role of proline oxidation in providing energy and carbon skeletons for recovery after stress, and for processes like pollen germination.

In this paper, we present evidence that T4C (L-thiazolidine-4-carboxylic acid) is a relatively specific inhibitor of proline oxidation in barley mitochondria. We have also looked at T4C's effect on proline levels and on proline metabolism in turgid and wilted barley leaves.

## MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare* var. Larker) and corn seedlings (*Zea mays* L. Mo17 x B73) were grown in the dark at  $30 \pm 2$  C in moist vermiculite. Mitochondria were isolated from shoots of 3- to 4-day old seedlings using a modification of the procedure of Day and Hanson (1977). Insoluble PVP (5%) was included in the grinding medium unless otherwise indicated, and the ratio of grinding medium to tissue (v/w) was increased to 4:1. Protein was estimated by the method of Lowry *et al.* (1951), using BSA (fraction V) as the standard. Assays were conducted at  $26 \pm 2$  C unless otherwise indicated and were initiated upon addition of substrate.

Oxygen uptake and mitochondrial swelling. These processes were measured simultaneously using an oxygen electrode (model 53, Yellow Springs Instrument Co.) mounted in the light path of a Cary 210 spectrophotometer (Varian Instruments). The spectrophotometer was used in the single beam mode, with a period of 10 seconds and a band width of 0.25 nm. A full scale of 0.1 relative  $A_{520}$  was obtained by adjusting the gain. The  $O_2$  electrode was mounted in the top of the magnetically stirred and temperature controlled 3 ml cuvette. The cuvette was placed within 1.5 cm of the detector window. Uptake of substances into the

mitochondrial matrix results in mitochondrial swelling and a decrease in relative  $A_{520}$ .

$O_2$  utilization within this cuvette was measured at  $26 \pm 1$  C in 2.2 to 2.5 ml of standard reaction medium (SRM) consisting of 250 mM sucrose, 30 mM MTT (10 mM each Mes, Tes, Tricine), 1 mM  $KH_2PO_4$ , 1 mM  $MgCl_2$ , and 1 mg/ml BSA (pH 7.2). ADP:O ratios, RCR, and  $O_2$  content of air-saturated water were determined according to Estabrook (1967). The following mM extinction coefficients were used:  $E_{340} = 6.22$   $mm^{-1} cm^{-1}$  for NADH, and  $E_{260} = 15.4$   $mm^{-1} cm^{-1}$  for ADP (Elthon and Stewart 1982).

Isolation and assay of P5C reductase from etiolated barley shoots. P5C reductase was isolated from barley using a modification of our procedure for etiolated mung bean hypocotyls (Elthon and Stewart 1982). The grinding medium used for this isolation, was the same as that used for mitochondrial isolation from barley. Barley P5C reductase was found to precipitate at a lower ammonium sulfate concentration (<40%) than that of mung bean (40 to 50%). P5C reductase activity from barley was only measurable after ammonium sulfate fractionation, indicating the presence of inhibitors within the crude cytoplasmic supernatant. P5C was synthesized and assayed as previously reported (Elthon and Stewart 1982).

Experiments with barley leaves. Barley plants were grown in soil in a growth chamber under a 16 h photoperiod ( $500 \mu\text{E m}^{-2} \text{sec}^{-2}$ ) at 21 C. After emergence, plants were watered daily with modified Hoagland solution (Johnson *et al.* 1957). Fully expanded second leaves from 2-week-old barley plants were excised at the base of the leaf blade. Prior to excision, plants were removed from the growth chamber to room light ( $11 \mu\text{E m}^{-2} \text{sec}^{-1}$ ) to reduce transpiration rates. Leaves were weighed, then placed individually in 1 x 75 cm vials with the cut end of the leaf in 1 ml of solution. The control leaves were placed in 50 mM sucrose and 1 mM glutamate. T4C treated leaves were placed in the same solution with 5 mM T4C. Leaves were allowed to take up the solution under room light.

Radioactive precursors were added to the cut end of the leaf in 5  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . Details of amount added, specific radioactivity, and length of pretreatment are given in the figure legends. Procedures for extraction, chromatography, counting, and proline determination were as previously described (Buhl and Stewart 1983).

## RESULTS AND DISCUSSION

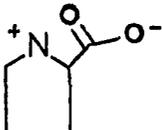
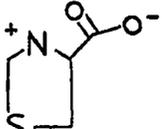
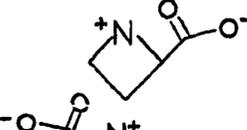
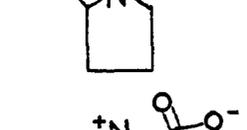
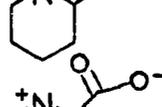
Mitochondrial experiments. Isolation of reasonably active mitochondria from etiolated barley shoots required the addition of insoluble PVP. Without addition of PVP, the oxidation of proline and other mitochondrial substrates did not show good respiratory control. An additional increase in mitochondrial quality was obtained by increasing the volume of the grinding medium used per gram of tissue (from 2:1 to 4:1).

When etiolated barley shoots are homogenized, the resulting slurry has a yellow color. This color is removed from solution to some extent by PVP. Our experience with the isolation of P5C reductase from this tissue, has shown that proteins are inactivated by complexing with this yellow substance. Reasonably active mitochondria can be isolated in the absence of PVP, only if very young tissue is used (shoots <1 cm in length).

The effect of a number of proline analogs on proline dependent  $O_2$  uptake was investigated (Table I). Assays were conducted under State 3 conditions (in the presence of ADP) to maximize substrate oxidation and the resulting flow of electrons through the electron transport chain. Under these optimum conditions, the various analogs would have their greatest influence on the oxidation rate. Of those tested,

**Table I. Effect of Various Proline Analogs on Proline  
Dependent Oxygen Uptake**

Assays were conducted in 2.2 to 2.5 ml of SRM, containing 2.5 mM ADP. Mitochondrial protein averaged 0.93 mg/assay. Average control rate with 10 mM proline was 61.4 natoms O/min mg protein. Inhibitors were used at 1 mM concentrations. Results are the mean of 3 separate experiments.

L-PRO	Analog	Inhibition
		%
		
	T4C	67
	PM	12
	A2C	8
	D-PRO	7
	PIP	5
	4-OH PRO	+2
	3,4-DEHYDRO PRO	+8

only T4C (L-thiazolidine-4-carboxylic acid) was particularly effective. T4C inhibited proline dependent  $O_2$  uptake an average of 67% when used at a concentration of 1 mM. Other experiments have shown that a concentration of only 0.2 mM results in over 50% inhibition. Addition of PM (2-pyrrolidine methanol), A2C (L-azetidine-2-carboxylic acid), D-PRO (D-proline), or PIP (L-pipecolic acid) resulted in little inhibition. Addition of 4-hydroxy-L-proline or 3,4-dehydro-DL-proline resulted in a stimulation of the  $O_2$  uptake rate.

Because of the stimulation of  $O_2$  uptake (by 3,4-dehydroproline in particular), we wanted to determine if any of these analogs would support  $O_2$  uptake themselves (Table II). Using analog concentrations of 10 mM, 3 were found to support  $O_2$  uptake. Oxidation of 10 mM 3,4-dehydro-DL-proline occurred at the same rate as oxidation of 10 mM L-proline. T4C inhibits the oxidation of 3,4-dehydroproline as well as it does that of proline. Surprisingly, T4C was found to support a reasonable rate of  $O_2$  uptake. Thus, its inhibitory effect on proline oxidation, may be even greater than indicated in Table I. A low rate of  $O_2$  uptake was also obtained when 4-hydroxy-L-proline was added. PM, A2C, D-PRO, and PIP did not support any measurable  $O_2$  uptake.

By following mitochondrial swelling at the same time as measuring  $O_2$  uptake, we were able to roughly correlate the

Table II. Oxygen Uptake and Mitochondrial Swelling by  
Various Proline Analogs

Assays were conducted in 2.2 to 2.5 ml of SRM containing 2.5 mM ADP. Mitochondrial protein was near 1.0 mg/assay. Substrates were used at 10 mM concentrations.

Substrate	Oxygen Uptake	Swelling
	atoms O/min·mg protein	
L-PRO	67.0	+++++++
3,4-DEHYDRO PRO	68.0	+++++++
T4C	12.9	++
4-OH PRO	6.47	+
PM	0.00	
A2C	0.00	
D-PRO	0.00	
PIP	0.00	

amount of substrate dependent  $O_2$  uptake to the degree of swelling. Since swelling indicates the entry of these substrates into the mitochondrial matrix (where oxidation via proline DH occurs), it is evident that the degree of oxidation of these substrates could depend upon their transport or permeability rate in addition to their degree of reactivity with the DH.

This result raises the possibility that T4C could inhibit proline dependent  $O_2$  uptake either by reducing the transport or permeability rate, or by interacting with the DH. When T4C is added to mitochondria oxidizing proline, a rapid reduction in the  $O_2$  uptake rate is observed. No sufficient correlating decrease in  $A_{520}$  is found. If transport was inhibited, the mitochondria would shrink due to depletion of the substrate within the matrix. This depletion of substrate would effect the inhibition of  $O_2$  uptake. Thus, the kinetics of T4C inhibition suggest that T4C is a DH inhibitor.

If T4C is to be useful in investigating mitochondrial proline oxidation, it must be relatively specific. To check this, we determined the effect of T4C on the oxidation of various mitochondrial substrates (Table III). P5C dependent  $O_2$  uptake was inhibited slightly, and the oxidation of malate + pyruvate to an even lesser degree. Succinate, exogenous NADH, and citrate dependent  $O_2$  uptake were all

Table III. Effect of T4C on the Oxidation of Various Mitochondrial Substrates

Assays were conducted in 2.2 to 2.5 ml of SRM containing 2.5 mM ADP. Mitochondrial protein averaged 0.94 mg/assay. The T4C concentration used was 1 mM. Results are the mean of three separate experiments.

Substrate	Control Rate natoms O/min·mg protein	Inhibition %
10 mM L-proline	51.1	69
10 mM DL-PSC	39.9	13
10 mM L-malate + 10 mM pyruvate	118	6
10 mM succinate	147	+11
1 mM NADH	138	+19
10 mM citrate	43.1	+20

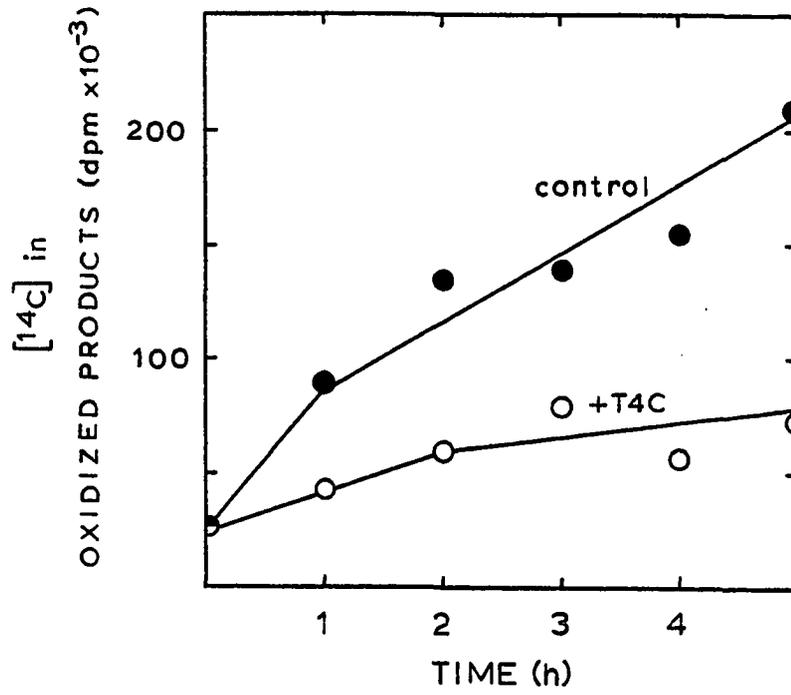
stimulated slightly by T4C. Thus, T4C's inhibitory affect is fairly specific (at least for the substrates tested).

Experiments with green leaves. We then investigated the effect of T4C on proline metabolism in green leaf tissue. The effect of T4C on proline oxidation in excised barley leaves is shown in Figure 1. The appearance of  $^{14}\text{C}$  into oxidized products (Stewart *et al.* 1977) of proline is markedly inhibited by T4C. The specific radioactivity of proline throughout the experiment was similar in the presence and absence of T4C. Thus, the slopes of the lines can be compared directly to estimate the relative oxidation rates of the two treatments. The calculated rates, using the slopes and the specific radioactivity (Stewart *et al.* 1977), are 0.3 and 0.09  $\mu\text{mol/h/g}$  fresh wt for the control and T4C treated leaves respectively. These values indicate an approximate 70 percent inhibition of proline oxidation by T4C. However, since the proline levels were somewhat higher in the T4C treated leaves compared to the controls (2.5 vs. 2.0  $\mu\text{mol/g}$  fresh wt), the degree of inhibition would be greater (Stewart *et al.* 1977). Thus, T4C is as effective an inhibitor of proline oxidation in intact leaves as it is in isolated mitochondria. The rate of 0.3  $\mu\text{mol/h/g}$  fresh wt for proline oxidation is higher than values previously reported for barley leaves, because of the higher proline levels present.

**Figure 1. Radioactivity (from  $^{14}\text{C}$ -proline) Recovered  
in Oxidized Products in Control and T4C Treated Turgid  
Leaves**

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After excision, control leaves were placed in 50 mM sucrose and 1 mM glutamate. T4C treated leaves were placed in the same solution containing 5 mM T4C. After a 10 h incubation period, 5  $\mu\text{l}$  of U- $^{14}\text{C}$ -L-proline (600,000 dpm,  $1.2 \times 10^6$  dpm/ $\mu\text{mol}$ ) was added to each leaf (0 time). Each value is the mean of three replicate samples.

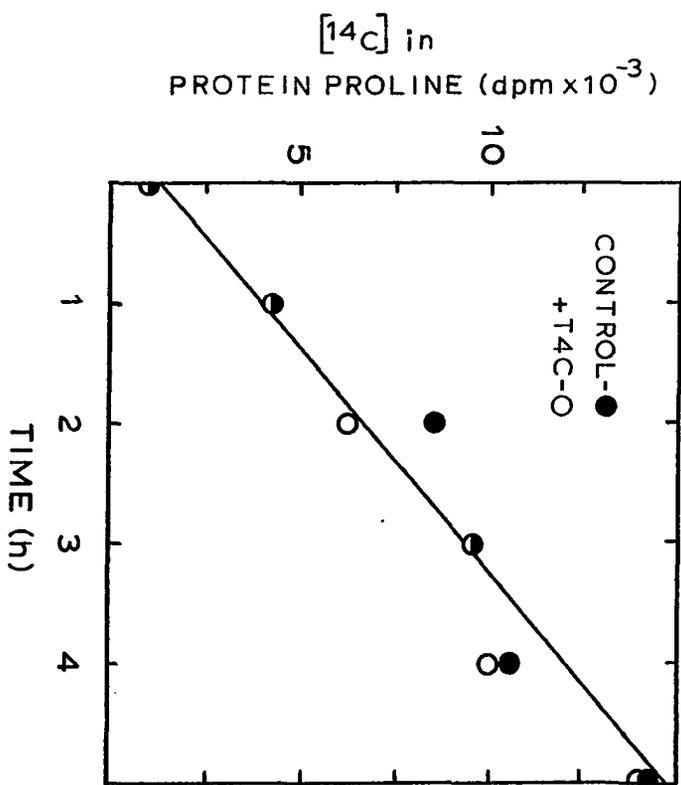


The effect of T4C on the incorporation of  $^{14}\text{C}$ -proline into protein is shown in Figure 2. There was no difference in the rate of incorporation in the two treatments. As stated above, since the specific radioactivity was the same in the two treatments, there was no effect of T4C on protein synthesis as measured by the incorporation of  $^{14}\text{C}$ -proline. Proline levels do not affect the rate of protein synthesis in leaves (Stewart *et al.* 1977).

The effect of T4C on proline synthesis from glutamate is shown in Figure 3. These leaves were wilted to make the leaves synthesize proline rapidly. Less conversion of  $^{14}\text{C}$ -glutamate to proline was observed in the T4C treated leaves than in the controls. This inhibition of proline synthesis could have resulted from inhibition of P5C reductase, since T4C has been reported to inhibit this enzyme (Miler and Stewart 1976). However, *o*-aminobenzaldehyde was included in the alcohol used to extract these leaves and no  $^{14}\text{C}$  was recovered in the P5C-*o*AB complex on the chromatograms.

Experiments with P5C reductase isolated from etiolated barley shoots, suggests that the effect of T4C on this enzyme is variable. When P5C reductase is first isolated, it is relatively insensitive to T4C. However, after a brief period of storage (5 days at  $-20\text{ C}$ ), the enzyme preparation has become relatively inactive and has developed sensitivity to T4C. Similar results were obtained with P5C reductase

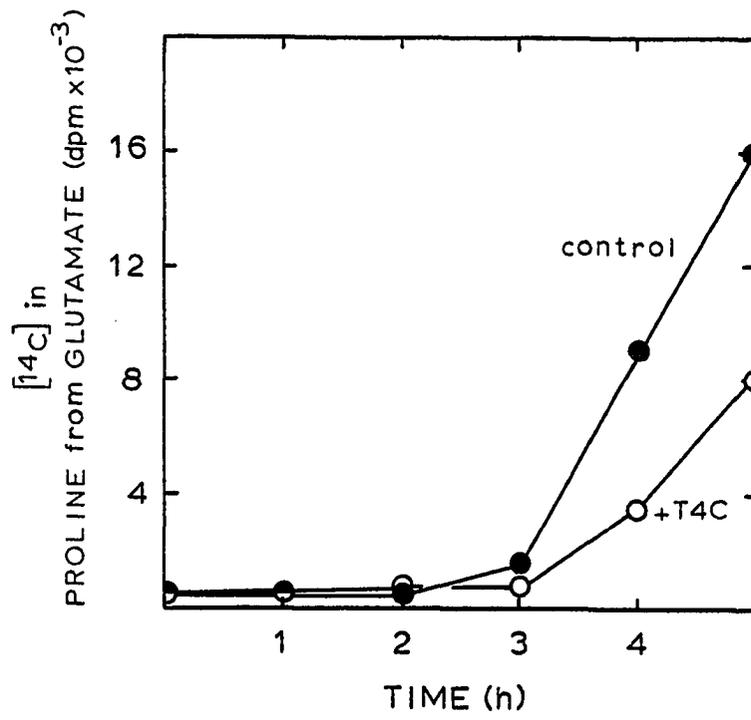
Figure 2. Radioactivity (from  $^{14}\text{C}$ -proline) Recovered  
in Protein-Proline from Leaves Described in Figure 1



**Figure 3. Radioactivity (from  $^{14}\text{C}$ -glutamate) Recovered in Proline from Control and T4C Treated Wilted Leaves**

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After excision, the control leaves were placed in 50 mM sucrose and 1 mM glutamate, and T4C treated leaves in the same solution containing 5 mM T4C. After 10 h incubation, the leaves were wilted and 5  $\mu\text{l}$  of U- $^{14}\text{C}$ -L-glutamate (670,000 dpm,  $>200 \mu\text{Ci}/\mu\text{mol}$ ) was added to each leaf (0 time). Each value is the mean of three replicate samples.



from etiolated mung bean hypocotyls, except that this enzyme preparation is relatively stable and actually increases in activity during the first few days of storage. The effect of T4C could also occur earlier in the proline biosynthetic pathway. Since proline synthesis is regulated in turgid leaves by feedback inhibition by proline, it is possible that T4C could have an analogous effect.

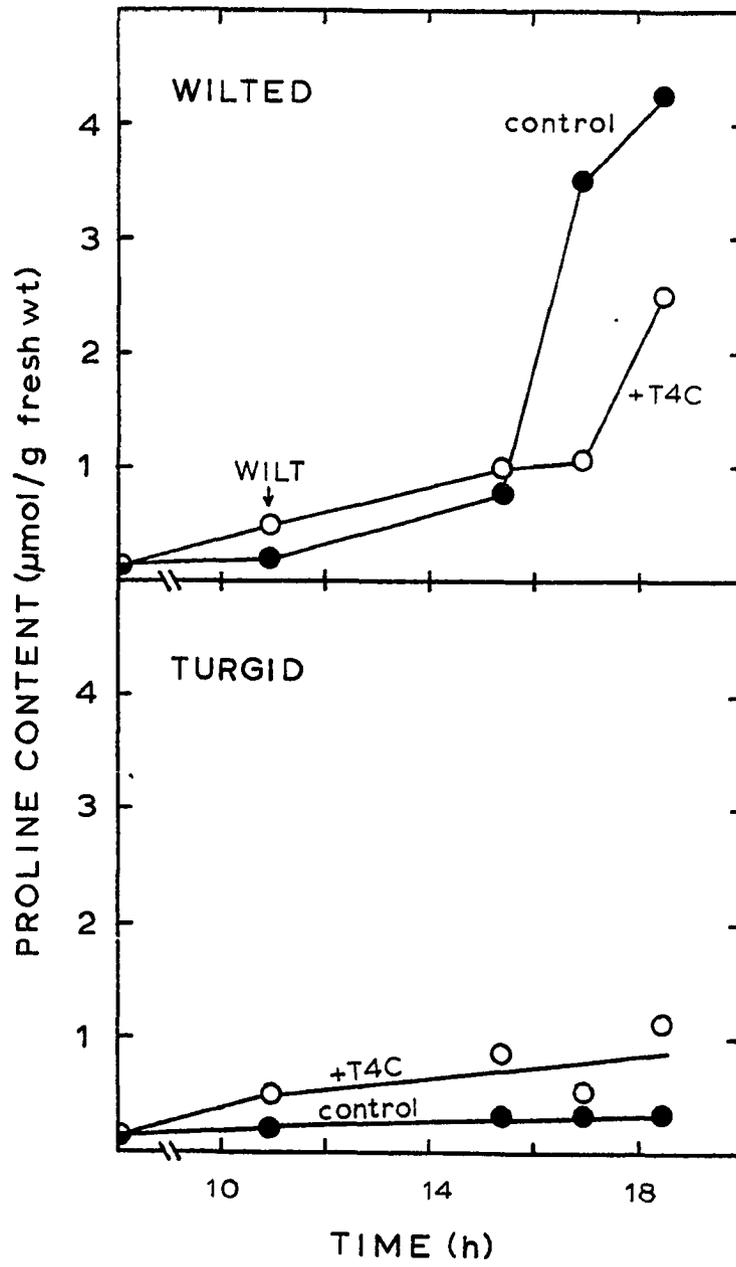
The effects of T4C on proline content in wilted and turgid leaves is shown in Figure 4. Addition of T4C caused a small increase in proline levels in turgid leaves. Thus, inhibition of proline oxidation (Figure 1), although essential for proline accumulation to occur, is not sufficient in itself to cause accumulation.

In wilted leaves that are rapidly synthesizing proline due to loss of normal feedback inhibition (Boggess *et al.* 1976a), addition of T4C resulted in less proline accumulation. Since proline oxidation is already inhibited to a considerable extent in wilted leaves (Stewart *et al.* 1977), the effect of T4C would be primarily on proline synthesis (Figure 3). This supports previous findings which have shown that accumulating proline arises primarily from stimulated synthesis (Boggess *et al.* 1976b).

**Figure 4. Effect of T4C on the Proline Content of Wilted and Turgid Leaves**

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After excision, the control leaves were placed in 50 mM sucrose and 1 mM glutamate. T4C treated leaves were placed in the same solution containing 5 mM T4C. After 11 h incubation, the leaves were rapidly wilted and transferred to water (turgid) or maintained in a humid chamber (wilted). Each value is the mean of three replicate samples.



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## CONCLUSIONS

In concluding, it may be useful to briefly summarize some of our findings. The first paper of this dissertation represents our current understanding as to how plant mitochondria may function chemiosmotically. Although this may seem to be just a literature review, it is in reality much more. This is the first time that the processes of proton translocation, utilization, and release in mitochondria have been integrated to show the effective proton gradient. In addition, the effects of electron transport, proton translocation, metabolite transport, and phosphorylation on the electrochemical gradient have seldom been balanced. Figure 1 illustrates how we envision the pH and electrical gradients during steady-state substrate oxidation and phosphorylation. This type of figure gives an instantaneous appreciation of chemiosmosis and its implications. We have used the traditional red and blue to indicate acid and base conditions. It is easily seen in this figure, that the functioning of the electron transport chain is a very dynamic process.

In our second paper, we further characterized the submitochondrial location and functioning of the enzymes achieving proline oxidation. We have summarized some of these findings in Figure 2. Both proline and P5C DH are

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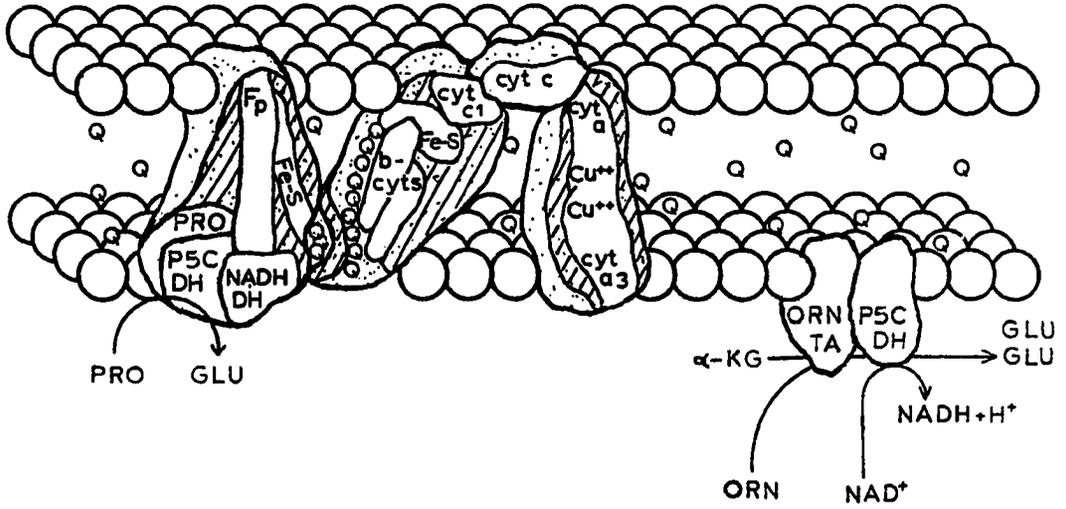
components of the electron transport chain. The fate of electrons from proline and P5C is similar to that of electrons from endogenous NADH. This suggests that these DHs are located physically close in the electron transport chain as illustrated. Figure 3 is the biochemical pathway that represents what is occurring in Figure 2. We have some evidence indicating that proline transport into the matrix is a carrier-mediated energy-dependent process. Further research is necessary to establish the nature of this transport system.

In our third paper, we have tried to find a useful inhibitor of proline oxidation. T4C should be very useful in investigation of proline oxidation in isolated mitochondria. T4C may also be useful in evaluating the roles of proline oxidation and proline synthesis in intact tissue, although this application will be limited since T4C inhibits both of these processes.

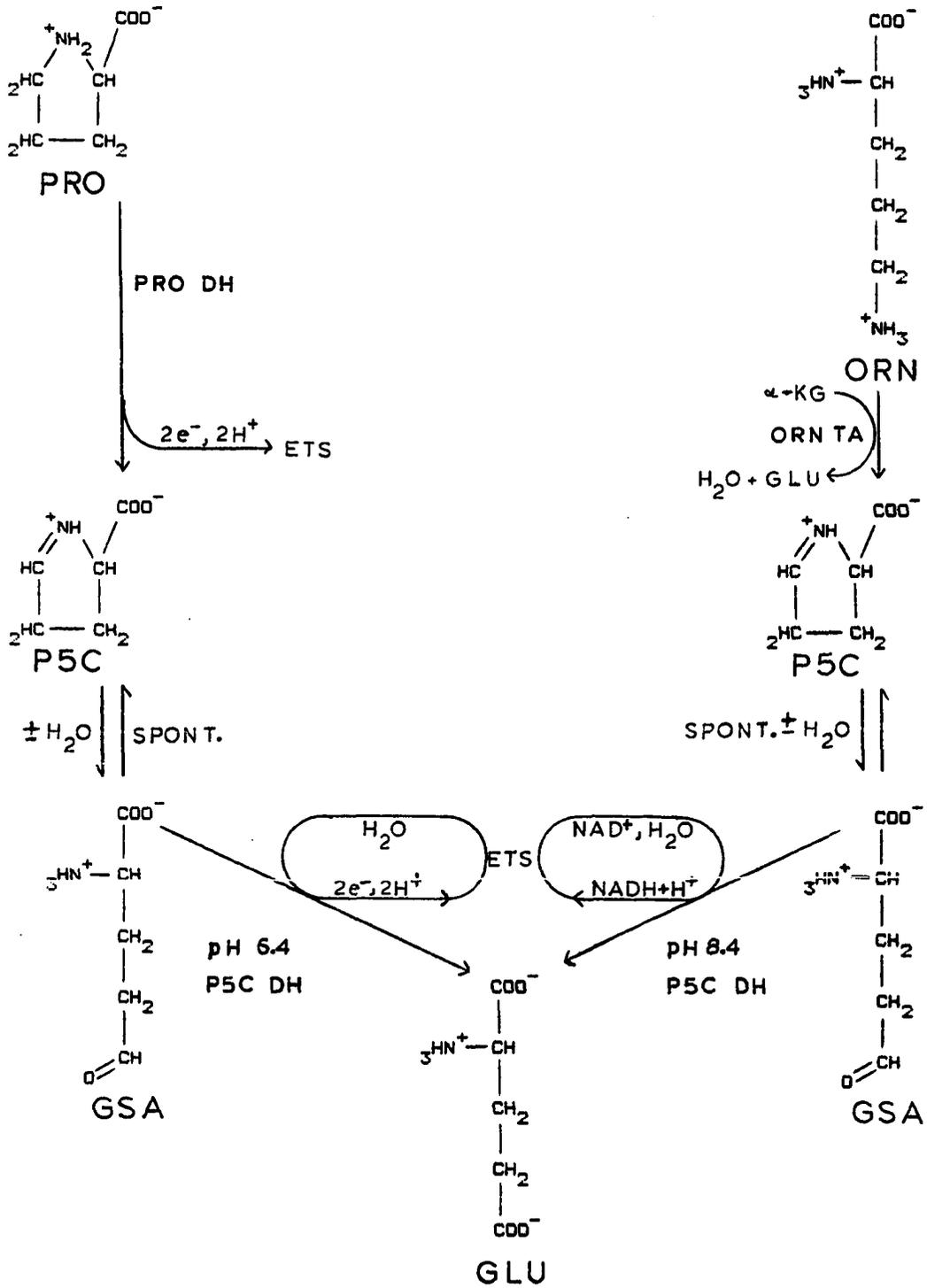
**Figure 1. The Electrochemical Gradient Across the Inner Membrane**



**Figure 2. Submitochondrial Location of Enzymes  
Oxidizing Proline and Ornithine**



**Figure 3. The Biochemical Pathway of Mitochondrial  
Proline and Ornithine Oxidation**



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**APPENDIX:**

**USE OF THE SMARTFACE ANALOG/DIGITAL CONVERTER**

## INTRODUCTION

During the last year, I have spent considerable time trying to computerize our laboratory. This has involved the selection of a computer system with both word processing, and data collection and manipulation capabilities. It is the purpose of this appendix to explain in some detail the use of this data collection system.

The least expensive way to collect and manipulate data from a variety of instruments is to use an analog/digital (A/D) converter in connection with a microcomputer. Thus, we selected an A/D converter called Smartface (Analytical Computers, Elmhurst, IL) that is capable of taking data from two instruments at once with reasonable accuracy. To use an instrument with this system, it must have a recorder output somewhere in the range of 0-400 mV (for the unit we purchased). Fortunately, this includes almost all instruments. The Smartface A/D converter also supports the capability of initiating data collection at the instrument. A switch can be mounted on the instrument to interrupt data collection, or to turn it on and off.

Most instruments send data to recorders as a linear variation of voltage, although there are a few with log outputs. For linear systems, a value of 50% would result in a voltage of 5 mV leaving an instrument with a 0-10 mV

recorder output range. An A/D converter converts this voltage into a digital number. The converter that we have purchased, converts voltage to a fraction of 4000. Thus the 5 mV voltage above would result in a value of 2000 in the 10 mV range. In addition to the 0-10 mV range, the Smartface also has ranges of 0-1 mV, 0-100 mV, and 0-400 mV.

As a result of the variability in recorder outputs, data obtained from the computer is only as precise as data that would have been obtained with a strip-chart recorder. Because of this, with instruments that have a dial (analog) or a digital meter, the value obtained from the computer will not always agree with the value on the instrument. My experience, is that an average variation of 3% is observed with our spectrophotometer. Thus, one may want to determine which is more precise, the instrument or the A/D converter. Since the instrument also uses a system to convert analog signals to data, a comparison of the precision of that system with the precision of the recorder output - A/D converter combination is necessary. Since this comparison is normally not possible, perhaps a useful gauge would be the price (quality) of the instrument.

## A SAMPLE PROGRAM

This is a description of a program written to take data from our spectrophotometer. It is an extremely complex program that uses a number of features found in our Apple II system. This program incorporates several features of programs which were purchased from Analytical Computers. It was my goal to modify the commercial programs to make them useful for the specific purpose of collecting data from our spectrophotometer. The result is a program that can handle data collection from all spectrophotometer modes. In addition, it can store, plot, or print data in the time required for preparation of the next sample (typically less than 90 seconds). This program provides an example that will be useful in writing programs for other instruments.

Figure A1 illustrates the flow chart for the program we have written. To run this program, you need to turn the computer on with the program disk in drive 1 and a data disk in drive 2. The program will automatically load, run itself and greet you.

HI THERE, I AM YOUR FRIENDLY  
SPECTROPHOTOMETER PROGRAM.

ALL CONNECTIONS BETWEEN THE SPEC AND  
THE SMARTFACE SHOULD BE MADE BEFORE  
EITHER THE SMARTFACE OR THE SPEC ARE  
TURNED ON.

IF YOU HAVE NOT DONE SO,  
TURN THE SMARTFACE AND THE SPEC OFF  
BEFORE MAKING THE CONNECTIONS.

PRESS RETURN TO CONTINUE.

Once that you are sure that you have everything hooked-up alright, you will be asked to select the spectrophotometer mode you wish to use. It is essential that you do not use a mode different than the one you have selected. Depending upon the spectrophotometer mode, the voltage output to the A/D converter changes.

SELECT SPECTROPHOTOMETER MODE:

1. 0-1 ABS,
2. 0-2 ABS,
3. 0-3 ABS,
4. 0-0.2 ABS,
  
5. 0-100 %T,
6. or 0-20 %T.

ENTER NUMBER FOR MODE.

Once the Smartface has been set to the appropriate input voltage, it is necessary to set its range (0 and 100%). Zero should be set with the cuvettes (containing the appropriate solvent) in place. When you hit return, the computer will take 40 data points from the spectrophotometer. It ignores the first 10 and averages the

last 30, storing this average as SA(1) in memory.

SET SPECTROPHOTOMETER MODE,  
AND WITH CUVETTES IN PLACE,  
SET SPECTROPHOTOMETER TO ZERO.

HIT RETURN WHEN SET, OR  
ENTER SKIP TO SKIP RANGE SETTING.

You then need to set the upper end of the range. This is achieved by using something to set 80 to 100% of the potential scale.

INSERT APPROPRIATE CARD  
TO ACHIEVE 80-100% OF THE POSSIBLE RANGE.

HIT RETURN WHEN SET.

After hitting return, the same process is repeated and the average is stored as SA(2). The program then stops and asks you to enter the current value on the spectrophotometer. It then calculates full scale from this.

ENTER SPECTROPHOTOMETER READING

The computer then informs you of the range that has been set.

YOU HAVE JUST SCALED THE  
COMPUTER FROM 0 TO 4000.  
THE UPPER VALUE MUST NOT  
EXCEED 4000. IF ABOVE 4000  
OR XXXX WAS OBTAINED

ENTER EXIT,  
OTHERWISE HIT RETURN.

If XXXX was obtained during data collection, you have exceeded the voltage range that you set and it is necessary to adjust the Smartface. Only set the Smartface if you are sure that you have selected the correct spectrophotometer mode. When you hit return, SA(1) is stored as ZN and SA(2) is stored as ZX. These values are the minimum and maximum values of the range.

The maximum value of the range will vary depending upon which spectrophotometer mode you are in. Approximate values of the range are given below.

0-1 ABS	0-4000
0-2 ABS	0-750
0-3 ABS	0-1100
0-0.2 ABS	0-750
0-100 %T	0-4000
0-20 %T	0-750

You can then set the appropriate data collection mode.

DO YOU WANT TO RUN:

1. KINETICS,
2. INDIVIDUAL SAMPLES,
3. OR EXIT THE PROGRAM?

ENTER NUMBER OF YOUR CHOICE.

Kinetics mode. Assuming that you have chosen to collect data over time, you need to answer 2 questions.

SELECT TIME INTERVAL BETWEEN POINTS:

0. TO RESET RANGE,
1. 0.5 SEC,
2. 1.0 SEC,
3. or 2.0 SEC.

ENTER NUMBER FOR DESIRED INTERVAL.

If you need other time intervals, you can modify the program to get 0.05, 0.1, and 0.25 seconds. You can get longer time intervals by ignoring every other point or so.

ENTER NUMBER OF DATA POINTS,

BETWEEN 1 AND 1000.

Actually, you can enter up to 10,000 data points.

However, for most applications, less than 1000 data points are sufficient. If you are unsure of the number of data points needed, enter a large number like 500. Later, you can use

CTRL-X to stop data collection when a sufficient number of points have been collected.

Before data collection actually begins, you must enter the name of the file you want to store the data in. You must be able to recall this later.

ENTER SAMPLE# TO BEGIN.

Data collection then begins. It will automatically end when all the points have been collected. If you want to stop data collection hit CTRL-X. The data are automatically stored as a sequential file on disk before continuing. This file contains the spectrophotometer mode, the number of data points, and all of the data points.

DO YOU WANT TO:

1. PLOT DATA,
2. PRINT DATA,
3. OR DO BOTH?

ENTER NUMBER OF YOUR CHOICE.

If you have chosen to do both, the data will first be plotted on the computer screen. It will then be dumped to the printer. After this has been completed, the contents of the data file will be printed. This will include the

filename, the number of data points, and all of the data points. The program then cycles back to run another sample.

Individual sample mode. In the individual sample mode, you only need to enter the number of samples you have to read (100 or less). The computer will then wait for you to press return before starting.

ENTER NUMBER OF SAMPLES,  
FROM 1 TO 100.  
PRESS RETURN TO BEGIN.

The computer will take 40 data points at 0.5 second intervals. It will average the last 30 and store the result in an array. It will do this for each sample, resulting in an array from SA(1) to SA(NS) where NS=number of samples.

Between samples, the computer will beep and then go through a timing sequence that will last for about 5 seconds. During this time, the screen will fill up with characters. You must open the door of the spectrophotometer during this 5 second interval. If you do not do this, the computer will start reading the same sample again. Data collection will not begin again until you close the door. After the total number of samples have been read, the computer will skip the timing sequence and continue.

DO YOU WANT TO USE A LINE  
EQUATION TO CONVERT YOUR DATA?  
YES OR NO.

If yes, you will be asked to enter the slope and y-intercept, and your data will be converted. In either case, you will then be able to store or print the data.

DO YOU WANT TO:  
1. STORE DATA ON DISK,  
2. PRINT DATA,  
3. OR DO BOTH?

ENTER NUMBER OF YOUR CHOICE.

If you have chosen to store the data on disk, you will then be asked to provide a filename for data storage. The data will be stored in a sequential file consisting of the spectrophotometer mode, the number of data points, and all of the data points. After completion of data recording, the program cycles back to set the range again. If you want to skip the range setting and read values in the same spectrophotometer mode, then enter skip and the number of samples to be read.

Plotting and printing stored data. An additional section has been added to the end of the program to allow printing or plotting of stored data. To use this portion of the program, stop the program using CTRL-C return. Then enter RUN 3000 and hit return.

If you are only going to print the data, you will have to set drive 2 as the active drive. To do this, type CATALOG,D2 and hit return. Then type RUN 3000 return.

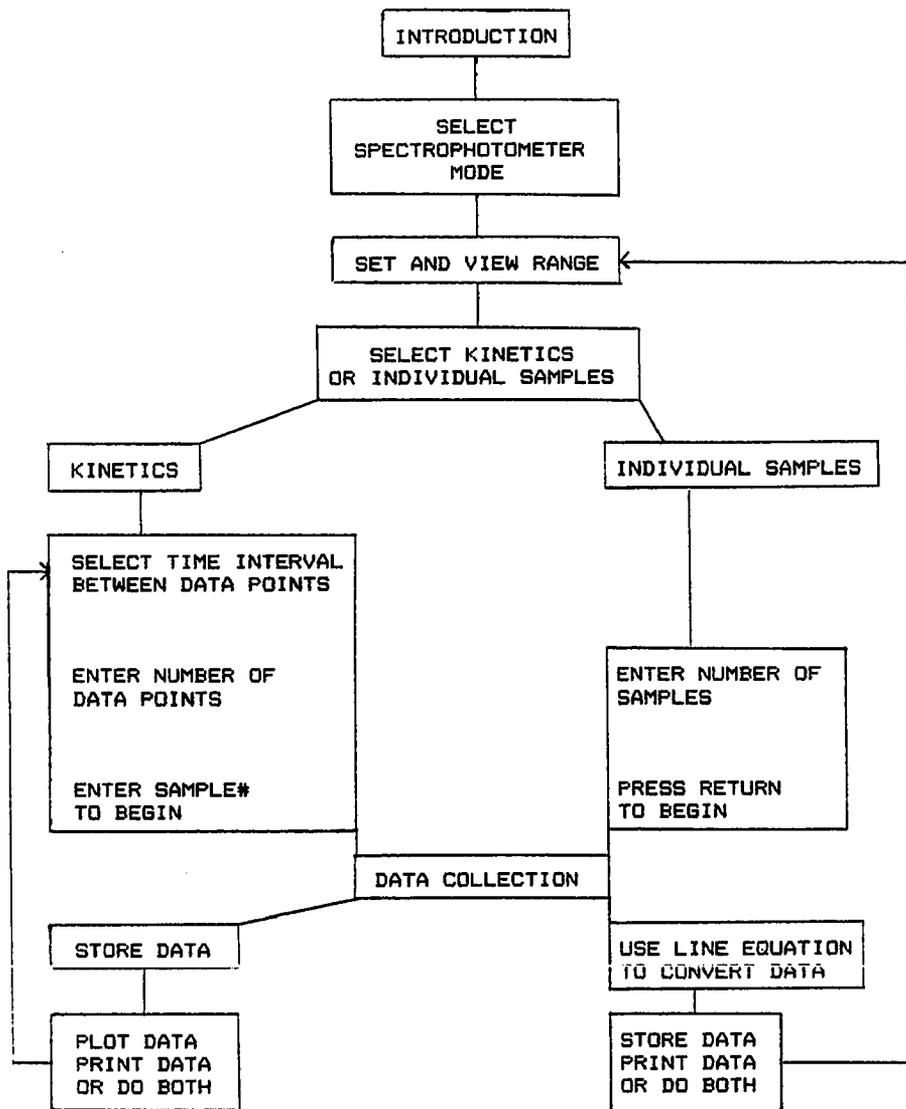


Figure A1. Flow Chart for Spectrophotometer Program

## VARIABLES USED IN THE PROGRAM

SA(J)	SAMPLE NUMBER
C\$	MODE SELECTION OR A JUNK VARIABLE, DEPENDING UPON USAGE
R1	SMARTFACE RANGE 1
R2	SMARTFACE RANGE 2
NS	NUMBER OF SAMPLES
SP	SPECTROPHOTOMETER MODE
NP	NUMBER OF DATA POINTS PER SAMPLE
T\$	TIME INTERVAL BETWEEN DATA POINTS
KI	KINETICS OR INDIVIDUAL SAMPLES
ZN	MINIMUM VALUE OF THE RANGE
ZX	MAXIMUM VALUE OF THE RANGE
FAC	FACTOR FOR CONVERSION OF DATA TO SPECTROPHOTOMETER MODE
SN\$	FILE FOR STORAGE OF KINETIC DATA
J,M,C,I	COUNTERS WITHIN THE PROGRAM
SL	APPLE II SLOT WHICH CONTAINS THE SERIAL INTERFACE
B\$	"666768"
A\$	HAS A VARIETY OF USES WITHIN THE PROGRAM
I,Y	MEMORY LOCATION COUNTERS
A	USED TO CONVERT T\$ FOR USE BY RS232.S0. IT ALSO HAS A VARIETY OF OTHER USES WITHIN THE PROGRAM.
A1,A2	USED TO STORE THE NUMBER OF DATA POINTS IN MEMORY
N1,N2	USED TO RETRIEVE THE NUMBER OF DATA POINTS FROM MEMORY

VS           SUM OF DATA POINT VALUES

V            VALUE OF AN INDIVIDUAL DATA POINT

T            VALUE FOR TIMER

Z\$           USE A LINE EQUATION TO CONVERT DATA? YES OR NO.

S8           VALUE OF SLOPE

I8           VALUE OF Y-INTERCEPT

PS           PRINT DATA, STORE DATA, OR DO BOTH (INDIVIDUAL  
              SAMPLES)  
              PLOT DATA, PRINT DATA, OR DO BOTH (KINETICS)

X            USED TO INPUT VALUES FROM FILES FOR PRINTING

Z            USED TO PRINT OR PLOT STORED FILES

SQ           SELECTION OF RANGE SETTING MODE

Q            VALUE OF SPECTROPHOTOMETER READING WHEN SETTING  
              80-90% OF POSSIBLE RANGE

CALL -958        USED TO CLEAR ALL CHARACTERS AHEAD OF THE  
                  CURSOR

CALL 8201        CALLS THE PROGRAM RS232.S0 WHICH WAS  
                  PURCHASED

RS232.S0        THE PROGRAM PURCHASED TO RUN THE SMARTFACE

PKWDATA         A PURCHASED GRAPHICS SHAPE TABLE

POKE 232,0      ?

POKE 233,112    ?

## VARIABLES USED IN THE PLOTTING SECTION OF THE PROGRAM

XL, YL	POSITION OF LEFT END OF X-AXIS
XR, YR	POSITION OF RIGHT END OF X-AXIS
X0	MINIMUM VALUE OF X-SCALE
X1	MAXIMUM VALUE OF X-SCALE
DX	LENGTH OF X-AXIS
NL	NUMBER OF LABELS
IX	INTERVAL BETWEEN LABELS
L	LENGTH OF LABEL
XX	NUMBER OF HGR POINTS PER DATA POINT ON THE X-SCALE
X3, Y3	X AND Y POSITIONS FOR PLOTTING
JX	INTERVAL FOR INTERVAL DIVISIONS
X2	A COUNTER
XB, YB	POSITION OF LOWER END OF Y-AXIS
XT, YT	POSITION OF UPPER END OF Y-AXIS
Y0	MINIMUM VALUE OF Y-SCALE
Y1	MAXIMUM VALUE OF Y-SCALE
YY	NUMBER OF HGR POINTS PER DATA POINT ON THE Y-SCALE
JY	INTERVAL FOR INTERVAL DIVISIONS
Y2	A COUNTER
ND	A COUNTER
D	AN INPUTTED DATA POINT

## PROGRAM LISTING WITH SOME EXPLANATION

Line 1000: This line sets the lower limit of memory available to the program. The program starts at location 38400 and ends near 25000. If more memory is needed, this location can be changed to 24576 but absolutely no lower because locations 16384 to 24575 are used for plotting in the graphics mode.

Lines 1010 to 1020: These lines contain the program's introduction.

Lines 1022 to 1028: Selection of the spectrophotometer mode is achieved through these lines. Through this, the user also selects the voltage range for the Smartface.

Lines 1030 to 1170: These lines are for setting the range. Line 1030 arranges for the zero value of the range to be taken. Line 1040 passes 5 variables to the data collection routine. It is essential that these variables be set before data collection. When data collection begins, 40 data points are taken at 0.5 second intervals. The last 30 are averaged and are stored in memory as SA(1). In line 1050, the upper value of the range is setup. The value of 80-100% was chosen to keep the precision as high as possible. Again, 30 data points are averaged, and are stored as SA(2). Lines 1051 to 1057 allow for input of the

current spectrophotometer reading, and for calculation of the 100% value of the range. Viewing of the scale is made possible by lines 1060 to 1065. Lines 1120 to 1170 set FAC, which is a variable for converting a value to the appropriate spectrophotometer mode.

Lines 1180 to 1240: These lines are for the selection of essential variables to run either kinetics or individual samples.

Lines 1300 to 1465: The data collection routine is contained within these lines. Lines 1312 to 1316 are repeats of lines 1026 to 1028. It is essential that these lines are repeated, since these variables sometimes get lost. In line 1370, CALL 8201 is the program we purchased that actually collects the data and stores it in memory. Line 1380 opens a file for storage of kinetic data, and prints to that file the spectrophotometer mode and the number of data points. Lines 1390 to 1452 remove the data from memory, convert it to the appropriate form, and store it (kinetic data only) in the file which was created in 1380. Line 1465 is the timer for the individual samples mode.

Lines 1470 to 1476: These lines effect the conversion of data using a line equation.

Lines 1480 to 1600: These lines give the user a choice

of printing or storing data from the individual samples mode.

Lines 1610 to 1800: These lines are for plotting kinetic data.

Lines 2010 to 2040: These lines are for printing kinetic data.

Lines 3000 to 3020: These lines give the user a choice of printing or plotting stored data.

```
1000 LOMEM: 25000: DIM SA(100):D
      $ = CHR$(4)
1010 HOME : PRINT "HI THERE, I A
      M YOUR FRIENDLY": PRINT "SPE
      CTROPHOTOMETER PROGRAM.": PRINT
      : PRINT "ALL CONNECTIONS BET
      WEEN THE SPEC AND": PRINT "T
      HE SMARTFACE SHOULD BE MADE
      BEFORE": PRINT "EITHER THE S
      MARTFACE OR THE SPEC ARE"
1020 PRINT "TURNED ON.": PRINT :
      PRINT : PRINT "IF YOU HAVE
      NOT DONE SO,": PRINT "TURN T
      HE SMARTFACE AND THE SPEC OF
      F": PRINT "BEFORE MAKING THE
      CONNECTIONS.": PRINT : PRINT
      : INPUT "PRESS RETURN TO CON
      TINUE.":C$
1022 HOME : PRINT "SELECT SPECTR
      OPHOTOMETER MODE": PRINT : PRINT
      "1. 0-1 ABS": PRINT : PRINT
      "2. 0-2 ABS": PRINT : PRINT
      "3. 0-3 ABS": PRINT : PRINT
      "4. 0-0.2 ABS": PRINT : PRINT
      : PRINT "5. 0-100 %T": PRINT
      : PRINT "6. 0-20 %T": PRINT
      : PRINT
1024 INPUT "ENTER NUMBER FOR MOD
      E.":SP
1025 IF SP < 1 OR SP > 6 THEN 10
      22
1026 IF SP = 1 OR SP = 3 OR SP =
      5 THEN 1028
1027 R1 = 52:R2 = 52: GOTO 1030
1028 R1 = 51:R2 = 51
```

Listing 1. A Program to Run a Spectrophotometer

```

1030 HOME : PRINT "SET SPEC MODE
      ": PRINT "AND WITH CUVETTES
      IN PLACE": PRINT "SET SPEC T
      O ZERO": PRINT : PRINT "HIT
      RETURN TO SET OR": INPUT "EN
      TER SKIP TO SKIP RANGE SETTI
      NG. ";C$: IF C$ = "SKIP" THEN
      1120
1040 NS = 2:SQ = 0:NP = 30:T$ = "
      1":KI = 2: GOTO 1300
1050 HOME : PRINT "INSERT APPROP
      RIATE CARD": PRINT "TO ACHIE
      VE 80-100% OF THE POSSIBLE R
      ANGE": PRINT : PRINT : INPUT
      "HIT RETURN WHEN SET. ";C$: GOTO
      1320
1051 HOME : PRINT : INPUT "ENTER
      SPEC READING";Q
1052 IF SP = 1 THEN SA(2) = SA(2
      ) * (1 / Q)
1053 IF SP = 2 THEN SA(2) = SA(2
      ) * (2 / Q)
1054 IF SP = 3 THEN SA(2) = SA(2
      ) * (3 / Q)
1055 IF SP = 4 THEN SA(2) = SA(2
      ) * (0.2 / Q)
1056 IF SP = 5 THEN SA(2) = SA(2
      ) * (100 / Q)
1057 IF SP = 6 THEN SA(2) = SA(2
      ) * (20 / Q)
1060 HOME : PRINT "YOU HAVE JUST
      SCALED THE COMPUTER": PRINT
      : PRINT "FROM "SA(1)" TO "SA
      (2)": PRINT : PRINT "THE UPPE
      R VALUE MUST NOT EXCEED 4000
      ": PRINT : PRINT "IF ABOVE 4
      000 OR XXXX WAS OBTAINED": PRINT
      : PRINT "ENTER EXIT": PRINT

1065 INPUT "OTHERWISE HIT RETURN
      . ";C$:ZN = SA(1):ZX = SA(2):
      IF C$ = "EXIT" THEN 1022

```

Listing 1. (continued)

```

1120 IF SP = 1 THEN FAC = 1
1130 IF SP = 2 THEN FAC = 2
1140 IF SP = 3 THEN FAC = 3
1150 IF SP = 4 THEN FAC = .2
1160 IF SP = 5 THEN FAC = 100
1170 IF SP = 6 THEN FAC = 20

1180 HOME : PRINT "DO YOU WANT T
O RUN:": PRINT : PRINT "1.
KINETICS,": PRINT : PRINT "2
. INDIVIDUAL SAMPLES,": PRINT
: PRINT "3. OR EXIT THE PRO
GRAM.": PRINT : PRINT : INPUT
"ENTER NUMBER OF YOUR CHOICE
.":KI
1190 SQ = 1: IF KI = 1 THEN 1220
1200 IF KI = 2 THEN 1240
1210 IF KI = 3 THEN END
1215 GOTO 1180
1220 HOME : PRINT "SELECT TIME I
NTERVAL BETWEEN POINTS": PRINT
: PRINT "0. TO RESET RANGE"
: PRINT : PRINT "1. 0.5 SEC
": PRINT : PRINT "2. 1.0 SE
C": PRINT : PRINT "3. 2.0 S
EC": PRINT : PRINT : PRINT :
INPUT "ENTER NUMBER FOR DES
IRED INTERVAL.":T$
1222 IF T$ = "0" THEN 1030
1230 HOME : PRINT "ENTER NUMBER
OF DATA POINTS.": PRINT : INPUT
"BETWEEN 1 AND 1000.":NP:NS =
1: HOME : PRINT : INPUT "ENT
ER SAMPLE# TO BEGIN.":SN$: GOTO
1310
1240 HOME : PRINT "ENTER NUMBER
OF SAMPLES,": PRINT : INPUT
"FROM 1 TO 100.":NS:NP = 30:
T$ = "1": PRINT : PRINT : PRINT
: INPUT "PRESS RETURN TO BEG
IN.":C$: GOTO 1310

```

Listing 1. (continued)

```

1300 PRINT D$;"BLOAD RS232.S0,D1
"
1310 J = 1
1312 IF SP = 1 OR SP = 3 OR SP =
5 THEN 1316
1314 R1 = 52:R2 = 52: GOTO 1320
1316 R1 = 51:R2 = 51
1320 TEXT : HOME :SL = 2: POKE 6
,16 * SL: POKE 32,5: POKE 84
36,R1: POKE 8445,R2:B$ = "66
6768":A$ = T$:A = VAL (A$) -
1 + 48:A$ = STR$ (A): IF A$
= "48" THEN A$ = "4653": GOTO
1340
1330 A$ = A$ + "00"
1340 B$ = B$ + A$:M = 1:Y = 8446 +
M
1350 A = VAL ( MID$ (B$,M,2)): POKE
Y,A: IF A = 00 OR A = 53 THEN
1365
1360 M = M + 2:Y = Y + 1: GOTO 13
50
1365 IF KI = 2 THEN A = NP + 10:
GOTO 1370
1366 A = NP
1370 A1 = INT (A / 256):A2 = A -
A1 * 256: POKE 8194,A2: POKE
8195,A1: TEXT : HOME : CALL
- 958: CALL 8201: IF KI = 2
THEN 1390
1380 A$ = SN$: PRINT D$;"OPEN SWI
TCH,D2": PRINT D$;"CLOSE SWI
TCH": PRINT D$;"OPEN":A$: PRINT
D$;"DELETE":A$: PRINT D$;"OP
EN":A$: PRINT D$;"WRITE":A$:
N1 = PEEK (8692):N2 = PEEK
(8693):NP = 256 * N2 + N1: PRINT
SP: PRINT NP

```

Listing 1. (continued)

```
1390 VS = 0:C = 0: IF KI = 2 THEN
    I = 8714: GOTO 1400
1395 I = 8694
1400 V = ( PEEK (I) * 256 + PEEK
    (I + 1)): IF V > 32768 THEN
    V = V - 65536
1410 IF SQ = 0 THEN 1420
1415 V = ((V - ZN) / (ZX - ZN) *
    FAC): IF V < .0001 THEN V =
    0
1420 IF KI = 2 THEN VS = VS + V:
    GOTO 1440
1430 PRINT V
1440 I = I + 2:C = C + 1: IF C =
    NF THEN 1450
1445 GOTO 1400
1450 IF KI = 1 THEN 1600
1451 SA(J) = VS / NF: IF Q = 0 THEN
    1453
1452 IF SA(J) < .0001 THEN SA(J)
    = 0
1453 J = J + 1: IF SQ > 0 THEN 14
    60
1454 IF J = 2 THEN 1050
1456 IF J = 3 THEN 1051
1460 IF J = NS + 1 THEN 1470
1465 PRINT CHR$(7): HOME :T =
    1: FOR TD = 1 TO 500: PRINT
    T:T = T + 1: NEXT TD: GOTO
    1320
```

Listing 1. (continued)

```

1470 HOME : PRINT : PRINT "DO YOU
      U WANT TO USE A LINE EQUATIO
      N": PRINT : PRINT "TO CONVER
      T YOUR DATA?": PRINT : INPUT
      "YES OR NO.": Z$: IF Z$ = "NO
      " THEN 1480
1474 HOME : INPUT "ENTER VALUE OF
      SLOPE.": S$: PRINT : PRINT
      : PRINT : INPUT "ENTER VALUE
      OF Y-INT.": I$
1476 FOR J = 1 TO NS: SA(J) = (SA
      (J) - I$) / S$: NEXT
1480 HOME : PRINT "DO YOU WANT T
      O": PRINT : PRINT "1. STOR
      E DATA ON DISK,": PRINT : PRINT
      "2. PRINT DATA,": PRINT : PRINT
      "3. OR DO BOTH.": PRINT : PRINT
      : PRINT : INPUT "ENTER NUMBE
      R OF YOUR CHOICE.": P$: IF P$
      = 2 GOTO 1520
1490 HOME : PRINT : INPUT "ENTER
      FILENAME FOR DATA STORAGE."
      : A$: PRINT D$; "OPEN SWITCH, D
      2": PRINT D$; "CLOSE SWITCH":
      PRINT D$; "OPEN": A$: PRINT D
      $; "DELETE": A$: PRINT D$; "OPE
      N": A$: PRINT D$; "WRITE": A$: PRINT
      SP: PRINT NS: J = 1
1500 FOR J = 1 TO NS: PRINT SA(J
      ): NEXT : PRINT D$; "CLOSE": A
      $: PRINT D$; "OPEN SWITCH, D1"
      : PRINT D$; "CLOSE SWITCH": IF
      P$ = 1 THEN 1030
1520 PRINT D$; "PR#1": IF P$ = 2 THEN
      A$ = "SAMPLES":
1525 HTAB 5: PRINT A$: HTAB 10: PRINT
      NS: FOR J = 1 TO NS: HTAB 10
      : PRINT J". "SA(J): NEXT : PRINT
      D$; "PR#0": GOTO 1030
1600 PRINT D$; "CLOSE": A$
1605 Z = 0

```

Listing 1. (continued)

```

1610 HOME : PRINT "DO YOU WANT T
D:": PRINT : PRINT "1. PLOT
DATA,": PRINT : PRINT "2.
PRINT DATA,": PRINT : PRINT
"3. OR DO BOTH.": PRINT : INPUT
"ENTER NUMBER OF YOUR CHOICE
.";PS: IF PS = 2 THEN 2010
1620 PRINT D$;"BLOAD PKWDATA,D1"
: PRINT D$;"OPEN SWITCH,D2":
PRINT D$;"CLOSE SWITCH": PRINT
D$;"OPEN";A$: PRINT D$;"READ
";A$: POKE 232,0: POKE 233,1
12: INPUT SP: INPUT NP
1625 DEF FN XN(X) = (X - X0) *
XX + XL: DEF FN YN(Y) = (Y -
Y0) * YY + YB
1630 HGR2 : SCALE= 1: ROT= 0: HCOLOR=
3
1632 XL = 29:YL = 155:XR = 279:YR
= YL: HPLOT XL,YL TO XR,YR
1634 X0 = 0:X1 = NP:DX = 274 - XL
:NL = 5:IX = INT (NP / NL):
XX = DX / (X1 - X0):Y3 = YL +
3: ROT= 16
1636 FOR X2 = X0 TO X1 STEP IX
1638 X2$ = STR$ (X2):L = LEN (X
2$):X3 = FN XN(X2): DRAW 2 AT
X3,YL
1640 X3 = X3 - 3: DRAW ASC (X2$)
AT X3,Y3: IF L = 1 THEN 164
4
1642 FOR I = 2 TO L: DRAW ASC (
MID$ (X2$,I,1)): NEXT
1644 NEXT X2
1650 JX = INT (NP / (NL * 2))
1652 FOR X2 = 0 TO X1 STEP JX: DRAW
1 AT FN XN(X2),YL: NEXT
1660 XB = XL:YB = YL:XT = XB:YT =
5: HPLOT XB,YB TO XT,YT

```

Listing 1. (continued)

```

1662 Y0 = 0: IF SP = 1 THEN Y1 =
      1
1664 IF SP = 2 THEN Y1 = 2
1666 IF SP = 3 THEN Y1 = 3
1668 IF SP = 4 THEN Y1 = .2
1670 IF SP = 5 THEN Y1 = 100
1672 IF SP = 6 THEN Y1 = 20
1674 DY = YT - YB:NL = 5:IY = Y1 /
      NL:YY = DY / (Y1 - Y0)
1676 FOR Y2 = Y0 TO Y1 STEP IY:Y
      3 = FN YN(Y2): DRAW 2 AT XB
      ,Y3
1678 Y2$ = STR$ (Y2):L = LEN (Y
      2$):Y3 = Y3 + 3:X3 = 1
1680 ROT= 0: DRAW ASC (Y2$) AT
      X3,Y3: IF L = 1 THEN 1684
1682 FOR I = 2 TO L: DRAW ASC (
      MID$ (Y2$,I,1)): NEXT
1684 NEXT Y2
1686 JY = Y1 / (NL * 2)
1688 FOR Y2 = 0 TO Y1 STEP JY: DRAW
      1 AT XB, FN YN(Y2): NEXT
1760 HPLOT XL,YB TO XL,YT TO XR,
      YT TO XR,YB TO XL,YB:ND = 1
1770 INPUT D:X3 = FN XN(ND):Y3 =
      FN YN(D): IF Y3 < YT THEN Y
      3 = YT
1780 DRAW 1 AT X3,Y3:ND = ND + 1
      : IF ND < NP + 1 THEN 1770
1790 PRINT D$:"CLOSE":A$: PRINT
      D$:"PR#1": HTAB 5: PRINT A$:
      PRINT CHR$ (9):"G2": PRINT
      : PRINT : PRINT : TEXT : IF
      PS = 3 THEN 2010
1800 PRINT D$:"PR#0": IF Z = 1 THEN
      3000
1810 GOTO 1220

```

Listing 1. (continued)

```
2010 HOME : PRINT D$;"PR#1"  
2020 PRINT D$;"OPEN";SN$: PRINT  
D$;"READ";SN$: HTAB 5: PRINT  
SN$: INPUT SP: INPUT NP: HTAB  
10: PRINT NP  
2030 FOR I = 1 TO NP: INPUT X: HTAB  
10: PRINT I". "X: NEXT  
2040 PRINT D$;"CLOSE";SN$: PRINT  
: PRINT : PRINT : PRINT D$;"  
PR#0": IF Z = 1 THEN 3000  
2050 GOTO 1220  
3000 HOME : PRINT "ENTER NAME OF  
FILE TO ACCESS OR": PRINT :  
INPUT "HIT RETURN TO END.";  
A$:SN$ = A$: IF A$ = "" THEN  
END  
3020 Z = 1:D$ = CHR$ (4): GOTO 1  
610
```

Listing 1. (continued)