THE EFFECTS OF AGING ON RAT HEART MITOCHONDRIAL CYTOCHROMES, RESPIRATION, OXIDATIVE PHOSPHORYLATION, AND PROTON TRANSPORT

By

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CHAPTER I

INTRODUCTION

The purpose of this investigation was an evaluation of the effects of aging on the components and activity of the electron transport chain in rat heart mitochondria. A diagram of the electron transport chain can be found in Figure 1.

The mitochondria are responsible for the synthesis of ATP and thus are the major supplier of energy to the cell. Obviously, if the mitochondria were not capable of adequately supplying the cell with ATP, the cell would be unable to function properly. Aging has been shown to have deleterious effects on different facets of the living organism. The effects of aging on mitochondria and the subsequent effect on the process of aging in the cell has been the subject of a number of investigations.

Floyd et al. (1984) and Harmon et al. (1987) studied the effects of aging on rat brain mitochondria. These investigators reported decreases in the content of all cytochromes and in respiration in senescent synaptic mitochondria. In addition, changes in the wavelength maxima of cytochrome b were observed. These results appear to indicate some type of relationship between aging and the activ-

ity of the electron transport chain in neuronal mitochondria. It was the results obtained from this study that prompted the investigation into heart mitochondria.

Two groups of data will be provided; one group represents an analysis of mitochondria isolated from rat hearts that had been stored in the freezer for approximately one year while the other group represents an analysis of mitochondria isolated from hearts of freshly sacrificed rats. These two groups will be referred to throughout the thesis as frozen rat heart mitochondria and fresh rat heart mitochondria. Quantities used consisted of 35 frozen hearts, 14 3-month-old, 14 12-month-old, and 7 28-month-old; and 21 fresh hearts, 7 of each age.

Parameters investigated of the frozen rat heart mitochondria were cytochrome content, maximum absorbance wavelength, and cytochrome b/a ratio. Parameters investigated of the fresh rat heart mitochondria were cytochrome content, maximum absorbance wavelength, state 3 respiration, state 4 respiration, respiratory control ratio (RCR = state 3/state 4), ADP/O ratio, and proton translocation (H^+/O).

CHAPTER II

LITERATURE REVIEW

Introduction

Numerous characteristics and functions of mitochondria have been investigated in terms of how they are affected by the aging process. Literature on such characteristics as the number of mitochondria, structure of mitochondria, membrane lipid content, cytochrome content, and the content, synthesis, and turnover of mitochondrial DNA and protein will be reviewed. Also, the mitochondrial function of phosphorylation coupled to respiration will be discussed.

Effects of Aging on the Heart

Frolkis and Bogatskaya (1968), Abu-Erreish et al. (1977), and Lakatta and Yin (1982) all reported a decrease of oxygen consumption in aging rat hearts. Along with the decreases in oxygen consumption, a decrease in palmitate oxidation (Lakatta and Yin, 1982), fatty acid oxidation (Abu-Erreish et al., 1977), the content of high-energy phosphate compounds (Frolkis and Bogatskaya, 1968; Lakatta and Yin, 1982), and ATP/ADP ratio (Lakatta and Yin, 1982), the levels of free carnitine and long-chain acyl carnitine (Lakatta and Yin, 1982; Abu-Erreish et al., 1977), and an

increase in the glycolytic rate (Frolkis and Bogatskaya, 1968; Lakatta and Yin, 1982) were also observed.

Lakatta and Yin (1982) revealed a 15% decline in the coronary blood flow of aged hearts, expressed per gram heart. A lowered performance of cardiac work and a decrease in cardiac efficiency, expressed as the rate of oxygen consumed per ventricular pressure developed, was observed in senescent hearts under different cardiac workloads (Abu-Erreish et al., 1977).

It appears from the above experiments that the aging heart is incapable of maintaining adequate functioning especially when subjected to high workloads. Additional evidence for the decrease in maintenance can be seen in the age-related changes that occur in oxidative phosphorylation and respiration (reported later in this review).

Effects of Aging on the Mitochondria

Characteristics

Number of Mitochondria. There are numerous reports of decreases in the total number of mitochondria with age. Decreases were observed in mouse liver (Wilson and Franks, 1975), myocardium (Herbener, 1976), and mouse and rat skeletal muscle (Tonna and Severson, 1971). Fixed postmitotic cells such as the neuron of the mouse pyramidal tract also showed a decrease in the number of mitochondria (Samorajski, 1971). A moderate decrease occurred in the intermitotic cells of mouse liver (Wilson and Franks, 1975;

Tate and Herbener, 1976) and human liver (Tauchi and Sato, 1968).

Structure of Mitochondria. Changes in the fine structure of mitochondria have also been reported. Tate (1973) revealed a decrease in the number of cristae of mouse myocardium. A decrease in the number of mitochondrial cristae was also observed in aging liver cells (Tate and Herbener, 1976). Aged mouse liver mitochondria demonstrated a great variation in size with an increase in the number of large mitochondria, a decrease in matrix density accompanied by vacuolation, and a loss of dense granules (Wilson and Franks, 1975). The changes in the matrix density could be a result of oxygen deficiency (King and King, 1971). An increase in the size of aging hepatocyte mitochondria was also reported by Tauchi and Sato (1968), and Tate and Herbener (1976). It is interesting that cells with a high replication rate demonstrate no fine structural changes in the mitochondria of aging organisms (Miquel et al., 1980). This phenomenon has been observed in the mitochondria of the seminiferous (Miquel et al., 1978) and gastrointestinal epithelia (Miquel et al., 1979).

An investigation into the effects of oxygen tension on the mitochondria of cardiac muscle and autonomic ganglion from young and aged rats was performed by Sulkin and Sulkin (1967). Aged mitochondria showed marked swelling and loss of cristae at low oxygen tensions. However, young mitochondria were relatively unaffected. The results from this study suggest that older mitochondria are more sensitive to

physiological stress.

Membrane Lipid Content. Membrane lipids contribute to membrane fluidity, hydrophobicity, permeability (Bangham, 1972; Lucy, 1974; Singer, 1974; Chapman, 1975), membrane chemical and functional asymmetry (Bretcher, 1972; Bretcher, 1973), and the activity of many membrane-bound enzymes by affecting enzyme thermal and kinetic behavior (Coleman, 1973; Raison, 1973; Fox, 1974). Information from numerous studies (Grinna and Barber, 1972, 1975a, 1975b; Grinna, 1976) lend credence to the theory that age-related lipid changes cause functional changes in the membrane. These functional changes result in alterations of the interactions between membrane lipids and proteins (Grinna, 1977).

Many changes in the lipid content of aging mitochondrial membranes have been reported. There is a significant increase in the ratio of cholesterol to phospholipid in the mitochondrial membranes of rat liver and kidney (Grinna, 1977), and rat heart (Lewin and Timiras, 1984). Lewin and Timiras (1984) reported as much as a 26% increase in cholesterol content. This increase in membrane cholesterol would defluidize the membrane reducing thermal motion (Lewin and Timiras, 1984). In addition, increased cholesterol content decreases membrane permeability and the expansion of phospholipid monolayers (Papahadjopoulos et al., 1973; Inoue and Kitagawa, 1976) and decreases the activities of some membrane-bound enzymes (Papahadjopoulos et al., 1973; Kimelberg and Papahadjopoulos, 1974).

There are several reports that the ratio of unsaturated to saturated fatty acids decreases with age. The fatty acids of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine exhibited an increase in saturation in rat heart mitochondria (Lewin and Timiras, 1984). Contradictorily, Grinna (1977) reported a slight increase in the unsaturation of fatty acids in rat liver and kidney mitochondrial membranes. Lewin and Timiras (1984) demonstrated an increased unsaturation/saturation ratio and increased chain length in cardiolipin (a phospholipid required for cytochrome c oxidase activity). These two alterations of the cardiolipin molecule result in opposing effects. The increase in unsaturation increases the fluidity of the membrane, while the increased chain length decreases the fluidity (Lewin and Timiras, 1984). Lewin and Timiras (1984) also found an increase in the content of most phospholipids except cardiolipin. Grinna (1977) found an age-related decrease in the content of phosphatidylethanolamine.

Overall, these studies suggest an age-dependent decrease in mitochondrial membrane fluidity (Grinna, 1977; Lewin and Timiras, 1984), and an alteration of asymmetry (Grinna, 1977). A decrease in membrane fluidity may result in impaired energy transduction (Lewin and Timiras, 1984).

Content, Synthesis, and Turnover of Mitochondrial DNA and Protein. Mitochondria contain unique genetic components (i.e. DNA, RNA, ribosomes) necessary for the synthesis of several inner membrane protein molecules (Marcus et

al., 1982a). The majority of mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol, and then transported into the mitochondria (Alberts, et al., 1983). The following section will look at the age-related changes in mitochondrial DNA (mtDNA) and protein content, synthesis, and turnover.

An initial increase in the mtDNA content of rat liver occurred between the ages of 3 and 12 months, followed by a significant decrease by 24 months (Stocco and Hutson, 1978). A decrease in mtDNA to protein ratio was observed with age in rat brain (Huemer et al., 1971). Polson and Webster (1982) also reported a decrease in the mtDNA to mitochondrial protein ratio in aging mouse whole brain, heart, liver, and kidney. Between the ages of 12 and 26 months, drastic decreases of 59% in brain, 64% in heart, 76% in liver, and almost 90% in kidney were observed (Polson and Webster, 1982). These authors stated that the decrease in ratio was probably due to mtDNA loss, not a decrease in the number of mitochondria.

Shima (1975) reported no age-dependent changes in liver mtDNA synthesis. Correspondingly, Piko and Matsumoto (1977) observed that age did not affect the mtDNA replication process in mouse whole brain, heart, kidney, and liver. Litoshenko and Levitsky (1981), however, revealed a decrease in the replication of mtDNA with age in rat liver. A subclass of mtDNA was identified in older rats by its altered ethidium bromide binding properties (Murray and Balcavage, 1982). This modified form of mtDNA impedes

changes in the winding number which could possibly inhibit replication. Piko et al. (1984) reported a small increase in the percentage of catenated mtDNA (interlinked molecules of two or more circular units) in senescent mouse and rat whole brain, heart, kidney, and liver. In addition, a large increase in the frequency of circular dimers occurred with age in mouse brain and rat kidney and to a lesser degree in mouse and rat heart (Piko et al., 1984). Piko et al. (1984) suggested that this phenomenon is indicative of an overall deterioration in tissue physiology, not senescent changes in mitochondria. A significant increase in the frequency of larger replicative forms was observed in aging mouse liver (Piko et al., 1984). These investigators stated that this replicative form may be due to changes in the rate of mtDNA replication and/or turnover with age. White and Bunn (1985) did not observe large deletions, insertions, rearrangements, or single base changes in the mtDNA of aging human diploid fibroblasts; as a result they suggested that the mtDNA was efficiently maintained and/or eliminated when damaged. This observation can only be made for cells that continually divide (White and Bunn, 1985). Those cells that are post-mitotic may not dispose of damaged mtDNA molecules.

Vorbeck and Martin (1976), and Stocco and Hutson (1978) observed a significant decrease in the quantity of rat liver mitochondrial protein with advancing age. Rat liver mitochondrial protein synthesis was shown to decrease with age by Marcus et al. (1982a,b). However, under high work-

loads, cardiac mitochondrial protein synthesis increased by as much 60% in 15-month-old rats (Starnes et al. 1981). Old rats demonstrated an inability to synthesize inner mitochondrial membrane proteins at the same rate as young rats (Marcus et al., 1982a). Also, old mitochondria were shown to be more susceptible to protein synthesis inhibitors (Marcus et al., 1982b).

Comolli et al. (1972) reported that rat liver mitochondrial protein turnover rates are slower than the rates of protein turnover for other organelles. These rates changed only slightly with age (Fletcher and Sanadi, 1961; Menzies and Gold, 1971; and Comolli et al., 1972). Menzies and Gold (1972) stated that the synchrony of synthesis and degradation of rat whole brain mitochondrial protein changed with age. Lavie et al. (1981) reported a decrease in the rate of mitochondrial protein degradation in aged rat liver. In light of the results from the study of mitochondrial protein synthesis and turnover, Menzies and Gold (1972) and Marcus et al. (1982b) concluded that an alteration in the control of mitochondrial protein synthesis was occurring in old animals.

A brief review of the literature concerning the age-related changes in cellular DNA, RNA, and protein is discussed for the purpose of relating mitochondrial changes with cellular changes. Liu et al. (1978) reported a decrease in the DNA content of rat testicular tissue between the ages of 3 and 20 months. This decrease was followed by an increase in content between the ages of 18 and 30 months

(Liu et al., 1978). A decrease in the replication of nuclear DNA with age was observed in rat liver by Litoshenko and Levitsky (1981). Shima (1975) found an age-dependent increase in the synthesis of DNA.

Kanungo et al. (1970) studied the age-related changes in total RNA content from various tissues. They found that in heart, skeletal muscle, kidney, and testis the RNA content increased continuously. In liver and spleen, the content did not change after 40 weeks of age (Kanungo et al., 1970). The content of RNA in whole brain tissue decreased from 11 to 40 weeks of age and then increased (Kanungo et al., 1970). Liu et al. (1978) reported a decrease in the quantity of RNA in rat testes from 3 to 20 months of age, followed by an increase in quantity from 18 to 30 months of age. The pattern of change observed in nuclear DNA was also observed in RNA, that is, an increase in synthesis until the point of maturation after which synthesis declined. This pattern was observed in rat liver nuclei by Richardson and Cheung (1982) and Castle et al. (1978). Castle et al. (1978) concluded that the decrease in synthesis after maturation was due to alterations in chromatin not to RNA polymerase activity.

Richardson and Cheung (1982) found that protein content from various rat tissues did not decline with age. Also, all research reported in the review article by Richardson and Birchenall-Sparks (1983) stated that protein content did not show an age-related change. However, Liu et al. (1978) reported a decrease in the protein content of rat

testes from age 3 to 20 months, followed by an increase in content.

A 47% age-related decline in cell-free protein synthesis was noted in rat testes by Liu et al. (1978). Ekstrom et al. (1980) reported that in rat whole brain a decrease in cell-free protein synthesis occurred between the ages of 6 and 32 months. More specifically, Starnes et al. (1983) observed an increase in rat heart protein synthesis from immaturity to maturity, followed by a significant decline. As mentioned above, Liu et al. (1978) observed the opposite trend with respect to protein content. In addition, Starnes et al. (1983) found that the decline in protein synthesis could not be reversed by endurance exercise training.

Blazejowski and Webster (1983) studied the age-related changes in protein synthetic activity of mouse heart, whole brain, kidney, liver, and skeletal muscle. All tissues except heart showed an age-dependent decrease in protein synthesis (Blazejowski and Webster, 1983). In fact, no more than a 10% decrease in protein synthesis was observed in heart muscle over the life span of the organism (Blazejowski and Webster, 1983). A review article by Richardson and Birchenall-Sparks (1983) lists the results of studies conducted on age-associated changes in the protein synthesis from various rodent tissues. Eighty-five percent of those studies reported a decrease in protein synthesis with age.

Ekstrom et al. (1980) concluded from the results of

their study that the decrease in protein synthesis was not related to RNase activity, but was partially associated with ribosomal activity. Gabius et al. (1983) observed that the decrease in synthesis was due to reduced binding of the aminoacyl-tRNA to the ribosome. In addition, Gabius et al. (1983) found the decrease in synthesis to also be due to reduced peptidyl transfer.

Rat liver protein degradation was observed to dwindle with age (Lavie et al., 1981). Crie et al. (1981) reported a 50% age-dependent decline in the turnover of cardiac protein. An age-associated decrease in the protein turnover of mouse liver cells was also found by Richardson and Cheung (1982). Richardson and Cheung (1982) concluded that the rate of protein degradation is associated with the age-related decline in protein synthesis, because protein content does not change. If this is not the case, than the half-life of protein must be extended (Richardson and Birchenall-Sparks, 1983).

The impact of the reported changes in the synthesis, content, and turnover of DNA, RNA, and protein is realized by the changes that occur in active protein molecules with advancing age. The protein structure of aldolase is altered in old rabbits (Demchenko et al., 1983). Changes in the activity of cytochrome oxidase, malate dehydrogenase, mitochondrial adenine nucleotide translocator, and brain superoxide dismutase have been reported by Abu-Erreish and Sanadi (1978), Gandhi and Kanungo (1974), Nohl and Kramer (1980) and Vanella et al. (1982), and Danh et al. (1983),

respectively.

Only one correlation can be made between mtDNA and nuclear DNA, and that is that both demonstrate a decrease in replication with age (Litoshenko and Levitsky, 1981). It is interesting that no changes in mtDNA synthesis were observed (Shima, 1975; Piko and Matsumoto, 1977) and that nuclear DNA synthesis actually increased with age (Shima, 1975). More literature on mtDNA and nuclear DNA synthesis will have to be reviewed to determine the validity of these findings.

There appears to be a potential relationship between the age-dependent changes in the synthesis and turnover of mitochondrial and cellular protein. Both demonstrated decreases to varying extents. There is a decrease in mitochondrial protein synthesis (Marcus et al., 1982a,b), and cellular protein synthesis (Liu et al., 1978; Ekstrom et al., 1980; Starnes et al., 1983; Blazejowski and Webster, 1983; Richardson and Birchenall-Sparks, 1983). Also, mitochondrial protein turnover (Comolli et al., 1972; Lavie et al., 1981) and cellular protein turnover (Lavie et al., 1981; Crie et al., 1981; Richardson and Cheung, 1982) declined with age.

Cytochrome Content. Abu-Erreish and Sanadi (1978) reported a 30% age-dependent decrease in the content of cytochromes aa_3, $c + c_1$, and b in rat heart and whole brain homogenate. A decrease in cytochrome oxidase activity was also found (Abu-Erreish and Sanadi, 1978). It seems likely that the changes in the cardiolipin molecule (mentioned

previously) have some bearing on the decreased oxidase activity. It is interesting that the turnover number of cytochrome c oxidase is unchanged during the aging process (Abu-Erreish and Sanadi, 1978). Miquel et al. (1980) reported the same results of oxidase activity in both rat heart homogenate and purified mitochondria. The content and activity of cytochrome aa_3 of total liver homogenate decreased in aged rats, whereas cytochrome aa_3 of the mitochondrial fraction remained unchanged (Vorbeck et al., 1982). The content of cytochrome aa_3 decreased a significant 15% with aging in rat heart mitochondria (Lemeshko et al., 1982). Starnes et al. (1983) reported a significant decrease in cytochrome c concentration between the ages of 9 and 25 months in rat heart mitochondria. This group also observed that the content of cytochrome c could be increased with endurance exercise training.

Respiration and Oxidative Phosphorylation

State 3. Chen et al. (1972) reported that the age-related changes in state 3 respiration were dependent on the substrate used. State 3 respiration declined, in rat heart mitochondria, after 20 months of age with the substrates glutamate-malate, glutamate-pyruvate, palmitylcarnitine, and β-hydroxybutyrate. The substrates pyruvate-malate, α-oxoglutarate, palmityl-CoA, succinate, and ascorbate-cytochrome c showed no significant decline. Skeletal muscle mitochondria demonstrated a decrease in

rate with the substrates glutamate-malate. However, palmityl-CoA, palmitylcarnitine, succinate, and ascorbate-cytochrome c-driven activities showed no decline in rate in skeletal muscle. The authors suggested that the decline in state 3 respiration may be due to alterations in the subtle regulation of mitochondrial oxidation. These alterations may be a result of certain ion transport mechanisms across the membrane or dehydrogenase activity.

Wilson et al. (1975) stated that no significant age-dependent change in the rate of state 3 respiration occurred in mouse liver mitochondria for the substrate succinate. These authors made the generalization that the overall respiratory capacity was unaffected during the aging process. This statement contradicts that made by Chen et al. (1972).

An age-related decline in state 3 respiration of rat heart mitochondria using the substrates glutamate-malate was reported by Murfitt and Sanadi (1978). These investigators isolated two distinct bands of mitochondria using isotonic density gradient centrifugation. The upper band of mitochondria are capable of normal respiratory activity in both young and old rats. The lower band, however, demonstrated depressed respiratory activity in old rats. They hypothesized that the lower band contained mitochondria that were in the process of degradation as a result of a normal turnover cycle. The depressed activity could be due to rapid and severe failing in enzyme activity prior to degradation.

An age-dependent decline in state 3 respiration using the substrates palmitoylcarnitine, palmitoyl-CoA-carnitine, pyruvate-malonate-carnitine, and octanoate occurred in rat heart mitochondria (Hansford, 1978). No change was observed with the substrates pyruvate-malate and glutamate-malate (Hansford, 1978). The lack of change observed with glutamate-malate is in contrast to the results of Chen et al. (1972) and Murfitt and Sanadi (1978).

Chiu and Richardson (1980) observed a decline in state 3 respiration in mitochondria isolated from rat hearts aged 12 to 32 months with the substrates β -hydroxybutyrate, glutamate, palmitylcarnitine, glutamate-malate, and pyruvate-malate. Comparing the rates at 3 and 32 months, a significant decrease is observed when the substrates β -hydroxybutyrate, glutamate, or pyruvate-malate were used. No significant rate changes occurred with succinate. Chiu and Richardson (1980) also looked at the rates of state 3 respiration in aging rat whole brain. The substrates glutamate and glutamate-malate demonstrated a decrease between 12 and 32 months. A comparison of the rates at 3 and 32 months indicated a significant decrease with the substrates glutamate, glutamate-malate, and pyruvate-malate. Again, no significant change was observed with succinate. The results obtained from both heart and brain mitochondria and the observation that the number of mitochondria in a tissue decreases with age (Tauchi and Sato, 1968; Wilson and Franks, 1975; Herbener, 1976; Tate and Herbener, 1976) lead Chiu and Richardson (1980) to the hypothesis that the mito-

chondria's capability for a high rate of ATP production may decline in senescent tissues.

Weindruch et al. (1980) studied state 3 respiration in mitochondria isolated from mouse liver, whole brain, and spleen. Using the substrates glutamate, malate-pyruvate, and succinate-rotenone, no rate changes were observed in brain whether the rate was expressed per mitochondrial protein or per unit of cytochrome c oxidase activity. The same substrates were tested with liver mitochondria and the same results were observed. However, state 3 respiration was observed to decline when the substrate β-hydroxybutyrate was used if the rate was expressed per mitochondrial protein. If the rate is normalized to cytochrome c oxidase activity, the degree of change is lessened. The lack of change with the substrates succinate-rotenone is in concordance with the results of Wilson et al. (1975). Spleen mitochondria demonstrated an apparent decrease in state 3 respiration with succinate-rotenone when the rate was expressed per mitochondrial protein.

Farrar et al. (1981) reported no age-related change in the rate of state 3 respiration of mitochondria isolated from rat skeletal muscle. However, when the rats were exposed to an endurance training program, state 3 respiration, as well as the content of mitochondrial protein, increased.

A decrease in state 3 respiration supported by pyruvate-malate occurred in aging rat brain synaptic and nonsynaptic mitochondria (Deshmukh and Patel, 1982). These

authors suggested that a decrease in pyruvate uptake may be one of the factors responsible for this decrease. Lemeshko et al. (1982) found no age-related changes in state 3 respiration with succinate or malate-glutamate as substrates in rat heart mitochondria. Contrary to the findings of Deshmukh and Patel (1983), Vitorica et al. (1985) reported no age-related change in state 3 respiration with pyruvate-malate or succinate in rat whole brain mitochondria. State 3 respiration did show a significant decrease when the substrates glutamate-malate were used (Vitorica et al., 1985). This result prompted Vitorica et al. (1985) to hypothesize that there is a deficiency in glutamate metabolism or uptake.

State 4. State 4 respiration has been viewed as a sensitive indicator of inner mitochondrial membrane damage (Horton and Spencer, 1981). Before the addition of exogenous ADP, the rate of state 4 respiration is limited by proton conductance, and in the absence of inorganic phosphate by phosphate availability (Vitorica et al., 1985). All but three of the following investigations measure the rate of state 4 respiration before the addition of exogenous ADP. The present investigation measured state 4 respiration after the addition of ADP, therefore those studies measuring respiration before ADP addition cannot be used for the purpose of drawing comparisons.

Chen et al. (1972) reported no age-dependent changes in the rate of state 4 respiration for rat heart and skeletal muscle mitochondria using the substrates malate-glutamate,

palmitylcarnitine, and palmityl-CoA-carnitine. Chen et al. (1972) did not state when respiration was measured, before the addition of exogenous ADP or after its utilization. State 4 respiration was also observed to not change with age by Wilson et al. (1975) in mouse liver mitochondria with the substrate succinate. Using the substrates succinate and glutamate-malate, Horton and Spencer (1981) reported that no age-related changes in respiration occurred in rat liver mitochondria.

Chiu and Richardson (1980) found that no changes in state 4 respiration occurred in aging rat heart mitochondria with the substrates succinate, palmitylcarnitine, glutamate-malate, and pyruvate-malate. State 4 respiration did decrease in heart mitochondria with β-hydroxybutyrate, and increased with glutamate. Aging whole brain mitochondria demonstrated no change in respiration with the substrate succinate. However, with glutamate-malate and pyruvate-malate an increase in brain mitochondrial respiration occurred, while a decrease was observed with glutamate.

Mouse brain mitochondria demonstrated no change in the rate of state 4 respiration using the substrates glutamate, malate-pyruvate, and succinate-rotenone (Weindruch et al., 1980). The same substrates used to measure state 4 respiration in brain were used in liver with the addition of β-hydroxybutyrate, and again no change was observed. A significant increase in state 4 respiration was observed, however, in spleen mitochondria with succinate when the

rate was expressed per cytochrome c oxidase activity. This investigation measured the rate of state 4 respiration after ADP utilization.

Vitorica et al. (1985) observed age-related changes in the rate of state 4 respiration before and after the addition of exogenous ADP in rat whole brain mitochondria. Before the addition of ADP, the rate was unchanged with the substrates glutamate-malate and succinate, and decreased with pyruvate-malate. After the conversion of ADP to ATP, the rate of state 4 respiration was significantly greater for all the substrates tested.

Respiratory Control Ratio. The respiratory control ratio is an indicator of the degree of coupling between oxygen uptake and oxidative phosphorylation (Milstein et al., 1968). Gold et al. (1968) reported a lack of age-associated decreases in RCR and that this result reflected favorably on the integrity of the mitochondrial membrane. Mitochondria were isolated from rat liver, heart, and kidney. The results of a study by Chen et al. (1972) indicated a decrease in RCR in aging rat heart mitochondria when the substrates glutamate-malate, glutamate-pyruvate, palmitylcarnitine, and β-hydroxybutyrate were used, and in skeletal muscle mitochondria with glutamate-malate. No age-dependent change in RCR was demonstrated in mouse liver mitochondria with the substrate succinate (Wilson et al., 1975).

The RCR values obtained from the study by Chiu and Richardson (1980) indicated that the RCR did not change

with the substrates succinate, palmitylcarnitine, and glutamate-malate in rat heart mitochondria, and succinate in rat whole brain mitochondria. The RCR values decreased with the substrates β-hydroxybutyrate, glutamate, and pyruvate-malate in heart and glutamate, glutamate-malate, and pyruvate-malate in brain.

Horton and Spencer (1981) reported a decline in the RCR values obtained from aging rat liver mitochondria using the substrates succinate and glutamate-malate. These results conflicted with those of an identical study by Vorbeck et al. (1982) in which no changes were observed with the substrate succinate (with rotenone).

ADP/O. The ADP/O ratio is the amount of ADP converted to ATP per oxygen atom consumed. ADP/O ratio is equivalent to the P/O ratio (Tzagoloff, 1982). Both ratios are a means of expressing the efficiency of phosphorylation in mitochondria (Tzagoloff, 1982).

All of the following studies reported no age-dependent changes in the ADP/O ratio. Weinbach and Garbus (1959) isolated mitochondria from rat whole brain and liver. The following substrates supported respiration, succinate in the study of brain tissue, and β-hydroxybutyrate, succinate, α-ketoglutarate, glutamate, and malate in the study of liver tissue. Chen et al. (1972) used rat heart and skeletal muscle with the substrates malate-glutamate, palmitylcarnitine, and palmityl-CoA-carnitine to observe changes in the ADP/O ratio. Inamdar et al. (1974) isolated mitochondria for this study from hamster heart, skeletal

muscle, and liver and used the substrates pyruvate-malate, succinate, and glutamate-malate.

Mitochondria from rat heart and the substrates succinate, β-hydroxybutyrate, palmitylcarnitine, glutamate, glutamate-malate, pyruvate-malate, and from rat whole brain and the substrates succinate, glutamate, glutamate-malate, and pyruvate-malate were used to measure ADP/O by Chiu and Richardson (1980). Weindruch et al. (1980) utilized mitochondria from rat liver with the substrates glutamate, pyruvate-malate, succinate-rotenone, and β-hydroxybutyrate and from rat brain with glutamate, pyruvate-malate, and succinate-rotenone. Vorbeck et al. (1982) observed phosphorylation in mitochondria obtained from rat liver along with the substrate succinate with the inhibitor rotenone present. Vitorica et al. (1985) used rat whole brain mitochondria and succinate, pyruvate-malate, and glutamate-malate as substrates.

CHAPTER III

EXPERIMENTAL PROCEDURE

Isolation of Rat Heart Mitochondria

Virgin female Fisher 344 rats were obtained from the National Institute of Aging colony at Harlan-Sprague-Dawley, Inc., Indianapolis, IN. Hearts were removed from the rats immediately following decapitation. Four hearts were pooled prior to isolation of mitochondria. Rat heart mitochondria were isolated using the procedure described by Chance and Hagihara (1963). An explanation of this procedure is provided below. The observed yield is approximately 2 mg of protein per heart. Solutions and instruments were kept on ice throughout the procedure.

A whole fresh or frozen heart was placed in 20 ml of MSE medium which contained 0.225 M mannitol, 0.075 M sucrose, and 0.2 mM EDTA at pH 7.4. The heart was cut into quarters to facilitate blood drainage. The MSE medium and blood were decanted. Another 20 ml of fresh MSE medium containing protease, 5 mg/heart, was added for proteolytic digestion. The heart tissue was then minced.

The mixture of minced heart and MSE-protease medium was homogenized until the tissue was further broken apart, equivalent to approximately four passes of the pestle up

and down the homogenizer. Homogenization was performed using a motor driven, loose-fitting teflon pestle and a size C, teflon-glass Potter-Elvehjem homogenizer. The motor was set at 250 rpm to execute homogenization. The pH of the homogenate was raised to 7.8 using Trizma base and then incubated at room temperature for 5 minutes while constantly stirring, after which, additional MSE medium was added. The incubated mixture was homogenized using a motor driven, tight-fitting, size C homogenizer. Two passes were sufficient.

The homogenate was centrifuged at 500 x g for 5 minutes. The supernatant was decanted into clean centrifuge tubes. Caution must be exercised to prevent pouring the pellet's loose top layer off with the supernatant. The supernatant was centrifuged at 10,000 x g for 10 minutes. After centrifugation, the inside of the centrifuge tube was wiped with a cotton swab to remove any fat. The centrifuge tube was gently shaken to remove the layer of fluff from the pellet, and the supernatant was discarded.

The pellet was resuspended in MSE medium and homogenized using a hand-held size A homogenizer. The homogenate was centrifuged at 11,670 x g for 10 minutes and the supernatant discarded. The pellet was resuspended and homogenized as described above. The homogenate was centrifuged at 26,260 x g for 10 minutes. The pellet was resuspended and homogenized again. This was the final product of suspended rat heart mitochondria.

Determination of Cytochrome Content and Wavelength Maxima

Cytochrome content and maximum absorbance wavelength of cytochromes c + c_1, b_{TOTAL} (+30 mV midpotential + -30 mV midpotential), b_{562} (+30 mV midpotential), b_{566} (-30 mV midpotential), and aa_3 were determined from a reduced MINUS oxidized difference spectrum (Figure 2). The spectrum was produced from a DBS-3 Johnson Research Foundation (University of Pennsylvania) scanning dual wavelength spectrophotometer. The cell path length was 1 cm. A 1.5:1 ratio of mitochondrial suspension to 50 mM sodium phosphate-0.25 M sucrose buffer (pH 7.4) was used for this procedure.

Millimolar extinction coefficients of 19.5 for cytochrome c + c_1 (552 minus 538 nm), 28 for the b cytochromes (562 or 566 minus 575 nm), and 24 for cytochrome aa_3 (605 minus 630 nm) were utilized in calculating cytochrome content. The succinate MINUS oxidized difference spectrum was used to determine the content of c + c_1, b_{562}, and aa_3 cytochromes. The dithionite MINUS oxidized difference spectrum was used to determine the content of total b cytochrome. The dithionite-reduced MINUS succinate-reduced spectrum was used for determination of b_{566} cytochrome content.

The same difference spectra were used in ascertaining the maximum absorbance wavelength for each cytochrome. Previous studies indicate that the maximum absorbance wave-

length for cytochrome c + c_1 does not change with age (Floyd et al., 1984 and Harmon et al., 1987). Based on that, cytochrome c + c_1 was used as an internal standard for the measurement of wavelength maxima of frozen rat heart mitochondria.

Determination of Oxidative Phosphorylation

Oxidative phosphorylation (respiration) was measured by a Clark oxygen electrode fitted to a glass water-jacketed chamber. The oxygen electrode was also attached to a strip chart recorder. A sample recording of respiration from which state 3, state 4, RCR, and ADP/O were calculated is shown in Figure 3. Temperature was held at 25^{0}C. Mitochondria were suspended in a medium consisting of 0.25 M sucrose, 40 mM Tris-SO_4 buffer (pH 7.4), and 1.25 mM sodium phosphate (mono- and dibasic). Malate (4.5 mM) PLUS glutamate (4.5 mM) and succinate (9 mM) were used as substrates. Electron transfer was initiated by the addition of Tris-ADP (final concentration of approximately 250 μM). Four parameters can be assessed from the rate of oxygen consumption: state 3 respiration, state 4 respiration, RCR, and ADP/O ratio.

Determination of Proton Translocation

Proton translocation or the H^{+}/O ratio was measured using the method described by Hinkle and Horstman (1971), except that whole mitochondria were used instead of submitochondrial particles. The apparatus consisted of a pH

electrode fitted to the top port of a glass water-jacketed chamber with a Clark oxygen electrode fitted to the side port. The oxygen electrode determines the aerobic state of the mitochondrial suspension. A sample recording of proton translocation from a strip chart recorder can be found in Figure 4. The temperature was held at 25^{0}C. The suspension consisted of 0.92 mM KCl, 4.38 mM glycylglycine, 7.69 mM succinate, and approximately 2 mg/ml of mitochondria. Following anaerobicity, valinomycin (1 mg/ml) and rotenone (10 µg/ml) were added to the suspension. The anaerobic suspension was pulsed with 10 µl of 0.1 M KCl. This procedure was repeated with the addition of an uncoupler, 1.92 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP), prior to pulsing with 0.1 M KCl. After all the oxygen had been consumed following the KCl pulse, a pulse of 2 µl of 0.01 N HCl was administered. The amount of hydrogen ions in the HCl pulse was known and thus the recording of this pulse was used as a standard measurement from which the number of translocated protons were measured.

CHAPTER IV

RESULTS

Frozen Rat Heart Mitochondria

Cytochrome Content

Although little change in the content of any of the cytochromes occurred from 3 to 12 months of age, changes were observed from 3 to 28 months of age (Table I). Cytochrome b_{562} decreased in content a statistically significant 37% from age 3 to 28 months. The content of cytochrome b_{TOTAL} decreased a statistically insignificant 19% from 3 to 28 months of age. A statistically insignificant 26% decrease in the content of cytochrome b_{566} was observed between 3 and 28 months of age.

Wavelength Maxima of Cytochromes

The value of the wavelength maximum of cytochrome c + c_1 was normalized to 552 nm. Determination of the wavelength maxima for the b- and a-type cytochromes was based on the correction factor used to normalize the wavelength maxima of cytochrome c + c_1. As shown in Table II, no significant changes in the maximum absorbance wavelength of any of the cytochromes were observed.

Cytochromes b/a Ratio

The ratio of the b cytochromes to cytochrome aa_3 was determined by dividing the contents of the b cytochromes by the content of cytochrome aa_3. Cytochrome aa_3 was used because no significant changes in content occurred with aging. Cytochrome b_{562}/a ratio decreased a statistically significant 28% from 3 to 28 months of age (Table III). The b_{566}/a ratio demonstrated an increase of 11% at 12 months versus 3 months of age. This increase was followed by a 24% decrease from age 12 to 28 months. This same pattern occurred with the b_{TOTAL}/a ratio. A 10% increase was observed from 3 to 12 months of age, followed by a 17% decrease from 12 to 28 months. The significance of these changes cannot be ascertained because of the large standard of error measurements.

Fresh Rat Heart Mitochondria

Cytochrome Content and Wavelength Maxima

The content of all the cytochromes did not change significantly with age (Table IV). The maximum absorbance wavelength also did not change significantly with aging for any of the cytochromes (Table V).

Oxidative Phosphorylation

State 3 and State 4 Respiration. The measurements of state 3 and state 4 respiration using succinate to drive respiration are shown in Table VI. State 3 respiration,

following the first addition of ADP, did demonstrate some changes with aging. However, due to the large standard of error measurements the importance of these changes cannot be determined. State 4 respiration following the first ADP addition demonstrated an insignificant 25% decrease from 3 to 12 months of age. Following the second addition of ADP, state 3 respiration declined 14% from age 12 months to 28 months. State 4 respiration showed a parallel decline of 26%. None of these changes were statistically significant.

Table VII presents the results of state 3 and state 4 respiration measurements when malate PLUS glutamate supported respiration. State 3 respiration following the first addition of ADP demonstrated a statistically insignificant 18% decrease from 3 to 28 months of age. While state 4 respiration remained unchanged. A 22% decrease was observed of state 3 respiration from age 3 to 28 months following the second addition of ADP. This decrease was also not significant. Again, state 4 respiration remained unchanged.

A statistically significant 24% decrease was observed in the state 3 rate from the first to second additions of ADP at 28 months of age when malate PLUS glutamate were used as substrates. An identical decline was observed for the state 4 rate.

Respiratory Control Ratio. The values of RCR calculated from state 3 and state 4 respiration with succinate as the substrate are presented in Table VIII. No changes were observed with age for either addition of ADP or from

first to second additions of ADP.

The calculated RCR when malate PLUS glutamate drove respiration can also be seen in Table VIII. RCR decreased 13% from age 12 months to 28 months following the first addition of ADP. This change was statistically insignificant. No other changes were observed either with age or between additions of ADP.

It seems unusual that there was not a change in RCR from the first to second additions of ADP at age 28 months when malate PLUS glutamate supported respiration. This would have been anticipated because changes did occur under these conditions for both state 3 and state 4 respiration.

ADP/O. The ADP/O ratio was calculated from the measurement of respiratory activity. Values are presented in Table IX. Changes were not observed between age groups or between additions of ADP when succinate supported respiration.

There were no age-related changes in ADP/O for each addition of ADP when malate PLUS glutamate supported respiration. However, when comparing values at each age for the first and second additions of ADP, ADP/O did increase following the second addition of ADP. At 3 months of age a 10% increase was observed, at 12 months a 14% increase, and at 28 months an 11% increase. Increases at 3 and 12 months were statistically significant, whereas the increase at 28 months was not.

Proton Translocation (H^+/O). The calculated H^+/O ratios versus time for each age group are presented in

Figures 5 through 10. These graphs represent the number of protons pumped per oxygen atom consumed with time. The rate of proton pumping decreased with time due to exhaustion of the oxygen supply. The value of importance is the number of protons pumped immediately following the oxygen pulse. This value is obtained by extrapolating from the plotted line to zero time. Mitochondrial suspensions were pulsed two times, noted as 1st and 2nd runs, and if quantities allowed, two mitochondrial suspensions were prepared from each mitochondrial isolation, noted as 1st and 2nd groups.

Some variability was observed within runs and between groups and also within and between ages. Generally, the second group tested is considered to be more accurate, possibly due to stabilization of the membrane. However, there appeared to be no age-related changes evidenced by the observed approximate H^+/O value of 4.5, which is the expected value, for each age. This result was anticipated because little change in respiration with age was observed.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

The data obtained from the analysis of frozen rat heart mitochondria will not be discussed in this chapter. The reasoning is that accurate information was probably not derived due to the fact that the hearts were frozen and for varying lengths of time. The following involves an evaluation of the results obtained from fresh heart mitochondria and a comparison of these results to similar studies found in the literature.

In this study, cytochrome content lacked changes associated with aging. These results contrast with the findings of Abu-Erreish and Sanadi (1978) who reported a 30% decrease in the content of cytochromes aa_3, $c + c_1$, and b with age, Lemeshko et al. (1982) who reported a 15% decrease in the content of cytochrome aa_3 with age, and Starnes et al. (1983) who reported a significant decrease in the content of cytochrome c with age. A decrease in cytochrome content was anticipated in this study based on the findings of these investigators.

Cytochrome wavelength maxima lacked age-dependent changes. Investigations by other researchers into wave-

length maxima changes of cytochromes with aging were not found in the literature.

State 3 and state 4 respiration demonstrated a lack of significant decline with age following each addition of ADP with either substrates succinate or malate PLUS glutamate.

The results of state 3 respiration concur with those of Chen et al. (1972) using the substrate succinate, Hansford (1978) using the substrates malate PLUS glutamate, Chiu and Richardson (1980) using the substrate succinate, and Lemeshko et al. (1982) using the substrates glutamate and malate. The results conflict with those of Chen et al. (1972), Murfitt and Sanadi (1978), and Chiu and Richardson (1980) using the substrates malate PLUS glutamate.

The results of state 4 respiration are in agreement with the two studies on cardiac mitochondria found in the literature, one using the substrates malate PLUS glutamate (Chen et al., 1972), and the other using the substrates succinate and malate PLUS glutamate (Chiu and Richardson, 1980).

The reported significant decreases in both state 3 and state 4 respiration following the second addition of ADP only when malate PLUS glutamate drove respiration and only at 28 months of age appears unique. Reports of similar comparisons were not found in the literature.

One possible reason for the changes in respiration rates is that the initial measurements made by the first addition of ADP were not reliable. The reasoning is that

the mitochondria may not be in state 3 at the onset and this would alter the recording of phosphorylation. In addition, the membrane covering the oxygen electrode may not have equilibrated completely in the suspension prior to the addition of ADP. Correction of these two factors would have been made following the completion of the initial conversion of ADP to ATP. Thereby, the second measurement of phosphorylation would be more accurate. However, that the changes occurred only under a specific set of conditions seems to discredit this theory. If the mitochondria were not completely in state 3 initially and/or if the membrane was not equilibrated then changes should have been observed throughout.

It is equally possible that some age-related factor is responsible for the decline in rate from first to second additions of ADP, based on the fact that changes did occur under a specific set of conditions. Perhaps there is an age-related change in the uptake of glutamate. This result has been reported by Vitorica et al. (1985).

Another explanation is that an alteration in the inner mitochondrial membrane occurred with aging. Changes in the composition or damage to the integrity of the membrane would result in improper functioning of the electron transport chain. More extensive investigation would be required to determine if in fact the observed changes in state 3 and state 4 respiration are meaningful and why they occur with age.

Age-related changes in RCR, either for each addition of ADP or when comparing the first to second additions of ADP were not observed. The reports of changes in the RCR of cardiac mitochondria in the literature are ambiguous. Chiu and Richardson (1980) reported a lack of age-associated decline in RCR with the substrates succinate and malate PLUS glutamate. Chen et al. (1972) reported a decrease in RCR with the substrates malate PLUS glutamate.

The results of the age-related effects of ADP/O demonstrated a lack of significant change with each addition of ADP. These results concur with those found in the literature by Chen et al. (1972) with the substrates malate PLUS glutamate, and Chiu and Richardson (1980) with the substrates malate PLUS glutamate and succinate.

The number of protons translocated per oxygen atom consumed or H^+/O was found to not be affected by aging. The evaluation of this mitochondrial parameter is novel in that similar investigations have not be performed.

Conclusions

This study has found that overall aging does not have a negative effect on the respiratory components or activity of rat heart mitochondria. Minor changes were noted throughout, but the only changes found to have any significance were those involving the values of state 3 and state 4 respiration following the second addition of ADP. These changes may in fact have some important consequences in

terms of the mitochondria's capability of maintaining ATP production in the aged organism. However, that outcome can only be proven by further investigation.

CHAPTER VI

DISCUSSION

Theories on Aging

To date, there are a number of theories as to why aging occurs. This chapter will provide a brief overview of those theories. A discussion of the possible relationships between the results of this study and one of the theories of aging, the free radical theory, will be included. Information on the theories was obtained from a review article by Hayflick (1985), and is the reference for the material covered in the first part of this chapter.

There are three basic groups of theories, organ, physiological, and genome-based. Each of these are further broken down into subgroups. Two theories make up the organ theory, immunological and neuroendocrine theories. Free radical theory, cross-linkage theory, and waste-product accumulation theory are components of the physiological theory. The genome-based theory consists of the somatic mutation theory, error theory, and program theory. There is both evidence for and against each of these theories. As yet no single theory can exclusively provide reasons for the process of aging.

The premise of the immunological theory of aging is

that as the organism ages it becomes increasing vulnerable to pathology and disease. This statement is primarily based on two observations. One, the immune system becomes less capable of producing antibodies, both in quantity and quality, with aging. Two, there is an increase in autoimmune diseases with aging due to a decline in the organism's ability to differentiate from self and nonself.

Though these observations are valid, it is unlikely that they could solely be the cause of aging. One reason is this theory cannot be applied universally. Organisms with immune systems inferior to the higher vertebrates or completely lacking in an immune system also age. Another reason is that the immune system is, to a certain degree, regulated by hormones. Perhaps aging of the immune system is influenced by aging of the endocrine system. Finally, it is probable that all cells undergo an aging process that is genetically based. Therefore, the cells of the immune system are likely to undergo the same process which would affect the overall functioning of the immune system. That the cells change due to aging may also explain the increased incidence of autoimmune disease. Alterations in the markers of the cell could cause it to become identified as antigenic, thereby eliciting attacks upon itself. These three reasons against the immunological theory indicate that it is unlikely the fundamental cause of aging in the organism.

The neuroendocrine theory of aging obtains some of its

support from the fact that few processes in the organism would be unaffected by alterations in the neuroendocrine system. It is known that a loss of neurons and endocrine cells occurs with aging. At this point it is uncertain what effect this loss would have on aging. There are known changes in responses to hormones with aging due to changes in receptor concentrations or disturbances in hormone regulation.

There are two arguments against the neuroendocrine theory. First, like the immunological theory, the neuroendocrine theory cannot be universally applied due to the fact that not all organisms that age have as extensive a neuroendocrine system. Second, also mentioned in regards to the immunological theory, the aging process may take place at the cellular level. It would appear unlikely that changes in the neuroendocrine system are the sole cause of aging.

The free radical theory of aging is a proposed explanation for age-related changes occurring at the cellular level. Free radicals are atoms or molecules with an unpaired electron. As a result, free radicals are highly reactive and have the potential to cause chain reactions. When a free radical reacts with a stable molecule it generates another free radical that can react with another stable molecule, and so on. This type of chain reaction often occurs with the phospholipids of membranes.

Free radical reactions generally only occur in mamma-

lian cells where O_2 is the initial source of the free radicals. Reactions can arise from exposure of cells or organelles to ionizing radiation, nonenzymatic reactions, or enzymatic reactions. Of the enyzmatic reactions, the one of relevance to this study is the reduction of O_2 to water carried out by the electron transport chain.

Damage from free radical reactions can occur as an accumulation of oxidative alterations in collagen, elastin, and DNA, oxidative degradation of mucopolysaccharides, build up of metabolically inert substances, such as age pigments, due to oxidative polymerization reactions, alterations in the membrane characteristics of mitochondria and lysosomes, and fibrosis of arterioles and capillaries due to peroxidation of serum and vessel wall components. Reports have shown a relationship between the cumulative effects of free radical reactions and diseases of the cardiovascular, immune, and central nervous systems, and cancer.

The most important support for the free radical theory of aging comes from the observation that free radical damage can be arrested by the administration of antioxidants. It has been shown that the life expectancy of mice, rats, and other organisms can be increased by the addition of dietary antioxidants. Geographical areas that contain high concentrations of the antioxidant selenium report a low incidence of cancer. Antioxidants have been shown to slow the development of autoimmune diseases, which would

seem to demonstrate a cellular influence to the immunological theory of aging.

The free radical theory of aging can be applied universally and there is much support for its existence. Whether free radical damage is the fundamental cause of aging has yet to be decided. This theory will be discussed in more depth in the second part of this chapter.

The cross-linkage theory of aging is based on the occurrence of age-related molecular changes in components of the extracellular compartment, DNA, and RNA. These molecular changes involve covalent or hydrogen bonds between two macromolecules, resulting in the macromolecules' immobilization and inability to function. An accumulation of linked macromolecules results in a decrease in intracellular space, hindering transport and operation of vital processes. If DNA is affected, mutation or cell death may occur. Cross-linking of components in the extracellular compartment can result in impedement of production and release of hormones, decreases in solubility, elasticity, and permeability, increase in viscosity, and impairment of the flow of nutrients and removal of waste.

Support for the cross-linkage theory is provided by the documentation of cross-linkage in collagen, proteins, DNA, and a number of naturally occurring substances in living tissues. In addition, this theory can be applied universally.

Arguments against the cross-linkage theory are three

fold. First, many vital molecules that have the potential to cross-link will turnover. It is possible, however, these renewable cells may contain a genome that has been altered resulting in a progeny of defective cells. Second, it has not been demonstrated that the quantity or quality of cross-linkage is sufficient to cause aging. Finally, controls of the rate of cross-linking have not be discussed and thus an explanation for the different life spans of organisms cannot be provided.

It has been stated that because free radical reactions can cause cross-linkage that the free radical theory is an example of the cross-linkage theory. It may be possible, however, for this interrelationship to support both theories.

The premise of the waste-product accumulation theory is based on the observation that with aging, terminally differentiated cells, such as neurons, and cells of skeletal and cardiac muscle, accumulate pigmented inclusion bodies. These pigmented inclusion bodies, or lipofuscin, are waste products that are highly cross-linked probably resulting from accumulative auto-oxidative reactions with lipid components of lysosomes and other membranous organelles.

Criticism against the waste-product accumulation theory is that it lacks universality, and its random distribution and rate of accumulation. It has been suggested that this theory has more of a secondary role in causing aging.

The somatic mutation theory was popular many years ago

but because of lack of significant studies of late it has essentially been discredited as playing a role in aging. The reasoning behind the theory was mutations in genes could accumulate in somatic cells to a sufficient level to cause physiological decrements seen with aging. Indisputable results were never obtained from the numerous studies performed to gain evidence for this theory.

The error theory of aging is similar to the somatic mutation in that both propose an accumulation of mutations or errors, but differ in that the errors are occurring in nonrepeated DNA sequences. It is proposed that when nonrepeated DNA sequences are lost the result is aging. This theory has also lacked evidence and therefore has not been considered an explanation for aging.

The rationale behind the program theory of aging is that like the developmental sequence of events is written in the genome, so is the sequence that leads to age changes. There is some belief that there is a selection process postponing the expression of deleterious genes until the post-reproductive period when expression would do less harm. This would mean that age changes due to the expression of deleterious genes is the price paid for reserving only beneficial gene expression early in life. Experimental data have been unable to support this theory and therefore will not be considered valid at this time.

The Free Radical Theory and Age-Related Changes In Respiration

It was previously stated in Chapter IV that state 3 and state 4 respiration demonstrated significant decreases from the first to second additions of ADP only when respiration was driven by malate PLUS glutamate and only at 28 months of age. One proposed explanation for the apparent changes in respiration is that the integrity of the inner mitochondrial membrane is altered because of free radical damage to the membrane.

Free radicals are atoms or molecules that contain an unpaired electron and are generated by, among other sources, mitochondrial respiration. Free radicals are known to react with polyunsaturated fatty acids, a component of membranes, ultimately yielding lipid peroxides (Leibovitz and Siegel, 1980). Lipid peroxides can decompose to aldehydes which can cross-link proteins, lipids, and nucleic acids resulting in a loss of cellular integrity (Leibovitz and Siegel, 1980). There are a number of types of free radicals, however, only the superoxide radical or $O_2^{\cdot}$ will be discussed because it is the free radical generated by the electron transport chain in mitochondria (Nohl and Hegner, 1978). Superoxide dismutase is the enzyme that quenches the superoxide radical, protecting the cell from toxicity (Guarnieri et al., 1981).

The concentration of superoxide radicals increases with

aging in rat heart mitochondria (Nohl and Hegner, 1978) while the concentration of superoxide dismutase remains unchanged (Nohl and Hegner, 1978; Nohl et al., 1979). Nohl and Hegner (1978) reported that superoxide dismutase is only able to quench 80% of the superoxides generated. Thereby, in the aged animal more than 20% of the superoxide radicals generated escape quenching because of an increased production of radicals but not of enzymes. In other words, as the animal ages the concentration of superoxide radicals produced exceeds the protective capacity of the cell (Nohl and Hegner, 1978).

The superoxide radicals that escape decomposition migrate across the inner mitochondrial membrane to the cytosolic side (Nohl and Hegner, 1978). The fact that peroxides were found present in the membrane indicates that chain reactions between superoxide radicals and polyunsaturated fatty acid side chains have occurred (Nohl and Hegner, 1978). With a greater concentration of free superoxide radicals, as seen with aging, greater damage to the inner mitochondrial membrane would be expected.

The electron transport chain is intimately associated and dependent upon the inner mitochondrial membrane. If the inner membrane is damaged by free radical reactions, as has been observed particularly in older animals, than alterations in respiration would be anticipated. Nohl et al. (1978) reported decreases in both RCR and P:O (or ADP/O) with aging independent of the substrate used. When super-

oxide dismutase activity was inhibited by diethyldithio carbamate (DDC), Guarnieri et al. (1981) reported decreases in oxygen uptake during state 3 respiration and a slower rate of ATP synthesis.

Malondialdehyde, a product of free radical oxidation of polyunsaturated fatty acids (Hayflick, 1985), production was inhibited 80 to 90% by the substrate succinate in rat liver mitochondria (Meszaros et al., 1982). It appears that succinate is capable, in some capacity, of preventing free radical reactions in the membrane.

Though limited in number, these studies provide support for the theory that free radical induced damage to the inner mitochondrial membrane has the potential to alter respiration and oxidative phosphorylation. Unfortunately, few relationships can be drawn between the results of the above investigations and the results presented in this study, outside of that they offer some validity to the theory that free radical damage may be the cause of the apparent changes in state 3 and state 4 respiration.

The validity of the changes observed in state 3 and state 4 respiration in this study remains uncertain. That free radical reactions can damage membranes certainly has a great deal of merit. Damage to the inner mitochondrial membrane would most likely cause alterations in the activity of the electron transport chain. Therefore, if the age-related changes observed in this study were found to be valid, through further experimentation, then the free radi-

cal theory of aging would certainly have to be considered a viable cause of these changes.

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APPENDICES

APPENDIX A

TABLES

TABLE I

EFFECT OF AGE ON CYTOCHROME CONTENT
OF FROZEN RAT HEART MITOCHONDRIA

Age (months)	Cytochrome Content (nmol/mg protein)				
	$c + c_1$	b_{562}	b_{566}	b_{TOTAL}	aa_3
3	0.76 ± 0.05(14)	0.32 ± 0.04(11)	0.34 ± 0.04(13)	0.57 ± 0.06(14)	0.54 ± 0.04(14)
12	0.79 ± 0.03(14)	0.31 ± 0.03(13)	0.33 ± 0.05(12)	0.57 ± 0.04(14)	0.54 ± 0.04(14)
28	0.75 ± 0.04(7)	0.20 ± 0.02(7)	0.25 ± 0.02(7)	0.46 ± 0.04(7)	0.49 ± 0.04(7)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE II

EFFECT OF AGE ON WAVELENGTH MAXIMA* OF CYTOCHROMES
OF FROZEN RAT HEART MITOCHONDRIA

Age (months)	Wavelength (nm)			
	b_{562}	b_{566}	b_{TOTAL}	aa_3
3	562.38 ± 0.34(11)	563.68 ± 0.32(13)	563.27 ± 0.28(14)	606.31 ± 0.35(14)
12	562.66 ± 0.25(13)	563.95 ± 0.59(12)	562.73 ± 0.30(14)	606.04 ± 0.28(14)
28	562.54 ± 0.33(7)	563.74 ± 0.78(7)	563 ± 0.42(7)	606.34 ± 0.28(7)

*Based on cytochrome c + c_1 wavelength normalized to 552 nm.
Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE III

EFFECT OF AGE ON CYTOCHROMES b/a RATIO
OF FROZEN RAT HEART MITOCHONDRIA

Age (months)	b/a Ratio		
	b_{562}	b_{566}	b_{TOTAL}
3	0.54 ± 0.04(11)	0.63 ± 0.09(13)	1.03 ± 0.09(14)
12	0.56 ± 0.02(13)	0.71 ± 0.14(12)	1.14 ± 0.13(14)
28	0.42 ± 0.03(7)	0.54 ± 0.07(7)	0.95 ± 0.06(7)

Number in parentheses denotes n = number of observations,
$P > 0.05$ for all values, values are means ± S.E.M.

TABLE IV

EFFECT OF AGE ON CYTOCHROME CONTENT
OF FRESH RAT HEART MITOCHONDRIA

Age (months)	Cytochrome Content (nmol/mg protein)				
	$c + c_1$	b_{562}	b_{566}	b_{TOTAL}	aa_3
3	1.09 ± 0.07(7)	0.34 ± 0.02(7)	0.10 ± 0.01(7)	0.39 ± 0.03(7)	0.60 ± 0.04(7)
12	1.01 ± 0.11(7)	0.34 ± 0.06(7)	0.10 ± 0.02(6)	0.39 ± 0.05(7)	0.50 ± 0.04(7)
28	1.00 ± 0.08(7)	0.31 ± 0.03(7)	0.10 ± 0.01(7)	0.37 ± 0.04(7)	0.55 ± 0.01(7)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE V

EFFECT OF AGE ON WAVELENGTH MAXIMA OF CYTOCHROMES
OF FRESH RAT HEART MITOCHONDRIA

Age (months)	Wavelength (nm)				
	$c + c_1$	b_{562}	b_{566}	b_{TOTAL}	aa_3
3	551.4 ± 0.1	561.2 ± 0.6	566.3 ± 0.4	561.4 ± 0.6	605.9 ± 0.2
12	551.3 ± 0.1	561.7 ± 0.2	566.1 ± 0.3	562.1 ± 0.2	606.4 ± 0.1
28	551.2 ± 0.1	561.8 ± 0.2	565.0 ± 1.0	561.8 ± 0.5	605.9 ± 0.2

n = 7 for all values, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE VI

EFFECT OF AGE ON SUCCINATE-DRIVEN RESPIRATION
OF FRESH RAT HEART MITOCHONDRIA

Age (months)	Respiration (nmol O/min per nmol heme a)			
	1st ADP addition		2nd ADP addition	
	State 3	State 4	State 3	State 4
3	838.8 ± 100.2(6)	337.7 ± 26.0(5)	684.0 ± 75.7(5)	272.0 ± 28.4(4)
12	949.0 ± 230.2(6)	251.8 ± 40.7(4)	659.8 ± 20.0(5)	205.4 ± 36.6(4)
28	719.2 ± 56.7(7)	315.2 ± 23.4(7)	564.7 ± 58.6(5)	278.3 ± 14.4(5)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE VII

EFFECT OF AGE ON MALATE PLUS GLUTAMATE-DRIVEN RESPIRATION OF FRESH RAT HEART MITOCHONDRIA

Age (months)	Respiration (nmol O/min per nmol heme a)			
	1st ADP addition		2nd ADP addition	
	State 3	State 4	State 3	State 4
3	490.5 ± 46.0(5)	137.11 ± 23.3(5)	394.8 ± 33.9(5)	92.1 ± 6.2(4)
12	473.1 ± 114.0(6)	121.56 ± 28.7(6)	382.9 ± 105.8(6)	79.1 ± 13.9(5)
28	403.8 ± 19.0(7)	122.30 ± 9.5(7)	306.6 ± 24.3(5)	92.5 ± 7.9(5)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE VIII

EFFECT OF AGE ON RESPIRATORY CONTROL RATIO (RCR) OF FRESH RAT HEART MITOCHONDRIA

Age (months)	Respiratory Control Ratio			
	Succinate		Malate + Glutamate	
	1st ADP addition	2nd ADP addition	1st ADP addition	2nd ADP addition
3	2.25 ± 0.23(6)	2.38 ± 0.12(4)	3.66 ± 0.23(6)	3.75 ± 0.39(5)
12	2.28 ± 0.09(6)	2.39 ± 0.14(6)	3.90 ± 0.09(6)	3.68 ± 0.15(6)
28	2.32 ± 0.17(7)	2.05 ± 0.21(5)	3.39 ± 0.24(7)	3.43 ± 0.38(5)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

TABLE IX

EFFECT OF AGE ON ADP/O RATIO OF
FRESH RAT HEART MITOCHONDRIA

Age (months)	ADP/O Ratio			
	Succinate		Malate + Glutamate	
	1st ADP addition	2nd ADP addition	1st ADP addition	2nd ADP addition
3	2.24 ± 0.07(5)	2.22 ± 0.07(4)	2.73 ± 0.1 (5)	3.03 ± 0.08(5)
12	2.40 ± 0.12(5)	2.35 ± 0.02(5)	2.69 ± 0.09(5)	3.13 ± 0.04(5)
28	2.24 ± 0.06(7)	2.26 ± 0.09(4)	2.74 ± 0.14(7)	3.07 ± 0.03(4)

Number in parentheses denotes n = number of observations, $P > 0.05$ for all values, values are means ± S.E.M.

APPENDIX B

FIGURES

Figure 1. The Electron Transport Chain

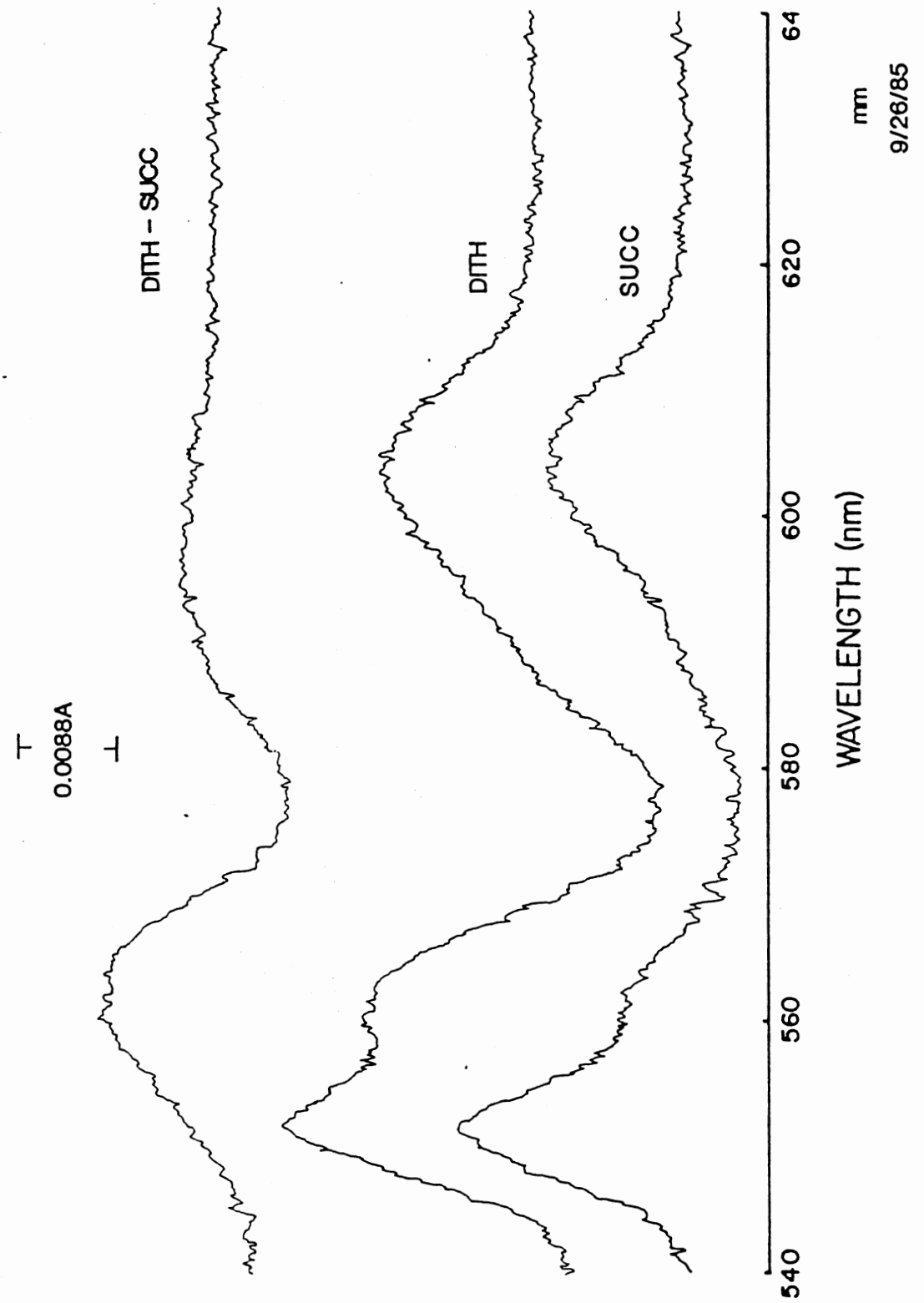

Figure 2. Difference Spectrum

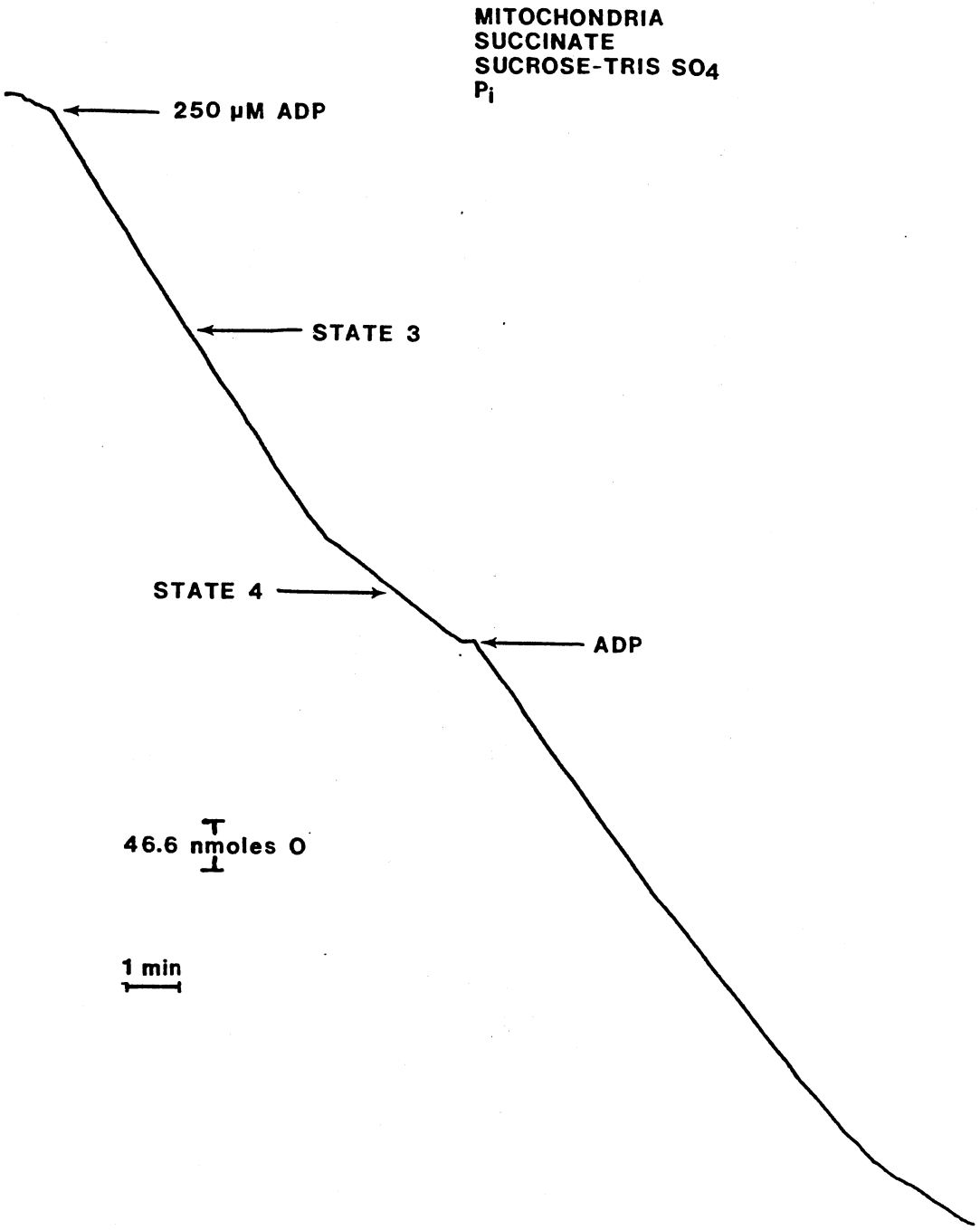

Figure 3. Recording of Respiration

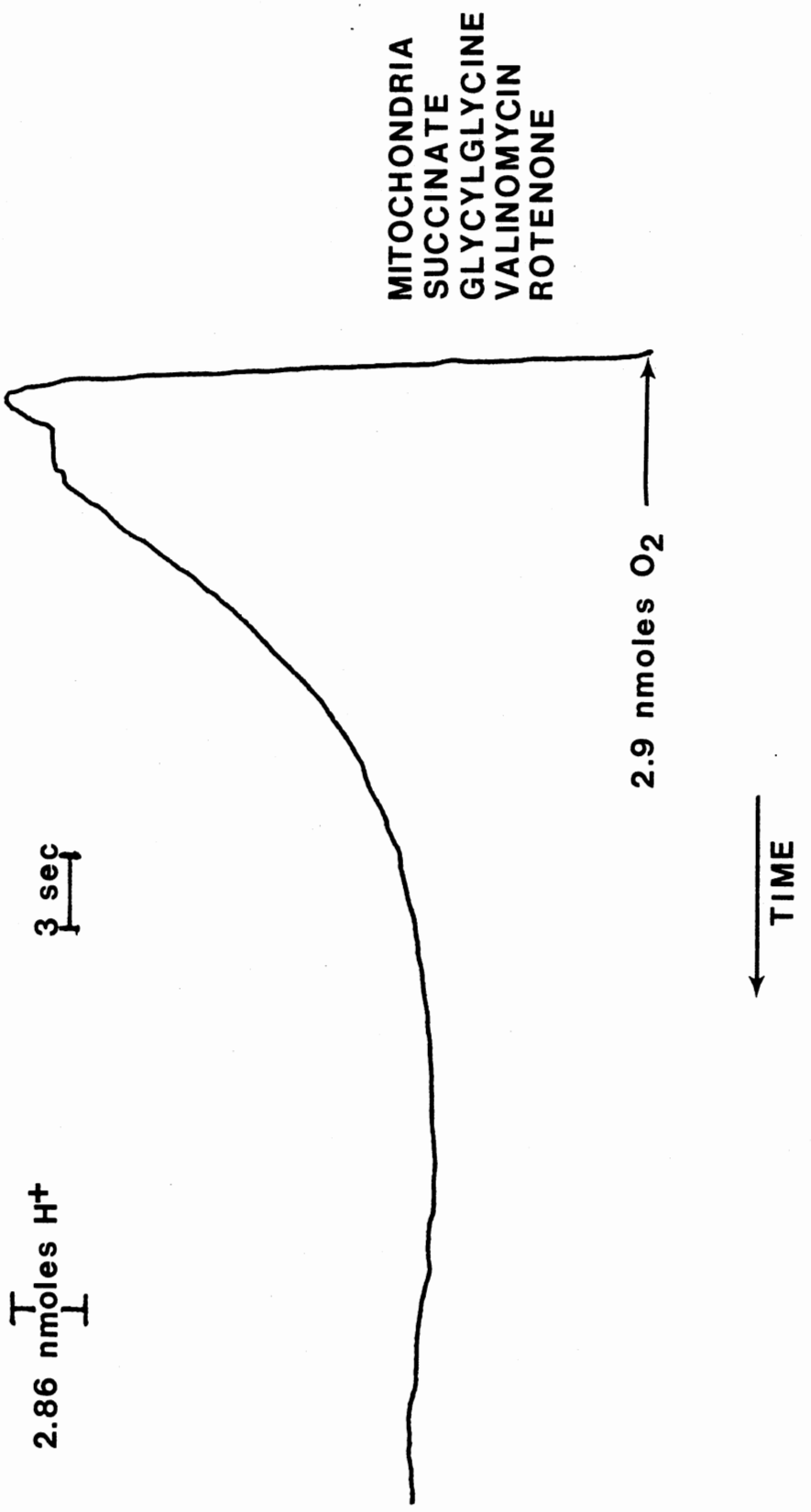

Figure 4. Recording of Proton Translocation

APPENDIX C

EFFECTS OF AGING ON PROTON TRANSLOCATION

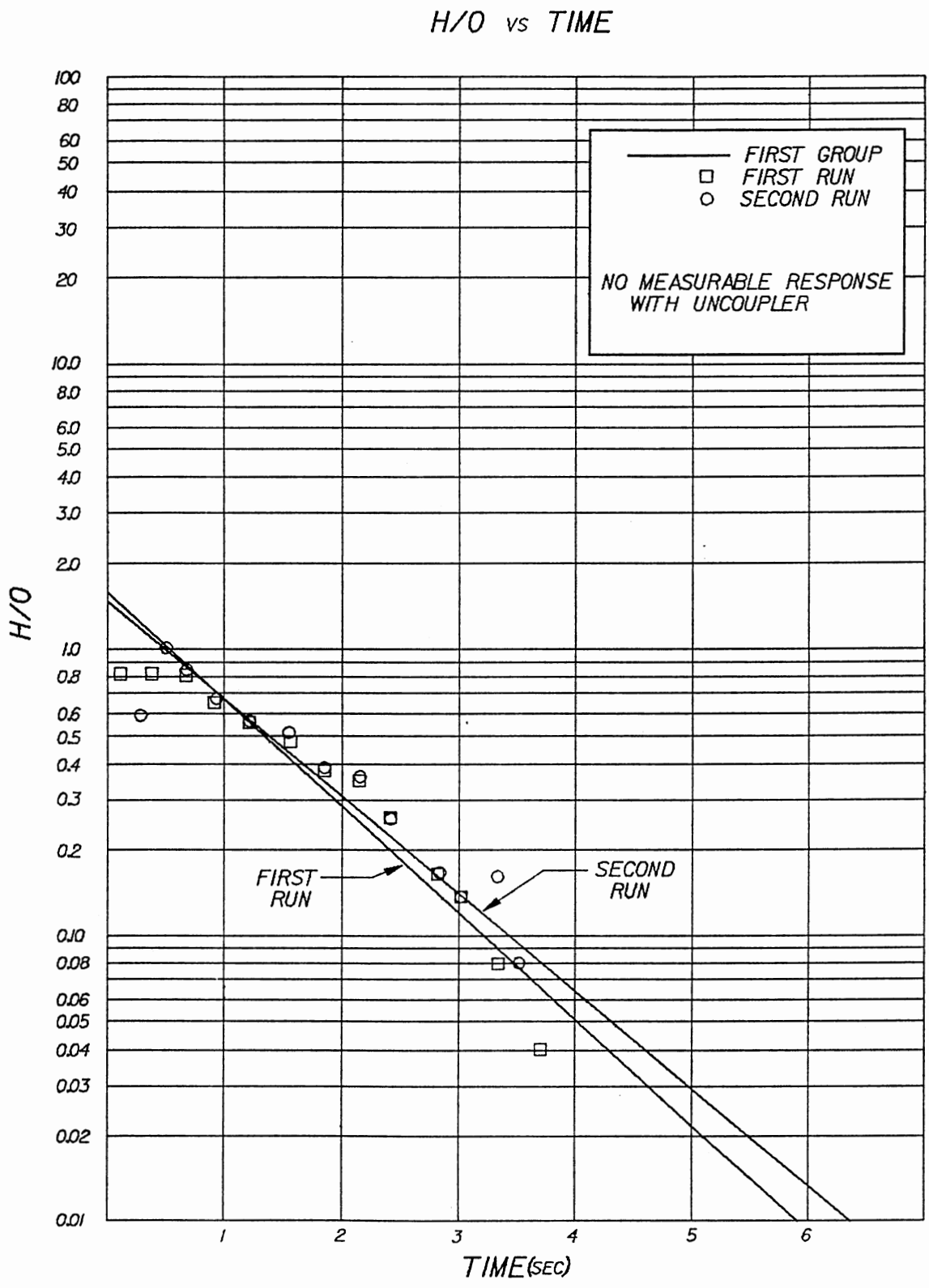

Figure 5. Cardiac Mitochondrial Proton Translocation of a 3-month-old Rat

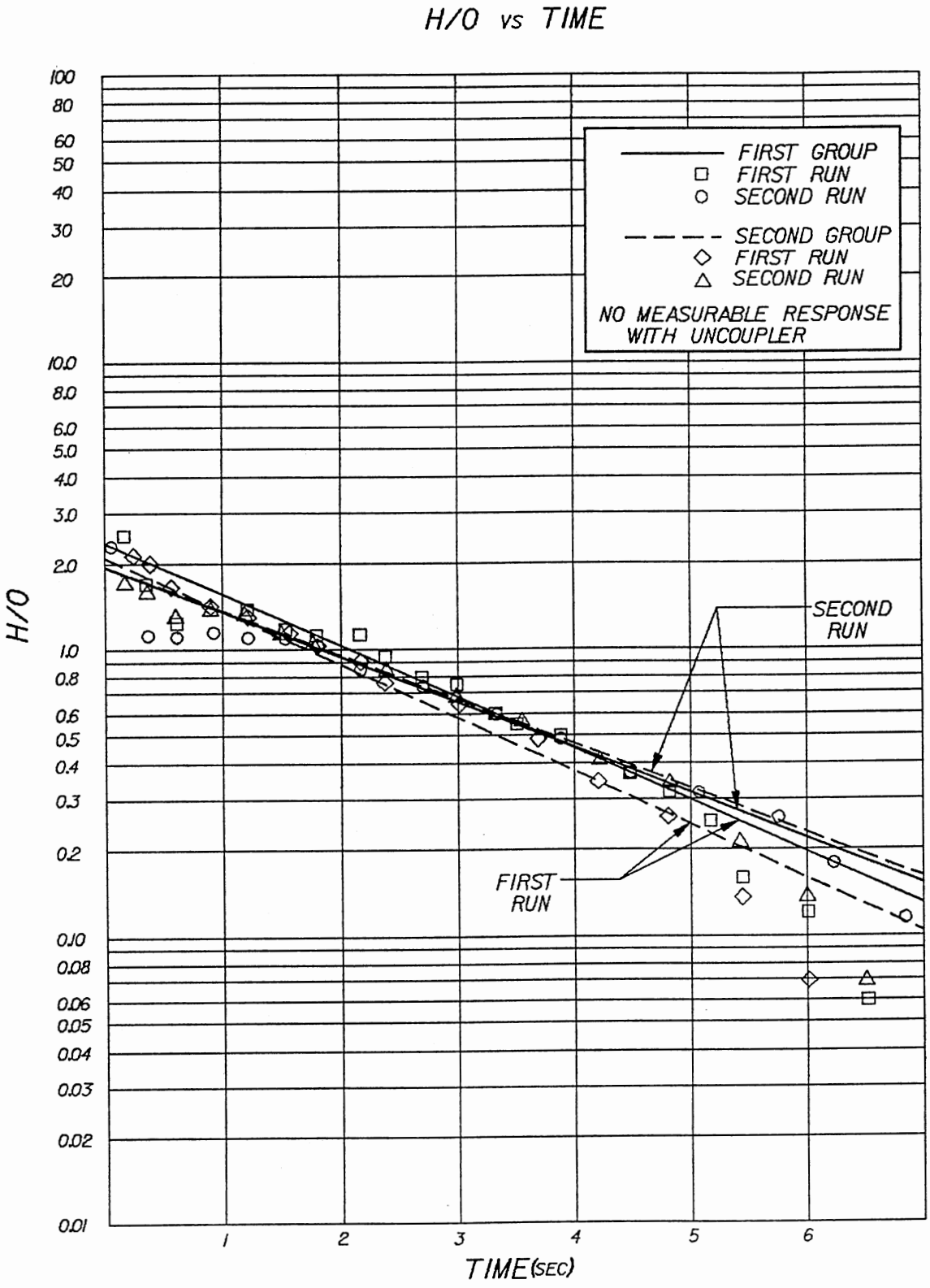

Figure 6. Cardiac Mitochondrial Proton Translocation of a 3-month-old Rat

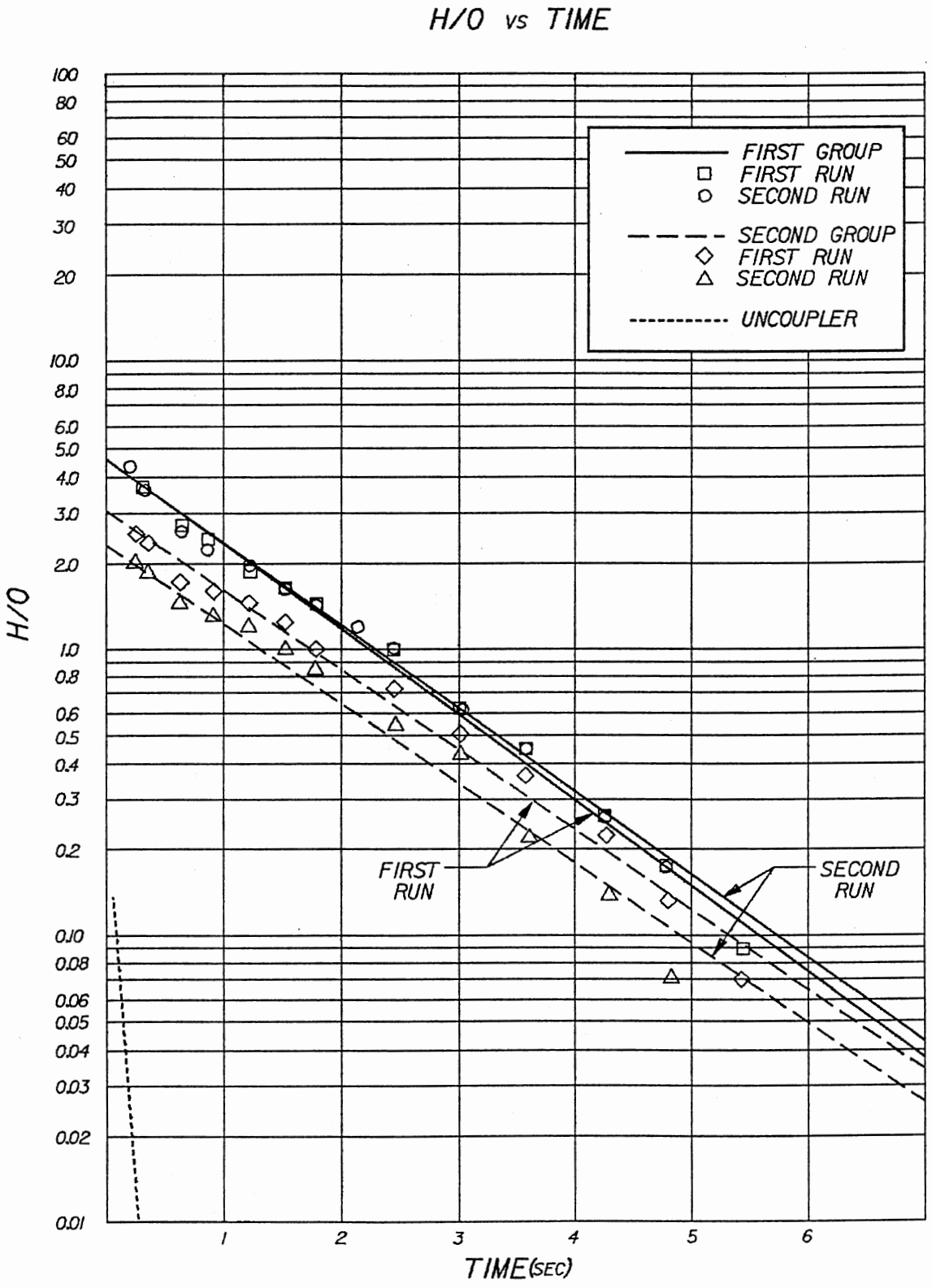

Figure 7. Cardiac Mitochondrial Proton Translocation of a 12-month-old Rat

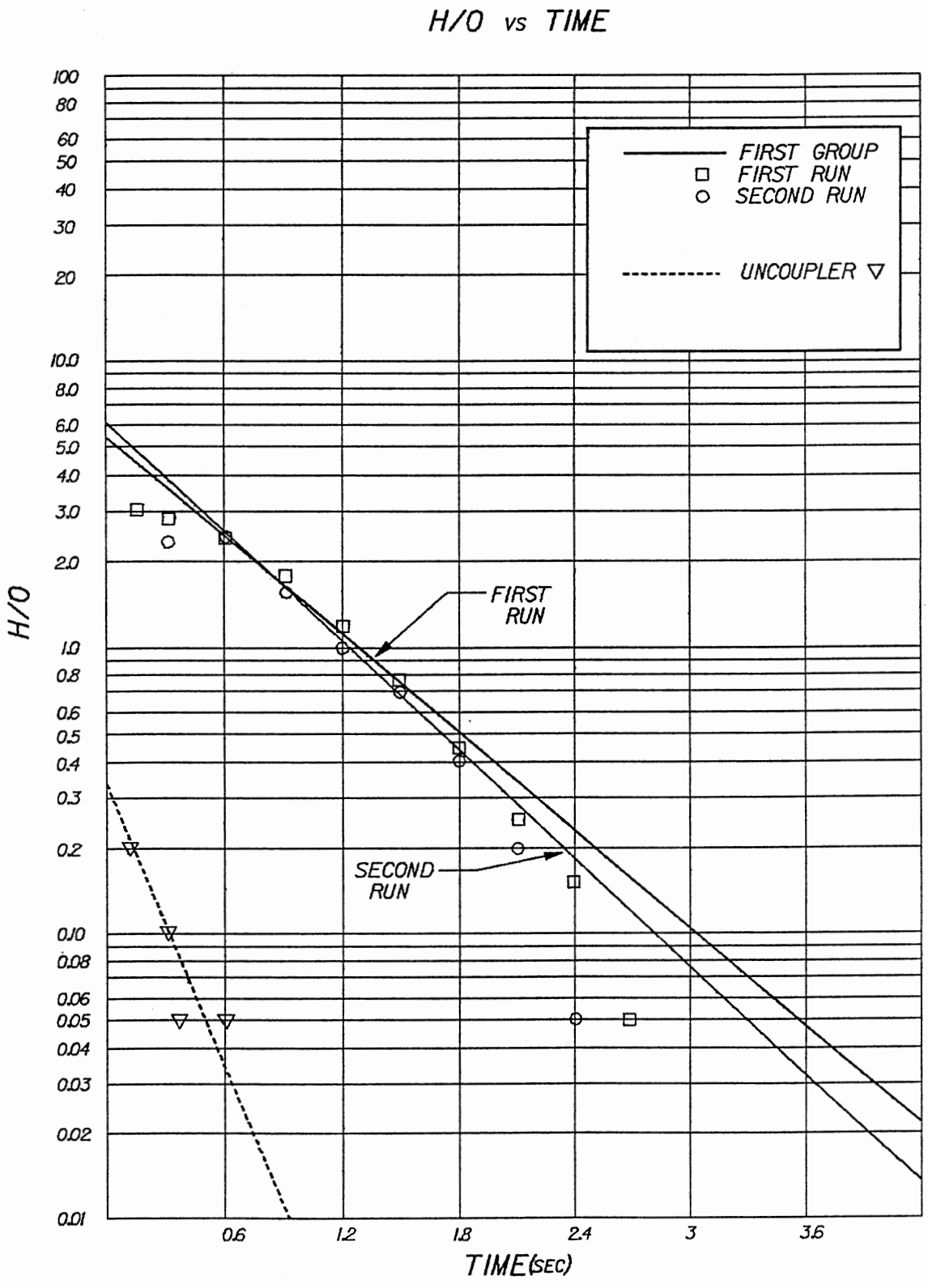

Figure 8. Cardiac Mitochondrial Proton Translocation of a 12-month-old Rat

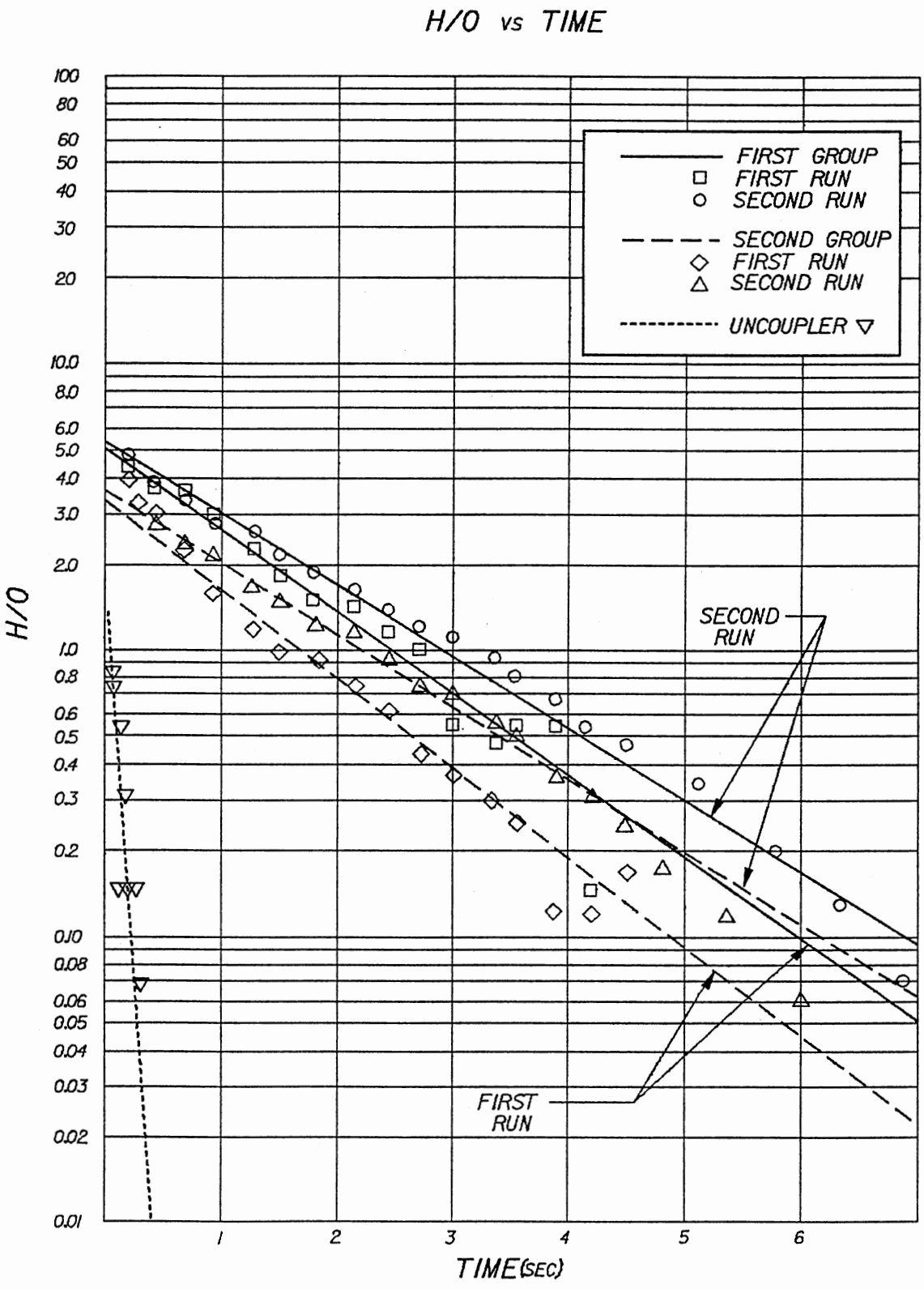

Figure 9. Cardiac Mitochondrial Proton Translocation of a 28-month-old Rat

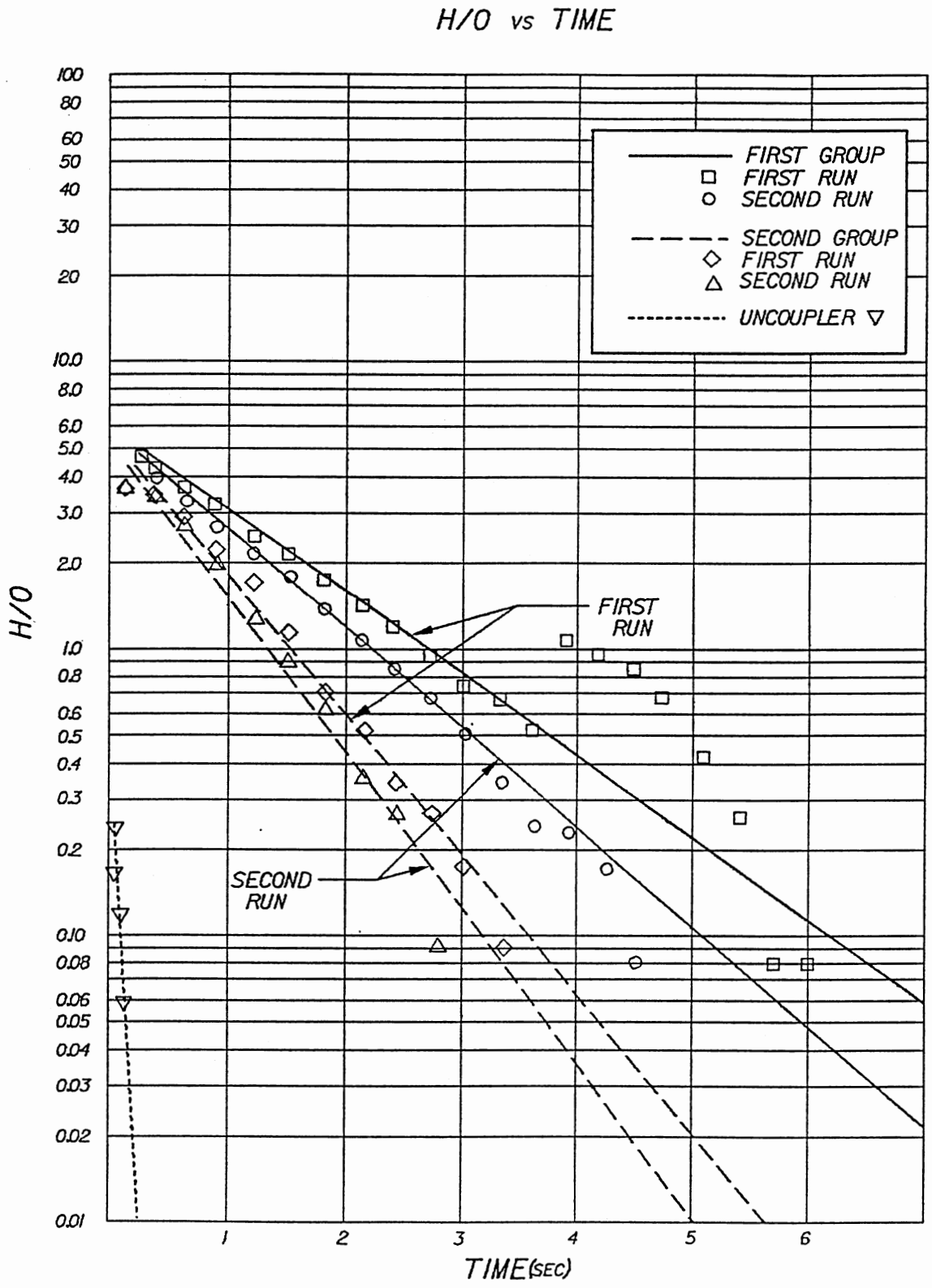

Figure 10. Cardiac Mitochondrial Proton Translocation of a 28-month-old Rat

VITA

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