A STUDY OF THE OXIDATION OF EXTRAMITOCHONDRIAL REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE BY PIGEON HEART MITOCHONDRIA

by

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This is a long story, which shouldn't be long, but it will take a long time to make it short.

- Henry David Thoreau

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ABSTRACT

ABSTRACT

Holl, F.B. A study of the oxidation of extramitochondrial reduced nicotinamide adenine dinucleotide in pigeon heart mitochondria.

The concept of mitochondrial impermeability to reduced nicotinamide adenine dinucleotide (NADH) has recently undergone a critical reappraisal in the literature. Dr. K. Wrogemann (unpublished results) has found that a procedural modification in the isolation of pigeon heart mitochondria produced a preparation with metabolic properties different to the usual "control" mitochondria isolated according to the method of Chance and Hagihara (76). The former or "modified" mitochondrial preparation devised by Dr. Wrogemann exhibited significantly lower rates of oxidation of pyruvate + malate and NADH and as well, lower respiratory control ratios (RCR's) and phosphorylation ratios (ADP:O).[1] The cause of these differences and the mode of oxidation of extramitochondrial NADH were investigated. The two types of mitochondria were isolated from a single heart or the pooled tissue of two hearts and tested polarographically at 28^0 C. Simultaneous measurements of oxygen consumption, NADH oxidation and swelling were also made in certain experiments. The kinetics of NADH oxidation were determined by fluorometric measurements of

[1] A more detailed definition of the "control" and "modified" preparations is presented in the Introduction.

physiological concentrations (1-10 μM) of this substrate. Morphological studies using electron microscopy and light scattering were also performed. The dissimilarities in oxidation of added NADH between control and modified mitochondria were not attributable to differences in either the initial volume after isolation or the volume changes upon substrate addition. However, in the electron micrographs the size of individual organelles in the two preparations was not identical and indicated the existence of heterogeneity in the control population which was supported by differential centrifugation studies. Electron microscopy also indicated a possible relationship between the observed functional differences and the organization of mitochondrial cristae. The pathway or mechanism of oxidation of NADH, the indigenous mitochondrial concentration of cytochrome c and the capacity of the respiratory chain were not responsible for the lower rates of oxidation of extra-mitochondrial NADH observed in the modified preparation. Data on the metabolic behaviour of the latter mitochondria with added pyruvate + malate, viz. lower oxidation, RCR's and ADP:O ratios did not necessarily reflect the same cause(s) as the lower rate of NADH oxidation in the preparation. The presence of proteinase in the isolation medium might have been involved in the lower rate of NADH oxidation but not in that of pyruvate + malate at proteinase

concentrations ≤0.5 mg/ml. At a proteinase concentration of 6 mg/ml in the medium the isolated mitochondria showed no coupling of oxidation and phosphorylation with pyruvate + malate. In other experiments, the lower rates of pyruvate + malate oxidation and lower RCR's in modified mitochondria could be increased by the addition of albumin to the media which suggested that fatty acids might be associated with the observed effects. No such relationship was indicated for NADH oxidation by modified mitochondria. Data is presented which indicated that lower NADH oxidation probably was directly related to membrane structural integrity whereas the slower pyruvate + malate utilization was associated with factor(s) influencing the mechanism(s) coupling oxygen consumption to oxidative phosphorylation.

Of the various hypotheses that have been suggested to account for the mitochondrial oxidation of cytoplasmic NADH, the malate/oxaloacetate shuttle can be eliminated in pigeon heart mitochondria since it could not be detected. This and other findings indicate that perhaps direct permeation is the normal route by which NADH enters the mitochondria to be oxidized. The physiological significance of the rates of oxygen consumption with NADH are discussed in relation to the known data on <u>in vivo</u> myocardial metabolism.

GLOSSARY

TERMS

ADP:O Ratio - The ratio of molecules of ADP esterified per atom of oxygen utilized by the mitochondrial respiratory chain. It is equivalent to the P:O ratio, having maximum observed ("theoretical") values of three for NAD-linked substrates and two for flavoprotein-linked substrates of the electron transport chain (31).

Control and Modified Mitochondrial Preparations - The two mitochondrial suspensions used in this study will be designated "control" and "modified". Control mitochondria are prepared by a modification of the method of Chance and Hagihara (76) using 3-4 g of heart tissue in 40 ml medium giving a tissue wet weight/homogenizing medium volume ratio of 1:10. Modified mitochondria are prepared by the same technique using 250 mg heart tissue per 40 ml medium giving a corresponding tissue wet weight/ homogenizing medium volume ratio of 1:160.

Coupling - "Coupling" of respiration and oxidative phosphorylation refers to the dependence of the respiratory rate on ADP. "Tightly coupled" mitochondria are those having high respiratory control ratios whereas "loosely coupled" mitochondria show little or no

dependence of respiration on the presence of phosphate acceptor (ADP).

External NADH - External NADH refers to NADH added to the mitochondrial preparations, as opposed to that generated within the mitochondria. The following terms will be used interchangeably to designate external NADH; exogenous, cytoplasmic, extramitochondrial and added.

Respiratory Control Ratio - The respiratory control ratio is defined as the ratio of the rate of respiration in the presence of phosphate acceptor (e.g. ADP) divided by the rate of respiration in the absence of phosphate acceptor (59).

i.e. it is given by the formula:

$$RCR = \frac{\text{state 3 respiration}}{\text{state 4 respiration}}$$

Slater's Factor - Slater postulated an essential electron transporting factor between diaphorase and cytochrome c which represents a common pathway of electron transport to cytochrome a from NADH or succinate (83). Cytochrome b in the intact system appears to include Slater's factor.

State 3 Respiration - All required components of respiration are present (oxygen, phosphate, phosphate acceptor, substrate) and the respiratory chain itself is the rate-

limiting factor. This is the period of "active" respiration in coupled mitochondria (59).

State 4 Respiration - This is known as the controlled or resting state. Phosphate acceptor (e.g. ADP) is lacking and respiration is slow in coupled mitochondria (59).

GLOSSARY

ABBREVIATIONS

ADP - adenosine-5'-diphosphate

ATP - adenosine-5'-triphosphate

EDTA - ethylenediamine tetracetic acid

GOT - glutamate:oxaloacetate transaminase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1)

MDH - malic dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37)

NAD^+ - nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide (reduced)

PHM - pigeon heart mitochondria

RCR - respiratory control ratio

Tris - Tris(hydroxymethyl)aminomethane

Tris-HCl - Tris(hydroxymethyl)aminomethane hydrochloride

INTRODUCTION

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INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+) was originally discovered by Harden and Young (1) as a coenzyme in yeast juice. It has since been found in practically all living tissues, but generally in low concentrations. Biologically, NAD^+ functions as a coenzymic hydrogen acceptor associated with dehydrogenases - being converted from the oxidized (NAD^+) to the reduced form (NADH) (Fig. 1).

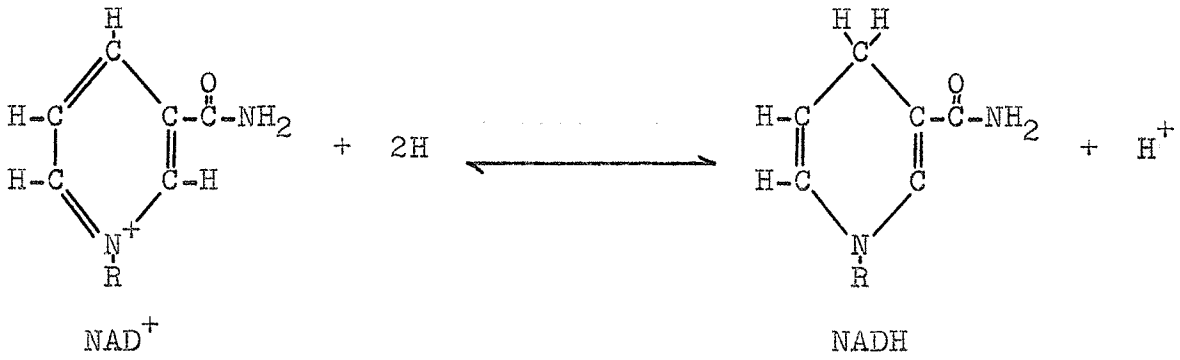


FIGURE 1. The interconversion of oxidized and reduced forms of nicotinamide adenine dinucleotide

R = -ribose-diphosphate-ribose-adenine

In the cytoplasm the reduced form of the coenzyme (NADH) is formed principally by certain reactions of glycolysis (Fig. 2). Increased production of NADH by active glycolysis tends to decrease the cytoplasmic NAD^+/NADH ratio. However, under anaerobic conditions this decrease in the ratio is minimized by the conversion of pyruvate to lactate

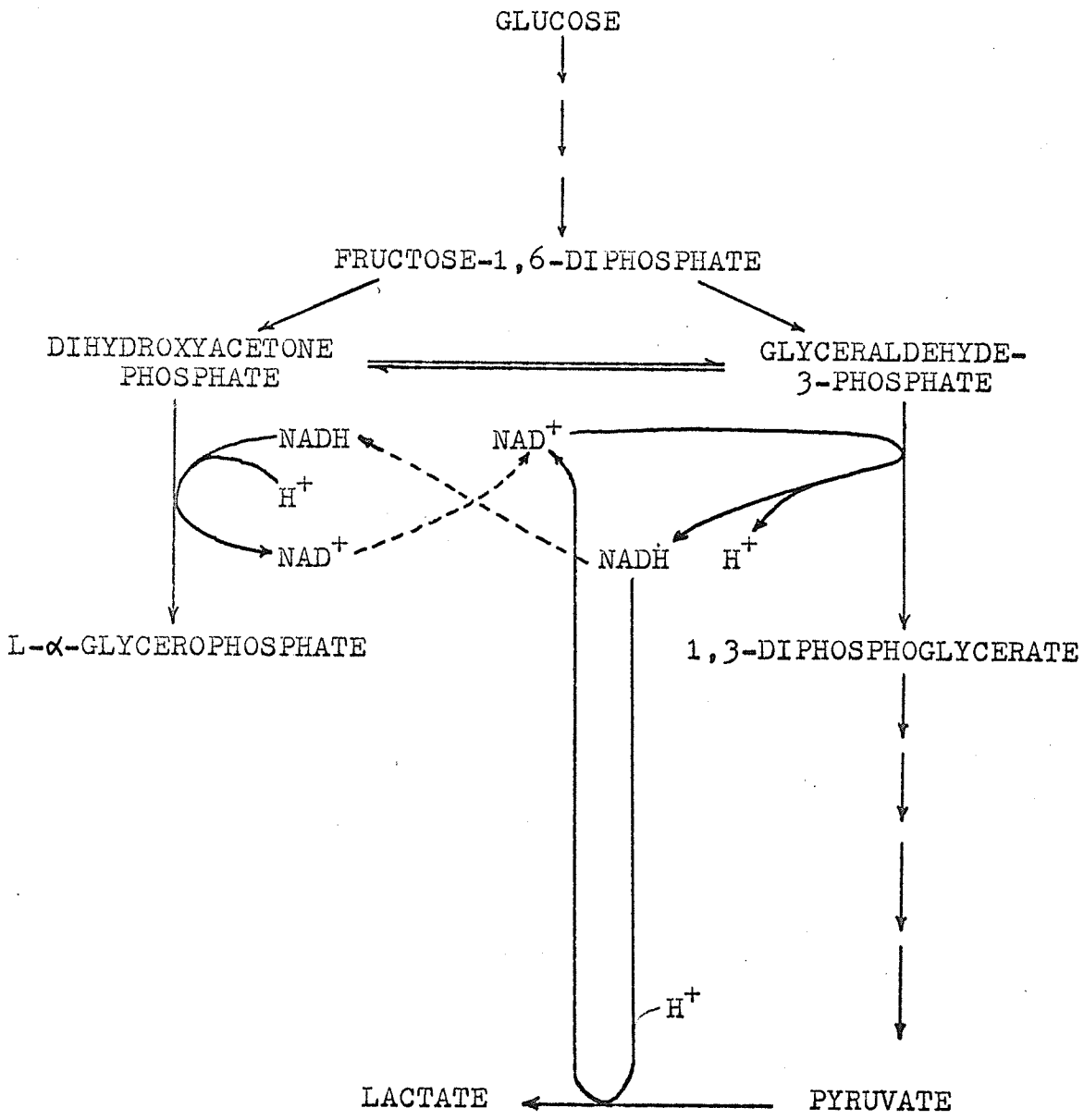
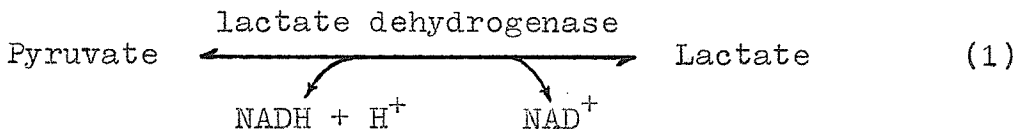
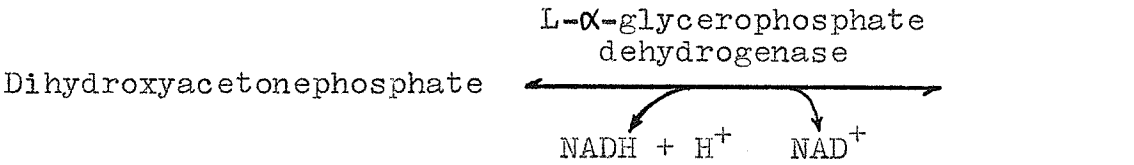


FIGURE 2. Glycolysis - indicating the production and utilization of NADH in the cytoplasm

(Equation 1) and by the formation of α-glycerophosphate (Equation 2).



(1)



(2)

During aerobic glycolysis, decreased lactate formation requires that an alternate route of NADH removal be identified. In heart tissue, lactate does not accumulate under aerobic conditions, indicating that the cytoplasmic NADH has been reoxidized by a system other than lactate dehydrogenase (e.g. α-glycerophosphate dehydrogenase). Ultimately, however, the NADH produced during aerobic glycolysis must directly or indirectly reach the mitochondrial respiratory chain. This electron transport system is illustrated in Fig. 3, showing the points of entry of various substrates, the localization of inhibitor and uncoupler activities and the phosphorylation sites for energy production. Reoxidation of cytoplasmic NADH by the respiratory chain must require direct or indirect permeability of the mitochondrial membrane system to this

FIGURE 3. Electron transport chain showing the points of entry of substrates and some inhibitor and uncoupler sites. The three phosphorylation sites are also indicated.

AH_2 = NAD^+- linked substrate

BH_2 = Flavoprotein-linked substrate

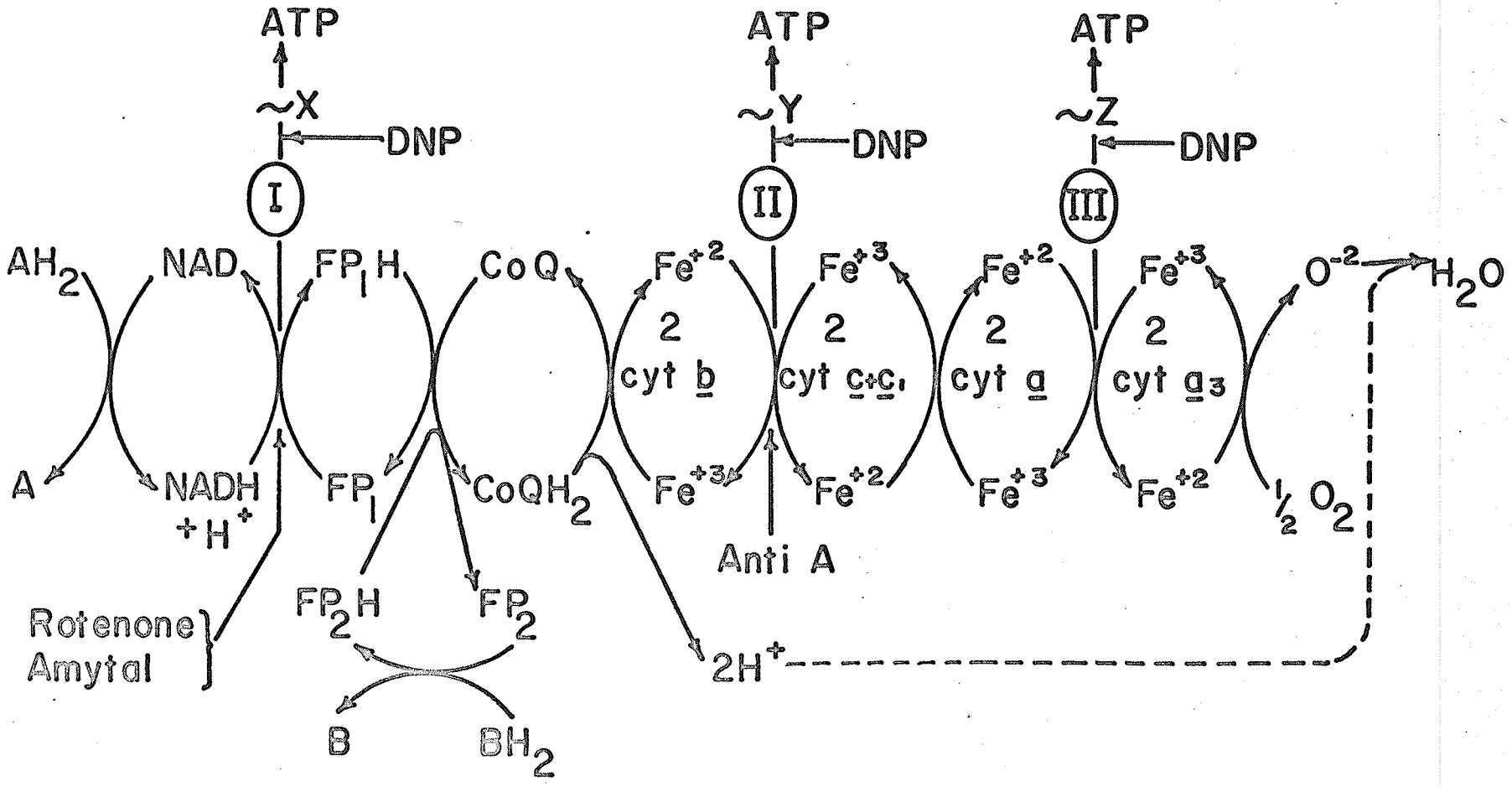
FP = Flavoprotein

CoQ = Coenzyme Q

cyt = cytochrome c

Anti A = Antimycin A

DNP = 2,4-dinitrophenol


ATP
~X
DNP
I
ATP
~Y
DNP
II
ATP
~Z
DNP
III
AH2
NAD
FP1H
CoQ
Fe+2
Fe+3
Fe+2
Fe+3
O-2
H2O
2
cyt b
2
cyt c+c1
2
cyt a
2
cyt a3
A
NADH
+H+
FP1
CoQH2
Fe+3
Fe+2
Fe+3
Fe+2
½ O2
Anti A
Rotenone
Amytal
FP2H
FP2
2H+
B
BH2

nucleotide.

The concept that intact mitochondria are impermeable to external NADH was originally suggested by Lehninger (2, 3, 4). This conclusion has gained wide acceptance (5-9, 16-18) although there appears to be increasing evidence (10-15) that neither the in vitro nor the in vivo situation can be classified as a case of complete permeability or impermeability. Any discussion of the degree of mitochondrial permeability in the oxidation of cytoplasmic NADH must, therefore, recognize three major factors: (i) the definition and significance of the term "impermeable", (ii) the criteria used to define "intactness" of a particular mitochondrial preparation in relation to its permeability to NADH, and (iii) the relevance of (i) and (ii) to the pertinent physiological data on the required minimum rate of NADH removal from the cytoplasm by the mitochondria of a given tissue. Much of the difficulty in interpreting permeability studies arises from the failure of most authors to define their limits of permeability relative to the existing physiological requirements of the particular tissue examined.

Permeability

Several frames of reference exist for the description of the permeability of a membrane to any particular substance.

In the instance of cytoplasmic NADH, permeability may be stated simply in terms of the absolute rate of uptake and oxidation of NADH by the mitochondria. Alternatively, the relative permeability can be stated as the ratio of the oxidation rates of external NADH relative to the maximum capacity of the respiratory chain to oxidize this substrate (assuming that no intramitochondrial NADH accumulation occurs), or relative to the oxygen consumption observed using other conventional mitochondrial substrates (e.g. pyruvate + malate). These criteria of permeability suffer from one common failure: the lack of any obvious relationship to physiological requirements.

Examining the possible physiological significance of cytoplasmic NADH oxidation raises the problem; is direct mitochondrial permeability to NADH necessary? Two hypotheses exist.

(a) Assuming NADH must penetrate the mitochondrial membrane directly, the question resolves itself to one of rate of penetration and relative permeability as defined above. Slow rates of oxidation of external NADH by isolated mitochondria cannot be unequivocally interpreted as implying membrane impermeability <u>in vivo</u>. Such rates, though slow, may merely reflect a minimal physiological requirement for NADH removal. If the maximum required rate of NADH removal from the cytoplasm is low, there would be little need to

have available the full NADH oxidizing capacity of the respiratory chain. Control of the rate *in vivo* could be exercised by appropriate changes in membrane permeability induced by the intracellular environment. Certain studies of pigeon heart mitochondria (11, 12, 13) have attempted to examine the problem of NADH entry and oxidation in a more physiological context.

The problem of interpreting data in a physiological context will be considered in greater detail in the Discussion. The relative physiological importance of NADH transport into liver and muscle (particularly heart) mitochondria will be considered in relation to the direction and size of electron flow as well as the cellular requirements for reoxidation of extramitochondrial NADH by the respiratory chain.

(b) Assuming that direct NADH entry into the mitochondrion does not occur, the removal of cytoplasmic NADH must be accomplished by some alternative mechanism(s). A number of shuttle systems (Fig. 4) which transfer the reducing equivalents of the coenzyme across the membrane barrier have frequently been proposed (17-27) but not always supported by experimental evidence. Such mechanisms require the presence, in cytoplasm and mitochondria, of the necessary enzymes in sufficiently high activity levels to carry out the reactions. Also, the rate of transfer of the intermediate carriers

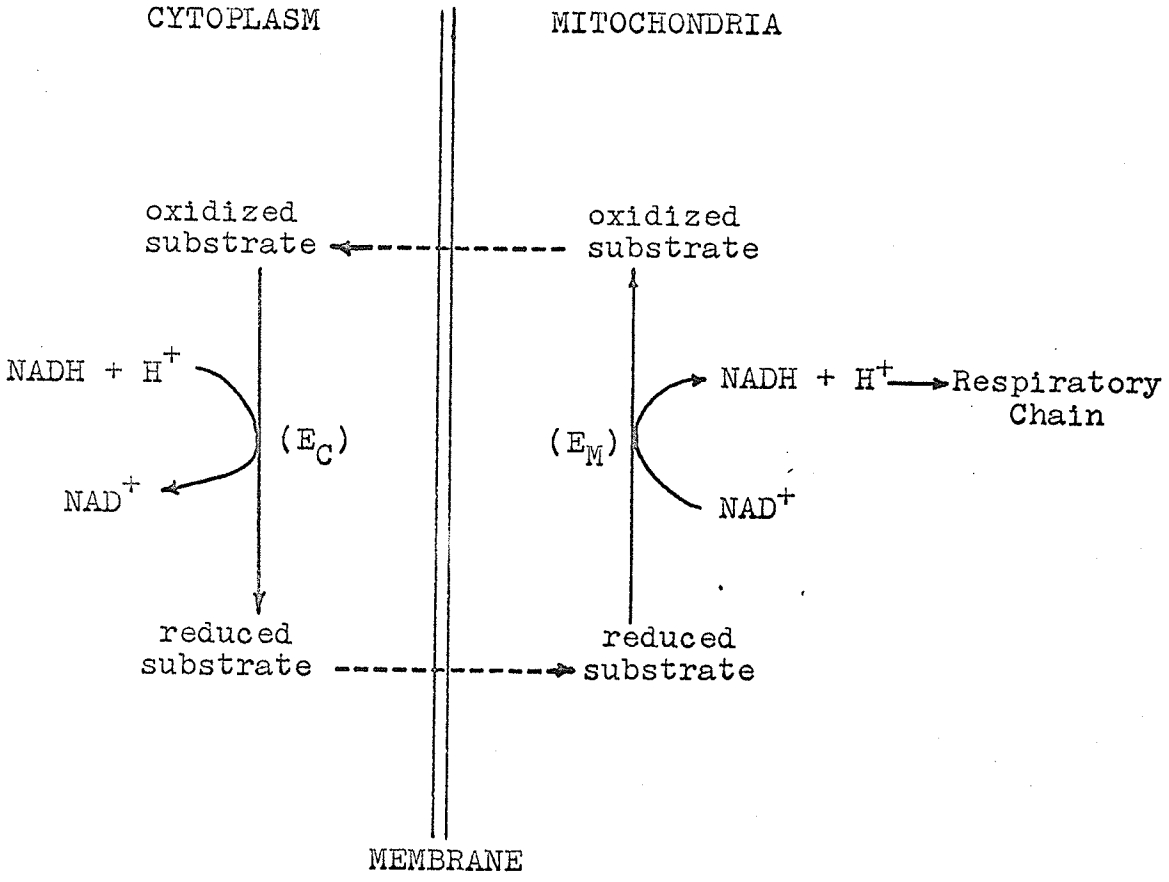


FIGURE 4. Generalized scheme of metabolite shuttle systems for the transport of reducing equivalents from the cytoplasm to the mitochondrial respiratory chain. The oxidized and reduced substrates must penetrate the mitochondrial membrane freely

(E_C) and (E_M) represent the cytoplasmic and mitochondrial forms respectively of the enzymes required to interconvert the oxidized and reduced substrates

across the mitochondrial membrane must not be a limiting factor. This latter consideration has often been ignored in studying shuttle mechanisms. Recently however, Haslam and Krebs (28), in characterizing a shuttle which has been proposed for heart mitochondria involving malate and oxaloacetate, observed a transport rate of malate which was only 3.5% of that required for the calculated minimum physiological rate. Their work re-emphasizes the importance of relating studies of permeability to the in vivo cellular requirements in consideration of either direct NADH permeability or transfer of intermediates in possible shuttle mechanisms.

Intactness

The criteria used to define "intactness" of isolated mitochondria are an integral part of any interpretation of data concerned with mitochondrial membrane permeability.

There are four main criteria which have been used to designate the degree of mitochondrial intactness.

1. Electron Microscopy

The use of the electron microscope is an obvious means of determining mitochondrial integrity. The technique can be used to detect obvious damage or morphological defects as well as contamination of individual preparations by mitochondrial fragments or other foreign material. However,

there are specific limitations to electron microscopic examination.

(a) The effects of fixation and staining processes on mitochondrial morphology are difficult to predict.

(b) Metabolic defects may be undetectable morphologically.

(c) Photomicrographs of selected areas of the embedded material may provide an inaccurate analysis, particularly in a heterogenous preparation.

2. Respiratory Control Ratios (RCR) (see glossary)

Tightly coupled mitochondria generally exhibit high respiratory control ratios since their state 3 respiration is dependent upon the presence of phosphate acceptor (59). Assuming that intact mitochondria are tightly coupled, high RCR values have been used as an indicator of mitochondrial integrity (31, 59).

However, RCR's may not be as definitive a criterion of structural integrity as had been assumed. The factors controlling respiratory control may be subject to environmental alteration exemplified by the increases in RCR which can be accomplished by addition of bovine serum albumin to loosely coupled preparations (84, 85). Such observations suggest that the mitochondrial environment (e.g. the presence of fatty acids) may play a significant role in determining the RCR and the observed coupling or

lack of it may not be a valid indicator of mitochondrial structure.

3. ADP:O Ratio (see glossary)

ADP:O ratios which approach the theoretical value (3.0 for NAD-linked substrates) have also been used as a criterion of mitochondrial intactness. However, several observations suggest this is a rather poor basis for defining integrity of the preparation.

(a) Presence of high ATPase activity may produce low ADP:O ratios even in mitochondrial preparations which are phosphorylating.

(b) Mitochondria with loose coupling (low RCR values) do not always show low ADP:O ratios.

(c) Mitochondrial preparations which exhibit coupling (see glossary) with some substrates (e.g. pyruvate + malate) may appear uncoupled when incubated with others. For example, "non-coupling" has been observed in preparations using NADH as substrate, even though phosphorylation could be demonstrated in these same preparations with pyruvate + malate (11).

4. NADH Utilization

This criterion is based on the assumption reiterated by Lehninger (31) that "impermeability to extramitochondrial NADH is a characteristic of virtually all mitochondria

whether *in vitro* or *in vivo*". Observed oxidation of NADH therefore is presumed to indicate damage to the mitochondrial membrane, permitting access to the respiratory chain, or alternatively, oxidation by another pathway in the mitochondrial preparation (e.g. shuttle systems) or by fragments or other particles contaminating the preparation.

The value of this criterion depends solely upon the validity of the initial assumption. If it is valid, then the criterion is an excellent one; if not, then using external NADH oxidation to gauge intactness becomes meaningless. Since mitochondrial studies are generally performed *in vitro*, criteria of physiological intactness must be arbitrarily chosen and may not necessarily reflect the true intracellular picture. They can, however, provide a basis for some comparative information, particularly between various mitochondrial preparations from the same tissue.

In earlier experiments in this laboratory (11, 12, 13) a direct uptake and oxidation of external NADH at physiological substrate levels was shown in pigeon heart mitochondria which exhibited the conventional criteria of intactness, viz. high RCR's and ADP:O ratios. The mitochondria in those studies were isolated by the method of Chance and Hagihara (76) in which 3-4 g of minced myocardium are homogenized in 40 ml of medium, followed by the other

steps described under Methods. The properties of mitochondria prepared in this way have been well-defined. In this thesis, such organelles are termed "control" mitochondria. Their properties, with reference to NADH oxidation and other metabolic parameters are compared in this work with those of "modified" mitochondria which are prepared by a procedure devised by Dr. K. Wrogemann (unpublished data). The latter method is identical to that used for control mitochondria except that only 0.25 g (rather than 3-4 g) of myocardium is homogenized in 40 ml of medium. Thus, in preparing modified mitochondria, the ratio of myocardium (g) to medium (ml) was 1:160 whereas the ratio in the control preparation was 1:10. It was recognized that the term "control" might be taken to imply, incorrectly, that such mitochondria were in some way prejudged to be superior to those of the other preparation. Similarly, "modified" might erroneously be understood to indicate that such preparations were a modification of the control mitochondria. The term "modified" refers strictly to the change in preparation of the organelles. In spite of these disadvantages, the two terms were retained since no more satisfactory ones could be found.

In preliminary unpublished studies Wrogemann demonstrated that the modified mitochondria oxidized added NADH much more slowly than control preparations but exhibited

lower RCR's and a slow oxygen consumption with pyruvate + malate as substrate. The work described in this thesis represents an attempt to determine the possible cause(s) of the behavioural differences observed between the control and modified mitochondrial preparations. The difference in oxygen consumption with added NADH as substrate was utilized as a basis for examining the requirements for and the mechanism(s) of cytoplasmic NADH oxidation in pigeon heart mitochondria.

LITERATURE REVIEW

LITERATURE REVIEW

Following the studies of Lehninger (2, 3, 4) in which the factors influencing the mitochondrial oxidation of externally added NADH were first examined, it has generally been accepted that intact mitochondria are impermeable to NADH. Lehninger's work on liver mitochondria suggested that extramitochondrial NADH does not penetrate to the internal site of electron transport, but is largely oxidized by a non-phosphorylating, cytochrome c-requiring "external" pathway. A variety of data has since been accumulated in support of this original concept of impermeability to NADH in intact mitochondria. Chance and Williams (5) observed evidence of a permeability barrier to external NADH in studies of guinea pig liver mitochondria. Low rates of oxidation were observed until treatment in hypotonic (0.11 osM) medium was employed to swell the mitochondria. This treatment produced a four to five fold increase in the NADH oxidation rate.

The discovery by Ernster and his coworkers (6) that Amytal (sodium amobarbital - an inhibitor of respiratory chain NADH oxidation (see Fig. 3)) only partially inhibits the oxidation of externally added NADH (non-phosphorylating) led these authors to postulate two alternative pathways in their rat liver mitochondrial preparations.

(a) Internal pathway - Amytal-sensitive
- phosphorylating
} i.e. the respiratory chain

(b) External pathway - Amytal-insensitive
- non-phosphorylating
- non-mitochondrial cytochrome c reductase
- bypasses Slater's factor (see glossary)
- cytochrome c-requiring

The latter pathway (b) is similar to that first suggested by Lehninger (3). A more detailed consideration of the external pathway will be presented later in this review.

Evidence for a cytochrome c-stimulated oxidation of extramitochondrial NADH has also been observed by Humphreys and Conn (32) in lupine mitochondria. Their results indicated that an amount of NADH was oxidized equivalent to the amount of cytochrome c added. Their inhibitor studies, however, established that the same pathway existed for the oxidation of internal or external NADH, and indicated that the observed cytochrome c stimulation is not necessarily related to an external oxidative pathway.

In studies on rat liver mitochondria, Maley (33) found both oxidation and phosphorylation with external NADH as substrate. The addition of cytochrome c to her preparations resulted in increased oxygen consumption and a corresponding decrease in phosphorylation (P/O ratios). Although these observations are compatible with permeability

of the mitochondrial membrane to NADH, Maley preferred to conclude that the enhanced oxidation was related to the extent of mitochondrial damage induced by the particular isolation procedure. She favours the Lehninger concept of an external pathway for NADH oxidation.

In preparations of beef heart and rat liver mitochondria isolated in 0.88 M sucrose, Ziegler and Linnane (35) could not detect oxidation of external NADH. They concluded that the use of hypertonic preparation media ensures intactness of the mitochondrial membrane. They subsequently concluded that no equilibrium exists between internal and external pyridine nucleotides in intact mitochondria. However, it has been noted by several authors (10, 11, 36) that the high tonicity of 0.88 M sucrose is inhibitory to mitochondrial oxidations, including external NADH oxidation. This observation constitutes an adequate explanation for the low rates of added NADH oxidation observed by Ziegler and Linnane (35).

Oxidation of reduced pyridine nucleotides (NADH and NADPH) coupled to phosphorylation has been examined by Joshi, Newburgh and Cheldelin (34). In a system consisting of beef heart mitochondria and a soluble fraction from heart they detected oxidative phosphorylation associated with oxidation of both NADH and NADPH. This phosphorylation was blocked by Antimycin A and 2,4-dinitrophenol (see Fig. 3).

Joshi et al. (34) attributed three possible effects to the soluble supernatant fraction; a permeability increase, a transport of reduced pyridine nucleotides by some factor, or a reduction of a supernatant compound which crosses the mitochondrial membrane and is subsequently oxidized by the electron transport system. This latter possibility is related to an alternative type of oxidative pathway postulated for the removal of cytoplasmic NADH (i.e. shuttle mechanisms which mediate the transport of reducing equivalents across the mitochondrial membrane). One of the better established of these mechanisms is the α-glycerophosphate shuttle (Fig. 5).

The α-glycerophosphate cycle of insect flight tissue has been examined by Zebe et al. (19), Sacktor and Estabrook (20), Estabrook and Sacktor (21), Vogell et al. (22) and Sacktor and Dick (23). Their results indicated that the enzymes necessary for this cycle occur in insect flight muscle in sufficient activity to account for the oxidation of cytoplasmic NADH. Sacktor and Dick (23) used teased muscle preparations which showed no oxidation with added NADH alone as substrate but responded with rapid oxygen consumption upon further addition of dihydroxyacetonephosphate. Other studies on the mechanism of the α-glycerophosphate shuttle were also performed by Delbrück et al. (24) and Young et al. (25) in mammalian systems and by

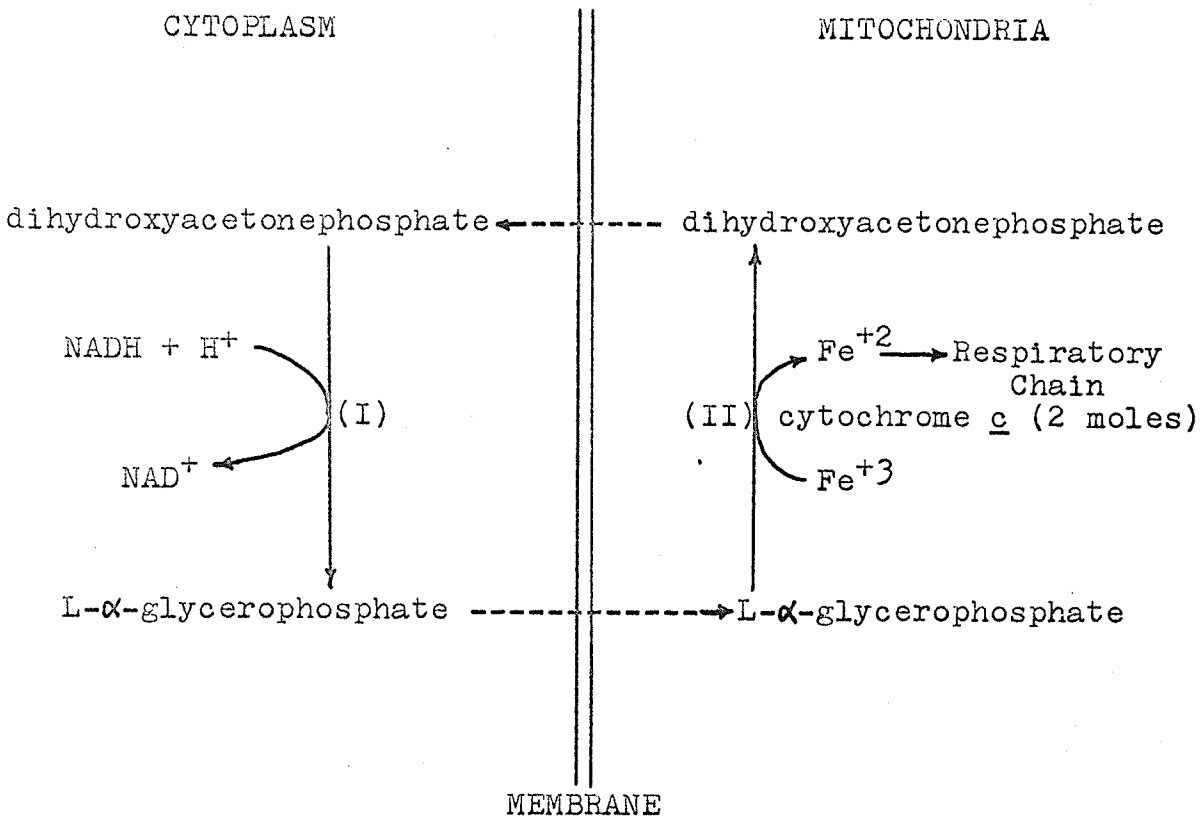


FIGURE 5. α-Glycerophosphate shuttle

(I) L-α-glycerophosphate dehydrogenase

(II) L-α-glycerophosphate oxidase

Klingenberg and Slenczka (26) in various tissues. The latter authors demonstrated that this shuttle system was not functional in heart tissue since the mitochondrial L-α-glycerophosphate oxidase (L-glycerol-3-phosphate: cytochrome c oxidoreductase, EC 1.1.2.1), one of the two enzymes required for cyclic operation, is not present in sufficient quantity in heart muscle. Suranyi et al. (27) reconstructed an α-glycerophosphate cycle for NADH oxidation using subcellular fractions from rat skeletal muscle. The system consisted of adding glycerol-3-phosphate and the cell sap obtained from the preparation of rat skeletal muscle mitochondria to the mitochondrial suspension. The NADH oxidation rates obtained with this system were the same as those observed when the cell sap was replaced with an excess of L-α-glycerophosphate dehydrogenase purified from rabbit skeletal muscle. Their observation that this oxidation of external NADH was insensitive to Amytal or rotenone inhibition, but sensitive to Antimycin A provided additional evidence to support the hypothesis of the operation of the α-glycerophosphate shuttle in their system (see Fig. 3 for inhibitor sites and points of substrate entry to the respiratory chain).

On examining the role of mitochondria in intracellular oxidation of cytoplasmic NADH, Devlin and Bedell (7) concluded that intact liver mitochondria are impermeable to

external NADH. Addition of catalytic quantities of acetoacetate or β-hydroxybutyrate increased oxidation of added NADH by rat liver mitochondria. A new "shuttle" mechanism was postulated by Devlin and Bedell (7) involving the transfer of reducing equivalents from NADH by β-hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate:NAD^+ oxidoreductase, EC 1.1.1.30) to β-hydroxybutyrate (Fig. 6). Almost immediately, evidence refuting the postulated cycle was presented by Lehninger et al. (37) who demonstrated that the D-β-hydroxybutyrate dehydrogenase of rat heart and rat liver mitochondria is unreactive with external NADH. Furthermore, the enzyme was not detectable in significant amounts in the soluble fraction of either rat liver or heart. Lehninger et al. (37) suggested that the mitochondria used by Devlin and Bedell (7) had been damaged during their isolation procedure.

The morphological relationship of isolated mitochondria to the oxidation of extramitochondrial NADH has been examined by Deshpande et al. (38) in preparations from rabbit heart muscle. The authors noted a variety of morphological differences between isolated mitochondria and the organelles in situ. These authors reported a slow rate of oxidation of extramitochondrial NADH by mitochondrial preparations essentially free of fragments. Pathways of oxidation such as the glycerophosphate cycle and the

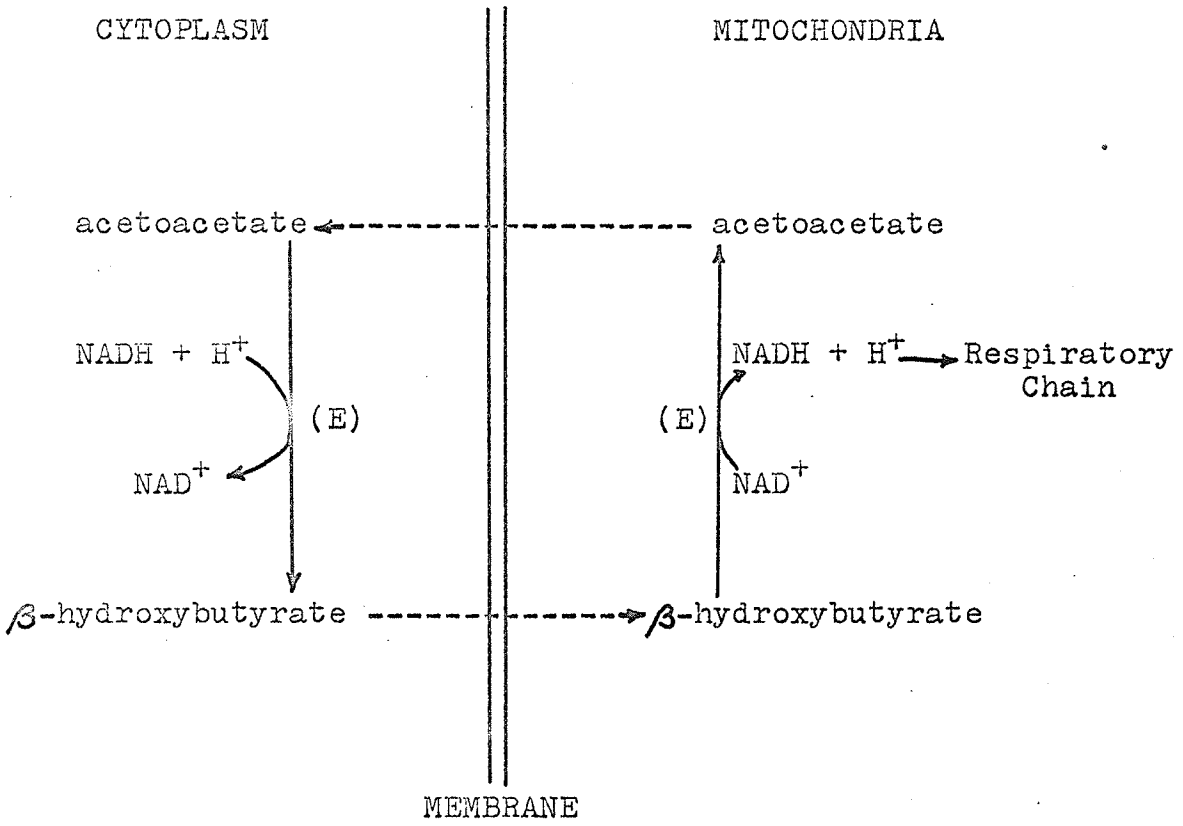


FIGURE 6. Acetoacetate/β-hydroxybutyrate shuttle

(E) D-3-hydroxybutyrate:NAD^+ oxidoreductase (EC 1.1.1.30)

acetoacetate/β-hydroxybutyrate cycle were not found to be functioning in their system. The data also indicated a similar rate of oxidation of free NADH and of enzyme-bound NADH by the mitochondrial suspensions. They concluded "in view of the uncertainties as to the structural and biochemical integrities" of isolated mitochondria, that it would be "unjustified to state that heart muscle mitochondria are incapable of catalyzing the oxidation of cytoplasmic NADH".

Studies of intracellular hydrogen transport by Boxer and Devlin (8) once again designate mitochondrial impermeability to NADH as a criterion of intactness in liver mitochondria, attributing low NADH oxidation rates to damage inflicted during preparation. The higher rates of oxygen consumption often observed in heart mitochondrial preparations implies, to Boxer and Devlin, an increased lability of the mitochondrial membrane of this tissue. These authors re-emphasize the significance of mechanisms such as the α-glycerophosphate cycle to account for physiological oxidation of cytoplasmic NADH.

Diphosphopyridine nucleotides (NAD^+ and NADH) were shown to enter liver mitochondria at a measurable rate <u>in vivo</u> and <u>in vitro</u> by Purvis and Lowenstein (39). However, their comparisons of the transport rate and respiration showed that the rate of transfer across the membrane could

in no way account for any significant portion of the respiratory rate. Lowenstein (9) reiterated the concept of mitochondrial impermeability to NADH and suggested carrier systems (shuttles) as the major mechanism of oxidation of this reduced coenzyme.

Oxidation of external NADH was studied in human and rat skeletal muscle mitochondria by Hedman, Suranyi, Luft and Ernster (40) who observed a cytochrome c stimulation of aerobic oxidation of NADH by mitochondria prepared in the Tris-KCl medium of Chappell and Perry (86). The cytochrome effect was completely suppressed by rotenone in the rat muscle preparation, but only partially affected in human muscle mitochondria. Finding no evidence that NADH oxidation proceeded by any of the proposed shuttle pathways (viz. β-hydroxybutyrate, malate/oxaloacetate or α-glycerophosphate shuttles), these authors concluded that skeletal muscle mitochondria contain an oxidative pathway for external NADH whose operation is dependent upon added cytochrome c and which is sensitive to inhibition by Amytal, rotenone and Antimycin A. Furthermore, certain water soluble CoQ homologues could be substituted for cytochrome c in some instances (see Fig. 3).

Borst (41), in his studies of NADH oxidation in Ehrlich ascites tumour cells, summarized four different types of pathways which had been postulated to that date

(1961) for the aerobic oxidation of NADH by the mitochondria of animal cells.

1. "Internal" pathway - Antimycin A-, Amytal-sensitive
   - phosphorylating
   - does not oxidize external NADH in intact mitochondria

2. "External" pathway I - Antimycin A-, Amytal-insensitive
   - non-phosphorylating
   - involves extramitochondrial cytochrome c

3. "External" pathway II - Amytal-insensitive
   - Antimycin A-sensitive or -insensitive
   - involves quinones and in some cases cytochrome c

4. Indirect oxidation by means of a substrate cycle (e.g. α-glycerophosphate cycle or β-hydroxybutyrate cycle)

In the Ehrlich cells, Borst found that none of the above pathways could satisfactorily account for the oxidation of extramitochondrial NADH, and concluded that this process involved an as yet unknown metabolic cycle.

Inhibitor studies by Ernster, Dallner and Azzone (42) demonstrated that oxidation of extramitochondrial NADH by rat liver mitochondria could be inhibited by rotenone (a partial inhibition dependent upon the presence of added cytochrome c). This observation provided further evidence for a separate cytochrome c-requiring external pathway of NADH oxidation.

Conover and Ernster (43) have studied the flavoenzyme DT diaphorase (reduced NAD(P):(acceptor) oxidoreductase,

EC 1.6.99.2) with respect to possible coupling of extra-mitochondrial NADH oxidation to the mitochondrial respiratory chain. They indicated the possibility that this vitamin K_3-requiring enzyme, found in rat liver cytoplasm, could mediate electron transfer from cytoplasmic NADH to the respiratory chain. The problem with this system, noted by the authors, lies in relating it to the physiological context since vitamin K_3 is an artificial electron acceptor. An evaluation of the possible physiological significance of this enzyme will necessitate identification of the natural substance which accepts electrons from this enzyme.

In 1963, Smith, Cheldelin and Newburgh (44) continued their earlier studies (34) on the effects of a soluble fraction from heart homogenates on oxidation and phosphorylation of external NADH by heart mitochondria. They examined the relationship of this soluble fraction to the permeability of mitochondria and the significance of NADH to this process. From the heat lability of the soluble fraction, the authors postulated the existence of an "active" protein component which implied a possible enzymatic alteration of an existing permeability barrier.

Blanchaer (10) reported studies of mitochondrial respiration from red and white guinea pig skeletal muscle. The findings in these experiments indicated that in white muscle, the α-glycerophosphate shuttle probably could couple

the oxidation of NADH in the cytoplasm to the mitochondrial respiratory chain. In contrast, the properties of the red muscle mitochondria suggested that a direct oxidation of NADH by the respiratory chain was more probable than a shuttle mechanism. This data is compatible with previous reports by Blanchaer et al. (45) and Van Wijhe et al. (46) on the quantitative and histochemical characteristics of red and white skeletal muscle. The authors also noted that the presence of both α-glycerophosphate oxidase (L-glycerol-3-phosphate:cytochrome c oxidoreductase, EC 1.1.2.1) and dehydrogenase (L-glycerol-3-phosphate:NAD^+ oxidoreductase, EC 1.1.1.8) in white fibres provided evidence for the coupling of the oxidation-reduction reactions of cytoplasmic glycolysis and mitochondrial respiration in this fibre type.

Margreth and Azzone (47) re-emphasized this distinguishing feature between red and white muscle types. They suggested that continually active muscles (i.e. red) may reoxidize NADH through the respiratory chain by either a substrate shuttle or some alternative unknown mechanism. The same authors published further evidence that glycolytically-generated, external NADH is oxidized by the mitochondrial respiratory chain through the same electron transfer pathway as that involved in the oxidation of intramitochondrial NADH. They discounted the possibility in their preparation of increased NADH oxidation as a result of

increased permeability caused by membrane damage. Furthermore, the inhibition of NADH oxidation demonstrated by the addition of oligomycin indicated that, for their system, the oxidative process may be compulsorily-linked to the formation of high energy intermediates. However, some difficulties arise in the interpretation of their oligomycin effects in homogenates. Drastic homogenization conditions or the addition of potassium chloride (10 mM) to the incubation medium greatly reduced or abolished the oligomycin inhibition. The presence and degree of inhibition also depended on the tissue used. These considerations suggest that the results of this type of experiment must be interpreted cautiously.

In a report on the mitochondrial NADH oxidase from brain mitochondrial extracts, Di Prisco et al. (48) proposed that some of the available experimental data on external NADH oxidation were not readily explained in terms of a permeability barrier to exogenous nicotinamide nucleotides. They refer to data from Ernster et al. (6), Vignais and Vignais (49) and Maley (33) in which NADH generated continuously by dehydrogenases added to the incubation media external to the mitochondria was readily oxidized by the Antimycin A-sensitive respiratory chain. The studies of brain mitochondrial extracts (48) provide evidence for the occurrence of component(s) which modify the NADH oxidative

rate by the respiratory chain. This "activating factor" was characterized as a heat stable, soluble, non-dialyzable, mitochondrial component. Disruption of the mitochondria releases the unknown factor into the soluble fraction. Recombination of particulate and soluble fractions produced increased NADH oxidase activity.

The role of magnesium in the oxidation of external NADH by rat liver mitochondria has been examined by Cereijo-Santalό (14). He emphasizes the possibility that variations in mitochondrial permeability to external NADH occur in the living cell and therefore deserve further consideration. Santalό (14) observed that rat liver mitochondria incubated with Mg^{+2} (>2 mM) did not oxidize added NADH. In comparing Santalό's data with the conditions utilized by Lehninger (2, 3), it is thus possible to explain the lack of extra-mitochondrial NADH oxidation in Lehninger's rat liver preparations by the presence of an inhibitory level (5 mM) of $MgCl_2$ in the Warburg medium. The presence of Mg^{+2} (>2 mM) in the incubation media used in experiments of other workers (16, 33) could also account for their reports of mitochondrial impermeability to added NADH. However, the Mg^{+2} concentration cannot account for the low oxygen consumption rates found by other workers (39). In the absence of added Mg^{+2}, Santalό found external NADH could be rapidly oxidized by liver mitochondria. The author attempts

to relate these observations to a possible mode of Mg^{+2} control over cellular NADH oxidation under physiological conditions. Magnesium, a known protector of mitochondrial structure, may be an important factor in the control of mitochondrial permeability. Whatever the mechanism, Santaló's data indicates that the free magnesium concentration in the medium is critical. The estimated concentration of total cellular magnesium (bound and free) in mitochondria is 3 mM and in cytoplasm is lower. The free magnesium concentration in the cytoplasm and its relationship to mitochondrial structure and to the magnesium-ATP binding is a significant aspect of the possible regulation of NADH entry into mitochondria.

Additional studies by Santaló (15) were reported on the respiratory-dependent swelling of rat liver mitochondria in sucrose and in potassium chloride media. He observed a maximal swelling rate at pH 7.0 in both sucrose and potassium chloride with NADH as substrate. The onset of swelling was delayed, however, in the potassium chloride medium. The NADH-induced swelling was resistant to rotenone, which was interpreted to indicate that the reduced pyridine nucleotide acts as a substrate of the respiratory chain. Santaló tried to relate the susceptibility of mitochondria to swelling to a proton pressure on the mitochondrial membranes exerted by the flow of electrons through the respiratory chain. He

suggested that respiratory swelling could be inhibited by an increased H^+ binding capacity (increased alkalinity or increased molarity), thus implying a link between electron transport and swelling through intramitochondrial proton production. However, insufficient evidence was presented to define a mechanism by which the proton pressure initiates the mitochondrial swelling.

An additional challenge to the theory of mitochondrial impermeability to external NADH was also reported by Cereijo-Santaló (50) in studies of aerobic glycolysis and mitochondrial swelling. He suggested glycolytically-induced mitochondrial swelling may render mitochondria permeable to NADH. A causal relationship was detected between mitochondrial swelling and the failure of glycolytic lactate formation. Cereijo-Santaló postulated that swelling increases permeability to NADH, inducing a competition for the cytoplasmic reduced coenzyme and consequently inhibiting lactate production.

Blanchaer, Lundquist and Griffith (11), using heart mitochondria, published further evidence compatible with direct mitochondrial permeability to external NADH, contrary to the generalization proposed by Lehninger (31). Studying pigeon heart mitochondria, Blanchaer and his coworkers found that physiological concentrations (1-10 μM) of NADH could be oxidized in preparations of acceptable metabolic integrity

(see Introduction). The oxidative process was modified by ATP, ADP, NAD^+ and P_i. The oxidation of external NADH observed which was sensitive to Amytal and Antimycin A, implied that the reaction pathway was that of normal respiratory electron transport. Further studies on the control of NADH oxidation in pigeon heart mitochondria were published by Blanchaer and Griffith (12) which indicated that NADH oxidation might be a normal feature of intact heart mitochondria. In these experiments, the authors demonstrated that accessibility of the added NADH to the mitochondria is dependent, at least in part, on the concentrations of Mg^{+2}, P_i, ADP, ATP and NAD^+. Blanchaer and Griffith (12) also proposed that access of physiological concentrations of NADH (<10 μM) to the respiratory chain may be controlled at the enzymatic level. They suggest that higher non-physiological NADH concentrations (0.1-1.0 mM) used in earlier studies may be a significant factor in the differences evident from work performed subsequently in the lower physiological range. The experimental evidence of Blanchaer *et al.* (11) and Blanchaer and Griffith (12) indicated that in pigeon heart mitochondria oxidation of cytoplasmic NADH could be a property of intact preparations. Griffith and Blanchaer (13) presented additional kinetic evidence for this system which is in agreement with a possible physiological role for direct oxidation of external

NADH by mitochondria. Their report described a potential regulatory mechanism for this oxidative process by the cellular ATP which was an effective inhibitor at concentrations within its reported intracellular range (72). The mode of inhibition is unlikely an effect on permeability since sonication did not alter the sensitivity to the nucleotide. Furthermore, the insensitivity of the inhibition to oligomycin or 2,4-dinitrophenol appears to eliminate an effect via the coupling enzymes of oxidative phosphorylation. Blanchaer (51), in later kinetic experiments on the effects of adenine nucleotides on the respiratory chain, has found using beef heart electron transport particles, that the ATP inhibition occurs in the respiratory chain at the NADH dehydrogenase level, but is more complex than a simple competitive mechanism.

Stopkie and Weber (52) studied the control of NADH oxidation using membranes from Mycoplasma laidlawii. Earlier experiments by Pollack et al. (53) had localized an NADH oxidase activity in the membrane fraction. The more recent experiments of this group (52) demonstrate an inhibitory effect of ADP observed on NADH oxidation which they interpret as a mode of control over the membrane-localized oxidase activity. The suppression of activity effected by ADP was to be associated only with the membrane fraction. There was no nucleotide effect observed on the

NADH oxidase in the *in situ* soluble fraction.

Substrate utilization (including NADH) has been examined by Papa *et al.* (16) in calf retina mitochondria. These preparations did not oxidize NADH directly, nor through the β-hydroxybutyrate or α-glycerophosphate cycles. The authors proposed the possibility of an oxidative mechanism involving an active NAD(P)H dehydrogenase in retina mitochondria in the presence of added 2-methyl-1,4-naphthoquinone (vitamin K_3). The enzyme studied by these authors appears similar to the DT diaphorase examined by Conover and Ernster (43). However, as has been noted above in relation to the work of the latter authors, vitamin K_3 is not a normally occurring electron acceptor. In both instances, the failure to relate the function of the enzyme to a natural electron acceptor casts some doubt on the physiological significance of the findings.

The validity of the cytochrome *c*-requiring external pathway of extramitochondrial NADH oxidation discussed earlier has been challenged by several workers including Schneider (54) and Schollmeyer and Klingenberg (55). Their findings indicated the absence of any cytoplasmic cytochrome *c*. This discovery should exclude the existence of any physiological cytochrome *c*-stimulated extra-mitochondrial pathway for NADH oxidation. That such an external system does or does not exist has yet to be firmly

established, since both views have received additional support. There is also some controversy as to whether the external pathway is, in fact, mitochondrial or resides in a contaminant present in the mitochondrial preparations. Thus, Sottocasa (56) has reported some of the biochemical properties of inner and outer mitochondrial membrane preparations from rat liver in which enzyme distribution data of the membrane sub-fractions indicated that the outer mitochondrial membrane contains the NADH-cytochrome c reductase system responsible for the "external" pathway of cytoplasmic NADH oxidation. Localization of the rotenone-insensitive NADH-cytochrome c reductase has been used by others as a marker for the outer mitochondrial membrane (57, 73-75).

However, very recently (1968) this assumption has been seriously questioned by work from the laboratory of D.E. Green (58). They present evidence that the rotenone-insensitive NADH-cytochrome c reductase activity is not an intrinsic property of the outer mitochondrial membrane. They attribute the "erroneous" conclusions in previous reports to incomplete separation of the various membrane and particulate sub-fractions examined biochemically. Green et al. (58) have concluded that the rotenone-insensitive NADH-cytochrome c reductase activity is a consequence of microsomal contamination of mitochondrial membrane

preparations, and is not a mitochondrial property. The findings reported in the present thesis are in agreement with this view on the localization of the cytochrome c-stimulated rotenone-insensitive pathway.

The problem of external NADH oxidation has also been dealt with in a variety of non-animal tissues. Ikuma and Bonner (60), using mitochondria isolated from mung bean hypocotyls, found that in contrast to many of the reports on animal mitochondria, NADH was oxidized at a substantial rate and exhibited respiratory control (RCR = 3-4.5). Ohnishi et al. (61) also reported coupled oxidation of NADH added to yeast mitochondria. The active oxidation of external NADH exhibited respiratory control and ADP:O ratios of about 1.9. The latter finding, and the insensitivity of respiration to Amytal and rotenone, suggested that phosphorylation site I (see Fig. 3) was absent from yeast mitochondria studied by Ohnishi. Oxidation of added NADH has been observed by Watson and Smith (62) in mitochondria isolated from Aspergillus niger. Both phosphorylation and respiratory control were reported. The oxidation was relatively insensitive to Antimycin A and oligomycin, but surprisingly could be uncoupled by 2,4-dinitrophenol. In a later report, Watson and Smith (63) proposed a mechanism involving a bypass of phosphorylation site I and hence of the rotenone and Antimycin A sites. The authors suggest that coupled

oxidation of exogenous NADH proceeds via a non-phosphorylating pathway from NADH to cytochrome b and thence through the normal respiratory chain. Although their mitochondria showed respiratory properties similar to those of intact yeast and mammalian preparations, the external pathway for Aspergillus niger suggested by Watson and Smith (63) exhibits a distinctive feature in that it displays both coupling and phosphorylation. This peculiarity, which has never been demonstrated in animal mitochondria, suggests the Aspergillus pathway is not necessarily analogous to the currently described mammalian system.

The problem of mitochondrial membrane permeability to reduced pyridine nucleotides also arises in circumstances requiring the production of increased quantities of cytoplasmic reducing equivalents (i.e. during active gluconeogenesis NADH is required as the hydrogen donor for several cytoplasmic reactions and thus a "reverse" flow of NADH (or reducing equivalents) is required from the mitochondria to provide the reducing power to the extra-mitochondrial system). An examination of the mode of generation of this cytoplasmic reducing power for gluconeogenesis by Krebs, Gascoyne and Notton (64) in kidney cortex slices contains reference to the impermeability of the mitochondrial membrane to pyridine nucleotides. Although their studies examined transport of NADH from the

mitochondria to the cytoplasm, the malate/oxaloacetate shuttle system they propose (Fig. 7) may have some relevancy to the reverse situation (i.e. removal of cytoplasmic NADH). Borst (65), discussing this pathway, indicated a major disadvantage to the malate/oxaloacetate system, viz. the sum equation (Equation 3) is zero, unlike that for the α-glycerophosphate shuttle which is highly exergonic (Equation 4).

$$NADH + NAD^+ \rightleftharpoons NAD^+ + NADH \qquad (3)$$

$$NADH + fp + H^+ \rightleftharpoons NAD^+ + fpH_2 \qquad (4)$$

Under these conditions the malate/oxaloacetate cycle could only function when a marked concentration difference can be maintained between the cytoplasm and the mitochondria for one of the metabolites in the cycle. This concentration gradient is required to provide the "push" necessary to overcome the thermodynamic disadvantages of the proposed mechanism. Sacktor et al. (87), studying rat leg muscle in situ demonstrated elevations in lactate (3-fold), α-glycerophosphate (4-fold) and L-malate (1.5-fold) during 100 contractions of the muscle. They suggest these three substrates contributed to increased oxygen consumption, a large proportion of which was mediated through the α-glycerophosphate cycle. Whether the increase in L-malate concentration observed is sufficient to provide the

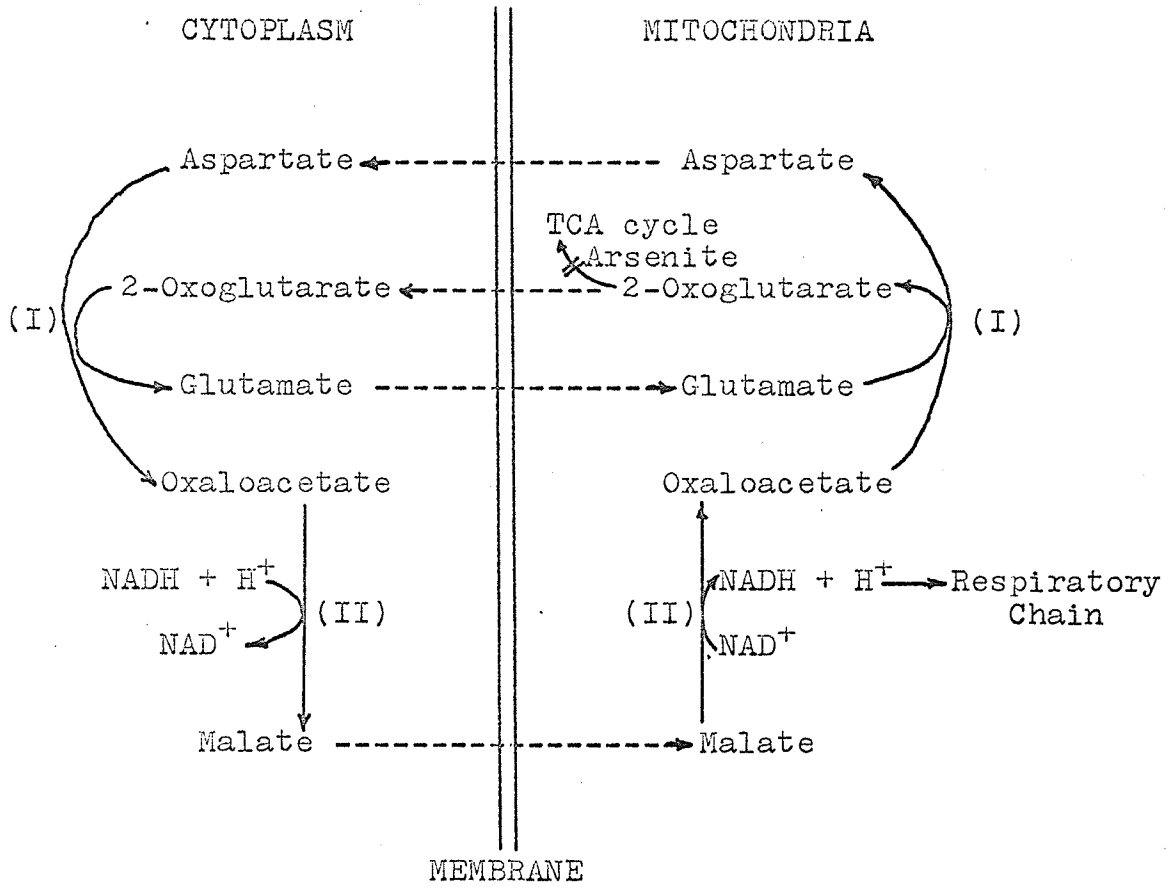


FIGURE 7. Malate/oxaloacetate shuttle

(I) Glutamate:oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1)

(II) Malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37)

To demonstrate the shuttle, it is necessary to add arsenite to block the side reaction of 2-oxoglutarate (α-ketoglutarate) into the tricarboxylic acid cycle (17).

thermodynamic "push" required to operate the malate/oxaloacetate shuttle is not known. However, doubt in this regard is raised by the results of Williamson (88) who has shown that incubation of perfused rat heart with pyruvate led to an increased mitochondrial NADH concentration and an increased cytoplasmic NAD/NADH ratio. This information suggests that the direction of movement of NADH (thermodynamically) would be from mitochondria to cytoplasm, rather than in the other direction. Williamson's data are therefore compatible with the proposal by Krebs et al. (64), discussed earlier, that the malate/oxaloacetate shuttle functions to transfer reducing equivalents from mitochondria to the cytoplasm during active gluconeogenesis.

More extensive studies on the role of various substrate cycles as hydrogen carriers in the transport of extramitochondrial NADH into mitochondria during aerobic metabolism have been published by Hassinen (17) using rat liver mitochondria. This work revealed that neither the acetoacetate/β-hydroxybutyrate pair nor the dihydroxyacetone-phosphate/α-glycerophosphate pair functioned as the mechanism for exogenous NADH oxidation in these preparations. However, Hassinen (17) reported evidence compatible with the operation of the malate/oxaloacetate shuttle (Fig. 7) in his preparations. The addition of aspartate and oxoglutarate to the mitochondrial suspension had no effect on respiration

with external NADH as substrate. However, in a system containing mitochondria, malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) and glutamate:oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) he observed a five-fold stimulation of NADH oxidation upon addition of oxaloacetate and aspartate, the substrates of the external enzymes. Arsenite was also present in the system to prevent removal of oxaloacetate through the tricarboxylic acid cycle. Hassinen (17) attributed this increase to the oxidation of malate formed extramitochondrially from oxaloacetate and NADH by the added malate dehydrogenase and concluded that the malate/oxaloacetate cycle plays a significant role in the transport of reducing equivalents from NADH in the cytoplasm into the mitochondria of rat liver. In spite of this evidence, the immediate acceptance of a physiological role for the malate/oxaloacetate shuttle as a mechanism of hydrogen transfer has been rendered somewhat more difficult by the recent data reported by de Haan and Tager (66). These workers demonstrated direct evidence for a permeability barrier to 2-oxoglutarate (α-ketoglutarate) in rat liver mitochondria. Such a barrier had earlier been suggested by the studies of Meijer and Tager (67) and Chappell *et al.* (68) in which a specific membrane transport system for 2-oxoglutarate was discussed. This obstacle to the passage of a shuttle

intermediate could establish a serious rate-limiting step in the function of the malate/oxaloacetate system. However, it was also observed (66) that L-malate increases mitochondrial permeability to 2-oxoglutarate. The concentration of L-malate in the cytoplasm might be sufficient to stimulate 2-oxoglutarate passage and maintain the operation of the shuttle mechanism. Azzi et al. (69) have revealed the existence of two more carrier systems involving L-glutamate and L-aspartate respectively. The latter system requires the presence of L-glutamate for its operation.

Haslam and Krebs (28, 70) and Griffiths and Haslam (71) have presented a thorough examination of the penetration of L-malate and oxaloacetate into mitochondria. They have reported (70) that although the mitochondrial membrane is permeable to both these substances, the diffusion rate of oxaloacetate is very slow. The authors (71) propose that oxaloacetate and L-malate penetration is an energy-facilitated process, requiring special permeability mechanisms. In correlating the rate of L-malate entry into mitochondria with the rate of oxygen consumption in rat heart preparations, Haslam and Krebs (28) found that the observed rate was only 3.5% that of the calculated minimum physiological requirement for transport of reducing equivalents into the mitochondria. It would appear that on the basis of these results (28, 66-71), the requirements for

a feasible malate/oxaloacetate cycle become much more complex. The simple cycling of metabolites is complicated by the imposition of barriers and metabolic controls over the individual components of the system. The possible operation of the malate/oxaloacetate shuttle for the transfer of reducing equivalents from the cytoplasm to mitochondria could therefore involve a complex interplay of a variety of physiological controls. Some of these relationships are illustrated in the system proposed by Chappell (18) for the oxidation of cytoplasmic NADH by mitochondria (Fig. 8). The movement of some or all of the substrate anions is linked to exergonic reactions and involves several transport systems.

Chappell's report (18) is compatible with the present status of shuttle systems as the mode of reoxidation of cytoplasmic NADH. Although it is doubtful that the acetoacetate/β-hydroxybutyrate shuttle has any physiological relevance (37), there is a variety of evidence to support the contention that the α-glycerophosphate shuttle may function as the pathway of oxidation in tissues such as liver (8) and skeletal muscle (10, 27), both of which contain the required complement of enzymes and intermediates. In other tissues such as myocardium, which lacks the mitochondrial α-glycerophosphate oxidase (26), the malate/oxaloacetate system has been suggested as a possible alternative (17, 64). Significant objection has been raised

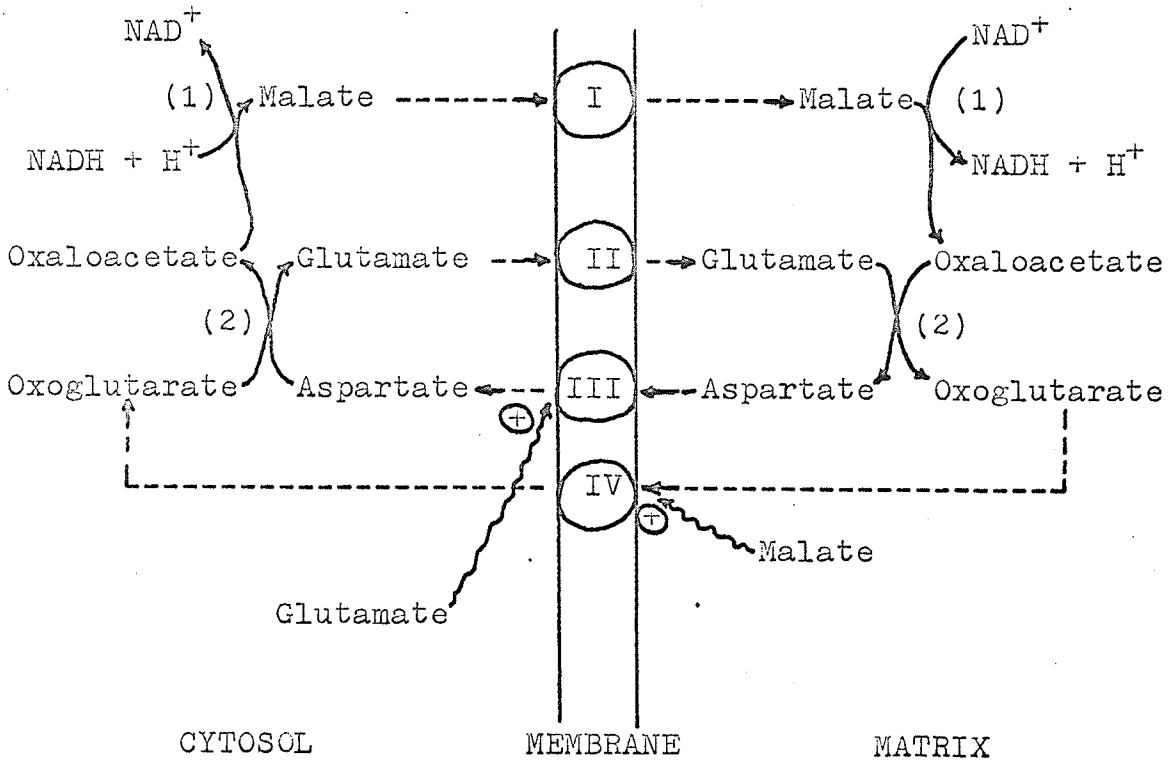


FIGURE 8. The role of some transporting systems in the oxidation of NADH by mitochondria (taken from Chappell (18))

I : malate transporter
II : glutamate transporter
III : aspartate transporter (glutamate-activated)
IV : oxoglutarate transporter (malate-activated)

(1) Malate dehydrogenase
(2) Aspartate aminotransferase

Activating effects of compounds on carriers are indicated by wavy arrows with positive signs.

against the physiological operation of this mechanism (65, 87, 88) including data in this thesis. Since no convincing evidence is available that any of the shuttles proposed in the literature are operative in heart tissue, alternative hypotheses must be considered. The oxidation of added NADH by an external cytochrome c-stimulated rotenone-insensitive pathway has also been suggested (3, 56, 57). However, in view of recent evidence in the literature (58, 91) and the data of this thesis, such oxidation is probably attributable to microsomal contamination of the preparations and is not a characteristic property of mitochondria. Direct permeability of myocardial mitochondria to NADH remains a valid hypothesis for which no well-documented alternative has yet been proposed.

It is clear from this review that the available evidence does not favour any one of the various theories suggested to explain mitochondrial oxidation of cytoplasmic NADH. Indeed, the possibility of as yet unknown mechanisms of reoxidation cannot be eliminated. The mode of oxidation may in fact be related to, or dependent upon, the source of the isolated mitochondria. The future examination of all potential mechanisms will require serious consideration of their relationship to the physiological requirements of the cell.

MATERIALS AND METHODS

MATERIALS

Chemicals

The following chemicals were obtained from the Sigma Chemical Company:

Adenosine-5'-triphosphate (ATP) from equine muscle, disodium salt (crystalline)

Antimycin A from *Streptomyces kitazawaensis* mycelia, crystalline, Type III

Bovine serum albumin, crystalline

Glutamic:oxaloacetic transaminase, ammonium sulphate, ketoglutarate, maleate suspension

Lactic dehydrogenase from rabbit muscle, crystalline suspension in ammonium sulphate, Type I

L-malic acid

D-mannitol, recrystallized from 0.1 mM EDTA before use

Nicotinamide adenine dinucleotide (reduced), disodium salt, Grade III

Pyruvic acid, sodium salt

Trizma base, Tris(hydroxymethyl)aminomethane, reagent grade

Trizma-HCl, Tris(hydroxymethyl)aminomethane hydrochloride, reagent grade

The following chemicals were obtained from British Drug

Houses Ltd.:

Ethylenediamine tetracetic acid, disodium salt, Analar reagent

Potassium chloride, Analar reagent

Sucrose, Analar reagent

The following chemicals were obtained from the companies indicated:

Adenosine-5'-diphosphate, sodium salt (P-L Biochemicals, Inc.)

Bovine albumin powder, Fraction V from bovine plasma (Armour Pharmaceutical Co.)

Amobarbital sodium U.S.P. (Eli Lilly and Co. (Canada))

Calcium chloride, dihydrate, granular (Baker Chemical Co.)

2,4-Dinitrophenol (Matheson, Coleman and Bell)

Malate dehydrogenase, suspension (Boehringer Mannheim Corporation)

Nagarse, crystallized lyophilized bacterial proteinase (Nagase and Co. Ltd., Osaka, Japan)

Rotenone (K + K Laboratories)

Sephadex, G-25, coarse (Pharmacia Ltd., Uppsala, Sweden)

Sodium dithionite, sodium hydrosulphite powder (Mallinckrodt Chemical Works Ltd.)

Composition of Media

The following media are those routinely used throughout this study in the preparation of pigeon heart mitochondria. Variations of these media or other special preparations will be described as part of the particular experiment(s) in which they occur. In each case final concentrations are given.

1. Stock medium (pH 7.4 at 22° C)
   - 0.21 M mannitol
   - 0.07 M sucrose
   - 0.10 mM EDTA
2. Homogenizing medium
   - 40 ml stock medium
   - 0.01 M Tris-phosphate (pH 7.6 at 22° C)
   - 20 mg Nagarse
3. Suspending medium
   - 25 ml stock medium
   - 0.01 M Tris-HCl (pH 7.4 at 22° C)
4. Cuvette medium (pH 7.2 at 22° C)
   - 0.23 M mannitol
   - 0.07 M sucrose
   - 0.02 M Tris-HCl (pH 7.2 at 22° C)
   - 0.02 M EDTA
   - 5.0 mM phosphate

Pigeons

The pigeons utilized in these experiments represented a random population. Only birds which were healthy in appearance and without visible heart defects were used. No attempt was made to classify the pigeons on the basis of age, sex or species, and all were used in unfasted condition.

METHODS

Preparation of Mitochondria (composition of reagents is given in Materials section)

Pigeon heart mitochondria were prepared according to the method of Chance and Hagihara (76). The pigeon was decapitated and the heart rapidly removed and placed in ice-cold stock medium. All subsequent manipulations were performed in the cold (0 - 4° C) using equipment and solutions pre-cooled on ice. After a second rinsing in ice-cold stock medium, the heart was thoroughly minced with scalpels (blade No. 22, Bard-Parker Co. Inc.).

In the preparation of control mitochondria (see glossary), 3-4 grams of minced tissue were used while the modified mitochondria were prepared with only 0.25 g of mince in 40 ml homogenizing medium.

The mince was incubated for 8 minutes in 40 ml of homogenizing medium in a size C glass grinding vessel (A.H. Thomas Co., Philadelphia, Pa.). The sample was then homogenized with six passes of a loose-fitting Teflon pestle (0.33 mm clearance). Following a second 8 minute incubation, the homogenate was diluted with 40 ml of stock medium and rehomogenized using six passes of a tight-fitting Teflon pestle (0.10 mm clearance). The resulting homogenate was then centrifuged at 480 x g (2000 rpm) for 5 minutes in

a Sorvall RC2-B refrigerated centrifuge with an SS-34 head. The supernatant was removed by pipette from the sedimented cellular debris and centrifuged again at 12,100 x g (10,000 rpm) for 10 minutes. The supernatant was discarded and the fluffy white layer rinsed from the crude mitochondrial pellet by swirling with 1-2 ml of suspending medium. The rinsed pellet was then resuspended in an aliquot of suspending medium using a Vortex mixer and centrifuged at 7,710 x g (8,000 rpm) for 5 minutes. The pellet remaining after the supernatant had been discarded was resuspended in suspending medium to give the protein concentration desired for that particular experiment. A flow scheme of the preparation technique is illustrated in Fig. 9.

Respiration is a major function of mitochondria, involving the transfer of electrons along the respiratory chain to the terminal acceptor, oxygen. The measurement of oxygen consumption with various substrates represents a convenient method of comparing mitochondrial preparations. Furthermore, other aspects of mitochondrial behaviour are readily calculated from measurements of oxygen consumption. A variety of techniques exists for determining respiration rates, including manometric and polarographic methods. The latter system was used extensively in the present study because of its convenience and simplicity.

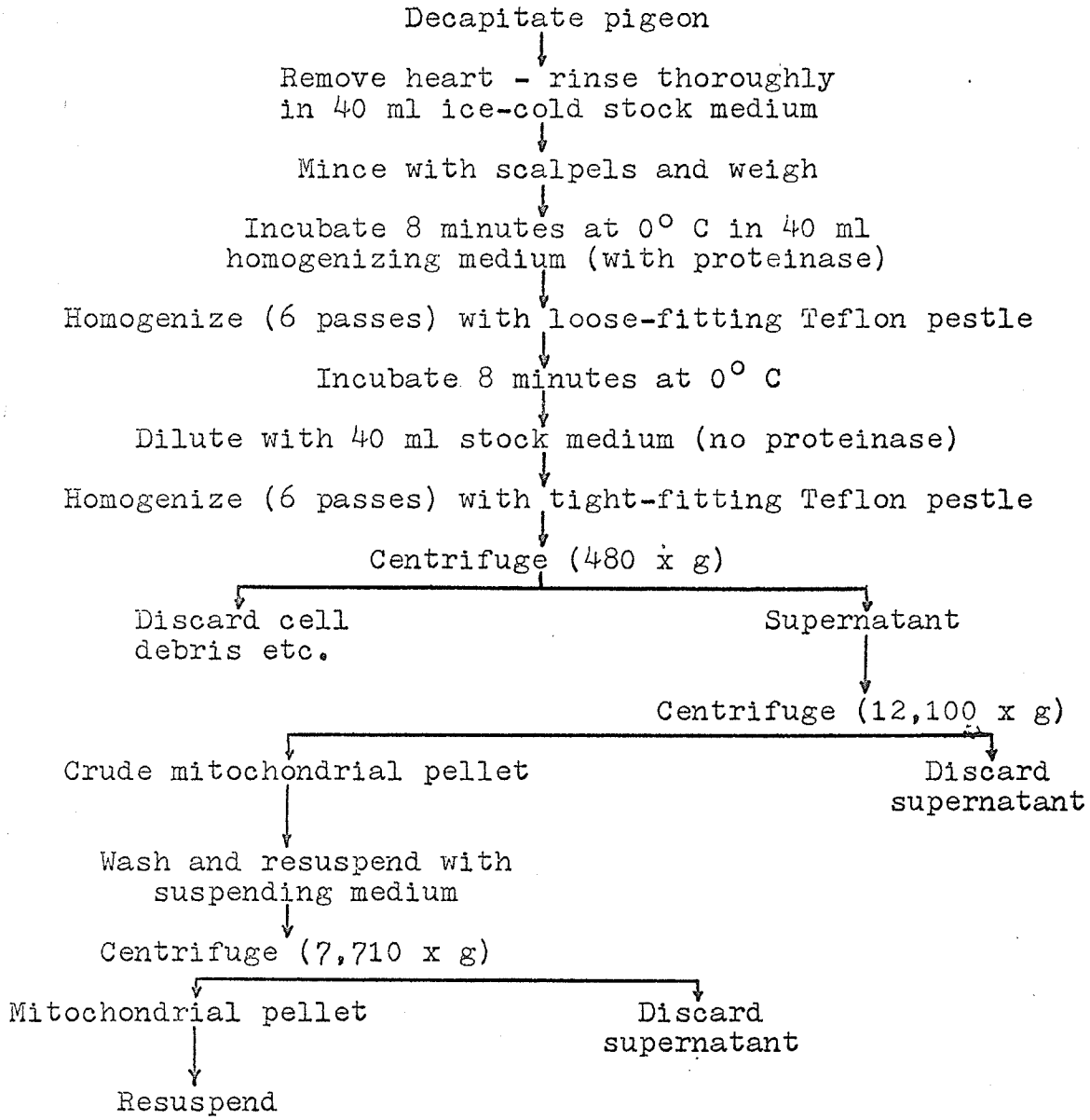


FIGURE 9. Flow scheme showing preparation of pigeon heart mitochondria by the method of Chance and Hagihara (76)

It was desirable in certain experiments to determine the relationship of substrate (NADH) utilization and oxygen consumption. Using a vibrating platinum electrode (American Instrument Co.) for oxygen measurements and a fluorometer (American Instrument Co.) for NADH oxidation, the two parameters could be monitored simultaneously and compared.

Kinetic studies were performed with physiological NADH concentrations (1-10 μM). To detect the oxidation of these low concentrations of NADH, the sensitivity available through fluorometric measurement is required. Parallel determination of oxygen consumption was not necessary in these experiments.

The experimental details of the oxygen and NADH measurements are given in the following sections.

Measurement of Oxygen Consumption

1. Polarographic

The application of the polarographic oxygen electrode technique to the study of respiration and oxidative phosphorylation was first described by Chance and Williams (77). They reported that a linear relationship existed between the current generated at the electrode and oxygen consumption ($\pm$ 10%).

Polarographic measurements in the present experiments

were made at 28° C on a Model KM Oxygraph (Gilson Medical Electronics) using a Clark (78) electrode assembly (Yellow Springs Instrument Co.) with a Teflon membrane and a polarizing voltage of 0.8 volts. A measured quantity of temperature-equilibrated cuvette medium was added to the reaction vessel with constant mixing by a Cole-Parmer Micro V magnetic stirrer and magnetic flea bar. The chart drive was adjusted to give the correct time scale for the measurements and the ordinate of the chart calibrated such that 36 divisions (18 cm) were equivalent to 250 μM O_2 (uncorrected for barometric pressure). Mitochondria containing 0.350 - 0.800 mg protein were added to the cuvette and the ground glass stopper was fixed in place in the cell. All further additions were made through the capillary tube in the cuvette stopper using Hamilton Microlitre syringes. The reaction vessel and electrode assembly are shown in Fig. 10. The recordings from typical control experiments with pyruvate + malate and NADH as substrates are illustrated in Fig. 11a and 11b.

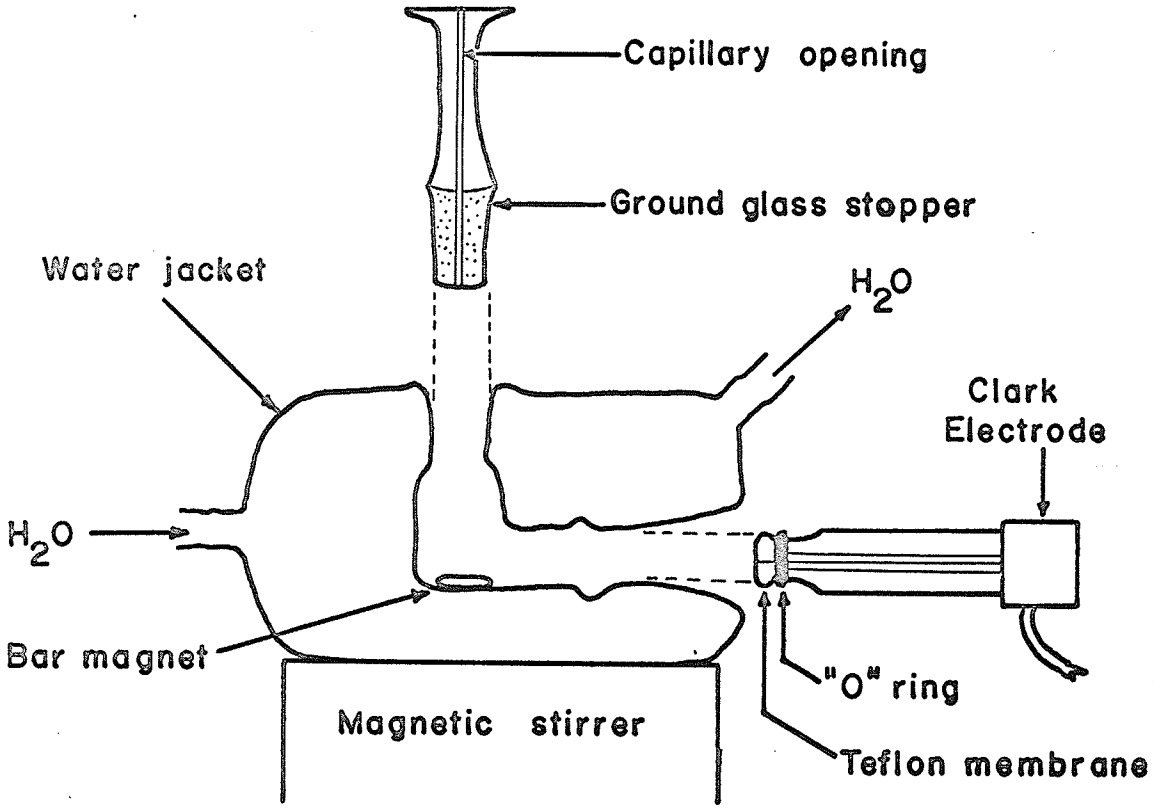


FIGURE 10. Reaction vessel and electrode assembly

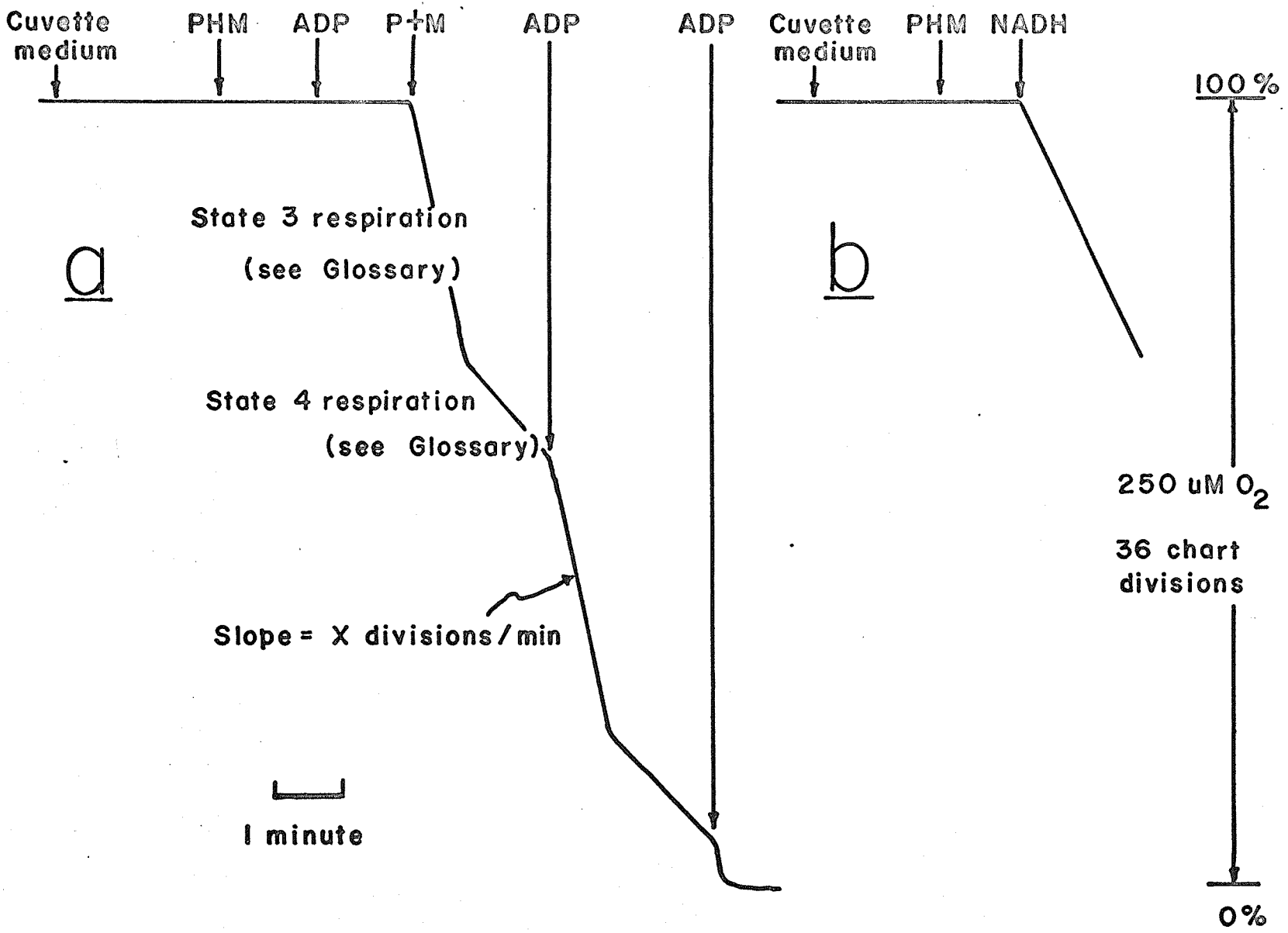
FIGURE 11. Typical control experiment showing polarographic measurement of oxygen consumption with pyruvate + malate and NADH as substrates.

a. Pyruvate + malate

b. NADH

PHM = pigeon heart mitochondria

P+M = pyruvate + malate


Cuvette medium
PHM
ADP
P+M
ADP
ADP
a
State 3 respiration
(see Glossary)
State 4 respiration
(see Glossary)
Slope = X divisions / min
1 minute
Cuvette medium
PHM
NADH
b
100 %
250 uM O_2
36 chart divisions
0%

2. Calculations (see Fig. 11)

(i) Oxygen consumption (polarographic)

$$\text{Respiration rate} = \frac{(O_2)(X)}{36} \cdot \frac{Y}{1000} \cdot \frac{1}{Z} \cdot 1000$$

$$= \mu\text{moles } O_2/\text{minute/g protein}$$

where X = slope of recorder tracing (divs/min)

Y = total volume in cuvette (ml)

Z = mitochondrial protein in cuvette (mg)

O_2 = oxygen concentration (μM) at 100% saturation (28° C), corrected for barometric pressure

36 = total number of chart divisions (ordinate)

(ii) Respiratory control ratio (see glossary)

The respiratory control ratio is calculated by the formula:

$$\text{RCR} = \frac{\text{Rate of state 3 respiration (divs/min)}}{\text{Rate of state 4 respiration (divs/min)}}$$

(iii) ADP:O ratio (see glossary)

The ADP:O ratios were calculated using a program prepared for the Programma 101 desk computer (Olivetti Underwood). The program was based on the following formula, assuming 36 ordinate chart divisions represent 100% oxygen and 10 μl ADP are added.

$$\text{ADP:O} = \frac{(0.01)(X)(36)}{(Y)(O_2)(2)(Z)}$$

where X = concentration of ADP (mM) added

Y = total number of divisions (ordinate) traversed in utilizing the ADP added

Z = total volume in cuvette (ml)

O_2 = oxygen concentration (μM) at 100% saturation (28° C), corrected for barometric pressure

36 = total number of chart divisions

2 = conversion factor for oxygen from molecules to atoms

0.01 = volume of ADP solution added (ml)

Combined Measurement of Oxygen Consumption and NADH Oxidation

As was indicated previously, it was desirable in certain experiments to monitor the oxygen consumption and NADH oxidation simultaneously. The former parameter was determined using a vibrating platinum electrode (American Instrument Co.). Both measurements were carried out at 28° C and recorded on a self-balancing potentiometric recorder (Texas Instruments Inc.). Mitochondria containing 0.500 - 1.50 mg protein were suspended in cuvette medium in 1 cm fluorometer cells. With the oxygen electrode in place, the vibration amplitude was set at 60 on an arbitrary scale of 100, and the sensitivity adjusted to give a chart span of 90 divisions (10.3 cm) equivalent to 250 μM O_2 (uncorrected for barometric pressure). All further additions were performed through the top of the electrode assembly using Hamilton Microlitre syringes with manual stirring for a few seconds

after each addition. Oxygen consumption rates, RCR's and ADP:O ratios were calculated as described previously in these Methods.

The simultaneous oxidation of NADH was measured by the use of the fluorescent properties of this reduced pyridine nucleotide. Excitation of NADH by light of 365 mμ produces a fluorescence spectrum at longer wavelengths. Since NAD^+ does not exhibit this property, the oxidation of NADH can be followed by the decrease in fluorescence emission at 436-466 mμ. A non-linear response to the concentration of NADH was observed and therefore a calibration curve (Fig. 12) over the range of NADH concentrations studied was prepared for each experiment. The sensitivity, dark current, zero and blank subtract adjustments were all made with 0.500 - 1.500 mg of mitochondrial protein suspended in 2.5-3.0 ml final volume in the reaction cuvette. After setting the chart speed, depending on the rate of oxidation, NADH was added to the cuvette by Hamilton Microlitre syringe with manual stirring for a few seconds. The rates of oxidation were calculated by replotting the observed rates using the calibration curve to give a linear function (Fig. 13). The calibration curve shows a plot of the actual NADH concentration (μM) versus the number of ordinate chart divisions. The number of ordinate chart divisions at arbitrary points on the recorder tracing are used in

FIGURE 12. Typical calibration curve of NADH fluorescence over the concentration range 0-50 μM.

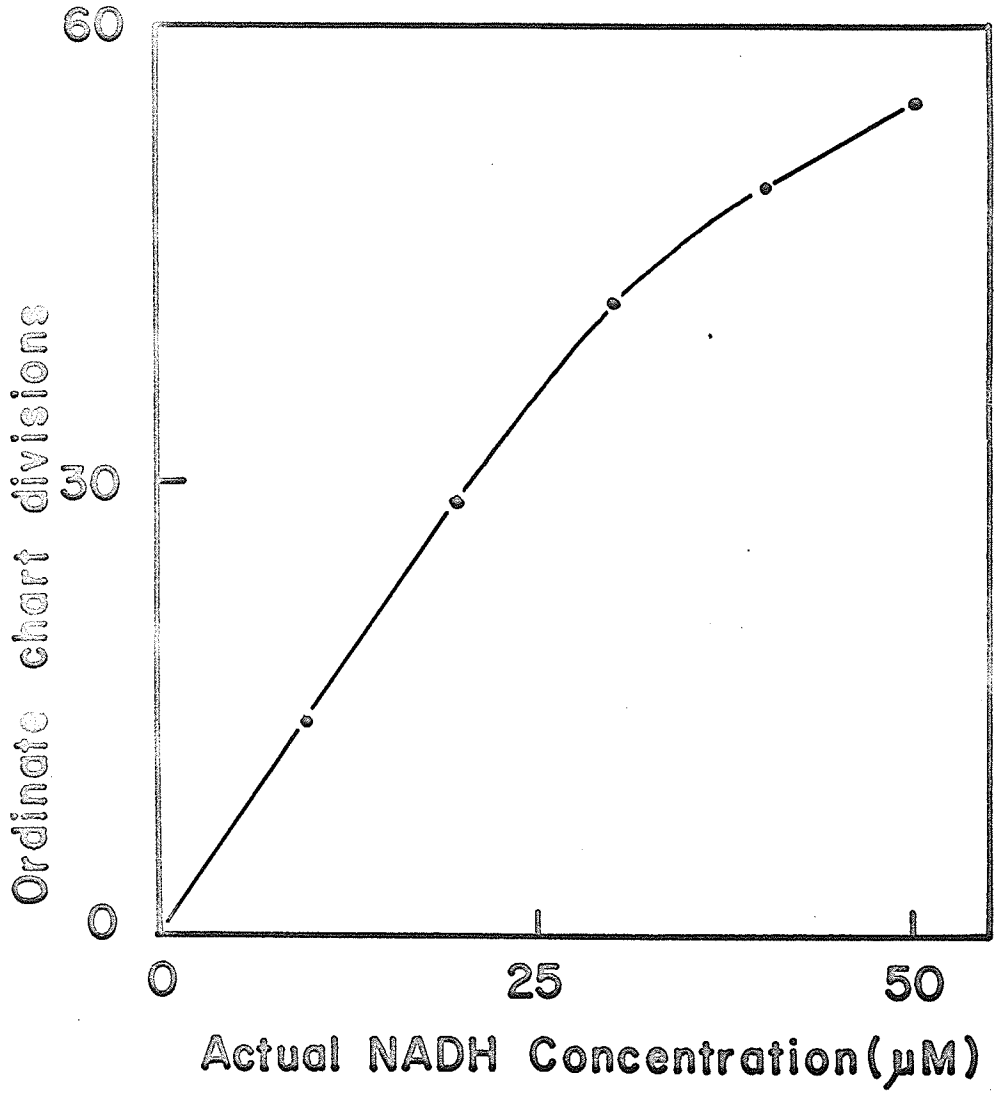
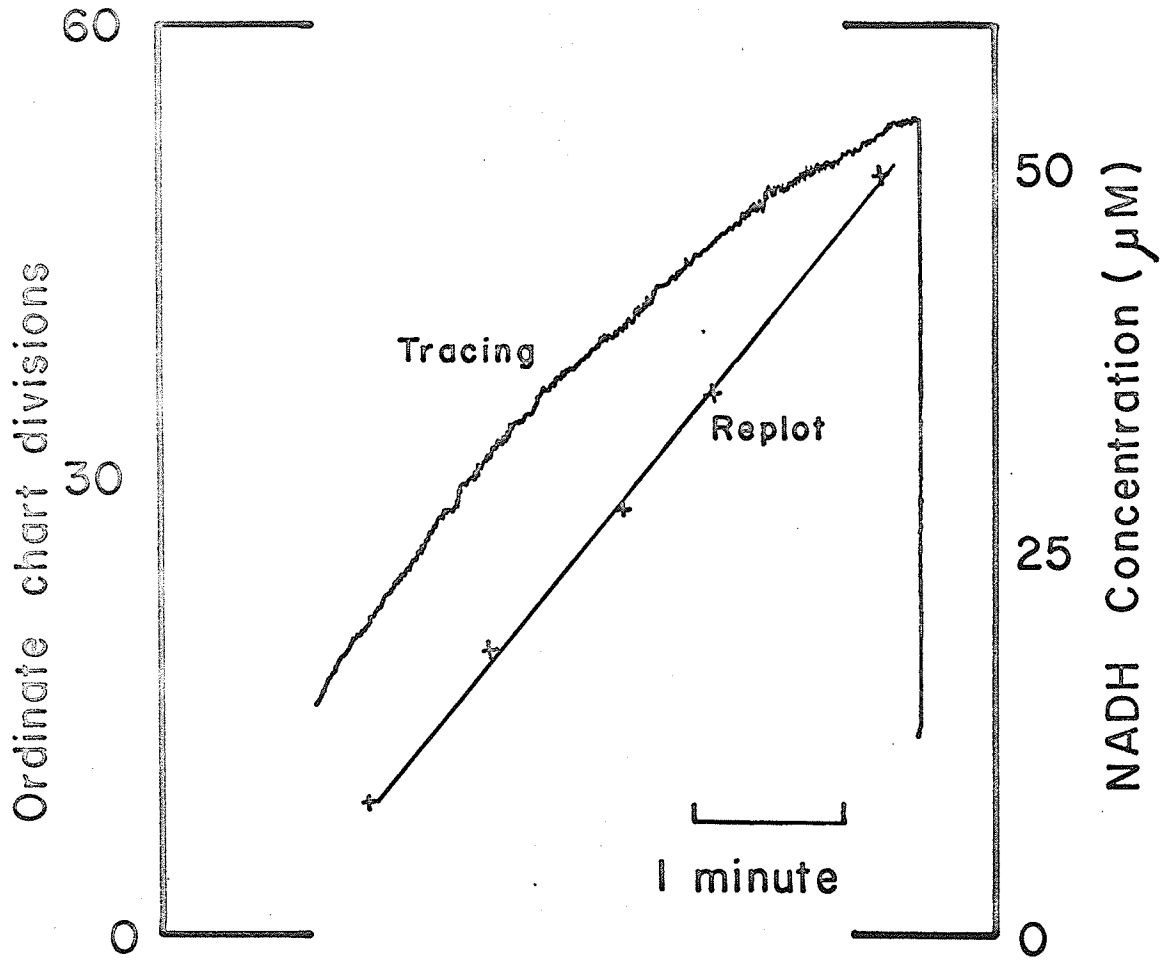

60
30
0
Ordinate chart divisions
0
25
50
Actual NADH Concentration (μM)

FIGURE 13. Fluorescence tracing and replot of oxidation of 50 μM NADH.


60
30
0
Ordinate chart divisions
Tracing
Replot
1 minute
50
25
0
NADH Concentration (μM)

conjunction with the calibration curve to obtain the corresponding NADH concentration (μM) at these points. These values can be replotted directly below the fluorescence tracing by converting the vertical scale into concentration units. Using the appropriate factors for dilution and mitochondrial protein, the NADH rate (μmoles/minute/g mitochondrial protein) can be calculated from the straight line plot of concentration changes (see Figs. 12 and 13).

Fluorometric Measurement of Oxidation of Physiological NADH Concentrations

All studies of NADH kinetics in the control and modified pigeon heart mitochondrial preparations were performed on the fluorescence attachment of the Eppendorf 1100M photometer. The decrease in fluorescence as NADH was oxidized was recorded on a Honeywell Electronik 19 recorder (50 mv). In all experiments, the primary exciting wavelength was obtained using a Hg 313 + 366 mμ filter in conjunction with a secondary filter of 470-3000 mμ.

Mitochondria (0.300 - 0.500 mg protein) were added to cuvette medium (28° C) (total volume 3.0 ml) in a quartz cuvette and a baseline established on the recorder chart. NADH (1-10 μM final concentration) was added by Hamilton Microlitre syringe, followed by brief rapid mixing. The reaction was followed until the NADH had been completely

utilized.

For inhibitor experiments with ADP (12, 13), the nucleotide was added to the reaction vessel before the mitochondria. After addition of the mitochondria, the reaction mixture was incubated for 1 minute before addition of NADH. The method of initial velocity calculation is described in section I of the Appendix.

Swelling Measurements

1. Turbidity

Measurements of mitochondrial swelling were made using the Beckman DU spectrophotometer at a wavelength of 520 mμ where an inverse relationship exists between optical density and volume (92).

Suspensions of mitochondria (0.050 - 0.300 mg protein) were added to 3.0 ml of cuvette medium (28° C) and the optical density read at 520 mμ against a blank containing cuvette medium only. Comparisons between samples were only valid when the protein concentrations, measured subsequently by the method of Lowry et al. (79), were similar (± 5.0%).

2. Volume changes

The absorbancy difference at the two wavelengths λ_1 = 540 mμ and λ_2 = 630 mμ on the Aminco-Chance dual

wavelength spectrophotometer was used to follow mitochondrial swelling changes during various states of respiration. The operating theory of this instrument has been described by Chance (93). Preliminary experiments performed by Miss B. Jacobson (personal communication) had indicated that the absorbancy difference with the wavelength pair 540-630 mμ reflected mitochondrial volume changes without interference from respiratory chain redox effects. All measurements were made at 28° C. Variations in mitochondrial volume were indicated by a deflection of the recorder tracing during the reaction after the initial baseline adjustment.

Cytochrome c Estimation (spectrophotometric)

Estimation of the cytochrome c concentration in mitochondrial preparations was performed on the Aminco-Chance dual wavelength spectrophotometer with the wavelength pair 550-540 mμ (59). The operating theory of this instrument has been described by Chance (93).

Mitochondria (0.350 - 1.500 mg protein) were added to cuvette medium (28° C) in a 1 cm glass cuvette. Following addition of an appropriate substrate, the mitochondria were permitted to respire to anoxia. At 0% oxygen, deflection of the recorder tracing indicated the reduction of cytochrome c. To ensure complete reduction of the cytochrome c present, a small amount of dithionite was added to the reaction vessel

on the end of a stirring rod. Further deflection of the recorder tracing revealed a more complete reduction of the complement of cytochrome c in the cuvette. On the basis of this reduction, the concentration of cytochrome c in the mitochondria was calculated (Appendix, section III).

Sonication

The maximum capacity of the mitochondrial respiratory chain for NADH oxidation was examined by disrupting the membrane barrier with sonic irradiation (Bronwill Biosonik Probe). To avoid heat damage, the mitochondrial suspensions (kept on ice) were sonicated for periods of 15 seconds each, separated by intervals of 15-30 seconds during which time both the sample and the probe were cooled on ice. The sonicator was operated at maximum power for all experiments (20 kc, delivering 120 W).

Assay Procedures

1. Protein estimation

The protein concentration of all mitochondrial preparations was determined by the method of Lowry et al. (79). Standards were prepared in duplicate for each estimation in the range 0-200 μg using 0.25% bovine serum albumin (Sigma). The samples consisted of undiluted

mitochondrial suspensions prepared in triplicate. Both standards and samples were incubated overnight with 1.0 N NaOH before the estimation was carried out. The error between replicated readings by this method was ± 2.0%.

2. NADH concentration

Assay of the NADH concentration used in the experiments was carried out enzymatically using the conversion of pyruvate to lactate by lactic dehydrogenase. The extent of reaction was determined by measuring the decrease in optical density at 340 mμ in the Beckman DU spectrophotometer. At pH 7.2, the equilibrium of the reaction is far toward the production of lactate and NAD^+, and it is therefore assumed that the reaction proceeds to completion, oxidizing all the NADH to NAD^+ (94). The samples (duplicates) and blank were prepared according to the scheme in Table I. The sample cuvettes were read at 340 mμ against the blank. After addition of 0.03 ml of 1 M pyruvic acid to all cuvettes, the samples were again read against the blank at 340 mμ. The NADH concentration was determined by the following calculations:

(O.D. reading 1 - O.D. reading 2)(mean of duplicates) =

$$\Delta \text{ O.D.}_{340\ m\mu}$$

At 340 mμ, 0.1 mM NADH has an absorbancy of 0.622 (82).

Table I

Composition of blank and sample cuvettes for enzymatic assay of NADH.

Addition	Sample	Blank
Double distilled water	2.65 ml	2.66 ml
1 M $K.PO_4$ buffer (pH 7.2)	0.30	0.30
Lactic dehydrogenase	0.01	0.01
NADH (approximately 25 mM)	0.01	-
Total volume	2.97 ml	2.97 ml

Including the factors for dilution, the concentration is determined by the formula:

$$\frac{\text{O.D.}_{340\ m\mu}}{0.622} \times \frac{3000}{10} \times 0.1 = \text{NADH (mM)}$$

3. ADP assay

The concentration of ADP used in respiration experiments was estimated by ultraviolet assay on the Beckman DU spectrophotometer at 260 mμ (81). A 5 μl sample of ADP (50 mM by weight) was added to 6 ml of 0.02 M $K.PO_4$ buffer (pH 7.2). The diluted samples were read in duplicate against a blank of 0.02 M $K.PO_4$ buffer. The ADP concentration was calculated as follows. From the data on the ultraviolet absorption spectrum provided by P-L Biochemicals Inc., it can be calculated that 63.5 μM ADP produces an O.D. reading of 0.99 at 260 mμ (82). Adjusting for dilution (5 μl in 6 ml), the concentration is calculated by the formula:

$$\frac{\text{O.D.}_{260\ m\mu}}{0.99} \times 63.5 \times \frac{1200}{1000} = \text{ADP (mM)}$$

RESULTS

RESULTS

Control and modified preparations of pigeon heart mitochondria were examined polarographically to determine their metabolic behaviour with pyruvate + malate (final concentrations 9.5-10.5 mM and 1.9-2.1 mM respectively) and NADH (final concentration 150-200 μM) as substrates. The parameters measured were oxygen consumption, RCR's and ADP:O ratios (these terms are defined in the Glossary). Fig. 14 illustrates a typical Oxygraph experiment using the above substrates.

Oxygen consumption measurements (μmoles O_2/minute/g mitochondrial protein) can be used as a comparative basis to determine (a) the accessibility of the substrate to the mitochondrial tricarboxylic acid cycle and/or to the respiratory chain, and (b) the ability of these pathways to oxidize the added substrate. Comparisons of the rates of oxygen consumption were used to assess permeability and/or oxidative ability of the control and modified mitochondrial preparations.

RCR's and ADP:O ratios (see Fig. 14) can also be used as criteria for comparing the relative structural integrity of the mitochondrial preparations (see Introduction). In the experiments reported in this thesis high RCR's are taken as indicative of a high degree of structural integrity.

FIGURE 14. Typical oxygraph tracing showing oxidation of pyruvate + malate and NADH by pigeon heart mitochondria.

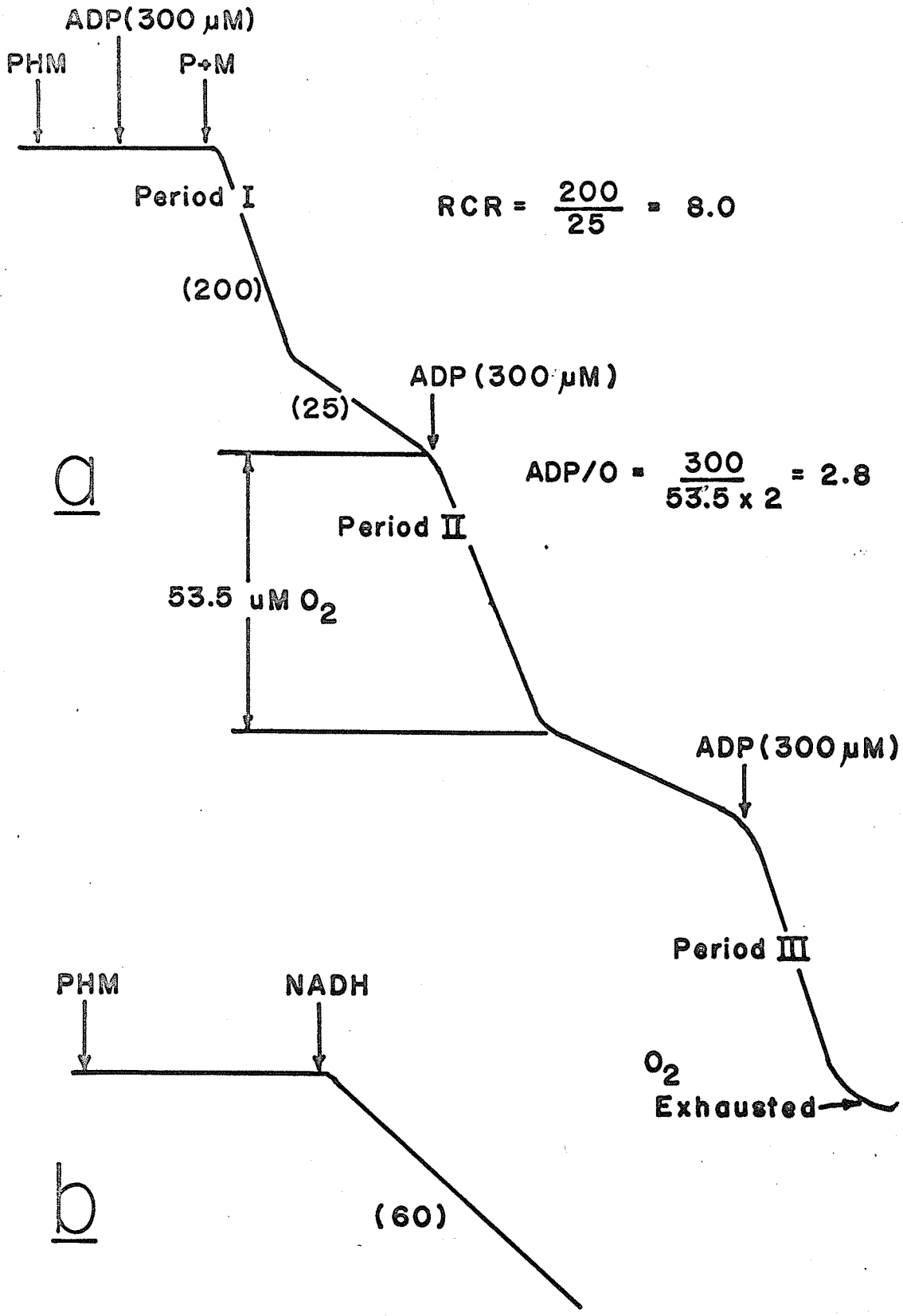
Calculation of RCR's and ADP:O ratios is indicated. Oxygen consumption (μmoles/minute/g mitochondrial protein) is given in bracketed numbers.

a. Pyruvate + malate

b. NADH

PHM = pigeon heart mitochondria

P+M = pyruvate+malate


ADP(300 µM)
PHM
P→M
Period I
RCR = 200/25 = 8.0
(200)
ADP (300 µM)
(25)
a
ADP/O = 300/53.5 x 2 = 2.8
Period II
53.5 uM O2
ADP(300 µM)
Period III
PHM
NADH
O2
Exhausted
b
(60)

The ADP:O ratios, which are representative of the phosphorylating efficiency of a mitochondrial preparation can be used as supporting evidence for structural intactness suggested by high RCR's. However, high ADP:O ratios do not appear to be as definitive a criterion as RCR's (see Introduction).

Data on oxygen consumption with pyruvate + malate as substrate are presented throughout this thesis with reference to respiration periods I, II and III. This terminology refers to three periods of state 3 respiration (see Glossary) induced by the additions of ADP to the reaction vessel. The addition of a limiting amount of phosphate acceptor (ADP) to coupled mitochondria in the presence of orthophosphate and substrate (pyruvate + malate) results in a rapid burst of respiration which continues until the ADP has been exhausted. Three successive additions of ADP were routinely made, resulting in the three bursts of oxygen consumption that are designated respiration periods I, II and III. The rate of oxygen consumption, RCR's and ADP:O ratios were calculated for each of these periods.

In all comparisons of control and modified mitochondria, both preparations were isolated from the mince of a single pigeon heart or from the pooled myocardial mince of two hearts.

The differences between duplicate estimations made

occasionally during the course of the work are presented in Table II as an indication of the precision (reproducibility) of the parameters measured.

Metabolic Characteristics of Control and Modified Preparations of Pigeon Heart Mitochondria

The raw data obtained from polarographic studies of control and modified mitochondrial preparations is presented in Tables IIIa, IIIb and IV. These measurements represent the data from paired control and modified preparations isolated from single pigeon hearts or a pooled tissue sample from two hearts. The material summarized in these tables was calculated from results of the polarographic studies similar to those shown in Fig. 14.

The means and standard deviations of oxygen consumption, RCR's and ADP:O ratios for the control and modified preparations are presented in Table V. As indicated by the results of these tables, the measurements studied showed a wide range of variability. Only oxygen consumption was recorded in the NADH experiments since respiratory control could not be demonstrated with either preparation using added NADH as noted in the literature (11).

To compare the behaviour of the two types of mitochondria statistically, paired data from control and modified preparations were analyzed using the Olivetti

Table II

Mean percentage difference between duplicates*.

Mitochondrial Preparation	Pyruvate + malate				NADH
	O_2	RCR	ADP:O		O_2
Control (14)**	16.9% ± 14.1	19.7% ± 13.5	8.0% ± 4.5	(10)	6.7% ± 5.1
Modified (8)	13.1% ± 7.5	8.2% ± 5.9	4.4% ± 2.2	(11)	17.4% ± 9.7

*means of percentage differences ± standard deviation
**numbers in brackets represent the number of experiments performed in duplicate

Table IIIa

Oxygen consumption (μmoles O_2/minute/g mitochondrial protein), RCR's and ADP:O ratios of control pigeon heart mitochondria with pyruvate + malate as substrate (final concentrations, 9.5-10.5 mM pyruvate and 1.9-2.1 mM malate).

Experiments with 41 pigeons are presented.

Period of Respiration								
I			II			III		
O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O
239.4	7.1	3.0	241.0	10.0	3.0	205.0	12.0	3.0
248.3	9.7	3.3	284.4	10.2	2.9	236.3	9.9	2.9
256.9	8.0	2.8	290.9	9.8	2.9	233.1	8.6	2.7
305.3	8.4	2.9	307.2	8.4	2.5	278.7	9.1	2.7
250.0	5.6	3.2	258.2	6.7	3.0	244.5	7.0	2.8
268.5	7.7	2.8	309.4	9.3	2.6	271.9	9.2	2.7
249.8	6.4	2.5	377.1	8.7	2.4	262.0	9.2	2.3
341.2	10.2	2.5	360.5	17.8	2.6	-	-	-
401.3	7.0	2.3	422.0	7.3	2.2	-	-	-
313.9	3.1	2.1	297.1	4.2	2.2	276.3	4.9	2.3
333.0	7.6	3.4	344.6	7.8	3.6	305.5	8.7	3.4
217.8	7.3	1.5	202.1	7.2	2.8	181.8	-	-
195.6	7.8	2.8	171.7	9.4	3.5	166.0	10.3	3.4
154.8	5.3	3.5	173.1	8.3	3.5	146.4	7.0	3.5
190.7	6.1	3.4	199.0	7.6	3.3	196.7	6.6	3.4
211.9	7.1	3.2	219.9	8.3	3.0	197.9	7.9	2.9
130.7	5.0	3.4	112.7	5.5	3.1	98.3	6.5	3.1
115.7	2.7	3.2	136.0	3.2	3.2	133.0	3.8	3.1
213.5	6.9	3.2	233.5	8.0	3.3	198.6	6.3	3.3
212.6	7.1	2.9	220.4	9.4	3.1	217.5	10.1	3.0
240.1	6.6	3.0	247.0	7.8	3.0	238.0	9.0	2.9
283.4	7.8	3.0	283.3	7.7	3.1	244.3	7.3	3.2
272.0	7.4	3.2	262.4	9.2	3.0	229.6	7.3	3.0
281.2	5.0	2.4	325.7	6.4	2.3	279.4	-	-
331.2	7.8	3.1	360.3	8.4	3.0	341.0	7.0	3.1
221.8	6.4	2.9	244.4	8.5	3.1	251.8	10.8	2.6
240.6	7.9	3.0	275.2	10.8	2.7	233.4	10.1	3.3
228.6	7.1	3.3	192.6	7.7	2.9	163.7	7.2	3.0
189.5	7.6	2.8	140.5	7.0	2.8	138.9	6.9	2.8
248.9	10.5	3.2	210.7	11.8	3.0	152.0	12.3	3.1
298.9	10.3	2.8	298.3	10.3	2.6	251.4	11.4	2.5
249.7	8.4	3.1	255.5	7.5	2.9	231.4	7.7	3.0
287.5	7.5	3.2	276.4	8.2	3.3	265.1	8.5	3.1
396.4	8.4	2.8	404.8	7.6	2.7	368.5	8.8	2.6
291.8	6.5	2.6	299.0	7.5	2.5	282.0	8.1	2.5
239.4	7.2	2.6	268.5	9.1	2.3	244.3	8.8	2.4
293.5	9.1	2.5	291.7	11.6	2.4	253.6	10.8	2.4
279.6	6.7	2.8	246.8	8.3	3.0	234.9	7.2	2.9
420.8	8.0	2.9	486.1	10.8	2.7	428.7	8.9	2.7
297.3	6.7	2.4	284.2	7.1	2.4	208.3	4.9	2.4
199.6	7.1	3.1	197.1	8.7	3.4	164.0	7.2	3.1

Table IIIb

Oxygen consumption (μmoles O_2/minute/g mitochondrial protein), RCR's and ADP:O ratios of modified pigeon heart mitochondria with pyruvate + malate as substrate (final concentrations, 9.5-10.5 mM pyruvate and 1.9-2.1 mM malate).

Experiments with 41 pigeons are presented.

Period of Respiration								
I			II			III		
O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O
163.8	4.6	3.3	196.0	8.0	2.9	195.0	8.7	3.1
211.5	7.3	3.2	237.8	9.5	2.7	205.7	9.8	2.8
179.3	5.3	2.9	226.8	7.8	2.7	191.4	7.8	2.8
254.4	6.3	2.7	290.1	6.8	2.3	293.7	7.8	2.4
166.0	3.7	2.9	198.0	5.2	2.7	206.5	6.8	2.8
157.5	5.0	2.7	210.0	7.1	2.6	215.3	9.0	2.6
153.0	2.8	2.2	203.0	4.7	2.2	-	-	-
125.8	2.1	1.7	126.7	2.9	2.0	-	-	-
126.7	2.7	1.6	138.6	3.6	1.8	-	-	-
171.6	4.4	2.5	221.6	7.8	2.5	234.4	8.2	2.5
142.9	4.8	3.2	158.9	5.3	3.0	152.3	8.0	-
93.4	2.6	3.2	114.1	3.7	3.2	126.2	5.1	2.9
87.1	3.7	3.6	106.3	6.7	3.2	107.0	6.7	3.4
121.0	4.7	3.1	173.0	6.4	3.1	163.2	7.5	3.1
160.8	4.6	3.0	202.6	7.1	3.1	177.3	7.3	3.1
151.8	4.4	2.7	147.3	4.9	2.6	145.9	6.3	2.6
67.7	3.0	3.1	73.9	4.4	2.8	65.2	5.0	2.9
120.0	2.5	3.0	129.5	3.3	2.7	139.0	-	-
-	-	-	218.1	6.8	3.0	213.2	6.6	3.1
106.8	2.9	2.3	125.8	5.1	2.8	138.9	6.4	2.7
198.1	4.5	3.0	216.7	5.9	3.0	241.6	7.2	2.9
212.6	5.6	3.0	235.8	6.6	2.6	215.2	7.8	2.7
210.1	3.6	2.9	184.1	3.6	3.1	205.9	6.0	3.3
226.1	2.9	2.4	251.8	3.9	2.1	238.8	-	-
157.5	3.8	3.0	191.8	6.6	3.0	205.6	7.0	3.0
171.2	3.7	2.9	207.1	5.4	2.5	194.8	-	2.5
213.2	5.1	2.7	222.8	7.3	2.5	210.2	8.3	2.6
131.4	3.7	3.6	162.8	5.8	3.0	148.5	5.8	2.8
206.5	5.5	2.9	195.3	5.6	2.7	146.1	5.3	2.6
200.4	6.5	3.1	177.0	7.2	3.1	136.7	6.6	3.0
267.1	7.0	2.7	319.2	10.2	2.3	265.7	10.0	2.5
189.5	4.6	3.0	230.5	5.2	2.7	234.1	6.5	2.7
280.5	6.6	3.2	288.8	7.5	3.1	275.6	8.5	3.2
327.1	5.0	2.7	384.6	6.3	2.5	362.0	7.3	2.6
204.4	3.9	2.5	229.1	4.7	2.4	246.2	7.9	2.5
167.7	3.8	2.6	206.4	7.2	2.2	-	-	-
273.1	8.3	2.9	252.8	8.1	2.3	-	-	-
181.3	3.7	3.1	170.8	5.0	2.9	167.6	5.6	2.8
245.7	5.7	2.8	252.1	7.9	2.6	218.1	7.5	2.5
186.9	4.1	2.7	209.5	5.2	2.3	189.2	5.8	2.3
150.0	4.6	2.9	176.9	7.3	2.9	111.7	5.3	3.4

Table IV

Respiration rate of control and modified pigeon heart mitochondria utilizing external NADH (150-200 μM) as substrate. Oxygen consumption is given in μmoles O_2/ minute/g mitochondrial protein.

The data are presented as paired measurements of control and modified mitochondria prepared from 61 pigeon hearts.

Respiration Rate			
Control PHM	Modified PHM	Control PHM	Modified PHM
33.1	8.8	43.1	12.9
33.1	8.7	72.9	25.9
37.6	8.9	78.1	25.9
51.7	6.6	62.9	12.2
40.2	8.7	52.4	12.1
58.8	10.2	46.2	15.5
39.4	9.6	51.0	17.1
92.8	20.0	50.6	17.1
52.0	24.3	67.1	13.7
24.8	11.6	68.1	17.5
48.6	16.8	41.7	15.0
49.5	5.4	49.0	24.9
80.6	11.1	75.3	22.0
40.5	18.9	75.5	14.0
37.0	14.7	65.9	16.0
49.0	13.0	132.4	25.7
35.4	8.8	102.4	23.7
35.7	11.9	86.3	21.7
24.0	9.9	100.7	27.1
50.3	22.6	114.3	27.2
55.3	10.2	83.1	32.1
68.0	29.4	50.1	23.1
41.6	25.2	52.0	25.2
24.0	8.4	54.9	14.3
53.0	9.4	52.7	12.5
26.6	11.9	65.2	12.6
58.4	30.1	76.2	22.9
54.1	22.9	66.4	12.6
43.0	8.1	63.2	13.3
67.1	26.6	36.7	8.3
		77.6	13.6

Table V

Respiratory behaviour of control and modified pigeon heart mitochondria with pyruvate + malate and added NADH as substrates. Summary of the raw data of Tables IIIa, IIIb and IV. Means ± standard deviation (S.D.) are given for oxygen consumption (μmoles O_2/minute/g mitochondrial protein), RCR's and ADP:O ratios.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate)									NADH
	I			II			III			
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control Mean	260	7.2	2.9	269	8.5	2.9	232	8.3	2.9	58
±S.D.	±66	±1.6	±0.4	±78	±2.3	±0.4	±65	±1.9	±0.3	±22
Modified Mean	179	4.5	2.8	202	6.1	2.7	194	7.1	2.8	17
±S.D.	±56	±1.4	±0.4	±59	±1.7	±0.4	±58	±1.3	±0.3	±7

Underwood Programma 101 desk computer. The results of these calculations are summarized in Table VI. They indicate that the modified mitochondria isolated with a lower ratio of tissue wet weight (g)/homogenizing medium (ml) were significantly less active ($P<0.01$) in all aspects of metabolic behaviour examined, and particularly in the oxidation of NADH (Table V). The difference in RCR's, although apparent in all three periods of respiration, became less obvious during periods II and III (see Table V). It is apparent from Table VI that the ADP:O ratios are not significantly different ($P>0.01$) during period I, whereas they become so following the second and third additions of ADP as shown in the analysis of paired data (Table VI). However, such differences could not be detected by an examination of the pooled (i.e. unpaired) data in Table V because of the variability of the measurements in the preparations from different pigeons.

As discussed in the Introduction, relative rates of oxygen consumption of two substrates might be used as a means of expressing permeability. The ratios of the oxygen consumption rates with NADH to those with pyruvate + malate are 23% and 10% in control and modified preparations respectively (calculated from Table V). The lower ratio in the modified mitochondria reflects the striking decrease from the control value which was observed in the rate of

Table VI

Summary of statistical analysis of the paired*** raw data comparing the behaviour of control and modified pigeon heart mitochondria with pyruvate + malate and NADH as substrates.

	Period of Respiration (Pyruvate + malate)									NADH
	I			II			III			
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
d.f.* = n-1	39	39	39	40	40	40	35	31	31	60
t (calculated)	8.61	10.25	0.72	6.03	6.17	5.66	4.51	3.67	3.03	16.99
t (theoretical)	2.71	2.71	2.71	2.70	2.70	2.70	2.72	2.74	2.74	2.66
Significance** ($P<0.01$)	S	S	NS	S	S	S	S	S	S	S

*d.f. = degrees of freedom

**S = significant

NS = non-significant

***data used for analysis was that of Tables IIIa and IIIb for pyruvate + malate and Table IV for NADH. The data of the control and modified preparations were paired for these calculations.

NADH oxidation by that preparation. Two kinds of criteria of intactness have been independently suggested for mitochondria (see Introduction): (a) low NADH oxidation and (b) high oxygen consumption with "coupled" substrates (e.g. pyruvate + malate), accompanied by high RCR's and ADP:O ratios. On this basis, the observation of decreased NADH utilization by the modified preparation suggests, at first thought, that these mitochondria were more intact than the controls with a resultant decrease in permeability to NADH. At the same time however, these mitochondria were less intact according to criterion (b) (Table V). This contradiction implies that either (a) or (b) is not a valid reflection of mitochondrial integrity. On the basis of evidence from earlier studies of pigeon heart mitochondria (11, 12, 13) and data reported later in this thesis, mitochondrial impermeability to NADH cannot be considered a valid criterion of structural integrity.

The remainder of the findings reported in this section deal with various attempts to determine the source of the metabolic differences between control and modified mitochondria, particularly in relation to their ability to oxidize extramitochondrial NADH.

Morphological Characteristics of Control and Modified Preparations of Pigeon Heart Mitochondria

It is conceivable that the difference in metabolic performance of the two mitochondrial preparations might be attributable to gross morphological dissimilarities, visible differences in structural integrity or to varying degrees of contamination by mitochondrial fragments and other particles. The morphological characteristics of the two preparations were therefore examined.

1. Electron Microscopy

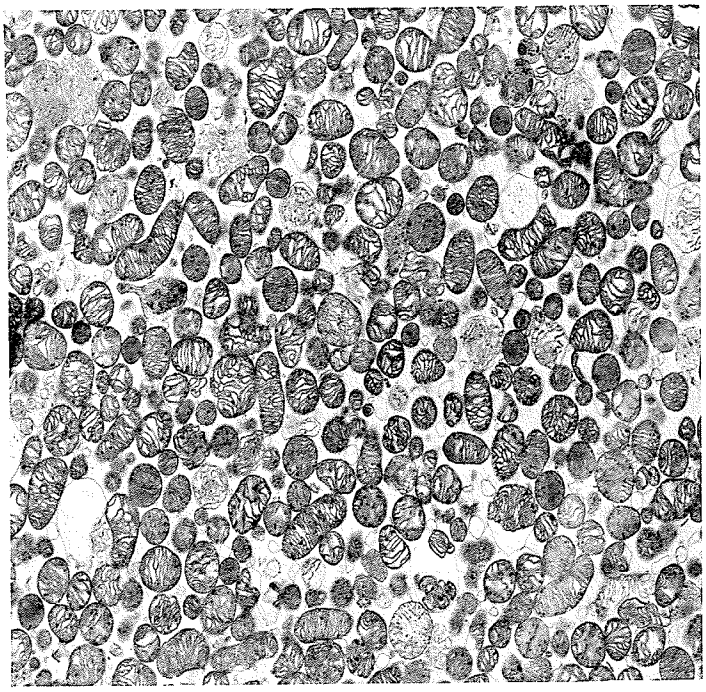
Control and modified pigeon heart mitochondria were isolated and prepared for electron microscopy. Photomicrographs of the two preparations are illustrated in Figs. 15a and 15b. There is no evidence in the preparations of any extensive degree of contamination by either mitochondrial fragments or microsomes. In both preparations, the inner and outer membrane systems appear to be intact.

The two photographs do, however, reveal certain morphological differences between the two preparations. The cristae of the modified preparation (Fig. 15b) are more irregular than those of the control and are associated with prominent intramitochondrial vacuoles. The apparent difference in organization of the cristae may have greater significance in view of the recent work on the relationship

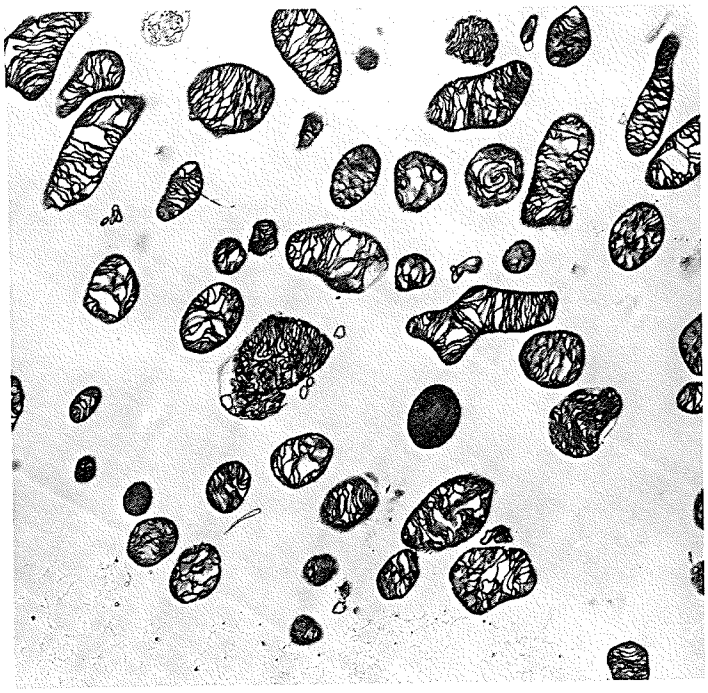
FIGURE 15. Electron micrographs of two preparations of pigeon heart mitochondria prepared by the modified method of Chance and Hagihara (76).

a. Control mitochondria (magnification 7500x)

b. Modified mitochondria (magnification 7500x)



a



b

of cristae structure and morphology to "energized" and "non-energized" forms of mitochondria (95, 96), but was not examined further in this study.

The electron micrographs also illustrate a difference in cross-sectional diameters between the two preparations. There is a distinct heterogeneity of this parameter in the control mitochondria, while the modified preparation appears to be a more homogeneous population of larger cross-sectional diameters. Since the electron micrographs represent a section through the preparation in which the mitochondria are probably randomly oriented, the average cross-sectional diameter may be indicative of the actual mitochondrial size in the two preparations. It was of interest, therefore, to examine the possibility of a relationship between size and metabolic function.

2. Segregation of mitochondrial populations

As discussed above, electron microscopy revealed a possible size difference between the two types of mitochondria based on observations of their cross-sectional diameters. The existence of a size/function relationship was examined by differential centrifugation. Peter and Lee (80) have observed that higher speeds in the initial centrifugation used to remove cellular debris can result in a significant loss of mitochondria. It seemed possible therefore that centrifugation of the myocardial homogenate

at 480 x g might produce a partial fractionation of a heterogeneous mitochondrial population and that this might be affected by the isolation conditions (e.g. the amount of muscle tissue homogenized). This hypothesis was tested by varying the centrifugal force used for the initial centrifugation of the myocardial homogenates. The effect of changing the usual 480 x g centrifugation to 270 x g or 755 x g was tested on both control and modified mitochondrial preparations. The remainder of the isolation procedure was unchanged. The oxidative activity of the resulting preparations was examined polarographically at 28° C with pyruvate + malate and NADH as substrates. The results of these experiments (Table VII) indicate that the rate of centrifugation may influence in part some of the properties of the resulting preparations.

The comparisons of modified mitochondria isolated with different rates of initial centrifugation suggested that this type of mitochondrial preparation consisted of a more homogeneous population. The observed differences in oxygen consumption, RCR's and ADP:O ratios with pyruvate + malate as substrate were not significant (see Table II). Similarly, no change in the rate of NADH oxidation was produced by centrifugation of the modified preparation at 755 x g. The use of percentage change comparisons can be misleading in cases where the original measurements are low

Table VII

The effect of varying the initial centrifugation rate on the oxidation of pyruvate + malate and NADH by control and modified mitochondria.

Paired comparisons are made for each type of preparation, between the usual procedure (480 x g) and the test methods (270 x g or 755 x g). The control and modified preparations are not paired. Oxygen consumption is given in μmoles O_2/minute/g mitochondrial protein.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate) I			II			NADH	
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	*Δ%
Control (480 x g)	239	7.2	2.6	269	9.1	2.3	51	+67%
(270 x g)	257	6.4	2.6	286	9.8	2.2	85	
Control (480 x g)	300	7.2	2.7	265	9.2	2.9	91	-28%
(755 x g)	227	3.9	2.4	220	4.0	2.3	66	
Modified (480 x g)	273	8.3	2.9	253	8.1	2.3	13	-32%
(270 x g)	248	7.6	2.7	228	9.1	2.4	9	
Modified (480 x g)	167	3.8	2.6	206	7.2	2.2	23	+4%
(755 x g)	155	3.3	2.3	194	6.4	2.1	24	

*Δ% = percentage increase or decrease in oxygen consumption with NADH as substrate compared to the rate in the (480 x g) preparation used as the comparison

and variable since a relatively small absolute difference can produce a large percentage variation. Consequently, the apparently large decrease (32%) in the rate of NADH oxidation in the modified (270 x g) preparation was not a statistically significant difference. The results of the initial centrifugation changes on modified mitochondria are, therefore, compatible with previous data which suggested a homogeneous preparation.

The observed differences in oxygen consumption, RCR's and ADP:O ratios with pyruvate + malate between the control (480 x g) and control (270 x g) preparations were not significant. The latter preparation was characterized by the generally accepted criteria of mitochondrial intactness, viz. high RCR's, ADP:O ratios and oxidation of pyruvate + malate. However, the concomitant increase in the rate of NADH oxidation (67%) was, in this instance, not attributable to experimental error alone (see Table II). These observations suggested that the lower rate of initial centrifugation used in the isolation of control (270 x g) mitochondria had resulted in a preparation containing a high proportion of intact mitochondria. Furthermore, they are compatible with previous proposals in the literature (11, 12, 13) that direct oxidation of added NADH may be a property of intact pigeon heart mitochondria. The results from the control (755 x g) preparation experiments provided additional

support to the possibility that the control (480 x g) preparation was a heterogeneous population which could be fractionated by differential centrifugation. With pyruvate + malate as substrate, the significant decrease in the rate of oxygen consumption, RCR's and ADP:O ratios of the control (755 x g) preparation suggested the proportion of intact mitochondria in the preparation was lower. It is unlikely, therefore, that the concomitant slower rate of NADH oxidation by the control (755 x g) preparation could be attributed to a decreased membrane permeability associated with increased structural integrity. The differences between the control (480 x g) and the control (755 x g) preparations are similar to the relationship observed between control and modified mitochondria, i.e. the control (755 x g) and modified preparations exhibit decreased rates of oxygen consumption with pyruvate + malate and NADH, and lower RCR's and ADP:O ratios than the control mitochondria. It is possible, therefore, that modified mitochondria might be the product of the segregation of a specific homogeneous fraction from the heterogeneous control population.

3. Spectrophotometric estimation of relative mitochondrial volume

It seemed possible that the faster rate of oxidation of NADH by control mitochondria noted above might be a consequence of mitochondrial swelling during the isolation

procedure such that penetration of the membrane by NADH was facilitated. Since direct measurements of mitochondrial volume are difficult in the quantities available in these preparations, an indirect method dependent upon the optical density measurement at 520 mμ was used. At this wavelength the optical density of a mitochondrial suspension is decreased if the mitochondria are made to swell. While this appears to be a valid assumption in sequential comparisons of a single preparation, or of different preparations containing the same number of mitochondria, the electron micrographs in this study suggested that perhaps, even though the optical density tests at 520 mμ were set up with similar protein concentrations (± 5%), this might result in a difference in the number of mitochondria added in a fixed amount of protein. Thus, if the modified mitochondria are larger, it would be expected that the optical density measurements at 520 mμ would be lower in such preparations independent of any swelling which might have occurred during the isolation procedure. With these reservations in mind, the data of Table VIII can be considered. Analysis of the paired data indicates that, in fact, the modified mitochondria had a significantly lower ($P<0.01$) optical density at 520 mμ. In accordance with the above considerations, it follows that this lower value could be due to the presence of fewer mitochondria which, in their

Table VIII

Optical density (O.D.) of pigeon heart mitochondrial (PHM) suspensions* measured at 520 m **.

Measurements are given in O.D. units.

Control PHM	Modified PHM
0.145	0.110
0.096	0.069
0.345	0.300
0.071	0.073
0.328	0.289
0.320	0.280

*comparisons were only made when the control and modified mitochondrial protein concentrations were similar. The data represents paired preparations from 6 pigeon hearts.

**higher $O.D._{520\ m}$ reflects a smaller mitochondrial volume

original state, are larger than those of the control preparation and/or swollen mitochondria. The relative contribution of these two factors cannot be assessed from the available data. The original hypothesis presented at the beginning of this section attributed the faster rate of NADH oxidation by the control mitochondria to mitochondrial swelling in this preparation. That the latter was not the case was shown by the higher optical density values at 520 mμ in the control preparations (Table VIII).

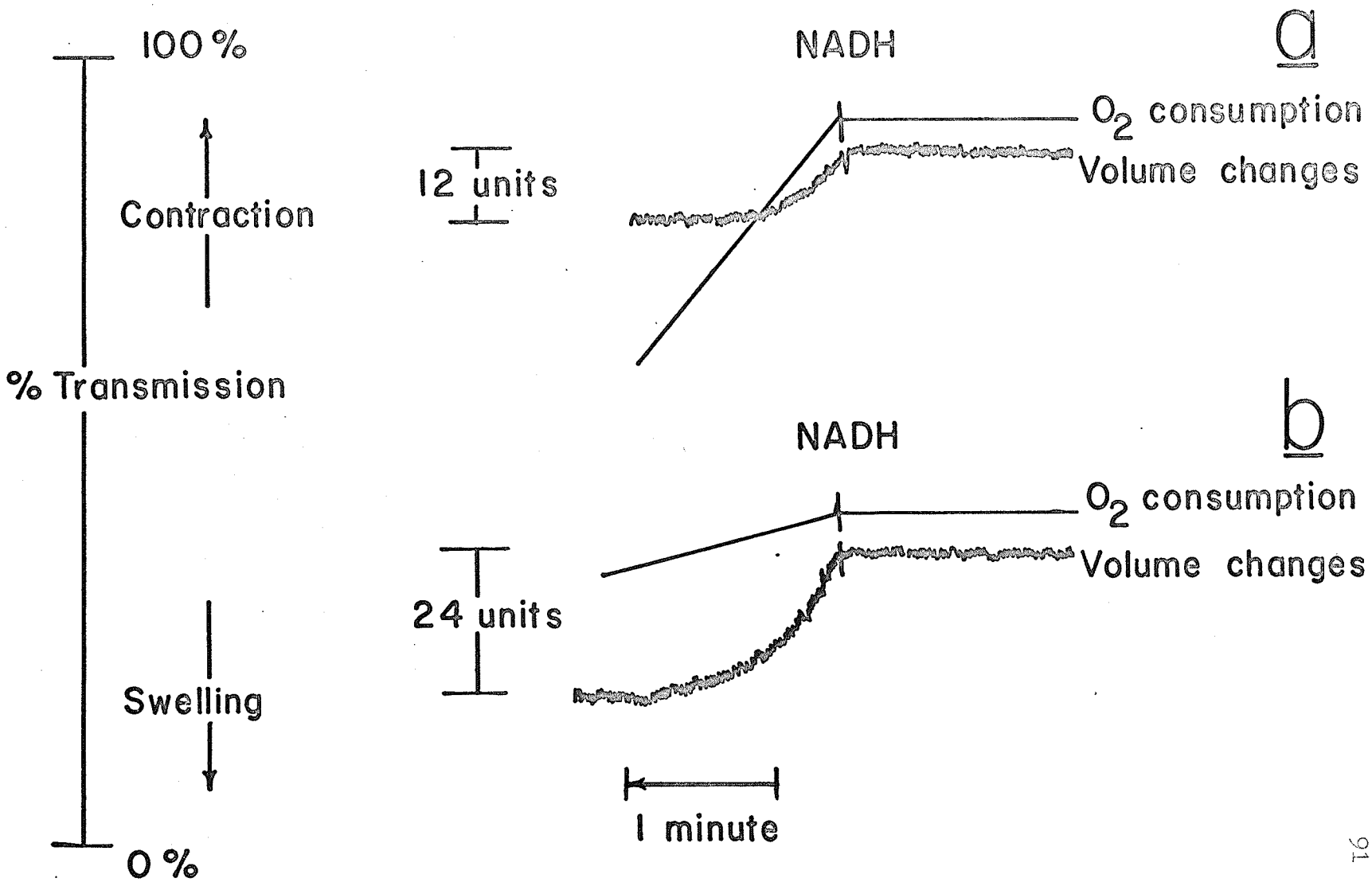
4. Volume changes

The discussion above considered the possible contribution of a volume difference to the rates of NADH oxidation in control and modified mitochondria. The purpose of these experiments was to detect swelling which might have occurred during isolation. However, it is known (31) that swelling may also occur when substrates are added to mitochondria. It seemed possible that the difference in rate of NADH oxidation observed in the two preparations might be due to a different degree of swelling induced by addition of NADH, which in turn might lead to a faster oxidation of this substrate in the control mitochondria. Therefore, swelling/contraction cycles were followed on the Aminco-Chance dual wavelength spectrophotometer using the light transmission difference at the wavelength pair 540-630 mμ (see Methods). Figs. 16a and 16b illustrate two of

FIGURE 16. Spectrophotometric measurement of volume changes in control and modified pigeon heart mitochondria upon addition of NADH.

a. Control mitochondria

b. Modified mitochondria


100 %
Contraction
% Transmission
Swelling
0 %
12 units
NADH
a
O_2 consumption
Volume changes
24 units
NADH
b
O_2 consumption
Volume changes
1 minute

the recorder tracings obtained with control and modified mitochondria respectively, utilizing NADH (150-200 μM) as substrate. It is apparent that during oxidation of external NADH, the modified mitochondria underwent a volume change (swelling) of approximately two times that of the control preparation with no concomitant change in the rate of NADH oxidation. Although the extent of the observed changes varied in different experiments, the examples illustrated are typical of the comparative relationship found in all experiments with the two preparations. It is clear, therefore, that the entry of NADH is not necessarily dependent upon a mitochondrial swelling during isolation (see discussion of Table VIII) or during respiration with NADH as substrate.

Characterization of the Pathway of Oxidation of Extra-Mitochondrial NADH by Control and Modified Preparations of Pigeon Heart Mitochondria

Borst (41), in reviewing the existing data (1961) on the oxidation of extramitochondrial NADH, discussed the four following types of mechanisms:

1. Internal phosphorylating pathway - respiratory chain
2. External I, non-phosphorylating, cytochrome c-stimulated pathway
3. External II, quinone involvement

4, Metabolite shuttle systems

In view of these possibilities, some characteristics of the pathway of oxidation of external NADH in the control and modified preparations were compared. Three approaches to this problem were undertaken; (a) studies with "effectors" that alter respiratory chain activity, (b) kinetic studies of physiological NADH concentrations (1-10 μM), and (c) studies of metabolite shuttle systems.

(a) "Effector" studies

A variety of substances which act at various locations on the respiratory chain (see Fig. 3, Introduction) were tested on the control and modified mitochondrial preparations. These effectors represented three operational classes of substances; inhibitors (Antimycin A, Amytal, rotenone), activators (cytochrome c) and uncouplers (2,4-dinitrophenol). The action of such compounds on the oxidative behaviour of mitochondria can be used to identify the pathway of oxidation of a particular substrate. All experiments were performed at 28° C using oxygen consumption measurements.

The results of these experiments are summarized in Table IX. The oxidation of external NADH in both control and modified mitochondria was sensitive to Amytal, Antimycin A and rotenone, indicating that the pathway of oxidation was the mitochondrial respiratory chain. Cytochrome c addition almost doubled oxygen consumption in

Table IX

Oxygen consumption of control and modified pigeon heart mitochondria with added NADH (150-200 μM final concentration) under the influence of several respiratory chain effectors.

Final concentrations are given with the respective effectors. The numbers in brackets are the number of experiments performed. Values for oxygen consumption are given as the mean ± standard deviation of μmoles O_2/minute/g mitochondrial protein.

Addition	Control PHM			Modified PHM		
	-Effector	+Effector	Δ%	-Effector	+Effector	Δ%
Amytal (2 mM)	71 ± 27 (5)	0	-100%	14 ± 9 (4)	0	-100%
Antimycin A (1-2 μM)	58 ± 22 (4)	0	-100%	17 ± 10 (4)	0	-100%
Rotenone (2-3 μM)	72 ± 18 (10)*	0	-100%	20 ± 7 (8)*	0	-100%
Cytochrome c (40 μM)	57 ± 27 (11)	93 ± 93	+64%	17 ± 8 (10)	30 ± 16	+67%
2,4-Dinitrophenol (30 μM)	49 ± 14 (4)	49 ± 14	0%	13 ± 4 (4)	13 ± 4	0%

*includes experiments in which rotenone was added after cytochrome c stimulation

both preparations. This increased oxidation rate implied the possible contribution of an external cytochrome c-stimulated rotenone-insensitive pathway as reported in the literature (3, 6, 56, 57). However, this was ruled out by several experiments in which the effect of rotenone on the cytochrome c-stimulated oxidation was examined (see note to Table IX). In all such instances rotenone produced complete inhibition of oxygen consumption. Thus, although cytochrome c could stimulate oxidation of added NADH, it would appear to do so by supplementing the endogenous cytochrome c of the respiratory chain and not through an external pathway. The uncoupler, 2,4-dinitrophenol, had no effect on NADH oxidation by either the control or modified mitochondrial preparations. This observation indicates that the oxidation of added NADH is probably not compulsorily coupled to oxidative phosphorylation, and that the mitochondrial NADH uptake prior to its oxidation is not necessarily an energy-linked function. These effector studies demonstrate that NADH added to control and modified preparations is oxidized by the mitochondrial respiratory chain. However, the results do not eliminate a role for an additional separate metabolite shuttle system in the oxidation of cytoplasmic NADH.

(b) Kinetic studies

To characterize further the behaviour of control and

modified mitochondrial preparations, the kinetics of oxidation of exogenous NADH were examined using the Eppendorf 1100M photometer fluorescence attachment. All measurements were made at 28° C using 1 cm quartz cuvettes containing 0.3-0.5 mg mitochondrial protein suspended in 2.5 ml cuvette medium. Initial velocities of NADH oxidation were calculated (see Appendix, section I) in the presence and absence of ADP (5 mM, 10 mM) over a physiological range of NADH concentrations (1-10 μM).

The calculations are summarized in Table X and presented graphically as Lineweaver-Burk (89) double reciprocal plots in Figs. 17 and 18 for the control and modified preparations respectively. The reciprocal plots in these two figures were eye-fitted. The kinetic parameters determined from the graphs are summarized in Table XI. The inhibitor constant (K_i) was calculated by the intercept method (97) using the following equation and solving for K_i:

$$\text{Intercept (abscissa)} = \frac{1}{K_m\left(1 + \frac{i}{K_i}\right)}$$

where i = concentration of inhibitor

ADP (5 mM, 10 mM) exerts a strong inhibition on mitochondrial oxidation of NADH in both control (73-93%) and modified (70-85%) preparations at the four substrate concentrations examined. The calculated K_m and K_i values

Table X

The effect of ADP on the oxidation of physiological concentrations of NADH (1-10 μM).

The rates are shown as absolute values of oxygen consumption in μmoles O_2/minute/g mitochondrial protein. The rates in the presence of ADP are also shown as percentages of those in the absence of ADP.

	1 μM NADH			2 μM NADH			4 μM NADH			10 μM NADH		
Mitochondrial Preparation	no ADP	5mM ADP	10mM ADP	no ADP	5mM ADP	10mM ADP	no ADP	5mM ADP	10mM ADP	no ADP	5mM ADP	10mM ADP
Control												
Absolute	39.3	5.6	3.1	69.9	10.5	6.2	108.4	18.0	11.4	165.3	44.6	23.4
%	100	14	18	100	15	9	100	17	12	100	27	14
Modified												
Absolute	6.6	1.3	-	10.0	2.7	1.5	16.0	3.6	2.5	25.0	2.7	4.7
%	100	20	-	100	27	15	100	23	16	100	31	19

FIGURE 17. Double reciprocal plots of velocity versus varying NADH concentrations in control mitochondria.

Experiments were performed in the presence and absence of added ADP (5mM, 10mM).

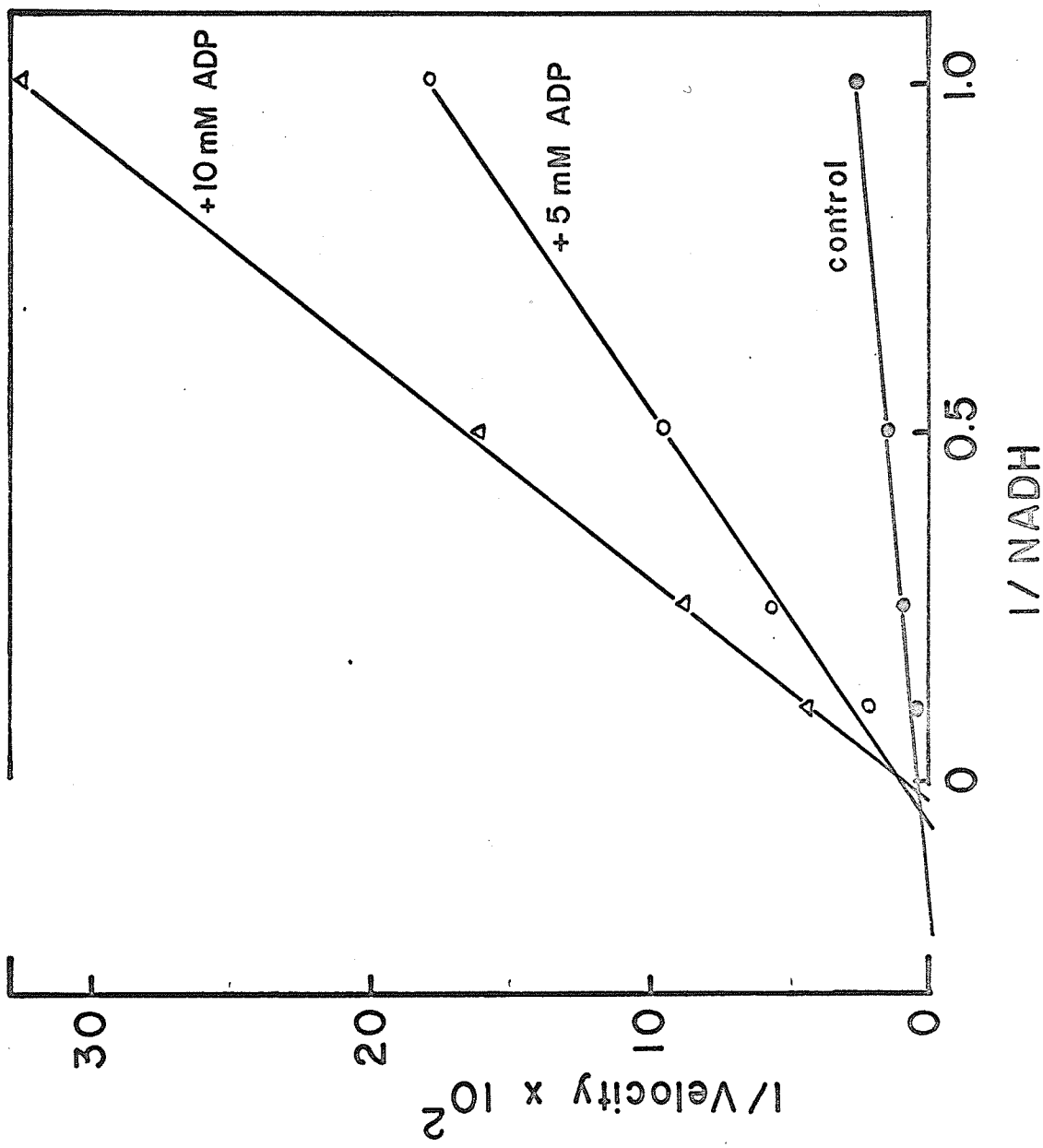

+10 mM ADP
+5 mM ADP
control
1/ Velocity x 10^2
30
20
10
0
0
0.5
1.0
1/ NADH

FIGURE 18. Double reciprocal plots of velocity versus varying NADH concentrations in modified mitochondria.

Experiments were performed in the presence and absence of added ADP (5mM, 10mM).

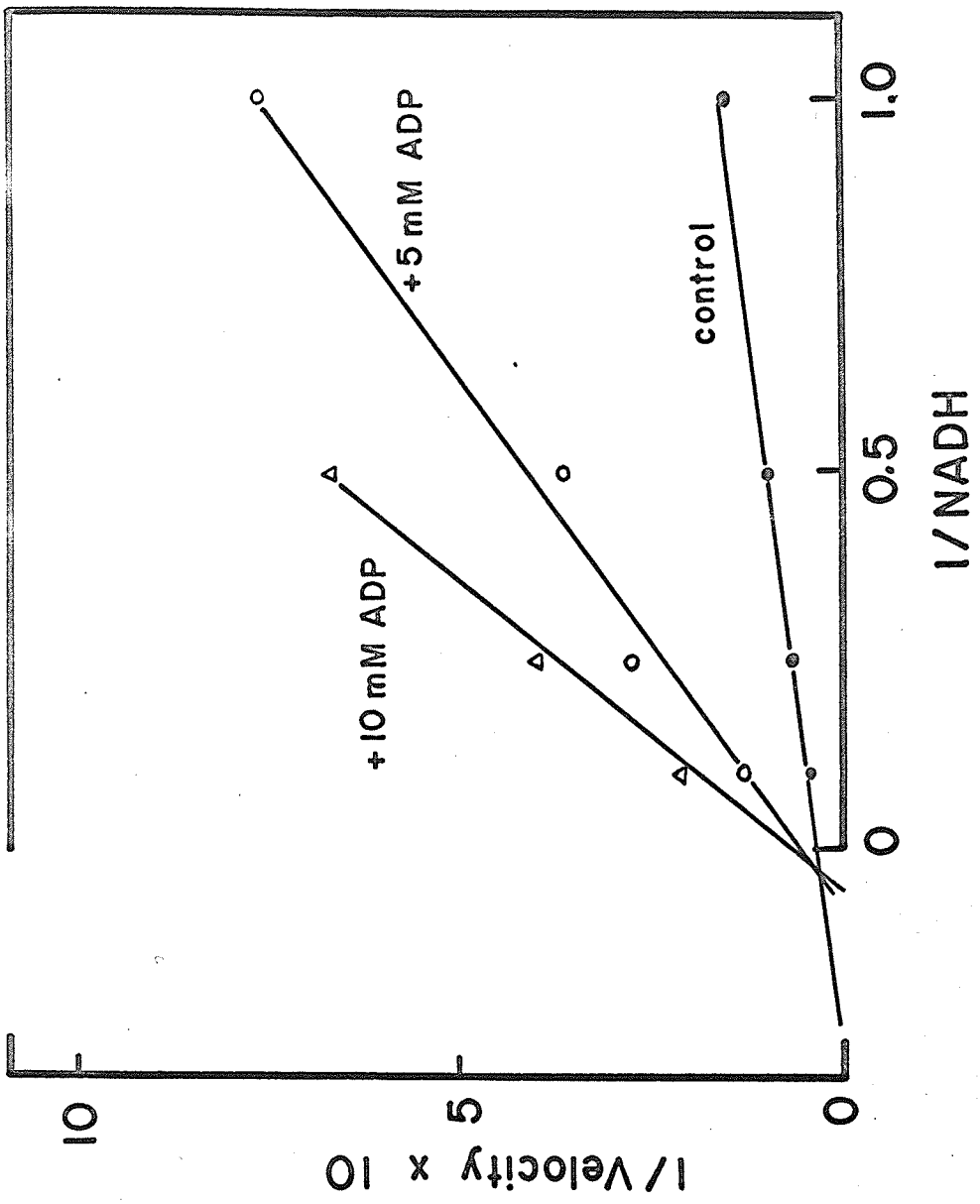

1/ Velocity x 10
10
5
0
0
0.5
1.0
1/NADH
+10 mM ADP
+5 mM ADP
control

Table XI

Summary of kinetic data for control and modified pigeon heart mitochondria with NADH (1-10 μM final concentration) as substrate.

Mitochondrial Preparation	K_m (μM)	V_{max} (μmoles NADH/minute/g mitochondrial protein)			K_i (mM)	
		Control	5mM ADP	10mM ADP	5mM ADP	10mM ADP
Control	5.6	277	114	100	1.1	5.1
Modified	5.0	33	22	17	1.5	4.3

(Table XI) are in good agreement with those previously reported (51). If the V_{max} values were nearly identical in the presence and absence of ADP, this would be suggestive of a competitive inhibition. However, the V_{max} values were in fact not identical, suggesting that the inhibition was of a mixed type (97). This is compatible with previous evidence in the literature (51) which has indicated a complex mechanism of inhibition by ADP exists. A detailed examination of the kinetics of ADP inhibition was considered beyond the scope of this study since the kinetics were performed for comparative purposes only, to detect similarities or differences between the behaviour of control and modified mitochondria with physiological concentrations of added NADH. Certain kinetic parameters of the two systems, viz. K_m and K_i appear to be very similar in both systems. This indicated that the oxidation of NADH was probably proceeding by the same mechanism in both preparations.

(c) Metabolite shuttles

The fourth general type of mechanism described by Borst (41) to account for the oxidation of cytoplasmic NADH by mitochondria was the metabolite shuttle. Since it is unlikely that the acetoacetate/β-hydroxybutyrate shuttle or the α-glycerophosphate shuttle are functional in myocardium (37, 26), the malate/oxaloacetate system (Fig. 7, Literature

Review) seemed the most promising alternative to examine. Because modified mitochondria exhibit relatively low membrane permeability to the direct entry and oxidation of extra-mitochondrial NADH, they were chosen as the test system for the malate/oxaloacetate shuttle mechanism. The experiments were performed at 28° C using the fluorescence attachment of the Aminco-Chance dual wavelength spectrophotometer to measure NADH oxidation and a vibrating platinum electrode for simultaneous monitoring of oxygen consumption. The latter parameters were examined in the presence and absence of the externally added enzymes (malate dehydrogenase and glutamate:oxaloacetate transaminase) and the intermediates (aspartate, glutamate + malate and 2-oxoglutarate) required for the operation of the shuttle. The components of the various test systems examined are summarized in Table XII. Diagrams reproducing (on a reduced scale) the fluorometer and spectrophotometer tracings are presented in Fig. 19a-h. Section II of the Appendix illustrates a full-scale copy of a portion of the original tracing corresponding to the experiment shown in Fig. 19a.

The control experiments (i.e. in the absence of externally added enzymes) are reproduced in Fig. 19a, c, e and g. NADH was the first addition to the mitochondria in all the experiments. The subsequent addition of aspartate (2.4 mM final concentration) produced no change in the rate

Table XII

Composition of test systems in the study of the malate/oxaloacetate shuttle.

Final concentrations in the reaction vessel are indicated for the various components.

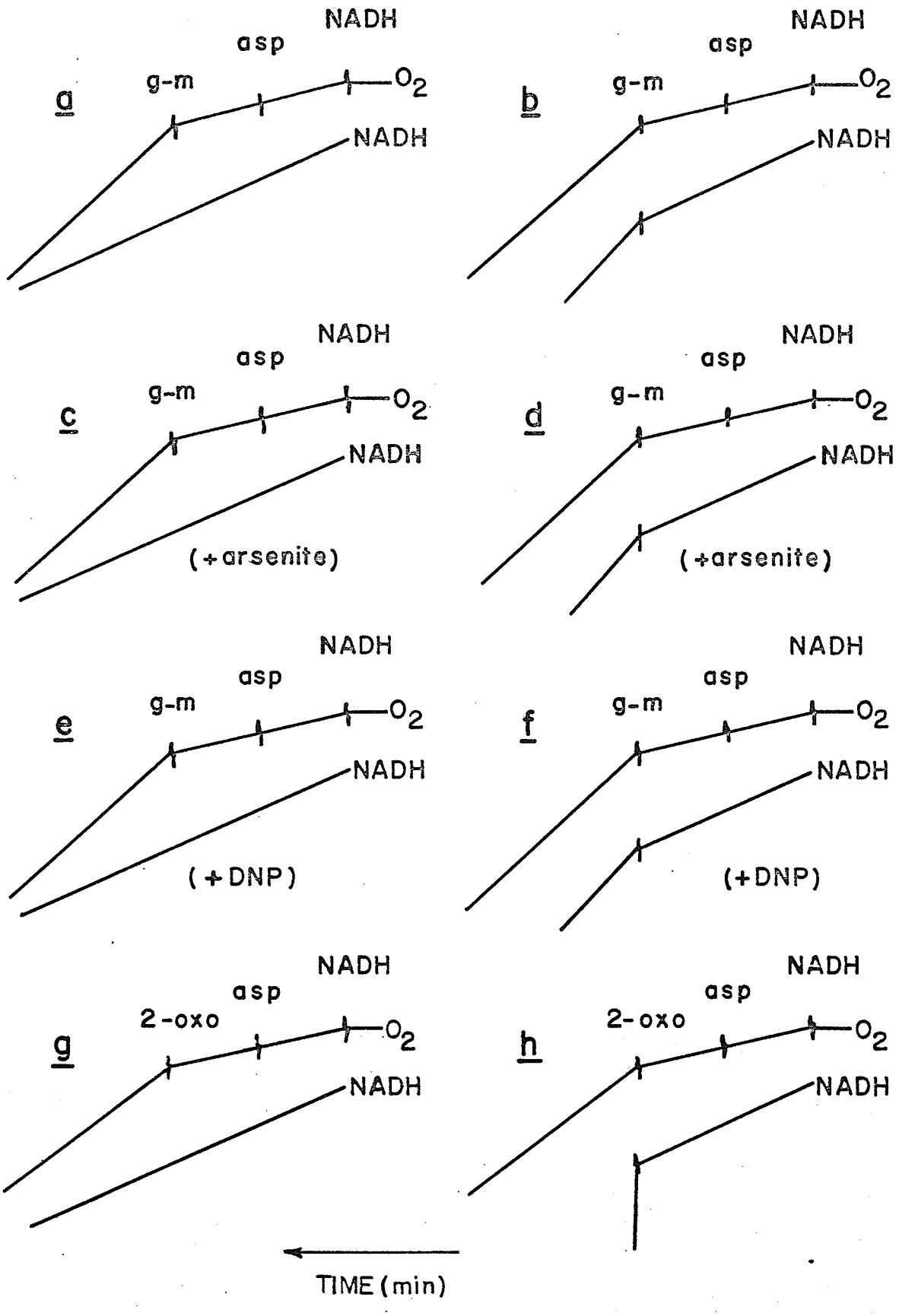
Figure 19 Reference	GOT* MDH	Arsenite 3.5 mM	DNP 30 μM	NADH 50 μM	Aspartate 2.4 mM	Glutamate-Malate 4.8 mM-4.8 mM	2-Oxoglutarate 2.4 mM
a	-	-	-	+	+	+	-
b	+	-	-	+	+	+	-
c	-	+	-	+	+	+	-
d	+	+	-	+	+	+	-
e	-	-	+	+	+	+	-
f	+	-	+	+	+	+	-
g	-	-	-	+	+	-	+
h	+	-	-	+	+	-	+

*GOT = glutamate:oxaloacetate transaminase
MDH = malate dehydrogenase
The enzymes were desalted by gel filtration on Sephadex G-25 (medium) before use. Both enzymes were added in excess of the calculated physiological activities to ensure that enzyme concentrations in the extramitochondrial portion of the postulated cycle were not rate-limiting.

FIGURE 19. Fluorometer and spectrophotometer recordings (reduced scale) of oxygen consumption and NADH oxidation in malate/oxaloacetate shuttle experiments with modified mitochondria.

g-m = glutamate + malate

asp = aspartate


External Enzymes Absent
External Enzymes Present
a
b
c
d
e
f
g
h
g-m
asp
2-oxo
NADH
O_2
(+arsenite)
(+DNP)
TIME (min)

of oxygen consumption or of NADH oxidation in any of the experiments in the absence of added enzymes. The further addition of glutamate + malate (4.8 mM each) resulted in a 3-5 fold stimulation of oxygen consumption (Fig. 19a, c, e) even in the absence of GOT and MDH. However, there was again no change observed in the rate of NADH oxidation corresponding to the increased oxygen consumption. The latter rate, therefore, presumably reflected only the oxidation of the glutamate + malate by way of transamination and the tricarboxylic acid cycle. Fig. 19c and e illustrates the effects of arsenite (3.5 mM) and 2,4-dinitrophenol (30 μM) on the control system. The former substance blocks the oxidation of 2-oxoglutarate and therefore should decrease the contribution to the oxygen consumption of the oxidation of glutamate + malate and thus make any stimulation of the oxygen rate as a result of the cycle mechanism more apparent. Also, arsenite by inhibiting the oxidation of 2-oxoglutarate might be expected to increase the rate of diffusion of this substrate from the mitochondrion so that it could participate in the external portion of the system. Although these expected effects of arsenite have been observed with liver mitochondria (17), they are clearly absent in the present experiments and no stimulation of NADH oxidation by arsenite was observed.

The possible effect of 2,4-dinitrophenol on NADH

oxidation by the malate/oxaloacetate shuttle was tested because this uncoupler has recently been shown (98) to influence the rate of outward movement across the mitochondrial membrane of 2-oxoglutarate. The 2,4-dinitrophenol had no effect on the rate of oxygen consumption or on the rate of NADH oxidation in the system when the externally added enzymes are not present. The addition of 2-oxoglutarate after aspartate to the control system, containing mitochondria, NADH and aspartate, produced a 3-fold stimulation of oxygen consumption but with no corresponding effect observed in the rate of NADH oxidation (Fig. 19g).

The above lack of stimulation of NADH oxidation by the various metabolite additions was not unexpected since the malate/oxaloacetate shuttle could not be expected to operate without the addition of the two external enzymes to the mitochondrial system. The test systems with GOT and MDH present are illustrated in Fig. 19b, d, f and h. The order of addition of the reactants was the same as in experiments without the external enzymes. Aspartate, in the presence of added enzymes, again had no effect on the rate of either oxygen consumption or NADH oxidation. Glutamate + malate addition (Fig. 19b) elicited a response in both the rate of oxygen consumption (3-fold) and the rate of NADH oxidation (3-fold). However, this cannot be assumed to indicate the operation of the shuttle, since the increase in oxygen

consumption was no greater than that observed with glutamate + malate in the absence of GOT and MDH (Fig. 19a). Thus, the enhanced NADH oxidation in Fig. 19b was presumably the result of its external reaction with oxaloacetate via malate dehydrogenase, the oxaloacetate being formed externally by transamination of the added aspartate with 2-oxoglutarate released from the mitochondria as a result of the internal transamination of glutamate and oxaloacetate. However, the malate formed externally from the reduction of oxaloacetate by NADH was either insufficient in amount or did not penetrate the mitochondrial membrane rapidly enough to enhance the rate of oxygen consumption through coupling of its oxidation to the respiratory chain. Had the latter occurred, an increase in the oxygen consumption rate could be expected in Fig. 19b over that in Fig. 19a approximately equivalent to the increase in the rate of NADH oxidation observed in the presence of the external enzymes (GOT, MDH). From the foregoing, it is clear that the acceleration of NADH oxidation seen in the presence of externally added glutamate; oxaloacetate transaminase and malate dehydrogenase occurred external to the mitochondria and no activity of the postulated malate/oxaloacetate shuttle was detected. The same test system, repeated in the presence of arsenite (Fig. 19d) and 2,4-dinitrophenol (Fig. 19f), showed no changes in behaviour from the effects discussed above. The

arsenite experiment indicated that 2-oxoglutarate oxidation in the tricarboxylic acid cycle was not a limiting factor in the operation of the shuttle. The absence of a 2,4-dinitrophenol effect might suggest that the concentration of uncoupler added was ineffective, that 2,4-dinitrophenol does not increase permeability of the membrane to 2-oxoglutarate or that 2-oxoglutarate movement from the mitochondria was not a limiting factor in the cyclic mechanism. Fig. 19h illustrates the effect of 2-oxoglutarate addition to the test system in the presence of the external enzymes. The same increase in the rate of oxygen consumption was observed as in the control (Fig. 19g) accompanied by a very rapid oxidation of NADH. This evidence suggests that a barrier to the passage of 2-oxoglutarate from the mitochondria exists, since its addition to the external system results in such a rapid oxidation of NADH (compare with Fig. 19g). The failure of the shuttle to operate was also emphasized by rotenone (see Fig. 3) experiments (not illustrated in Fig. 19). Sufficient rotenone was added to block oxygen consumption in the test system containing enzymes (GOT and MDH), mitochondria, NADH, aspartate and glutamate + malate. However, the increased external oxidation of NADH noted above in Fig. 19b, d and f was completely rotenone-insensitive. These results are compatible with the conclusion that in the test system studied in these

experiments, NADH was oxidized by an extramitochondrial reaction and was not dependent on the cyclic operation of the malate/oxaloacetate shuttle. The results reported in this experiment have been confirmed by Mrs. Yolanda Rigault of this laboratory using various concentrations of NADH and substrates with the modified mitochondria. It may therefore be concluded that the malate/oxaloacetate shuttle does not satisfactorily account for the oxidation of extramitochondrial NADH by modified pigeon heart mitochondria observed in these studies. Comparable experiments were not done on the control preparation because the high direct entry of NADH into these mitochondria would obscure any evidence of the shuttle mechanism.

Spectrophotometric Estimation of Cytochrome c Content in Preparations of Control and Modified Pigeon Heart Mitochondria

The rotenone-sensitive cytochrome c stimulation of exogenous NADH oxidation in the two preparations observed in the effector studies described above suggested that endogenous mitochondrial cytochrome c might be a rate-limiting factor in the oxidation process. The limitation imposed by the cytochrome c level might be expected to be greater in the modified preparation since the observed rate of NADH oxidation was lower.

To test this hypothesis, the cytochrome c content of control and modified mitochondria was estimated using the Aminco-Chance dual wavelength spectrophotometer with wavelength pair 550-540 mμ as described under Methods. The cytochrome c concentrations were calculated as μmoles/g mitochondrial protein (see Appendix, section III). A statistical analysis was carried out on the paired control and modified data presented in Table XIII. It is clear that the difference in oxidation rates with added NADH cannot be attributed to the cytochrome c levels since the concentration of this carrier is not significantly different ($P > 0.01$) in the two preparations. Although added cytochrome c was previously shown in these results to stimulate NADH oxidation, the effect was similar for both control and modified mitochondria. A relatively lower indigenous cytochrome c level, therefore, does not account for the slower NADH oxidation rate in the modified mitochondria.

Comparison of the Respiratory Chain Capacity for Oxidation of NADH by Control and Modified Preparations of Pigeon Heart Mitochondria

The lower respiration rate observed with added NADH in modified mitochondria (relative to the control preparation) may be associated with a membrane and/or a respiratory chain phenomenon. To distinguish between these effects, the

Table XIII

Cytochrome c concentration of control and modified preparations of pigeon heart mitochondria (PHM) determined by spectrophotometric estimation.

Cytochrome c (μmoles/g mitochondrial protein)			
Control PHM		Modified PHM	
-dithionite*	+dithionite**	-dithionite	+dithionite
0.293	0.388	0.331	0.468
0.270	0.378	0.197	0.372
0.627	0.627	0.624	0.733
0.597	0.597	0.604	0.656

*reduced cytochrome c measured in mitochondria which have respired to anoxia in the presence of excess substrate (state 5 (31))

**dithionite was added to reduce residual oxidized cytochrome c that persisted in state 5

maximum oxidative capacity of the respiratory chain of the two preparations was examined after disruption of the membrane barrier with sonic irradiation (Bronwill Biosonik Probe). In preliminary experiments, the optimum time of sonication was determined for the two preparations (Fig. 20). A total sonication time of 30 seconds was sufficient to elicit the maximum rate of NADH oxidation.

The data obtained on the effects of sonication on the control and modified preparations is presented in Table XIV with the statistical analysis of the paired data. The significant difference in oxygen consumption between intact control and modified mitochondria before sonication disappeared after only 15 seconds of sonication. No difference was found between the maximum capacities of the respective respiratory chains to utilize NADH added to a saturating concentration. The large increase in oxidation rate after sonication demonstrates that a membrane barrier to NADH penetration exists in both preparations. The difference between oxidation of exogenous NADH by control and modified mitochondria before sonication must then be attributed to differences in the structural and/or functional properties of their membrane systems and not to differences in the capacity (V_{max}) of the NADH dehydrogenase and the subsequent carriers of the respiratory chain.

The observations thus far have indicated that the

FIGURE 20. The effect of time of sonication on the rate of oxygen consumption in control and modified mitochondria.

●—● - Control mitochondria

○—○ - Modified mitochondria

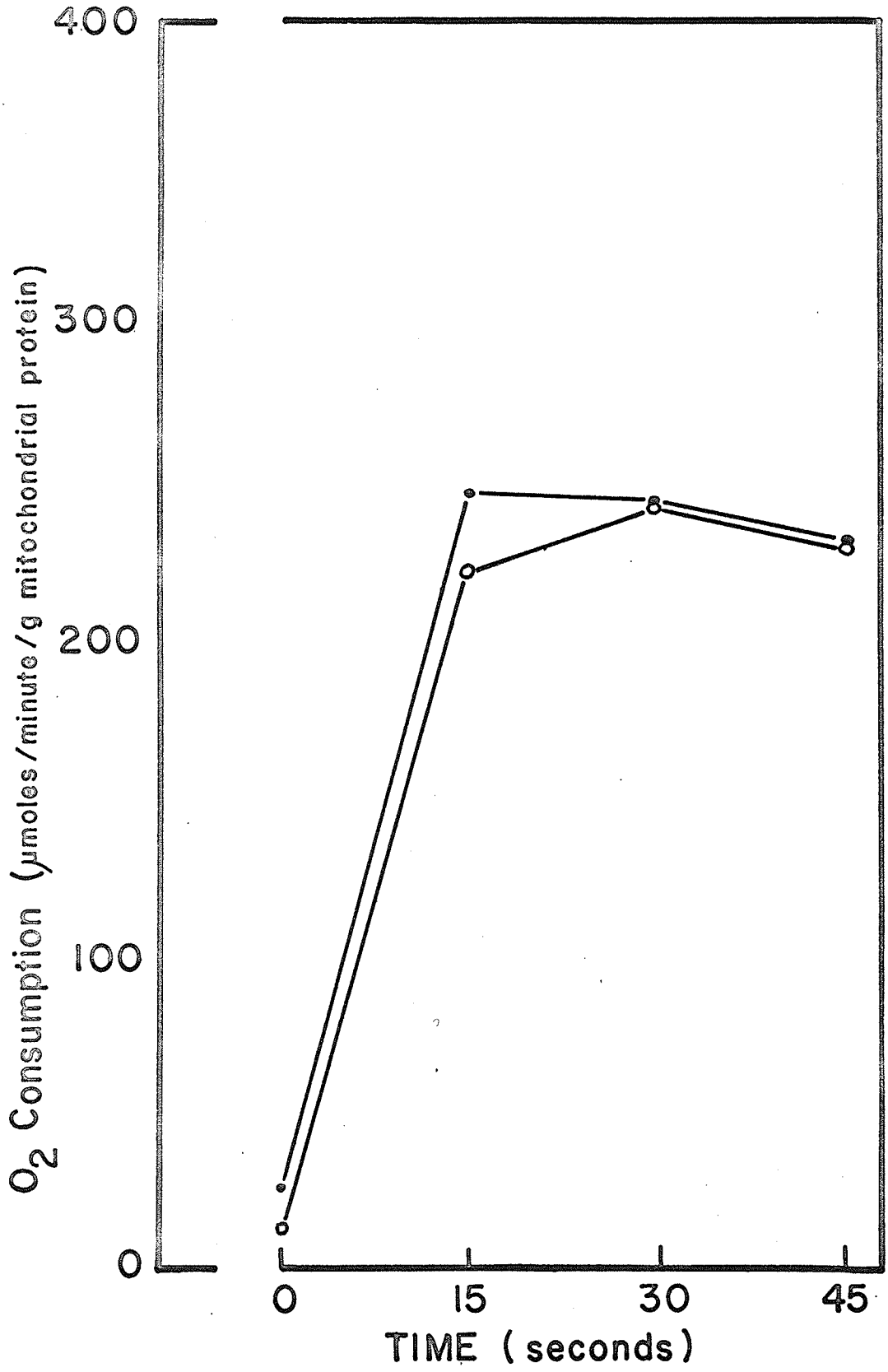

400
300
200
100
0
O_2 Consumption (μmoles/minute/g mitochondrial protein)
0
15
30
45
TIME (seconds)

Table XIV

The effects of sonication on the oxidation of externally added NADH (150-200 μM final concentration) by control and modified preparations of pigeon heart mitochondria measured polarographically at 28° C.

Respiration Rate (μmoles O_2/minute/g mitochondrial protein)								
Time of Sonication (seconds)								
0		15		30		45		
C*	M*	C	M	C	M	C	M
17	9	202	172	216	228	223	213
17	11	211	175	179	214	169	197
24	12	231	187	258	237	187	255
24	10	188	207	223	245	248	247
39	23	297	235	325	283	331	224
39	32	278	201	294	298	300	294
t (calculated)	6.575	2.875		0.162		0.203	
t (theoretical)	4.032	4.032		4.032		4.032	
Significance** (P 0.01)	S	NS		NS		NS	

*C = control
M = modified
**S = significant
NS = non-significant

differences between control and modified mitochondria are not related to either the respiratory chain or to a cytochrome c-linked external pathway of oxidation linked to the respiratory chain. It appears likely then that the altered metabolic behaviour is related to membrane structure and/or function. Preliminary experiments were carried out on four other aspects of the problem which are more directly concerned with mitochondrial structural integrity.

1. Dilution and mechanical effects
2. Proteinase (Nagarse) effects
3. Albumin effects
4. Supernatant experiments

1. Dilution and mechanical effects

The lower ratio of tissue wet weight (g)/volume of homogenizing medium (ml) used in the preparation of modified mitochondria might introduce two types of effects; (a) those associated primarily with the higher dilution of the myocardium during homogenization ("dilution" effects) and (b) those associated primarily with the changes in the mechanical properties of the system as a result of homogenizing a small quantity of tissue ("mechanical" effects). In the dilute system used to prepare modified mitochondria the individual organelles might be exposed to an increased shear force during homogenization. Changes induced by the mechanical action of this increased force

would be of type (b) above. In addition to the mechanical stress exerted on the mitochondria, the actual dilution of the tissue homogenized might also have some effect on the resultant preparation. The extent of removal from the mitochondria of any factor(s) necessary for efficient oxidation of substrates may be dependent upon the establishment of a dynamic steady state. The high volume of medium, relative to the mitochondrial concentration, during isolation of modified mitochondria would thus necessitate more complete removal of such a factor in order to attain the steady state condition. This removal may be reflected in conformational or functional changes in the isolated mitochondria.

To obtain a general picture of the effects on NADH oxidation obtainable by varying the amount of myocardium in a fixed volume of homogenizing medium (40 ml), a series of mitochondrial preparations were isolated using a range of ratios of tissue wet weight/homogenizing medium volume from 1/10 to 1/160. The rate of oxygen consumption by these preparations with added NADH as substrate (150-200 μM final concentration) was measured polarographically at 28° C. As an independent means of assessing mitochondrial integrity, the "tightness" of the coupling of oxidation and phosphorylation was tested using pyruvate + malate as substrate. All preparations used in the NADH oxidation rate

comparisons showed essentially normal oxidation rates with pyruvate + malate and exhibited RCR's of 4.5 or greater.

Fig. 21 shows the oxygen consumption (μmoles O_2/minute/g mitochondrial protein) plotted against concentration (increasing amounts of myocardium in a fixed volume of homogenizing medium). The concentrations of the control and modified mitochondrial preparations are indicated on the graph. The initial increases in tissue concentration appeared to produce a linear response in the rate of oxygen consumption. At higher tissue concentrations (>20 mg/ml), the response was non-linear and suggested an asymptotic plot, plateauing at some higher rate of oxygen consumption. These changes in NADH oxidation illustrated in Fig. 21 reflect the combined action of dilution and mechanical factors described on the previous page.

The asymptotic relationship described above was examined further by a double reciprocal plot (Fig. 22) of the reciprocal of oxygen consumption versus "dilution" (the reciprocal of concentration). The eye-fitted linear plot indicates an inverse relationship between the rate of oxygen consumption and dilution over the range studied. From the graph of the reciprocals, the "maximum" asymptotic rate of oxygen consumption was calculated using the intercept at the ordinate. The resultant value of 117 μmoles O_2/minute/g mitochondrial protein is compatible with the original data

FIGURE 21. Oxygen consumption as a function of the concentration (g/ml) of myocardium homogenized.

The concentrations used for control and modified preparations are indicated.

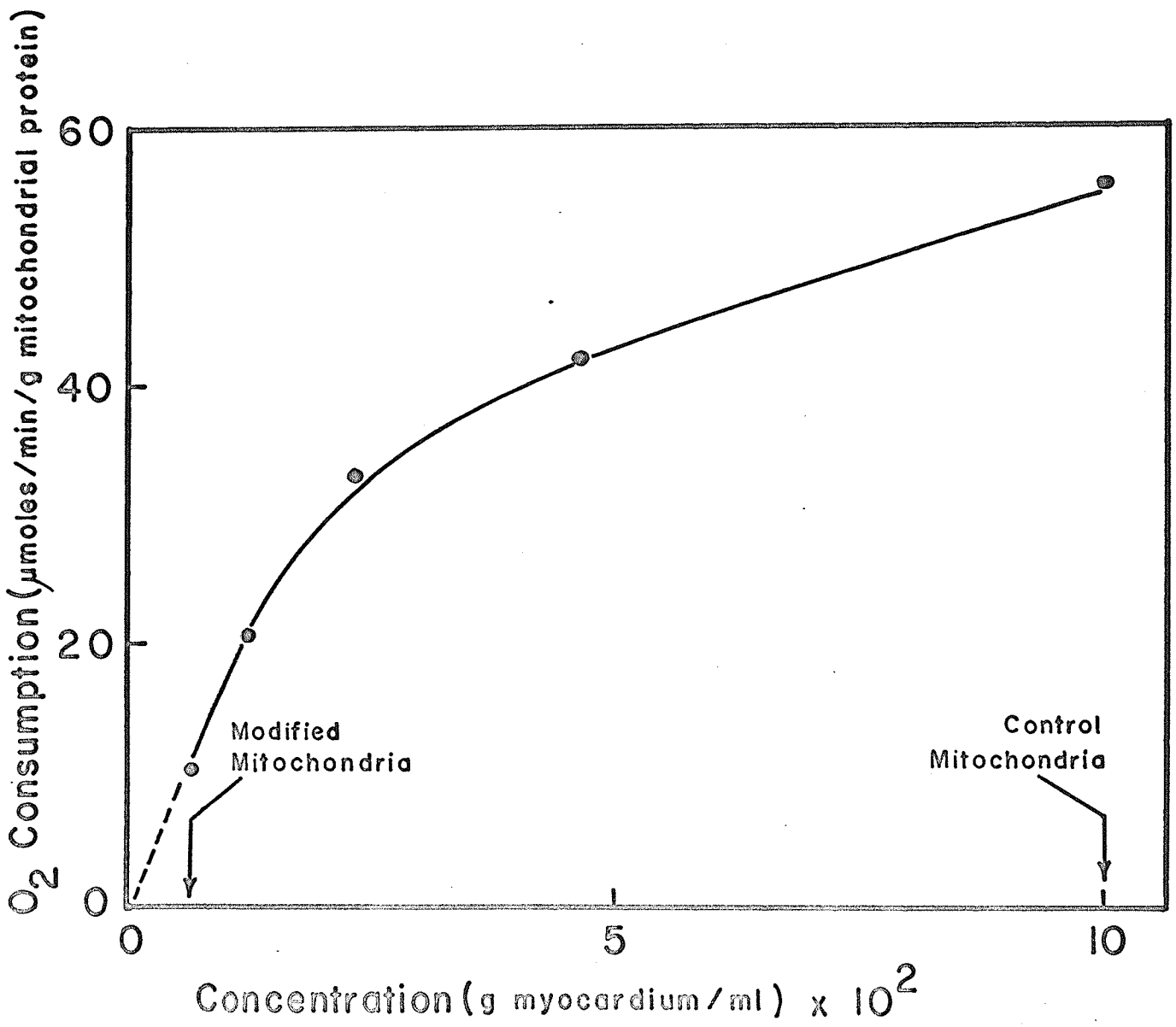
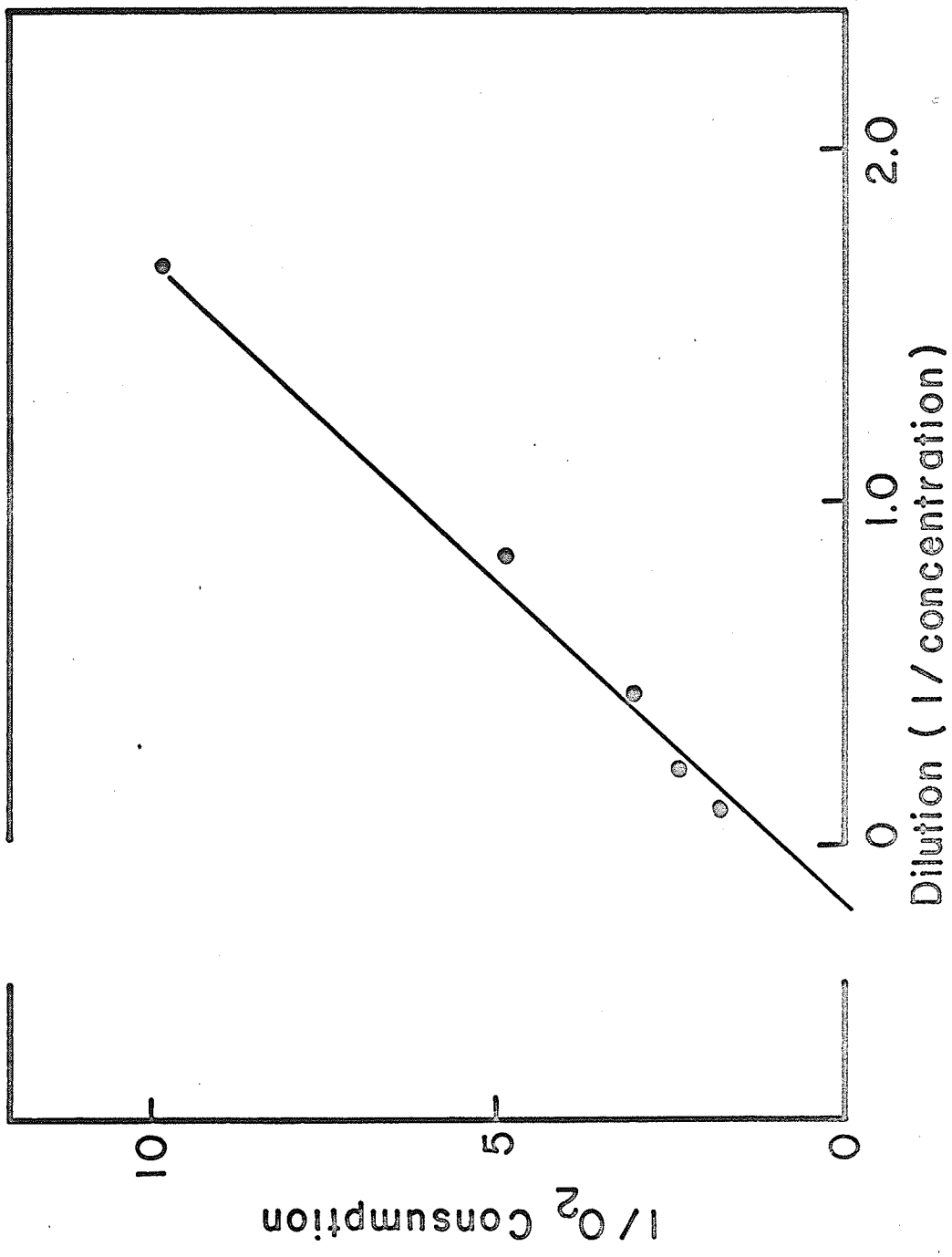

O_2 Consumption (μmoles/min/g mitochondrial protein)
60
40
20
0
0
5
10
Concentration (g myocardium/ml) x 10^2
Modified Mitochondria
Control Mitochondria

FIGURE 22. Double reciprocal plot of oxygen consumption versus varying concentration (g/ml) of myocardium homogenized.


1/O2 Consumption
10
5
0
0
1.0
2.0
Dilution (1/concentration)

of Fig. 21 which indicated that a plateau in the rate of oxygen consumption might occur at some point higher than the 60 μmoles O_2/minute/g mitochondrial protein. The additional observation that the calculated maximum rate (117 μmoles O_2/minute/g mitochondrial protein) was far below that of sonicated mitochondria (shown earlier in these results) suggested that the physiological rate of NADH oxidation might not necessarily utilize the maximum capacity of the respiratory chain.

As discussed earlier in this section, the metabolic differences between control and modified mitochondria might be related to either dilution effects or the altered mechanical properties of the system used in isolating the latter mitochondria. The small quantity (0.25 g) of myocardium initially homogenized in this system results in a homogenate which is 16-fold more dilute (with respect to tissue concentration) than the control preparation. To eliminate the contribution of mechanical effects to the properties of the resultant preparation, fractions of a control myocardial homogenate were diluted 16-fold at various stages during the isolation procedure. The remainder of the method following each particular dilution remained unchanged and the resulting preparations were tested polarographically at 28° C with pyruvate + malate and NADH as substrates. For comparison with the control and modified

mitochondria, the following preparations were isolated:

(a) Control (h); diluted 1/160 ratio of tissue wet weight/homogenizing medium volume after the first homogenization

(b) Control (2h); diluted to the ratio of the modified preparation after the second homogenization

(c) Control (2hs); diluted as in (b), followed by 10 minutes of mechanical stirring

The experimental results of analysis of these preparations are shown in Table XV. Dilution of the myocardial homogenate between the two homogenization steps (control (h) preparation) resulted in a preparation which was not significantly different from the usual modified mitochondria in any of the parameters measured. A similar situation was observed in the case of pyruvate + malate oxidation in the control (2h) and control (2hs) preparations. However, both these mitochondrial types had significantly faster (>2-fold) rates of NADH oxidation than the modified preparation. Since similar decreases in pyruvate + malate oxidation, RCR's and ADP:O ratios were observed in the modified, control (h), control (2h) and control (2hs) preparations (relative to control mitochondria), it would appear that the major factor causing these differences was related to dilution of the original tissue or muscle homogenate. However, the oxidation of extramitochondrial NADH did not follow the same pattern. The lower rates of oxidation of NADH in the modified

Table XV

Dilution and mechanical effects on the oxidation of pyruvate + malate and NADH by control and modified pigeon heart mitochondria.

Mitochondrial Preparation		Period of Respiration (Pyruvate + malate) I			II			III			NADH
		O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control	(5)**	***252	7.2	2.9	272	8.5	2.8	246	8.2	2.8	55
Modified		181	4.1	2.8	194	5.2	2.8	200	5.9	2.8	24
Control (h)*		177	4.3	2.8	204	5.3	2.8	193	6.7	2.6	31
Control	(4)	262	6.8	2.9	273	8.2	2.9	243	7.9	2.9	54
Modified		194	4.3	2.7	203	5.1	2.6	202	6.1	2.8	19
Control (2h)*		180	3.8	2.5	210	5.8	2.6	198	7.8	2.6	39
Control	(3)	264	6.3	2.9	278	7.8	2.8	249	7.4	2.7	57
Modified		211	3.7	2.7	218	4.5	2.7	229	5.7	2.9	21
Control (2hs)*		190	4.2	2.5	211	4.7	2.4	221	6.2	2.4	46

*for a description of these preparations, see text
**numbers in brackets represent the number of experiments in each comparison
***oxygen consumption (μmoles O_2/minute/g mitochondrial protein), RCR's and ADP:O ratios given are mean values

preparation were only duplicated by the control (h) preparation. This observation suggested that, in contrast to the situation with pyruvate + malate as substrate, the changes in NADH oxidation might be related to the altered mechanical properties of the system diluted before at least one of the homogenization steps. Thus, the changes observed in the oxidation of the two substrates, although showing similar trends, might be attributable to different causes.

2. Proteinase (Nagarse) effects

The use of proteolytic enzymes in the isolation procedure of muscle mitochondria has been described by Chance and Hagihara (76). The use of a brief digestion period with bacterial proteinase before homogenization probably results in less mechanical trauma to the mitochondria.

The ratio of tissue wet weight (mg)/proteinase (mg) is 150-200/1 in the isolation procedure for control mitochondria, whereas the use of a smaller quantity of myocardium in the preparation of modified mitochondria reduces the ratio to 12.5/1. This higher level of proteinase, relative to the mitochondrial concentration, might contribute to the observed metabolic behaviour of the modified mitochondria. To test this possibility, control and modified mitochondria were prepared with tissue wet weight/proteinase ratios according to the scheme

outlined in Table XVI. The four preparations described in Table XVI were examined polarographically using pyruvate + malate and NADH as substrates. The results of these experiments are shown in Table XVII. The data are compared in two pairs; control versus modified (C) and control (M) versus modified. The control and modified (C) comparison demonstrates a similar relationship with pyruvate + malate as substrate to that observed in the usual control and modified preparations. This is compatible with the earlier data that suggested the cause(s) of changes in pyruvate + malate oxidation were related primarily to dilution. Since no change in these preparations was made in the usual ratio of tissue wet weight/homogenizing medium volume, it might reasonably be suggested that the increased concentration of proteinase, relative to the amount of tissue homogenized in the modified preparation was not responsible for the changes observed in oxidation of pyruvate + malate. Absence of a proteinase effect cannot, however, be eliminated in the case of oxidation of added NADH since the rates of oxygen consumption of the control and modified (C) preparations with extramitochondrial NADH were not significantly different. Two possible interpretations were suggested on the basis of this data:

(a) The higher ratio of tissue wet weight/proteinase used in these preparations (control and modified (C))

Table XVI

Quantities of myocardium and Nagarse used to prepare pigeon heart mitochondria to compare the effects of the tissue/proteinase ratio.

Mitochondrial Preparation	Tissue wet weight (mg)	Nagarse (mg)	Nagarse (mg/ml)	Tissue wet weight / Nagarse
Control	3000	20	0.5	150/1
Control (M)*	3000	240	6.0	12.5/1
Modified	250	20	0.5	12.5/1
Modified (C)**	250	1.66	0.04	150/1

*control mitochondria prepared with a tissue wet weight/proteinase ratio of 12.5/1 usually used for modified mitochondria

**modified mitochondria prepared with a tissue wet weight/proteinase ratio of 150/1 usually used for control mitochondria

Table XVII

The effects of varying the ratio of tissue wet weight/proteinase during homogenization on the oxygen consumption, RCR's and ADP:O ratios with pyruvate + malate and NADH as substrates.

Oxygen consumption is given in μmoles O_2/minute/g mitochondrial protein.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate) I			II			NADH
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control	**259	7.1	2.7	343	9.0	2.5	37
Modified (C)	147	3.5	2.5	173	4.6	2.4	29
Control (M)	82*	-	-	-	-	-	25
Modified	179	4.5	2.5	228	5.8	2.4	16

*these preparations were completely uncoupled in all experiments

**values in the table are the means of three experiments

provides insufficient enzyme to digest the tissue, resulting in increased mechanical trauma during homogenization and a subsequent faster rate of NADH penetration and utilization.

(b) The higher ratio in these preparations maintains the structural integrity of the mitochondrial membrane and the resulting faster rate of oxidation of external NADH is a property of these intact mitochondria. A logical corollary to this hypothesis would be that in the preparation of modified mitochondria, the proteinase (acting on a smaller amount of tissue) damages the mitochondrial membrane in such a way as to hinder NADH penetration.

Insufficient data is available to reject either hypothesis. Furthermore, if the causes of differences in pyruvate + malate and NADH oxidation between control and modified mitochondria were not the same, interpretation of the results would be complicated further.

A comparison of control (M) and modified mitochondria is also shown in Table XVII. The former preparation was completely uncoupled in all experiments with pyruvate + malate as substrate. In this case, although the ratio of tissue wet weight/proteinase was 12.5/1 (as in the modified preparation), the high concentration of Nagarse (6 mg/ml) in the medium probably resulted in damage to the mitochondria, particularly in relation to the coupling mechanism. It is therefore surprising that the rates of NADH oxidation by

control (M) and modified mitochondria are not significantly different if, as has been suggested (3, 33), membrane damage necessarily increases NADH penetration. The similarity in rates might indicate that, in fact, the reverse is true (i.e. oxidation of added NADH is a property of intact mitochondria which can be inhibited by altering the structural integrity of the membrane under certain conditions).

The data in these experiments indicated that pyruvate + malate oxidation in control and modified mitochondria was probably not affected by the different ratios of tissue wet weight/proteinase. At high proteinase concentrations (6 mg/ml), however, the rate of pyruvate + malate oxidation in the control preparation could be significantly reduced and uncoupled, suggesting that some structural damage might have occurred. The results presented on oxidation of NADH provide additional evidence that decreases in this parameter observed in modified mitochondria might be attributable to different causes than those with pyruvate + malate as substrate. The concentration of proteinase (relative to the amount of tissue homogenized) appeared to affect the rate of NADH oxidation in one of two ways; (a) less tissue digestion with lower amounts of Nagarse resulting in increased mechanical damage and higher NADH rates or (b) higher amounts of Nagarse acting on the mitochondrial structure and producing lower NADH rates. No conclusive evidence exists

for either proposal.

3. Albumin effects

The inclusion of albumin in the media during the isolation and assay procedures has been shown to increase the RCR's of mitochondrial preparations (84, 85). This effect has been attributed to the binding by albumin of free fatty acids in the environment which affect the structural integrity of the mitochondria and often result in uncoupling. It was considered possible that the preparative change used in the isolation of modified mitochondria might have resulted in an increase in the quantity of free fatty acids, relative to the mitochondrial concentration. The action of these fatty acids might be responsible for the observed behaviour of the modified preparation. The effect of albumin on the oxidation of extramitochondrial NADH by modified mitochondria was therefore examined. Two types of preliminary experiments were conducted; (a) the preparation of modified mitochondria in the usual media supplemented with 0.5% or 1.0% bovine serum albumin and (b) the preparation of modified mitochondria in which the final washing and resuspension were carried out with suspending medium containing 1.0% bovine serum albumin. The results of polarographic analyses of these preparations are summarized in Tables XVIII and XIX.

The addition of the lower albumin concentration (0.5%) to the mitochondrial preparation media increased the rate of

oxygen consumption with pyruvate + malate and the RCR's of the modified mitochondria (Table XVIII). These effects were accompanied by a concomitant increase in the rate of oxygen consumption with NADH and suggest that albumin, as well as decreasing the uncoupling effect of free fatty acids might also modify mitochondrial structural integrity in such a way as to cause increased penetration of exogenous NADH. This type of result suggested further evidence for a possible differential effect involving the behaviour of the control and modified preparations with the two substrates.

In view of the effects of 0.5% albumin, the results with media containing 1% albumin were, therefore, somewhat surprising. Modified mitochondria prepared in the latter medium had low rates of pyruvate + malate oxidation and poor RCR's. Two possible explanations of this behaviour were suggested.

(i) The albumin in the medium might already have contained bound fatty acids which were released during the mitochondrial preparation procedure producing the slower uncoupled rate of oxidation of pyruvate + malate.

(ii) The 1% albumin provided the Nagarse in the homogenizing medium with a preferential protein substrate. Since the tissue would therefore not be predigested to the same extent, increased mechanical trauma to the mitochondria in the modified preparation might result in uncoupling of

Table XVIII

The effects of albumin in the isolation media of modified pigeon heart mitochondria on the oxidation of pyruvate + malate and added NADH.

Oxygen consumption is given in μmoles O_2/minute/g mitochondrial protein.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate)									NADH
	I			II			III			
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control	289	4.5	2.3	439	7.4	2.4	293	-	-	76
Modified	216	3.2	2.2	246	3.5	2.2	279	-	-	17
Modified*	257	3.8	2.0	256	4.0	1.9	-	-	-	45
Control	280	6.7	2.8	247	8.3	3.0	235	7.2	2.9	65
Modified	181	3.7	3.1	171	5.0	2.9	168	5.6	2.8	23
Modified**	47	2.5	2.3	37	1.8	2.4	-	-	-	21

*preparation media supplemented with 0.5% bovine serum albumin
**preparation media supplemented with 1.0% bovine serum albumin

pyruvate + malate oxidation. The observation that the rate of oxygen consumption with added NADH was not significantly changed by the inclusion of 1% albumin is additional evidence that the differences between control and modified mitochondria in utilization of the two substrates might not be attributable to the same cause(s).

The effect of 1% bovine serum albumin included in the final washing and resuspension medium for modified mitochondria was also investigated (Table XIX). Although the albumin preparation showed somewhat faster oxygen consumption with pyruvate + malate and significantly increased RCR's over the non-albumin modified mitochondria, little change in the rate of oxygen consumption with NADH was observed. These observations also suggested that the albumin-reversible uncoupling effects associated with pyruvate + malate oxidation in modified mitochondria (relative to control mitochondria) might be a separate phenomenon from the difference in NADH oxidation observed between the two preparations. These experiments, therefore, indicated in a preliminary manner that free fatty acids might be involved in the differences in oxidation of pyruvate + malate between control and modified mitochondria. It was unlikely that such free fatty acids were associated with the observed dissimilarities in the rate of oxygen consumption with extramitochondrial NADH.

Table XIX

The effect of washing and resuspension of modified mitochondria in medium containing 1% bovine serum albumin on the oxidation of pyruvate + malate and added NADH*.

Oxygen consumption is given in μmoles O_2/minute/g mitochondrial protein.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate)									NADH
	I			II			III			
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control	310	7.3	2.8	323	8.9	2.8	267	7.0	2.7	72
Modified	194	4.8	2.8	213	6.8	2.6	173	6.2	2.7	13
Modified**	194	8.2	2.5	249	10.6	2.6	202	9.0	2.6	19

*the values in the table represent the mean of three experiments
**modified mitochondrial preparations washed and resuspended in medium containing 1% bovine serum albumin

4. Supernatant experiments

Decreasing the concentration of myocardial tissue homogenized in the isolation of the modified preparation might have facilitated the removal from these mitochondria of a factor essential for the efficient oxidation of pyruvate + malate and NADH. In view of some of the evidence in the literature (48, 90), such an unknown factor, if it exists, could be protein in nature. A demonstrable effect of the supernatant of a mitochondrial preparation on the rates of oxygen consumption with pyruvate + malate and added NADH in modified mitochondria would indicate that such a factor had been removed from the mitochondria, or that it was indigenous to the supernatant fraction. The 12,100 x g supernatant normally discarded during the isolation of control mitochondria was used as the source material for the supernatant experiments. Two types of experiments were undertaken using this 12,100 x g supernatant:

(a) a comparison of control and modified mitochondria with a second modified preparation isolated using the 12,100 x g supernatant as the homogenizing medium

(b) a study of the effects of the 12,100 x g supernatant as the cuvette medium in polarograph experiments with modified mitochondria

In type (a) experiments, the 12,100 x g supernatant was retained from the second centrifugation of the muscle homogenate in the isolation procedure for control

mitochondria. This supernatant was used as the homogenizing medium for a modified mitochondrial preparation. Before use, 20 mg of Nagarse were added to 40 ml of the supernatant material to ensure that the concentration of fresh proteinase in this homogenizing medium was at least 0.5 mg/ml. All other aspects of the isolation procedure remained unchanged. The resulting preparations were examined polarographically at 28° C using pyruvate + malate and NADH as substrates. The results of these experiments are presented in Table XX. Use of the 12,100 x g supernatant (with 20 mg proteinase added) as the homogenizing medium for isolation of modified mitochondria had no significant effect on the rate of oxygen consumption with pyruvate + malate or added NADH, or on the RCR's or ADP:O ratios relative to the usual modified preparation. These observations may be interpreted in various ways:

(i) no factor has been removed from the mitochondria, or exists in the supernatant which alters the oxidation of pyruvate + malate or NADH

(ii) such a factor did exist but was destroyed by the experimental manipulations, or was unable to restore activity once the latter had been decreased

The data of Table XX is insufficient to suggest which of the explanation(s) might be correct.

In type (b) experiments, the 12,100 x g supernatant was used as the reaction medium for polarographic analysis.

Table XX

The effects of supernatant homogenizing medium (see text) on the oxidation of pyruvate + malate and NADH by modified pigeon heart mitochondria.

Oxygen consumption is given in μmoles O_2/minute/g mitochondrial protein.

Mitochondrial Preparation	Period of Respiration (Pyruvate + malate)									NADH
	I			II			III			
	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2	RCR	ADP:O	O_2
Control	**331	7.8	3.1	360	8.4	3.0	341	7.0	3.1	63
Modified	157	3.7	3.0	186	5.9	3.0	187	6.0	3.0	27
Modified (SN)*	137	3.5	2.6	160	5.4	2.8	171	7.5	3.0	38

*Modified (SN) = modified mitochondria prepared in supernatant homogenizing medium (see text)

**oxygen consumption, RCR's and ADP:O ratios are given as the mean values of four experiments

Thus, the presence of a supernatant component(s) associated with the oxidation of pyruvate + malate or extramitochondrial NADH might be indicated by a direct effect on these processes. This approach was found to have numerous obstacles, notably the effects of endogenous substrates and uncouplers. Overnight dialysis against double distilled water did not completely eliminate the former effect which suggested it might have been an action of the supernatant on indigenous substrates in the mitochondrial preparations. Consequently the polarographic measurements of oxygen consumption in these experiments are of questionable value and will not be considered further.

DISCUSSION

DISCUSSION

Control and modified pigeon heart mitochondrial preparations were examined in this study with two objectives:

(a) To determine the source(s) of the observed differences in oxidation of pyruvate + malate and extramitochondrial NADH between control and modified mitochondria

(b) To evaluate the mechanism proposed for extramitochondrial oxidation of NADH by pigeon heart mitochondria

In view of the above aims, this discussion will be presented in three main sections.

(1) a brief consideration of the interpretations made directly from the data with respect to the differences between control and modified mitochondria, and the oxidation of extramitochondrial NADH

(2) a discussion of myocardial metabolism with particular reference to the substrates utilized and the requirements for oxidation of cytoplasmic NADH

(3) the physiological significance of the experimental results in view of the data in section (2)

(1) Both biochemical and morphological aspects of the two preparations have been examined to resolve the differences in oxidative behaviour observed between control and modified mitochondria. Morphological investigation (electron microscopy) demonstrated differences in the organization of the mitochondrial cristae (Fig. 15a and b). Evidence in the literature (95, 96) has been presented to correlate conformational changes in the inner mitochondrial membrane

and cristae to the functional state of the organelles. A detailed examination of the application of this theory to the results above was beyond the scope of the present study, except to note that some relationship might exist between cristae morphology and the functional properties of the control and modified mitochondria.

Mitochondrial volume studies were carried out (Table VIII, Figs. 16a and b) in attempting to correlate metabolic differences between control and modified preparations to mitochondrial swelling. However, the rate of oxidation of added NADH was unrelated to either the initial volume of freshly isolated organelles or the volume changes following addition of the substrate. The modified mitochondria, in fact, appeared to be larger in volume than those of the control preparation. Considered in conjunction with the electron micrographs, the apparently larger volume of the modified mitochondria might be a reflection of their larger size (indicated by their larger cross-sectional diameters). In the subsequent investigation of a possible size/function relationship using differential centrifugation, it was evident that the control preparation was a heterogeneous population while the modified mitochondria could not be detectably fractionated. The heterogeneity of the former preparation might be related to the functional properties of the mitochondria (i.e. the morphological sub-fractions could

also differ in their metabolic behaviour). The modified mitochondria might represent a specific homogeneous sub-fraction of the control population produced by the particular isolation conditions. A note of caution must be interjected at this point however. The heterogeneity of the control preparation observed experimentally might, in fact, be an artifact of the isolation technique in which a certain fraction of the original mitochondrial population could have been altered both morphologically and functionally. This type of alteration is inevitable to some extent in all preparations. However, in the present situation it appeared that a selectively greater effect had been exerted on the original population during preparation of modified mitochondria. Thus, the latter preparation might conceivably consist almost entirely of this specific mitochondrial type. Upon review of the findings, it was apparent that no single all-inclusive hypothesis could be proposed to explain on a purely morphological basis the differences in the oxidative properties of control and modified mitochondria with pyruvate + malate and added NADH as substrates.

In search of an alternative explanation for the differences between the two preparations certain biochemical aspects of control and modified mitochondria were investigated. No dissimilarities could be demonstrated in their pathway or kinetics of oxidation of extramitochondrial

NADH, in their cytochrome c concentrations or in their respiratory chain capacity to utilize added NADH (see Tables IX, X, XI and XIV). The evidence presented is compatible only with the hypothesis that with NADH as the mitochondrial substrate, the lower rates of oxidation in the modified preparation are related to membrane structure and/or function.

The experiments designed to establish a structure/function relationship cannot be considered more than exploratory in nature. The studies of dilution and mechanical effects (Table XV) indicated that both these factors were contributing to the behaviour of the isolated mitochondria. The meaning of the terms "dilution" and "mechanical" has been described previously in the Results. It appeared that lower rates of pyruvate + malate oxidation in the modified mitochondria might be related to the increased volume of medium per gram tissue rather than any mechanical action (see Table XV). In contrast, the differences in the oxidation of NADH could only be attributed to the different mechanical properties of the isolation system for modified mitochondria. These experiments gave the first indication that, although the rates of pyruvate + malate and added NADH oxidation are lower in modified mitochondria, the factors causing these lower rates are not necessarily the same for each substrate. Pyruvate + malate

oxidation might be influenced by an effect on the coupling mechanism, since RCR's and ADP:O ratios were also lower in modified mitochondria. The rate of NADH oxidation, however, could reflect differences in membrane integrity. The data implied that increased NADH oxidation was associated with intact rather than damaged mitochondrial membranes.

A differential effect on pyruvate + malate and NADH oxidation was also implied by the evidence of the proteinase and albumin experiments. Variation in the concentration of proteinase (Tables XVI and XVII) produced a statistically significant change in the oxidation of NADH but not in pyruvate + malate when the enzyme concentration was $\leq$0.5 mg/ml. At a proteinase concentration of 6 mg/ml, pyruvate + malate oxidation was slow and the mitochondria were uncoupled. At the lower more usual enzyme concentrations (approximately 0.5 mg/ml), no effect could be demonstrated regardless of the amount of tissue present.

Data obtained from albumin experiments with the two mitochondrial systems is also compatible with the hypothesis that the differences in pyruvate + malate oxidation and NADH oxidation between the control and modified mitochondria are not necessarily attributable to the same cause (Tables XVIII and XIX). The results with added albumin suggested that fatty acids contributed to the observed dissimilarities in pyruvate + malate oxidation but not to those with NADH as

substrate.

The possible loss of a mitochondrial "factor" into the supernatant which was required for efficient metabolic behaviour was also examined. However, as noted in the discussion accompanying Table XX, the absence of a supernatant effect does not necessarily eliminate the possibility that NADH penetration is inhibited by the removal of a labile mitochondrial substance essential for its transport or by a factor present in the cytoplasm which might have a similar effect.

In attempting to define the source of the differences between control and modified mitochondria, information directly concerned with the more general problem of the oxidation of extramitochondrial NADH was also obtained. The effector studies reported demonstrated that the oxidation of NADH observed was mediated by the mitochondrial respiratory chain. Inhibitor experiments with rotenone eliminated the existence of the proposed (2, 6, 33) external cytochrome c-stimulated rotenone-insensitive alternative route. Since the above results could also be interpreted as compatible with a metabolite shuttle mechanism, the feasibility of the malate/oxaloacetate shuttle was examined in modified mitochondria. Under the conditions used in these experiments, no evidence was obtained to support the operation of the malate/oxaloacetate shuttle as the mediating

system for oxidation of cytoplasmic NADH. The major block in the operation of the mechanism appears to be associated with the passage of 2-oxoglutarate out of the mitochondria. It would appear then, that in myocardial mitochondria, direct permeability of extramitochondrial NADH must be retained as a possible pathway for the removal of the nucleotide from the cytoplasm to maintain the normal NAD^+/NADH ratio.

The results of the sonication experiments used to investigate the capacity of the respiratory chain for NADH oxidation provided additional evidence that low rates of oxidation are a reflection of the properties of the mitochondrial membrane. In the control preparation which exhibited the criteria of intact mitochondria, viz. high RCR's and ADP:O ratios in the oxidation of pyruvate + malate, an obvious limited permeability of the mitochondrial membrane to NADH was demonstrated. The observed rates of oxidation in "intact" control preparations was only 20-30% of that in the sonicated material. The physiological importance (if any) of these low rates of NADH oxidation by the intact organelles will be considered in the final section of the Discussion. The evidence thus far described in this thesis is, however, compatible with a limited but direct penetration of extramitochondrial NADH by intact pigeon heart mitochondria.

(2) To interpret the physiological significance of the data reported in this thesis, the general pattern of myocardial metabolism must be considered. To estimate the minimum requirement for the oxidation of cytoplasmic NADH requires a review of the substrates used by the myocardium and their mode of oxidation.

In 1956, Bing (99) described the variability of the myocardial fuel supply which acts as the source of cardiac energy. The extraction of individual carbohydrate metabolites from the blood was dependent upon their arterial concentrations. In contrast, the carbohydrate/non-carbohydrate relationship was influenced by the relative availability of the two classes of substances. The data discussed by Bing (99) is summarized in Table XXI. It is obvious that the bulk of the cardiac energy supply is derived from a fatty acid source. Glucose and lactate, the major carbohydrate substrates, contribute only 35% of the total oxygen consumption. Since the production of cytoplasmic NADH is a result of glycolytic degradation of carbohydrates, it is evident that a large requirement for NADH reoxidation might not arise in myocardial tissue.

The *in vivo* studies of Bing *et al.* (29, 100) on carbohydrate and fat metabolism in human heart demonstrated the variability of substrate oxidation dependent upon the availability and concentrations of carbohydrate and non-

Table XXI

In vivo myocardial oxygen usage attributable to various substrates (adapted from Bing (99)).

Carbohydrate		Non-carbohydrate	
Substrate	%	Substrate	%
Glucose	18	Fatty acids	67
Pyruvate	0.5	Amino acids	6
Lactate	17	Ketones	4

carbohydrate sources, as well as on the metabolic requirements of the tissue. For the purposes of this discussion certain assumptions are necessary to define the conditions of uptake and oxidation of substrates in the myocardium. These assumptions are:

(a) The energy requirements of the working heart muscle are met mainly by the oxidation of carbohydrate and non-carbohydrate substrates obtained from the circulating blood.

(b) Carbohydrate sources contribute 35% of the total myocardial oxygen consumption recorded.

(c) The oxidation of non-carbohydrates (fatty acids) does not contribute to the production of cytoplasmic NADH which must subsequently be oxidized.

(d) The NADH produced in the cytoplasm by glycolysis must be reoxidized to maintain a steady state $NAD^+/NADH$ ratio that will allow the NAD^+-requiring reactions in the cytoplasm to continue.

The oxidative pathway of both glucose ($C_6H_{12}O_6$) and lactate ($C_3H_6O_3$) in heart forms the intermediate pyruvate ($C_3H_4O_3$) which is then converted to CO_2 and H_2O by the tricarboxylic acid cycle. The relationship between oxygen consumption and NADH production with these two carbohydrate substrates is indicated in the scheme below.

Glucose:

$$C_6H_{12}O_6 + O_2 \longrightarrow 2\ C_3H_4O_3 + 2\ NADH + 2\ H^+$$

$$2\ C_3H_4O_3 + 5\ O_2 \longrightarrow 6\ CO_2 + 4\ H_2O$$

Two molecules of NADH are produced with the consumption of 6 molecules of oxygen ($NADH/O_2 = 1/3$).

Lactate:

$$C_3H_6O_3 + 1/2\ O_2 \longrightarrow C_3H_4O_3 + NADH + H^+$$

$$C_3H_4O_3 + 2\ 1/2\ O_2 \longrightarrow 3\ CO_2 + 2\ H_2O$$

One molecule of NADH is produced with the consumption of 3 molecules of oxygen ($NADH/O_2 = 1/3$).

Thus, for either glucose or lactate as substrate one-third of the oxygen consumption is required for the reoxidation of the NADH produced in the cytoplasm.

(3) The rate of oxygen consumption reported (30) for working rat heart is 13 μmoles O_2/minute/g fresh weight. On the basis that 3% of the total fresh weight is mitochondrial protein (28) this value can be recalculated to give a maximum rate of approximately 450 μmoles O_2/minute/g mitochondrial protein. In the above discussion it was noted that only 35% (approximately 160 μmoles O_2/minute/g mitochondrial protein) of this rate is attributable to carbohydrate sources. This fraction of the total rate is associated with the reoxidation of cytoplasmic NADH. Since only one-third of the rate of oxygen consumption with carbohydrates can be assigned to the reoxidation of cytoplasmic NADH, the required rate of this process would be $160/3 \simeq 55$ μmoles O_2/minute/g mitochondrial protein. This calculated rate is far below the maximum respiratory chain capacity revealed by sonication (Table XIV). However, it is compatible with the rates of oxygen consumption with added

NADH observed regularly in the control pigeon heart mitochondrial preparations (see Table V). The close agreement in these values suggests that the control mitochondria might be a "physiologically intact" preparation in which the rate of direct entrance and oxidation of extramitochondrial NADH by such mitochondria is sufficient to account for the physiological requirements. It would appear on this basis that the modified preparation may not be as physiologically competent as the control organelles. The technique used in the preparation of modified mitochondria might be responsible for alteration of the membrane structure thus affecting NADH penetration. No evidence as to the mechanism of direct entry (e.g. simple diffusion, energy-independent facilitated diffusion) of NADH has been presented in this work. Interpretation of the experimental results in their physiological context, the importance of which was emphasized in the Introduction, has been attempted in the present thesis. Low absolute rates of NADH oxidation have previously been taken by workers to indicate that the mitochondrial membrane is not permeable to this reduced pyridine nucleotide (2, 39). However, it is evident from the discussion above that the observed rates of oxidation can, in fact, adequately account for the required removal of NADH from the cytoplasm <u>in vivo</u>, and that direct permeability to NADH is probably a characteristic property of intact

pigeon heart mitochondria.

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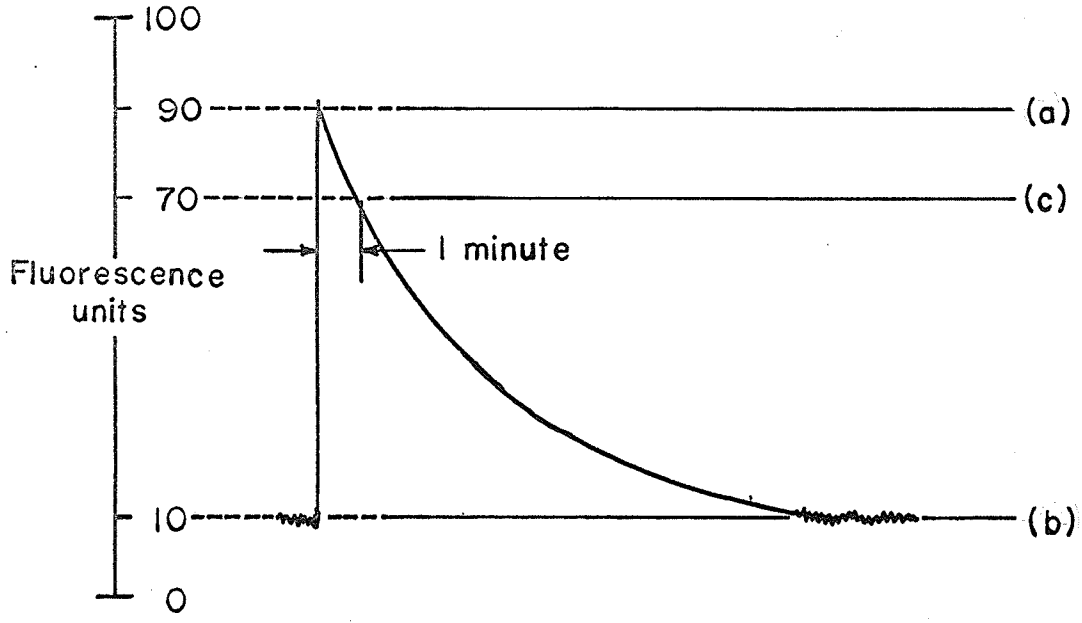
APPENDIX

APPENDIX

I. Calculation of initial velocity of NADH oxidation.



The accompanying diagram illustrates a typical fluorometer tracing for NADH oxidation indicating the fluorescence readings at the peak (a), baseline (b) and after 1 minute (c).

The amount of NADH remaining after 1 minute (Y) is calculated by the equation:

$$Y = \frac{(c) - (b)}{(a) - (b)} \times A$$

where A = concentration of NADH added (M)

The linear logarithmic plot of a first-order curve (as in the above diagram) is given by the equation:

$$2.303 \log Y = 2.303 \log A - kt$$

where k = constant

t = time (minutes)

Solving for k:

$$k = \frac{2.303}{t}(\log A - \log Y)$$

The initial velocity (V_o) can then be calculated using:

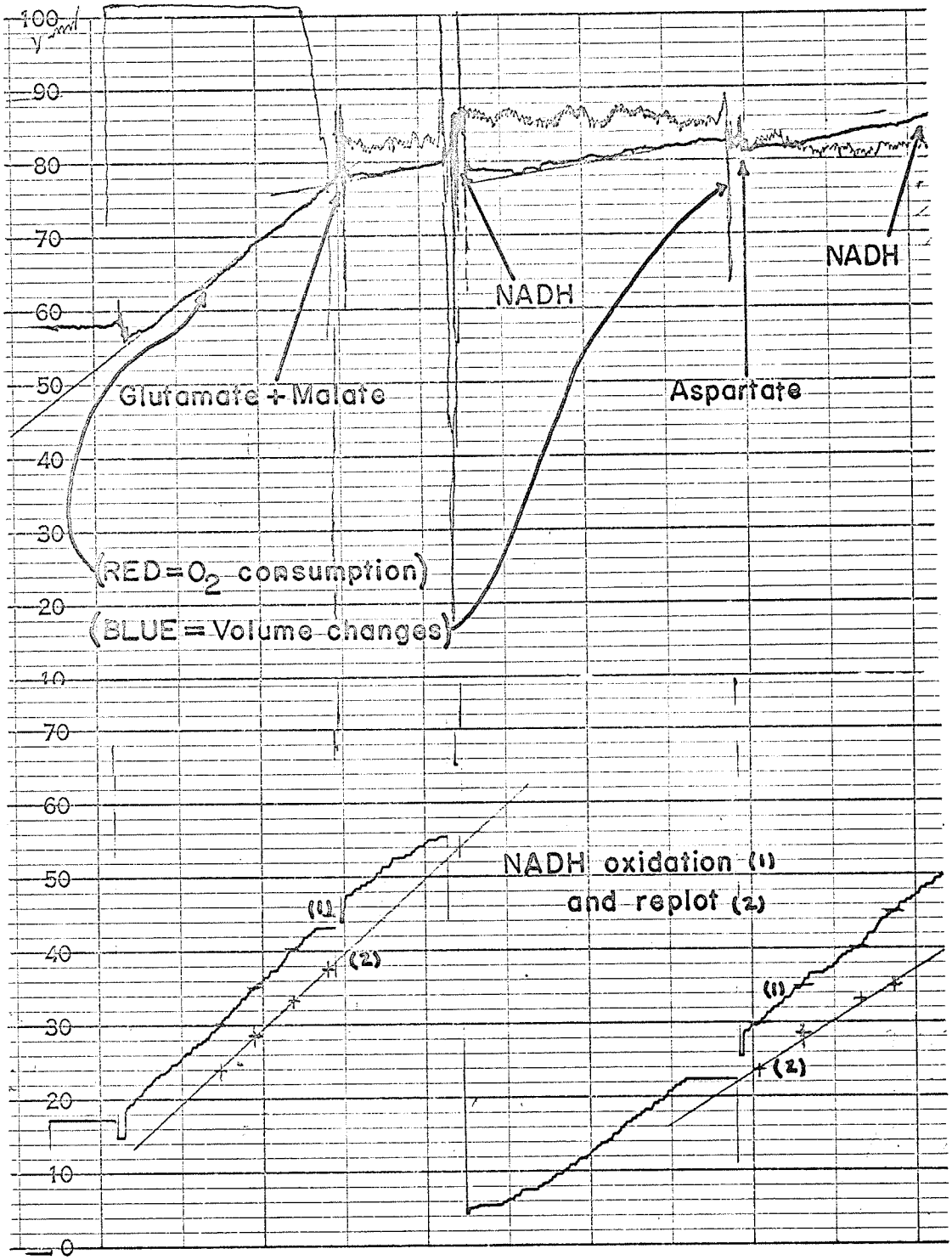
$$V_o = A(k) = \mu M/\text{minute}$$

The velocity can be converted to a rate expression by the following equation:

$$\text{Initial rate} = V_o \times \frac{1}{\text{mitochondrial protein (g)}} \times \text{volume in cuvette (ml)}$$

$$= \mu\text{moles NADH/minute/g mitochondrial protein}$$

II. Full-scale copy of metabolite shuttle experiment (corresponding to reduced copy in Fig. 19a).



III Calculation of mitochondrial cytochrome c concentration.

Cytochrome c concentration is calculated on the basis of the transmission change during reduction and the millimolar extinction coefficient. The latter value was taken as $19(cm - mM)^{-1}$ (81).

The formula used in calculating the concentration was:

Cytochrome c (μmoles/g mitochondrial protein) =

$$\frac{\text{\% Change in \% Transmission}}{\text{millimolar extinction coefficient}} \times 0.434 \times \text{volume in cuvette (ml)} \times \frac{1}{\text{mitochondrial protein (g)}}$$

Sample Calculation:

Transmission Change = 0.64%

Millimolar extinction coefficient = $19(cm - mM)^{-1}$

Volume in cuvette = 3.03 ml

Mitochondrial protein = 0.0015 g

$$\text{Cytochrome c} = \frac{0.64/100 \times 0.434}{19} \times 3.03 \times \frac{1}{0.0015}$$

$$= 0.293 \ \mu\text{moles/g mitochondrial protein}$$