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Biochemical Effects of Chronic Alcohol Ingestion on Cardiac Muscle of the Rat

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BIOCHEMICAL EFFECTS OF CHRONIC ALCOHOL INGESTION ON CARDIAC MUSCLE OF THE RAT

by

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ABSTRACT

In order to determine the effects on heart muscle of chronic ingestion of ethanol, Sprague-Dawley rats about twenty days old were divided into control and experimental groups. The experimental animals were given ten percent ethanol instead of drinking water for three weeks, fifteen percent ethanol for the next three weeks and twenty percent ethanol until sacrifice. Both groups of animals were fed with regular rat chow ad libitum.

Measurements of alcohol dehydrogenase activity in homogenates of fresh rat heart tissue showed that methods developed for this enzyme assay in rat liver homogenates could not be used with rat heart tissue because of interfering enzyme activities. This is not a problem with liver tissue. If the heart tissue were frozen and stored for several weeks, the assay method was usable on supernatants of heart homogenate, and demonstrated the presence of low levels of alcohol dehydrogenase in this tissue. Within the experimental limits, chronic ingestion of ethanol had no significant effect on the amount of this enzyme demonstrable in rat hearts which had been frozen and stored similarly prior to assay.

A ten to twenty percent increase in succinate dehydrogenase activity was found in homogenates of fresh heart tissue from rats which had received ethanol continuously for six months compared with tissue from control rats which had not been given ethanol. This would be in accord with a report that chronic

ethanol ingestion resulted in an increase in the number of mitochondria in rat heart tissue, providing that such treatment did not change either the mitochondrial content of succinate dehydrogenase, nor the extraction of that enzyme from heart tissue. Further experiments showed that extraction of enzyme activity by the procedure used was far from complete, therefore conclusions as to the relative numbers of mitochondria in heart tissue on the basis of these enzymic measurements are subject to some uncertainty. Measurements of oxidative phosphorylation by the mitochondrial fraction of rat heart muscle showed that the ingestion of ethanol over a period of eight to twelve months did not affect the rates of oxidation of a number of substrates, nor did it change the ADP/O ratio or the respiratory control ratio. The interpretation of these experimental observations is complicated by the difficulty which was encountered in isolating mitochondria from older rats. Using a procedure developed for use with heart muscle and in general use for that purpose, one can obtain clean preparations of mitochondria from young rats. With the same procedure and tissue from rats 12 months or older, the mitochondrial fraction is contaminated by a large amount of gel, which is presumably partially hydrolysed collagen. The increase in the amount of connective tissue is probably the reason for the decrease in specific activity of the mitochondrial fraction noted over a period of five months, since gel protein is included in "mitochondrial" protein measurements.

LIFE

Daniel Yue-King Chan was born in Hong Kong, China, December 15, 1941. He was graduated from Hope College, Holland, Michigan, U.S.A., June, 1964, with the degree of Bachelor of Science.

In September, 1964 he began further studies in chemistry at Miami University, Oxford, Ohio. In September, 1965 he began graduate studies at Loyola University in the Department of Biochemistry and Biophysics and received his M.S. Degree in June, 1967.

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Daniel Yue-King Chan

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CHAPTER I

INTRODUCTION

Alcoholism was considered as a disease as early as 1785 by Benjamin Rush, a physician who wrote a paper entitled "An Inquiry into the Effects of Ardent Spirits upon the Human Body and Mind with an Account of the means of Preventing and of the Remedies for Curing them" (Catanzaro, 1968). It is the name of the chronic disease from which the alcoholic suffers.

The modern concept of an alcoholic is a person who has become dependent on the drug, alcohol, much more than the socially accepted norm for his culture. This leads to the damage of the body, and a deterioration in the relationship with his family, friends and job. Alcoholism is one of the oldest diseases known to mankind. Histories of ancient Egypt, Greece, Roman Empires and China recorded that alcoholism was prevalent.

Enormous efforts have been put into the study of the metabolism of ethanol in humans and animals. It has long been known to be oxidized in the liver to carbon dioxide and water (Himwich, 1933). This process, however, consists of several steps: 1. Ethanol is converted into acetaldehyde; this is the slowest reaction and the rate limiting step. 2. Acetaldehyde is converted to acetyl CoA, but the pathway has not been definitely established (Himwich, 1933). It may take place directly or via acetic acid. 3. Acetyl CoA is oxidized via the citric acid cycle to carbon

dioxide and water. The entire process may be represented as

Ethanol ⟶ acetaldehyde ⟶ acetyl CoA ⟶ $CO_2 + H_2O$

Chronic alcoholics have been known to suffer from heart and liver damage. The genesis of idiopathic cardiac disease due to chronic ingestion of ethanol is not understood although the relationship has been observed for more than 100 years. The disease is not acute; it arises from unknown factors which either interfere with or influence the normal cardiac function. It has a protracted incubation period and an insidious onset, Hibbs, 1963).

In the last ten years, many studies of alcoholic myocardial disease have been published. Hibbs (1963) reported the loss of mitochondrial cristae; decrease in enzymic activity, deformation and fragmentation of heart mitochondria in alcoholic patients. Szanto et al (1967) carried out myocardial biopsy in chronic alcoholic patients with primary myocardial disease. Electron microscopic studies revealed distinctive ultrastructural changes i.e. dilation on profiles of sarcoplasmic reticulum and increase in number and size of mitochondria. Changes were observed by Szanto et al (1967) in the hearts of rats given 20% ethanol in their drinking water for more than four months. Alexander (1967) performed cardiac biopsies on sixty alcoholic patients. He reported mitochondriosis, increased distance between contractile elements, disappearance of myofibrils, sarcoplasmic reticulum missing from some area, enlargement and serration of nuclei. The

results were similar to those observed at postmortem by Hibbs et al (1963). Wendt, Schwartz and Lee (1966) reported loss of striation, dissociation and desegmentation in muscles of failing hearts from dogs, guinea pigs and cats. They also found significant depression of myocardial contractility and increase in cardiac tissue mass. The mitochondria isolated by Schwartz and Lee (1962) from chronic failing heart of guinea pig exhibited a significant depression of metabolic activity. Tobian (1960) and Meerson (1964) found that the number and size of mitochondria was increased in rabbit heart by inducing artificial heart failure (aortic stenosis). Meerson also observed swollen and degenerated mitochondria, with undefined membranes and an increase in the number of cristae. These phenomena also existed in chronic alcoholic patients as reported by Webb (1965), Alexander (1966) and Ferrans (1966). Alexander (1967) reported the condensation in outer and inner mitochondrial membrane which he suggested the involvement of permeability or response to ATPase. There was focal and general condensation of matrix in the mitochondria. There were other changes arising along with the mitochondrial alterations, such as dense mitochondrial inclusions and a change of the cellular environment in the neighbourhood of the mitochondria. Ferrans et al (1965) and Hickie (1960) and Hibbs (1963) concomitantly found, in postmortem myocardial tissue of alcoholic patients, an aggregation of lipid droplets (0.1 to 3 microns in diameter), primarily triglycerides in large quantity in the myocardial fibers.

Opie (1969) demonstrated the increase of myocardial triglyceride synthesis from non-lipid sources such as glucose after an infusion of alcohol into the dog. Marciniak et al (1968) observed a three-fold increase in triglyceride content in cardiac tissue of chronic alcoholic dog. Pearse (1960) found in cardiac muscular dystrophy, the feature of unusual aggregation of glycogen accompanying the mitochondriosis. The myofibrils were abnormally short and there were sites of proliferation of connective tissue leading to fibrosis and elastosis. Regan (1964) has demonstrated the acute effect of ethanol on myocardium of dog. There were marked depression of fatty acid uptakes, increase of triglyceride content in myocardium, leakage of phosphate, potassium and reduction of left ventricular contractility accompanied by a low cardiac output. Ferrans (1965) reported deposits of lipid, primarily triglycerides, particularly in cardiac muscle fibers in the atria and ventricles of human heart failure. He explained the abnormal mitochondrial cristae as a result of fatty acids preferentially esterified into triglycerides rather than into mitochondrial phospholipids.

Parameters such as the level of high energy phosphates, contractility and energy production in the myocardium have been another target for the investigators. Schwartz and Lee (1962) reported that mitochondria obtained from chronic failing heart of guinea pig were uncoupled with respect to oxidative phosphorylation, with a value of 43% of the control. The preparation also

exhibited low oxygen consumption in the presence of glutamate, succinate and α-ketoglutarate, although there was no change in ATPase. Wendt et al (1964) declared that the myocardial oxygen extraction and myocardial oxygen consumption were normal in alcoholic patients with primary myocardial disease without hepatic dysfunction. In patients with Laennec's cirrhosis, coronary blood flow and myocardial oxygen consumption were both diminished but became normal on exercise. Inorganic phosphate was liberated by the myocardium in both groups. From the morphological finding in the electron micrograph done by Alexander (1967), this implies metabolic derangement of both oxidative and inotropic mechanism, losses of supply of phosphate-bond energy. Lochner (1969) reported that heart contents of ATP, ADP, creatine phosphate and inorganic phosphate were unchanged in rat heart (after prolonged ingestion of 20% ethanol) when perfused with 100 mM ethanol, but a significant depression in ATP and inorganic phosphate contents were seen with 200 mM ethanol.

In heart mitochondria from alcohol patients, Meerson (1964) noted the loss of mitochondrial enzymes, such as malic dehydrogenase and α-ketoglutaric dehydrogenase; Gould (1970) found a consistent decrease in isocitric dehydrogenase and malic dehydrogenase. Marciniak et al (1968) detected in chronic alcoholic dogs, a significant increase of malic dehydrogenase in heart muscle. Other enzymes compared showed differences which may not be significant: aldolase, glyceraldehyde phosphate dehy-

drogenase, lactic dehydrogenase, α-glycerophosphate dehydrogenase, isocitrate:NADP dehydrogenase, glutamic oxalic transaminase and glucose-6-phosphate dehydrogenase showed an increase; isocitrate: NAD dehydrogenase showed a slight decrease. Pearse (1960) has made a survey of some obstructive myocardiopathies. The level of mitochondrial oxidative enzymes rose while ATPase decreased moderately in affected myofibrils. Phosphorylases (a + b) mainly b, were found to rise considerably in the large perinuclear spaces. Monoamine oxidase (MAO) and glycogen were found to be higher, with monoamine oxidase being higher in the left ventricle than on the right ventricle. Muller and Pearse (1964) suggested the possibility of correlation between the levels of MAO and the glycogen level in the cardiac muscle, and significant increase in MAO may reflect the mitochondriosis. Ferrans et al (1965) reported the decrease in intensity of oxidative enzymes in histochemical staining. In the heart tissue of alcoholic patients, the most apparent decreases were in the lactic dehydrogenase, succinic dehydrogenase and isocitric dehydrogenase. In the study of ethanol on myocardium of dog, Regan (1964) detected the leakage of glutamic oxaloacetic transaminase, glutamic dehydrogenase and malic dehydrogenase, prior to cardiac failure. In chronic ingestion of ethanol by rats over 8 to 12 months, Miles et al (1967) observed depressed ATPase activity in myofibrils.

There is disagreement about the presence of ADH in cardiac tissue. Results of Lochner et al (1969) indicated that ethanol-2-

^{14}C was not metabolized to any measurable extent by either the perfused rat heart or tissue slices, while Bartlett and Barnet (1949) found that uniformly labeled ethanol was oxidized to carbon dioxide by slices of rat heart. The rate of oxidation was, however quite slow compared with that observed with slices of rat kidney and liver. The detailed mechanism of the oxidation is, at present, unknown. Keilin and Hartree (1945), Griffaton and Lowy (1964) suggested the oxidation of alcohol in heart through a mechanism involving the combination of catalase and sources of hydrogen peroxide such as xanthine oxidase. Mezey et al (1968) reported that there was no alcohol dehydrogenase in rat heart supernatant. Wendt et al (1966) claimed that ADH was present in the myocardium of rats and in the coronary sinus blood of chronic alcoholics.

Attention has also been focused on the coenzymes and intermediates in the oxidation of ethanol. Opie (1969) found an increase in the NADH/NAD ratio in the infarcted area of heart. Gould (1970) also noted a rise of the NADH/NAD ratio in human heart and liver due to chronic ethanol ingestion. Cherrick and Leevy (1965) found an increase of the NADH/NAD ratio in heart, liver and kidney from rats on prolonged ethanol treatment. Since neither kidney nor heart oxidizes in vivo significant amounts of ethanol, these changes are suggested to be due to the utilization by these organs of metabolites with nicotinamide-adenine dinucleotide reducing potential which are derived from ethanol. He

emphasized that the effect of ethanol on the hepatic NADH/NAD ratio was dose-related, not all-or-none. He was unable to find ADH in rat heart with methods which were developed to assay the enzyme in liver. Forsander et al (1958) has shown that the increased hepatic NADH/NAD ratio in fed rats was solely because of a decrease in NAD concentration. Moreover, while Smith and Newman (1959) found that on acute ingestion of 3 gm ethanol per Kg body weight reduced the NADH/NAD ratio in liver of fed rats and reversed it in fasting rats. Raiha and Oura (1962) reported a greater fall in the ratio in fed animals than in fasting animals. The variability may be due to various methods of tissue handling and pyridine nucleotide extraction employed.

The accumulation of glucose, lactate and acetoacetate in the myocardium of alcoholic patients were interpreted by Wendt et al (1966) in terms of the changes of redox potentials in myocardium and in the coronary-venous blood specimens. The redox potentials were determined by the ratios of lactate/pyruvate, β-hydroxybutyrate/acetoacetate or α-glycerol phosphate/dihydroxy acetone phosphate. Unal et al (1965) performed similar experiments and found that the myocardial utilization of free fatty acids decreased considerably and lactic acid level rose in spite of a slight increase in utilization. Progressive metabolic acidosis developed, which suggested that alcohol may interfere with aerobic metabolism.

The release of catecholamines as a result of alcohol in-

gestion was demonstrated by Mallov et al (1967), which may account for some of the apparent effects of ethanol. Opie (1969) has found that catecholamines were lost from the infarcted area in heart tissue. Large doses of alcohol produced a liberation of adrenaline from adrenal gland as noted by Perman (1961). However, Czaja and Kalant (1961) have shown that adrenal stimulation was not a constant concomitant of alcohol depression but rather depends on the route of administration and on the speed by which the blood alcohol concentration rose. Acetaldehyde, a product of the oxidation of alcohol, was also known to increase the release of catecholamines (Truitt, 1967).

A considerable amount of research has been published concerning the effects of ethanol on liver and its metabolism. This may provide a guide to the studies of ethanol effects on heart. Ethanol has been known to be metabolized mainly extramitochondrially in liver (Nyberg et al, 1953). However the effect of ethanol on ADH in liver is still controversial. Dajani (1963) reported that rat liver ADH increased with age, but more rapidly in rats given 20% ethanol instead of water. Similar results were reported with by Mirone (1965). Ugarte (1967) found that in liver biopsies, ADH activity was significantly lower in alcoholic patients than in the control or moderate drinkers. In addition, there was difference in ADH activity between the alcoholics with normal livers and those with diseased livers. In human alcoholics, Figueroa and Klotz (1962) found that liver ADH activity was

markedly decreased, while serum ADH was unchanged, but there was a significant elevation of serum isocitric dehydrogenase, glutamic pyruvate transaminase and glutamic oxalacetate transaminase. Dajani and Kouyoumjian (1966), using male rats fed with ethanol for 28 weeks, found an increase of isocitric dehydrogenase and glutamic dehydrogenase in liver tissue. Succinic dehydrogenase was elevated slightly. Kiessling and Pilstrom (1966) demonstrated that there was a sex difference in the response to prolonged ethanol consumption by rats. They found that the rate of oxidation of succinate in liver homogenate of male rats was decreased by 50%, but was unchanged in the female. Lactate dehydrogenase in female liver and phosphofructokinase in male liver showed a significant increase in activity after ethanol treatment whereas the activity of aldolase, glutamic dehydrogenase, malate dehydrogenase and isocitric dehydrogenase were not affected. Greenberger (1965) found that chronic administration of ethanol to female rats did not enhance the oxidation of ethanol-1-^{14}C, besides, there was no significant change between control and experimental rats of ADH activity in liver supernatant. Forsander et al (1965) showed that addition of ethanol stopped the production of carbon dioxide and ketone bodies in the incubation of liver slices. Lundsgaard (1938) found that the respiratory quotient decreased in liver when ethanol was added to the perfused blood. The ratios of lactate/pyruvate and β-hydroxybutyrate/acetoacetate increased in liver in the oxidation of ethanol. Ethanol ingestion was also found to

decrease pyruvate markedly in liver. The reason was not known. Moreover, ethanol when added to liver slices, also depressed the activity of the citric acid cycle enzymes and changed the rate of glycolysis in liver (Forsander, 1964). Cherrick and Leevy (1965) and Raiha (1962) found that ethanol increased the NADH/NAD ratio in fasting rat liver, kidney and heart, but had no effect on the ratio in the blood or brain. The total NADH + NAD was increased, while Mirone (1965) reported a decrease in total NAD + NADH in chronic alcoholic male mice. Lieber and Schmidt (1961) showed that ethanol stimulated fatty acid synthesis and also the incorporation of acetate in fatty acids in incubating liver slices. A relatively larger fraction of acetyl CoA was directed to fatty acid synthesis and less was metabolized via TCA cycle. Ethanol also inhibits the oxidation of fatty acids (Forsander, 1966). Beard and Barboriak (1965) showed that, on short-term administration of ethanol, a rise in plasma unesterified fatty acids, triglycerides, total cholesterol, and accumulation of liver lipids in dog. Zakim (1965) observed that ethanol ingestion lowered the level of long chain acyl CoA in rat liver and raised the level of α-glycerol phosphate.

Using earlier studies as a guide, the author studied the effects of chronic ethanol ingestion on succinic dehydrogenase and other mitochondrial enzymes of rat cardiac muscle. In addition, the possible presence of alcohol dehydrogenase in rat heart was investigated.

CHAPTER II

EXPERIMENTAL

ANIMAL HANDLING:

Several groups of rats of modified Sprague Dawley strain were studied. When the rats were 20 days old, the experimental animals were given 10% (v/v) ethanol in their drinking water for the first three weeks, 15% ethanol for the second three weeks and 20% ethanol until sacrificed. The controls received tap water. Both groups of animals were fed ad libitum with regular rat chow, Dajani (1963).

DETERMINATION OF ALCOHOL DEHYDROGENASE IN LIVER AND HEART OF RAT:

The levels of alcohol dehydrogenase (ADH) in rat heart and liver were determined by a spectrophotometeric technique as described by Bonnichsen and Brink (1951). The crude tissue method was modified in several respects in order to achieve a more accurate measurement of the enzyme in rat heart and liver.

REAGENTS:

1. NaOH-glycine buffer at pH 9.60 containing 5.44×10^{-4} M NAD
2. NaOH -- Solid, Mallinckrodt, Lot. 7708
3. Glycine -- Eastman Organic Chemical, 445
4. NAD -- Pabst Laboratories, Lot. 312
5. 95% Ethanol -- U.S. Industrial Chemical Co. New York.

APPARATUS:

Roller Smith Balance -- Bethlehem, Pa., 700437

Spectrophotometer -- Beckman DU, Ser. No. 29587, National Technical Laboratory, Pasadena, California

Homogenizer -- Arthur H. Thomas Co. Philadelphia, Pennsylvania

Centrifuge -- Pr-2 refrigerated centrifuge, Clinical centrifuge-Z4859, International Equipment Co. Boston, Massachusettes, and Servall automatic refrigerated centrifuge, Ivan Sorvall, Inc., Norwalk, Connecticut

PROCEDURE:

Rats were starved overnight and sacrified by decapitation. Liver, heart and kidney, were removed immediately, washed in cold physiological saline and frozen in a dry ice bath. The frozen organs were stored in a freezer at -20 ^{o}C. The apex and the base of the heart were removed and discarded. The rest of the heart was cut horizontally. Frozen tissues were stored for ADH determination and for histochemical staining.

About 150 to 200 mg of frozen tissue were weighed on a Roller Smith balance, and transferred to a homogenizing vessel placed in an ice bath. Cold sucrose solution (0.25 M) was added in the homogenization in an amount twenty times the wet weight of tissue. The process was terminated as soon as visible tissue fragment disappeared. The homogenate was transferred and the debris was separated by means of a clinical centrifuge by spinning for 60 seconds at room temperature. The upper layer looked turbid

and red in color, and was stored cold for enzyme analysis.

Supernatants of these homogenates were prepared, when needed, by centrifuging at 12,000 x G (tip) at 0 ^{o}C for 15 minutes. The supernatant from this procedure was re-centrifuged twice under the same conditions.

SPECTROPHOTOMETRIC MEASUREMENT:

The amount of alcohol dehydrogenase can be calculated from the change in optical density of the reaction mixture taken at 340 nm. In this case the enzyme was measured in terms of the rate of appearance of NADH. The molar extinction coefficient of NADH at 340 nm, pH 7.5 is 5.76×10^{3}. The spectrophotometer was set at a slit opening of 0.04 mm, the cell compartment maintained at 25 ^{o}C.

0.5 ml of cold supernatant or homogenate was pipetted into the cuvette which contained 2.5 ml of glycine-NaOH buffer, pH 9.60. Final concentrations of the components in each cuvette were 0.527 M ethanol, 5.44×10^{-4} M NAD, 0.1 M glycine-NaOH, final pH 9.0. The blank cuvette contained everything except ethanol. Absorbance of the mixture was taken at 340 nm at half minute intervals for eight minutes.

HISTOCHEMICAL METHODS: Method of Nachlas et al (1957)

REAGENTS:

1. Nitro BT -- 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride, Sigma, Lot. 77B-5140
2. Isopentane -- Eastman Organic Co., Lot. 2370
3. Phosphate buffer -- 0.20 M pH 7.6 Mallinckrodt
4. Xylol -- Merck 6962
5. Ethanol -- U.S. Industrial Chemical Co.
6. Acetone -- Mallinckrodt, 2440
7. Saline -- 0.85% ice cold, Mallinckrodt

PROCEDURE:

Rats from both groups were sacrificed by decapitation. Liver, heart and kidney were removed immediately and washed in physiological saline. The tissues were cut into small blocks about 3 to 5 mm thick and were rapidly frozen by immersion in isopentane kept at -70 ^{o}C with a mixture of acetone and dry ice. The blocks were stored in the deep freeze at -20 ^{o}C. Using the cryostatic rotary microtome, sections about 6 microns thick were cut and mounted upon clean glass slides. The block of tissue retained activity for at least two three months.

A stock solution of buffered succinate was kept cold in the refrigerator. This solution was prepared by combining equal volumes of 0.20 M phosphate buffer pH 7.6 and 0.20 M sodium

succinate. When an incubation solution was needed, 10 ml of the buffered substrate reagent were added to 10 ml of an aqueous solution containing 10 mg of Nitro-BT. The final concentration of substrate was 0.05 M succinate.

Four to six mounted sections were placed in a Coplin jar containing 20 ml of the succinate-tetrazole mixture. The incubation was carried out at 25 ^{o}C for five minutes. Adequate color was noted at the end of incubation. The sections were washed in saline for 1 minute and then dehydrated by passing through graded alcohol solutions each for 5 minutes, using 30%, 50%, 70%, 80%, 95% and absolute ethanol. The slides were immersed in xylol for 10 minutes and then mounted with Permount.

In an attempt to demonstrate the presence of ADH, a similar experiment was done with 0.01 M ethanol as substrate. No significant color was observed at 25 ^{o}C or even at 34 ^{o}C for one hour.

ASSAY OF SUCCINIC DEHYDROGENASE: Method of Singer and Kearney (1957)

REAGENTS:

1. Kreb's manometric fluid (density = 1.033 g/ml at 20 °C):
   44 g anhydrous sodium bromide, Mallinckrodt
   0.3 g Triton X-100, Rohm and Haas Co.
   0.3 g Acid Fuchsin, W. H. Kessel and Co. C.I. 692
   1000 ml redistilled water
2. Phenazine methosulfate 1%, Aldrich Chemical Co., Milwaukee, Wis.
3. Potassium phosphate buffer pH 7.6, 0.3 M, Mallinckrodt
4. Sodium succinate, 0.2 M, neutralized
5. HCN, 0.01 M, pH 8, prepared from KCN by addition of 0.85 equivalent of HCl

PROCEDURE:

An albino rat 500-600 g was sacrificed by decapitation. The heart was removed immediately, washed in cold saline and diced. To the heart tissue was added twenty times its weight of 0.25 M sucrose solution for homogenization. The homogenate was centrifuged once with the clinical centrifuge to remove cell debris. The Warburg manometric instrument (Gilson Medical Electronics, Middleton, Wis.) was used for the assay of succinic dehydrogenase (SDH). The main compartment of the Warburg flask contained 0.5 ml phosphate buffer pH 7.6, 0.5 ml of heart homogenate, 0.3 ml

HCN and 1.2 ml distilled water. The side arm of the flask contained 0.3 ml succinate and 0.2 ml phenazine methosulfate. A thermobarometer was run with the sample manometers at a shaking speed of 120 cycles per minute. After 7 minutes of thermoequilibration at 38 ^{o}C, the reagents in the compartment and the side arm were mixed by tipping. The pressure was read at constant volume at two minute intervals. Protein was determined by the biuret method.

OXIDATIVE PHOSPHORYLATION BY MANOMETRIC TECHNIQUE: Hunter (1955)

Mitochondria and substrate were incubated in a medium containing inorganic orthophosphate and a phosphate acceptor system. Oxygen consumption and the removal of inorganic phosphate were measured. A Wartburg apparatus was used for the incubation. Reagents are listed in TABLE AA and TABLE AB.

PREPARATION OF RAT HEART MITOCHONDRIA: Tyler and Gonze (1967)

Five adult albino rats weighing 500-600 g were sacrified rapidly by decapitation. The hearts, rinsed in cold mannitol-sucrose-EDTA (MSE) solution, 0.3 M, were minced and washed again until fluid was clear. For homogenization, 5 ml of proteolytic enzyme solution, 2 mg/ml of Nargase, was added to the tissue and homogenized for 30 seconds, then diluted quickly to 20 ml with MSE and continued to homogenize until a smooth fluid was obtained. The preparation was diluted to 40 ml with MSE and centrifuged for

REAGENTS FOR MANOMETRIC MEASUREMENTS OF OXIDATIVE PHOSPHORYLATION

TABLE AA

Components in main compartment of flask

	Conc./2 ml	Final Conc./2.8 ml	Osmolar
1. K-phosphate buffer, pH 7.0	0.020 M	0.0143 M	0.0429
2. $MgCl_2$	0.010	0.0071	0.0214
3. Na_2-ATP	0.0025	0.0018	0.0034
4. Mannitol-sucrose(MS)	0.090	0.0643	0.0643
5. α-Ketoglutarate neutralized	0.015	0.0100	0.0321
6. Mitochondria in 0.3 M MS	0.5 ml	0.0536	0.0536
7. Bovine albumin	2 mg		
8. KOH in center well 5N	0.2 ml		

TABLE AB

Components in side arm of flask

	Conc./.3ml	Final conc./2.8 ml	Osmolar
1. Hexokinase	0.3 mg/ml	0.03 mg/ml	
2. Glucose	0.3 M	0.0321 M	0.0321
3. Mannito-sucrose	0.6 M	0.0643	0.0643
4. Bovine albumin	1 mg/ml		

Total osmolarity per flask was 0.348 at pH 7.0

10 minutes at 10,000 x G (tip). The supernatant containing the proteinase was discarded. The pellet was suspended in 40 ml MSE and centrifuged at 700 x G for 10 minutes. The supernatant was centrifuged at 10,000 x G for 10 minutes and the pellet was saved and washed by suspension in 40 ml MSE. The process was repeated three times. The pellet was resuspended in 2 ml Mannitol-sucrose solution. The mitochondrial protein was about 17 mg/ml as determined by the biuret method.

INCUBATION PROCEDURE:

The flasks were kept in cracked ice while being prepared. Except the hexokinase and glucose, all components in a volume of 2 ml were placed in the main compartment of the vessel, with 0.5 ml mitochondria preparation to be added at the last moment. The phosphate acceptor system (0.3 ml of hexokinase-glucose) was pipetted into the side arm and tipped in after 7 minutes equilibration at 30 ^{o}C. A thermobarometer was also equilibrated. Oxygen consumption was read every 2 minutes. At the end of 10 to 15 minutes incubation, the reaction was terminated by pipetting 2 ml of reaction mixture into 4 ml of cold 15% $HClO_4$. A control flask without substrate was deproteinized first to give the true zero-time phosphate value.

PHOSPHATE DETERMINATION:

REAGENTS:

A. Sulfuric acid, 5 N, Mallinckrodt, Lot. WSTE

B. Ammonium molybdate, 2.5% (w/v), Lot. NBX

C. Reducing reagent -- A freshly prepared solution made by adding 2 ml of distilled water to a 50 mg mixture of 1-amino-2-napthol-4-sulfonic acid, sodium bisulfite, sodium bisulfate (1:6:6).

PROCEDURE: A modification of the method of Fiske and Subbarow (1925)

A color reagent was prepared by mixing 20 ml of solution A with 20 ml of solution B and 2 ml of solution C in an Erlenmyer flask, just prior to use.

At the end of incubation 2 ml of reaction mixture was pipetted into a chilled 12 ml centrifuged tube containing 4 ml of ice-cold perchloric acid, 15%. The tube was spun in an International Clinical centrifuge for 5 minutes. The supernatant was saved.

A measured quantity, usually 100-200 µl of perchloric acid supernatant was pipetted into a graduated colorimeter tube containing 7.5 ml of water. The micropipette was washed out twice with the resulting solution. After all tubes were prepared, a timer was started and 2 ml of the color reagent was pipetted into the first tube and mixed, 30 seconds later, the second tube, and so on. The solution in each tube was then diluted to 10 ml mark

and mixed thoroughly. Exactly 15 minutes from the addition of color reagent, the absorbance was read on the Klett-Summerson colorimeter using No. 60 filter.

OXIDATIVE PHOSPHORYLATION - ALTERNATE METHOD:

A modification of the method of Chance and Hagihara (1967) was used to isolate mitochondria from heart.

REAGENTS:

1. Mannitol-sucrose-EDTA medium (MSE) -- 0.225 M mannitol, 0.075 M sucrose, 0.05 mM EDTA, pH 7.4
2. Unneutralized tris buffer -- Tris (hydroxymethyl) amino methane Sigma, Lot. 123B-5060
3. Crystalline bacterial proteinase -- "Nagarse" U.S. distributor: Enzyme Development Corp. 64 Wall St. New York, N.Y. Nagarse and Co. LTD, Osaka, Japan
4. Chance and Hagihara reaction medium:

   Sucrose, 0.070 M

   Mannitol, 0.225 M

   EDTA 1 mM

   Potassium phosphate buffer, 0.010 M, pH 7.2

PROCEDURE:

All containers were kept cold and surrounded with ice. The Servall centrifuge model was set to run at 0 °C. The rat was sacrified by decapitation and allowed to bleed for 30 seconds. The heart was removed immediately and washed in cold MSE medium, blotted dry weighed, and minced with a scalpel. The tissue was then washed with MSE until the washing fluid looked clear and colorless. An enzyme solution containing 5 mg of Nagarse, 2.5 ml MSE and 50 µl of 1 M Tris base (unneutralized) was added to the tissue. The mixture was transferred to a glass homogenizer fitted with Teflon pestle, 30 seconds after addition of enzyme solution, 20 ml MSE was added and homogenized until a smooth uniform homogenate was obtained. The tissue must not be exposed to the concentrated proteinase for more than 30 seconds. The homogenate was centrifuged immediately.

CENTRIFUGATION OF THE HOMOGENATE:

1. The homogenate was then diluted to 40 ml, divided equally into two tubes, and spun at 14,500 x G (measured at the tip of the centrifuge tube) for 10 minutes using Servall head type SS-34 at 11,000 RPM. The supernatant, which contained the proteinase, was discarded. The whole pellet was resuspended in 40 ml MSE by hand with the aid of a teflon pestle in a glass homogenizer.
2. After a second centrifugation at 800 x G for 10 min, the sup-

natant was divided into two tubes. The pellet, which contained nuclei, red cells and debris, was discarded.

3. The third centrifugation at 14,500 x G for 10 min, yielded a brown pellet (mitochondrial fraction) covered by a loosely packed fluffy layer and a supernatant which was discarded. The tubes were shaken gently with a small volume of MSE to remove the fluffy layer as completely as possible from the tightly packed brown pellet.
4. The mitochondrial fraction was gently broken up with a glass rod, resuspended in 20 ml MSE. The preparation was centrifuged at 14,500 x G for 10 min. This step was repeated two times.
5. The mitochondria were then suspended in 0.6 to 1.0 ml MSE and placed in an ice bath. The mitochondrial protein was determined by the biuret method, about 20 mg/ml.

INCUBATION OF MITOCHONDRIA:

Instruments:

Clark electrode: Model 5331 oxygen probe, Yellow Spring Instrument.

Reaction bath : Model 5301 bath assembly, 4 reaction chambers, YSI.

Recorder : Servo recorder, Model EU-20 B, Heath.

PROCEDURE:

Five ml of reaction medium was pipetted into each reaction chamber and equilibrated at 25 °C with stirring. The lucite plunger was inserted above the surface. 25% oxygen or air was bubbled through the medium using a flexible plastic needle (CRC Lab Apparatus) for at least 30 min. After equilibration, the oxygen needle was pulled out and the Clark electrode was inserted into the plunger body and tightened. Air bubbles were eliminated through the vertical vent in the lucite plunger. The reaction medium must be always below the top of the vent.

The recorder was adjusted to 85% of full scale when the reaction medium was equilibrated with the gas used. The paper speed was adjustable, usually 2 min per inch. 100 µl of mitochondrial suspension containing 1-3 mg protein was injected with plastic needle and syringe. After a two minute incubation, 100 µl of substrate was injected and followed by 50 µl of ADP (1.23 µmoles) or other reagent and oxygen consumption measured. Another two or three additions of ADP were usually required to bring the solution to the anaerobic state. α-Ketoglutarate, pyruvate-malate and succinate were used as substrates. Time, atmospheric pressure and temperature were recorded.

DETERMINATION OF ADP:

Test Principle:

ADP was determined by using enzymes from C.P. Boehringer und Soehne. G.m.b.H. Mannheim, Germany.

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{PK}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{MK}} 2\ \text{ADP}$$

Assay Mixture:

V = 3 ml $\quad\quad$ λ = 340 nm

d = 1 cm $\quad\quad$ T = 25 °C

1. 2.760 ml Tris buffer, 0.1 M, pH 7.6
2. 0.150 ml PEP, phosphoenolpyruvate, $C_3H_4O_6PNa.H_2O$, 5 mg/ml dissolved in 0.5 M $MgSO_4$/2 M KCl.
3. 0.060 ml $NADH.Na_2(3.5\ H_2O)$ MW = 771, Sigma Lot. 67B-6440, 98% purity, 6 mg/ml in distilled water.
4. 0.010 ml LDH, lactic acid dehydrogenase, 5 mg/ml.
5. 0.010 ml PK, pyruvate kinase, 10 mg/ml.
6. 0.010 ml MK, myokinase, 2 mg/ml.
7. 0.010 ml $ADP.Na_2$ Sigma, Lot. 129B-7291, (0.246 µmoles/10µl)

PROCEDURE:

Except for ADP and myokinase, the reaction mixture was pipetted into a cuvette and mixed. The initial optical density was taken, 10 µl of ADP were added and the contents gently mixed by inversion. The second reading of optical density was recorded one minute later, 10 µl of myokinase were added and mixed gently. The third reading was taken 15 minutes later. The addition of myokinase permits calculation of the quantity of AMP present.

DETERMINATION OF OXYGEN CONTENT IN THE REACTION MEDIUM:

PREPARATION OF MITOCHONDRIAL ENZYME (Estabrook, 1958):

A fed, normal rat, weighing 600 g was decapitated. The liver weighing about 13 g, was removed, washed and chilled immediately with 0.25 M sucrose which was used as preparative medium throughout the procedure. The liver was chopped, washed, homogenized and diluted to 130 ml (1:10). The homogenate was divided evenly into tubes and centrifuged at 1000 x G for 15 minutes twice to remove cell debris, nuclei. The supernatant was centrifuged at 14,500 x G for 15 minutes. The mitochondria were washed three times with 26 ml of sucrose (1:2).

The mitochondria were lysed by suspending in 40 ml of cold redistilled water. After 2 hours at 4 ^{o}C, the mixture was centrifuged at 14,500 x G for 20 minutes. The yellow supernatant was discarded. The pellet was suspended in 40 ml of 0.85% saline

and centrifuged at 14,500 x G for 10 minutes. This washing was repeated once. The mitochondrial pellet was finally resuspended in 0.1 M sodium-potassium phosphate buffer pH 7.4 so that 1 ml of suspension represented 5 g of liver, or 2.5 ml of buffer for 13 g of liver. The mitochondrial protein was about 20 mg/ml determined by biuret method.

DETERMINATION OF NADH:

Since NADH is stable in solution for only a few hours, it was used soon after preparation. NADH.Na.(3.5 H_2O), Lot. 67B-6440, 98% pure, was obtained from Sigma. Two batches were prepared, each weighing 0.0155 g/ml, about 0.02 M, dissolved in 0.05 M Na-K-phosphate buffer, pH 7.5. The amount of NADH was determined directly with a Beckman DU spectrophotometer. 20 µl of NADH solution were added to 3 ml of 0.1 M Na-K-phosphate buffer pH 7.5. Phosphate buffer was used as blank of which the optical density was negligible. The absorbance was read at 340 nm and 25 °C. The results were shown in TABLE AC.

MEASUREMENT OF NADH CONTENT OF STOCK SOLUTIONS

TABLE AC

Optical density of NADH solution

NADH-1			NADH-2	
Cell 19	Cell 11	Blank	Cell 20	Cell 18
0.588	0.588	-0.002	0.670	0.668
0.590	0.588	-0.004	0.670	0.668
0.587	0.590	-0.003	0.670	0.668
0.587	0.589	-0.003	0.670	0.668
Average = 0.588			Average = 0.669	

Calculated values:

NADH-1	NADH-2
0.0154 M	0.0175 M
0.0154 μmoles/μl	0.0175 μmoles/μl
0.771 μmoles/50 μl	0.877 μmoles/50 μl

INCUBATION PROCEDURE:

The method by Chappell (1964) was followed. 5 ml of Chance and Hagihara reaction medium was pipetted into each reaction chamber and saturated with 25.7% oxygen. After 30 min of equilibration at 25 °C, 100 µl of lysed mitochondria was injected. A known amount of NADH was injected 2 min later in 50 µl volume and the decrease in oxygen tension was recorded. The process was repeated until the medium was anaerobic. The deflexions observed were summarized in TABLE AD.

Calculation:

Average of three trials = 0.589 µgatom O_2/ml

Correction for 98% purity of NADH

= 0.589 x 0.98

= 0.577 µgatom O_2/ml

Oxygen content in 5 ml reaction medium

= 0.577 µgatom/ml x 5ml

= 2.89 µgatom

TABLE AD

Determination of oxygen content

	NADH µl	NADH µmoles	R'	R	r	O_2 µgatom/ml
1.	50	0.771	22.9	90.2	4.4	0.561
	50	0.771	23.2	90.2	4.4	0.548
	50	0.771	21.1	90.2	4.4	0.597
2.	50	0.771	21.0	90.6	4.7	0.612
	50	0.771	21.0	90.6	4.7	0.606
	50	0.771	20.7	90.6	4.7	0.609
	50	0.771	20.4	90.6	4.7	0.613
3.	50	0.877	26.1	90.5	5.2	0.557
	50	0.877	24.8	90.5	5.2	0.580
	50	0.877	23.6	90.5	5.2	0.604

Calculation:

$$O_2 = \frac{(R-r)(\mu\text{moles NADH})}{R' \times V} \ \mu\text{gatom/ml}$$

R = Initial deflexion

R'= deflexion on addition of NADH

r = Final deflexion

V = Total volume of reaction medium in ml

ATMOSPHERIC PRESSURE CORRECTIONS:

The atmospheric pressure was obtained daily from the National Weather Service Broadcast. This value has been corrected to sea level and, after conversion to millimeters, was re-corrected to the local altitude by subtracting 18 mm Hg. Total gas pressure in the mixture was then calculated by subtracting the water vapor pressure at the experimental temperature. Unsettled and stormy weather was avoided so that fluctuations in pressure during experiments were negligible.

PROTEIN DETERMINATION: BIURET METHOD

BIURET REAGENTS:

A. Copper sulfate, 0.375

B. Sodium potassium tartarate, 1.5 g

C. Sodium hydroxide, 50%, 15 ml

D. Dissolved A, B, C in redistilled water and diluted to 250 ml mark in volumetric flask.

PROCEDURE:

Twenty microliters of protein solution were added to a microtube, 20 µl distilled water were added to a similar tube (blank). To both tubes, 200 µl of biuret reagent were added and mixed. After 30 minutes standing at room temperature, the absorbance was taken at 540 nm. A standard curve was prepared with a range of 10 to 30 mg/ml of protein, using bovine serum albumin.

CHAPTER III

RESULTS AND DISCUSSION

It has been reported that, although ethanol is oxidized by heart tissue (Bartlett and Barnet, 1949) the pathway is different from that in liver because of the reported absence of alcohol dehydrogenase (Mezey et al, 1968) and the possibility of oxidation via catalase and H_2O_2 produced in the tissue (Griffaton and Lowy, 1964). Since the purpose of this research was to determine the effects of chronic ingestion of ethanol on the biochemistry of cardiac tissue it was important to determine the presence or absence of alcohol dehydrogenase (ADH), the enzyme which has been shown to oxidize the major amount of ethanol in liver (Dajani et al, 1963).

Since the levels of alcohol dehydrogenase in heart are expected to be low, it is important to be sure that the ADH assay method actually measures ADH. Therefore, the published method for ADH measurement in liver tissue was re-examined (Bonnichsen, 1955). The procedure depends on the measurement of the difference in absorbance at 340 nm between solutions which contain ethanol, tissue preparation, buffer etc., and solutions identical except the omission of ethanol. The value for ΔD_{340} at two minutes indicates the amount of alcohol dehydrogenase present. For homogenate of frozen liver tissue, ΔD_{340} increases with time (FIGURE A-1), but this increase is due to a less rapid decrease of D_{340} in

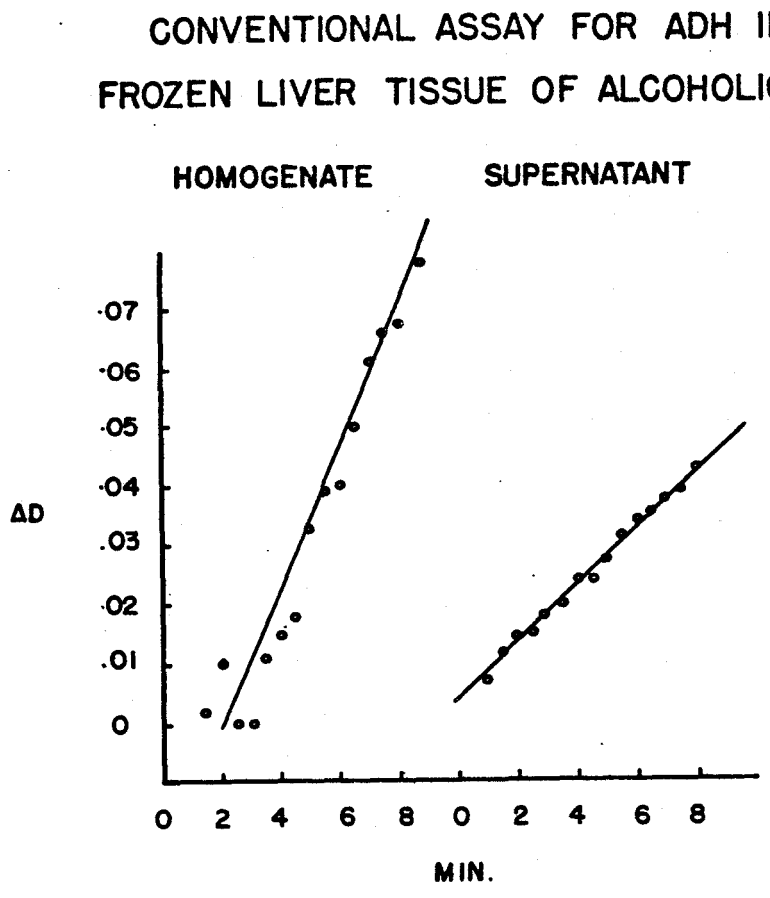

FIGURE A-1

Homogenate and supernatant were prepared from frozen liver of rat at 35 weeks of exposure to ethanol. The tissue was homogenized in 80 ml of 0.25 M sucrose per gm wet weight. At zero time, 0.5 ml of homogenate was added to a cuvette which contained 2.5 ml of 0.1 M glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD. 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was taken at half a minute intervals for eight minutes.

TABLE A-1a

Absorbance at 340 nm in the assay of alcohol dehydrogenase in homogenate from frozen liver tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.668		
	0.660	0.658	0.002
2	0.660	0.650	0.010
	0.640	0.640	0.000
3	0.633	0.633	0.000
	0.635	0.624	0.011
4	0.635	0.620	0.015
	0.635	0.617	0.018
5	0.640	0.607	0.033
	0.639	0.600	0.039
6	0.640	0.600	0.040
	0.650	0.600	0.050
7	0.650	0.589	0.061
	0.650	0.584	0.066
8	0.652	0.584	0.068
	0.659	0.581	0.078
9	0.664	0.587	0.077

the solutions containing ethanol than in those which do not (TABLE A-1a). The 12,000 x G supernatant from this preparation has, what seems to be, a lower value of ADH per ml., but the values of D_{340} rise in both ethanol containing and non-ethanol solutions, and the ΔD_{340} is really an increase in NADH due to the presence of ethanol. Since ADH is not found in the mitochondria or bound to large particles, removal of these components should not affect the assay for ADH. The change observed in homogenate is due to the multiple reactions in supernatant, mitochondria etc., which can either produce or remove NADH (and NADPH). Removal of the mitochondria and debris reduces both NADH removal and NADH production. Thus ΔD_{340} of supernatant should give a better measurement of ADH. For comparison, crystalline equine ADH (liver) was assayed using the solutions (FIGURE A-2, TABLE A-2).

This approach was used with supernatant from homogenates of frozen rat heart except that it was not possible to dilute the tissue as much because the ADH activity was so much lower. From a number of experiments similar to those in FIGUREs A-3 and A-4, it seemed reasonable to expect that the slope of the best straight line through the first three or four points should give the same value for alcohol dehydrogenase activity as the value at two minutes as recommended by Bonnichsen (1955). The use of the slope also protects against an incorrect reading at two minutes. Even with this procedure, the values of duplicate analyses sometimes varied considerably. FIGURE A-5 shows the individual analyses of

TABLE A-1b

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from frozen liver tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.125	0.118	0.007
	0.130	0.119	0.011
2	0.135	0.121	0.014
	0.138	0.123	0.015
3	0.143	0.125	0.018
	0.147	0.127	0.020
4	0.152	0.128	0.024
	0.155	0.131	0.024
5	0.160	0.133	0.027
	0.165	0.134	0.031
6	0.169	0.135	0.034
	0.173	0.138	0.035
7	0.177	0.140	0.037
	0.181	0.142	0.039
8	0.186	0.143	0.043

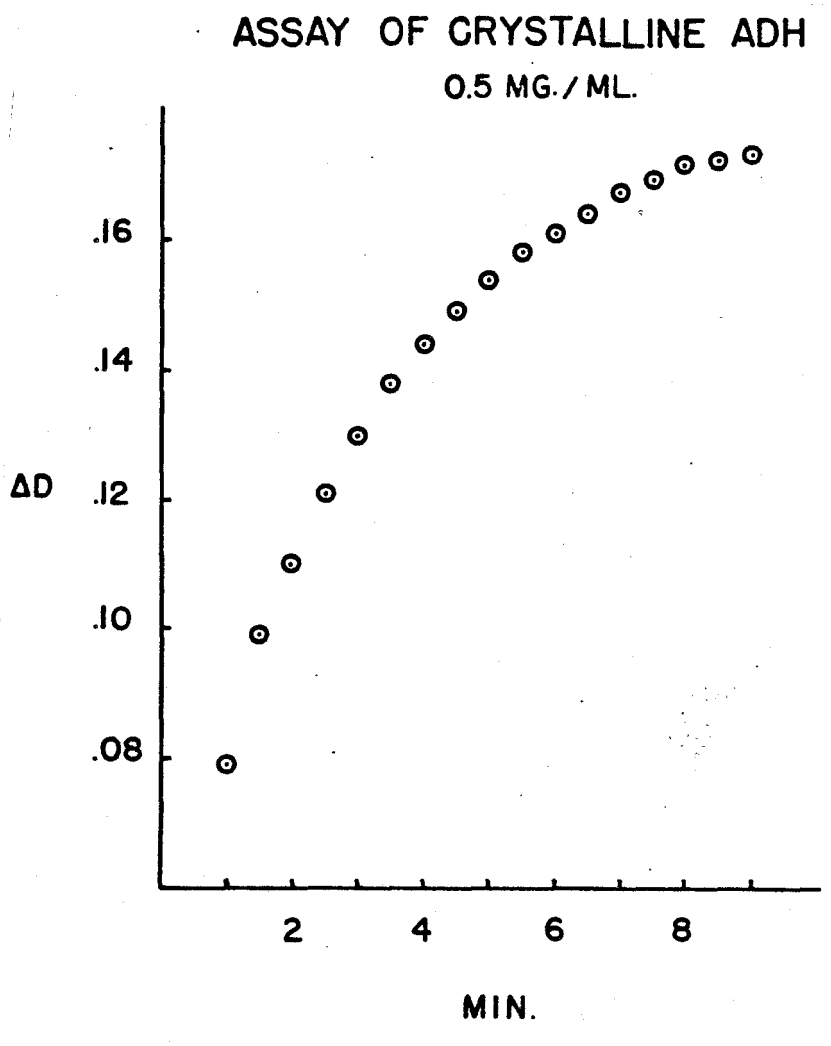

FIGURE A-2

Crystalline alcohol dehydrogenase, Sigma Co., Lot. 66B-0460, was dissolved (0.5 mg/ml) in cold 0.01 M phosphate buffer, pH 7.4. At zero time, 0.5 ml of enzyme solution was added to a cuvette which contained 2.5 ml of 0.1 M glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD, 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was taken at half a minute intervals for nine minutes.

TABLE A-2

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase (0.5 mg/ml)

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.308	0.223	0.085
	0.330	0.228	0.102
2	0.344	0.232	0.112
	0.357	0.234	0.123
3	0.369	0.235	0.134
	0.376	0.235	0.141
4	0.382	0.235	0.147
	0.388	0.236	0.152
5	0.392	0.236	0.156
	0.395	0.237	0.156
6	0.400	0.237	0.163
	0.402	0.237	0.165
7	0.404	0.237	0.167
	0.406	0.237	0.169
8	0.408	0.237	0.171
	0.409	0.237	0.172
9	0.410	0.237	0.173

CONVENTIONAL ASSAY FOR ADH IN FROZEN HEART TISSUE OF NORMAL RAT

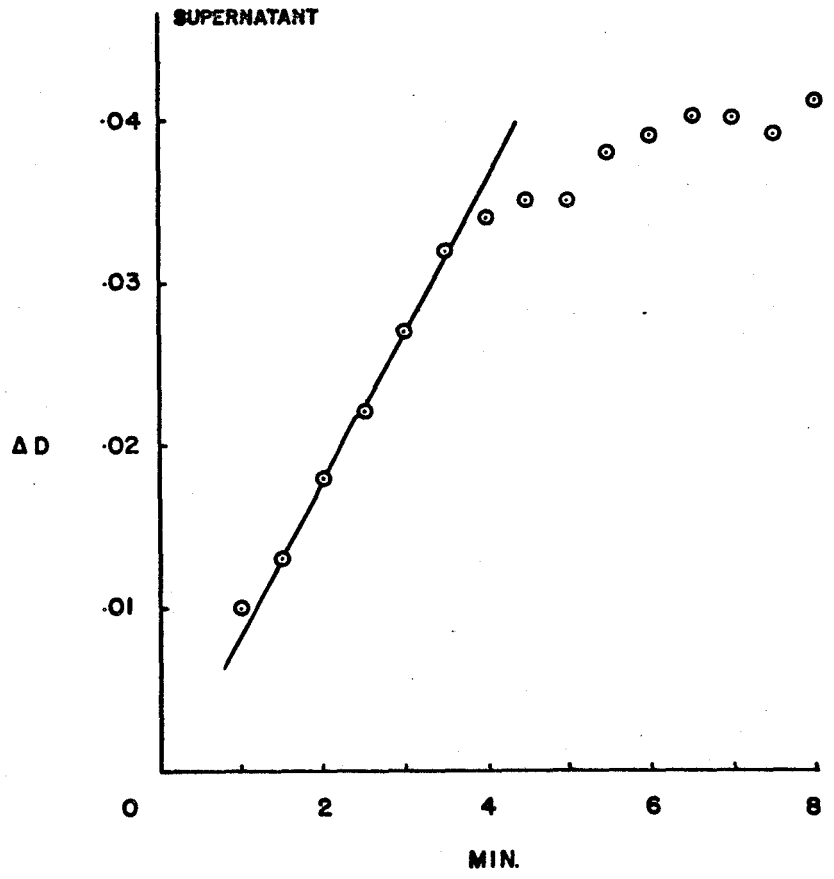

FIGURE A-3

Supernatant of frozen heart tissue was prepared from control rat at the same time as ethanol-treated rat in FIGURE A-1. The tissue was homogenized in 20 ml 0.25 M sucrose per gm wet weight. At zero time, 0.5 ml of supernatant was added to a cuvette which contained 2.5 ml of 0.1 M glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD, 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was taken at half a minute intervals for eight minutes.

CONVENTIONAL ASSAY FOR ADH IN
FROZEN HEART TISSUE OF ALCOHOLIC RAT

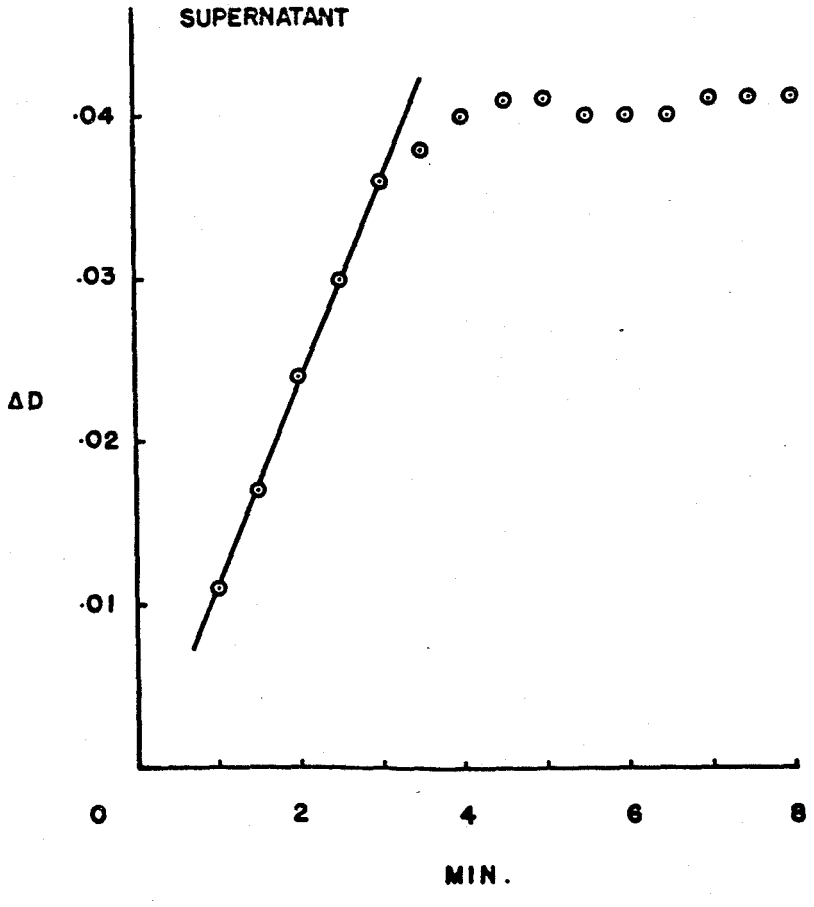

FIGURE A-4

Supernatant of frozen heart tissue was prepared from rat after 14 weeks of exposure to ethanol. The tissue was homogenized in 20 ml of 0.25 M sucrose per gm wet weight. At zero time, 0.5 ml of supernatant was added to a cuvette which contained 2.5 ml of 0.1 M glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD, 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was taken at half a minute intervals for eight minutes.

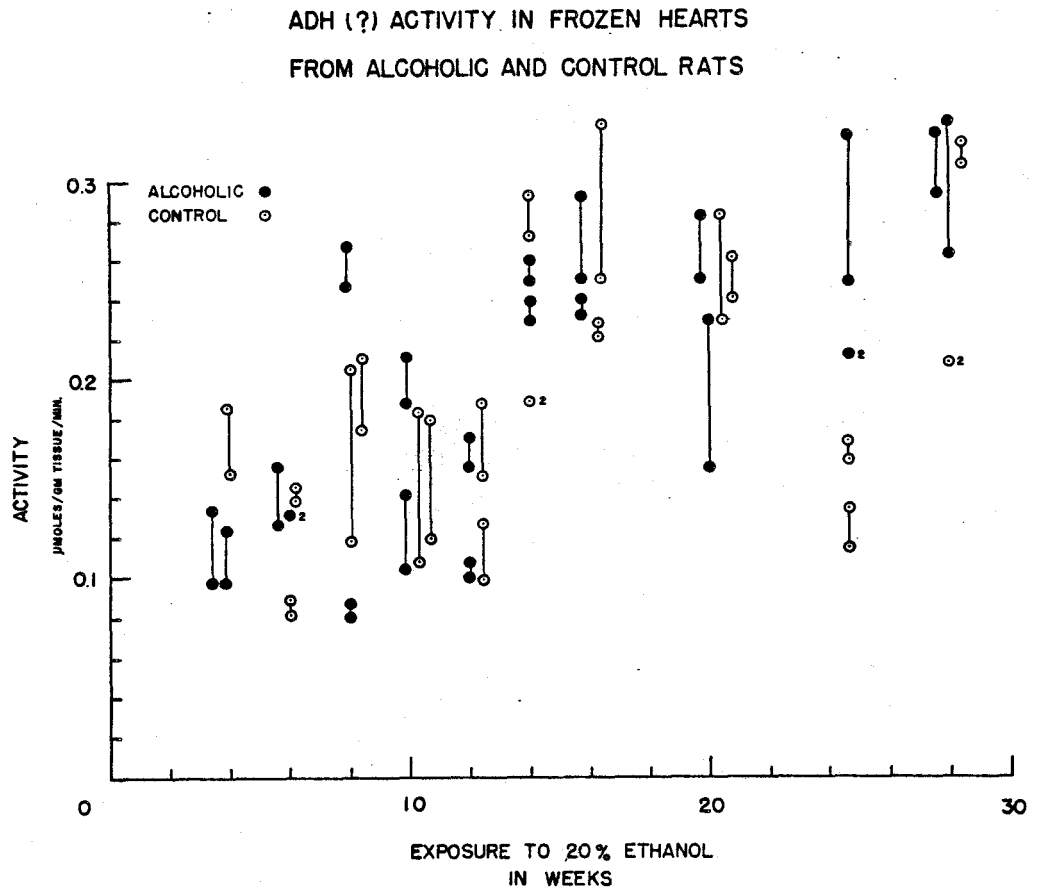

FIGURE A-5

The effect of age and ethanol ingestion on rat heart alcohol dehydrogenase in 12,000 x G supernatants of homogenates of tissue 5:1 (v/w) in 0.25 M sucrose. Tissues were quick-frozen and stored 2-4 weeks prior to homogenization and assay.

tissue from two control rats and two experimental rats, sacrificed at intervals over a period of twenty eight weeks of exposure of experimental animals to 20% ethanol. Within the limits of the assay method, ADH activity seems to be affected more by age than by exposure to ethanol.

One possible influence on the measurement of ADH activity was the quick-freezing process used to preserve the tissue. The effect of this process was explored using fresh rat heart tissue. In FIGURE A-6, it can be seen that D_{340} of the non-ethanol solution decreases steadily from the beginning. This indicates that NADH removing enzymes are predominant in the supernatant solution. For comparison a similar experiment was done with fresh rat liver (TABLE A-7b, p. 88). The supernatant of fresh rat liver homogenate contains a slight excess of NADH producing activities, and that is suitable for the assay of ADH (FIGURE A-7). In contrast, the supernatant from fresh heart tissue homogenate contains so much NADH removing activity (TABLE A-7a, p. 87) that assay for ADH is not feasible. This conclusion was reinforced by adding crystalline equine liver ADH to heart supernatant, (FIGURE A-8, A and A'). The ΔD_{340} due to crystalline ADH plus ethanol is obviously greater than the value obtained when an equal amount of ADH is added to supernatant from rat heart homogenate.

These rather confusing results can be understood when one recalls the differences between heart tissue and liver tissue. The latter is soft and easy to homogenize and few mitochondria are

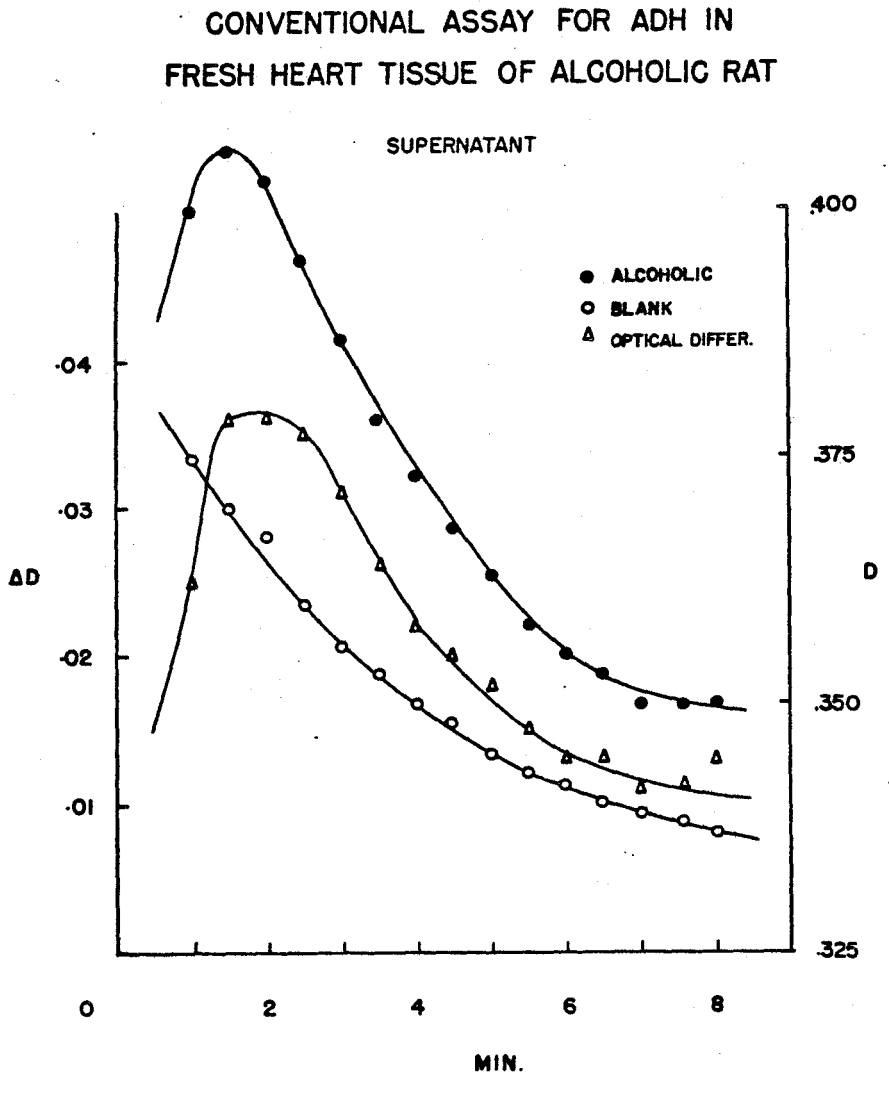

FIGURE A-6

Supernatant was prepared from fresh heart tissue homogenate (5:1 v/w) of rat at 40 weeks ethanol ingestion. The reaction was initiated by adding 0.5 ml of supernatant to a cuvette containing 2.5 ml of 0.1 M glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD, 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was measured at half a minute interval for eight minutes.

CONVENTIONAL ASSAY FOR ADH IN FRESH LIVER AND HEART SUPERNATANT OF ALCOHOLIC RAT

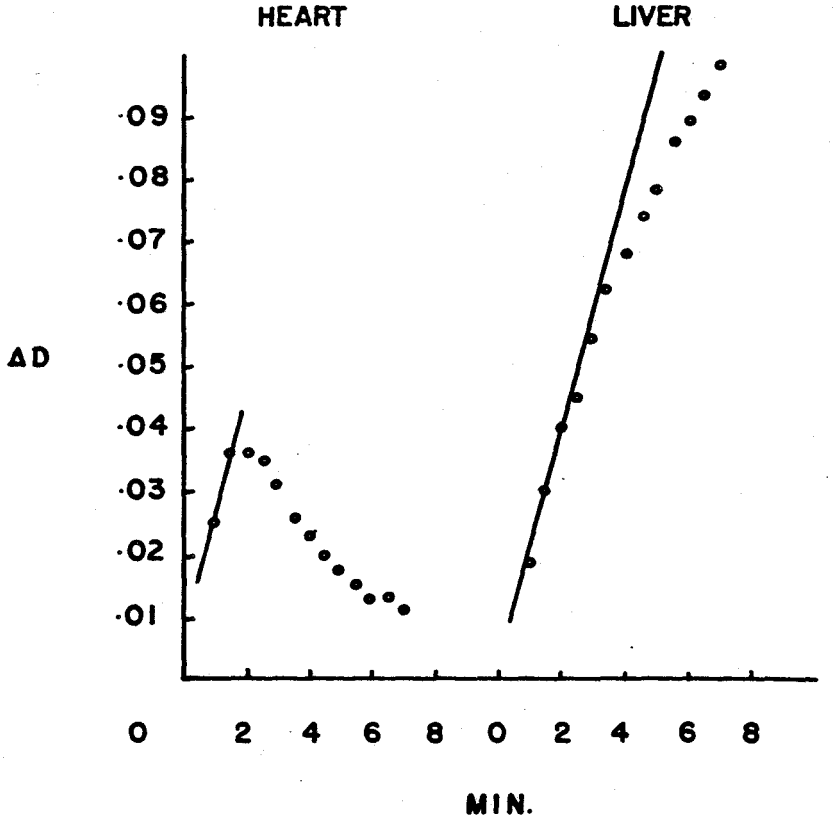

FIGURE A-7

Supernatants were prepared from fresh liver and heart tissues of rat at 40 weeks of ethanol ingestion. The tissues were both added with 0.25 M sucrose for homogenization (liver 20:1, heart 5:1, v/w). The homogenates were centrifuged at 12,000 x G at 0 °C. The reaction was initiated by adding 0.5 ml supernatant to a cuvette containing 2.5 ml of glycine-NaOH buffer, pH 9.6, 5.44×10^{-4} M NAD, 0.5 M ethanol. The blank contained everything except ethanol. The absorbance at 340 nm was measured at half a minute intervals for eight minutes.

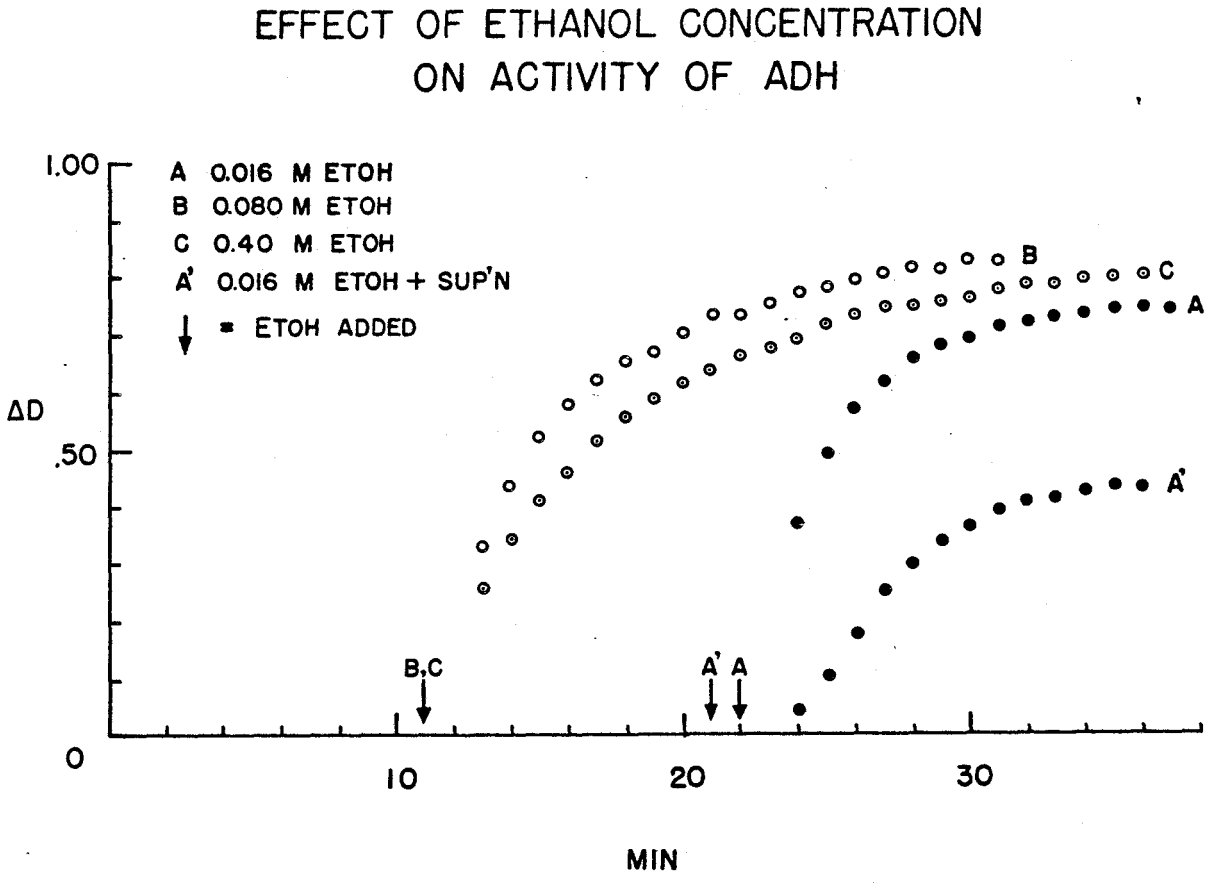

FIGURE A-8

Crystalline equine liver alcohol dehydrogenase (Sigma) was dissolved (0.2 mg/ml) in 0.01 M phosphate buffer, pH 7.0. The assay medium contained 1.5 ml 0.1 M glycine-NaOH buffer pH 9.6, 5.44×10^{-4} M NAD, 1 ml of enzyme solution. After temperature equilibration, aqueous ethanol was added as indicated to give the final concentrations shown. Absorbance was measured at 340 nm at intervals. Final concentration of crystalline ADH in each cuvette was 0.067 mg/ml.

broken in the process. Therefore the 12,000 x G supernatant from a fresh liver homogenate has little NADH oxidase activity and an assay for ADH is possible although not necessarily correct. On the other hand, heart tissue is difficult to homogenize and the 12,000 x G supernatant from fresh heart homogenate contains a considerable number of broken mitochondria - a significant amount of enzymes which catalyse the oxidation of NADH. Therefore a meaningful assay for ADH activity by this method is not feasible in fresh heart tissue. Another possible sequence of reactions which might be important in the removal of NADH is the "microsomal ethanol oxidizing system" (MEOS) described by Lieber and DeCarli (1970) in liver. This involves the reaction of NADPH with ethanol to form acetaldehyde and NADP. Catalysts required would be trans-hydrogenase and MEOS, and the activity would be increased by ethanol. If these systems were present in heart tissue, they could form part of the NADH removing activity which complicates measurement of ADH activity in supernatants of fresh heart homogenates.

Further experiments were undertaken in an effort to find a method which could be used with fresh heart tissue. von Wartberg (1965) recommended pre-incubation of tissue extract with the buffer-NADH reaction medium, with ethanol added to initiate the reaction. When this method was used with fresh heart tissue, the previously observed drop in ΔD_{340} was observed during the first ten minutes in all samples (TABLE A-8D, p. 93), but there was no

significant difference between blank and experimental cuvettes after the addition of ethanol to one and water to the other. The presence of some ADH-like activity in this preparation is indicated by the data in TABLE A-8E, p. 94 in which ethanol was added to the experimental cuvette immediately after the tissue preparation. This suggests that pre-incubation with the NADH-containing reaction mixture either removes or inhibits ADH activity. This conclusion was confirmed by experiments with crystalline equine liver ADH shown in FIGURE A-8, which also confirmed the fact that 0.016 M ethanol gives higher values of ΔD_{340} at two minutes than 0.08 or 0.40 M ethanol. Using the best level of ethanol (0.016 M), the activity of crystalline ADH (measured two minutes after addition of ethanol) is virtually completely removed by pre-incubation with heart supernatant and the NADH-containing reaction mixture (curve A' compared with A in FIGURE A-8). An effort was made to avoid this problem by incorporating a reducing agent which is particularly effective in maintaining -SH groups in reduced form (Cleland's Reagent, dithiothreitol: Cleland, 1964). FIGURE A-9 demonstrates the lack of success, and the probability that oxidation of the -SH groups of ADH is not the basis for the suppression of ADH activity by the supernatant of homogenates of fresh rat hearts.

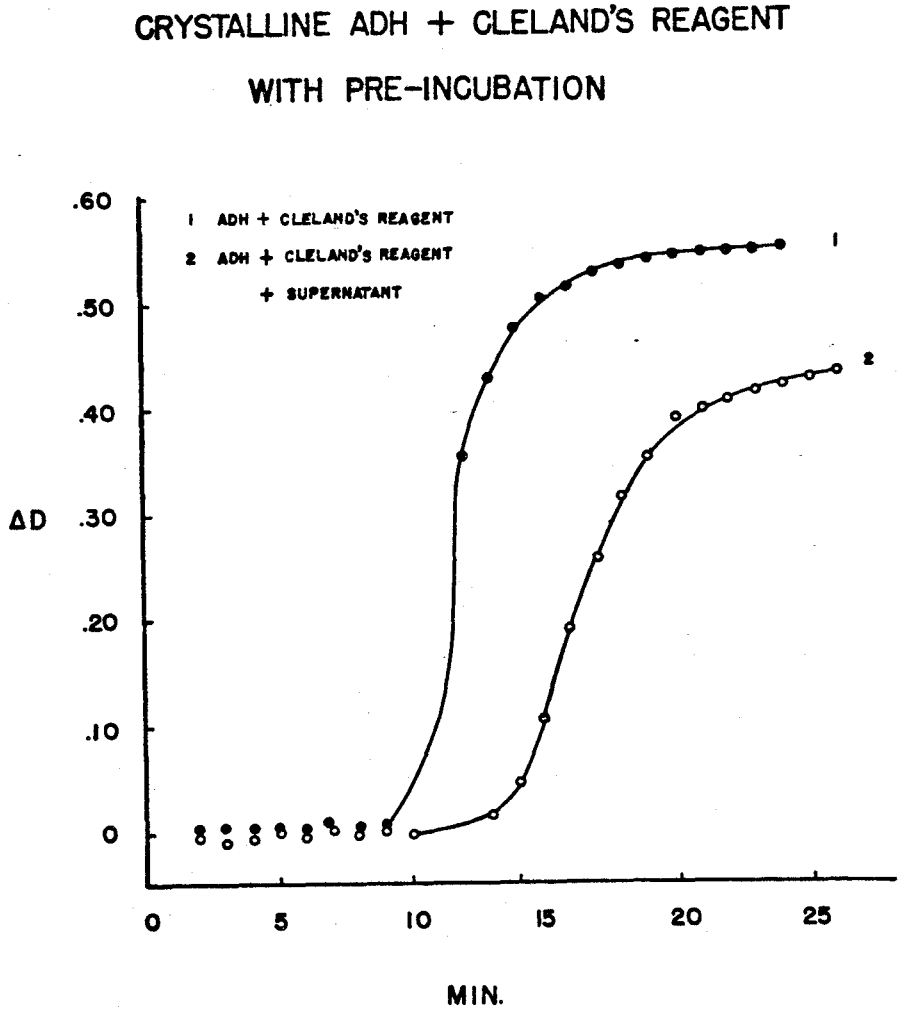

FIGURE A-9

Crystalline equine liver alcohol dehydrogenase (Sigma) was pre-incubated at 25 °C in buffer, NAD, and Cleland's reagent with or without addition of 0.5 ml rat heart supernatant. One half ml of diluted ethanol or water was added at t = 10 minutes and the absorbance at 340 nm measured as a function of time. The final concentrations were: 0.05 M buffer, 2.72×10^{-4} M NAD, 5×10^{-5} M Cleland's reagent, 0.016 M ethanol, 0.067 mg/ml ADH.

Frozen tissues are somewhat different. There is considerable loss of mitochondrial oxidative activity on freezing so that a crude assay for ADH in supernatant of frozen heart is possible. The conclusion to be drawn from these measurements is that ADH is present in heart tissue, but is difficult to demonstrate in fresh tissue because of interference from enzymes which rapidly remove the product being measured. Furthermore, its quantitative measurement in heart tissue by accepted methods is not possible.

It is generally accepted that succinic dehydrogenase (SDH) is confined to mitochondria (Singer and Kearney, 1957). It is therefore reasonable to assume that an increase in the number of normal mitochondria in a tissue would result in a corresponding increase in the succinic dehydrogenase per gram of that tissue.

Preliminary histological experiments were done on sections of freshly frozen heart, liver and kidney tissue using the procedure of Nachlas et al (1957). With 0.05 M succinate as the added substrate, each of the tissues showed easily detectable precipitates of the nitro-formazan after five minutes of exposure at 25 ^{o}C. However, no difference in color was observed when tissues from five control rats were compared with tissues from the same organ from five rats which had been given 20% as drinking fluid for six to eight months. Rats maintained on the regime used in this research were reported by Porta and Gomez-Dumm (1968) to consume approximately 17 gm of ethanol per Kg per day. This

indicates that a substantial ethanol intake for a period of four weeks does not markedly affect the activity of succinic dehydrogenase as measured by this histological technique. If the increase in the number of mitochondria in heart, reported by Szanto et al (1967), is accompanied by normal enzyme content, an increase in SDH would be expected.

In principle, this staining method should be usable for demonstration of alcohol dehydrogenase activity, because the NADH produced could be reoxidized by the mitochondrial electron transport system, transferring the electrons to the nitrotetrazolium to give the formazan precipitate. A pilot experiment on heart tissue, using 0.01 M ethanol gave no color (compared to incubation solutions without ethanol) at 34 ^{o}C for time periods up to 60 minutes. The conclusion is that ADH in rat heart is very low.

Experiments using the assay method of Singer and Kearney (1957) showed that approximately 20% of the succinic dehydrogenase (SDH) activity of rat heart homogenate remained in the 12,000 x G supernatant (FIGUREs B-1, B-2). This indicates considerable damage to heart mitochondria and, although this does not affect the assay for SDH, it supports the interpretation given earlier for difficulties in the assay for alcohol dehydrogenase in similar heart supernatants. (The SDH activity is undoubtedly bound to mitochondrial fragments which also retain NADH oxidase activity).

TABLE B shows that hearts of rats given 20% ethanol for four to five months have more SDH per milligram homogenate protein

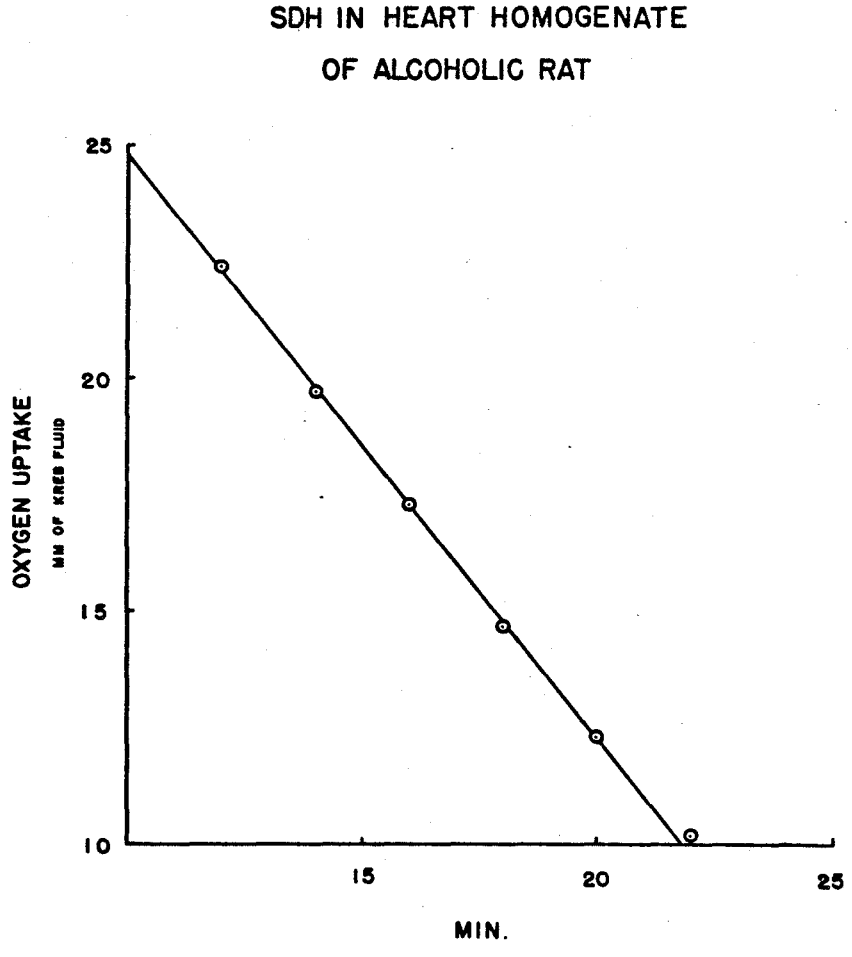

FIGURE B-1

Succinic dehydrogenase was assayed with heart homogenate from rat on 42 weeks of ethanol ingestion. The protein concentration of the homogenate was 5.45 mg/ml. The oxygen consumption in mm of Kreb's fluid was plotted versus time in minutes.

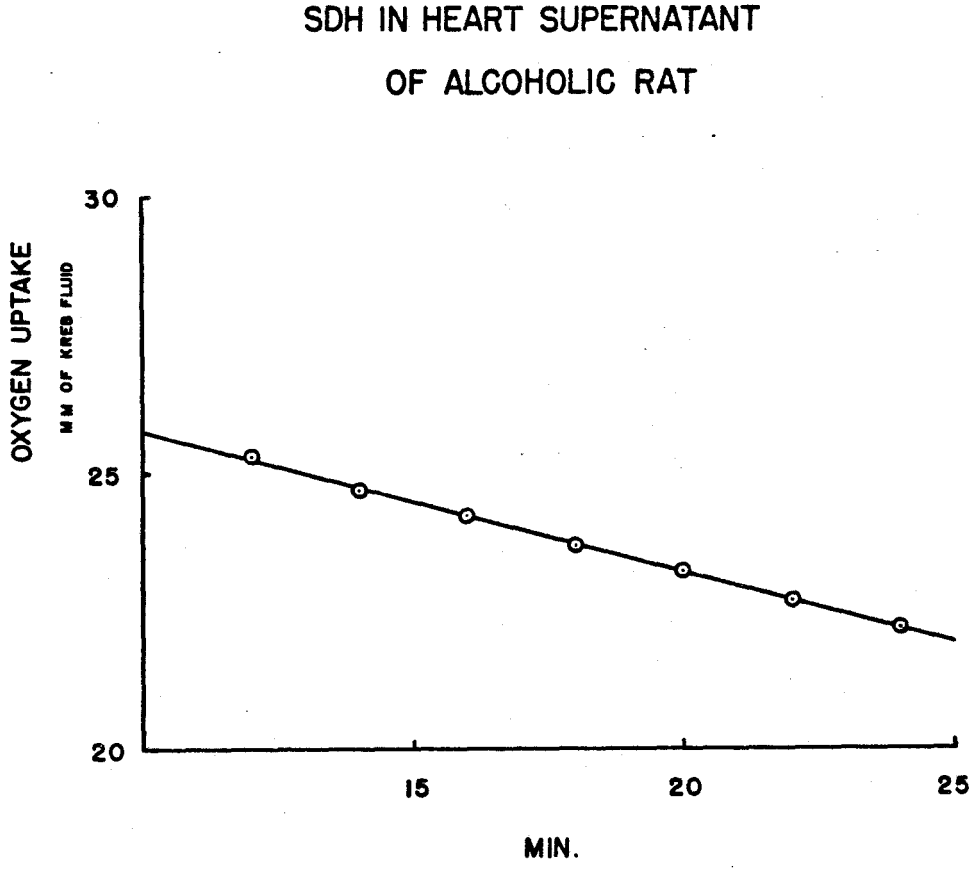

FIGURE B-2

Succinic dehydrogenase was assayed in fresh heart supernatant from rat on 42 weeks of ethanol ingestion. The protein in the homogenate was 1.5 mg/ml. The oxygen consumption in mm of Kreb's fluid was plotted as a function of time.

TABLE B

Activity of succinate dehydrogenase in homogenate of fresh tissue of rat heart

Rat	Age weeks	Body g	Hom. Prot. mg/ml	µl O_2/10/mg
CR94	28	540	6.60	43.0
CR81	29	488	7.48	42.3
CR91	30	495	8.86	37.5
AR113	28	543	6.22	47.0
AR118	29	507	6.44	46.8
AR117	30	503	6.96	45.0

AR rats were treated with ethanol for 4 to 5 months

than the paired control rats. These results seem to indicate an increase in mitochondria in the hearts of rats treated with ethanol for a substantial period of time. The data in TABLE B are consistent with the suggestion by Szanto et al (1967) that mitochondriosis develops in rats ingesting alcohol over a period of months, since an increase in SDH would accompany such an increase in normal mitochondria.

Because of the findingsthat assay for alcohol dehydrogenase in supernatants from fresh rat hearts were markedly different from the results of identical experiments using fresh frozen rat hearts which had been stored for several weeks, an experiment was designed to evaluate the effect of frozen storage on the assay for succinic dehydrogenase. Part of a rat heart was homogenized and SDH measured. The remainder of the heart was quick frozen and stored at -20 ^{o}C for five weeks. Assay for SDH after storage in the refrigerator showed a 40% increase in SDH! Storage of the homogenate overnight reduced the SDH activity measurably (FIGURE B-3), in contrast to the observed effect of similar overnight storage on SDH activity of heart homogenates (FIGURE B-4).

These unexpected findings are consistent with modern knowledge of the problems of extracting undamaged mitochondria from heart tissue. Damaged mitochondria show a higher SDH activity than do intact mitochondria. With fresh heart tissue, considerable amounts of undamaged mitochondria are present in the homogenate and further damage during overnight storage is greater

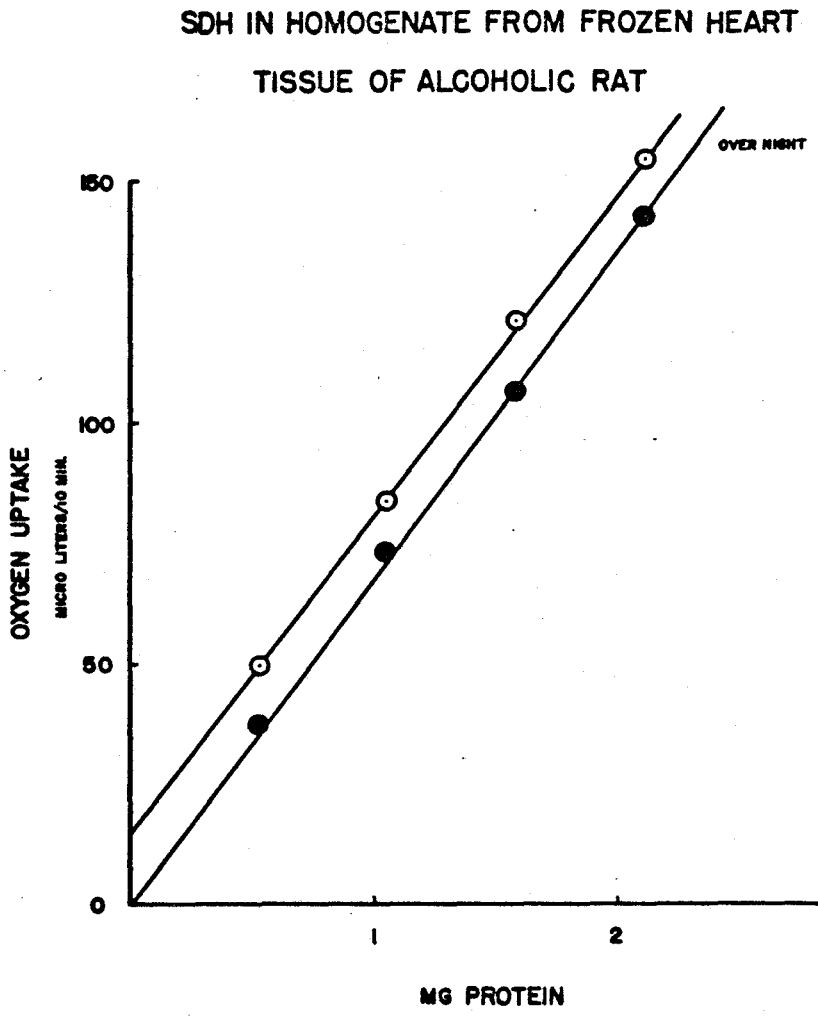

FIGURE B-3

Succinic dehydrogenase was assayed with homogenate prepared from heart tissue frozen for one month from rat in FIGURE B-1 on 42 weeks of ethanol ingestion. The protein concentration was 5.28 mg/ml. Oxygen consumption in μl per 10 min was plotted as a function of homogenate protein. The line with black symbols was the assay done on preparation standing over night at 4 ^{o}C.

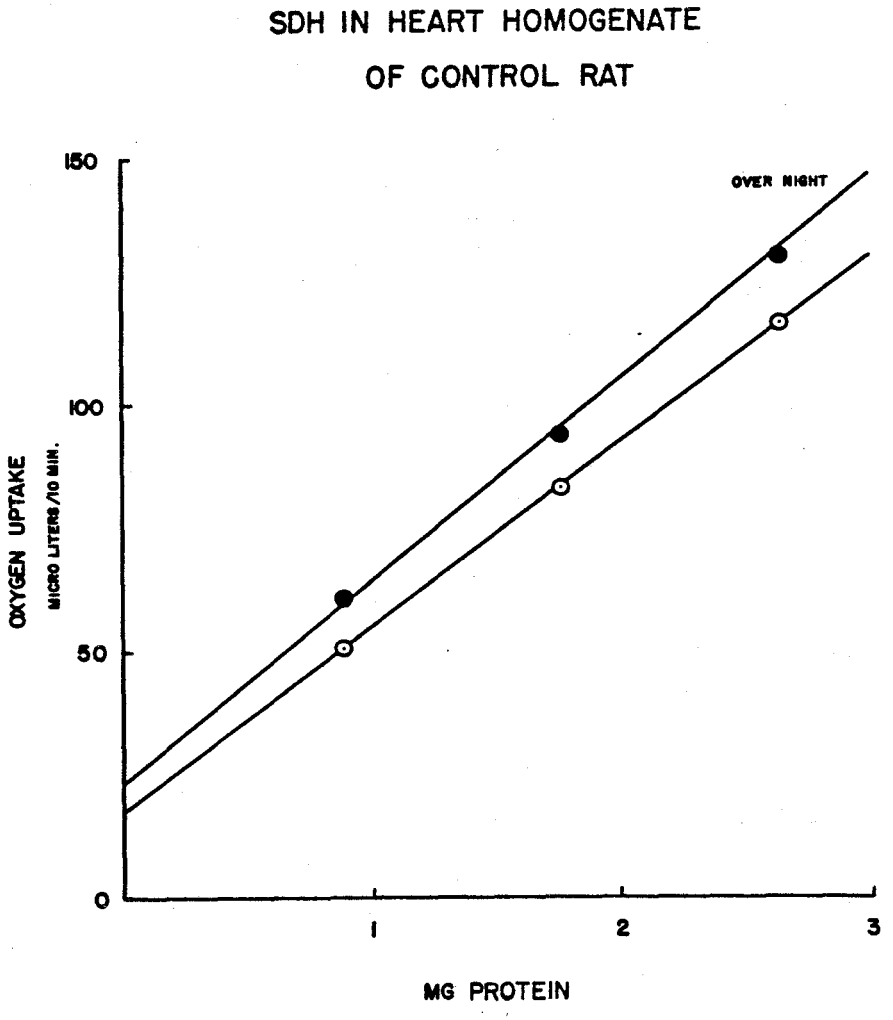

FIGURE B-4

Assay of succinic dehydrogenase was done on fresh heart homogenate of control rat, 30 weeks old. The protein in the homogenate was 8.86 mg/ml. The oxygen consumption in ul was plotted as a function of homogenate protein in mg. The line with black symbols represents sample standing over night at 4 ^{o}C.

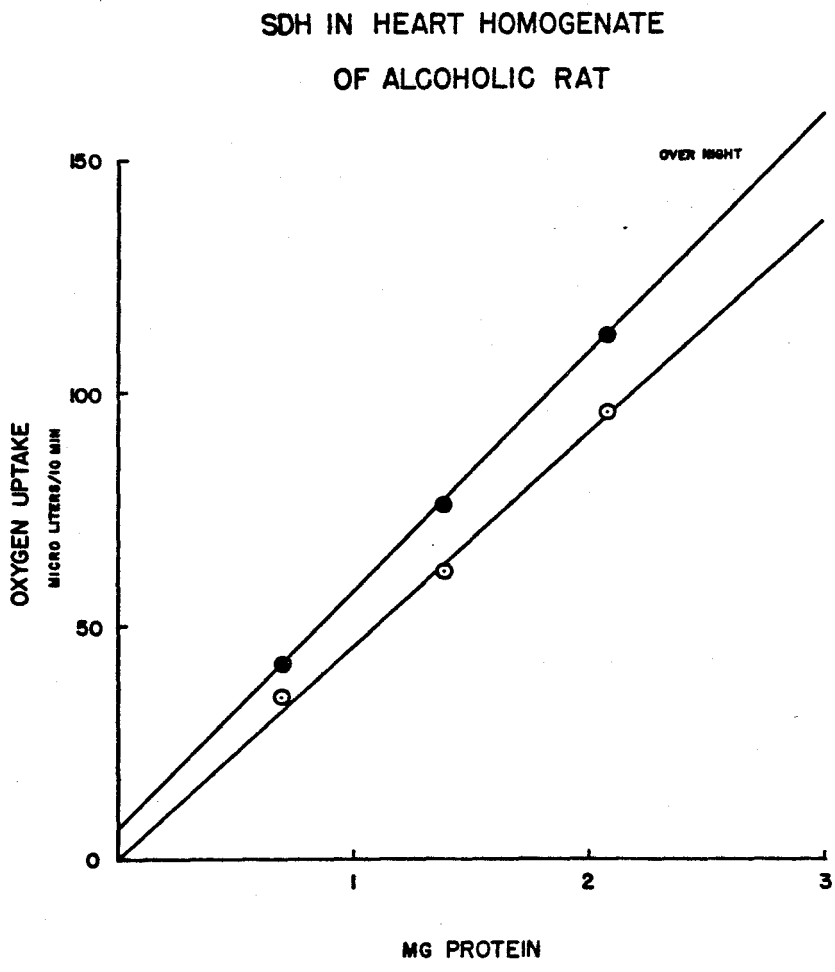

FIGURE B-5

Succinic dehydrogenase was assayed with fresh heart homogenate of rat on 27 weeks of ethanol ingestion. The homogenate protein was 6.96 mg/ml. The oxygen consumption in µl was plotted as a function of homogenate protein in mg. The line with black symbols represents sample standing over night at 4 ^{o}C.

than loss of succinate dehydrogenase activity during the same period -- the net effect is an experimental increase of SDH. With frozen tissue, most mitochondria, if not all, are damaged. Most of the SDH present is available and can be measured. Overnight storage reveals the loss of SDH activity. Sardesai (1969) has reported that mitochondrial SDH was increased considerably by disruption of mitochondrial membrane through repeated freezing and thawing technique. This indicates that the assay of SDH activity is affected by factors such as the permeability of mitochondrial membrane to substrate and electron acceptors, and by the fragility or integrity of the mitochondria. The increase of SDH activity in cardiac tissues of prolonged ethanol-treated rats does not necessarily lead to the conclusion of mitochondriosis since evidence of increase in mitochondrial membrane permeability resulting from prolonged ethanol ingestion has been reported (Kiessling and Pilstrom, 1966).

These findings show that previous measurements of SDH activity in homogenates are quite likely to underestimate the actual amount of enzyme present in the preparation. The meaning of such measurements should be carefully reconsidered by anyone making significant use of effects of experimental variables on SDH in heart tissue.

To investigate the effect of chronic ethanol intake on the efficiency of oxidation of rat heart mitochondria, comparisons were made of the oxidation of α-ketoglutarate using mitochondria

prepared from five control rats and (separately) from five rats given ethanol continuously for ten months. TABLE C shows that ethanol intake gives rise to a decrease in phosphorylation efficiency P/O = 2.2 as compared with 2.5 for the controls. Also the effectiveness of the mitochondrial protein is reduced: ΔO_2/mg protein/min is only 0.054 μgatoms compared with 0.066 for the controls.

This preliminary experiment also showed that the manometric method was not suitable for collecting the amount of data needed to make an adequate survey of the oxidative capacities of rat heart mitochondria. Too many rats would be required. Further experiments were deferred until a Clark oxygen electrode and associated apparatus could be obtained.

Preliminary experiments with the Clark electrode were not satisfactory, since the respiratory control values were very poor. After a number of trials, it was found that a major difficulty was the isolation procedure for the mitochondria. According to Tyler and Gonze (1967), rat heart mitochondria were separated by centrifugation at 8,000 x G for ten minutes. In my hands, using a Servall centrifuge with a number SS-34 head, the use of a speed which gave 10,000 x G at the tip, gave unsatisfactory preparations of both liver and heart. When the speed was increased to yield 14,500 x G at the tip, mitochondria with satisfactory properties were obtained from either liver or heart. The problem is apparent-ly one of communication. The question is, what did Tyler and

TABLE C

Oxidative phosphorylation by manometric method

Pi Uptake*	O_2 Uptake*	P/O	Mito**	Oxid'n Rate***
Control Rats				
42.21	15.90	2.65	21.2	0.063
43.05	19.14	2.25	21.2	0.076
40.95	15.15	2.70	21.2	0.060
Ethanol-treated Rats				
26.25	12.50	2.27	23.2	0.054
26.55	12.64	2.10	23.2	0.055
26.46	12.00	2.21	23.2	0.052

* Pi uptake in μmoles, O_2 uptake in μgram atoms, per flask
** Mitochondrial protein mg/ml
*** μgram atoms O_2/min/mg mitochondrial protein
Incubation time: 12 min for control rats, 10 min for ethanol-treated rats

Gonze mean by "8,000 g" ? Was this the force at the tip, the mid point of the solution in the tube, or the force at the upper surface of the solution? In the preparation method which was successful the appropriate values at 11,000 rpm were: RCF at tip: 14,500 x G; at mid point: 11,440 x G; at the top surface of the solution: 8,600 x G.

With mitochondria from the livers of young rats, values for ADP/O and RCR with α-ketoglutarate, pyruvate-malate, succinate, etc., were comparable with previously published work (Chappell, 1964). "Satisfactory properties" include a relatively slow oxidation rate in the absence of added ADP, a marked response to ADP which returns to the initial rate after the added ADP is phosphorylated. FIGUREs D-1 through D-4 show typical experimental recordings. The difference between succinate as substrate and either α-ketoglutarate or pyruvate-malate is partly as expected and partly due to the greater age of rat AR 213 compared with AR 203 at the time of sacrifice. Values in TABLE G-1, p. 111 for ADP/O ratios are comparable with those reported by Tyler and Gonze (1967) for pyruvate-malate using mitochondria from much younger rats. The results are summarized in TABLE D-1 (details are in the APPENDIX, pp. 97-120 TABLEs D-2, E 1-6, F 1-6, G 1-5, H 1-5). After 34 to 37 weeks of 20% ethanol treatment, there was no significant difference between mitochondria from control rats and experimental rats as measured by the oxidation of α-ketoglutarate and pyruvate-malate. After 43-44 weeks, there was no change in

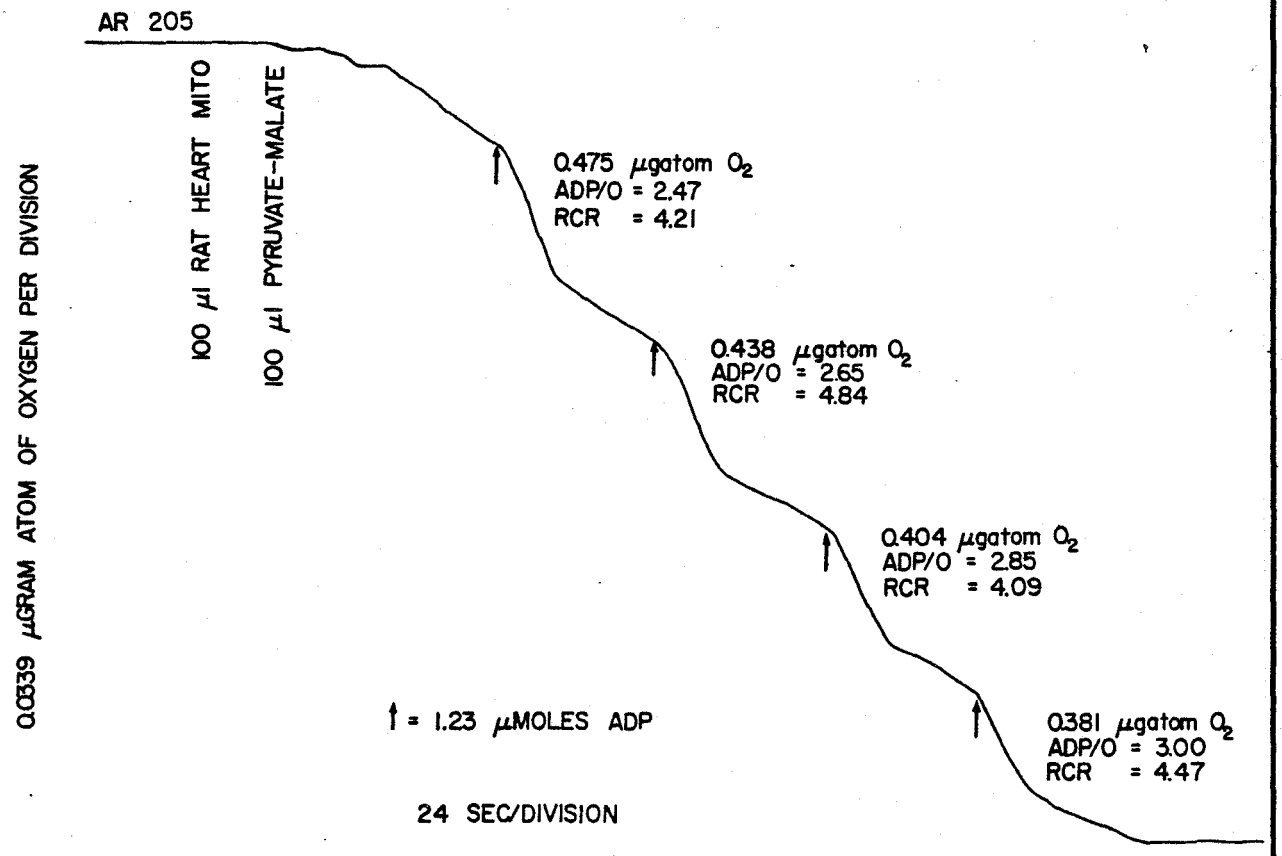

FIGURE D-1

The oxidative phosphorylation of heart mitochondria obtained from rat at 34 weeks of ethanol ingestion. The mitochondrial protein was 15.0 mg/ml. The metabolic process was carried out in 5 ml of Chance and Hagihara medium purging with 25% oxygen at 25 °C. The final concentration of substrate contained 4 mM pyruvate and 1 mM MALATE. Each 50 ul injection contained 1.23 µmoles ADP.

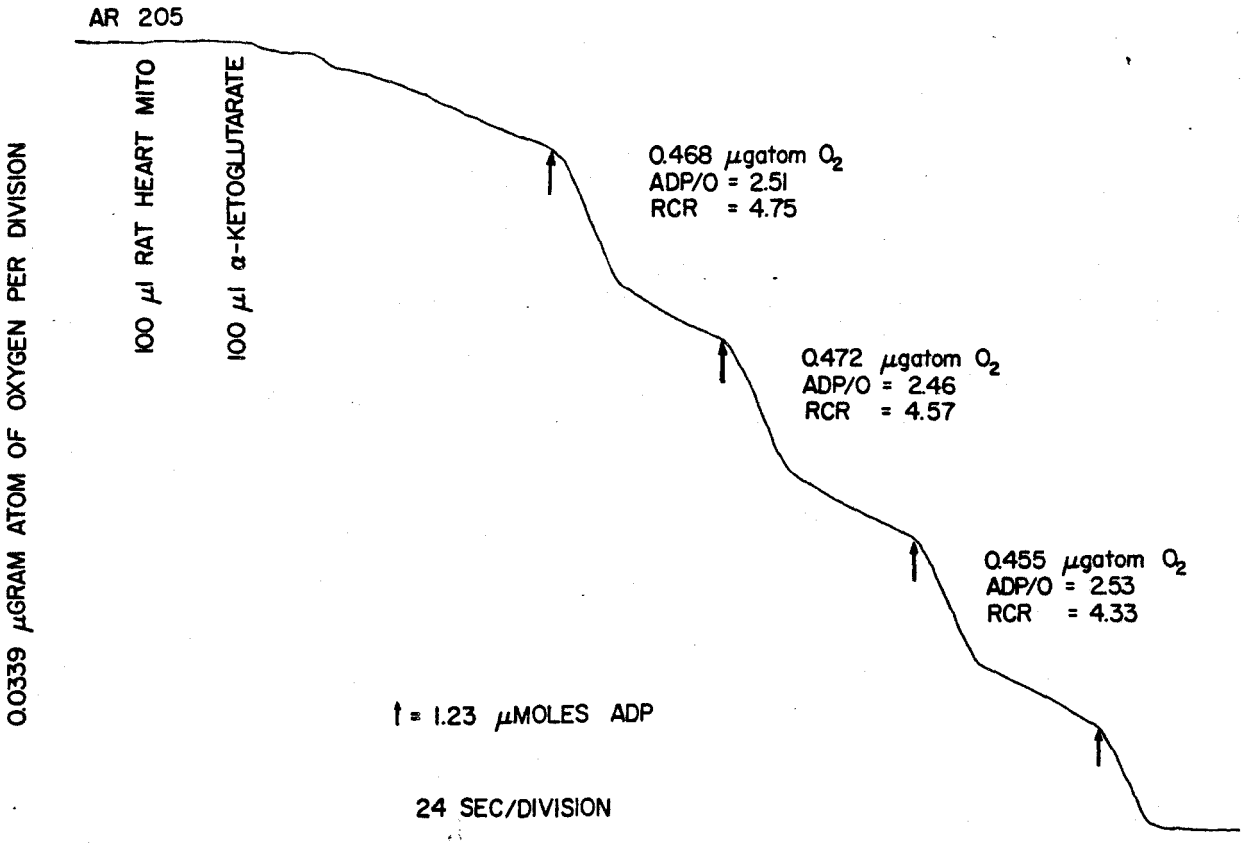

FIGURE D-2

The oxidative phosphorylation of heart mitochondria obtained from rat at 34 weeks of ethanol ingestion. The mitochondrial protein was 15.0 mg/ml. The metabolic process was carried out in 5 ml of Chance and Hagihara medium purging with 25% oxygen at 25 ^{o}C. The final substrate concentration was 5 mM α-ketoglutarate. Each 50 µl injection contained 1.23 µmoles ADP.

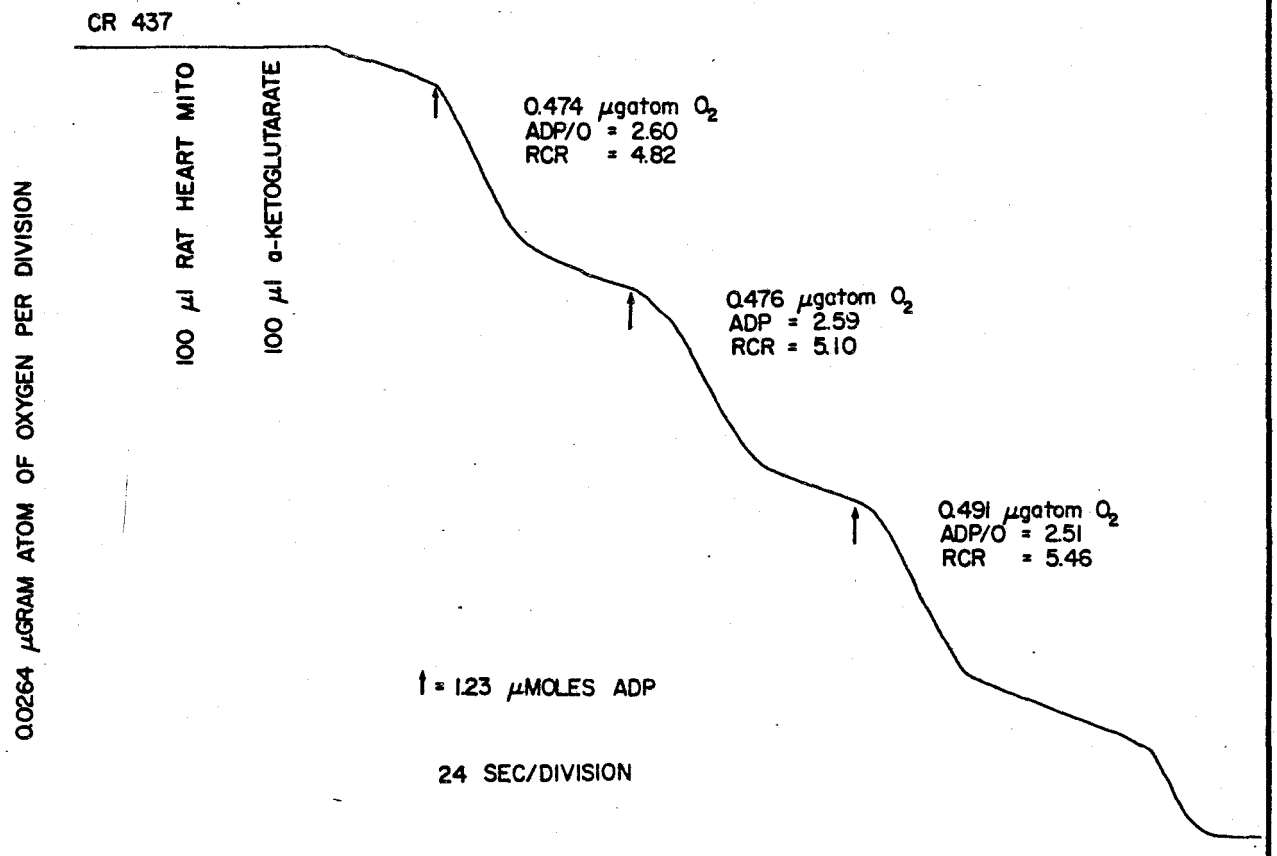

FIGURE D-3

The oxidative phosphorylation of heart mitochondria obtained from rat at 47 weeks on regular diet, and tap water as drinking fluid. The mitochondrial protein was 9.8 mg/ml. The metabolic process was carried out in 5 ml of Chance and Hagihara medium purging with 25% oxygen at 25 ^{o}C. The final substrate concentration was 5 mM α-ketoglutarate. Each 50 µl injection contained 1.23 µmoles ADP.

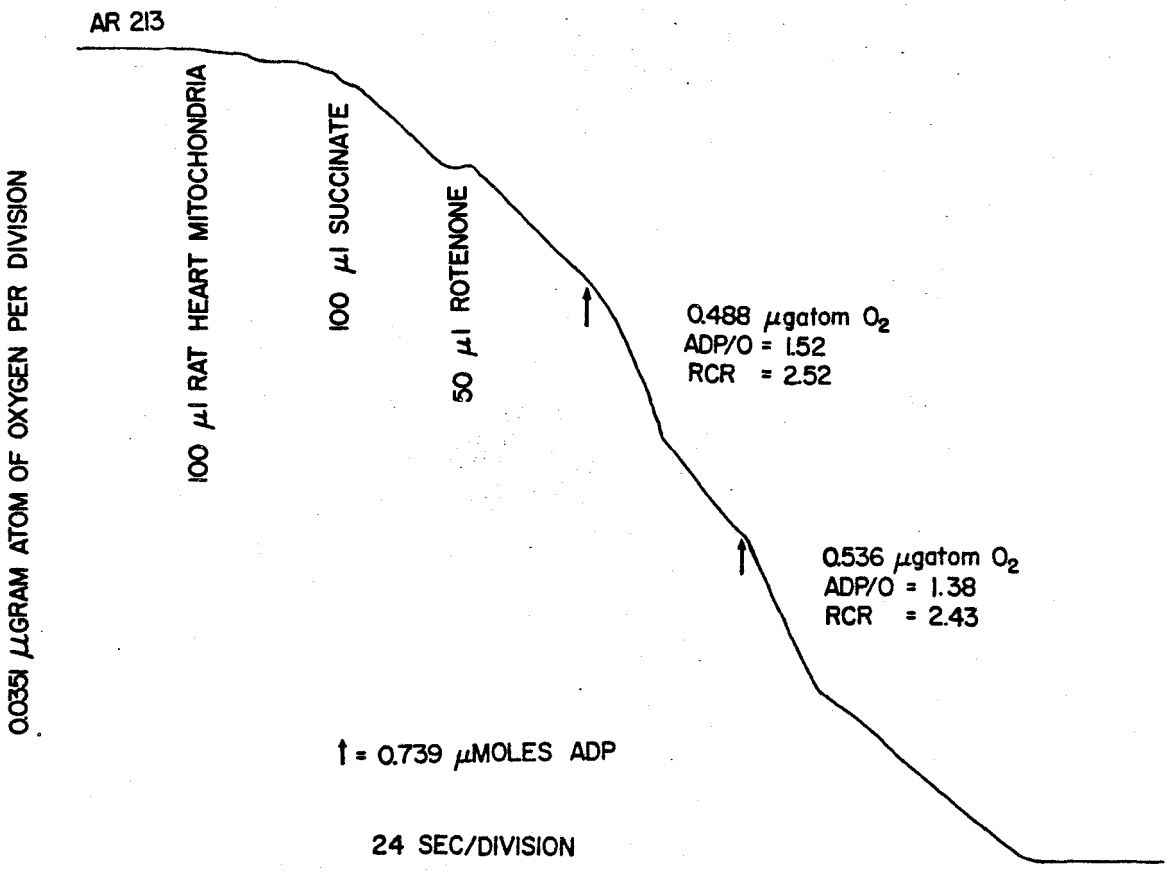

FIGURE D-4

The oxidative phosphorylation of heart mitochondria obtained from control rat, with 62 weeks on regular diet. The mitochondria (15.6 mg/ml) was incubated in 5 ml of Chance and Hagihara medium purging with 25% oxygen at 25 °C. The final substrate concentration was 5 mM succinate. Each 50 µl injection contained 1.23 µmoles ADP.

TABLE D-1

The effect of ethanol ingestion on oxidative process in rat heart mitochondria

Weeks after 20% ethanol	Number of rats*	Substrate	ADP/O	RCR	Max. Oxid. rate**
34-37	4 C	α-Ketoglutarate	2.56±.04	5.7±.7	.29±.03
	4 E		2.55±.14	5.9±1.1	.29±.04
34-37	4 C	Pyruvate-Malate	2.50±.12	4.1±.5	.32±.04
	4 E		2.62±.23	4.4±.6	.33±.08
43-44	5 C	α-Ketoglutarate	2.49±.25	4.2±.9	.27±.03
	5 E		2.61±.33	4.1±.8	.26±.08
43-44	5 C	Pyruvate-Malate	2.56±.15	3.4±.6	.27±.03
	5 E		2.61±.14	3.2±.4	.26±.03
55-56	3 C	α-Ketoglutarate	2.54±.09	3.8±.4	.24±.03
	3 E		2.39±.05	3.6±.3	.21±.01
55-56	3 C	Succinate	1.24±.11	1.8±.3	.31±.03
	3 E		1.24±.09	1.9±.1	.28±.03
57-58	3 C	α-Ketoglutarate	2.55±.11	4.1±.5	.24±.02
	3 E		2.77±.31	4.4±1.0	.23±.02
57-58	3 C	Succinate	1.28±.13	1.8±.2	.31±.02
	3 E		1.22±.17	1.9±.2	.30±.03

* C = Control, E = Experimental

** Maximum oxidation rate in μgram atoms of oxygen per minute per mg mitochondrial protein.

RCR = Respiratory Control Ratio

the ADP/O ratio, but there was a reduction in the oxidation rate and the respiratory control ratio. Since this change was observed in both control and experimental groups, it is presumed to be due to aging, not ethanol. Further aging (55-58 weeks) resulted in further decrease in the rate of oxidation of α-ketoglutarate, but no significant change in the RCR. Oxidation of succinate at 55-58 weeks of 20% ethanol treatment showed the same rates, values for ADP/O and RCR in experimental as in control animals, but these numbers are lower than have been observed by others in much younger animals.

The special properties of heart tissue markedly affect the results of biochemical experiments carried out with preparations from this tissue. There are numerous fibers in the tissue, which makes the preparation of homogenates very difficult. Various approaches have been developed, some of which have been used in these experiments. For example, the use of a tissue press permits the removal of most fibers and subsequent homogenation with less trouble than with minced tissues. However, considerable damage is done to formed elements, and opinions differ as to the desirability of this procedure. Others mince the tissue with a scalpel prior to homogenizing with loose-fitting pestle. Chance and Hagihara (1961) pioneered a valuable approach to the isolation of relatively undamaged mitochondria from heart tissue. They used a bacterial collagenase in high concentration for a short period of time as part of the homogenization of minced tissue. This

hydrolysed some of the fibers present and thus reduced both the difficulty of homogenizing the tissue and the damage to mitochondria. In our hands the method was reasonably satisfactory except that a jelly-like material was found as the upper part of the mitochondrial pellet in most experiments. This is, presumably, the partially hydrolysed collagen plus varying amounts of mitochondria. In the early experiments the gel was separated from the more normal appearing pellet. Since this leads to loss of some of the mitochondria and to erratic recoveries of "mitochondrial protein" per gram rat heart (TABLEs E-1, F-1, G-1, H-1, APPENDIX) the jelly was included in the mitochondrial preparation in the last three experiments (bottom portion of TABLEs D-1 and G-1). This change may account for the marked increase in the ADP/O ratio for α-ketoglutarate observed with experimental rats at 55-58 weeks. When the jelly was discarded (TABLE G-1, top) the recovery of mitochondrial protein was about 6 mg/gm rat heart, the ADP/O value was 2.54 $\pm$ 0.09, while when the jelly was retained, the mitochondrial protein averaged 17 mg/gm heart and the ADP/O value was 2.77 $\pm$ 0.3 .

These results illustrate the difficulty encountered in attempting to evaluate the amount of mitochondrial protein (and thus the number of mitochondria) in these rat hearts. It is obvious that the "mitochondrial pellet" is composed of two quite different layers. With old rats, if the upper layer is discarded, recoveries of protein are markedly reduced. However, when the

upper layer is retained there is, if anything, an improvement in the "mitochondrial properties" of the residue.

Within the limitations of this data, there are no significant differences in the biochemical properties of rat heart mitochondria prepared from rats which have ingested 20% ethanol for periods of 34 to 58 weeks and mitochondria from control rats given water for similar periods.

When this research was being planned, arrangements were made with another staff member to carry out electron microscope studies on the hearts of rats from the same experimental groups to verify the published observations about changes in heart mitochondria and to correlate structural changes with biochemical measurements. Unfortunately, when the first animals were ready for sacrifice, the collaboration was no longer available, and it was not possible to arrange collaboration with other staff members with the requisite skill and access to equipment.

At a time when it seemed that such cooperation might still be obtained, the hearts of two freshly killed rats were prepared for electron microscopy by the method of Pease (1965) and with the advice of Dr. Wen Chang, Department of Pathology, Evanston Hospital Evanston, Illinois, and Mrs. Maria Mezari, Department of Pathology, Loyola Medical Center, Maywood, Illinois. Sections of the left and right ventricles were photographed with a Siemens electron microscope at a total magnification of 8000. FIGUREs I-1 to J-3 are typical of the results obtained, and are in general accord

with literature descriptions. Comparison of FIGURE I-3 from the lower portion of the left ventricle of a rat (AR 206 sacrificed after 8.5 months of 20% ethanol ingestion) with FIGURE I-2 from the middle portion of the same ventricle of the same rat illustrates the difficulty of estimating changes. In FIGURE I-3 numerous mitochondria with well developed cristae are seen in the middle of the figure, but very much enlarged mitochondria with relatively poor cristae are seen on the right -- in addition to structures which appear to be mitochondria without cristae at the right and upper left. FIGURE I-2 does not contain many easily recognizable mitochondria and looks more like a section from the middle left ventricle of the control rat (FIGURE I-1) than it does the lower section of the left ventricle of the same rat (FIGURE I-3).

FIGURE J-2 and J-3 are different slices from the middle part of the right ventricle of the experimental rat. Both show significant numbers of enlarged and damaged mitochondria. The mitochondria in a similar section from the middle right ventricle of the control rat also shows considerable numbers of damaged mitochondria with possibly more organized cristae and fewer vacuoles. These differences between the parts of the same heart indicate the dimensions of the problem of determining significant differences in mitochondria counts -- and of interpreting the results of experiments which depend upon the isolation of mitochondria from homogenates of such hearts. It seems quite

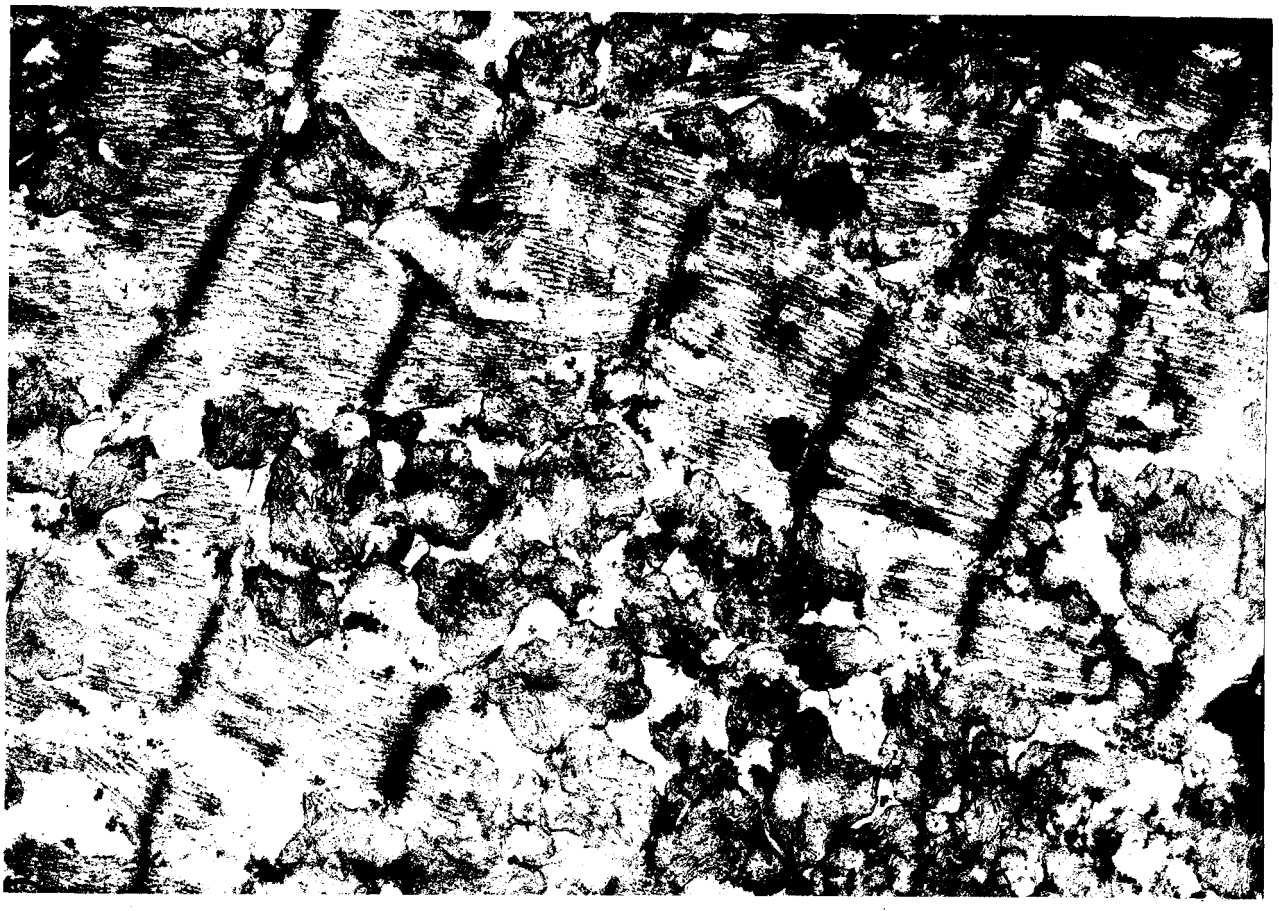

FIGURE I-1

"Control" myocardium of middle section of left ventricle. Myofibrils and Z-lines are well defined. The mitochondria are normal. Rat is about 11 months old. 8000x magnification.

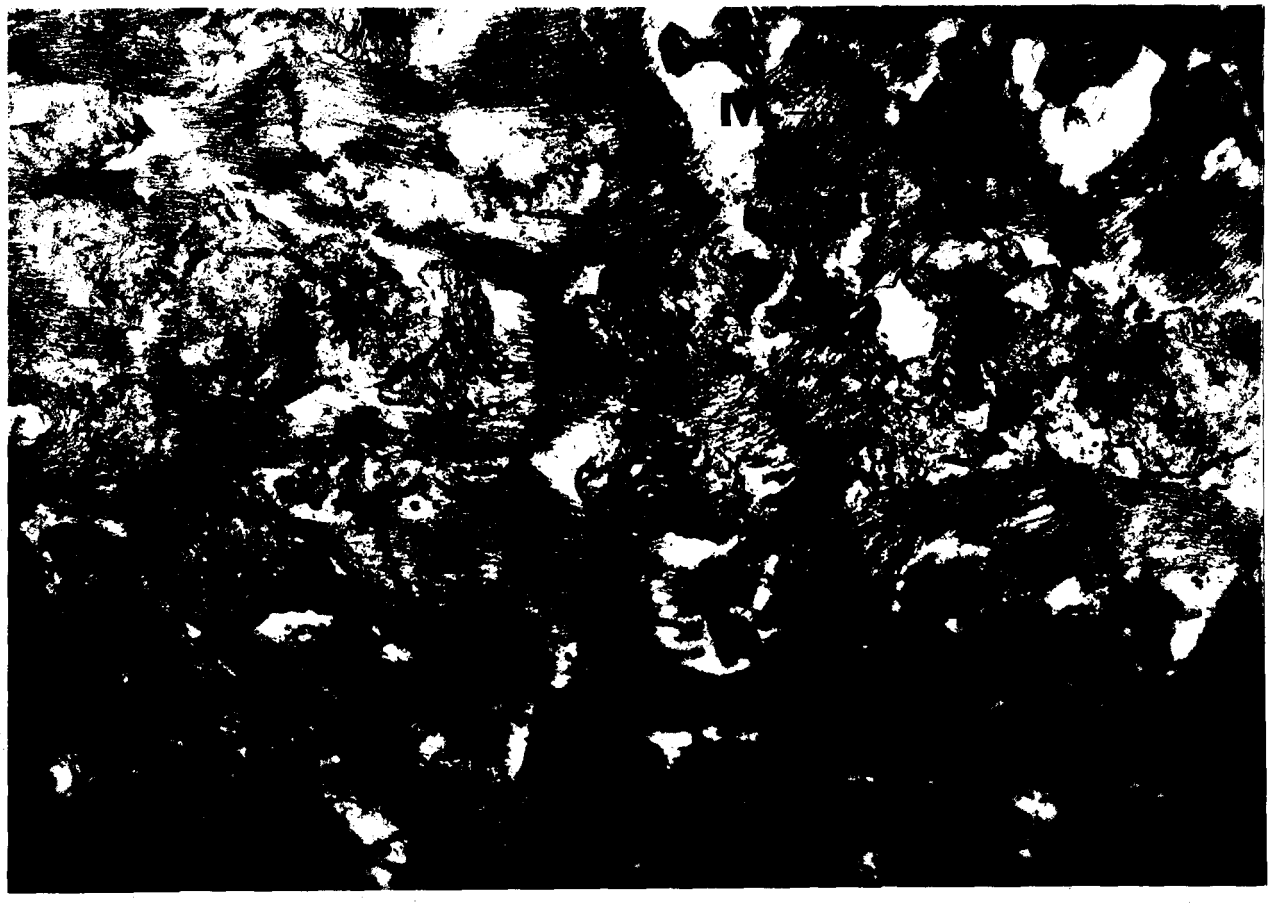

FIGURE I-2

Myocardium obtained from middle section of left ventricle of experimental rat, on 8.5 months of 20% ethanol ingestion. Myofibrils are short, fragmented and very poorly defined, and invaded by mitochondria. Moth-eaten mitochondria (M) are seen. 8000x magnification.

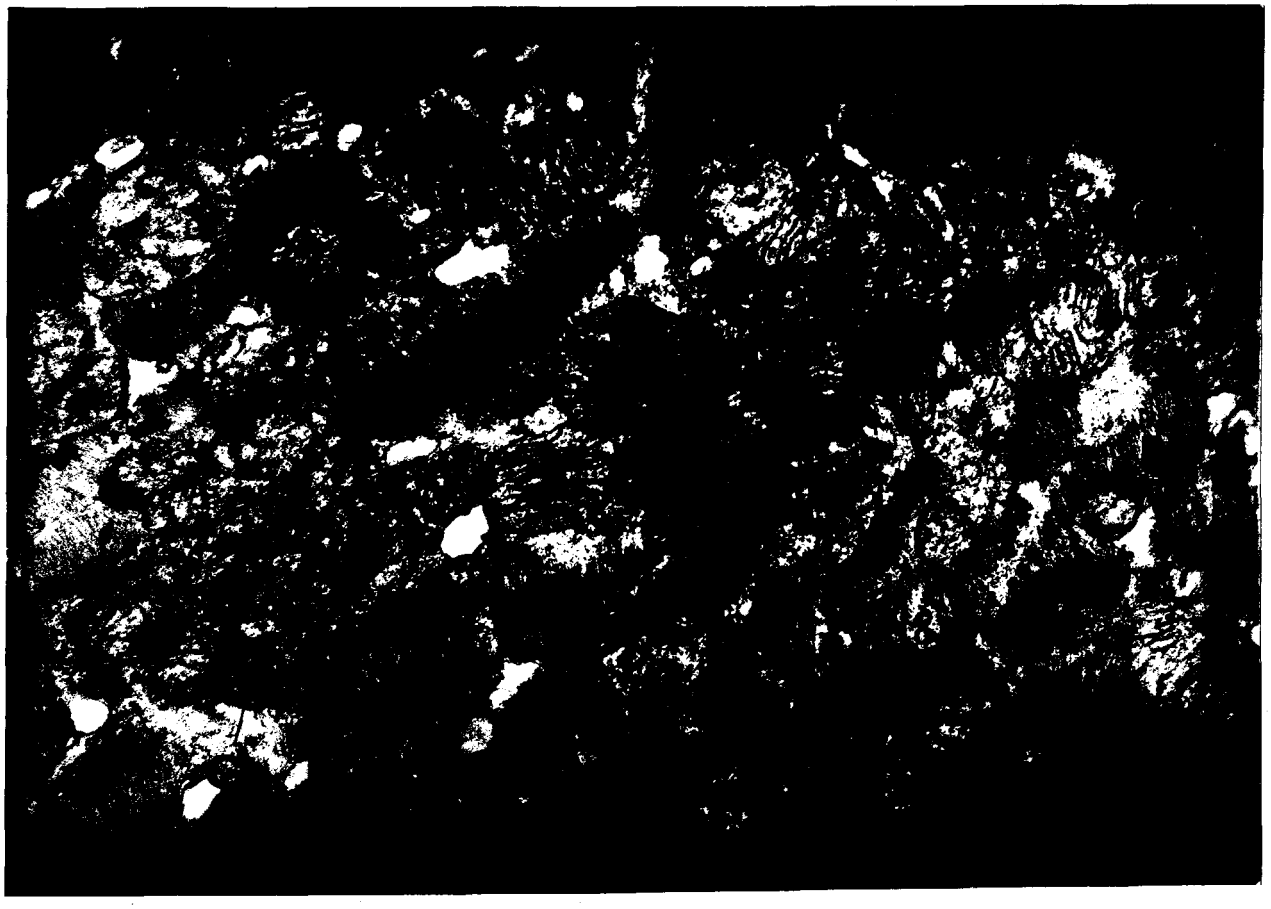

FIGURE I-3

Myocardium of lower section of left ventricle of experimental rat on 8.5 months of 20% ethanol ingestion. Mitochondria of bizarre-shape (M_1), enlarged in size (M_2) and with undefined cristae (M_3). Cluster of glycogen is also seen outside the mitochondria. 8000x magnification.

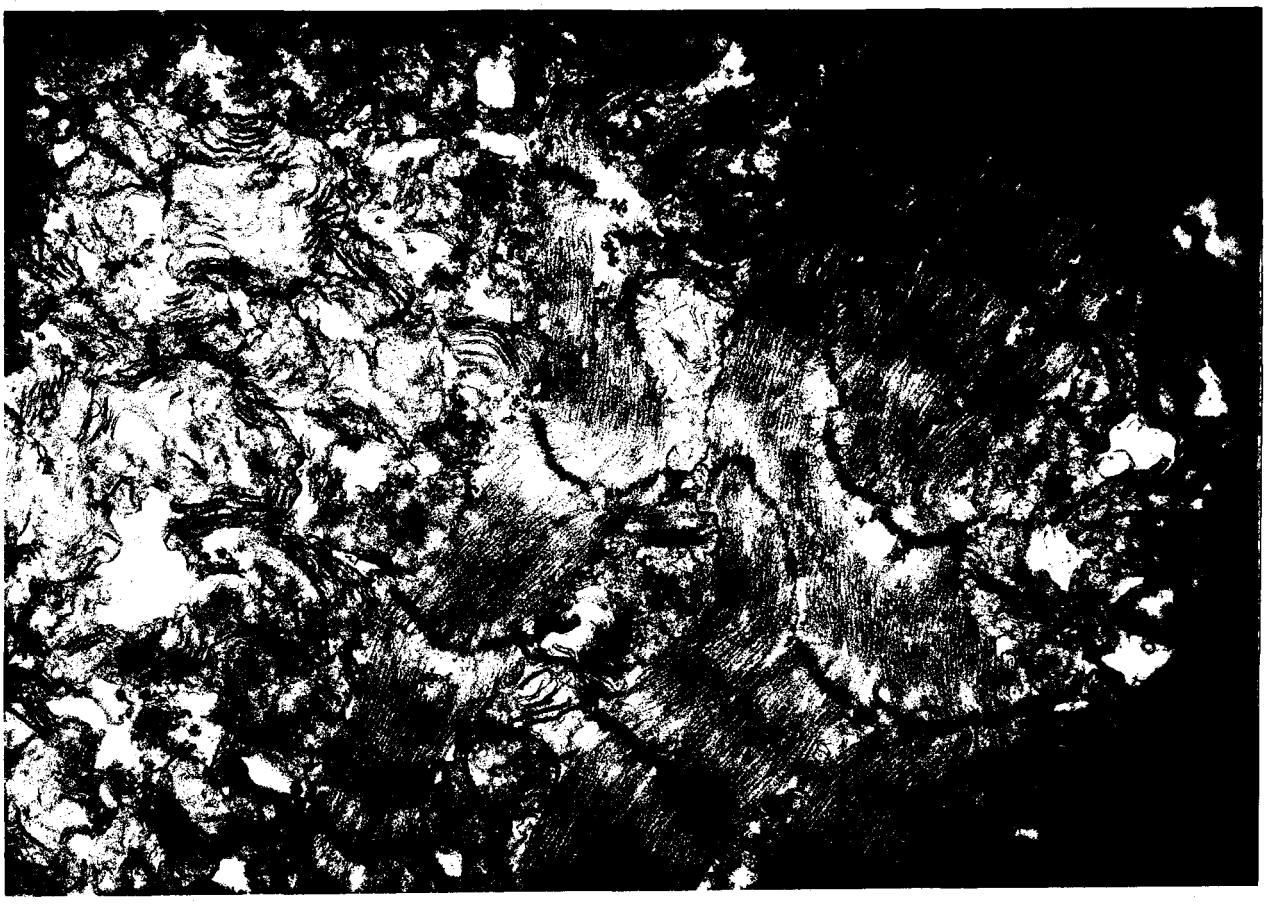

FIGURE J-1

'Control' myocardium of middle section of right ventricle. Myofibrils are clearly defined and the mitochondria and cristae look normal. 8000x magnification.

FIGURE J-2

Myocardium of middle section of right ventricle from experimental rat on 8.5 months of 20% ethanol ingestion. Myofibrils are filled with moth-eaten mitochondria (M) which are vacuolarized, deformed, with undefined or broken membrane contour and disappearance of cristae. 8000x magnification.

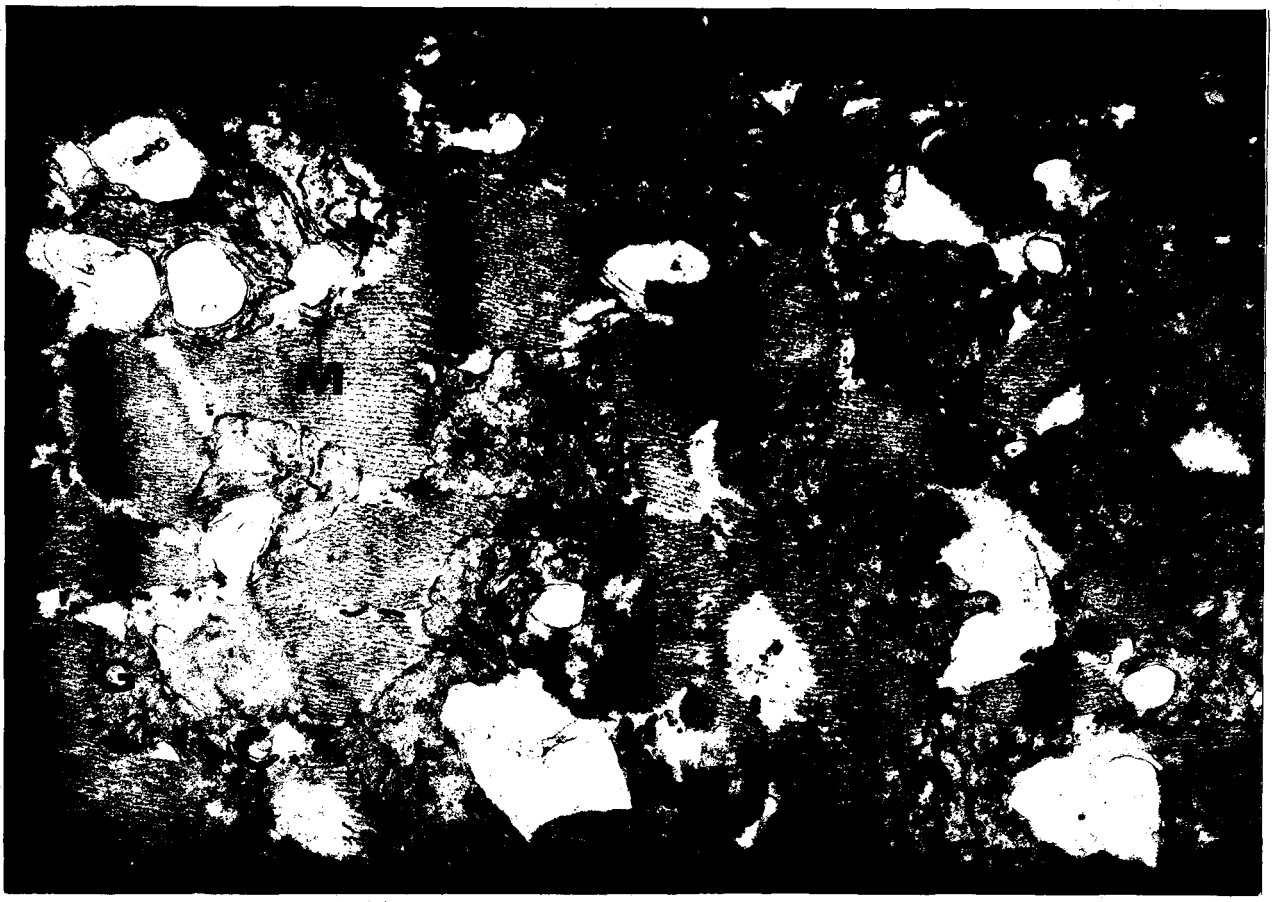

FIGURE J-3

Myocardium of middle section of right ventricle from experimental rat on 8.5 months of 20% ethanol ingestion. Enlarged mitochondria (M) contain vacuoles, broken membrane and deformed in shape. Granular glycogen (G) is seen outside mitochondria.
8000x magnification.

reasonable to assume that the enormous mitochondrion in the right lower border in FIGURE I-3 might be found in a different centrifugal portion from that of the relatively "normal" mitochondria in the center. The average enzymic content of mixture of the "normal" sized particles with well developed cristae and the particles of similar size with few or no cristae might be expected to differ from those from a younger rat.

CONCLUSIONS

The experiments reported in this chapter indicate strongly that alcohol dehydrogenase is found in rat heart, although experimental difficulties due to other enzymic activities prevent quantitative measurement of ADH activity. Within the experimental limits, there is no demonstrable effect of chronic ethanol ingestion on ADH activity in rat heart.

Other experiments show that succinic dehydrogenase activity, measured by standard methods, is increased by ingestion of ethanol over a period of about six months. This is what would be expected if the number of mitochondria with normal SDH were increased, as was reported by Szanto et al (1967). However, the methods do not assay the actual amount of enzyme present in the preparation.

A different view of the subject was taken in the last series of experiments. A group of rather old rats (250-350 gm) were divided into experimental and control groups and the experi-

mental group allowed to ingest ethanol for a long period of time, Measurements of the efficiency of mitochondrial oxidative phosphorylation after ten or more months of exposure to ethanol showed no significant differences between experimental and control groups, although there were differences in oxidation rate and respiratory control between groups with ten months elapsed time and those with fourteen months elapsed time. This in accord with the findings of Szanto et al (1967) who found that changes due to age obscured those due to chronic ethanol after ten to twelve months.

APPENDIX

TABLE A-3

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from frozen heart tissue of control rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.509	0.490	0.009
	0.510	0.500	0.010
2	0.520	0.507	0.013
	0.525	0.507	0.018
3	0.532	0.510	0.022
	0.537	0.510	0.027
4	0.540	0.508	0.032
	0.543	0.509	0.034
5	0.545	0.510	0.035
	0.548	0.510	0.038
6	0.549	0.510	0.039
	0.549	0.509	0.040
7	0.550	0.510	0.040
	0.550	0.511	0.039
8	0.552	0.511	0.041

TABLE A-4

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from frozen heart tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.508	0.497	0.011
	0.521	0.504	0.017
2	0.532	0.508	0.024
	0.540	0.510	0.030
3	0.547	0.511	0.036
	0.550	0.512	0.038
4	0.552	0.512	0.040
	0.554	0.513	0.041
5	0.554	0.513	0.041
	0.554	0.514	0.040
6	0.554	0.514	0.040
	0.555	0.515	0.040
7	0.556	0.515	0.041
	0.556	0.515	0.041
8	0.556	0.515	0.041

TABLE A-5a

Activity of alcohol dehydrogenase in frozen rat heart tissue (μmoles/min/g tissue)

Exposure week	Age day	ETOH-treated Rat	Activity	Control Rat	Activity
4	57	53	0.097	45	-----
			0.124		
		55	0.124	47	0.153
			0.134		0.188
6	71	54	0.132	49	0.145
			0.132		0.139
		56	0.129	51	0.088
			0.157		0.082
8	85	57	0.080	34	0.206
			0.086		0.118
		58	0.247	36	0.174
			0.264		0.209
10	99	59	0.123	38	0.183
			0.105		0.108
		60	0.190	40	0.119
			0.211		0.119
12	113	61	0.157	42	0.152
			0.170		0.189
		62	0.105	44	0.127
			0.101		0.099

TABLE A-5b

Activity of alcohol dehydrogenase in frozen rat heart tissue (μmoles/min/g tissue)

Exposure week	Age day	ETOH-treated Rat	Activity	Control Rat	Activity
14	127	63	0.240	46	0.188
			0.229		0.188
		64	0.260	48	0.271
			0.250		0.292
16	141	66	0.250	50	0.229
			0.292		0.223
		67	0.333	52	0.250
			0.396		0.327
20	169	19	0.250	1	0.229
			0.292		0.292
		20	0.156	4	0.271
			0.229		0.240
25	204	21	0.333	5	0.167
			0.250		0.135
		22	0.211	6	0.114
			0.211		0.160
28	225	23	0.292	7	0.208
			0.333		0.208
		24	0.337	8	0.316
			0.261		0.305

TABLE A-6

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from fresh heart tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.400	0.375	0.025
	0.406	0.370	0.036
2	0.403	0.376	0.036
	0.395	0.360	0.035
3	0.387	0.356	0.031
	0.379	0.353	0.026
4	0.373	0.350	0.023
	0.368	0.348	0.020
5	0.363	0.345	0.018
	0.358	0.343	0.015
6	0.355	0.342	0.013
	0.353	0.340	0.013
7	0.350	0.339	0.011
	0.350	0.338	0.012
8	0.350	0.337	0.013

TABLE A-7a

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from fresh heart tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.400	0.375	0.025
	0.406	0.370	0.036
2	0.403	0.367	0.036
	0.395	0.360	0.035
3	0.387	0.356	0.031
	0.379	0.353	0.026
4	0.373	0.350	0.023
	0.368	0.348	0.020
5	0.363	0.345	0.018
	0.358	0.343	0.015
6	0.355	0.342	0.013
	0.353	0.340	0.013
7	0.350	0.339	0.011
	0.350	0.338	0.012
8	0.350	0.337	0.013

TABLE A-7b

Absorbance at 340 nm in the assay of alcohol dehydrogenase in supernatant from fresh liver tissue of alcoholic rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1	0.444	0.425	0.019
	0.468	0.438	0.030
2	0.488	0.448	0.040
	0.500	0.455	0.045
3	0.515	0.461	0.054
	0.527	0.465	0.062
4	0.538	0.470	0.068
	0.548	0.474	0.074
5	0.556	0.478	0.078
	0.566	0.480	0.086
6	0.573	0.848	0.089
	0.580	0.487	0.093
7	0.588	0.490	0.098
	0.594	0.492	0.102
8	0.598	0.495	0.103

TABLE A-8A

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase (0.2 mg/ml) with pre-incubation

Min	Ethanol	No Ethanol	ΔAbsorbance
11	0.100	0.098	0.002
12	0.100	0.098	0.002
13	0.101	0.099	0.002
14	0.102	0.099	0.003
15	0.102	0.099	0.003
16	0.103	0.099	0.004
17	0.104	0.100	0.004
18	0.104	0.100	0.004
19	0.103	0.101	0.002
20	0.104	0.102	0.002
21	0.104	0.101	0.003
22	0.5 ml ETOH	0.5 ml H_2O	
23	-----	-----	-----
24	0.459	0.086	0.373
25	0.583	0.085	0.498
26	0.650	0.086	0.564
27	0.700	0.086	0.614
28	0.744	0.086	0.658
29	0.760	0.086	0.675
30	0.781	0.086	0.695
31	0.796	0.086	0.710
32	0.808	0.086	0.722
33	0.814	0.086	0.728
34	0.820	0.086	0.734
35	0.828	0.086	0.742
36	0.830	0.086	0.744
37	0.832	0.085	0.747
38	0.832	0.085	0.747

TABLE A-8A'

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase pre-incubated with supernatant from fresh heart tissue of ethanol-treated rat

Min	Ethanol	No Ethanol	ΔAbsorbance
9	0.631	0.640	-0.009
10	0.634	0.648	-0.014
11	0.638	0.650	-0.012
12	0.645	0.657	-0.012
13	0.651	0.660	-0.009
14	0.656	0.666	-0.010
15	0.660	0.670	-0.010
16	0.660	0.670	-0.010
17	0.660	0.670	-0.010
18	0.665	0.671	-0.006
19	0.665	0.670	-0.005
20	0.664	0.672	-0.008
21	0.5 ml ETOH	0.5 ml H_2O	
24	0.588	0.545	0.043
25	0.648	0.540	0.108
26	0.720	0.538	0.182
27	0.789	0.538	0.251
28	0.840	0.540	0.300
29	0.880	0.540	0.340
30	0.909	0.541	0.368
31	0.928	0.544	0.384
32	0.945	0.544	0.401
33	0.958	0.545	0.413
34	0.970	0.546	0.424
35	0.972	0.547	0.425
36	0.978	0.548	0.430

Final ethanol concentration = 0.016 M

TABLE A-8B

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase (0.2 mg/ml) with pre-incubation

Min	Ethanol	No Ethanol	ΔAbsorbance
5	0.097	0.096	0.001
6	0.097	0.095	0.002
7	0.097	0.096	0.001
8	0.098	0.097	0.001
9	0.098	0.097	0.001
10	0.098	0.097	0.001
11	0.5 ml ETOH	0.5 ml H_2O	
12	-----	-----	-----
13	0.439	0.110	0.329
14	0.544	0.107	0.437
15	0.618	0.105	0.513
16	0.674	0.101	0.573
17	0.720	0.100	0.620
18	0.756	0.102	0.654
19	0.780	0.101	0.679
20	0.804	0.103	0.701
21	0.822	0.098	0.734
22	0.839	0.102	0.737
23	0.850	0.100	0.750
24	0.870	0.097	0.773
25	0.876	0.093	0.783
26	0.882	0.090	0.792
27	0.908	0.088	0.820
28	0.895	0.086	0.809
29	0.900	0.084	0.816
30	0.900	0.084	0.816
31	0.908	0.083	0.825

Final ethanol concentration = 8×10^{-2} M

TABLE A-8C

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase (0.2 mg/ml) with pre-incubation

Min	Ethanol	No Ethanol	ΔAbsorbance
8	0.099	0.097	0.002
9	0.101	0.098	0.003
10	0.100	0.098	0.002
11	0.5 ml ETOH	0.5 ml H_2O	
13	0.342	0.083	0.259
14	0.427	0.083	0.344
15	0.495	0.084	0.411
16	0.550	0.083	0.467
17	0.595	0.083	0.512
18	0.634	0.083	0.551
19	0.667	0.083	0.584
20	0.698	0.083	0.615
21	0.720	0.083	0.637
22	0.744	0.084	0.660
23	0.762	0.085	0.677
24	0.781	0.083	0.698
25	0.799	0.084	0.715
26	0.814	0.083	0.731
27	0.828	0.083	0.745
28	0.832	0.084	0.748
29	0.840	0.084	0.756
30	0.850	0.084	0.766
31	0.858	0.084	0.774
32	0.872	0.085	0.787
33	0.872	0.083	0.789
34	0.880	0.084	0.796
35	0.884	0.085	0.799
36	0.890	0.084	0.806

Final ethanol concentration = 0.40 M

TABLE A-8D

Absorbance at 340 nm in the assay of alcohol dehydrogenase with pre-incubation in supernatant from fresh heart tissue of control rat

Min	Ethanol	No Ethanol	ΔAbsorbance
8	0.632	0.642	-0.010
9	0.623	0.631	-0.008
10	0.618	0.626	-0.008
11	0.612	0.620	-0.008
12	0.608	0.618	-0.010
13	0.599	0.609	-0.010
14	0.600	0.610	-0.010
15	0.603	0.610	-0.007
16	0.605	0.615	-0.010
17	0.5 ml ETOH	0.5 ml H_2O	
18	-----	-----	-----
19	0.509	0.518	-0.009
20	0.507	0.517	-0.010
21	0.507	0.517	-0.009
22	0.506	0.515	-0.009
23	0.505	0.513	-0.008
24	0.504	0.511	-0.007
25	0.503	0.509	-0.006
26	0.500	0.506	-0.006
27	0.499	0.507	-0.008
28	0.498	0.505	-0.007
29	0.497	0.506	-0.009
30	0.496	0.506	-0.010

TABLE A-8E

Absorbance at 340 nm in the assay of alcohol dehydrogenase without pre-incubation in supernatant from fresh heart tissue of control rat with ethanol added last

Min	Ethanol	No Ethanol	ΔAbsorbance
2	0.664	0.639	0.025
3	0.618	0.591	0.027
4	0.597	0.572	0.027
5	0.578	0.556	0.022
6	0.560	0.543	0.017
7	0.556	0.534	0.016
8	0.540	0.525	0.015
9	0.532	0.522	0.010
10	0.527	0.516	0.011
11	0.523	0.513	0.010
12	0.521	0.510	0.011
13	0.516	0.507	0.009
14	0.515	0.508	0.007
15	0.513	0.507	0.006
16	0.511	0.504	0.007
17	0.509	0.504	0.005
18	0.509	0.503	0.006

TABLE A-9a

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase pre-incubated with Cleland's reagent

Min	Ethanol	No Ethanol	ΔAbsorbance
1			
2	0.132	0.128	0.004
3	0.133	0.128	0.005
4	0.133	0.128	0.005
5	0.133	0.128	0.005
6	0.133	0.129	0.004
7	0.134	0.130	0.004
8	0.135	0.130	0.005
9	0.136	0.132	0.004
10	0.5 ml ETOH	0.5 ml H_2O	
12	0.463	0.111	0.352
13	0.540	0.112	0.428
14	0.588	0.113	0.475
15	0.616	0.123	0.503
16	0.636	0.123	0.513
17	0.649	0.124	0.525
18	0.656	0.124	0.532
19	0.662	0.125	0.537
20	0.665	0.125	0.540
21	0.669	0.125	0.544
22	0.670	0.126	0.544
23	0.673	0.125	0.548
24	0.672	0.124	0.548

TABLE A-9b

Absorbance at 340 nm in the assay of crystalline equine liver alcohol dehydrogenase pre-incubated with enzyme solution containing Cleland's reagent and supernatant from fresh heart tissue of control rat

Min	Ethanol	No Ethanol	ΔAbsorbance
1			
2	0.794	0.795	-0.001
3	0.770	0.780	-0.010
4	0.760	0.766	-0.006
5	0.750	0.750	0.000
6	0.740	0.746	-0.006
7	0.737	0.737	0.000
8	0.732	0.735	-0.003
9	0.728	0.730	-0.002
10	0.720	0.722	-0.002
11	0.5 ml ETOH	0.5 ml H_2O	
13	0.624	0.606	0.018
14	0.650	0.605	0.045
15	0.704	0.600	0.104
16	0.786	0.597	0.189
17	0.854	0.597	0.257
18	0.910	0.597	0.313
19	0.949	0.597	0.352
20	0.977	0.597	0.380
21	0.992	0.597	0.385
22	1.000	0.597	0.403
23	1.008	0.596	0.412
24	1.015	0.596	0.419
25	1.018	0.596	0.422
26	1.026	0.596	0.430

TABLE D-2

Rat diet schedule and date of sacrifice

Control			Experimental		
Rat	Date sacrificed	Days on diet	Rat	Date sacrificed	Days on* diet
414	6/2/70	284	204	6/2/70	283
413	6/5/70	287	205	6/4/70	286
412	6/10/70	292	227	6/8/70	290
404	6/25/70	307	228	6/23/70	305
437	8/3/70	346	207	8/4/70	347
436	8/6 70	349	208	8/7/70	350
434	8/12/70	355	209	8/11/70	354
433	8/17/70	360	210	8/13/70	356
432	8/21/70	364	211	8/20/70	363
438	10/27/70	431	214	10/28/70	432
440	10/29/70	433	218	10/30/70	434
448	11/2/70	437	223	11/3/70	438
439	11/9/70	444	212	11/11/70	446
443	11/12/70	447	213	11/13/70	448
441	11/17/70	452	217	11/18/70	453

120 albino rats arrived on Aug. 22, 1969, from Simionsan Laboratory, California. 60 rats were assigned arabic numbers starting with 200 as the experimental rats, the other 60 rats beginning with 400 as the control group. The experimental group was treated with 10% ethanol on October 5, 1969 (see page

* The exact date of birth for these rats is not known. Therefore, time is counted from the date of their arrival. The length of alcohol treatment for the experimental group is found by subtracting 45 days from the corresponding "Days on diet"

TABLE E-1

Weight of rat heart and mitochondrial concentration

Rat	Heart g	Mito Protein mg/ml	Mito Protein per heart mg	Mito Protein per g heart mg
CR414	1.21	19.6	15.7	13.0
CR413	1.20	24.8	19.8	16.5
CR412	1.19	17.5	14.0	11.8
CR404	1.44	23.4	18.7	13.0
AR204	1.10	21.8	17.4	15.9
AR205	1.20	15.0	12.0	10.0
AR227	1.15	23.0	18.4	16.0
AR228	1.90	15.1	12.1	6.3

TABLE E-2

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of control rats

Rat	α-Ketoglutarate		Pyruvate-Malate	
	ADP/O	RCR	ADP/O	RCR
414	2.53	6.21		
	2.54	5.93		
	2.57	6.20		
413	2.59	4.89	2.31	3.82
	2.64	5.31	2.49	4.27
	2.77	6.00	2.50	4.17
			2.63	4.40
			2.40	4.40
			2.67	4.75
412	2.52	5.89	2.40	4.71
	2.51	6.57	2.62	3.67
	2.64	6.32	2.66	4.21
	2.66	7.00		
404	2.39	4.80	2.56	3.69
	2.45	5.78	2.48	4.15
	2.48	5.62	2.57	3.00
	2.50	4.81		
	2.59	4.50		
	2.53	5.28		

TABLE E-3

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of ETOH-treated rats

Rat	α-Ketoglutarate		Pyruvate-Malate	
	ADP/O	RCR	ADP/O	RCR
204	2.30	5.26		
	2.49	5.44		
	2.32	6.58		
205	2.51	4.47	2.47	4.21
	2.46	4.57	2.65	4.84
	2.53	4.33	2.85	4.09
			3.00	4.47
227	2.58	5.87	2.67	4.39
	2.65	6.76	2.14	4.23
	2.63	7.60	2.48	6.04
	2.52	4.63	2.30	3.63
	2.80	5.50		
	2.78	5.55		
228	2.53	7.06	2.50	4.16
	2.55	6.53	2.77	3.73
	2.67	6.57	2.50	4.18
			2.68	4.38
			2.80	4.20
			2.74	4.20

TABLE E-4

Maximum oxidation rate per mg mitochondrial protein of control rats

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
414	α-Ketoglutarate	0.587	1.96	0.299
		0.550		0.281
		0.545		0.278
		0.475		0.242
		0.495		0.253
		0.477		0.243
413	α-Ketoglutarate	0.721	2.48	0.291
		0.774		0.312
		0.827		0.333
	Pyruvate-Malate	0.778		0.314
		0.786		0.317
		0.781		0.315
		0.765		0.308
		0.828		0.334
		0.757		0.305
412	α-Ketoglutarate	0.566	1.75	0.323
		0.584		0.334
		0.551		0.315
		0.538		0.307
	Pyruvate-Malate	0.726		0.415
		0.559		0.319
		0.613		0.350
404	α-Ketoglutarate	0.712	2.34	0.304
		0.765		0.327
		0.696		0.297
		0.623		0.266
		0.585		0.250
		0.681		0.291
	Pyruvate-Malate	0.712		0.304
		0.742		0.317
		0.545		0.233

TABLE E-5

Maximum oxidation rate per mg mitochondrial protein of ethanol-treated rats

Rat	Substrate	µgatom O_2/min	Mito mg	µgatom O_2/min/mg
204	α-Ketoglutarate	0.748	2.18	0.343
		0.725		0.335
		0.748		0.343
205	Pyruvate-Malate	0.455	1.50	0.303
		0.427		0.285
		0.393		0.262
		0.342		0.228
	α-Ketoglutarate	0.423		0.282
		0.411		0.274
		0.393		0.262
227	α-Ketoglutarate	0.568	2.30	0.247
		0.550		0.239
		0.555		0.241
		0.496		0.216
		0.659		0.287
		0.659		0.287
		0.690		0.300
	Pyruvate-Malate	1.248		0.543
		1.117		0.486
		0.715		0.331
		0.629		0.273
228	α-Ketoglutarate	0.473	1.51	0.313
		0.454		0.301
		0.455		0.301
	Pyruvate-Malate	0.463		0.307
		0.479		0.317
		0.469		0.311
		0.532		0.352
		0.531		0.352
		0.525		0.348

TABLE E-6

SUMMARY OF OXIDATIVE PHOSPHORYLATION EXPERIMENTS*

	Control		Alcoholic	
	αKG	Pyr-mal	αKG	Pyr-mal
ADP/O				
n	16	12	16	14
mean	2.56	2.50	2.55	2.62
S.D.	0.092	0.119	0.136	0.232
S.E.	0.023	0.034	0.034	0.062
RCR				
n	16	12	16	14
mean	5.69	4.10	5.88	4.35
S.D.	0.710	0.492	1.068	0.560
S.E.	0.177	0.142	0.267	0.152
Max O_2/min/mg mito protein				
n	19	12	16	14
mean	0.291	0.319	0.286	0.334
S.D.	0.030	0.041	0.038	0.085
S.E.	0.007	0.012	0.010	0.023
Min O_2/min/mg mito protein				
n	19	12	16	14
mean	0.057	0.085	0.049	0.074
S.D.	0.015	0.014	0.011	0.023
S.E.	0.004	0.004	0.003	0.006

S.D. = Standard deviation
S.E. = Standard error of the mean
n = Number of observations
RCR = Respiratory control ratio
*Medium equilibrated with 25.7% oxygen

TABLE F-1

Weight of rat heart and mitochondrial concentration

Rat	Heart g	Mito Protein mg/ml	Mito Protein per heart mg	Mito Protein per g heart mg
CR437	1.05	9.8	9.8	9.3
CR436	1.20	15.1	15.1	12.6
CR434	1.10	13.2	11.9	10.8
CR433	1.34	10.0	10.0	7.5
CR432	1.48	15.2	10.6	7.2
AR207	1.40	16.6	16.6	11.9
AR208	1.46	14.3	14.3	9.8
AR209	1.01	12.9	11.6	11.5
AR210	1.37	8.9	8.0	5.9
AR211	1.10	9.0	8.1	7.4

TABLE F-2

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of control rats

Rat	α-Ketoglutarate		Pyruvate-Malate	
	ADP/O	RCR	ADP/O	RCR
437	2.86	4.22	2.77	3.13
	2.57	5.32	2.71	3.71
	2.56	5.24	2.61	3.90
	2.60	4.82	2.72	3.73
	2.59	5.10	2.67	4.29
	2.51	5.46	2.63	3.81
436	2.70	2.76	2.47	2.45
	2.64	3.34	2.49	2.75
	2.42	3.50	2.51	3.33
	2.46	2.73	2.35	2.39
	2.49	3.14	2.41	2.65
			2.51	3.00
434	2.17	4.80	2.78	3.08
	1.92	4.17	2.48	4.00
	2.41	4.35	2.32	4.01
	2.14	4.67		
	2.10	4.88		
433	2.77	3.02		
	2.86	3.42		
	2.79	3.15		
432	2.48	4.95		
	2.36	5.33		
	2.39	5.00		

TABLE F-3

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of ETOH-treated rats

Rat	α-Ketoglutarate		Pyruvate-Malate	
	ADP/O	RCR	ADP/O	RCR
207	2.56	3.19	2.80	2.65
	2.67	3.23	2.71	3.35
	2.44	3.60	2.36	3.70
	2.64	3.39	2.74	3.24
	2.70	3.53	2.68	3.16
	2.53	4.43	2.75	3.52
	2.67	3.16		
	2.50	3.26		
	2.56	3.78		
208	2.65	3.86	2.54	2.78
	3.22	4.77	2.51	2.93
	2.69	4.49	2.43	2.67
	2.58	3.98	2.59	3.00
	2.79	4.81	2.61	2.89
	2.56	4.29		
	2.57	3.34		
	2.64	3.77		
	2.79	3.08		
209	2.58	4.85	2.51	3.11
	2.68	5.25	2.69	3.50
	2.62	5.56	2.86	4.00
	2.58	4.86	2.39	3.24
	2.58	4.62	2.62	3.28
	2.71	5.57	2.75	3.75
210	3.96	4.07	2.71	3.00
	1.75	4.77	2.61	3.12
	2.04	4.80	2.69	3.87
	2.60	4.22	2.36	2.92
	2.39	4.39	2.55	3.20
	2.40	4.80	2.55	2.83
211	2.45	3.60		
	2.53	3.78		
	2.43			

TABLE F-4

Maximum oxidation rate per mg mitochondrial protein of control rats

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
437	α-Ketoglutarate	0.257	0.98	0.289
		0.265		0.298
		0.271		0.304
	Pyruvate-Malate	0.184		0.207
		0.240		0.270
		0.239		0.269
		0.312		0.351
		0.310		0.348
		0.279		0.313
436	α-Ketoglutarate	0.330	1.51	0.220
		0.375		0.250
		0.366		0.244
		0.334		0.223
		0.360		0.240
	Pyruvate-Malate	0.344		0.229
		0.392		0.261
		0.407		0.271
		0.317		0.211
		0.388		0.259
		0.420		0.280
434	α-Ketoglutarate	0.373	1.32	0.283
		0.363		0.275
		0.339		0.272
		0.381		0.289
		0.357		0.270
	Pyruvate-Malate	0.357		0.270
		0.404		0.306
		0.400		0.303
433	Pyruvate-Malate	0.258	1.00	0.258
		0.252		0.252
		0.214		0.214
432	α-Ketoglutarate	0.454	1.52	0.299
		0.476		0.313
		0.469		0.309

TABLE F-5a

Maximum oxidation rate per mg mitochondrial protein of ethanol-treated rats

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
207	α-Ketoglutarate	0.401	1.66	0.242
		0.405		0.244
		0.409		0.246
		0.355		0.214
		0.395		0.234
		0.421		0.254
		0.337		0.203
		0.348		0.210
		0.368		0.288
	Pyruvate-Malate	0.276		0.166
		0.391		0.236
		0.427		0.257
		0.387		0.233
		0.418		0.252
		0.440		0.265
208	α-Ketoglutarate	0.376	1.43	0.263
		0.369		0.258
		0.361		0.252
		0.370		0.259
		0.394		0.276
		0.346		0.242
		0.315		0.220
		0.329		0.230
		0.310		0.217
	Pyruvate-Malate	0.430		0.301
		0.432		0.302
		0.383		0.268
		0.422		0.295
		0.375		0.265

TABLE F-5b

Maximum oxidation rate per mg mitochondrial protein of ethanol-treated rats

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
209	α-Ketoglutarate	0.377	1.29	0.292
		0.375		0.291
		0.360		0.279
		0.341		0.264
		0.346		0.268
		0.349		0.271
	Pyruvate-Malate	0.333		0.258
		0.360		0.276
		0.364		0.282
		0.359		0.278
		0.363		0.281
		0.367		0.284
210	α-Ketoglutarate	0.224	0.89	0.252
		0.227		0.255
		0.217		0.244
		0.226		0.254
		0.216		0.243
		0.218		0.245
	Pyruvate-Malate	0.236		0.265
		0.256		0.288
		0.229		0.257
		0.238		0.267
		0.241		0.271
		0.225		0.253
211	α-Ketoglutarate	0.382	0.90	0.424
		0.413		0.459

TABLE F-6

SUMMARY OF OXIDATIVE PHOSPHORYLATION EXPERIMENTS*

	Control		Alcoholic	
	αKG	Pyr-mal	αKG	Pyr-mal
ADP/O				
n	22	15	33	23
mean	2.49	2.56	2.61	2.61
S.D.	0.247	0.148	0.339	0.140
S.E.	0.053	0.038	0.038	0.029
RCR				
n	22	15	32	23
mean	4.24	3.35	4.12	3.20
S.D.	0.934	0.619	0.784	0.375
S.E.	0.199	0.160	0.139	0.078
Max O_2/min/mg mito protein				
n	16	18	32	23
mean	0.274	0.270	0.262	0.265
S.D.	0.030	0.042	0.052	0.028
S.E.	0.008	0.010	0.009	0.006
Min O_2/min/mg mito protein				
n	19	18	32	23
mean	0.064	0.082	0.064	0.084
S.D.	0.009	0.010	0.016	0.012
S.E.	0.002	0.002	0.003	0.003

S.D. = Standard deviation
S.E. = Standard error of the mean
n = Number of observations
RCR = Respiratory control ratio
*Medium equilibrated with air

TABLE G-1

Weight of rat heart and mitochondrial concentration

Rat	Heart g	Body g	Heart: Body	Mito Prot. mg/ml	Mito Prot. mg/ml	Mito Prot. mg/g heart
Body weight below 600 g						
CR438	1.00	559	0.00179	16.5	11.6	11.6
CR440	1.21	590	0.00205	12.2	9.8	8.1
CR448	1.12	594	0.00189	15.6	11.4	10.2
Mean	1.11	581	0.00191	14.8	10.9	9.9
AR214	1.36	592	0.00230	9.0	8.1	6.0
AR218	1.29	567	0.00226	10.4	7.2	5.7
AR223	1.14	577	0.00198	11.0	6.6	5.8
Mean	1.26	579	0.00218	10.1	7.3	5.8
Body weight over 600 g						
CR439	1.40	748	0.00187	17.2	24.1	17.2
CR443	1.50	819	0.00183	20.3	28.4	19.0
CR441	1.28	645	0.00199	15.3	21.4	16.7
Mean	1.39	737	0.00190	17.6	24.6	17.6
AR212	1.17	667	0.00176	12.3	15.4	13.1
AR213	1.57	724	0.00217	19.0	34.2	21.8
AR217	1.29	661	0.00195	18.0	23.4	18.1
Mean	1.34	684	0.00196	16.4	24.3	17.7

TABLE G-2

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of control and ethanol-treated rats of body weight below 600 g

Rat	Succinate		α-Ketoglutarate	
	ADP/O	RCR	ADP/O	RCR
CR438	1.28	2.22		
	1.11	1.85		
	1.21	2.10		
CR440	1.15	1.70	2.55	3.66
	1.08	1.88	2.58	3.75
	1.17	1.77	2.48	4.62
	1.15	1.78	2.69	3.36
CR448	1.15	1.86	2.56	3.54
	1.21	1.57	2.50	3.90
	1.33	1.57	2.40	4.07
	1.35	1.68		
	1.38	1.27		
	1.36	1.92		
	1.38	2.12		
AR214	1.32	1.99		
	1.27	2.10		
	1.34	2.07		
	1.19	2.17		
	1.41	2.11		
	1.40	2.02		
	1.24	1.93		
	1.25	1.76		
AR218	1.16	1.86	2.46	3.33
	1.20	1.89	2.38	3.44
	1.19	1.75	2.39	3.60
	1.14	1.83	2.34	4.00
AR223	1.22	1.81		
	1.18	1.85		
	1.20	1.83		
	1.13	1.94		

TABLE G-3

Maximum oxidation rate per mg heart mitochondrial protein of control rats of body weight below 600 g

Rat	Substrate	µgatom O_2/min	Mito mg	µgatom O_2/min/mg
438	Succinate	0.583	1.65	0.353
		0.480		0.291
		0.598		0.362
440	Succinate	0.391	1.22	0.320
		0.400		0.328
		0.394		0.323
		0.382		0.313
	α-Ketoglutarate	0.280		0.230
		0.276		0.226
		0.268		0.220
		0.252		0.207
448	Succinate	0.498	1.56	0.319
		0.501		0.321
		0.481		0.308
		0.466		0.299
		0.472		0.303
		0.425		0.272
		0.401		0.257
	α-Ketoglutarate	0.461		0.296
		0.385		0.247
		0.365		0.234

TABLE G-4

Maximum oxidation rate per mg heart mitochondrial protein of ethanol-treated rats of body weight below 600 g

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
214	Succinate	0.269	0.90	0.299
		0.258		0.287
		0.285		0.317
		0.253		0.281
		0.560	1.80	0.311
		0.475		0.264
		0.277	0.90	0.308
		0.260		0.289
218	Succinate	0.324	1.04	0.312
		0.307		0.295
		0.308		0.296
		0.283		0.272
		0.226		0.217
		0.199		0.191
	α-Ketoglutarate	0.218		0.210
		0.214		0.206
		0.213		0.205
		0.224		0.215
223	Succinate	0.334	1.10	0.304
		0.309		0.281
		0.320		0.291
		0.316		0.287

TABLE G-5

SUMMARY OF OXIDATIVE PHOSPHORYLATION EXPERIMENTS*

	Control		Alcoholic	
	αKG	Succ	αKG	Succ
ADP/O				
n	7	14	4	16
mean	2.54	1.24	2.39	1.24
S.D.	0.091	0.107	0.050	0.087
S.E.	0.043	0.029	0.025	0.022
RCR				
n	7	14	4	16
mean	3.84	1.81	3.95	1.93
S.D.	0.419	0.249	0.293	0.131
S.E.	0.159	0.067	0.147	0.033
Max O_2/min/mg mito protein				
n	7	14	4	18
mean	0.237	0.312	0.209	0.283
S.D.	0.029	0.028	0.005	0.032
S.E.	0.011	0.007	0.002	0.008
Min O_2/min/mg mito protein				
n	7	14	4	18
mean	0.060	0.176	0.067	0.150
S.D.	0.006	0.028	0.193	0.014
S.E.	0.002	0.008	0.010	0.003
Initial rate/mg mito protein				
n		9		8
mean		0.116		0.111
S.D.		0.014		0.008
S.E.		0.005		0.003
Rate after addition of rotenone/mg mito protein				
n		9		8
mean		0.136		0.130
S.D.		0.178		0.011
S.E.		0.006		0.004

*Reaction medium equilibrated with 25.4% oxygen, body weights of rats under 600 g.

TABLE H-1*

ADP/O ratios and respiratory control ratios in the metabolism of heart mitochondria of control rats
Body weight over 600 g

Rat	Succinate		α-Ketoglutarate	
	ADP/O	RCR	ADP/O	RCR
439	1.12	1.63	2.49	3.99
	1.34	1.66	2.61	4.13
	1.32	1.69	2.66	4.55
	1.30	1.62	2.77	4.78
	1.20	1.72		
	0.99	2.00		
	1.40	1.88		
	1.13	1.93		
	1.34	1.33		
443	1.17	1.55	2.39	3.36
	1.32	1.82	2.51	4.45
	1.35	1.83	2.43	4.91
	1.31	2.14		
	1.23	1.58		
	1.21	1.72		
441	1.44	1.55	2.54	3.47
	1.60	1.91	2.57	3.84
	1.30	1.86	2.51	4.04
	1.31	1.93		
	1.27	1.84		
	1.31	1.89		

* See TABLE G-1

TABLE H-2

ADP/O ratios and respiratory control ratios in the metabolism of heart mito chondria of ETOH-treated rats
Body weight over 600 g

Rat	Succinate		α-Ketoglutarate	
	ADP/O	RCR	ADP/O	RCR
212	1.39	1.84	3.32	6.67
	1.23	1.88	2.95	8.84
	1.12	1.67	2.80	15.81
	1.21	1.90	2.87	14.30
	1.30	1.81	2.91	9.52
	1.31	1.82		
	0.93	1.80		
	0.89	2.05		
	0.97	1.92		
	0.94	1.96		
213	1.36	1.81	2.89	4.24
	1.34	1.87	2.84	4.94
	1.52	2.52	2.86	5.34
	1.38	2.43	2.79	5.91
	1.27	1.72		
	1.39	2.03		
217	1.29	1.67	2.42	3.24
	1.23	1.72	2.34	3.42
	1.21	1.75	2.19	3.47
	1.24	1.72		
	1.21	1.60		
	1.17	1.69		

TABLE H-3

Maximum oxidation rate per mg heart mitochondrial protein of control rats of body weight over 600 g

Rat	Substrate	µgatom O_2/min	Mito mg	µgatom O_2/min/mg
439	Succinate	0.537	1.72	0.312
		0.451	1.37	0.329
		0.446		0.326
		0.480		0.350
		0.448		0.327
		0.460		0.336
		0.398		0.291
		0.380		0.277
		0.350		0.256
	α-Ketoglutarate	0.369	1.72	0.215
		0.389		0.226
		0.378		0.220
		0.365		0.212
443	Succinate	0.685	2.03	0.337
		0.711		0.350
		0.507	1.62	0.313
		0.450		0.278
		0.526		0.325
		0.505		0.312
	α-Ketoglutarate	0.434	2.03	0.214
		0.501		0.247
		0.543		0.267
441	Succinate	0.485	1.53	0.317
		0.495		0.324
		0.381	1.22	0.312
		0.382		0.313
		0.370		0.303
		0.356		0.292
	α-Ketoglutarate	0.381	1.53	0.249
		0.414		0.271
		0.388		0.254

TABLE H-4

Maximum oxidation rate per mg heart mitochondrial protein of ethanol-treated rats of body weight over 600 g

Rat	Substrate	μgatom O_2/min	Mito mg	μgatom O_2/min/mg
212	Succinate	0.355	1.23	0.289
		0.342		0.278
		0.403		0.328
		0.423		0.343
		0.446		0.363
		0.460		0.374
		0.347		0.282
		0.323		0.263
		0.336		0.273
		0.321		0.261
	α-Ketoglutarate	0.295		0.240
		0.316		0.260
		0.278		0.226
		0.279		0.227
		0.257		0.209
213	Succinate	0.634	1.90	0.334
		0.616		0.324
		0.442	1.52	0.291
		0.374		0.246
		0.458		0.301
		0.449		0.295
	α-Ketoglutarate	0.437	1.90	0.230
		0.405		0.213
		0.416		0.219
		0.409		0.215
217	Succinate	0.440	1.44	0.306
		0.440		0.306
		0.410		0.285
		0.411		0.285
		0.412		0.286
		0.418		0.290
	α-Ketoglutarate	0.342	1.80	0.238
		0.345		0.240
		0.346		0.240

TABLE H-5

SUMMARY OF OXIDATIVE PHOSPHORYLATION EXPERIMENTS*

	Control		Alcoholic	
	αKG	Succ	αKG	Succ
ADP/O				
n	10	21	12	22
mean	2.55	1.28	2.77	1.22
S.D.	0.111	0.126	0.307	0.166
S.E.	0.352	0.028	0.089	0.035
RCR				
n	10	21	12	22
mean	4.15	1.77	4.36	1.87
S.D.	0.521	0.188	0.105	0.228
S.E.	0.165	0.051	0.398	0.049
Max O_2/min/mg mito protein				
n	10	21	12	22
mean	0.238	0.313	0.230	0.300
S.D.	0.023	0.024	0.015	0.033
S.E.	0.007	0.005	0.004	0.007
Min O_2/min/mg mito protein				
n	10	21	12	22
mean	0.058	0.179	0.042	0.162
S.D.	0.009	0.023	0.021	0.027
S.E.	0.003	0.005	0.006	0.006
Initial rate/mg mito protein				
n		12		11
mean		0.111		0.104
S.D.		0.012		0.014
S.E.		0.004		0.004
Rate after addition of rotenone/mg mito protein				
n		12		11
mean		0.131		0.127
S.D.		0.006		0.016
S.E.		0.002		0.005

*Reaction medium equilibrated with 25.4% oxygen, body weights of rats exceed 600 g.

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APPROVAL SHEET

The dissertation submitted by Daniel Yue-King Chan has been read and approved by five members of the Faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given approval as regards content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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DATE

SIGNATURE OF ADVISOR