EFFECTS OF CYCLOPROPANE ON RAT BRAIN AND LIVER MITOCHONDRIA

by

Glauce Socorro de Barros Viana
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TO MY HUSBAND, WITH WHOM I SHARED THE EXPERIENCE OF WRITING THIS THESIS.

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INTRODUCTION

Despite the many advances in the studies of the physiology of the central and peripheral nervous systems and much investigative work on the mechanism of action of anesthetic drugs, it is still impossible at the present time to present a unified theory of narcosis. The several existent ones either relate anesthesia to similarities in the physical or chemical properties of anesthetic agents or describe biochemical or physiological phenomena occurring during anesthesia (18).

Several investigators have sought to explain anesthesia in terms of biochemical phenomena. Quastel (43, 44) demonstrated *in vitro* inhibition of oxygen uptake by the brain after exposure to barbiturates, choral hydrate or urethane. He proposed that barbiturates interfere with the reoxidation of NADH, which in turn depresses the function of the citric acid cycle. Barbiturates can interfere with the synthesis of high-energy compounds by uncoupling oxidation from phosphorylation.

In vivo and *in vitro* studies in cats have shown decreased synaptic transmission in the superior cervical ganglion caused by chloroform, ether, ethanol or barbital; at the same time, axonal conduction remains unimpaired (27, 28).

Various workers have attempted to relate anesthetic potency to the thermodynamic activity or the molecular size of the agent. According to the Ferguson principle, the anesthesic effect will depend upon a definite concentration of anesthetic molecules in some cellular phase. For ideal solutions this concentration will be some particular fraction of the saturated vapor of the anesthetic liquid. Anesthetics that form non-ideal solutions with the cell phase are expected to constitute

exceptions to the Ferguson principle (36). Increased anesthetic potency has also been correlated with a variety of physiochemical properties of anesthetics such as molecular weight, adsorption, oil solubility and Van der Waals correlation factors that relate to molecular volume and to attraction between molecules (6, 64, 13).

In 1961 Pauling (40) proposed that during anesthesia hydrated microcrystals of the clathrate type are formed which are stabilized by proteins. This results in an increase in the impedance of nerve tissue such that the level of electrical activity of the brain is restricted to that of anesthesia. At the same time, Miller (34) proposed a similar theory. He suggested that although gas anesthetic hydrates are not normally formed until excessive pressures are reached, an anesthetic may increase the number of "icebergs" in water. An "iceberg" is defined as an area of highly ordered water surrounding a dissolved gas molecule. Such an ice cover could lower the conductance and occlude the pores of cell membranes (4).

All these data and theories do not present an experimentally verifiable fundamental mechanism by which the anesthetic agents produce their effects.

An unresolved question in medical research is how anesthetics induce narcosis. Especially interesting are the volatile and gaseous anesthetics that have no common molecular structure or size, yet compounds as unrelated as halothane, diethyl ether, cyclopropane, nitrous oxide and xenon produce similar results on brain function. It has been recognized that the usual drug-receptor models cannot accomodate these drugs (17).

Featherstone and Muehlbacher (13) include among "inert gases" those which are generally considered to exert their biological effects without undergoing any change in their own chemical structures or modifying the primary chemical structure of other substances.

It is almost certain that no chemical reaction takes place between an anesthetic molecule and a receptor or molecule in the organism. There are no specific chemical groupings that are required if a molecule is to be an anesthetic. It is the physical rather than the chemical properties of a molecule which enable one to predict anesthetic action (36).

Since anesthetics affect such diverse cellular processes as electrical excitability, cell division and substrate transfer into mitochondria, the interference of these agents with such processes might be utilized as models systems to study anesthetic action.

The work of Hodgkin and Huxley (25) has been an important contribution to the understanding of the processes involved in nerve excitation. Normally, excitation in the nervous fiber results from a highly specific change in the membrane permeability for Na^+ and K^+. This increase in sodium permeability soon decays and is followed by a large and specific increase in the K^+ permeability of the fiber membrane. While the detailed molecular mechanisms involved in the transient increase in Na^+ permeability and the sustained increase in K^+ permeability of the fiber are unknown, there are reasons for thinking that the ion flows involved in nerve excitations take place through aqueous channels or pores in the cell membrane.

The work of several investigators cited by Mullins (36) showed that anesthetics interfere with the movement of both Na^+ and K^+ as they

cross the membrane during an action potential. Using the voltage clamp technique it is possible to measure not only the effects of anesthetics on the Na^+ and K^+ currents of the nerve fiber, but also to measure the kinetic parameters that turn on the Na^+ conductance, turn it off, and turn on the K^+ conductance. It has been found that the kinetic parameters involving the rates of increase in ion conductance are not affected by anesthetics nor is the extent to which the Na^+ conductance mechanism is activated by the membrane potential. The two Hodgkin-Huxley parameters which are affected by anesthetics applied to nerves are the maximum Na^+ conductance and the maximum K^+ conductance developed by the fiber. Such experimental findings, according to Mullins (36), can be interpreted in two ways: either the anesthetic blocks some of the channels that would carry Na^+ and K^+, or it uniformly lowers the rate at which Na^+ and K^+ can be carried through the existing channels.

It is known that in the superior cervical ganglion the fibers synapsing in the ganglion are considerably more sensitive to anesthetics than the axons that run straight through the ganglion. The synaptic transmission seems to occur in the following steps:

Metabolism	→	Transmitter synthesis in nerve terminals	→	Packaging of transmitter in vesicles →
Transmitter release on nerve excitation	→	Combination of transmitter with post-synaptic membrane receptor	→	Induction of permeability change in post-synaptic membrane

According to data in the literature it is the last step in this sequence (the induction of permeability change) that seems to be the one most sensitive to anesthetic action. If this is the case, it would make

synaptic excitation somewhat analogous to nerve excitation in that in both cases it is the flow of ionic current that is affected by the anesthetic agent (36).

The maintenance of a low internal Na^+ concentration in excitable cells has been shown to be dependent on the so-called Na^+ pump mechanism. A possible scheme for the function of the enzyme system is:

$$Na^+(in) + ATP + E \rightarrow E\cap P + ADP + Na^+(out)$$

$$E\cap P + K^+(out) \rightarrow E + Pi + K^+(in)$$

The hydrolysis of ATP to ADP + Pi causes the exchange of Na^+ from the inside for K^+ from the outside of the cell membrane. Inhibition of the Na^+ pump can be expected to allow Na^+ accumulation in the cell and to cause a loss of K^+ inside the cell and thus to abolish the ionic gradient across the cell membrane, giving as a consequence, the inhibition of nerve excitability. Anesthetic agents have been shown to interfere with the Na^+ pump mechanism (53).

The non-polar regions of the protein molecules can be considered potential sites for the action of anesthetics. According to the work of some investigators (36), proteins have the ability to bind substantial quantities of pure hydrocarbons such as butane, as well as more polar anesthetic agents, resulting in a conformational change in the protein molecule.

Schoenborn et al (49, 50) showed an exceptionally strong binding between xenon (Xe) and myoglobin as well as hemoglobin. These X-ray diffraction studies located the site of Xe binding as a natural cavity in the myoglobin molecule. The Xe interaction is due to a Van der Waals' type of binding and apparently does not affect the ability of hemoglobin

as an oxygen carrier. Further exploration of this hemoglobin "pocket" has shown that cyclopropane will bind in it, although nitrous oxide will not, probably due to the linear shape of the nitrous oxide molecule, so that it would not be expected to fit the cavity as does a spherical molecule like cyclopropane or Xe.

One obvious way to regulate cellular activity is by affecting the rate of chemical catalysis of intracellular enzymes. A mitochondrion can be viewed as an array of enzymes embedded in a lipid matrix. Anesthetics have been shown to affect several mitochondrial functions including enzyme activity (36).

The mitochondria are perhaps the best understood of the cell organelles in terms of ultra structure, molecular organization, and functional role in cell metabolism. Although it has been known for several years that the mitochondria are the "power plants" of the cell, recent research on mitochondria structure has opened many new challenges: the role of membrane structure in the mechanism of phosphorylation, the compartmentation of enzymatic reactions within mitochondria, the interplay between glycolysis and respiration, and the role of mitochondria in ion transport processes (29).

The mitochondrial membranes generally contain 35 to 40% lipid and 60 to 65% protein. More than 90% of the membrane lipid is phospholipid. Analyses have shown that more than 25% of the total membrane protein may consist of respiratory enzymes (57).

Mitochondria from different tissues differ in characteristic ways both with respect to details of structure (shape, size, numbers and organization of crystae) and functional properties (41). Mitochondria

in any one tissue may also show changes in their structure and enzymatic properties in response to alterations in physiological conditions. Heterogeneity of structure and composition of mitochondria found in similar cells of a tissue has been reviewed by Novikoff (39).

Evidence that mitochondria form a heterogeneous population has come from three approaches: 1) the isolation from rat liver of two mitochondrial fractions with somewhat different enzymatic compositions; 2) histochemical observations on the distributions of mitochondrial enzymes throughout the liver lobule; 3) determination, for several enzymes, of the relationship between enzyme activity and mitochondrial size in a sample of rat liver mitochondria (31).

According to Lusena and Depocas (32), the heterogeneity of mitochondria in cells of the hepatic lobule, examined by electron microscopy, conflicts with the observed homogeneity of mitochondria isolated from the whole organ.

In the same way methods involving centrifugation of brain mitochondria preparations in continuous sucrose density gradients and the determination of enzyme activities throughout the gradient, have yielded evidence of mitochondria heterogeneity in brain (37).

Experiments (58) using rat brain mitochondria suggested: a) a heterogeneity of the physical composition of the mitochondrial pellet obtained from different brain regions under standard experimental conditions; b) that the amount of enzymes in analogous particles is different, indicating a constitutional heterogeneity of the mitochondria, or c) the nature of the enzyme protein could be qualitatively different.

The basic biochemical properties of the brain mitochondrial fraction

appear to be comparable with those of mitochondria from liver and other tissues (e.g. phosphorylation quotient and respiratory control), but there are certain characteristic differences. Some of these differences may be due to contamination with non-mitochondrial components, e.g. the high Mg^{++} stimulated ATPase activity and the presence of glycolytic enzymes in brain mitochondria (2).

Turnover rates of the protein components differ according to the mitochondrial origin. Studies by Fletcher and Sanadi (14), and Beattie et al (3) have shown that in liver mitochondria all protein components have an identical half-life of 8.5 days. In kidney mitochondria, the water-soluble proteins have a half-life of 5.9 days, which is significantly shorter than that of the whole mitochondria which is 8.6 days. In brain mitochondria, the water-soluble proteins have a half-life of 17.9 days as compared to 26.3 days for the whole mitochondria. These water-soluble proteins appear to be synthesized extramitochondrially, and then are subsequently integrated into the mitochondrial structure.

Menzies and Gold (33) studied the effects of aging in the turnover rates of mitochondria from a variety of rat tissues. The turnover rates of mitochondria from liver, brain, heart and testes of young adult (12 months old) and aged (24 months old) rats were measured by following the loss of radioactivity from proteins of purified mitochondrial preparations after initial labeling with 3H-leucine. No significant differences were found for any time between the two age groups.

On the other hand, some metabolic characteristics of mitochondria were studied by Von Korff et al (60). Their work showed that mitochondria from rabbit brain and heart differ significantly in their metabolic

properties. The main objective of this study was to carry out a long term investigation on the vulnerability of brain to anoxia. It is well known that the brain consumes 20% of the basal oxygen requirement of an individual. According to these authors the high rate of oxidation must involve the mitochondria in energy-yielding processes.

There are many studies in the literature on mitochondria from several sources, and the recent improvement of isolation techniques has produced the so-called "washed" preparations freed of many contaminants formerly present in the "crude" ones.

The mitochondrion is now a relatively well known cellular organelle and so it constitutes a good biological model for studies at the molecular level. In addition this biological system might be a useful tool for studies on mechanisms of anesthetic action.

Cyclopropane was chosen in the present study for the following reasons: a) it is a small molecule relatively chemically inert and so it doesn't undergo structural changes or participate in any reaction in the biological system; b) the literature contains few studies on gaseous anesthetics at the molecular level, probably due to technical problems involved with experimental gas work; c) anesthetic gases seem to act in the cellular membrane by means of weak electrostatic forces (Van der Waals type binding), and thus it is easy to get reversibility of the biologic effect.

The investigator should, however, be aware of the fact that the action of anesthetics may be a pluralistic one, perhaps affecting many cellular processes by different mechanisms.

The main goal of the present work was to study:

a) Interaction of the gas anesthetic with a specific component(s)

in mitochondria;

b) Possible interference with energy producing mechanisms and its relationship to anesthetic action;

c) Comparison of the cyclopropane effects on rat brain and liver mitochondria.

MATERIALS AND METHODS

I. Materials

A. Chemicals

All the reagents used were obtained from commercial sources. The gases came from Ohio Medical Products, Madison, Wisconsin and Liquid Carbonic (Division of General Dynamics). The water used was redistilled in glass apparatus in the laboratory.

B. Apparatus

Spectrophotometers-- For all the enzymatic assays a Perkin-Elmer model 356 two-wavelength double-beam spectrophotometer connected to a Perkin-Elmer model 165 recorder was used.

Modes of operation of spectrophotometer: Double-beam spectroscopy was used for obtaining oxidized-reduced difference spectra of the cytochromes. In this procedure the two beams are set at the same wavelength and then scanned over the desired range. Because of the turbidity, the samples were placed close to the photomultiplier, thereby decreasing the light loss due to scatter.

Double wavelength recording with (λ_1) and (λ_2) fixed but different (dual mode) was used for measuring small changes in absorption with time. The absorption of λ_1 relative to λ_2 was then monitored with the recorder.

Most of the work was done at 25° C, which was maintained using a temperature controlled water bath from Wilkens-Anderson Company, Chicago, Illinois, connected to the primary cell compartment of the spectrophotometer.

For protein and ADP determinations a Beckman DB spectrophotometer

or a Hitachi-Perkin-Elmer spectrophotometer, model 139 UV-VIS was used.

Mixing Chamber and Flowmeter-- The gas-mixing apparatus was made in the laboratory using a Simet Gas Mixer from Veriflo Corporation, National Welding division, Richmond, California, and a precision spherical float flowmeter from Roger Gilmont Instruments, Inc., Great Neck, New York (Figure 1). Connections were made using swaglock and plastic tubing. Up to four gases may be mixed together. The precision of the gas mixtures was verified using a GC-2A Gas chromatograph from Beckman-Scientific and Process Instruments division. The chromatographic column used was a 70021 with a 6' length molecular sieve, 13x, 42/60 mesh. The variability in the mixtures was less than 10%.

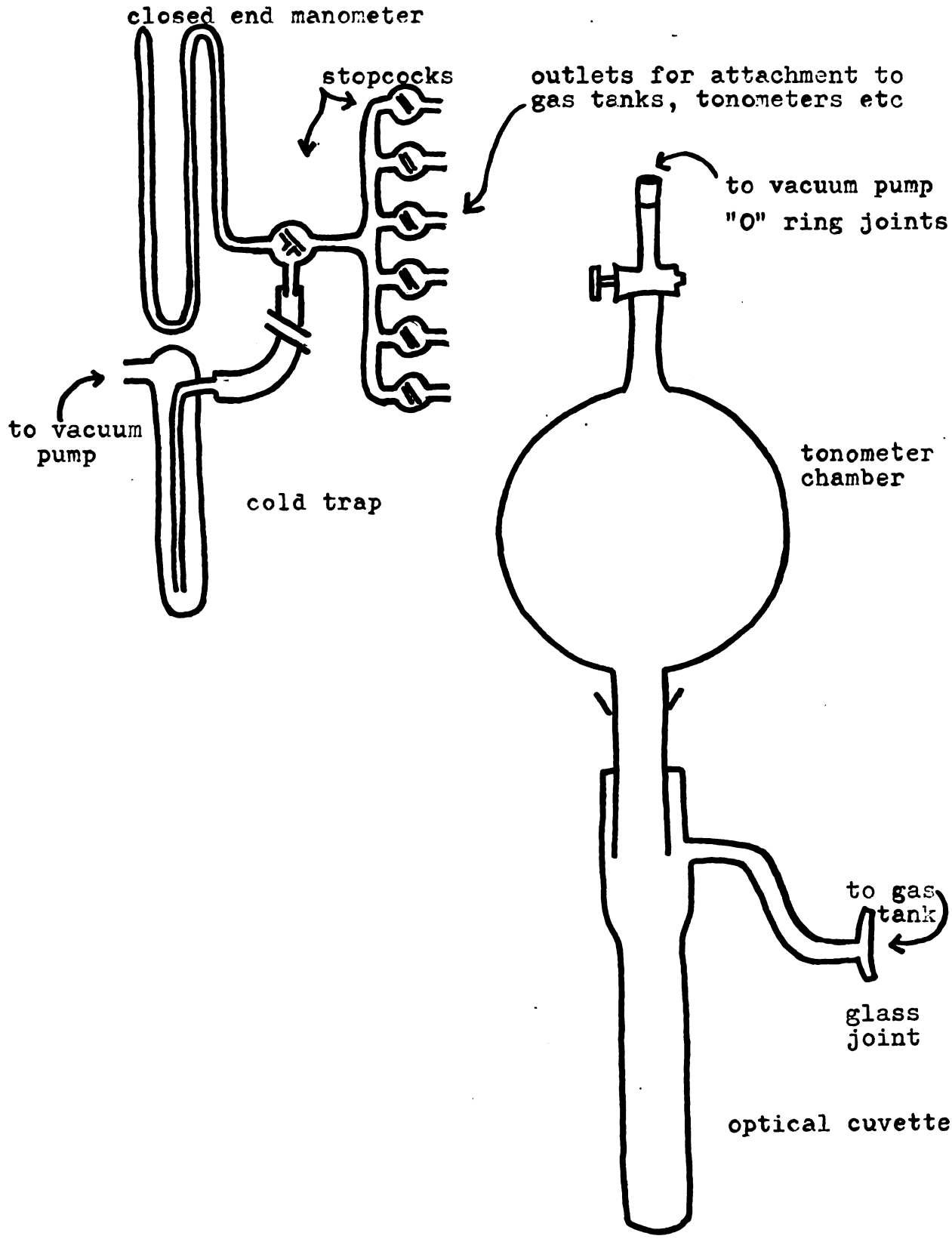
Degassing Apparatus Below 1 Atmosphere--For pressures less than 1 atmosphere the apparatus shown in Figure 2 was used to easily transfer gases at pressures 0 to 1300 mm Hg. Gas tanks, gas storage vessels, and tonometers were attached to the set of stopcocks permitting all the connecting tubing to be completely evacuated before introduction of the desired gas. The gas partial pressure can be monitored at all times. The gas pressure was measured to the nearest millimeter by means of a meter scale attached to a closed end manometer.

Oxygraph and Clark Electrode--The model KM Oxygraph from Gilson Medical Electronics, Middletown, Wisconsin and a Clark type electrode (model YSI 4004) assembly available from Yellow Springs Instrument Co., Yellow Springs, Ohio, connected to the Oxygraph apparatus was used for the studies on oxidative phosphorylation.

The Oxygraph, a recording oxygen cathode, is a specific application of polarographic analysis which depends upon the properties of current-

Fig. 1- Mixing Chamber and Flowmeter

Fig. 2- Degassing Apparatus used to transfer gases below 1 atm.



potential curves obtained with suitable electrodes. The Clark electrode used has the cathode, anode and KCl bridge contained in a single compartment and separated from the reaction medium by a Teflon membrane. The membrane is permeable to oxygen, and the diffusion potential is created by a magnetic stirring bar.

II. Methods

A. Isolation of Liver Mitochondria

The Schnaitman and Greenawalt procedure (47) slightly modified, as described below, was followed.

Two adult male albino rats (Sprague-Dawley strain), weighing 200-250 g were used. The animals were sacrificed by a blow on the head and exsanguinated, the livers were quickly removed and placed in ice-cold medium (isolation medium A), containing 220 mM D-mannitol, 70 mM sucrose, 2 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, and 0.5 mg/ml bovine serum albumin (BSA). The pH of the medium was adjusted to 7.4 with NaOH 1N prior to use. The livers were washed with isolation medium, minced, weighted, and suspended in approximately 2 volumes of isolation medium. First the mince was homogenized manually with a Dounce homogenizer tissue grinder (4 up and down strokes) followed by 4-6 passes in a motor-driven Potter-Elvehjem Teflon, tissue grinder (total clearance between pestle and tube 0.004"-0.006") and diluted with isolation medium to give a 10% homogenate. The homogenate was centrifuged at 560 x g for 15 minutes and the pellet was discarded. The supernatant was centrifuged at 7,000 x g for 15 minutes. The fluffy layer was carefully discarded, and the pellet was washed twice by resuspension and hand homogenization first in one-half and then in

one-fourth of the original volume of isolation medium, followed by centrifugation at 7,000 x g and 8,000 x g for 15 minutes respectively. The final mitochondrial pellet was resuspended in isolation medium without BSA. All operations were carried out at 0-4°C and the centrifugations were performed in a Lourdes Beta-fuge model A-2.

B. Isolation of Brain Mitochondria

The Clark and Nicklas procedure (10) was used.

Six to eight Sprague-Dawley male rats, weighing 200-250 g, were decapitated, the cerebral hemispheres rapidly removed and placed in ice-cold isolation medium B (0.25 M sucrose, 10 mM Tris, 0.5 mM K^+ EDTA, pH 7.4). Blood was removed with a cotton swab, and the tissue transferred into a 40 ml cold isolation medium and weighed. The tissue was chopped finely with scissors, and homogenized manually in a Dounce homogenizer tissue grinder (2 up and down strokes). A further 20 ml of ice-cold isolation medium was added and the total homogenate was submitted to 4-6 up and down strokes with a motor driven Potter-Elvehjem Teflon tissue grinder (total clearance between pestle and tube 0.004"-0.006"). The homogenate was centrifuged for 3 minutes at 2,000 x g and the supernatant was centrifuged for 3 minutes at 12,500 x g (Lourdes Beta-fuge model A-2 at 0-4°C). The crude mitochondrial pellet was resuspended to a final volume of 10 ml in a 3% Ficoll medium (3% Ficoll, 0.12 M mannitol, 0.03 M sucrose, 25 μM K^+ EDTA, pH 7.4). The suspension was carefully layered onto 20 ml of 6% Ficoll medium (6% Ficoll, 0.24 M mannitol, 0.06 M sucrose, 50 μM K^+ EDTA, pH 7.4) and centrifuged for 30 minutes at 11,500 x g in a Spinco ultra-centrifuge, model L at 0°C. The supernatant was decanted and the fluffly layer removed from the

pellet. The mitochondrial pellet was resuspended in isolation medium and recentrifuged for ten minutes at 12,500 x g in a Lourdes Beta-fuge model A-2 at 0-4° C.

C. Chemical and Enzymatic Analysis

Difference Spectra--The oxidized-reduced spectra of the cytochromes was obtained by scanning the sample (reduced) and reference (oxidized) cuvettes in the primary compartment, through the experimental spectral range (650-350 mμ), in a split mode operation using the Perkin-Elmer model 356 double-beam spectrophotometer at 25° C.

The optical sample cuvette was a modified Thunberg tube (Figure 3) containing phosphate buffer, 0.1 M, pH 7.4, mitochondria suspension (5-10 mg protein) and substrate (5-10 μmoles) in a final volume of 2.0 ml. The reference cuvette contained everything except substrate.

For the difference spectra in the presence of gas, the gassing procedure to be described in Methods, section D, was used throughout.

Succinic-Dehydrogenase (SDH) assay--The system which oxidizes succinate with O_2 as hydrogen acceptor (succinoxidase) includes the enzymes of the respiratory chain. Like cytochrome oxidase it has been used as a "mitochondrial marker." The choice of a hydrogen acceptor for assays of succinic dehydrogenase is somewhat problematical, since the natural acceptor (probably cytochrome b or ubiquinone) is not clearly demonstrated. Assays present several problems: rate limitation by slow penetration of the acceptor must be avoided and also errors due to inhibition by the oxaloacetate which may accumulate if malate dehydrogenase is present in the same preparation (31).

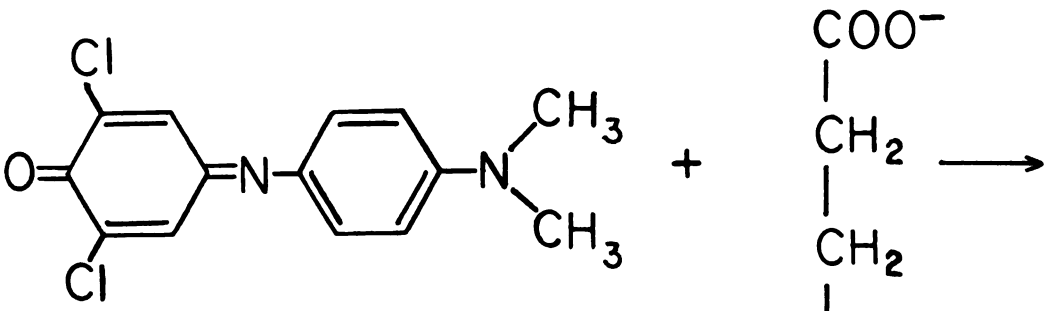
It is generally agreed that the succinic dehydrogenase in mitochondria is bound to the membranes, being in close relationship with

the enzymes of the respiratory chain.

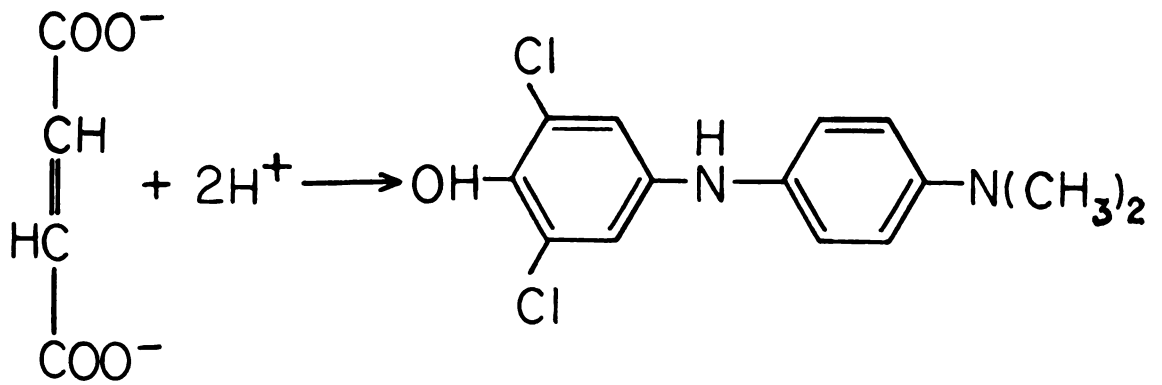
The redox reaction in SDH assays follows the general pattern:

Succinate + Acceptor = Fumarate + Reduced Acceptor

In the present case, the method used was based in the substrate-dependent reduction of a blue dye, sodium 2,6 dichlorophenol-indophenol (DCPIP), to its colorless form. The enzymatic oxidation of succinate by indophenol in the presence of cyanide was followed spectrophotometrically at 600 mμ (38, 20). The overall reaction is:



blue form of 2,6 DCPIP succinate



fumarate colorless form of 2,6 DCPIP

The reaction medium contained 2.0 ml phosphate buffer (0.1 M, pH 7.4),

5-10 µl mitochondria (containing 100-400 µg protein), 10 µmoles sodium cyanide, 0.057-0.114 µmoles DCPIP and 0.1-10.0 µmoles succinate, unless otherwise specified. The blank contained everything but succinate. Changes of absorbance were recorded after the addition of substrate, using the model 165 Perkin-Elmer recorder.

Specific activity is defined as micromoles of indophenol reduced per mg of protein per minute at 25° C. The optical density change at 600 mµ divided by 20 (molar extinction coefficient of DCPIP), gives the value for micromoles of indophenol reduced (20, 1).

In experiments with less than 1 atm, gas pressure, the optical density change was followed in a model double-beam spectrophotometer, but in this case in a dual mode operation with λ_1 = 560 and λ_2 = 600 mµ and using only one cuvette.

Oxidative Phosphorylation assay--The assay procedure was that described by Sordahl et al (54). The final volume in the oxygen-electrode chamber was 1.0-1.5 ml and 1-2 mg of mitochondrial protein per ml was added to the reaction mixture.

Assay media

For liver mitochondria:
10 mM substrate (β-hydroxybutyrate or succinate as Na salt)
75 mM KCl
50 mM tris-HCl
12.5 mM K_2HPO_4
5 mM $MgCl_2$
1 mM EDTA
pH 7.4

For brain mitochondria:
10 mM substrate (same as liver)
0.3 M mannitol
10 mM KCl
10 mM tris-HCl
5 mM K_2HPO_4
0.2 mM EDTA
1 mg/ml of BSA
pH 7.4

The recorder pen full range was assumed to be 480 nanoatoms atmospheric oxygen per ml in solution at 25° C. The bottom of the tracing at zero current corresponds to zero oxygen concentration.

From the experimental polarographic data the ADP:O ratio (eg. to the P:O ratio) and the oxygen consumption (QO_2) were calculated. ADP:O ratio or moles of ADP added per atom of oxygen taken up, measures the amount of oxygen consumed during the state 3 "burst" per nanomoles of ADP added. The rate of oxygen consumption (QO_2) was calculated from state 3 respiration and is an expression of the activity of the enzymatic protein (nanoatoms oxygen/min/mg protein).

In order to produce the state 3 "burst" or respiration, 200-500 nanomoles of ADP were added. The exact concentration of the ADP solution was determined by reading the optical density in a spectrophotometer at 259 mμ and using the millimolar extinction coefficient of 15.4.

For the oxidative phosphorylation studies with gases, the desired gas was introduced with a syringe of 10 ml capacity through the capillary bore stopper in the Clark type electrode assembly. The desired gas mixtures were taken directly from the mixing chamber connected to the gas tanks. 80% Cyclopropante-20% Oxygen and 80% Nitrogen-20% Oxygen mixtures gently bubbled into the jacketed cell (reaction chamber) were used, allowing 5 minutes for equilibration. When necessary, air was bubbled under the same conditions as control.

In all cases the mitochondrial suspension was used immediately after isolation.

Protein determination--Protein was determined by the Biuret method (61), using bovine serum albumin as standard. The readings were carried out at 750 mμ and at room temperature in Beckman DB or Hitachi-Perkin-Elmer UV-VIS spectrophotometers.

D. Gassing Procedure

For the gassing procedures a modified Thunberg tube (Figure 3) was used. These tubes permit evacuation of the air in the tube, replacement with the desired gas mixture and also use of higher pressures if required. The procedure included evacuation on the house vacuum line, the tubes were subsequently shaken for one minute, evacuated again and the desired gas mixture from a mixing chamber was admitted. This procedure was followed in all measurements carried out at 1 atm. pressure.

For less than 1 atm. the special degassing apparatus was used. The reaction mixture in the optical cuvette (Thunberg tube connected to a tonometer) was submitted to vacuum, and gases were admitted to the desired pressure. Control experiments followed the same procedure using air instead of gas.

E. Statistical Analysis

Statistical Analysis was performed by Analysis of Variance and by Scheffé Test according to the particular needs in each case.

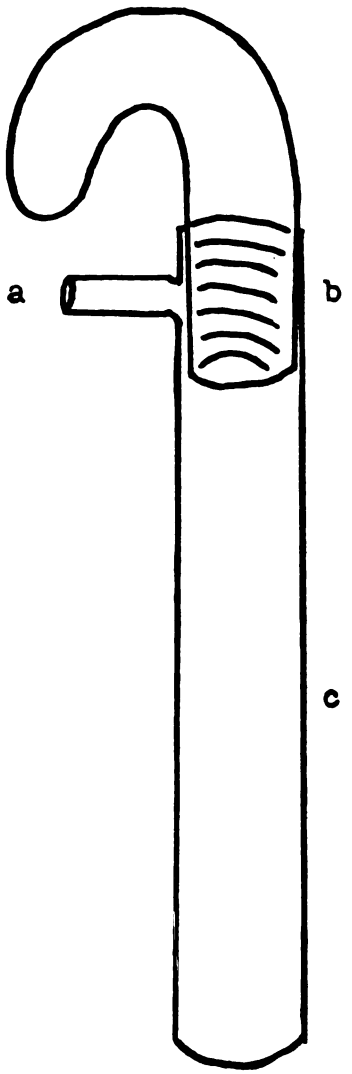


Fig. 3- Thunberg tube
a. outlet for evacuation and replacement of gas
b. ground glass
c. optical cuvette

RESULTS

A. Characteristics of the "Washed" Mitochondria from Rat Liver and Brain

"Washed" preparations reduce contamination from cytoplasmic components, synaptosomes and other membraneous fragments. The pellet suspensions contained 15-30 mg of protein/ml.

The difference spectra (Figure 4) with succinate as substrate show that both brain and liver mitochondria preparations contain the normal complement of mammalian cytochromes absorbing at wavelengths which are similar to those of cytochromes present in other tissues, as well as the other components of the respiratory chain (flavoprotein and NADH).

There are qualitative and quantitative similarities between the two preparations, but the major difference is the time for appearance of the reduced cytochromes peaks, usually around 15 minutes for liver and 30 minutes for the brain preparation in air.

The presence of cyclopropane causes different effects depending on mitochondrial origin. An example (one of 5 experiments) is shown in Tables 1 and 2, the appearance of all but the γ bands is delayed by cyclopropane as compared with nitrogen in the liver preparation. In brain mitochondria the reduced a + a3 peaks developed at the same time for both cyclopropane and nitrogen. Reduced cytochrome peaks (with the exception of a3α and the Soret band) decreased faster, within 30 minutes, when compared with nitrogen.

Cyclopropane effects must be compared with those of nitrogen because the difference spectra studies in both cases were done in anaerobic and so comparable experimental conditions.

In rat liver mitochondria (Figure 5), cyclopropane generally

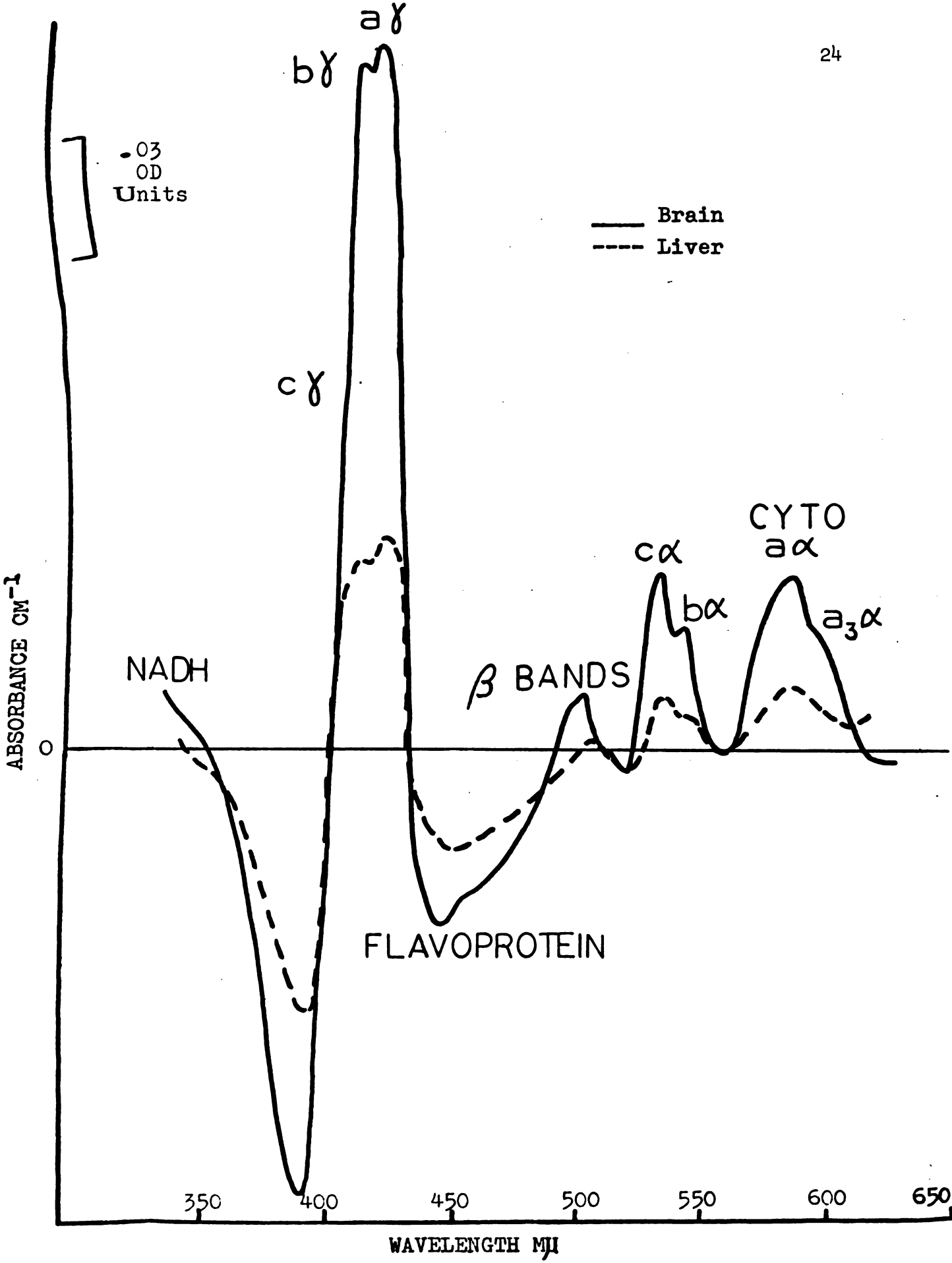


Fig. 4- Difference Spectra of Rat Brain and Liver Mitochondria with succinate. Readings taken at 30 minutes.

TABLE 1 - EFFECTS OF CYCLOPROPANE AND NITROGEN ON THE REDUCED CYTOCHROME PEAKS OF A DIFFERENCE SPECTRA OF RAT LIVER MITOCHONDRIA AT DIFFERENT TIMES

Peak Ident.	Peak Height in mm/mg Protein (%)								
	Air Time in Min.			Cyclopropane Time in Min.			Nitrogen Time in Min.		
	5	15	30	5	15	30	5	15	30
a + a3	-	17.9	17.9	-	-	17.9	-	17.9	17.9
a3 α	-	35.7	35.7	-	-	35.7	17.9	35.7	17.9
b α	-	17.9	89.3	-	-	89.3	-	392.9	267.9
c α	-	17.9	89.3	-	-	89.3	35.7	392.9	285.7
β bands	-	17.9	53.6	-	-	89.3	89.3	339.3	267.9
γ bands	285.7	1625.0	2000.0	464.3	875.0	1964.3	107.0	2464.3	2482.1

The optical sample cuvette (reduced) contained: Phosphate Buffer, pH 7.4, 0.1 M, mitochondria suspension (5.6 mg Protein) and 5.0 µmoles succinate in a final volume of 2 ml. The reference cuvette (oxidized) contained everything except substrate. The measurements were done at different times at control atmospheric conditions and submitting the cuvettes to the desired gas by the gassing procedure.

TABLE 2 - EFFECTS OF CYCLOPROPANE AND NITROGEN ON THE REDUCED CYTOCHROME PEAKS OF A DIFFERENCE SPECTRA OF RAT BRAIN MITOCHONDRIA AT DIFFERENT TIMES

Peak Ident.	Peak Height in mm/mg Protein (%)								
	Air Time in Min.			Cyclopropane Time in Min.			Nitrogen Time in Min.		
	5	15	30	5	15	30	5	15	30
a + a3	97.2	138.9	111.1	97.2	166.7	125.0	97.2	180.6	180.6
a3 α	-	-	27.8	-	97.2	97.2	-	83.3	97.2
b α	-	-	138.9	-	263.9	236.1	-	277.8	263.9
c α	-	-	152.8	-	180.6	152.8	-	166.7	180.6
β bands	-	-	111.0	-	125.0	69.4	-	55.6	111.0
γ bands	541.7	583.3	1972.0	764.0	2444.0	2638.9	555.6	2431.0	2597.2

The optical sample cuvette (reduced) contained: Phosphate Buffer, pH 7.4, 0.1 M, mitochondria suspension (7.2 mg Protein) and 5.0 µmoles succinate in a final volume of 2 ml. The reference cuvette (oxidized) contained everything except substrate. The measurements were done at different times at control atmospheric conditions and submitting the cuvettes to the desired gases by the gassing procedure.

increased the time for appearance of the reduced peaks (5-15 minutes in aerobic conditions and in the presence of nitrogen, and about 30 minutes in the presence of cyclopropane).

One possible explanation may be that, in liver mitochondria, cyclopropane interferes in some way with electron transport through the chain, thus slowing cytochrome reduction. Another possibility is that in this system the substrate concentration is low compared to the amount of protein used and so cyclopropane is acting in the range of its inhibitory effect on SDH. (See next item on SDH assays.)

On the other hand, in brain mitochondria (Figure 6), cyclopropane favored reduction of the components of the respiratory chain, the reduced cytochrome peaks appearing rapidly under the gas influence (around 15 minutes), and also declining faster compared with nitrogen (Table 2). This effect could be attributed to SDH stimulation by cyclopropane.

Nitrogen in both preparations, as it would be expected due to the lack of oxygen, increased the reduction state of the cytochromes, the fully reduced state being reached between 15-30 minutes.

These differential effects on brain and liver mitochondria may be partially explained by the differences in intensity of stimulatory action on SDH activity produced by cyclopropane in the two mitochondrial systems, thus accelerating in a greater or smaller manner the electron transfer through the chain. Another possibility, at least with the brain preparation, is that the effects are non specific and caused by the lack of oxygen, as probably happens with nitrogen.

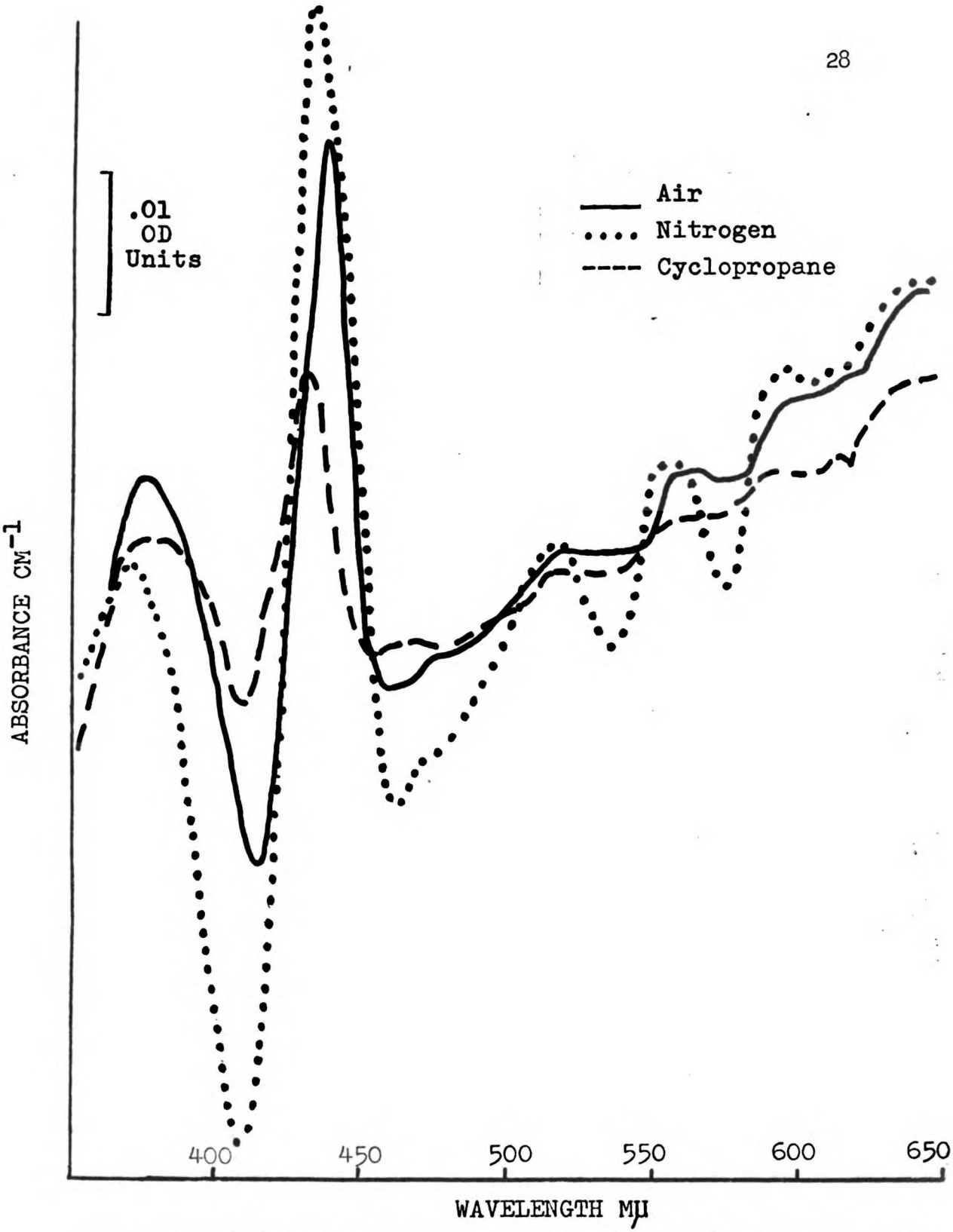


Fig. 5- Difference Spectra of Rat Liver Mitochondria at atmospheric conditions and in the presence of gases. Readings taken at 15 minutes with succinate as substrate.

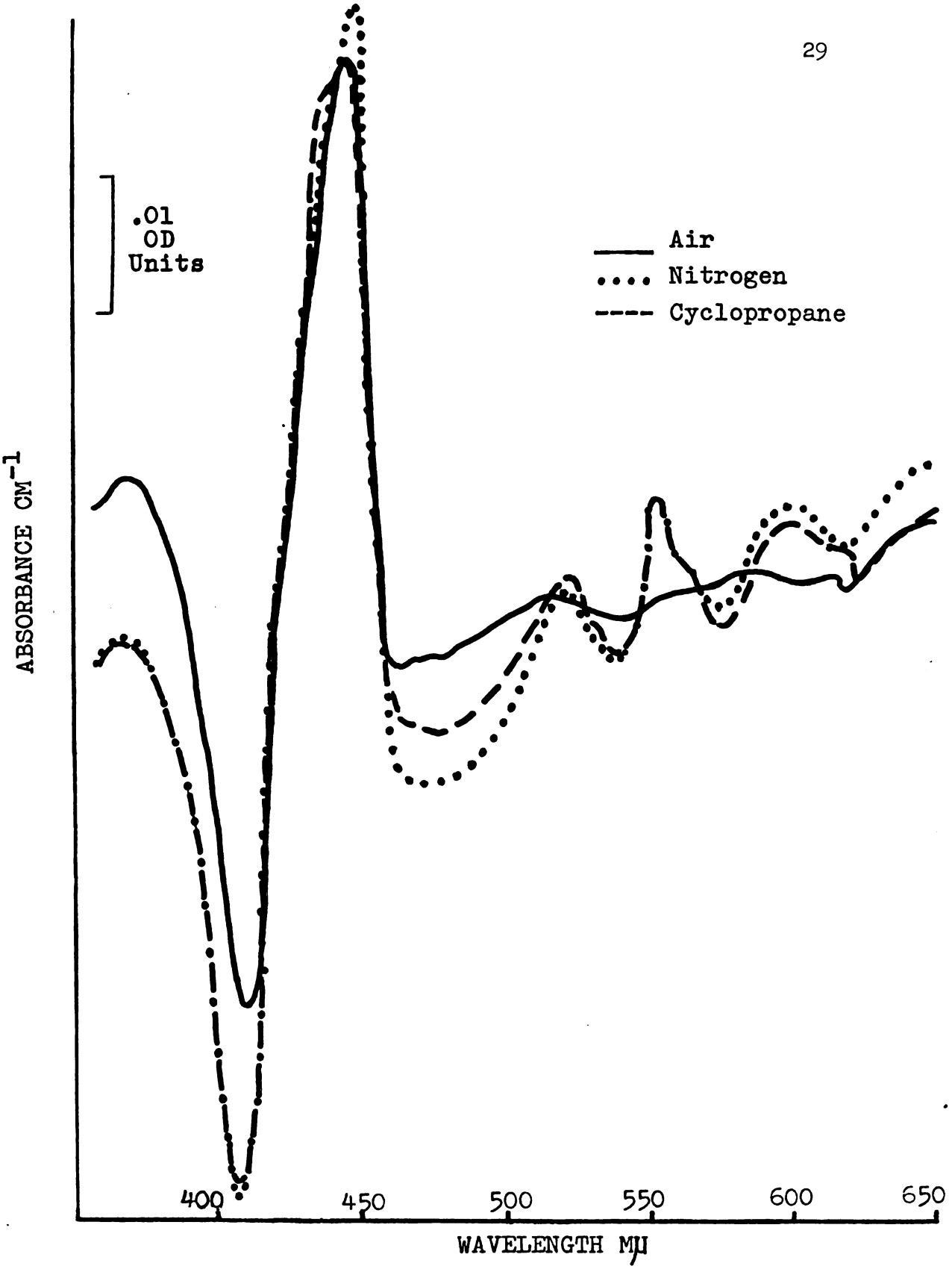


Fig. 6- Difference Spectra of Rat Brain Mitochondria at atmospheric conditions and in the presence of gases. Readings taken at 15 minutes with succinate as substrate.

The use of other substrates such as β-hydroxybutyrate, pyruvate or malate, did not give large difference spectra as with succinate (Figures 7 and 8). With β-hydroxybutyrate (Figure 7), the spectra, although qualitatively similar, showed smaller peak height for both liver and brain mitochondria. Perhaps in these conditions some factor is required for optimum activity. For example, the respiration rate with pyruvate as the only substrate is very low, because oxaloacetate is required with several mitochondrial preparations for pyruvate to enter the TCA cycle, and the low respiration may be attributed to a lack of oxaloacetate (2).

B. Properties of the "Particulate" Succinic Dehydrogenase (SDH)

Succinic dehydrogenase is routinely used as a mitochondrial "marker." Enzymatic experimental conditions were established, studying the SDH activity at different temperatures, and also in the presence of different substrate concentrations in rat liver and brain mitochondria.

All the measurements were done at 25° C in the presence of cyanide to block the last step in the respiratory chain (cytochrome oxidase), and avoid reoxidation of the chain components. The mitochondrial protein concentration provides an index of the optimal amount of SDH for the assay. The protein concentration for the SDH assay usually ranged between 100-200 µg protein/ml for liver and 200-400 µg protein/ml for brain mitochondria.

Substrate dependence--A concentration of 2.5 mM succinate gave optimal (or close to optimal) enzyme activity in both preparations (see item C). At higher substrate concentrations (greater than 5 mM), substrate inhibition occurred.

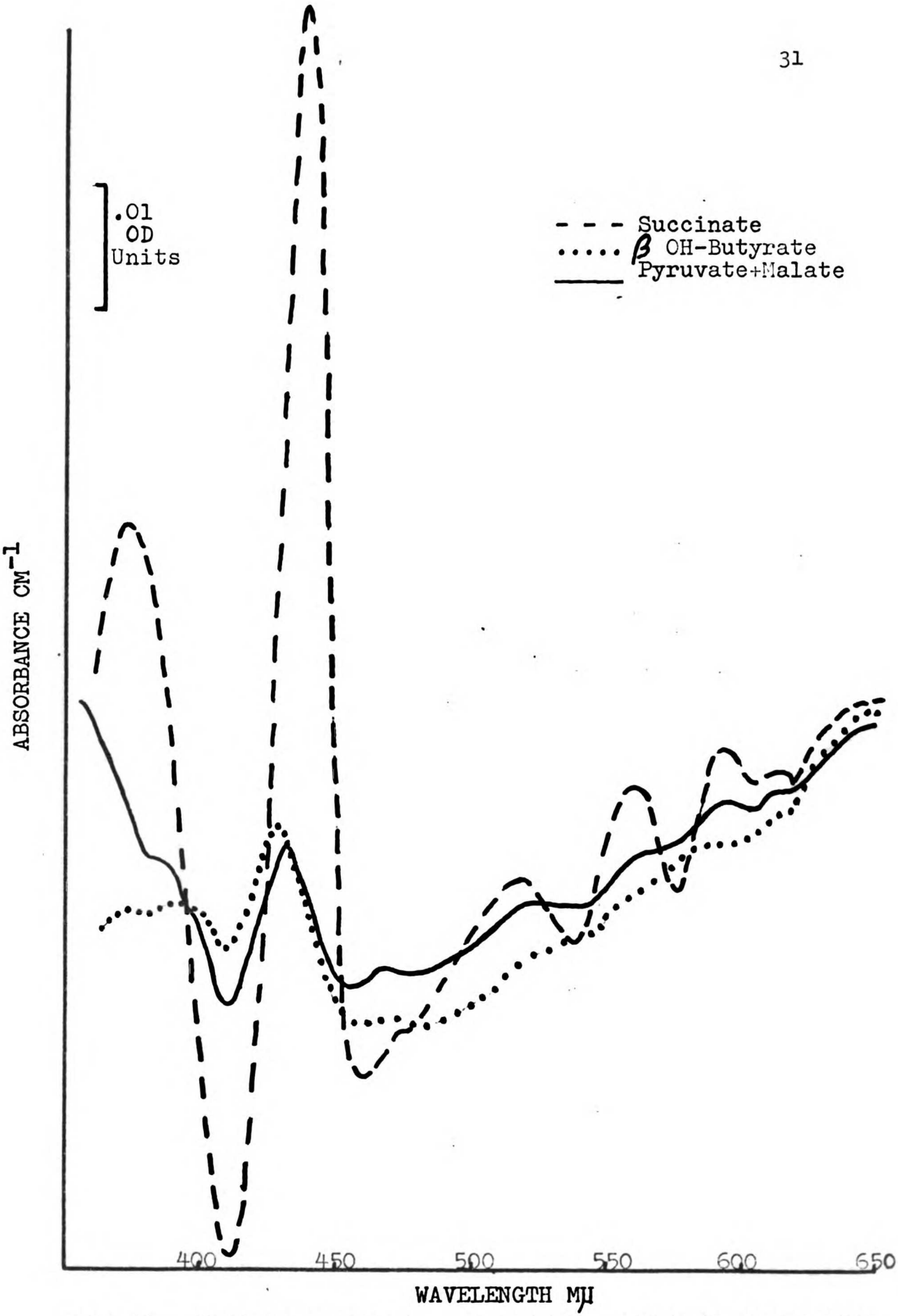


Fig. 7- Difference Spectra of Rat Liver Mitochondria with different substrates. Readings taken at 15 minutes.

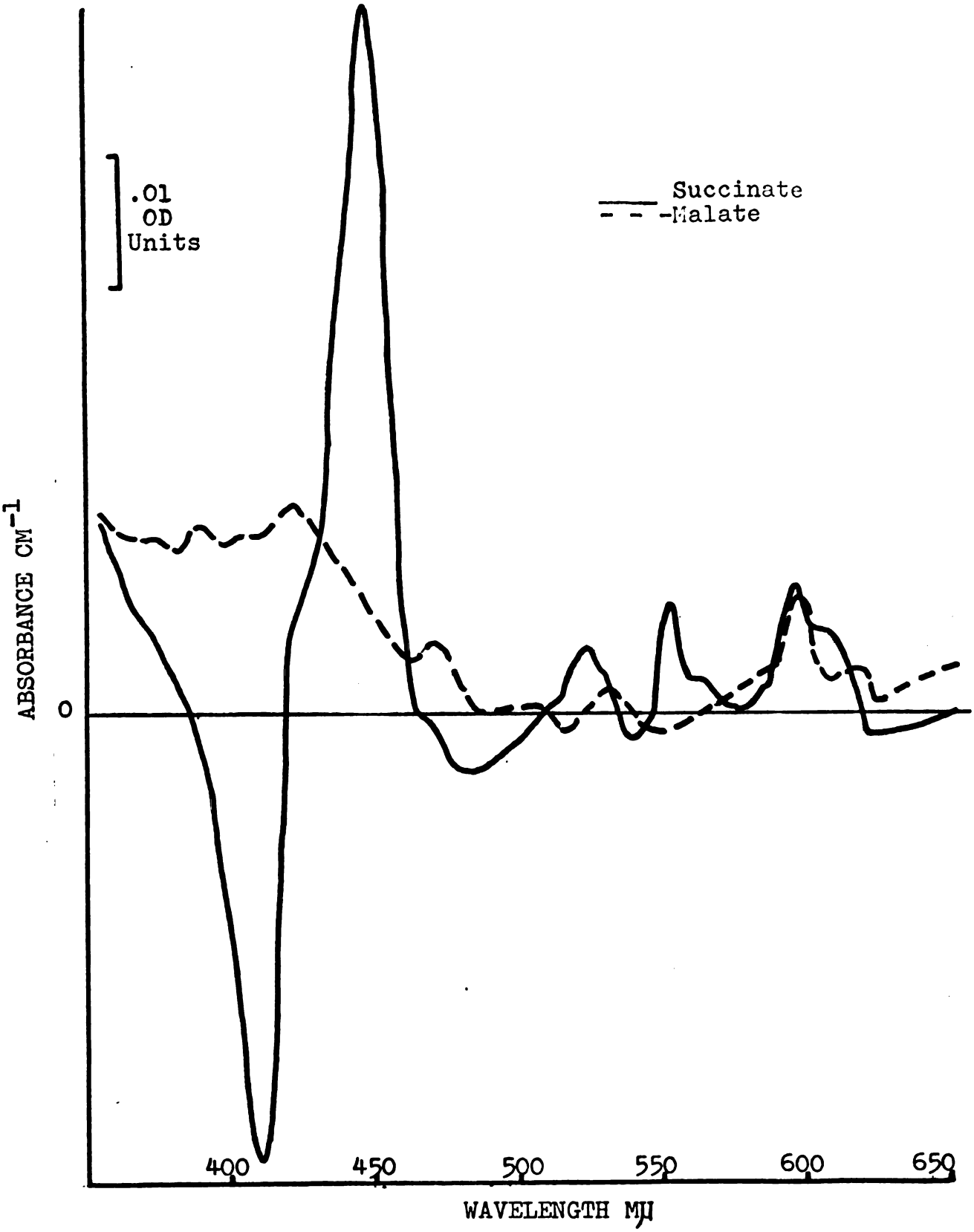


Fig. 8- Difference Spectra of Rat Brain Mitochondria with succinate and malate as substrates. Readings taken at 30 minutes.

Temperature dependence--Changes in SDH activity were examined at constant substrate concentration and at different temperatures, varying from 15° C to 35° C. The enzyme activity was linear for about 15 minutes at 25° C and 35° C and for 30 minutes at 15° C with liver (Figure 9), and up to 10 minutes with the brain enzyme in the range of temperatures used (Figure 10).

The enzymatic rate usually increased with increasing temperatures, as one would expect, with both preparations. However, the increase in rate between 25° C and 35° C was somewhat less with the liver enzyme.

C. Effect of Gases on SDH Activity at Various Substrate Concentrations

The effect of varying succinate concentration on the enzyme activity of the liver and brain enzyme at atmospheric pressure, in the presence of air (control) and in the presence of cyclopropane and nitrogen, is presented in Figures 11 and 12.

The results indicated that a concentration of 2.5 mM succinate gives optimal SDH activity and also maximal stimulation of enzyme activity of approximately 20% for liver and of 30% for brain mitochondria.

Reciprocal Lineweaver-Burk plots of the SDH activity and substrate concentrations are shown in Figures 13 and 14.

It must be realized that the reciprocal plot is usually used for inhibition type studies. However, it does not mean that this plot may not be used to interpret data that indicate activation or stimulation of enzyme activity (45).

In the brain mitochondria preparation, cyclopropane stimulated SDH activity over the range of substrate concentration used (Figure 13).

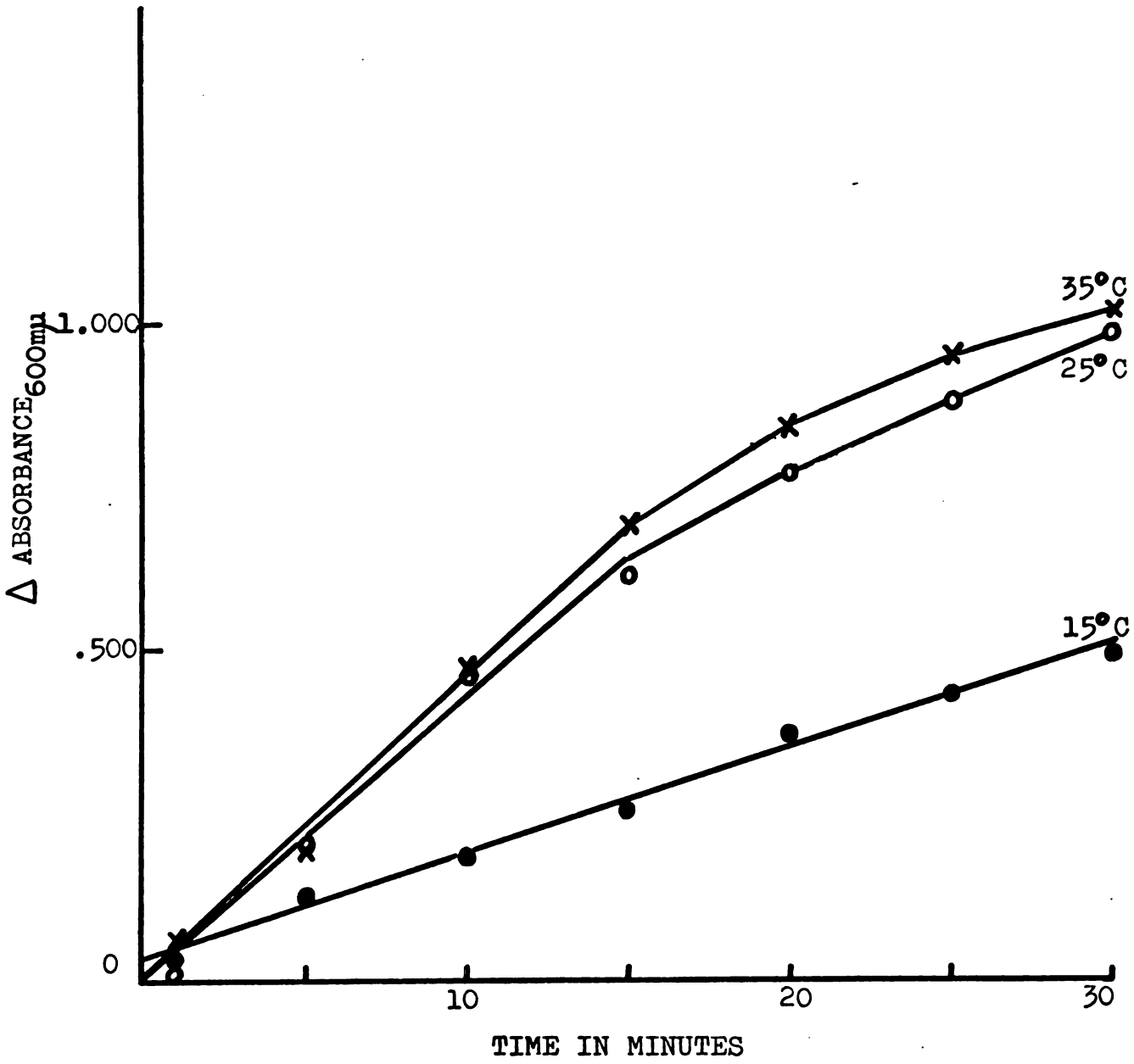


Fig. 9- Rat Liver Mitochondria: SDH activity as a function of time and temperature

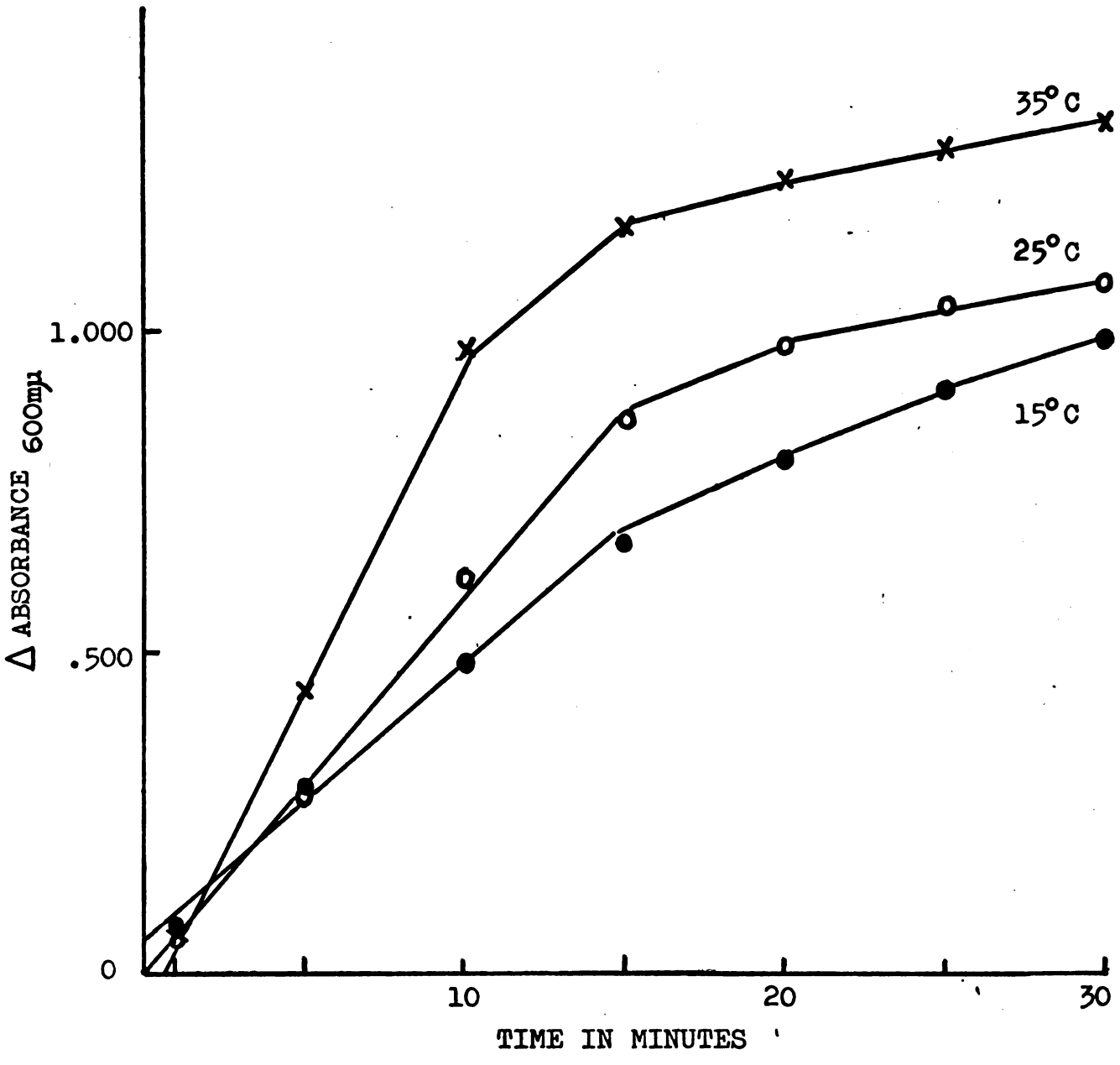


Fig. 10- Rat Brain Mitochondria: SDH activity as a function of time and temperature

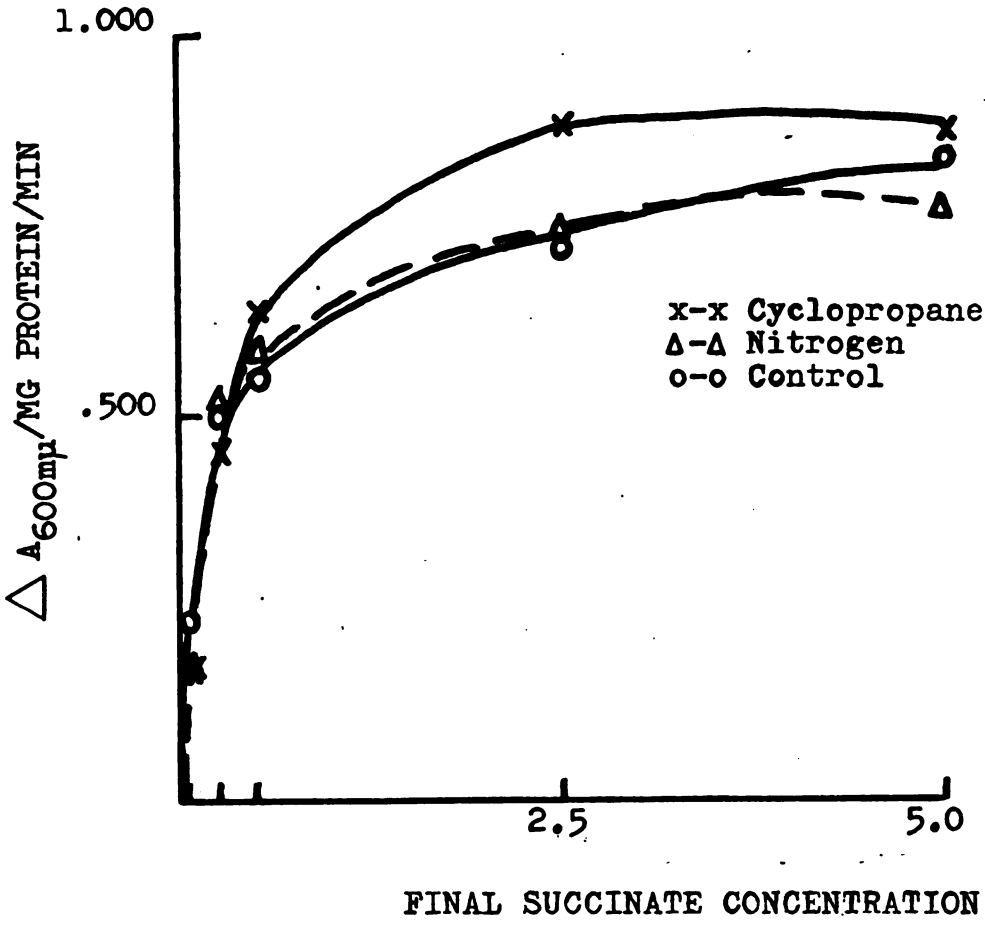


Fig. 11- Liver Mitochondria: Influence of succinate concentration on the cyclopropane and nitrogen effects on the enzyme activity at 25°C. Averages of at least three experiments.

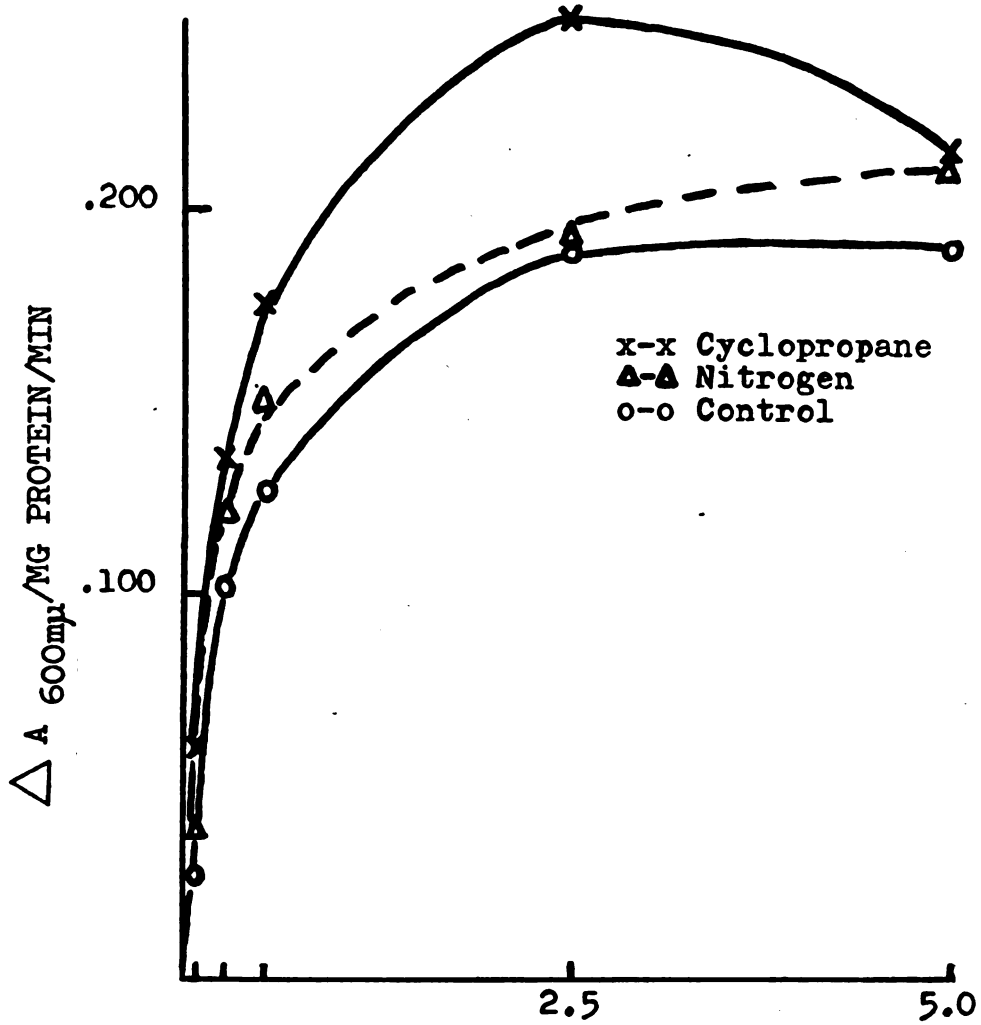


Fig. 12- Brain Mitochondria: Influence of succinate concentration on the cyclopropane and nitrogen effects on the enzyme activity at 25°C. Averages of at least three experiments.

In liver mitochondria (Figure 14), cyclopropane stimulated the enzyme activity at greater substrate concentration (2.5 mM) and caused an inhibition type effect with lower concentration of succinate (0.25 mM).

Nitrogen in both preparations had a stimulatory effect on enzyme activity, though smaller than that produced by cyclopropane.

Aging of the preparation altered the effect of cyclopropane on SDH activity. This is summarized for both liver and brain mitochondria in Tables 3 and 4. In the liver preparation (Table 3), age decreased the intensity of the stimulatory effect (% of change) of cyclopropane. In the brain (Table 4) the age of the preparation did not change significantly the relatively stimulatory gas effect. Nitrogen effects in both situations were not greatly age-dependent.

The effects of cyclopropane and nitrogen were examined on rat brain and liver mitochondria at atmospheric pressure and at different substrate concentrations, as percentage of change of enzyme activity. In rat liver mitochondria (Table 5), cyclopropane increased SDH activity (stimulatory effect) in concentrations equal to or greater than 2.5 mM succinate, presenting an inhibitory effect with lower substrate concentrations (0.05 mM). On the other hand, cyclopropane in brain mitochondria caused a significant stimulation of enzyme activity throughout the experimental range of different substrate concentrations utilized (Table 6). The percentage of change was approximately the same between 0.05-2.5 mM succinate, the stimulatory effect decreasing with higher substrate concentrations (5 mM). Perhaps substrate inhibition that appeared to happen with concentrations equal to or greater than 5 mM succinate could be responsible, at least in part, for the same phenomenon that also occurred with the liver enzyme.

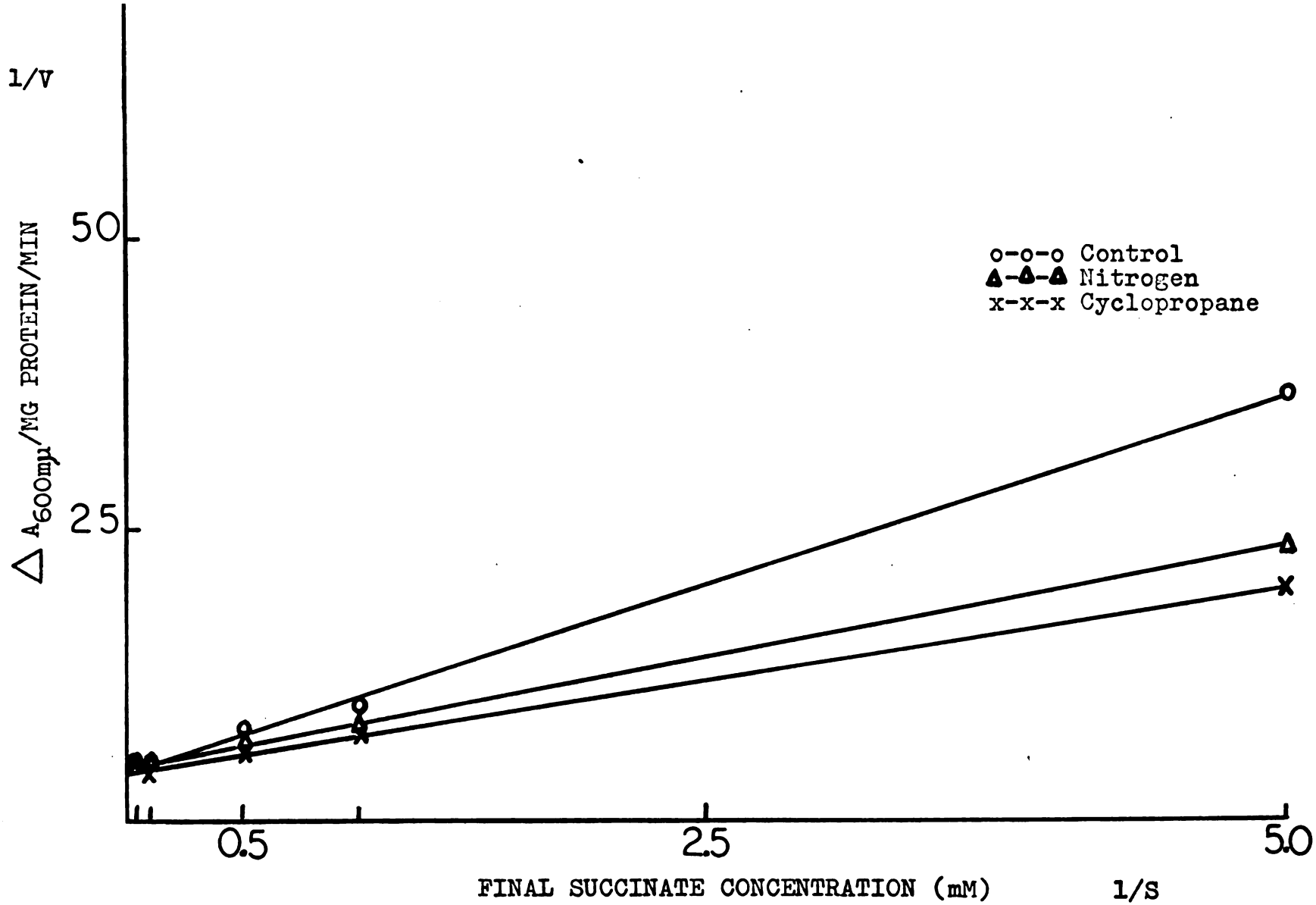


Fig. 13- Brain Mitochondria
Lineweaver-Burk Plot: reciprocal plot of changes in enzyme activity (1/V) at various substrate concentrations (1/S) in the control atmospheric conditions and in the presence of nitrogen and cyclopropane

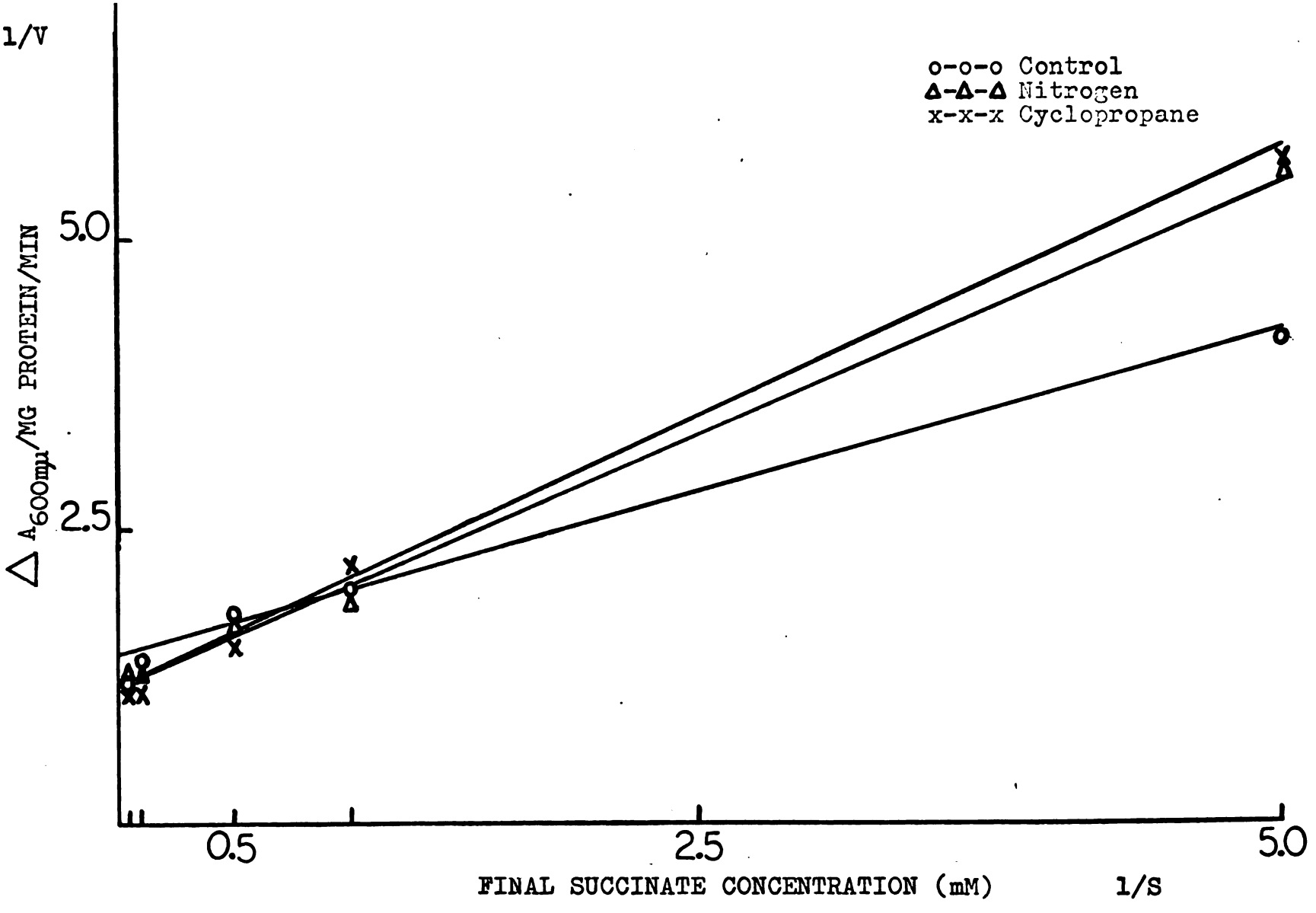


Fig. 14- Liver Mitochondria
Lineweaver-Burk Plot: reciprocal plot of changes in enzyme activity (1/V) at various substrate concentrations (1/S) in the control atmospheric conditions and in the presence of nitrogen and cyclopropane

TABLE 3 - INFLUENCE OF AGING ON CYCLOPROPANE AND NITROGEN EFFECTS ON SDH ACTIVITY AT ONE SUBSTRATE CONCENTRATION* AND AT ATMOSPHERIC PRESSURE IN RAT LIVER MITOCHONDRIA

Preparation Age	$\Delta A_{600m\mu}$/mg protein/min Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
Fresh	0.156±0.014(12)	0.212±0.008(12)	↑35.9	0.190±0.014(8)	↑21.8
Aged (24 hrs)	0.175±0.014(16)	0.225±0.013(16)	↑28.6	0.214±0.013(13)	↑22.3

*Substrate concentration = 2.5 mM succinate. The values are means ± S.E.M. The figures within parentheses represent number of experiments.

TABLE 4 - INFLUENCE OF AGING ON CYCLOPROPANE AND NITROGEN EFFECTS ON SDH ACTIVITY AT ONE SUBSTRATE CONCENTRATION* AND AT ATMOSPHERIC PRESSURE IN RAT BRAIN MITOCHONDRIA

Preparation Age	$\Delta A_{600m\mu}$/mg protein/min Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
Fresh	0.052±0.005(4)	0.067±0.006(4)	↑28.8	0.054±0.003(4)	↑3.8
Aged (24 hrs)	0.056±0.004(8)	0.073±0.006(8)	↑30.4	0.061±0.005(8)	↑8.9

*Substrate concentration = 2.5 mM succinate. The values are means ± S.E.M. The figures within parentheses represent number of experiments.

TABLE 5 - INFLUENCE OF SUCCINATE CONCENTRATION ON CYCLOPROPANE AND NITROGEN EFFECTS ON SDH ACTIVITY IN RAT LIVER MITOCHONDRIA

Succinate mM	Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
	$\Delta A_{600m\mu}$/mg protein/min				
0.05	0.238(8)	0.175(9)	↓26.5	0.177(7)	↓25.6
0.25	0.503(13)	0.458(11)	↓ 8.9	0.521(6)	↑ 3.6
0.50	0.550(7)	0.664(10)	↑20.7	0.579(6)	↑ 5.3
2.50	0.725(17)	0.881(17)	↑21.5	0.741(10)	↑ 2.2
5.00	0.839(9)	0.870(5)	↑ 3.7	0.775(3)	↓ 7.6

The values are averages. The figures within parentheses represent number of experiments. For statistical details see analysis of variance (Table 9).

TABLE 6 - INFLUENCE OF SUCCINATE CONCENTRATION ON CYCLOPROPANE AND NITROGEN EFFECTS ON SDH ACTIVITY IN RAT BRAIN MITOCHONDRIA

Succinate mM	Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
	$\Delta A_{600m\mu}$/mg protein/min				
0.05	0.027(4)	0.050(4)	↑85.0	0.041(5)	↑51.9
0.25	0.103(8)	0.136(7)	↑32.0	0.122(7)	↑18.4
0.50	0.128(9)	0.177(6)	↑38.3	0.148(8)	↑17.2
2.50	0.188(13)	0.247(13)	↑31.4	0.192(13)	↑ 2.1
5.00	0.191(5)	0.215(4)	↑12.6	0.210(4)	↑ 9.9

The values are averages. The figures within parentheses represent number of experiments. For statistical details see analysis of variance (Tables 8A and 8B).

Nitrogen was introduced as a secondary control and as shown (Tables 5 and 6) it also caused slight changes in SDH activity. Table 7 presents the effects of cyclopropane and nitrogen on SDH activity in rat brain and liver mitochondria at constant (2.5 mM) substrate concentration. In this set of conditions, the cyclopropane stimulatory effect was greater with the brain enzyme (30% stimulation) than with the enzyme from liver (20% stimulation). Nitrogen effects in both preparations were statistically insignificant.

The analysis of variance showed a statistically significant difference, at the 95% confidence level, between the effects of gases in brain mitochondria (Table 8A). The Scheffé Test (Table 8B) detected a significant contrast ($\alpha = 0.05$) between cyclopropane and the control.

There was no statistically significant difference between the effects of gases in the liver enzyme at the same confidence level (Table 9). This fact could be partially explained by the enzyme aging effect on the cyclopropane activity in the liver preparation (see Tables 3 and 4).

D. Effect of Varying Cyclopropane and Nitrogen Pressures on Enzyme Activity

Effects of different gas concentrations on enzyme activity were examined at different pressure conditions ranging from 0.063 atm. pressure to 1 atm.pressure and at one substrate concentration (2.5 mM succinate).

In liver mitochondria (Table 10), the stimulatory effect of cyclopropane, with one exception (0.75 atm. press. not statistically different), did not change very much over the range of pressure used

TABLE 7 - EFFECTS OF CYCLOPROPANE AND NITROGEN ON SDH ACTIVITY IN RAT LIVER AND BRAIN MITOCHONDRIA

Gas Phase	$\Delta A_{600m\mu}$/mg protein/min Liver	% Change	Brain	% Change
Air (Control)	0.725±0.047(17)		0.188±0.017(13)	
Cyclopropane	0.881±0.035(17)	↑21.5	0.247±0.026(13)	↑31.4
Nitrogen	0.741±0.078(10)	↑ 2.2	0.192±0.016(13)	↑ 2.1

The experiments were done at one substrate concentration (2.5 mM succinate) and at atmospheric pressure. The results are means ± S.E.M. The figures within parentheses represent number of experiments.

TABLE 8A - RAT BRAIN MITOCHONDRIA: ANALYSIS OF VARIANCE OF THE EFFECTS OF CYCLOPROPANE AND NITROGEN ON SDH ACTIVITY AT VARIOUS SUBSTRATE CONCENTRATIONS* AND AT ATMOSPHERIC PRESSURE

Source	Sum of Squares	D.F.	Mean Squares	F	F.95
Between concentrations	0.357215	4	0.089304	17.30	(3.65; 3.48)
Between gases	0.068098	2	0.034049	6.60	(4.98; 4.79
Interactions	0.009917	8	0.001240	0.24	(2.82; 2.66)
Error	0.490376	95	0.005162	--	--
"Total"	0.925607	109	--	--	--

*Substrate concentration = 0.05-5.00 mM succinate.

TABLE 8B - SCHEFFÉ TEST* ($\alpha = 0.01$)
CONTRAST AMONG GROUPS

Contrast	Confidence Limits	Result
Air-Cyclopropane	0.036 ± 0.023	Significant
Air-Nitrogen	0.014 ± 0.051	Not significant
Cyclopropane-Nitrogen	0.045 ± 0.053	Not significant

*Test performed with the data used in the Analysis of Variance shown above.

TABLE 9 - RAT LIVER MITOCHONDRIA: ANALYSIS OF VARIANCE OF THE EFFECTS OF CYCLOPROPANE AND NITROGEN ON SDH ACTIVITY AT VARIOUS SUBSTRATE CONCENTRATIONS* AND AT ATMOSPHERIC PRESSURE

Source	Sum of Squares	D.F.	Mean Squares	F.	F.95
Between concentrations	6.325048	4	1.581262	48.61	(2.45; 2.37)
Between gases	0.053215	2	0.026607	0.82	(3.07; 3.00)
Interactions	0.304856	8	0.038107	1.17	(2.02; 1.94)
Error	4.000930	123	0.032528	--	--
"Total"	10.684049	137	--	--	--

*Substrate concentration = 0.05-5.00 mM succinate.

(2.5-1 atm. press.). The maximum effect (30% stimulation) was already reached around 1/4 of atmosphere and so, close to the pharmacological concentration range.

With the brain enzyme (Table 11), the maximum percentage of change (30% stimulation) was reached at 1 atm. pressure.

Nitrogen again, in both cases, was introduced as a secondary control. It caused a slight stimulant effect (less than 10%) in brain mitochondria. The effect was somewhat greater (about 20% stimulation) with the liver enzyme. even though always smaller but not statistically different from that presented by cyclopropane.

Perhaps nitrogen effects were more pronounced here than in the previous conditions (different substrate concentrations and same pressure), due to the better technically controlled pressured conditions used and also to the predominance of fresh enzyme preparations.

The analysis of variance (Table 12A) showed statistically significant difference between the effects of the gases on rat brain mitochondria and no difference between atmospheric pressure, at a 95% confidence level. The Scheffé test applied detected a significant contrast between cyclopropane and the control (Table 12B).

In the liver preparation, however (Tables 13A and 13B), cyclopropane and nitrogen were significantly different from the control population, both causing stimulation of enzyme activity, even though cyclopropane had always a greater effect. Again, no difference was detected between pressures tested.

Future experiments will be done using different pressure conditions and different substrate concentrations.

TABLE 10 - RAT LIVER MITOCHONDRIA: EFFECT OF PRESSURE ON SDH ACTIVITY AT ONE SUBSTRATE CONCENTRATION

Pressure (Atm.)	Δ A560-600mμ/mg protein/min Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
0.25	0.169±0.018(12)	0.227±0.018(12)	↑34.3	0.204±0.017(8)	↑20.7
0.50	0.186±0.013(15)	0.231±0.012(15)	↑24.2	0.218±0.013(13)	↑17.2
0.75	0.158±0.020(9)	0.264±0.010(11)	↑67.1	0.188±0.024(8)	↑18.8
1.00	0.175±0.014(16)	0.225±0.013(16)	↑28.6	0.214±0.013(13)	↑22.3

The cuvette contained: Phosphate Buffer, pH 7.4, 0.1 M, mitochondria suspension (100-400 μg protein), 10 μmoles cyanide, 0.114 μmoles DCPIP and 5 μmoles succinate in a final volume of 2 ml. The reaction was followed spectrophotometrically in a dual mode operation with λ_1 = 560 mμ and λ_2 = 600 mμ. The values are means ± S.E.M. The figures within parentheses represent number of experiments.

TABLE 11 - RAT BRAIN MITOCHONDRIA: EFFECT OF PRESSURE ON SDH ACTIVITY AT ONE SUBSTRATE CONCENTRATION

Pressure (Atm)	Δ A560-600mμ/mg protein/min Air (Control)	Cyclopropane	% Change	Nitrogen	% Change
0.063	0.064±0.008(4)	0.072±0.007(4)	↑12.5	0.069±0.005(4)	↑7.8
0.125	0.062±0.009(4)	0.067±0.010(4)	↑ 8.1	0.065±0.012(4)	↑4.8
0.250	0.049±0.004(5)	0.054±0.006(5)	↑10.2	0.053±0.002(5)	↑8.2
0.500	0.056±0.005(7)	0.068±0.005(7)	↑21.4	0.060±0.006(7)	↑7.1
0.750	0.058±0.004(4)	0.065±0.003(5)	↑12.1	0.057±0.003(4)	↓1.7
1.000	0.056±0.004(8)	0.073±0.006(8)	↑30.4	0.061±0.005(8)	↑8.9

The cuvette contained: Phosphate Buffer, pH 7.4, 0.1 M, mitochondria suspension (100-400 μg protein), 10 μmoles cyanide, 0.114 μmoles DCPIP and 5 μmoles succinate in a final volume of 2 ml. The reaction was followed spectrophotometrically in a dual mode operation with λ_1 = 560 mμ and λ_2 = 600 mμ. The values are means ± S.E.M. The figures within parentheses represent number of experiments.

TABLE 12A - RAT BRAIN MITOCHONDRIA: ANALYSIS OF VARIANCE OF THE EFFECTS OF CYCLOPROPANE AND NITROGEN ON SDH ACTIVITY ON ONE SUBSTRATE CONCENTRATION* AND AT DIFFERENT PRESSURES

Source	Sum of Squares	D.F.	Mean Squares	F	F.95
Between Atm. Press.	0.002199	5	0.000440	2.279	(2.37; 2.29)
Between gases	0.001743	2	0.000872	4.515	(3.15; 3.07)
Interactions	0.000428	10	0.000043	0.222	(1.99; 1.91)
Error	0.015829	82	0.000193	--	--
"Total"	0.020200	99	--	--	--

*Substrate concentration = 2.5 mM succinate.

TABLE 12B - SCHEFFÉ TEST* ($\alpha = 0.05$)
CONTRAST AMONG GROUPS

Contrast	Confidence Limits	Result
Air-Cyclopropane	0.010 ± 0.008	Significant
Air-Nitrogen	0.003 ± 0.009	Not Significant
Cyclopropane-Nitrogen	0.007 ± 0.008	Not Significant

*Test performed with the data used in the Analysis of Variance shown above.

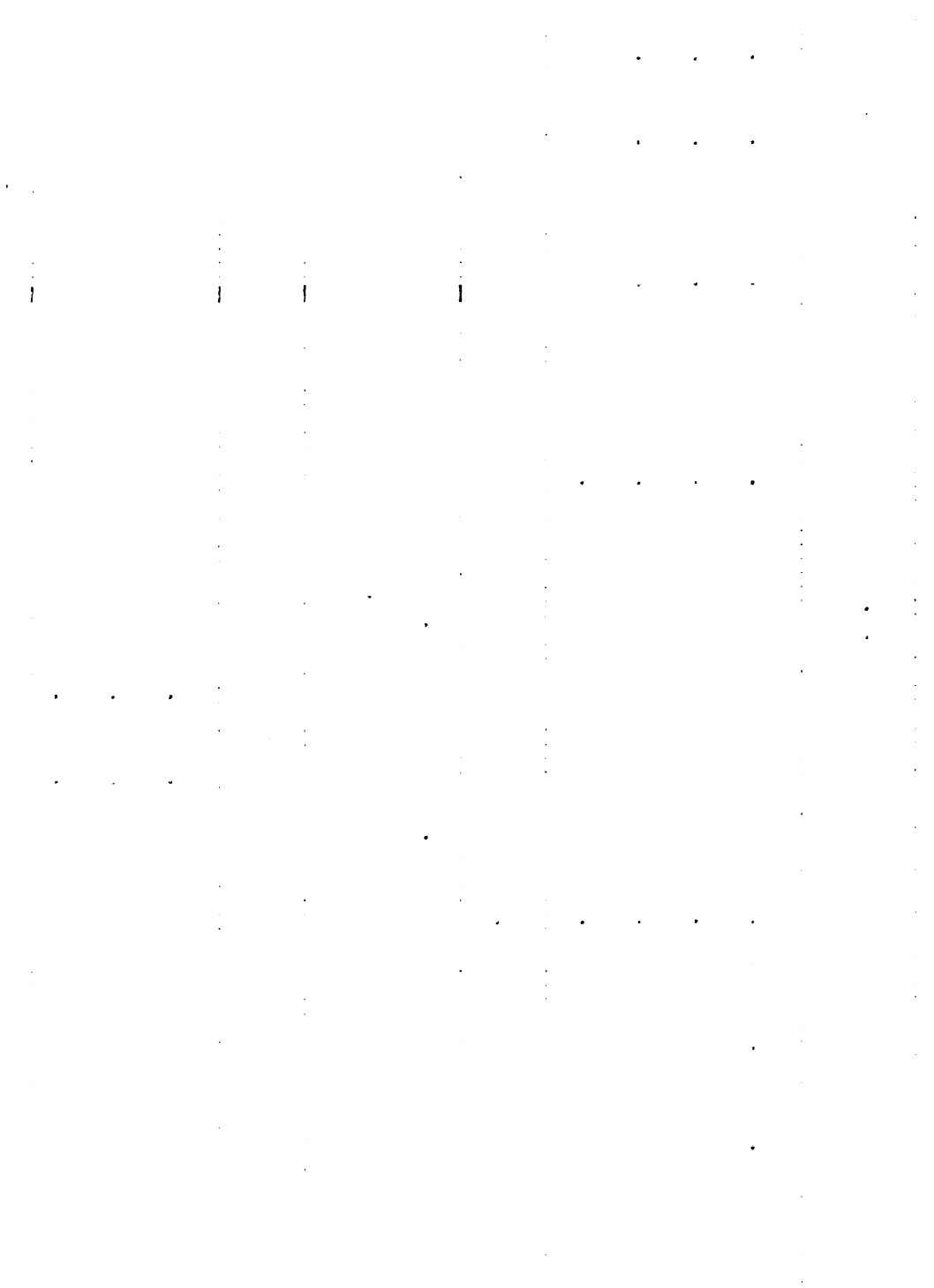


TABLE 13A - RAT LIVER MITOCHONDRIA: ANALYSIS OF VARIANCE OF THE EFFECTS OF CYCLOPROPANE AND NITROGEN ON SDH ACTIVITY AT ONE SUBSTRATE CONCENTRATION* AND AT DIFFERENT PRESSURES

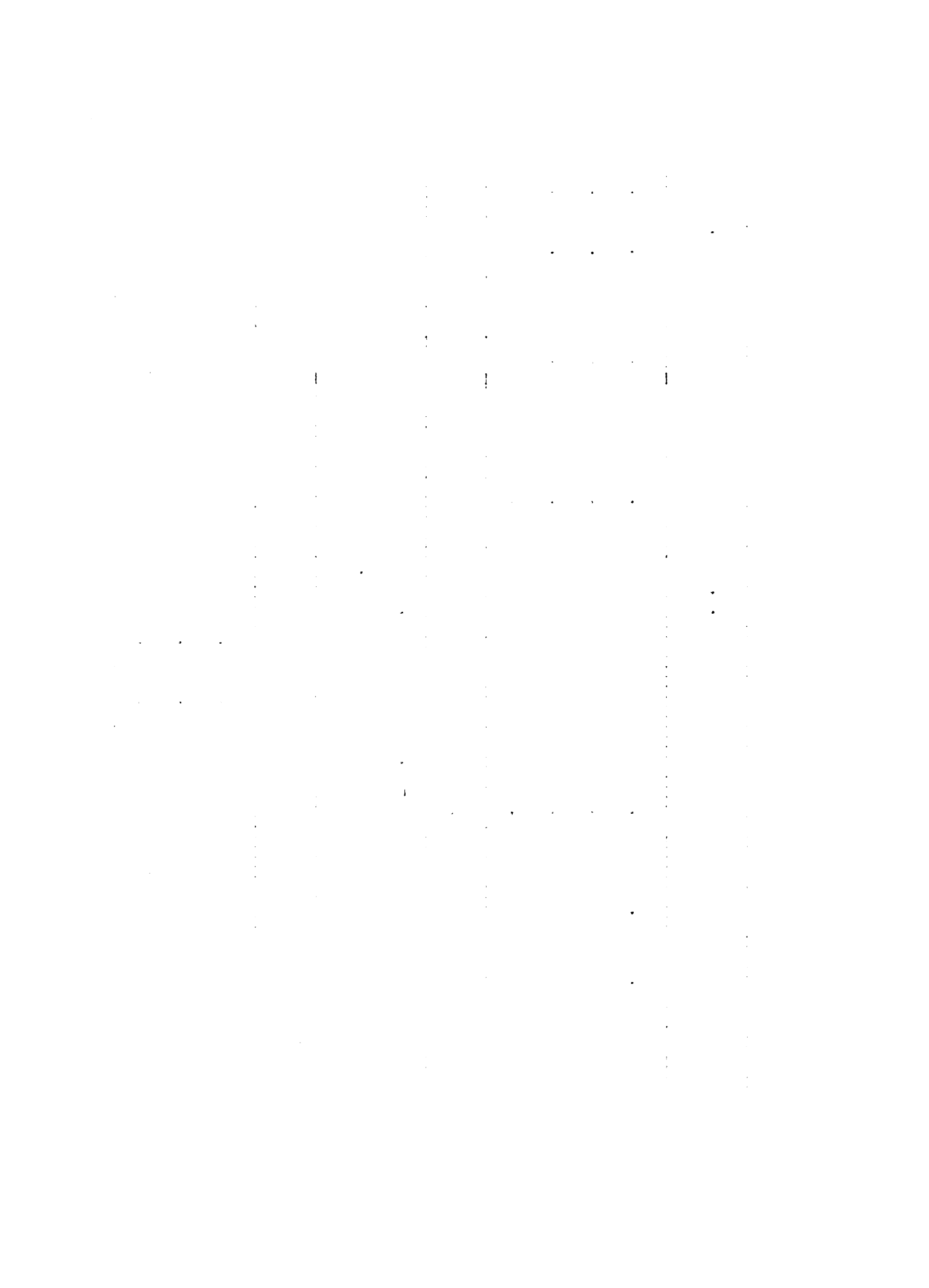
Source	Sum of Squares	D.F.	Mean Squares	F	F.95
Between Atm. Press.	0.002539	3	0.000846	0.331	(2.68; 2.60)
Between gases	0.086952	2	0.043476	17.023	(3.07; 3.00)
Interactions	0.009520	6	0.001587	0.621	(2.17; 2.10)
Error	0.349886	137	0.002554	--	--
"Total"	0.448896	148	--	--	--

*Substrate concentration = 2.5 mM succinate.

TABLE 13B - SCHEFFÉ TEST* (α = 0.05)
CONTRAST AMONG GROUPS

Contrast	Confidence Limits	Result
Air-Cyclopropane	0.057±0.024	Significant
Air-Nitrogen	0.038±0.026	Significant
Cyclopropane-Nitrogen	0.021±0.026	Not Significant

*Test performed with the data used in the Analysis of Variance shown above.



It would be interesting to see if higher pressures (up to 1 atm.) will increase the cyclopropane effects in rat brain and will maintain the observed gas effects without alteration in liver mitochondria.

The control was air using the same pressure conditions. The effect of vacuum was also examined and found to be close to the control.

E. Reversibility of the Enzyme System after Cyclopropane Effect

The reversibility experiment was done by submitting the reaction media, enzyme and substrate to the gas after evacuation. The reaction velocity started in the presence of the gas was measured for about 5 minutes. The cuvette was degassed and the reaction velocity measured again for 5 minutes in the absence of cyclopropane.

Exposure to cyclopropane at 25° C, at optimal substrate concentration and at atmospheric pressure had previously shown a stimulatory effect of approximately 30% on SDH activity in both liver and brain mitochondria.

However, in this single experiment the SDH activity ($\Delta A_{600m\mu}$/mg protein/min) was 0.077 and 0.935 for brain and liver mitochondria respectively, in the presence of cyclopropane, and 0.064 and 0.435 after degassing, and so approximately the same as the original control values (0.058 and 0.522).

Thus, the effect of cyclopropane on the enzyme activity in both preparations was reversible.

F. Normalization of Data

Due to the variability of SDH activity from one preparation to another, in part explained by the problems involved with enzymatic

assay, the percentual rate of the SDH activity obtained with the gas over the activity obtained with the control at the same conditions was calculated (ΔA600 mμ/mg protein/min (gas)/ΔA600 mμ/mg protein/min (control)). In rat liver mitochondria (Figure 15), cyclopropane showed an inhibition type effect with lower substrate concentrations, and caused enzyme stimulation with the higher ones. Nitrogen followed cyclopropane patterns, its effects, however, being closer to those presented by the control. In rat brain mitochondria (Figure 16), cyclopropane produced a stimulation of enzyme activity (with the exception of 5 mM succinate concentration), all over the range of substrate concentration used. Nitrogen values, except in the presence of very low substrate concentration (0.05 mM succinate), did not differ significantly from the control ones.

In order to avoid many of the problems involved with SDH assays, Bernath and Singer (5) recommend that the enzyme activity be determined at varying dye concentrations and extrapolated to Vmax with respect to the electron carrier. At fixed dye concentration an uncertain fraction of the activity is measured since the affinity of the dye for the enzyme may vary, depending on the treatment during isolation procedures.

G. Gas Effects on Oxidative Phosphorylation

Table 14 gives the representative values for oxidative phosphorylation as measured polarographically with rat liver and brain mitochondria in the presence of succinate and β-hydroxybutyrate as substrates. They are presented to show the behavior of the preparations concerning ADP:O ratio and QO_2, the figures representing means ± S.E.M.

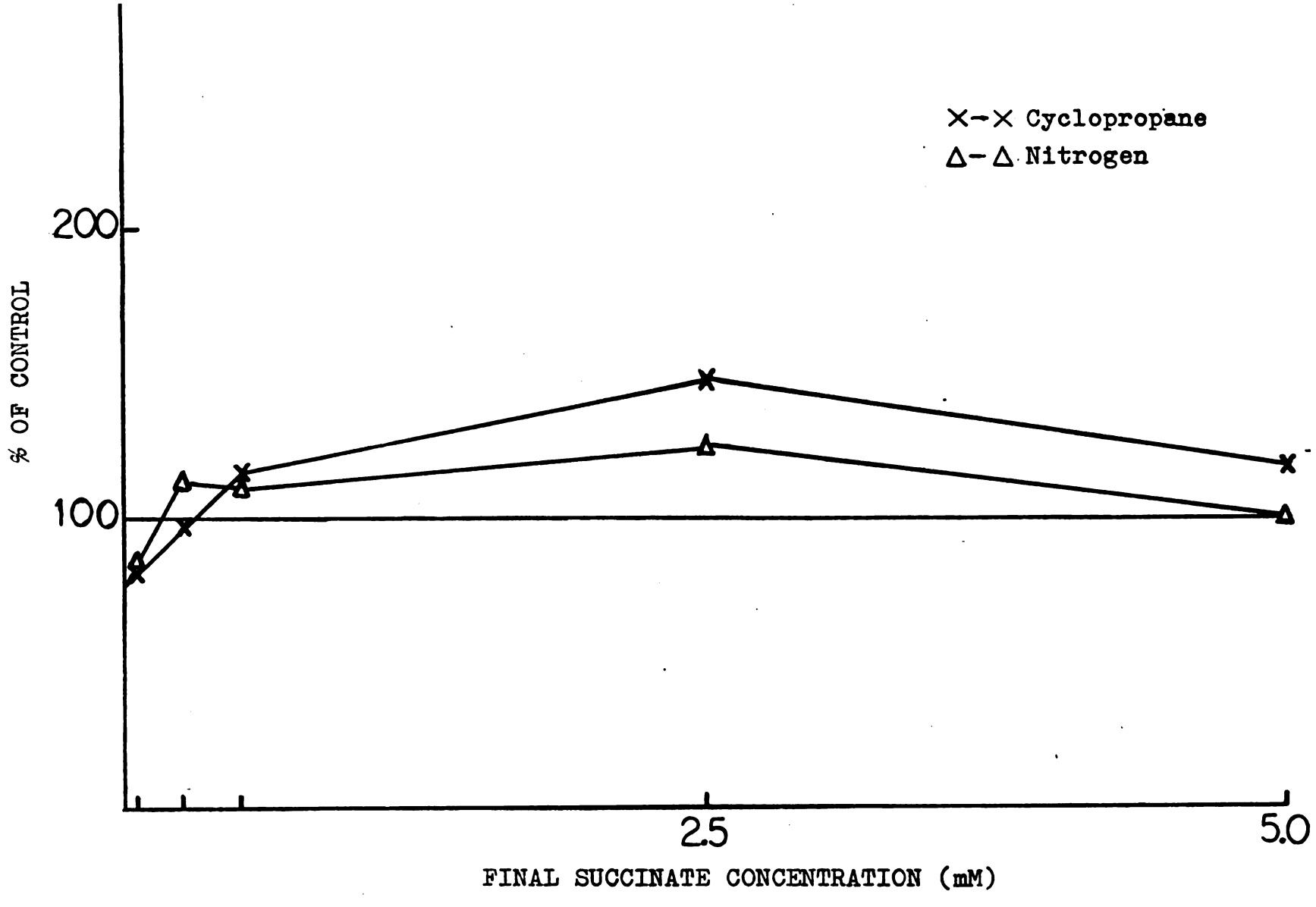


Fig. 15- Rat Liver Mitochondria: Normalization of Data
The straight line represent control levels (=100 per cent). The values are averages of at least three independent experiments.

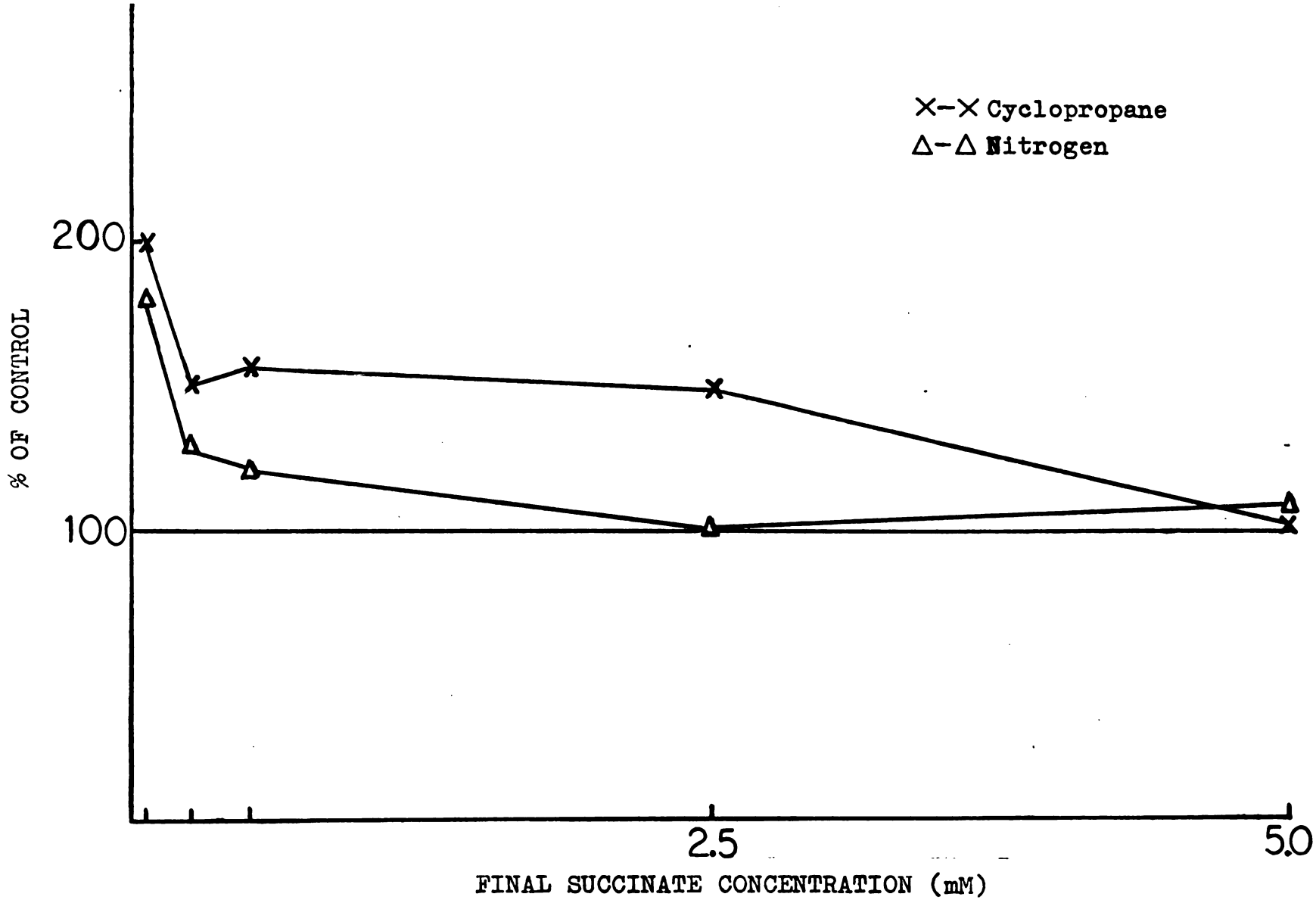


Fig. 16- Rat Brain Mitochondria: Normalization of Data
The straight line represent control levels (=100 per cent). The values are averages of at least three independent experiments.

TABLE 14 - REPRESENTATIVE VALUES FOR OXIDATIVE PHOSPHORYLATION AS MEASURED POLAROGRAPHICALLY WITH RAT LIVER AND BRAIN MITOCHONDRIA

Mitochondria Origin	Succinate		β-hydroxybutyrate	
	ADP:O	QO_2	ADP:O	QO_2
Liver	2.121±0.069(14)	115.143±9.669(14)	3.083±0.277(6)	52.583±7.578(6)
Brain	1.910±0.179(10)	57.040±7.914(10)	1.950±0.181(8)	49.913±3.648(8)

The values are means ± S.E.M. The figures within parentheses represent number of experiments.

The results presented with cyclopropane constitute preliminary studies done with different substrates. Future studies will involve the use of inhibitors to localize the site of anesthetic action in the respiratory chain.

Rat Liver Mitochondria--The results (Figures 17A and 17B) showed that in liver mitochondria cyclopropane did not change appreciably the parameters studied with succinate as substrate. There were, however, a slight increase in QO_2 and a decrease in ADP:O values compared to controls, which might be an indication of some uncoupling-like action of the drug. With β-hydroxybutyrate, cyclopropane appears to increase somehow the ADP:O ratio, with little effect on QO_2 values.

Rat Brain Mitochondria--In brain mitochondria (Figures 18A and 18B) cyclopropane did not change appreciably the ADP:O ratio with succinate and increased it with β-hydroxybutyrate as substrate. The QO_2 values were decreased with the two substrates used.

It is interesting to notice the increase in ADP:O ratio with β-hydroxybutyrate in the brain and liver preparations. This might be an indication of inhibition of substrate oxidation in both cases.

The inhibition caused by some anesthetic agents with NAD-linked substrates is a well documented fact (11, 35, 23).

Cyclopropane in both preparations was used in a gas mixture with oxygen (80% Cyclopropane-20% Oxygen). Nitrogen in the same set of conditions (80% Nitrogen-20% Oxygen) was introduced as a secondary control. It produces a slight effect on the parameters studied, compared to the control aerobic conditions, in both brain and liver mitochondria.

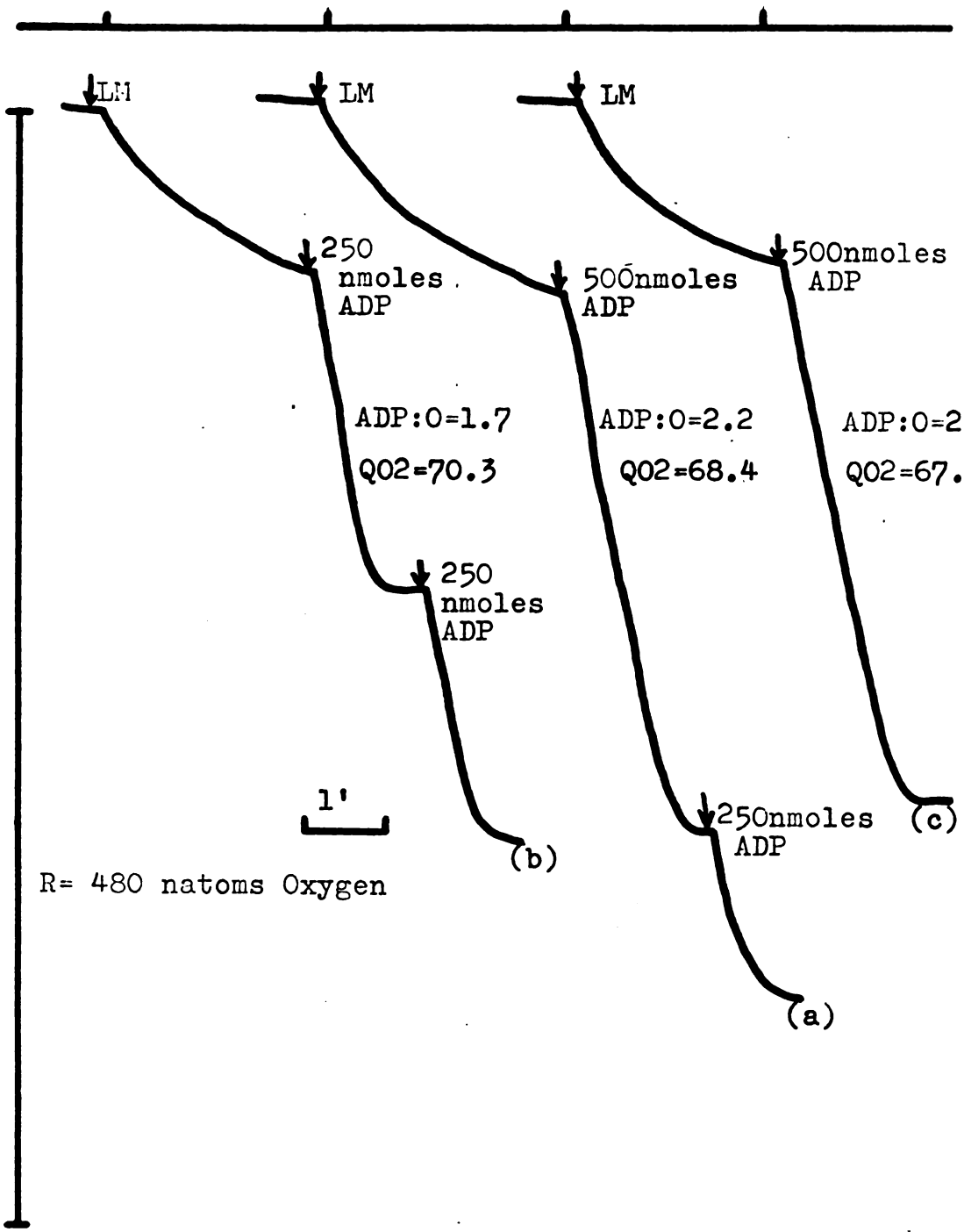


Fig. 17A- Polarographic trace of the oxidative phosphorylation of rat liver mitochondria with succinate as substrate in control conditions (a) and in the presence of cyclopropane (b) and nitrogen (c). The chamber contained: reaction media, mitochondria suspension (LM) correspondent to 2.3 mg protein and ADP added where indicated in a final volume of 1 ml. See "Methods".

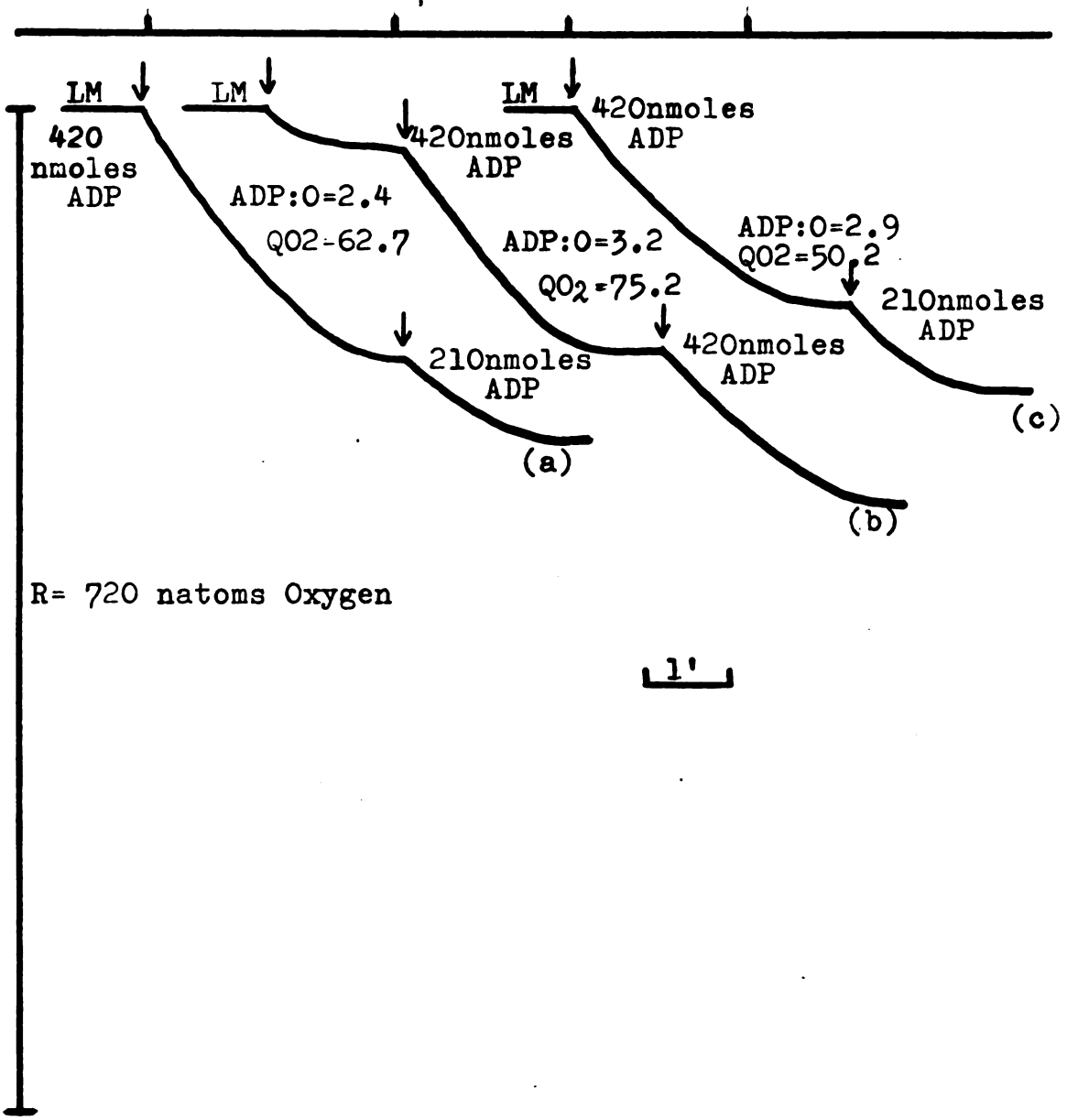


Fig. 17B- Polarographic trace of the oxidative phosphorylation of rat liver mitochondria with β-hydroxybutyrate in control conditions (a) and in the presence of cyclopropane (b) and nitrogen (c). The chamber contained: reaction media, mitochondria suspension (LM) correspondent to 1.22 mg protein and ADP added where indicated in a final volume of 1.5 ml. See "Methods" for details.

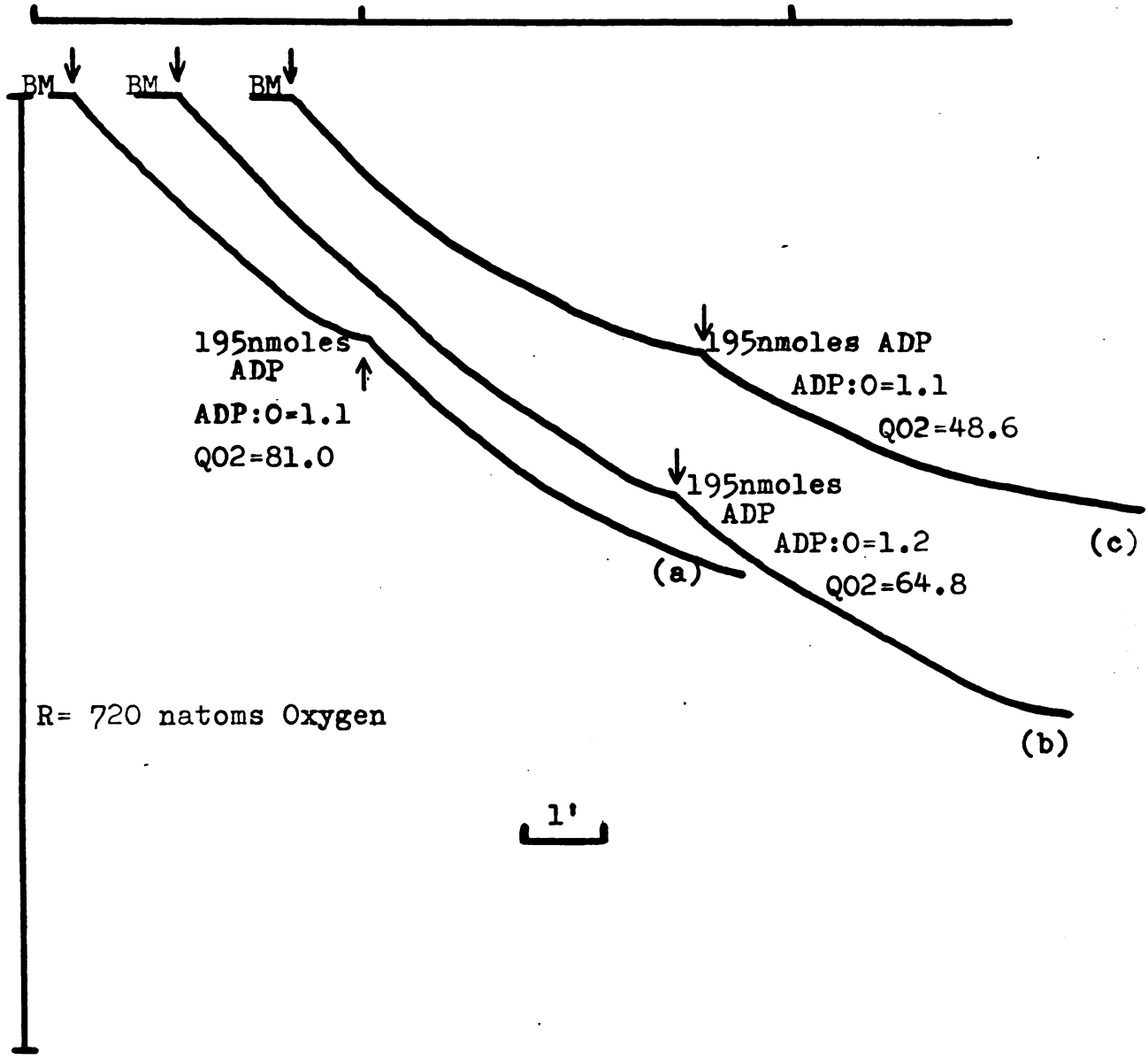


Fig. 18A- Polarographic trace of the oxidative phosphorylation of rat brain mitochondria in control conditions (a) and in the presence of cyclopropane (b) and nitrogen (c) with succinate. The chamber contained: reaction media, mitochondria suspension (BM) correspondent to 0.63 mg protein and ADP added where indicated in a final volume of 1.5 ml. See "Methods" for details.

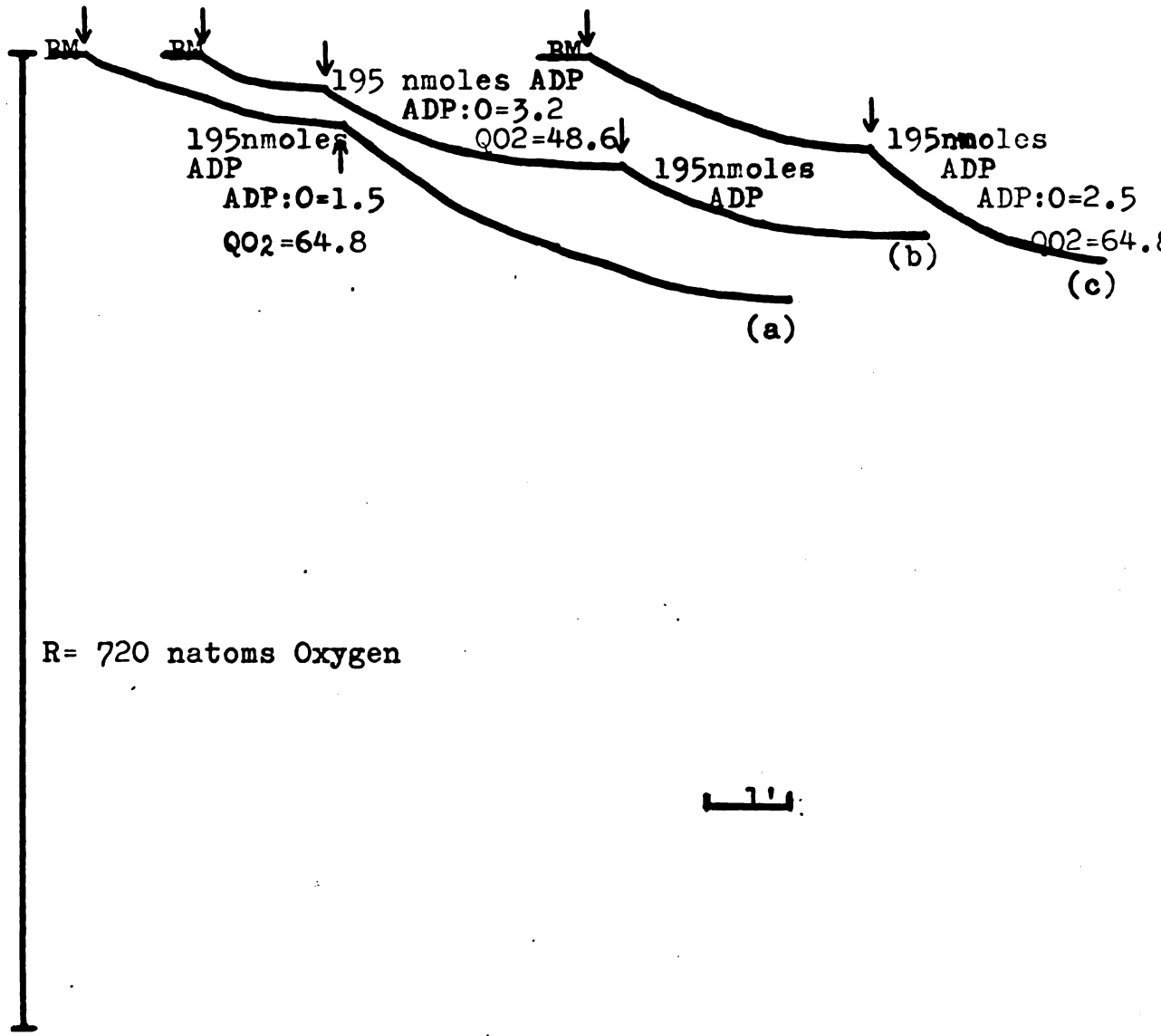


Fig. 18B- Polarographic trace of the oxidative phosphorylation of rat brain mitochondria with β-hydroxybutyrate in control conditions (a) and in the presence of cyclopropane (b) and nitrogen (c). The chamber contained: reaction media, mitochondria suspension (BM) correspondent to 0.63 mg protein and ADP added where indicated in a final volume of 1.5 ml. See "Methods".

DISCUSSION

The main findings of the present study may be summarized briefly as follows:

1. Cyclopropane caused stimulation of SDH activity in both brain and liver preparations, the effects being comparable at optimal substrate concentration (2.5 mM succinate);
2. In brain mitochondria, cyclopropane caused stimulation of enzyme activity over the entire range of substrate concentration used. In rat liver mitochondria, on the other hand, cyclopropane produced inhibition at lower substrate concentration (0.25 mM succinate);
3. The gas effects in both preparations were not appreciably pressure-dependent, the stimulation of enzyme activity appearing at very low pressure (0.25 atm. pressure);
4. In the difference spectra studies, cyclopropane slowed electron transport in liver mitochondria and favored cytochrome reduction in the brain preparation. The disappearance of the peaks in the latter was also faster;
5. Preliminary oxidative phosphorylation experiments showed an increase in the ADP:O ratios with β-hydroxybutyrate in brain and liver preparations. The oxygen consumption was not significantly altered in liver mitochondria and it was decreased in the presence of succinate and β-hydroxybutyrate in brain mitochondria. However, there was some indication of an uncoupling-like effect with succinate as substrate in liver mitochondria.

Cyclopropane produced a stimulatory effect on the mitochondrial marker succinic dehydrogenase, in both brain and liver preparations.

The enzyme from brain mitochondria appeared to be more sensitive to cyclopropane, showing a greater stimulation under the same set of conditions. This fact emphasizes mitochondrial heterogeneity and the qualitative differences and functional adaptations of this organelle.

As further evidence of heterogeneity, Gerlach et al (16) studied the different responses of adenine nucleotide synthesis in rat kidney and brain slices during aerobic recovery from anoxia and ischemia. They found that the inability of brain to recover after longer periods of anoxia was paralleled by its inability to increase "de novo" nucleotide synthesis. They suggested that organs capable of a complete post-anoxic, metabolic, and functional restoration were characterized by their ability to enhance "de novo" nucleotide synthesis subsequent to severe lack of oxygen.

Effects of cyclopropane on SDH and on the electron transport system are not unique to mammalian systems. Hegeman (24) studied the effects of xenon and cyclopropane on E. coli growth, cytochromes and flavoproteins. Cyclopropane and xenon prevent substrate-dependent reduction of the cytochrome chain. She suggested that SDH and NADH oxidase, which produce reduced flavins and pyridine nucleotides, are the primary sites of the inhibitory action of xenon and cyclopropane. Both NADH oxidase and SDH are flavoproteins and are membrane bound. She concluded after studying a soluble non-flavin dehydrogenase (lactic dehydrogenase), that the flavin or the intimate binding to the membrane, or both, are important factors in the inhibitory phenomenon presented by cyclopropane or xenon.

There are several possible mechanisms to explain the enzyme activation observed here:

a. cyclopropane may act as a binding link between enzyme and substrate, combining with both and so holding the substrate at the active site of the enzyme;

b. cyclopropane may change the equilibrium constant of the enzyme reaction perhaps by affecting the surface charge on the enzyme protein causing an easier interaction between enzyme and substrate;

c. cyclopropane may remove an inhibitor present in the enzyme preparation by forming a complex (12);

d. another possibility is that cyclopropane may nullify the effects of an inhibitor agent.

Some of these mechanisms (a, b, d) are more likely to occur in the present case. For instance, cyclopropane in a physicochemical type of interaction (electrostatic forces) might facilitate the formation of the enzyme-substrate complex. On the other hand, mechanism c is unlikely here, because of the relatively inert chemical properties of cyclopropane.

Since these studies are preliminary ones, only general and largely speculative comments about the nature of the effect of cyclopropane on SDH activity can be made and no definitive conclusions can be presented so far. However, the possibility remains that cyclopropane might act on the CoQred/CoQox ratio, thus affecting the state of enzyme activation. This possibility will be examined in future studies.

At 1 atm. pressure and in the presence of lower substrate concentration, the stimulatory effect was decreased, or even reversed, in mitochondria from hepatic origin.

An explanation for the gas effects on liver mitochondria could be that the accumulation of an endogenous inhibitor counteracts

somehow the cyclopropane stimulatory effect so that its action is seen only at higher substrate concentrations. It is known that oxaloacetate is a powerful SDH inhibitor and that this compound is present in mitochondrial preparations (5). Perhaps, at lower substrate concentration, the accumulation of oxaloacetate overcomes the stimulatory effect of succinate on SDH activity.

Similar studies have been done with 2,4 dinitrophenol (DNP) on rat liver mitochondria by Susheela and Ramasarma (56). They showed that low concentrations of DNP inhibited the basal activity of SDH, while at higher concentrations the kinetics were complicated by an apparent activation. At a given DNP concentration less inhibition was obtained at higher substrate concentration, leading to an increase in the apparent Km for succinate without affecting the Vmax.

There is strong evidence (36) that the actions of cyclopropane and other anesthetic agents are pluralistic ones, affecting many biological processes.

In addition to its essentially stimulatory effect on SDH, cyclopropane alters the properties of other enzymes as well. Trevor and Cummings (59) reported a reversible inhibition of ATPase-enzyme preparations by cyclopropane that could have significance regarding the anesthetic properties of the gas.

Effects of other "inert gases" on enzyme activities have also been reported. Gottlieb et al (19) showed that helium group gases inhibit active sodium transport systems in frog skin in a pressure-dependent manner.

Schreiner (48) observed that the helium-xenon series of elements

exert inhibitory effects on the activity of purified enzyme systems, such as oxygenases and hydrolases, including acetylcholinesterase. He postulated that the observed changes in enzymic activity may be the result of structural alterations in the enzyme molecules brought about by interaction with helium-group and other inert gases.

Schimassek et al (46) showed that mice treated with halothane have increased liver weight, while relative protein and glycogen content of the liver and the general enzyme pattern remained constant, meaning that a coordinate growth occurs. Of all the enzymes measured, only three showed a definite change in their activity; there was a decrease in the enzyme activity of pyruvate kinase and an increase of malic enzyme and glycerol-1-phosphate oxidase activity. These distinct effects make it possible to describe the changes observed as a special differentiation in the liver metabolism during chronic halothane treatment. They suggested that there are two general types of drug induced compensatory liver growth, one more or less affecting the oxidative metabolism--the mitochondria (halothane-type drugs), and the other affecting the biotransformation system--the microsomes (barbiturate-type drugs).

Brown (8) showed that halothane depresses the metabolism of several barbiturate drugs (type I substrate) by rat hepatic microsomal enzymes. This inhibition was dose-dependent, reversible, non-competitive, and independent of the lipid solubilities of the substrates. In contrast, the metabolism of aniline (type II substrate) was enhanced by halothane. He proposed that these actions may represent an effect of halothane on the terminal oxidase of the system, cytochrome P-450.

More recent studies (52) have examined the changes in lysosomal enzyme as an index for early detection of cell injury in the liver produced by anesthetics. The stability of the hepatic lysosome has been interpreted as an indication of cellular integrity in this organ. These investigators compared the effects of carbon tetrachloride with those of halogenated anesthetic compounds on the concentration of beta-glucuronidase and acid-phosphatase in the liver and plasma of rats. The two lysosomal enzymes were increased in most of the treatments.

Our studies also point to the fact that the action of cyclopropane is a pluralistic one.

Preliminary oxidative phosphorylation results with cyclopropane on liver mitochondria indicated an uncoupling-like effect with succinate as substrate, and a possible inhibition of oxidation with NAD-linked substrates. In brain mitochondria cyclopropane decreased the oxygen consumption with both substrates.

Uncoupling-like effects have already been observed on rat liver mitochondria, with some anesthetics in the presence of several substrates, including succinate. Snodgrass and Piras (53) showed that, when incubated with normal liver mitochondria, halothane uncoupled oxidative phosphorylation, induced irreversible swelling, abolished the 2,4 DNP-stimulated ATPase activity and caused a marked decrease in K-content. Using various substrates, they found that halothane uncouples at all three phosphorylation sites.

Miller and Hunter (35), working with isolated rat liver mitochondria, studied the alterations produced by halothane on electron transport, oxidative phosphorylation and membrane permeability. At

low concentrations (1.5-2.0%), they observed a rapidly appearing and reversible effect on electron transport in the region of NADH dehydrogenase. The oxidation of succinate does not appear to be altered in this concentration range, even though there is some evidence of a limited uncoupling effect. At higher anesthetic concentrations (3-4%) there is partial inhibition of succinate oxidation.

Our observed inhibition of NAD-linked substrate oxidation has been well studied with halothane. According to Cohen et al (11) in experiments on rat liver mitochondria, halothane (0-10%) had no effect on oxygen uptake with succinate. The effect of halothane on NADH oxidase was similar to that observed when glutamate was oxidized. In both cases, inhibition was dose-related and completely reversible provided that less than 3% halothane had been used.

Harris and coworkers (23), in experiments carried out with rat liver mitochondria, showed that the oxidation of NADH-linked substrates, but not of succinate, were markedly suppressed by low concentrations of halothane (2 mM) in a reversible manner. They suggested the rotenone-sensitive site of complex I (NADH-CoQ reductase) or nearby as the potential site for halothane action.

These findings are similar to our preliminary results using cyclopropane on rat liver and brain mitochondria.

Finally, we have observed that cyclopropane caused a decrease in oxygen consumption with succinate and also β-hydroxybutyrate on rat mitochondria. The brain preparation was more sensitive. These observations agree in part with those of some investigators using halogenated anesthetics (51).

In 1932, Quastel and Wheatley (42) showed that depression of brain oxidiation in vitro could be obtained with gaseous as well as non-gaseous anesthetic agents.

Other studies with barbiturates have comfirmed these findings (7, 43, 62, 63).

Hoech et al (26) investigated the effect of halothane on the oxygen consumption of rat brain, liver and heart slices. They showed that the oxygen consumption of rat brain slices was depressed significantly when exposed to 1% halothane, while the oxygen consumption of liver and heart slices was less sensitive. Studies of this type with other anesthetics are relatively few.

According to Levy and Featherstone (30), no significant differences were observed among the gases Xe, N_2O, N_2 or air in the "in vitro" oxidation of glucose or pyruvate or in oxidative phosphorylation by guinea-pig brain tissue.

Gatz and Jones (15) investigated the effects of chloroform, cyclopropane, diethyether, halothane, methoxyflurane and nitrous oxide on respiration and oxidative phosphorylation by rat brain mitochondria. Halothane and methoxyflurane were found to inhibit both oxygen uptake and oxidative phosphorylation when pyruvate, alpha-ketoglutarate and glutamate were used as substrates. However, only halothane evoked a 2,4 DNP-like effect in the presence of succinate.

In more recent work with several halogenated anesthetics, Schumer et al (51) showed that liver mitochondria obtained from rats exposed to either 3 or 5% halothane had significantly higher rates of oxygen than did those of a corresponding control series.

It is interesting to notice that the great number of studies done with anesthetic agents use halothane and other similar volatile compounds, the literature being deficient concerning inert gas anesthetics. The fact may be due to the popularity reached by halothane-type of anesthetics in recent years, and also to the difficulties involved with gas studies, mainly at the molecular level.

CONCLUSIONS

It must be realized that we cannot explain any phenomena observed in anesthesia based solely on SDH stimulation, but rather present it either as an additional effect or an integral part of the action of cyclopropane-like drugs.

The interesting point here is the influence cyclopropane and other anesthetic agents have on flavoprotein-type enzymes.

It is very common in Pharmacology to try to explain drug action in terms of enzyme inhibition, a stimulation of an enzyme activity being somewhat unusual.

It has been shown that SDH is transformed from an inactive to an active state in response to the metabolic state of the mitochondria. The major regulator of SDH activity in intact mitochondria is the red/ox CoQ_{10} ratio, since the percentage of reduction of CoQ_{10} changes 10-fold in transitions between metabolic states with consequent major changes in SDH activity (21, 22). These changes in SDH activity are sufficiently rapid at physiological temperatures to be of considerable importance in regulating SDH activity and therefore the rate of ATP synthesis. These observations lead Singer to propose SDH as a likely candidate for a role as a regulatory enzyme in the Krebs Cycle and hence in the overall rate of ATP synthesis (55).

Whenever there is less demand for high energy compounds, the succinate pathway becomes the important one. NAD is bypassed when succinate is oxidized to fumarate by SDH in the TCA cycle. The stimulation of SDH under the control of reduced CoQ_{10} levels will result in an accumulation of NADH. The increase in NADH slows down

the TCA cycle and thus decreases glucose oxidation.

It is well documented that the brain levels of glucose and also glycogen go up under the influence of anesthetic agents (9).

The Singer hypothesis may possibly be verified by using cyclopropane. The influence of cyclopropane on the levels of red CoQ_{10} and on NADH oxidase in the presence of activated and inactivated SDH should be examined.

Cyclopropane may thus be a potentially useful tool in disclosing the regulatory processes in the TCA cycle. One can further speculate that the effect of cyclopropane on the cellular process is one that interferes with normal metabolic regulation.

Direction of Future Studies--Future studies should be oriented toward:

1. further characterization of cyclopropane effects not only on the "particulate" SDH but also on the "soluble" enzyme;
2. influence of cyclopropne on red CoQ_{10} levels and thus on the succinic dehydrogenase activation state;
3. effects of cyclopropane on mitochondrial NADH oxidase activity;
4. oxidative phosphorylation experiments designed to aid in the localization of the site (s) of action of cyclopropane.

REFERENCES

1. Armstrong, J. McD. The Molar Extinction Coefficient of 2,6-Dichlorophenol Indophenol. Biochem. Biophys. Acta, 86:194-197, 1964.

2. Balázs, R., Biesold, D., and Magyar, K. Some Properties of Rat Brain Mitochondrial Preparations: Respiratory Control. J. Neurochem., 10:685-708, 1963.

3. Beattie, Diana S., Basford, R. E. and Koritz, Seymour B. The Turnover of the Protein Components of Mitochondria from Rat Liver, Kidney, and Brain. J. Biol. Chem., 242(20):4584-4586, 1967.

4. Bennett, P. B. The Aetiology of Compressed Air Intoxication and Inert Gas Narcosis. Pergamon Press, New York, First Ed., 1966, pp. 63-64.

5. Bernath, Paul, and Singer, Thomas P. Methods in Enzymology. Ed. by S. P. Colowick and N. O. Kaplan, vol. V, Academic Press, Inc., New York, 1962, pp. 597-614.

6. Brink, F., and Posternak, J. M. Thermodynamic Analysis of Relative Effectiveness of Narcotics. J. Cell Comp. Physiol., 32:211-234, 1948.

7. Brody, T. M., and Bain, J. A. Effect of Barbiturates on Oxidative Phosphorylation. Proc. Soc. Exper. Biol. & Med., 77(1):50-53, 1951.

8. Brown, Jr., Burnell R. The Diphasic Action of Halothane on the Oxidative Metabolism of Drugs by the Liver. Anesthesiology, 35(3):241-246, 1971.

9. Brunner, E. A., Passonneau, Janet V., and Molstad, C. The Effect of Volatile Anesthetics on Levels of Metabolites and on Metabolic Rate in Brain. J. Neurochem., 18:2301-2316, 1971.

10. Clark, John B., and Nicklas, William J. The Metabolism of Rat Brain Mitochondria. J. Biol. Chem. 245:4724-4731, 1970.

11. Cohen, P. J., Marshall, B. E., and Lecky, J. Effects of Halothane on Mitochondrial Oxygen Uptake: Site of Action. Anesthesiology, 30:337, 1969.

12. Dixon, M., and Webb, E. Enzymes. Academic Press, Inc., New York, 1964, pp. 420-449.

13. Featherstone, R. M., and Muehlbacher, C. The Current Role of Inert Gas in Search for Anesthetic Mechanisms. Pharmacol. Rev., 15:97-121, 1963.

14. Fletcher, Martin J., and Sanadi, D. R. Turnover of Rat Liver Mitochondria. Biochem. Biophys. Acta, 51:356-360, 1961.

15. Gatz, E. E., and Jones, J. R. Effects of Six General Anesthetics upon Mitochondrial Oxidative Phosphorylation: Possible Site and Mechanism of Malignant Hyperpyrexia. Fed. Proc., 28:356, 1969.

16. Gerlach, E., Marko, P., Zimmer, H. G., Pechan, I., and Trendelenburg, Ch. Different Response of Adenine Nucleotide Synthesis de novo in Kidney and Brain during Aerobic Recovery from Anoxia and Ischemia. Experientia, 27:876-878, 1971.

17. Goldstein, A., Aronow, L., and Kalman, S. M. Principles of Drug Action. Hoeber Med. Div., 1969, p. 100.

18. Goodman, L. S. and Gilman, A. The Pharmacological Basis of Therapeutics. Macmillan Co., New York, Fourth Ed., 1970, p. 45.

19. Gottlieb, Sheldon F., Cymernan, Allen, and Metz, Albert V. Jr., A New Physiological Effect of Xenon, Kripton and Argon: Inhibition of Sodium Active Transport. Fed. Proc., 27:768, 1967.

20. Green, D. E., Mii, S., and Kohout, P. M. Studies on the Terminal Electron Transport System. I. Succinic Dehydrogenase. J. Biol. Chem., 217:551-567, 1955.

21. Gutman, M., Kearney, Edna B., and Singer, T. P. Activation of Succinate Dehydrogenase by Electron Flux, from NADH and its Possible Regulatory Function. Biochem. Biophys. Res. Comm., 42(6):1016-1023, 1971.

22. Gutman, M., Kearney, Edna B., and Singer, T. P. Multiple Control Mechanisms for Succinate Dehydrogenase in Mitochondria. Biochem. Biophys. Res. Comm., 44:526-532, 1971.

23. Harris, R. A., Munroe, J., Farmer, B., Kim, K. C., and Jenkins, P. Action of Halothane upon Mitochondrial Respiration. Arch. Biochem. Biophys., 142:435-444, 1971.

24. Hegeman, Sally L. Sites of Action of the Inert Anesthetic Gases: Effect of Xenon and Cyclopropane on the Physiology and Biochemistry of Escherichia Coli. Ph.D. Thesis, U.C., San Francisco Medical Center, 1969, pp. 49-51.

25. Hodgkin, A. L., and Huxley, A. F. A Quantitative Description of Membrane Current and Its Application to Conduction and Excitation in Nerve. J. Physiol., 117,500, 1952.

26. Hoech, George P., Jr., Matteo, Richard S., and Fink, B. Raymond. Effect of Halothane on Oxygen Consumption of Rat Brain, Liver and Heart and Anaerobic Glycolysis of Rat Brain. Anesthesiology, 27:770-777, 1966.

27. Larrabee, M. G., and Holaday, D. A. Depression of Transmission through Sympathetic Ganglia during General Anesthesia. J. Pharmac. Exp. Ther., 105:400-408, 1952.

28. Larrabee, M. G., and Posternak, J. M. Selective Action of Anesthetics on Synapses and Axons in Mammalian Sympathetic Ganglia. J. Neurophysiol., 15:91-114, 1952.

29. Lehninger, A. Biochemistry. Worth Publ. Inc., New York, 1970. p. 395.

30. Levy, Louis, and Featherstone, R. M. The Effect of Xenon and Nitrous Oxide on In Vitro Guinea Pig Brain Respiration and Oxidative Phosphorylation. J. Pharm. Expt. Therap., 110:221-225, 1954.

31. Lowenstein, John M. Citric Acid Cycle: Control and Compartmentation. Marcel Dekker, New York, 1969, p. 37.

32. Lusena, C. V., and Depocas, Florent. Heterogeneity and Differential Fragility of Rat Liver Mitochondria. Canadian J. Biochem., 44:497-508, 1966.

33. Menzies, Robert A., and Gold, Philip H. The Turnover of Mitochondria in a Variety of Tissues of Young Adult and Aged Rats. J. Biol. Chem., 246:2425-2429, 1971.

34. Miller, S. L. A Theory of Gaseous Anesthetics. Proc. Nat. Acad. Sci., 47:1515-1524, 1961.

35. Miller, Robert N., and Hunter, F. Edmund, Jr. The Effect of Halothane on Electron Transport, Oxidative Phosphorylation, and Swelling in Rat Liver Mitochondria. Mol. Pharmacol., 6:67-77, 1970.

36. Mullins, L. J. Handbook of Neurochemistry. Vol. VI, Ed. by Abel Lajtha, Plenum Press, New York, 1971, pp. 395-421.

37. Neidle, A., Van Den Berg, C. J., and Grynbaum, A. The Heterogeneity of Rat Brain Mitochondria Isolated on Continuous Sucrose Gradients. J. Neurochem., 16:225-234, 1969.

38. Neufeld, Harold A., Scott, Charles R., and Stotz, Elmer. Purification of Heart Muscle Succinic Dehydrogenase. J. Biol. Chem., 210:869-876, 1954.

39. Novikoff, A. B. The Cell: Biochemistry, Physiology, Morphology. Vol. II. Academic Press, Inc., New York, 1961, p. 299.

40. Pauling, L. A. A Molecular Theory of Anesthesia. Science, 134:15, 1961.

41. Pollak, J. K., and Munn, E. A. The Isolation by Isopycnic Density-Gradient Centrifugation of Two Mitochondrial Populations from Livers of Embryonic and Fed and Starved Adult Rats. Biochem J., 117:913-919, 1970.

42. Quastel, J. H., and Wheatley, A.H.M. Narcosis and Oxidations of the Brain. Proc. Roy. Soc., London, B112:60, 1932.

43. Quastel, J. H. Biochemical Aspects of Narcosis. Curr. Res. Anesth. Analg., 31:151-163, 1952.

44. Quastel, J. H. Metabolic Activity of Neurons. Ann. N. Y. Acad. Sci., 109:436-450, 1963.

45. Reiner, John M. Behavior of Enzyme Systems. 2nd Ed., Van Nostrand Reinhold Co., New York, 1969, pp. 154-220.

46. Schimassek, H., Kunz, W., and Gallwitz, D. Differentiation of Liver Metabolism on the Molecular Level during Chronic Application of Halothane. Biochem. Pharmacol., 15:1957-1964, 1966.

47. Schnaitman, Carl, and Greenawalt, John W. Enzymatic Properties of the Inner and Outer Membranes of Rat Liver Mitochondria. J. Cell Biol., 38:158-183, 1968.

48. Schreiner, H. R. General Biological Effects of the Helium-Xenon Series of Elements. Fed. Proc., 27(3):872-878, 1968.

49. Schoenborn, B. P., and Nobbs, C. L. The Binding of Xenon to Sperm Whale Deoxymyoglobin. Mol. Pharmacol., 2:491, 1966.

50. Schoenborn, B. P., and Featherstone, R. M. Molecular Forces in Anesthesia. Ad. Pharmacol., 5:1-17, 1967.

51. Schumer, William, Erve, Peter R., Obernolte, Ronald P., Bombeck, C. Thomas, and Sadove, Max S. The Effect of Inhalation of Halogenated Anesthetics on Rat Liver Mitochondrial Function. Anesthesiology, 35:253-255, 1971.

52. Serrou, Bernard C., Aldrete, J. Antonio, and Schultz, Richard L. Effect of Anesthetic Drugs on Lysosomal Enzymes in the Rat. Proc. Soc. Exp. Biol. Med., 137(4):1389-1395, 1971.

53. Snodgrass, Philip J., and Piras, Martha M. The Effects of Halothane on Rat Liver Mitochondria. Biochem., 5:1140-1149, 1966.

54. Sordahl, Louis A., Johnson, Carl, Blailock, Zack R., and Schwartz, A. Methods in Pharmacology, Vol. I, Ed. by A. Schwartz, Appleton-Century Crofts, New York, 1971, pp. 247-283.

55. Singer, T. P. Biological Oxidations. Ed. by T. P. Singer, Wiley, New York, 1968, p. 339.

56. Susheela, L., and Ramasarma, T. Activation of Succinate Dehydrogenase by 2,4-Dinitrophenol--A Competitive Inhibitor. Biochem. Biophys. Acta, 242:532-540, 1971.

57. Tapley, D. F., Kimberg, D. V., and Buchanan, J. L. The Mitochondrion. New England J. Med., 276:1124-1132, 1967.

58. Tolani, Ambika J., and Talwar, G. P. Differential Metabolism of Various Brain Regions: Biochemical Heterogeneity of Mitochondria. Biochem. J., 88:337-362, 1963.

59. Trevor, A. J., and Cummins, J. T. Properties of Sodium- and Potassium-Activated Adenosine Triphosphatases of Rat Brain--Effect of Cyclopropane and other Agents Modifying Enzyme Activity. Biochem. Pharmacol., 18:1157-1167, 1969.

60. Von Korff, R. W., Steinman, Sondra, and Welch, Annemarie S. Metabolic Characteristics of Mitochondria Isolated from Rabbit Brain. J. Neurochem., 18:1577-1587, 1971.

61. Weichselbaum, T. E. Determination of Proteins in Blood Serum and Plasma. Am. J. Clin. Path., 16:40, 1946.

62. Wilkins, D. S., Featherstone, R. M., Gray, C. E., Schwidde, J. T., and Brotman, M. Studies on the Depression of Brain Oxidations. I. Biopsy Technique and Analysis of Variance in the Selection of a Pentobarbital Concentration. J. Lab. & Clin. Med., 34:846, 1949.

63. Wilkins, D. S., Featherstone, R. M., Schwidde, J. T., and Brotman, M. Studies on the Depression of Brain Oxidations. II. Comparative Influence of Pentobarbital upon Dog Cortex, Caudate Nucleus, and Hypothalamus. J. Lab. & Clin. Med., 35:411, 1950.

64. Wulf, R. J., and Featherstone, R. M. Correlation of Van der Waals' Constants with Anesthetic Potency. Anesthesiology, 18:97-105, 1957.