# EARLY CARDIAC EFFECTS OF ALCOHOL IN THE RAT

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by

LAWRENCE E. HOESCHEN

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ABSTRACT

The effects of chronic alcohol ingestion on the myocardium are diffuse and a search for some basic mechanism common to many forms of damage may prove fruitful. The effects of ethanol as a solvent on sarcolemmal and mitochondrial membranes, with consequent leakage of small particles such as ions may be one such mechanism. Another mechanism may be the demonstrated ability of chronic alcohol ingestion to produce a specific magnesium deficiency, for it is well known how vital magnesium is to mitochondrial and sarcolemmal membrane structure and function. The purpose of these experiments was to study the effect of chronic alcohol ingestion on cardiac mitochondrial respiration, and intracellular ion concentrations, especially magnesium, and their potential for reversibility if detected in the early stages.

Male Long-Evans rats weighing 200 g were given ethanol in water solutions ad lib as their sole source of liquid, and laboratory chow ad lib. For the first 4 weeks the ethanol concentration was increased twice weekly from 5% to 25% and remained at 25% for the next 8 weeks. The control animals were given tap water ad lib and chow pellets with dextrose instilled in them in amounts iso-caloric with the alcohol calories consumed by the alcohol-fed rats, which had been determined in preliminary 12 week trials. One group of control and alcohol-fed rats was selected for mitochondrial respiration studies and mitochondrial magnesium and calcium content. This group was further divided, one week before killing, into magnesium supplemented and non-supplemented groups, each with alcohol-fed and appropriate control rats. To further assess the reversibility of the mitochondrial dysfunction, all groups of animals were assayed for mitochondrial function in the presence of *in vitro* 5 mM $MgCl_2$, as well as 100 mM KCl, magnesium and potassium being the two most

abundant intracellular cations. Another group of control and alcohol-fed rats was selected for estimation of cardiac tissue water and electrolyte distribution, as well as an assessment of sarcolemmal permeability. This group was further divided, by allowing a number of control and alcohol-fed rats to return to standard lab chow and water ad lib for another 8 weeks before killing.

Analysis of the mitochondria showed a decrease in the respiratory control index associated with a drop in the mitochondrial magnesium content in the alcohol-fed rats, and these differences were abolished with the in vivo magnesium supplements. Analysis of the tissue water and electrolyte data showed an increase in sarcolemmal permeability in the alcohol-fed rats, with slight, but statistically non-significant decrease in intracellular potassium and increase in intracellular sodium and calcium and a shift of water from outside to inside the cell. All these changes were reversed with abstention from alcohol.

Chronic alcohol ingestion produces deficient mitochondrial respiration associated with a decrease in mitochondrial magnesium. In addition it produces an increase in sarcolemmal permeability with its attendant ion and water shifts. Both these processes, if detected early enough, are reversible, the former with magnesium supplements and the latter with abstention from alcohol.

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# I. INTRODUCTION

A. HISTORICAL PERSPECTIVE

The association between heavy alcohol ingestion and cardiac disease has been well documented since the mid-nineteenth century. The earliest report (Wood, 1855), as well as subsequent ones (Walshe, 1873), including the "Munich beer heart" (Bollinger, 1884) and others (Steell, 1893; MacKenzie, 1902), made no distinction between nutritional factors and a direct effect of alcohol in the development of alcoholic cardiomyopathy. In 1929, Aalsemeer and Wenckebach described a distinct type of heart disease (beri beri) in non-alcoholic orientals which was due to a nutritional deficiency of thiamine. Weiss and Wilkins, in 1937, also described beri beri heart disease and noted a connection with chronic alcoholism. Thus, at this time it was generally thought that alcoholic heart disease was due to a deficiency of thiamine (i.e. beri beri heart disease). However, Blankenhorn (1946) described twelve alcoholics with heart disease who did not respond to thiamine, and Brigden (1957) described thirteen cases of alcoholic heart disease which also did not respond to thiamine and presented in a low cardiac output state. By the late 1950's, over one hundred years after the first report of alcohol-associated heart disease, there was enough evidence in the literature to support the idea of an alcoholic cardiomyopathy as a clinical entity distinct from beri beri heart disease.

The clinical picture of alcoholic cardiomyopathy is not specific and appears the same as any congestive cardiomyopathy, with the features of a large heart on chest X-ray and signs and symptoms of congestive heart failure. The criteria for diagnosis have been outlined by McDonald et al. (1971) and Demakis et al. (1974); they consist of a) signs and symptoms of congestive heart failure, b) no known cardiac

disease and c) a history of heavy alcohol intake. The diagnosis of alcoholic cardiomyopathy is essentially one of exclusion of other forms of heart disease and documentation of heavy drinking. Because of these two features, it has been difficult to establish the true incidence of the disease. Schenk and Cohen (1970), in post mortem examination of 97 alcoholics, found 15 with clinical evidence of heart failure and no other cardiac diseases to account for it. Several authors have approached the problem the other way by reporting the incidence of alcoholic cardiomyopathy in the congestive cardiomyopathies. Burch and Giles (1973), summarizing their previous work, stated that 34 of the 123 patients referred to their bed rest program had alcoholic cardiomyopathy. Earlier, Brigden (1957) had diagnosed alcoholic cardiomyopathy in 13 of the 50 patients he studied. More recently Koide et al. (1980) reported that 10 of their 36 cases of cardiomyopathy were due to alcohol. From the latter three studies one could estimate the incidence at 26-28% of all cases of cardiomyopathy.

B. ACUTE EFFECTS OF ALCOHOL

Although extrapolation of the data on the acute effects of alcohol on the heart to the chronic effects is dangerous, a brief summary of the acute experiments may give some insight into the pathogenesis of the disease produced by chronic ingestion. First, the results of experiments on left ventricular function are confusing because different parameters of function are used in different experiments. Generally speaking, however, alcohol can be said to be a general myocardial depressant after acute ingestion. Acute in vivo hemodynamic experiments in man have shown an increase in cardiac output associated primarily with a rise in heart rate (Juchems and Klobe, 1969; Riff et al., 1969; Blomquist et al., 1970) and a mild decrease in peripheral vascular resistance. An earlier report by Wendt et al. (1966) showed no change in these parameters but a depression in myocardial metabolism. Patients with ischemic heart disease have demonstrated a decrease in cardiac contractility with no change in heart rate after acute alcohol ingestion (Conway, 1968; Gould et al., 1971). More recent studies with more sophisticated techniques using systolic time intervals (Ahmed et al., 1973) and echocardiography (Delgado et al., 1975) have proven that acute alcohol ingestion does produce a definite depression of left ventricular contractility in normal volunteers. All of the above studies were performed in man with blood alcohol levels in the range of 50-200 mg/dl produced via the oral route. Acute in vivo studies in animals have been done mostly in dogs and the early reports (Loomis, 1952; MacGregor, 1964) showed no depression of cardiac function until lethal levels of alcohol were achieved. Regan et al. (1966), infusing ethanol to attain blood levels of 100-200 mg/dl, demonstrated a decrease in left ventricular

contractility, associated with a leakage of myocardial cell constituents into the coronary sinus in dogs. A strain gauge analysis of left ventricular performance in dogs by Newman and Valicenti (1971) showed that myocardial depression was accompanied by an increase in preload and that this effect could be reversed by ouabain. Although there is general agreement about the myocardial depressant effect of alcohol, some of the inconsistencies in the literature from the in vivo experiments were thought to be caused by the influences of an intact autonomic nervous system. In addition, it has been shown that some of the effects of acetaldehyde, a product of alcohol metabolism, are mediated via catecholamine release (Walsh et al., 1969; Nakano and Prancan, 1972). Nakano and Prancan (1972) demonstrated that reserpine, propranolol, and phenoxybenzamine all blocked the cardiostimulatory effects of low levels of alcohol in dogs. Similar results have been obtained by other authors in dogs (Wong, 1973; Horwitz and Atkins, 1974) and humans (Child et al., 1979). Despite the influence of the autonomic nervous system, the contractility of cardiac muscle is depressed directly by alcohol as demonstrated by in vitro experiments in guinea pig myocardium (Nakano and Moore, 1972), isolated rat ventricle (Hirota et al., 1976), cat papillary muscle (Mason et al., 1978), and blood perfused canine atrium (Kobayashi et al., 1979).

The acute effects of alcohol on the electrophysiologic properties of the heart in vivo have been studied by James and Bear (1967) who perfused the sinus node artery in dogs and produced no chronotropic changes with ethanol except in doses beyond the lethal level, but showed marked acceleration of heart rate with acetaldehyde. However, Kostis et al., (1977) produced an increase in the atrial fibrillation threshold

in dogs when perfusing with ethanol, obtaining blood levels of 300 mg/dl. Kentala et al. (1976) produced periods of ventricular ectopic beats and sinus arrest in non-alcoholic patients with a history of myocardial infarction while they ingested alcohol. The most consistent effect produced by alcohol in the in vitro preparations on the action potential in cardiac muscle is a shortening of the action potential duration with a moderate decrease in its amplitude (Gimeno et al., 1962; Fisher and Kavaler, 1975; Williams et al., 1980; Snoy et al., 1980). The mechanisms of the electrophysiologic changes have not been elucidated.

Another important factor in the mechanical function of the heart is its blood supply and several authors have examined the acute effect of alcohol on the coronary circulation. Studies in dogs have produced no change (Willard and Horvath, 1964), a decrease (Webb and Degerli, 1965), and an increase (Ganz, 1963; Pitt et al., 1970; Mendoza et al., 1971; Abel, 1980) in coronary blood flow. Lindeneg et al. (1964) and Mendoza et al. (1971) found no change in coronary blood flow in humans, but the blood alcohol levels were only 3.16 mM ( 15 mg/dl) and 36 mg/dl respectively. Interest in the acute effects of alcohol on coronary blood flow has stemmed from its early use as an anti-anginal agent, probably more for its psychological effect than anything else. A recent interesting study by Friedman et al. (1981) in dogs with a ligated left anterior descending coronary showed that alcohol caused an increase in coronary blood flow in the healthy tissue and a further decrease in the ischemic tissue, producing in effect a "coronary steal". The implications in these experiments for patients with angina are quite serious.

The volume of literature on the acute metabolic effects of alcohol on the myocardium is very large. In vivo experiments in man have demonstrated a decrease in the uptake of free fatty acids (Lindeneg et al., 1964), or no change (Regan et al., 1966; Wendt et al., 1966). Acute alcohol infusions in rabbits have produced an increase in myocardial triglycerides associated with a decrease in fatty acid oxidation (Kikuchi and Kako, 1970; Kako and Kikuchi, 1972). Kikuchi and Kako (1970) also showed no change in lipoprotein lipase activity, an enzyme which enhances triglyceride accumulation. However, Mallov and Cerra (1967) had previously shown that acute alcohol ingestion or injection in rats produced a rise in cardiac lipoprotein lipase which was mediated via catecholamine release. Alterations in lipid metabolism have been attributed to mitochondrial dysfunction but Gvozdjak et al. (1973a,b,1976,1977) have found no change in mitochondrial respiration in rat myocardium after intraperitoneal injections of alcohol. They did, however, find a decrease in the activities of mitochondrial and sarcoplasmic $Mg^{+2}$-$Ca^{+2}$-activated ATPase (1977). In vitro experiments on canine myocardium have shown that ethanol inhibits calcium binding and uptake by sarcoplasmic reticulum but the concentrations of ethanol were far beyond the lethal level (Swartz et al., 1974; Retig et al., 1977). Williams et al. (1975) with similar levels of alcohol showed an inhibition of sarcolemmal Na-K-ATPase in guinea pigs. However, recently Kobayashi et al. (1979) demonstrated a uniform suppression of the positive staircase phenomenon in dog right atrium with very low concentrations of ethanol (1-3 m mol) indicating an interference in the excitation-contraction apparatus and calcium fluxes. Acute in vitro studies on protein synthesis have shown that alcohol, at a concentration

of 250 mg/dl at 37°C, had no effect on $^{14}$C-leucine incorporation in perfused guinea pig heart, but that acetaldehyde did (Schrieber et al., 1972, 1974, 1976). Gailis (1976) showed that this $^{14}$C leucine incorporation was temperature dependent and at higher temperatures alcohol can inhibit protein synthesis. Based on these studies, Rettig et al. (1979) examined ornithine decarboxylase activity, a rate limiting enzyme in protein synthesis, in rat hearts, and found that acetaldehyde, but not alcohol, inhibited the enzyme.

We can summarize the acute effects of alcohol on the heart by stating that alcohol depresses contractility, causes arrhythmias, shortens the action potential duration, increases coronary blood flow, increases triglyceride accumulation, and inhibits protein synthesis. These effects have been shown with clinically relevant concentrations of alcohol, and some are likely caused by acetaldehyde directly or indirectly via catecholamine release.

C. EFFECTS OF ACETALDEHYDE

Acetaldehyde, a metabolite of ethanol, accumulates in the blood after ethanol infusions in normal man, and in greater amounts in alcoholic man (Korsten et al., 1975). Therefore the role of acetaldehyde in the pathogenesis of any alcohol-induced disease process must be considered. Most of the experiments have examined the acute effects, because of the technical difficulties in chronically administering acetaldehyde. One study by Nagano et al. (1978) demonstrated a possible chronic effect of acetaldehyde, using Tolbutamide which is an acetaldehyde dehydrogenase inhibitor producing elevated blood levels of acetaldehyde. In rats given either 10% ethanol alone, 10% ethanol plus Tolbutamide, or Tolbutamide alone, for 8 to 20 weeks, the only cardiac damage produced, in the form of decreased oxidative phosphorylation in mitochondria, was in the ethanol-Tolbutamide group. Furthermore this group had higher blood acetaldehyde levels than the rats given ethanol alone, although acetaldehyde levels were not stated. In addition, no mention was made of the acetaldehyde levels produced by Tolbutamide alone. Weishaar et al. (1978) also showed decreased mitochondrial function, protein synthesis, and contractility in rats fed ethanol as 36% of their daily calories for 3 to 4 weeks, with some of them receiving pargyline, a drug known to elevate acetaldehyde levels in animals fed ethanol. Parglyine alone had no effect. The acetaldehyde levels produced with ethanol and pargyline were far too high (80 mM) to draw any conclusions. Clinically relevant blood levels of acetaldehyde are not always used in the experiments so that interpretation of the data is sometimes difficult. The usual range of acetaldehyde levels after acute ingestion in non alcoholics is from 0.023 mM (Truitt and Walsh, 1971) to 0.028 mM (Korsten et al., 1975) and in alcoholics is 0.043 mM

(Korsten et al., 1975). The following discussion concerns only acute experiments. As mentioned earlier, the in vivo experiments easily demonstrated the cardiostimulatory effects of acetaldehyde as mediated by catecholamine release (James and Bear, 1967; Nakano and Prancan, 1972; McCloy et al., 1974; Alexander et al., 1978), but the concentrations of acetaldehyde were 0.2 mM or greater. Recent in vivo experiments by Friedman et al. (1979) in dogs, have separated the cardiac depressant effects of alcohol from the stimulant effects of acetaldehyde within the same animal, using acetaldehyde concentrations of 0.02 to 0.04 mM. In vitro studies on the effect of acetaldehyde have used concentrations too high for appropriate interpretation of the data. J.S. Williams et al. (1975) showed that acetaldehyde (5 mM) inhibited sarcolemmal Na-K-ATPase in guinea pigs. E.S. Williams et al. (1980), using acetaldehyde at a concentration of 0.8 mM, produced a prolongation of the action potential duration of canine Purkinje fibers in contrast to the shortening produced by ethanol. Mitochondrial respiration in rat hearts can be depressed by acetaldehyde in vitro but the lowest concentration used was 0.3 mM (Segal and Mason, 1979). The ability of mitochondria from guinea pig hearts to accumulate calcium was not impaired (Nayler and Fassold, 1978) until the acetaldehyde concentration reached 10 mM, but the $Ca^{+2}$-dependent myofibrillar ATPase activity was depressed by acetaldehyde in the range of 0.02 mM-0.30 mM. Apart from the sympathomimetic effects on the hemodynamics of cardiac muscle, the role of acetaldehyde in the acute cardiac effects of alcohol still seems unclear, and its role in the chronic effects even moreso.

D. CHRONIC EFFECTS OF ALCOHOL

The literature on the chronic effect of alcohol on the heart is even more extensive than the acute studies. Inconsistent results have occurred because of many factors, such as duration and quantity of alcohol administration, the method of administration, the type of animal used, the type of diet, the use of pair-feeding techniques, and the different methods of isolation of organelles used, to name a few. Since the definition of the disease was finally developed by the late 1950's, there have been many reviews written on the subject of alcoholic cardiomyopathy (Burch and Walsh, 1960; Brigden and Robinson, 1964; Burch and DePasquale, 1969; Goodwin, 1977; Regan et al., 1977; Bing and Tillmanns, 1977; Bing, 1978; Rubin, 1979). The general conclusion is that alcohol has diverse effects on the myocardium, i.e. structural, mechanical and electrical function, biochemical in the form of lipid metabolism, enzyme activities, mitochondrial respiration, calcium metabolism, protein synthesis, and membrane composition. In the following discussion we will deal with each of these aspects separately while recognizing that they are interdependent.

D.i. ANATOMY

Mice fed 15% ethanol for 3 months (Sohal and Burch, 1969) developed widening of the intercalated disc space in the area of insertion of the myofibrils. Mice fed varying concentrations of ethanol (5-20%), beer, and wine for 4 to 10 weeks (Burch et al., 1971) showed no changes under light microscope, but electron microscopy revealed mild lipid accumulation, dilated T tubules, swollen sarcoplasmic reticulum and mitochondria, ruptured myofibrils, and a dehiscence of intercalated discs. The least damage appeared in the animals that had been consuming

the highest alcohol concentrations, but the shortest duration (4 weeks). A study of longer duration (24 weeks) by these same authors (Burch et al., 1971) produced the same lesions but to a more severe degree, irrespective of the type of beverage consumed. Alexander et al. (1977b)produced similar lesions in mice after 15 and 25 weeks of ethanol which were present under electron microscopic examination but not under light microscopy. Monkeys fed alcohol daily via stomach tube as 40% of their daily calories for 3 months developed a three fold rise in myocardial triglyceride content (Vasdev et al, 1975) with only mild structural damage. Segel et al. (1975) fed rats alcohol in 5%, 10% and 25% concentrations for 25-45 weeks, with and without vitamin supplements, and found the usual structural alterations described above as early as 7 weeks of drinking 5% ethanol. There were no changes in myocardial hydroxyproline or catecholamine content, and vitamin supplements did not protect the heart from structural damage. A morphometric study of mitochondrial ultrastructure in the myocardium of rats fed ethanol as 33% of their calories for 33 weeks demonstrated a decrease in the number of mitochondria but a doubling of the volume of an average mitochondrion, and most of this increase in volume was in the mitochondrial matrix space (Mall et al., 1980). Recently it has been shown that cardiac catalase may have a protective effect in the myocardium of rats fed ethanol for 5 weeks (Kino, 1981). The structural abnormalities found in animals have also been found in humans. Postmortem analysis of alcoholics who died in heart failure showed an increase in lipid content, the presence of lipofuscin granules thought to be related to lysosomal activity, and damaged mitochondria, all under light microscopy (Ferrans et al., 1965). Further analysis by electron microscopy revealed mitochondrial and sarcoplasmic reticular swelling and degenerating myofibrils (Hibbs et al., 1965).

Myocardial biopsy specimens obtained from patients with a history of alcoholism revealed the same lesions as found in the animal models (Alexander, 1966, 1967). It is interesting to note that even as early as 1966, Alexander commented on the similarity of potassium deficiency and magnesium deficiency hearts compared to alcoholic hearts. Several other authors have provided confirmation of the structural changes (Schenk and Cohen, 1970; Bulloch et al., 1972; Hognestad and Teisberg, 1973) with a little more patchy fibrosis present than in other studies. Generally speaking, the histologic changes caused by chronic alcohol consumption consist of dehiscence of intercalated discs, swollen mitochondria and sarcoplasmic reticulum, dilated T tubules, and ruptured myofibrils. These changes have been noted in varying degrees in other cardiac diseases and are likely a non specific response to chronic injury, except for the triglyceride accumulation which is a little more specific to alcohol effects. Obviously, many electrical, mechanical and biochemical changes can occur before the structural changes occur.

D.ii. VENTRICULAR FUNCTION

Most of the chronic alcohol studies in animals have been done in rats. Maines and Aldinger (1967) fed 25% alcohol to rats for a maximum of 28 weeks and found that the isometric systolic tension of the heart began to decrease at 16 weeks, and vitamin supplements did not protect the heart. However Lochner et al. (1969) fed rats 15% ethanol for 18 months and found no change in cardiac contractility, oxygen consumption, or high energy phosphate content. The work of Segel et al. (1975) showed that rats fed 5% ethanol for 25 weeks showed no deterioration in isolated papillary muscle mechanics, but the rats fed 10% ethanol and 25% ethanol for 45 and 26 weeks respectively developed a decrease in the time to peak tension

(TTPT) with no change in dT/dt, and vitamin supplements were not protective. Additional work by Segel et al. (1979) with isolated perfused hearts from rats fed ethanol as 38% of the daily calories, and with a low fat or high fat diet demonstrated no changes in any of the indices of contractility studied, regardless of diet. However, when the alcoholic hearts were challenged with dobutamine, they responded with a significantly smaller increase in contractility than controls. Experimental hypoxia in these hearts produced the same pattern, but even more pronounced in the low-fat treated hearts. These findings may explain why certain alcoholic animal models produce no change in myocardial contractility and others do, and that the depressive effects of chronic alcohol may only be manifested under stressful conditions. More recently, Whitman et al. (1980b) could not produce changes in contractility or metabolism in rats fed ethanol as 55% of their daily calories for 12 to 14 weeks. Chronic experiments in dogs have produced different results, depending on the duration of alcohol consumption. Dogs fed alcohol as 36% of their daily calories for 22 months showed significant changes in end diastolic pressure and volume with increases in both pre-load and afterload (Regan et al., 1974), while Pachinger et al. (1973) could produce no change in contractility in dogs fed 25% ethanol for 14 weeks. However, Bing et al. (1974) in extending this same animal model to 6 months of ethanol ingestion, did produce a decrease in left ventricular end-diastolic pressure (LVEDP) and an increase in the maximum rate of rise of left ventricular pressure (dp/dt max). Further extension of this model to 29 months (Sarma et al., 1976) paradoxically showed no hemodynamic changes between alcohol fed and controls in vivo, but a depression of Vmax in an in vitro preparation of glycerinated heart muscle fibers from the alcohol-fed animals. Thus,

animal experiments have produced different results because of different species, strains, alcohol quantity and duration, and the different parameters studied.

The hemodynamic data from chronic alcoholics has been obtained in various ways, leading to the general conclusion that chronic alcohol ingestion does depress myocardial contractility, even in patients who have not yet developed symptoms of cardiac disease. Studies performed by cardiac catheterization have demonstrated a deterioration in various hemodynamic measurements in alcoholics (Wendt et al., 1965; Regan et al., 1969; Asokan et al., 1972). Systolic time intervals have been shown to be abnormal in alcoholics, many of whom had no clinical evidence of heart disease (Spodick et al., 1972; Zambrano et al., 1974; Levi et al., 1977). Echocardiography performed on alcoholics without overt cardiac disease has shown hypertrophy of the left ventricle (Askanas et al., 1980; Koide et al., 1981) but no change in the echocardiographic indices of contractility (Askanas et al., 1980). However, alcoholics with previously diagnosed cardiomyopathy had depressed contractility as shown by echocardiography (Askanas et al., 1980). Overall, the evidence for alcohol-induced depression of myocardial contractility has been fairly consistent in humans, but less so in animal studies. A good, complete animal model of alcoholic cardiomyopathy has yet to be developed.

D.iii. ELECTROPHYSIOLOGIC FUNCTION

Originally, Evans (1959) described what was thought to be electrocardiographic changes secondary to alcoholic cardiomyopathy, specifically T wave changes. However, several reviews over the years have examined the evidence (Brigden and Robinson, 1964; Goodwin, 1974; Regan et al., 1977) and the consensus is that the electrocardiographic

changes found in alcoholic cardiomyopathy are general signs of myocardial muscle cell and conduction tissue damage. However, there are reports of magnesium depletion in alcoholics producing slightly more specific changes in the ST segments and T waves (Seelig, 1969; Luomanmaki, 1975). A chronic experiment in dogs, which ingested 40% ethanol for 7 to 33 months, produced no change in electrical conduction times in the hearts of animals ingesting alcohol for less than 1 year, but showed a prolongation of HQ time and QRS duration in animals drinking alcohol an average of 19 months, (Ettinger et al., 1976). Complete heart block has developed in an alcoholic with no other signs of heart disease (Leier et al., 1974), and the conduction defect was found to be in the region of the A-V node. In patients diagnosed as having alcoholic cardiomyopathy, frequent conduction disturbances were found at multiple sites in the atrioventricular system (Luca, 1979). Ventricular arrhythmias have developed in chronic alcoholics after heavy acute ingestion (Singer and Lundberg, 1972; Greenspon et al., 1979), and Ettinger et al. (1978) have described the "holiday heart" syndrome where chronic alcoholics who have consumed more than usual on a weekend binge have developed atrial arrhythmias in the absence of any overt heart disease. The mechanism of arrhythmias and conduction disturbances produced by chronic ethanol ingestion has not been established.

D.iv. CARDIAC METABOLISM

Many researchers have investigated the effects of chronic alcohol ingestion on mitochondrial function in the heart emphasizing different points such as intramitochondrial enzymes, calcium binding and uptake, and respiratory function. Depression of mitochondrial respiration has been demonstrated by many authors, particularly in rats. Gvozdjak et al. (1973a, 1973b, 1976) produced a decrease in the respiratory control

ratio of cardiac mitochondria in rats given 20% ethanol intraperitoneally for 10 weeks, with no change in ATP, creatine phosphate or inorganic phosphate content. Segel et al. (1975) found no defect in mitochondrial function in rats fed 25% ethanol until they had been drinking at least 19 weeks, at which time there was a significant decrease in the respiratory control ratio and oxygen consumption. However, Weishaar et al. (1977) showed a depression in oxygen consumption of cardiac mitochondria in rats fed 25% ethanol for only 8 weeks, which returned to normal after 8 weeks of abstention from alcohol. Rats fed alcohol as 36% of their daily calories for 7 weeks did not show any changes in the respiratory control ratio, but showed a decrease in oxygen consumption only with pyruvate as substrate, one of four substrates used (Williams and Li, 1977). In addition there was no change in fatty acid incorporation into the mitochondria. Furthermore, Whitman et al. (1980b),feeding alcohol to rats as 55% of their calories for 12 to 14 weeks, did not produce a change in the respiratory control ratio, but there was a decrease in state 3 and state 4 oxygen consumption only with glutamate as substrate, again one of four substrates tested. In this study there was no change in ATP, creatine phosphate, or triglyceride content in the heart. Rats fed ethanol as 36% of their diet were divided additionally into high fat plus ethanol and high protein plus ethanol and fed for 8 weeks. The high protein plus ethanol diet did not affect mitochondrial function, but the high fat plus ethanol diet produced a depression of both state 3 and state 4 respiration, but no change in respiratory control ratio (Wahid et al., 1980). Cardiac mitochondrial respiratory function has also been studied in dogs fed 25% ethanol for 14 weeks by Pachinger et al. (1973), who showed a diminution only in NAD specific isocitrate dehydrogenase activity, one of nine intracellular

enzymes studied, as well as a decrease in the respiratory control ratio with several substrates used. Extension of the duration of ethanol feeding in these animals to 6 months (Bing et al., 1974) and 29 months (Sarma et al., 1976) has confirmed this.

Because the heart has been shown to accumulate triglycerides after chronic alcohol ingestion (Alexander et al., 1977a), there has also been an interest in the effects of chronic alcohol ingestion on myocardial lipid metabolism. As mentioned earlier, acute alcohol can cause an accumulation of triglyceride, associated with a decrease in the β-oxidation of fatty acids (Kako et al., 1973). Relevant to this finding, in the chronic animal it has been shown that rats fed 20% ethanol for 6 weeks developed a large decrease in carnitine acyltransferase activity, specifically those of palmitoleate and arachidonate, in the myocardium (Parker et al., 1974). This enzyme is largely responsible for the initiation of the β-oxidation of fatty acids. Conversely, Williams and Li (1977) found no change in palmityl carnitine transferase activity in the hearts of rats fed ethanol as 36% of their calories for 7 weeks. Several authors have shown a general pattern of fatty acid metabolism in the myocardium after chronic alcohol ingestion, namely that of decreased β-oxidation and increased incorporation into various lipid fractions. This has been shown in rats for linoleic acid (Reitz et al., 1973), and oleic acid (Regan et al., 1974). Further analysis of this rearrangement of lipid fractions reveals that along with the increase in triglyceride accumulation there is a decrease in cardiac phospholipid content (Reitz et al., 1973; Regan et al., 1974). However, mice given ethanol by the inhalation technique for 10 days to produce dependence showed no change in myocardial phospholipid concentration (Littleton et al., 1979). Another experiment in mice given 20% ethanol

by mouth for 10 days showed a decrease in myocardial total lipid, all in the neutral lipid fraction, with no change in phospholipid or triglyceride fractions (Crane et al., 1981). Although a decrease in cardiac phospholipid content after chronic alcohol ingestion has not been conclusively proven, the significance of this proposed defect in terms of membrane function (mitochondrial or sarcolemmal) is worth noting. An interesting experiment by Wojcicki et al. (1975) demonstrated that a diet supplemented with essential phospholipids protected the myocardium of rats fed 15% ethanol for 8 months from structural damage and leakage of intracellular enzymes. In addition, the alcohol-induced elevation of free fatty acid levels in the blood was normalized by the essential phospholipid diet. These experiments suggest an increase in membrane permeability and evidence to support this is given by the work of Wendt et al. (1965) who demonstrated a leakage of intramitochondrial and cytosolic enzymes into the coronary sinus of chronic alcoholic patients undergoing right heart catheterization. In addition, Regan et al. (1969) demonstrated leakage of transaminase, potassium, and phosphate into the coronary sinus of alcoholics who had ingested 12 ounces of Scotch whiskey acutely.

Because of the well documented changes in cardiac contractility and mitochondrial function, there has been some interest in myocardial calcium metabolism. Bing et al. (1974), with dogs ingesting 25% ethanol for 6 months, in addition to the depressed mitochondrial function already described, showed a decrease in calcium binding and uptake by both mitochondria and sarcoplasmic reticulum, with no change in the associated $Ca^{+2}$, $Mg^{+2}$-ATPase activities. Endogenous calcium content of both organelles was significantly decreased in the alcoholic animals. In the same animal model Pachinger et al. (1975) found no change in myocardial high energy

phosphate levels. It is interesting to note that Sarma et al. (1976), in extending this animal model to 29 months, produced the same metabolic changes, but found no change in the hemodynamic properties in vivo. However, the in vitro study of glycerinated heart muscle fibers showed a depression of Vmax in the alcohol-fed animals. These experiments presumably measured the maximum velocity of contractile force uninfluenced by membranous structures which could alter the calcium levels.

Although no changes in high energy phosphate levels in the myocardium have been found, a few authors have examined the enzymes responsible for the metabolism of high energy phosphates. As previously mentioned there was no change in sarcoplasmic or mitochondrial $Ca^{+2}$-$Mg^{+2}$-ATPase activities in the dog heart after chronic alcohol consumption (Pachinger et al., 1975). However, Gvozdjak et al. (1977) found an increase in mitochondrial and sarcoplasmic ATPase activities in the myocardium of rats given 20% ethanol intraperitoneally daily for 10 weeks. Segel et al. (1975) found a decrease in myofibrillar ATPase associated with changes in isolated papillary muscle mechanics, but only in those rats consuming the highest concentration, namely 25%. Sharma and Banerjee (1977a,1977b) and Banerjee and Sharma (1978) in a series of experiments, showed a relationship between $Na^{+}$-$K^{+}$-ATPase activity, noradrenaline uptake, and chronic alcohol ingestion in the myocardium of cats. First they showed a direct relationship between $Na^{+}$-$K^{+}$-ATPase activity and noradrenaline uptake in the myocardium of cats, using $^{3}H$-ouabain binding studies (1977a). They also showed that 80% of these specific $^{3}H$-ouabain binding studies were located in the sympathetic nerve endings (1977b). They then demonstrated that cats fed 20% ethanol for 5 weeks via stomach tube developed an increase in the number of $Na^{+}$-$K^{+}$-ATPase molecules in the microsomal fraction

of the myocardial cells, suggesting an increase in the turnover of noradrenaline, but noradrenaline tissue levels were not measured. However, rats fed 32% ethanol for 16 weeks developed increased levels of myocardial noradrenaline (Rossi and Oliveira, 1976), but this effect was later shown to be diet related (Rossi, 1980). Catecholamine levels may be altered by acetaldehyde rather than alcohol itself, as mentioned before. Morvai et al. (1979) fed rats ethanol as 36% of their daily calories for 8 weeks and showed that there was a slight decrease in catecholamines in the noradrenergic nerve fibers in the heart, but an increase in the myocardial cell catecholamine level was demonstrated by a histofluorescent method. They suggested a chronic release of catecholamines from the nerve endings, possible mediated by acetaldehyde. More recently, experiments by Sabourault et al. (1981) showed that rats given ethanol by vapor to produce blood alcohol levels of 70 mg/dl for 3 weeks did not produce any change in the number and affinity of $\alpha$ and $\beta$ receptors in the heart.

The effect of chronic ethanol ingestion on myocardial protein synthesis has been examined by a few authors, including Rawat (1979a) who fed 6% ethanol to rats for 4 weeks and demonstrated a decrease in total RNA content, but not DNA, and a decrease in $^{14}$C-leucine incorporation into whole heart homogenate as well as isolated ribosomal and pH5 enzyme fractions. Similar results were shown in fetal and neonatal rat hearts from rats of mothers fed alcohol in the same manner (Rawat, 1979b). In contrast, rats fed ethanol as 25% of their daily calories for 7-10 weeks, showed no change in myocardial RNA, DNA or total protein content (Smith-Kielland and Morland, 1979). The work of Whitman et al. (1980a) demonstrated that chronic alcohol ingestion by rats as 55% of their daily calories for 14 weeks did not affect the increase in RNA or total myocardial protein

content induced by aortic banding. These rats had maintained a normal ability to develop cardiac hypertrophy in response to a pressure overload, in spite of chronic alcohol ingestion.

A special category of the metabolic effects of alcohol on cardiac muscle is the unique syndrome of cobalt cardiomyopathy. This was a form of alcoholic cardiomyopathy which occurred in epidemics in the mid 1960's in beer drinkers (Sullivan et al., 1969; Morin et al., 1969). However, the causative agent was found to be the cobalt in the beer, which was used as a foam stabilizer. The toxic effects of cobalt are probably due to its blockage of the oxidation of pyruvate to acetyl-CoA and of α-ketoglutarate to succinyl-CoA (Bing and Tillmanns, 1977).

Another aspect to be considered in the development of alcoholic cardiomyopathy, or any cardiomyopathy, is the role of the coronary circulation. In other words, is ischemia the cause of all the contractile, metabolic, and structural changes seen? Much of the histologic evidence has shown normal coronary arteries in chronic alcoholics (Ferrans et al., 1965; Schenk and Cohen, 1970; Goodwin, 1974; Burch and Giles, 1977). Furthermore, there have been several reports of a negative statistical correlation between alcohol consumption and ischemic heart disease (Klatsky et al., 1974; Stason et al., 1976; Yano et al., 1977; Evans et al., 1980; Blackwelder et al., 1980). Barboriak et al. (1977) supported this further with coronary angiography studies. The biochemical changes responsible for this phenomenon may be the elevated levels of high density lipoproteins found in chronic alcoholics (Danielsson et al., 1978; Devenyi et al., 1980). Using coronary angiography, Barboriak et al. (1979) demonstrated an inverse relationship between high density lipoproteins (HDL) and coronary artery occlusion and between alcohol intake

and coronary artery occlusion, as well as a positive association between HDL and alcohol intake. However, Factor (1976) examined the hearts of nine alcoholics below the age of 45 and found normal coronary arteries, but microscopic examination of the intra-myocardial small vessels revealed considerable damage. In addition, Tibblin et al. (1975) found a positive correlation between alcohol consumption and ischemic heart disease. The entire question of the relationship between coronary artery disease, alcohol consumption, and high density lipoproteins has been examined in detail in a recent symposium edited by Kaelber and Barboriak (1981), but the problem remains unsolved.

The effects of chronic alcohol ingestion on the heart are diverse. Alcohol causes structural damage in the form of swollen mitochondria and sarcoplasmic reticulum, dilated T tubules, ruptured myofibrils, dehiscence of intercalated discs, and accumulation of triglyceride. It depresses mitochondrial function, alters lipid metabolism by re-routing fatty acid metabolism, interferes with calcium binding, and decreases and increases various ATPase activities. It also causes increased membrane permeability, depresses protein synthesis, and has a negative association with coronary artery disease. Is there a common explanation for all these effects?

## E. ANIMAL MODELS OF CHRONIC ALCOHOL INGESTION

There are two major objectives in developing animal models of chronic alcohol ingestion. The first one is to produce addiction and study the neurochemical and behavioral changes related to the addiction process. This involves feeding the animal in such a way so that eventually the animal will choose to drink alcohol even when it is offered an alternative. It also involves the production of true withdrawal signs when alcohol is removed from the diet. The second objective is to develop an animal model of chronic ingestion which produces significant organ damage to allow investigation of the pathophysiology of the specific disease process, such as cirrhosis, hepatitis, cardiomyopathy, neuropathy, and others. The production of addiction is not important in this case. This thesis involves the production of an animal model with the second objective in mind. The literature is full of a variety of animal models which involve feeding by stomach tube, by mouth, intraperitoneal injection, inhalation of ethanol vapors, solid and liquid diets, pair feeding, ad lib feeding, free choice between ethanol and water, and ethanol as the sole source of liquid. These methods have been employed in varying durations, from a few days to several years, and in a variety of animals such as rats, mice, guinea pigs, hamsters, rabbits, cats, dogs, monkeys and baboons. Because of the variety of models it has been difficult to compare results and arrive at solid conclusions about a specific disease process. This is especially true of alcoholic cardiomyopathy. Although there have been many forms of pathology produced, no animal model of congestive heart failure secondary to chronic alcohol ingestion has been developed in an intact animal. In fact, some of the chronic experiments of very long duration have failed to show any convincing hemodynamic changes.

Sarma et al. (1976) failed to show any in vivo hemodynamic changes in dogs consuming 25% ethanol for 29 months, while Regan et al. (1974) did show some mild hemodynamic changes in dogs consuming alcohol as 36% of their calories for 22 months. Lochner et al. (1969) found no hemodynamic changes in rats consuming 15% ethanol for 18 months. Similarly Segel et al. (1979), in feeding ethanol as 38% of the daily calories to rats for 34 to 48 weeks, produced no hemodynamic changes until challenges such as dobutamine or hypoxia were introduced. However, in all of the above studies, significant biochemical abnormalities were produced. Interestingly, the experiments of Segel et al. (1979) showed a depression of contractility upon dobutamine challenge when the fat content in the diet was lowered, independent of any alcohol effect. The effect of diets which differ in fat or carbohydrate content during chronic alcohol ingestion has been demonstrated before in rat heart (Alexander et al., 1977a, 1977b). With the exception of thiamine deficiency, the role of nutrition in the development of alcoholic cardiomyopathy has not been elucidated. Perhaps the reason why the full-blown picture of alcoholic cardiomyopathy with the anatomic, biochemical and physiologic changes, with the appearance of congestive heart failure has not been produced in the animal is because the diet of the alcohol-fed animal has all the nutritional elements well in excess of the requirements. Perhaps there truly is an element of malnutrition in the development of alcoholic cardiomyopathy. This problem, i.e. alcohol vs. malnutrition, has been debated over many years, particularly with respect to alcoholic liver disease. Indeed it was the original diet proposed by Lieber (Lieber et al., 1965, 1966; DeCarli and Lieber, 1967) which apparently eliminated the malnutrition factor and implicated alcohol alone as the cause of

cirrhosis. It was subsequently shown that well nourished primates could still develop cirrhosis with this diet (Rubin and Lieber, 1974). However, the presence of malnutrition via malabsorption or hyperexcretion of nutrients cannot be ruled out. More recently Patek (1979) has emphasized the role of malnutrition in alcoholic liver disease and the problem remains unsolved.

In addition to nutritional factors affecting organ damage there is evidence to suggest that the pattern of drinking may be important. According to Lelbach (1975a, 1975b), prolonged, continuous heavy drinking is more likely to produce cirrhosis than binge drinking which allows the liver some time to repair. An interesting clinical experiment by Mendelson et al. (1969) combines the concepts of drinking pattern with malnutrition. They found that a programmed drinking schedule given to chronic alcoholics (every four hours, day and night), which produced relatively stable continuously elevated blood alcohol levels, did not produce the magnesium deficiency state that was produced by free choice drinking or unrestricted drinking. The magnesium excretion in the urine occurred only when the blood alcohol level was rising (similar to anti-diuretic hormone activity's inhibition) and when the blood alcohol level was relatively stable, the magnesium excretion ceased. The frequently rising and falling blood alcohol levels in the free choice and unrestricted drinking patterns produced excretion of magnesium during the rising phase and too small or no compensatory retention during the falling phase. The net result with these latter two patterns of drinking was magnesium deficiency. The mechanism of this phenomenon is not known. Consequently animals given their diet as liquid, combining the foodstuffs with alcohol, will naturally achieve high and relatively stable levels of alcohol because they consume the

ethanol with their daily nourishment without choice. Even if there were some fluctuation, the nutrients are provided in excess. This need not be the case but in order to more closely simulate the alcoholic's drinking pattern, an animal should be allowed to feed ad lib and have food and alcohol from separate sources. To prevent the dietary intake from being the source of malnutrition, one should ensure that the animal eats enough food to obtain the minimum daily nutritional requirements. Obviously, this does not control the loss of nutrients via malabsorption or hyperexcretion. The animal model used in the following experiments is based on these concepts. The role of magnesium deficiency in the development of alcoholic cardiomyopathy and the role of alcohol itself, with respect to membrane permeability changes in alcoholic cardiomyopathy will be discussed later. An interesting observation made in the past has been the inverse relationship between the presence of cardiomyopathy and cirrhosis (Goodwin, 1977; Regan, 1977) although the evidence is weak. Perhaps the occurrence of the two disease processes depends partly on the drinking pattern.

F. DEFINING THE PROBLEM

There are certain relationships between the effect of alcohol on membranes, mitochondrial function, and leakage of intracellular components which come to mind when reviewing the foregoing discussion. One could postulate that alcohol, by altering the phospholipid content of any membranous structure, could cause mitochondrial dysfunction as well as leakage of small molecules across these membranes. Alternatively the mitochondrial dysfunction, by its inability to meet the energy requirements of the cell, could cause a breakdown of membrane function which would manifest itself in the form of increased permeability. Or, alcohol could produce the increased membrane permeability which would cause loss or entry of small molecules, such as ions, which in turn would cause mitochondrial dysfunction. For example, loss of the major intracellular cations, magnesium and potassium, or gain of calcium would have catastrophic effects on cell metabolism. In vitro studies on the general properties of alcohols have shown a correlation between their carbon chain length and the effect on proteins (Bull and Breese, 1978), lipids in isolated membranes, specifically mitochondrial membranes (Lenaz et al., 1976), and isolated organ function (Rang, 1960), all of which indicate an alcohol-membrane interaction. It would therefore seem logical that chronic alcohol ingestion might affect the permeability of the sarcolemma or the mitochondrial membrane or both, which would result in a loss or gain of small molecules such as ions from the mitochondrial matrix and/or the entire cell itself. The following is a presentation of some of the evidence to support these concepts.

The cardiomyopathy produced by magnesium deficiency in rats (Heggtveit et al., 1964, Heggtveit, 1965) displays the same morphologic

changes as alcoholic cardiomyopathy, and this similarity was noted by Alexander as early as 1966, particularly the presence of swollen mitochondria. Admittedly this may be a non-specific end-stage condition. However, other studies in magnesium deficient animals showed swollen, poorly respiring mitochondria in rats (Nakamura et al., 1961) and ducks (DiGiorgio et al., 1962) and reversal of this damage with magnesium supplements (Nakamura et al., 1961). In addition magnesium salts have been shown to protect against experimentally induced myocardial cellular (Lehr et al., 1975) and mitochondrial (Mudd et al., 1955) damage. Indeed magnesium is required for the structural integrity of mitochondria and sarcolemma (Seelig, 1972a, 1972b; Woods et al., 1979). The effects of calcium excess in myocardial cells also causes swollen, poorly respiring mitochondria and has been well documented (Dhalla, 1978). Finally, there is abundant evidence that alcoholics are magnesium deficient (Heaton et al., 1962; Sullivan et al., 1969; Jones et al., 1969) as mentioned earlier. The work of Mendelson et al. (1969) partly described the mechanism of this phenomenon, and the importance of the drinking pattern in this process has already been discussed.

Therefore several questions arise concerning the pathogenesis of alcoholic cardiomyopathy. First, does alcohol cause the damage by initially increasing membrane permeability, causing a loss or gain of ions, and a consequent derangement of mitochondrial function and other metabolic functions? Secondly, does alcohol first cause a magnesium deficiency which then produces mitochondrial dysfunction which in turn causes increased permeability to the sarcolemma? Thirdly, does alcohol cause a magnesium deficiency first, which then directly causes increased sarcolemmal permeability and a loss or gain of ions with further metabolic

derangements? The answers to these questions are obviously all interrelated and it is likely a combination of all three processes.

Because the signs and symptoms of alcoholic cardiomyopathy are reversible in some cases (Gunnar et al., 1975; Schwartz et al., 1975; Baudet et al., 1977; Reeves et al., 1978; Hung et al., 1979), there must be some point in the development of the disease where functional changes, as in membrane permeability or mitochondrial respiration, have occurred before any permanent structural damage is evident. As mentioned earlier, depressed myocardial contractility can be demonstrated before any overt signs of heart disease occur.

The objectives of the following experiments are:

1. To produce an animal model of chronic alcohol ingestion which allows ad lib consumption of food and liquid from separate sources for all animals, maintains good nutrition and weight gain, and produces metabolic derangements which can be studied before any structural damage occurs.
2. To assess mitochondrial function, mitochondrial calcium and magnesium content, and the effect of magnesium supplements in vivo and in vitro on the alcohol-induced mitochondrial dysfunction.
3. To assess sarcolemmal permeability changes with respect to sodium, potassium, calcium, magnesium and water movement across the membrane and ascertain the reversibility of these changes with abstention from alcohol.

# II METHODS

A: ANIMAL MODEL

Materials

1. Male Charles River Long-Evans rats, weighing 200 grams, from Canadian Breeding Farm and Laboratories, Ltd., St. Constant, Quebec.

2. Wayne Lab-Blox F6 laboratory chow from Allied Mills, Inc., Libertyville, Illinois, U.S.A. (See Figures 1 and 2 for ingredients).

3. Dextrose: D-glucose, anhydrous, from Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.

4. Alcohol: 95% ethanol from Commercial Alcohols Limited, Gatineau, Quebec. All percentages of ethanol specified throughout this thesis take into account a correction factor for 95% ethanol, and therefore indicate the total amount of ethanol given.

Feeding procedure

Male Long-Evans rats weighing approximately 200 g at the outset were caged individually. For the experiments outlined in parts B and C a total of 80 animals were used, 32 for part B and 48 for part C. Prior to these experiments a feeding schedule was developed and rats were given ethanol in their drinking water in increasing amounts over 4 weeks to 25%. For another 8 weeks they were given a constant 25% ethanol. All food and liquid intakes were measured. There were no diet controls in this trial, as the animals were being used for other experiments and the purpose was to determine whether they would actually consume 25% alcohol as their sole source of liquid and to determine the amount of alcohol consumed as a percentage of their daily calories. Once the percentage of alcohol calories was determined, a second batch of animals was again fed alcohol, but this time a control group was given dextrose in the

lab chow pellets in amounts equal to the calculated calorie percentage of alcohol in the original alcohol-fed animals. These animals were fed for 12 weeks and a comparison between the caloric intake of the alcohol-fed rats and the control rats was made and they were found to be almost equal. In both these preliminary 12 week trials, it was found that the alcohol-fed rats consumed 36-38% of their calories as alcohol in the last 8 weeks of their feeding schedule and the percentage of dextrose calories in the controls (in the second preliminary experiment) was comparable. At this point, it was decided that the experiments outlined in parts B and C could be undertaken. The details of the feeding schedule are as follows.

The alcohol-fed rats were given Wayne Lab Blox F6 chow ad lib, and for the first week, were given a 5% ethanol in water solution to drink as their sole source of fluid. Throughout the schedule, they were allowed chow ad lib. In the first half of the second week, the ethanol solution was increased to 7.5% ethanol, and to 10% in the second half. In the first half of the third week, the concentration was increased to 12.5% and to 15% in the second half. In the first half of the fourth week, they drank 17.5% ethanol and 20% in the second half. At the start of the 5th week, and through to the end of the 12th week, they were given 25% ethanol to drink (see Figure 3). The liquid was measured and changed twice weekly, the chow was measured once weekly, and the animals were weighed once every 4 weeks. From these measurements, the total amount of food, liquid, and calories were calculated, as well as a breakdown of calories into carbohydrate, protein, fat, and alcohol. These measurements were added up in 2 week totals and broken down to daily averages by dividing by 14, and expressed both as consumption per rat and per 100 g of rat, to account for the lesser weight gain in the alcohol-fed rats.

**Guaranteed Analysis**

Crude Protein.....(Min.) 24.0%
Crude Fat.....(Min.) 6.0%
Crude Fiber.....(Max.) 4.5%

**Ingredients**

Soybean meal, corn and wheat flakes, wheat middlings, ground corn, fish meal, cane molasses, soybean oil, dried whey, brewers dried yeast, animal liver meal, vitamin A palmitate, D-activated animal sterol, vitamin E supplement, menadione sodium bisulfite (source of vitamin K activity), riboflavin supplement, niacin, calcium pantothenate, choline chloride, thiamine, ground limestone, dicalcium phosphate, salt, manganous oxide, copper oxide, iron carbonate, ethylene diamine dihydriodide, cobalt carbonate and zinc oxide.

**WAYNE LAB-BLOX F-6**

**Directions For Use** – This is a complete diet to be fed free choice in a self-feeder. It contains all known nutrients essential for growth, maintenance and reproduction. Keep a constant supply of fresh water available. Formulation and texture are constant. Blox contain no added antibiotics. The product is tested regularly to assure the absence of estrogenic activity and Salmonella.

FIGURE 1. Nutrient content and additives of Wayne Lab Blox F6 chow pellets.

## AVERAGE NUTRIENT COMPOSITION WAYNE LAB-BLOX F6

| | |
|---|---|
| Protein, % | 24.48 |
| Fat, % | 6.48 |
| Fiber, % | 3.40 |
| Ash, % | 7.66 |
| Nitrogen-Free Extract % | 47.98 |
| Gross energy, kcal/gm | 4.18 |
| *Digestible energy, kcal/gm | 3.43 |
| *Metabolizable energy, kcal/gm | 3.19 |

| Amino Acids (% of diet) | |
|---|---|
| Threonine, % | 1.03 |
| Valine, % | 1.35 |
| Cystine, % | 0.40 |
| Methionine, % | 0.46 |
| Isoleucine, % | 1.38 |
| Leucine, % | 1.98 |
| Phenylalanine, % | 1.17 |
| Lysine, % | 1.64 |
| Histidine, % | 0.62 |
| Arginine, % | 1.54 |
| Tryptophan, % | 0.31 |

| Minerals | |
|---|---|
| Calcium, % | 1.20 |
| Phosphorus, % | 0.99 |
| Potassium, % | 0.96 |
| Magnesium, % | 0.22 |
| Sodium, % | 0.39 |
| Chlorine, % | 0.50 |
| Iron, PPM | 340.00 |
| Zinc, PPM | 60.00 |
| Manganese, PPM | 210.00 |
| Copper, PPM | 20.00 |
| Cobalt, PPM | 0.70 |
| Iodine, PPM | 1.50 |

| Vitamins | |
|---|---|
| Carotene, PPM | 2.00 |
| Vitamin A, I.U./gm | 15.00 |
| Vitamin D, I.U./gm | 4.41 |
| Alpha-tocopherol, PPM | 35.00 |
| Thiamine, PPM | 14.00 |
| Riboflavin, PPM | 6.50 |
| Niacin, PPM | 60.00 |
| Pantothenic Acid, PPM | 18.00 |
| Choline, PPM | 1780.00 |
| Pyridoxine, PPM | 8.13 |
| Folic Acid, PPM | 1.60 |
| Biotin, PPM | 0.15 |
| Vitamin $B_{12}$, mcg/lb. | 13.60 |
| Menadione Sodium Bisulfite, PPM | 2.80 |

*Digestible and metabolizable energy values obtained by using values listed for swine in Atlas of Nutritional Data on United States & Canadian Feeds, National Academy of Sciences.

FIGURE 2. Micronutrient content of Wayne Lab Blox F6 chow pellets.

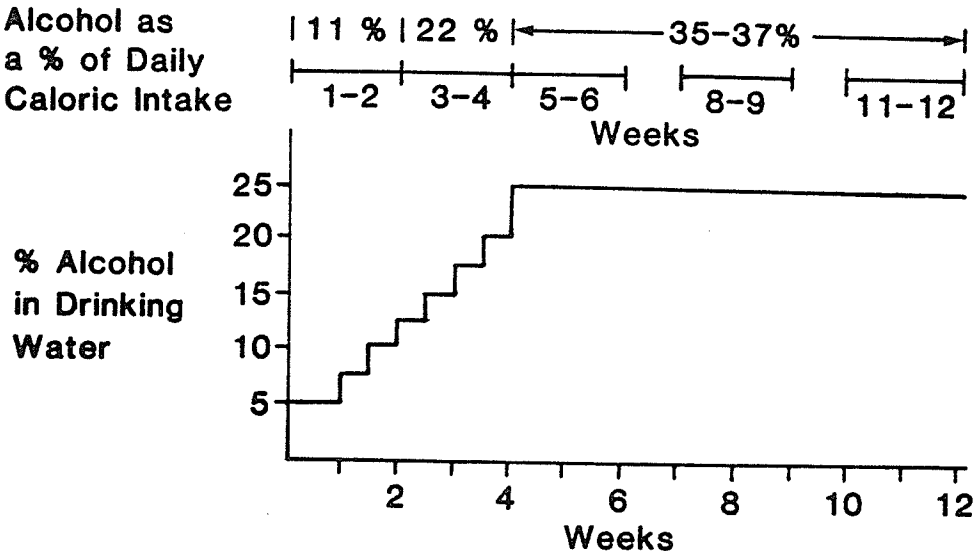



FIGURE 3. Alcohol feeding schedule illustrating the bi-weekly increments in concentration, expressed as a % solution in the drinking water, and as a % of the average total daily caloric intake.

Sample calculation of calories

First 2 weeks of feeding in an alcohol-fed rat. Daily fluid intake: 26.4 ml.

Average ethanol concentration over 2 weeks (5%, 7.5%, 10%) is 7%.

Amount of ethanol = 7% x 26.4 ml = 1.9 ml.

Specific gravity of ethanol at room temperature = 0.8

amount of ethanol = 1.9 x 0.8 = 1.5 g

Alcohol contains 7.1 K cal/g.

no. of calories of ethanol = 1.5 x 7.1 = 10.5 cal

Daily chow intake: 20.4 g.

Total chow as calories (3.43 K cal/g. - as digestable energy)

= 3.43 x 20.4 = 70.1 calories

Fat: (6.48% of total g)

= 6.48% x 20.4 = 1.3 g

Fat as calories (9.1 K cal/g)

= 1.3 x 9.1 = 12.0 calories

Protein (24.48% of total g)

= 24.48% x 20.4 = 5.0 g

Protein as calories (4.1 K cal/g)

= 5.0 x 4.1 = 20.5 calories

Carbohydrate as calories (CHO)

= Total calories - (Fat + protein calories)

= 70.1 - (12.0 + 20.5) = 37.6 calories

Daily caloric intake: (for one rat)

| | | %
|---|---|---|
| FAT | 12.0 | 15 |
| PROTEIN | 20.5 | 25 |
| CHO | 37.6 | 47 |
| ALCOHOL | 10.5 | 13 |
| TOTAL | 80.6 | 100 |

For the second 2 week period (3rd and 4th weeks) similar calculations were made, using an average ethanol concentration of 16% (12.5%, 15%, 17.5%, 20%). For the 5th-12th weeks, 3 periods of 2 week blocks were used for the calculations, with ethanol as a 25% solution in all (i.e. 5-6 weeks, 8-9 weeks, 11-12 weeks).

The control animals were given tap water to drink ad lib throughout the 12 weeks. Based on the calculations described above from the two preliminary trials of alcohol-fed rats, the average daily ethanol intake as a percentage of daily calories was determined, and dextrose was instilled into the chow pellets of the control animals in iso-caloric amounts. A batch of the original chow pellets was ground up, and dextrose added (in grams equivalent to the average caloric content of alcohol) and mixed and re-constituted.

Sample calculation of dextrose instillation into chow pellets

First 2 weeks:

From the previous alcohol-fed rats, it was determined that the average daily alcohol intake in calories was 9.2 (11% of total calories). Therefore dextrose (4.1 cal/g) was added to the chow as calculated in grams 9.2/4.1 = 2.23 g.

The average daily chow intake for the controls in the second preliminary experiment had been calculated as 21.5 g per day. Therefore dextrose was added to the ground up chow as 2.23 g for every 21.5 g of pellet chow, or for every 100 g of pellet chow, 10.4 g of dextrose was added (a ratio of 9.62:1).

For the 3rd and 4th week block a ratio of 100 g of pellet chow to 23.5 g of dextrose was mixed (4.25:1) equivalent to 18.9 calories,

or 22% of total. For the 5th-12th weeks, a mixture of 100 g of pellet chow and 46.9 g of dextrose (2.13:1) was prepared, equivalent to 35 calories, or 36% of the total. Now that both the alcohol concentration for the alcohol-fed rats and the dextrose concentration for the control rats had been pre-set, it remained to be seen whether in fact both groups of animals would consume iso-caloric amounts if fed ad lib, (see part A of results). A total of 80 rats were used for these experiments, 32 for the mitochondrial analysis (part B), and 48 for the water and electrolyte analysis (part C).

Histological assessment

During the mitochondrial isolation procedure outlined in part B, a section of left ventricle was taken from a control rat and alcohol-fed rat on 3 separate occasions at random. These samples were prepared for electron microscopy and assessed for structural damage. The tissue was placed in a solution of 5% glutaraldehyde buffer and 0.1 M Sorensen's phosphate buffer and fixed for 5 hours. It was then washed with a solution of 5% sucrose and Sorensen's buffer for 1 hour and cut into 1 mm cubes during the process. The tissue was then post-fixed in 1% osmium tetroxide in Sorensen's buffer, after which it was dehydrated with increasing concentrations of ethanol solutions and finally with methanol and propylene oxide. The sample was embedded in Araldite and cured for 48 hours at 60°C. Thin sections of this block of embedded tissue were cut (600-700 Å) with an LKB Ultra microtome, and placed on Formovar coated copper grids. They were double stained for 1 hour with saturated aqueous uranyl acetate and 5 minutes in lead citrate. The grids were viewed on a Phillips EM 201 electron microscope.

Blood alcohol levels

To avoid the stress of obtaining tail vein samples of blood from the rats used in the experiments of parts B and C, blood alcohol levels were taken from the tail vein samples of the rats used in the preliminary experiments, to assess whether they were indeed drinking any alcohol. Samples were taken at 6 weeks and 12 weeks, and analyzed immediately by an enzymatic method (Bernt and Gutman, 1974) on a Dupont Automatic Clinical Analyzer.

B: MITOCHONDRIAL ANALYSIS

Male Long-Evans rats were fed as described and 32 were taken for mitochondrial respiration studies; 16 were given the ethanol diet and 16 were given the dextrose diet. One week before the rats were killed, they were further divided in 4 groups of 8 animals each. Group $C_1$ was designated as a control group given the dextrose diet and group $A_1$ was given the alcohol. Group $C_2$ was identical to $C_1$ except that for 6 days prior to being killed, these animals received daily subcutaneous injections of 25 mg of Mg $SO_4$, a dose approximating the magnesium supplements used in magnesium deficiency experiments (Mishra, 1960a, 1960b; Heggtveit et al.,1964). Group $A_2$ was fed as $A_1$, but given the same injections as $C_2$. Alcohol was removed from the diet and replaced with water 24 hours before the rats were killed.

B i) Isolation and Preparation of Mitochondria

Mitochondrial preparations were made according to the method of Chance and Hagihara (1963) with modifications according to Wrogemann and Blanchaer (1968) and Thakar et al. (1973).

Reagents

1. Stock medium (0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA).
2. Homogenizing medium: 50 ml of stock containing 0.01 M tris-Pi, pH 7.6 plus 25 mg Nagase proteinase plus 2.5 μM ruthenium red.
3. Suspending medium: 20 ml of stock containing 0.01 M tris-Cl, pH 7.4 plus ruthenium red (2.5 μM).

Procedure

All equipment was kept ice cold and the procedures carried out at 0-4°C.

The rats were killed by guillotine and the hearts quickly excised and placed in ice cold stock medium. After being trimmed of fat the atria were removed and the heart was bisected and blotted dry. It was then finely minced and weighed and placed into ice cold homogenizing medium with a ratio of 300 mg tissue/20 ml medium. This was gently mixed with a glass stirring rod for 8 minutes at 0°C, after which time the first homogenization was carried out in a Thomas size C glass vessel with the loose teflon pestle (0.33 mm clearance). This homogenate was then incubated for another 8 minutes, and then diluted with an equal volume of stock medium, after which it was homogenized with the tight teflon pestle (0.10 mm clearance). The homogenate was then centrifuged at 400 g for 5 minutes and the supernatant was carefully pipetted off and centrifuged at 12,000 g for 10 minutes. The supernatant from this was discarded and the fluffy white layer on top of the mitochondrial pellet was removed by gentle swirling with stock medium. The walls of the tubes were wiped to remove any fat. Suspending medium was added in a ratio of 3-5 ml/3 g of muscle (i.e. 0.4 ml for each of 4 tubes per rat heart). The pellet was then loosened from the tube wall with a glass

rod and mixed on the vortex machine to achieve a homogeneous suspension. The suspension was centrifuged at 8,000 g for 5 minutes and the supernatant decanted and the tube walls dried. The pellets were re-suspended in a total volume of 1 ml of suspending medium to yield a protein concentration of approximately 25-30 mg/ml. From this 1 ml of mitochondrial suspension several aliquots were taken.

a) Triplicate samples of 25 µl, to which were added 0.5 ml NaOH, were stored at 0-4°C for later analysis of protein.

b) 50 µl aliquots were used the same day as prepared for the mitochondrial respiration studies.

c) The remainder of the samples was saved and frozen for later ion analysis (> 0.5 ml).

B ii) Analysis of Mitochondrial Magnesium and Calcium Content

Reagents

Concentrated perchloric acid (B + A Allied Chemical, Morristown, New Jersey, U.S.A.).

Concentrated nitric acid (Fisher Scientific Co.).

Calcium standard (Fisher Scientific Co.).

Magnesium standard (Fisher Scientific Co.).

Lanthanum chloride (Fisher Scientific Co.).

Preparation of samples

0.5 ml aliquots of mitochondrial suspension were incubated with an equal volume of a 1:1 mixture of concentrated nitric and concentrated perchloric acid overnight in covered, acid washed, plastic tubes at 60°C. Similarly treated was a blank consisting of 0.5 ml of

the suspension medium and 0.5 ml of acid mixture. From these, 0.2 ml aliquots were taken and diluted to 2.0 ml with a 1% lanthanum solution for calcium readings. For magnesium readings, another 0.2 ml aliquot was taken from each of the calcium samples and further diluted to 2.0 ml with the 1% lanthanum.

Preparation of standards

Calcium: A 1 mM solution of calcium was prepared. 0.4 ml of calcium standard was diluted to 10 ml with 1% lanthanum. From this 10 ml solution the following was taken.

a) 0.25 ml diluted to 25 ml with 1% lanthanum to obtain a 0.01 mM solution of calcium.

b) 0.625 ml diluted to 25 ml ..... (0.025 mM).

c) 1.25 ml ..... (0.05 mM).

d) 2.5 ml ..... (0.10 mM).

Magnesium: A 1 mM solution of magnesium was prepared. 0.24 ml of magnesium standard was diluted to 10 ml with 1% lanthanum. From this 10 ml solution the following was taken.

a) 0.10 ml diluted to 25 ml with 1% lanthanum to obtain a 0.004 mM solution of magnesium.

b) 0.25 ml ..... (0.01 mM).

c) 0.5 ml ..... (0.02 mM).

d) 0.625 ml ..... (0.025 mM).

Preparation of 1% lanthanum

A stock solution of 5% lanthanum chloride was made. 12.72 g of $LaCl_3.6H_2O$ was dissolved in 100 ml $H_2O$ and diluted 1:5 to prepare a 1% solution fresh daily.

Reading

For calcium determinations the wave length was set at 211 in the visual range, and for magnesium the wave length was 285.2 in the ultraviolet range. The readings were performed on a Perkin-Elmer Atomic Absorption Spectrophotometer. The samples were analyzed at two different times. Groups $A_1$ and $C_1$ were analyzed together, while groups $A_2$ and $C_2$ were analyzed at a later date. There was not enough sample material to perform duplicate assays.

B iii) Protein Assay

The Lowry method of determination of soluble protein was used for the protein assay (Lowry et al., 1951).

Reagents

1. Bovine serum albumin (Sigma). A stock solution of 200 mg/ml $H_2O$ was prepared.
2. Phenol reagent (B.D.H. Chemicals). A 1:3 dilution with $H_2O$ was prepared fresh.
3. Carbonate buffer: A mixture of 50 ml of 2% sodium carbonate (Fisher) plus 0.5 ml of 1% copper sulfate (Fisher) plus 0.5 ml of 2% sodium potassium tartrate (Fisher) was prepared.

Preparation of standards

1 ml of stock albumin plus 1 ml 2 N NaOH was diluted to 20 ml with $H_2O$. This was prepared fresh daily.

Dilutions were prepared as follows:

(absolute amount of protein in parentheses)

0.1 ml protein standard plus 0.9 ml $H_2O$ (10 µg)

0.2 ml ..... 0.8 ml $H_2O$ (20 µg)

0.4 ml ..... 0.6 ml $H_2O$ (40 µg)

0.6 ml ..... 0.4 ml $H_2O$ (60 µg)

0.8 ml ..... 0.2 ml $H_2O$ (80 µg)

0.9 ml ..... 0.1 ml $H_2O$ (90 µg)

1.0 ml ..... (100 µg)

The $H_2O$ was added after 30 minutes solubilization with NaOH.

Procedure

Mitochondrial samples were assayed in triplicate. A 25 µl aliquot (x3) was diluted to 1.0 ml with distilled $H_2O$. 0.1 ml of this diluted sample was placed in a glass test tube and 0.5 ml of 1N NaOH was added to each sample as well as each standard (as described above) and allowed to stand for 30 minutes. After 30 minutes 0.9 ml of $H_2O$ was added to each sample and the appropriate volume added to each standard (as described above). 5.0 ml carbonate buffer was added to standards and samples and they were allowed to stand for 10 minutes. 0.5 ml of 1:3 phenol solution was added to each tube and the tubes were allowed to stand for 30 minutes, after which time they were read on a Bausch and Lomb Spectrophotometer and the optical density was recorded at 550 mµ.

B iv) ADP (and ATP) Ultraviolet Absorption Assay

Principle: At pH 7.0 and $\lambda$ max. of 259 mµ the molar absorbancy index for ATP and ADP is $15.4 \times 10^{-3}$. Therefore $E_{mM} = 15.4$ (Burton, 1969).

Reagents

1. Phosphate buffer (0.02 M, pH 7.0). A solution was prepared by titration of 0.02 M potassium phosphate monobasic (Fisher) with 0.02 M potassium phosphate dibasic (Fisher) to a pH of 7.0.
2. A stock solution of ADP (Sigma, grade I, sodium salt) approximately 50 mM was prepared.

Procedure

Aliquots of 15 µl, 20 µl and 25 µl of stock solution of ADP were taken and to each were added approximately 20 ml of phosphate buffer in 25 ml volumetric flasks, swirled to mix, and diluted to 25 ml with buffer and mixed again. The $\lambda$ max = 259 mµ and therefore the DU spectrophotometer was set at wave length 259 mµ ultraviolet and adjusted to zero with a buffer blank, and the absorbance of each sample was read.

Calculation

The maximum absorbance reading was obtained and compared with the $E_{mM}$ and corrected for the appropriate dilutions to obtain the concentration of ADP in the stock solution.

$$\text{ADP: } \frac{\text{absorbance unknown x1 x25}}{15.4 \qquad 0.02}$$

= mM ADP

B v) Analysis of Mitochondrial Respiration

The following definitions are based on the work of Chance and Williams (1955), Packer (1960), Estabrook (1967), and Munn (1974).

The integrity of isolated mitochondria is assessed by measuring a) their ability to consume oxygen, with and without ADP, in the presence of appropriate substrates e.g. pyruvate and malate and b) their ability to consume ADP (by converting it to ATP) in a stoichiometric relationship

to oxygen consumption, and c) their "efficiency" of oxygen consumption when changing from a relative resting state (state 4) without ADP, to an active state (state 3) when ADP is added, and this is termed the respiratory control index. These parameters of mitochondrial respiration are defined in the following way.

1. Respiratory quotient (or state 3 oxygen consumption):

The amount of oxygen consumed, in micromoles, per gram of mitochondrial protein per minute, when the mitochondria have abundant supplies of oxygen, substrates and ADP.

2. State 4 respiration:

The amount of oxygen consumed, in micromoles, per gram of mitochondrial protein per minute when the mitochondria has abundant oxygen and substrate, but no added ADP.

3. Respiratory control index:

The ratio of state 3 respiration $O_2$ consumption to state 4 respiration $O_2$ consumption.

4. ADP:O

The ratio of the amount ADP consumed in micromoles, per gram of mitochondrial protein per minute to the amount of $O_2$ consumed, in micromoles per gram of mitochondrial protein per minute (in state 3). This is a measure of the mitochondria's ability to phosphorylate ADP in the presence of oxygen. Ideally, with substrates which produce coupling at all 3 sites, this ratio is 3:1 (succinate is 2:1).

Prior to the mitochondria and substrates being added to the oxidative phosphorylation medium in the 1.5 ml cuvette, the medium is bubbled and equilibrated at 28°C with room air which contains 21% oxygen.

Therefore, the absolute amount of oxygen which is dissolved in the medium (1.5 ml) can be calculated. The oxygen electrode (Transidyne) inserted in the cuvette then measures the $O_2$ concentration from the initial base-line level of 21% on graph paper, recorded on a Fisher Omniscribe recorder, to a final $O_2$ concentration of zero.

Calculation of $O_2$ content of cuvette (Umbreit, 1964).

EXAMPLE:

Obtain partial pressure of $O_2$ in air for that particular day

i.e. 0.21 x station barometric pressure

0.21 x (e.g. 985.5 millibars)

a) ml gas dissolved per 1 ml fluid when gas is at 1 atmosphere pressure = 0.027.

b) constant (at 1 atmosphere): (1013 m bar = 1 atmosphere)

$$\frac{1013}{.027} = 37{,}518.52$$

Find amount of $O_2$ dissolved in 1 ml of fluid

$$0.21 \times 985.5 = 206.955 \ (pO_2)$$

$$\frac{206.955}{37{,}518.52} = 0.00550683 \text{ ml } O_2/\text{ml fluid}$$

Cuvette has 1.5 ml fluid

$$1.5 \times 0.00550683 = 0.00881093$$

Change to STP conditions in order to convert to moles

$$V_{O_{(STP)}} = 0.00881093 \times \frac{273}{(273+28^{o}C)} \times \frac{985.5}{1013}$$

$$= 0.00777437 \text{ ml } O_2$$

1 mole occupies 22.4 l at STP

$$\# \text{ moles} = \frac{7.77437 \times 10^{-3} \text{ ml}}{22.4 \times 10^{3} \text{ ml}}$$

$$= 0.34707009 \times 10^{-6} \text{ moles } O_2 \text{ in } 1.5 \text{ ml}$$

concentration (per litre)

$$= \frac{0.34707009 \times 10^{-6} \times 10^{3}}{1.5}$$

$$= 0.216918 \times 10^{-3} \text{ moles/litre}$$

$$= 216 \text{ } \mu \text{ Molar}$$

Calculation of respiratory parameters: paper speed 2.5 cm/min.; $O_2$ consumption: 1 cm/1%

EXAMPLE (see Figure 4):

1. State 3 oxygen consumption:

The concentration of $O_2$ in cuvette = 216 µM, which when $O_2$ goes from 21% to 0%, it is recorded as a 21 cm drop. Therefore, the amount of $O_2$ consumed in 1 minute (2.5 cm)

$$= \frac{9.6}{21} \times 216 \text{ µmoles per litre}$$

$$= \frac{9.6}{21} \times \frac{216}{1000} \text{ µmoles in 1 } \underline{\underline{\text{ml}}}$$

for 1.5 ml cuvette $= \frac{9.6}{21} \times \frac{216}{1000} \times 1.5 \text{ ml}$

per gram of protein $= \frac{9.6}{21} \times \frac{216}{1000} \times 1.5 \frac{1}{31.2/1000}$

e.g. 31.2 mg $= \frac{31.2}{1000}$ g

But 31.2 mg is in 1 ml and only 50 µl (ie. 1/20 ml) was used

$$\frac{9.6}{21} \times \frac{216}{1000} \times 1.5 \times \frac{1000}{31.2} \times 20$$

$$= \frac{9.6}{21} \times \frac{216}{31.2} \times 1.5 \times 20$$

$$= 95 \text{ µmoles/minute/gram of mitochondrial protein}$$

2. State 4 oxygen consumption:

instead of 9.6, insert 1.3

$$= \frac{1.3}{21} \times \frac{216}{31.2} \times 1.5 \times 20$$

$$= 13 \text{ µmoles/minute/gram of mitochondrial protein}$$

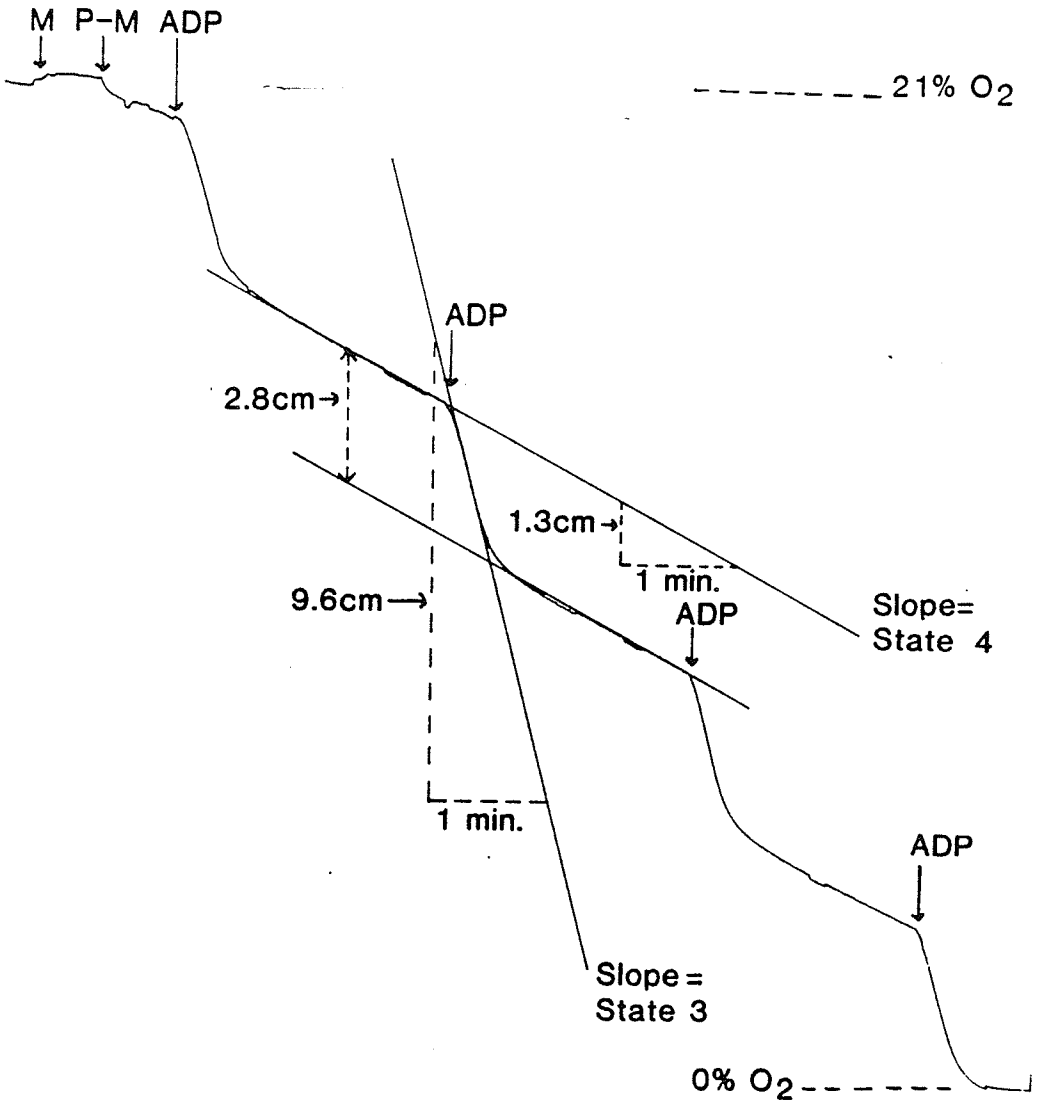



FIGURE 4. Sample assay of mitochondrial respiratory function. M = mitochondrial sample added to cuvette (50 µl aliquot); P-M = pyruvate-malate substrate (15 µl aliquot); ADP = adenosine diphosphate (7 µl aliquot). Paper speed 1 inch (2.5 cm)/min.

3. Respiratory control index:

State 3/State 4

= 95/13

= 7.3

4. ADP:O ratio:

Amount of ADP used (i.e. 7 µl of a 50 mM solution):

$= \frac{38 \times 7}{1000}$ µmoles

U.V. assay showed ADP to be 38 mM in this case

Amount of $O_2$ used:

$\frac{2.9}{21} \times \frac{216}{1000} \times 1.5$ µmoles

$$\frac{ADP}{O} = \frac{ADP}{O_{2/2}} = \frac{\frac{38x7}{1000}}{\frac{2.9}{21} \times \frac{216}{1000} \times 1.5 \times 2}$$

$$= \frac{38 \times 7 \times 21}{2.9 \times 216 \times 1.5 \times 2}$$

$$= 2.97$$

B vi) Preparation of Solutions

1. Tris: a 0.5 M solution was made by dissolving 6.055 g of Trizma base (Sigma) in 100 ml $H_2O$.
2. Phosphoric acid (Ortho 85%, Fisher): a 0.5 M solution was made by diluting 1 ml of 15 M acid to 30 ml with $H_2O$.
3. Tris phosphate: 50 ml of tris solution was titrated with 0.5 M phosphoric acid to pH 7.6. To obtain 0.01 M tris phosphate in the homogenizing medium, 1 ml of 0.5 M tris phosphate was dissolved in 50 ml of stock solution.
4. Tris chloride: 50 ml of tris solution was titrated with 0.5 M hydrochloric acid (Fisher) to pH 7.4. To obtain a 0.01 M tris chloride solution in the suspending medium, 0.4 ml of the 0.5 M tris chloride was dissolved in 20 ml of the stock solution.

5. EDTA (Analar): a 0.1 mM stock solution was made by dissolving 37.225 mg in 1 litre of $H_2O$.

6. Ruthenium Red (B.D.H. Chemicals): for a final concentration of 2.5 µM, 689 µg was dissolved in 500 ml of homogenizing medium and 27.57 µg in 20 ml of suspending medium.

7. Mannitol (Fisher): a 0.21 M solution was prepared by dissolving 38.2557 g in 1 litre $H_2O$.

8. Sucrose (Fisher): a 0.07 M solution was prepared by dissolving 23.961 g in 1 litre $H_2O$.

9. Stock medium: 38.2557 g of mannitol plus 23.961 g of sucrose were dissolved in 800 ml $H_2O$ and mixed. 37.225 mg EDTA were added to the solution after pH had been adjusted to between 7.0 and 7.6 with a concentrated tris solution. A final volume of 1 litre was prepared and stored in the refrigerator.

10. Oxidative phosphorylation medium. A 0.23 M solution of mannitol was made by dissolving 8.3812 g in 200 ml $H_2O$. A 0.07 M sucrose solution was made by dissolving 4.7922 g in 200 ml $H_2O$. A 0.02 M tris chloride solution was made by diluting 4 mls of 1 M tris chloride, pH 7.2 in 200 ml. The 1 M tris chloride was made by dissolving 1.211 g of Trizma base in 4 ml 1 N HCl, and the pH brought to 7.2 with 2 N HCl and made up to 10 ml with $H_2O$. A 0.02 mM solution of EDTA was prepared by diluting 1 ml of a 4 mM EDTA solution in 200 ml. The 4 mM EDTA was made by dissolving 0.01489 g EDTA in 10 ml $H_2O$. A 5 mM phosphorous solution was made by titration of 1M $K_2HPO_4$ with 1M $KH_2PO_4$ to a pH of 7.2. 1 ml of the latter solution was diluted to 200 ml. All of the above reagents were combined in 160 ml $H_2O$ and the pH adjusted to 7.2 if necessary. This was then diluted to 200 ml with $H_2O$ and was prepared fresh daily.

11. Additions to oxidative phosphorylation medium in the cuvette (1.5 ml):

a) ADP (Sigma): a 50 mM solution was made by dissolving 0.0755 g in approximately 2 ml $H_2O$, and the pH was adjusted to 7.2 with 1N NaOH, and the solution was diluted to a final volume of 3 ml with $H_2O$. A 7 µl aliquot of this solution was added to the 1.5 ml cuvette to initiate phosphorylation and state 3 respiration, which gave a final concentration of 250 µM in the cuvette.

b) 500 mM pyruvate - 100 mM L-malate: 0.1101 g pyruvic acid (Sigma) was weighed directly into a 2 ml volumetric flask, and 1 ml $H_2O$ added and mixed. To this was added 0.4 ml of a freshly prepared solution of 500 mM L-malate (Sigma), and the solution was diluted to a final volume of 2 ml and stored in the freezer. This solution was prepared fresh every 2 weeks.

c) Potassium chloride (Fisher): 22.365 g of KCl was dissolved in 100 ml $H_2O$ to produce 300 millimoles in 100 ml. A 50 µl aliquot of this solution was added to the cuvette to give a final KCl concentration of 100 mM.

d) Magnesium chloride (Analar): 15.248 g of $MgCl_2$ was dissolved in 100 ml $H_2O$ to produce 75 millimoles in 100 mls. A 10 µl aliquot of this solution was added to the cuvette to give a final $MgCl_2$ concentration of 5 mM.

C: TISSUE WATER AND ELECTROLYTE ANALYSIS

Male Long Evans rats were fed as described; 48 were taken for myocardial tissue water and electrolyte analysis and were divided into 4 groups of 12 animals each. 12 animals were fed the alcohol diet for 12 weeks (A) and another 12 were fed the dextrose control diet (C). To assess

the reversibility of any changes found, another group of 12 was fed the alcohol diet but after 12 weeks was placed back on tap water and standard lab chow for another 8 weeks until they were killed (alcohol-recovery, $A_R$). Another 12 were fed the control diet for 12 weeks and also placed back on tap water and standard lab chow for another 8 weeks (control-recovery, $C_R$). The alcohol was removed from the appropriate animals and replaced by water 24 hours before they were killed. The methods to be described for the preparation of tissue, morphometric analysis, and calculation of water and electrolyte distribution have been previously reported (Polimeni, 1974).

C i) Procedure

The animals were weighed just before the procedure began. They were anesthetized with ether and a bilateral nephrectomy was done via a retroperitoneal approach. The rat was then placed on its back and a 0.6 - 1.0 ml injection of 0.1 μM $Na_2SO_4$ containing 50-90 μCi of $^{35}S$ (Amersham) was given via the exposed femoral vein. The animal was then returned to its cage and the $^{35}S$ sulfate allowed to equilibrate. The procedure was carried out on 12 rats (6 controls and 6 alcohol-fed) each day for 2 consecutive days. On each day the first rat nephrectomized was the last to be killed for analysis, and the last rat nephrectomized was the first to be killed, with about a one-half hour delay. This was done so that the time for equilibration of $^{35}S$ sulfate ranged from ½ to 6 hours. After the appropriate equilibration times, the rats were anesthetized with ether, and a thoractomy was performed. 5 ml of blood was drawn from the inferior vena cava and centrifuged, and its plasma separated and stored at $4^{o}C$ for later analysis. The heart was excised and the atria, fat, connective tissue, and right ventricle removed. A thin slice of left ventricle was taken and

immersed and fixed in a cold glutaraldehyde solution iso-osmotic with rat plasma for microscopic analysis. The remaining left ventricle and interventricular septum was divided into two pieces, each of which was gently blotted and weighed. One piece was placed in a tared quartz crucible and dried overnight in a partial vacuum at 95°C and re-weighed to obtain the tissue water content. This same piece of tissue was then placed in a 0.1% lanthanum - 0.03 M trichloroacetic acid solution for several days (60 hours) in a polyethylene container for analysis of calcium by atomic absorption spectrophotometer. A second piece was extracted in 5 ml of 0.1 N $HNO_3$ for 48 hours and later analyzed for sodium and potassium content by emission spectrophotography, and for magnesium content by atomic absorption spectrophotometry, as well as scintillation counting of $^{35}SO_4$ for extracellular space determinations.

The extracellular space was measured by two different methods, morphometric and tracer. Two methods were used because accurate determination of this space is essential for correct intracellular and extracellular calculations of ion concentrations. In addition it was important to determine whether there were any changes in sarcolemmal permeability as evidenced by a larger ECS measured by tracer when compared to ECS measured by morphometry.

C ii) Procedure for Preparation of Tissue for Morphometric Analysis of Extracellular Space.

This method has been reported previously (Polimeni, 1974). A small portion of left ventricle was taken and cut into 15-20 one millimeter cubes with a scalpel. Each cube was placed in a glass vial of chilled, buffered 2% glutaraldehyde and fixed for 1 hour at 4°C. The glutaraldehyde

was decanted and the fixed tissue was rinsed 3 times with 50 mM Na cacodylate buffer, pH 7.2. The tissue was then dehydrated with ethanol in serial washings with increasing concentrations of ethanol. It was then infiltrated under vacuum overnight with a solution of glycol methacrylate and 2-butoxyethanol with benzoyl peroxide as catalyst, and embedded with polyethylene glycol 400, and N,N-dimethyl aniline in preparation for sectioning. The embedded tissue was sectioned with a Sorvall MT2-B "Porter-Blum" Ultramicrotome, placed on a glass slide and stained with methylene blue (.13 g/ml) pH 5.5 at room temperature. A photomicrograph of the stained section was obtained with a Zeiss Photomicroscope. Thirty-six 8 x 10 photomicrographs were taken from each section and approximately 20-30 prints were selected for point counting.

To quantify the extracellular space histologically as a fraction of the total ventricular muscle volume, the prints of the histological sections were overlaid with a grid composed of lines 1 cm apart and the number of grid intersections, or points, over the extracellular regions were counted. Intersections falling on the contiguities of the cellular and extracellular compartments or on ambiguous points were counted as half points. The number of points counted over the non cellular components of the tissue divided by the total number of points overlying the tissue yielded the fraction of extracellular space for that print. The fraction of ventricular extracellular space did not significantly change after about 1,000 points were counted.

C iii) Procedure for Calcium Determination

Reagents

1. Calcium AA standard solution (Aldrich Chemical Co.).

2. 1% $LaCl_3$, prepared from $La_2O_3$ and HCl (Fisher).
3. 1M NaCl and 0.3M TCA (Fisher).
4. Calcium extraction buffer solution prepared from #2 and #3 and diluted 10 x to give a 0.1% lanthanum solution.

Procedure

Each plasma and tissue sample was done in duplicate. From the tissue sample which had been extracted for 60 hours in the Ca extraction buffer, 2.0 ml was taken and centrifuged for 30 minutes at 2,000 rpm. The supernatant was diluted in duplicate to 3 different concentrations(1/5,1/10,1/20) with the extraction buffer. Plasma samples were thawed and mixed and 0.2 ml aliquots were diluted 50 x in duplicate with extraction buffer. The plasma and tissue samples and the prepared standards were analyzed for calcium content on a Jarrell Ash 850 atomic absorption spectrophotometer with a wave length of 4227 Å.

C iv) Procedure for Magnesium Determination

Reagents

1. Magnesium AA standard solution (Aldrich Chemical Co., Milwaukee, Wis.).
2. 1% $LaCl_3$ prepared from $La_2O_3$ and HCl (Fisher) and diluted 10 x to give a 0.1% La solution.

Procedure

Each plasma and tissue sample determination was done in duplicate. From the tissue sample which had been extracted in 0.1N $HNO_3$ for 48 hours, duplicate aliquots of 50 µl were taken and diluted 100 x to 5.0 ml with 0.1% $LaCl_3$. Similar 50 µl duplicate aliquots from plasma were diluted 100 x with 0.1% $LaCl_3$. Plasma and tissue samples, and

prepared magnesium standards were analyzed for magnesium content on a Jarrell Ash 850 atomic absorption spectrophotometer with a wave length of 2852 $\overset{o}{A}$, and a magnesium lamp.

C v) Procedure for Sodium and Potassium Determination

Reagents

1. Sodium, potassium and lithium standard solutions (Instrumentation Lab. Inc., Lexington, Ma.).
2. 0.1N $HNO_3$ (Fisher).

Procedure

From the tissue sample which had been extracted in 0.1N $HNO_3$ for 48 hours, duplicate aliquots of 0.10 ml were taken and diluted with 4.90 ml of Lithium blank (50 x dilution). From the plasma sample, duplicate aliquots of 60 µl were first diluted with 180 µl 0.1N $HNO_3$ (1:4). From this a 0.10 ml aliquot was taken and diluted with 4.90 mls of Lithium blank (1:50) for a total dilution of 1:200. The lithium blank was prepared at a concentration of 15 mEq/L. The standards for the plasma determinations of Na and K were prepared at a concentration of 140 and 5 mEq/L respectively. The tissue Na and K standards were prepared in equal concentrations of 25, 10 and 5 mEq/L. All tissue and plasma samples and standards were analyzed on an 1L 443 model flame photometer (Instrumentation Laboratories, Inc., Lexington, Ma.) with Lithium as the internal standard.

C vi) Procedure for $^{35}SO_4$ Determination Using Liquid Scintillation Counting

Reagents

1. 10% TCA (Fisher).
2. 0.1N $HNO_3$ (Fisher).
3. Scintillation cocktail (Beckman General Purpose).

Procedure

Duplicate 0.50 ml extracts of the tissue which had been digested in 0.1N $HNO_3$ for 48 hours were taken and mixed with 0.10 ml 10% TCA and left for 30 minutes. This mixture was centrifuged for another 30 minutes at 2000 rpm at room temperature. A 250 µl aliquot of the supernatant was placed in a scintillation vial and 10 ml of cocktail added. 0.1 ml of thawed plasma sample was mixed with 1.0 ml 10% TCA and 0.9 ml of 0.1N $HNO_3$ and centrifuged as the tissue was. A 250 µl aliquot was also taken and to it was added 10 ml of scintillation cocktail. The samples and appropriate blanks were analyzed on a Beckman Model L50 scintillation counter.

C vii) Calculations

a) Extra cellular space (ECS) by tracer method:

(1) Total tissue water = wet weight - dry weight

$$(H_2O)_m = W_w - W_d$$

grams of tissue $H_2O$ per gram of muscle (m)

(2) Radioactivity of $^{35}SO_4$

counts per minute per ml of ECF (extracellular fluid)

$$[Q]_o = (Q_o)/V_o$$

$$= (Q)_m/V_o$$

where [ ] = concentration
( ) = content
o = outside cell
where Q = radioactivity confined to ECS

$$Q_o = Q_m$$

and $[Q]_o = (Q)_o/V_o$

(3) $[H_2O]_o = (H_2O)_o/V_o$

i.e. concentration = content per unit of volume

Definition: $\delta_o$ (extracellular water)

$$\delta_o = (H_2O)_o/(H_2O)_m$$

grams of extracellular $H_2O$ per gram of muscle $H_2O$ (or total tissue $H_2O$)

substituting:

$$= \left[H_2O\right]_o V_o/(H_2O)_m$$

rearranging:

$$= \frac{\left[H_2O\right]_o \cdot (Q)_m}{\left[Q\right]_o \cdot (H_2O)_m}$$

Assuming that the radioactivity in ECS equilibrates with radioactivity in the plasma such that:

$$\frac{\left[H_2O\right]_o}{\left[Q\right]_o} = \frac{\left[H_2O\right]_p}{\left[Q\right]_p}$$

Then: (from the definition of $\delta_o$)

$$\delta_o = \frac{\left[H_2O\right]_p (Q)_m}{\left[Q\right]_p \cdot (H_2O)_m}$$

$$Q_m = \{R_m - R_b\} e^{\lambda tm}$$

where $R_m$ = muscle counts
$R_b$ = background counts
$e^{\lambda tm}$ = decay factor for muscle

$$\left[Q\right]_p = \{R_p - R_b\} e^{\lambda tp}/vp$$

$R_p$ = plasma counts
$v^p$ = plasma volume
$e^{\lambda tp}$ = decay factor for plasma

Substituting:

$$\delta_o = \frac{\left[H_2O\right]_p \{R_m - R_b\} e^{\lambda tm} \cdot D}{(H_2O)_m \{R_p - R_b\} e^{\lambda tp}/vp}$$

where D = dilution factor relating to assay procedures

D has been derived from the plasma volume vp, the volume of muscle in tissue extract, $v_e$, and the tissue water $(H_2O)_m$ and a dilution factor d.

$$\delta_o = \frac{d \cdot v_p \{v_e + (H_2O)_m\}\{R_m - R_b\} e^{\lambda(tm-tp)} \left[H_2O\right]_p \phi}{\{R_p - R_b\} \quad (H_2O)_m}$$

$\phi$ is a correction factor for the amount of $^{35}SO_4$ lost by uptake into red blood cells or other tissues, and has the value of 0.975 (Polimeni, 1974).

$[H_2O]_p$ is a known constant, and for rat plasma it is 0.946 grams of water per ml of plasma.

By inserting the measured values and the constants ($d, v_p, v_e, R_b$, $e^{\lambda(tm-tp)}$, $[H_2O]_p$ and $\emptyset$), the value of $\delta_o$, i.e. the amount of ECW expressed as a fraction of the total tissue (or muscle) water, can be calculated. The morphometric (histologic) analysis also provided the value of ECS as a fraction of tissue (ie. $ECS_H$; $cm^3/cm^3$). The value of $\delta_o$ is related to $ECS_H$ by the equation

$$\delta_o \cdot fH_2O = ECS_H$$

where $fH_2O = (H_2O)_m/Ww = (Ww-Wd)/Ww$.

b) Ion concentrations

Calculation of the extracellular ion concentration is straightforward enough, as it is equal to the plasma concentration. Calculations of the intracellular ion concentrations is based on the calculated value of intracellular water.

$$ECW = \delta_o \cdot \text{total tissue or muscle water}$$

i.e. $$(H_2O)_o = \delta_o (H_2O)_m$$

$$ICW = \text{total} - ECW$$

i.e. $$(H_2O)_i = (H_2O)_m - (H_2O)_o$$

For example, the concentration of intracellular Na ($[Na]_i$):

$$[Na]_i = \frac{\{(Na)_m - (H_2O)_o [Na]_o\}}{(H_2O)_i}$$

where $(Na)_m$ is the measured content of Na in the tissue sample and $[Na]_o$ is the measured plasma Na concentration, which is equal to extracellular Na concentration.

D. STATISTICAL ANALYSIS

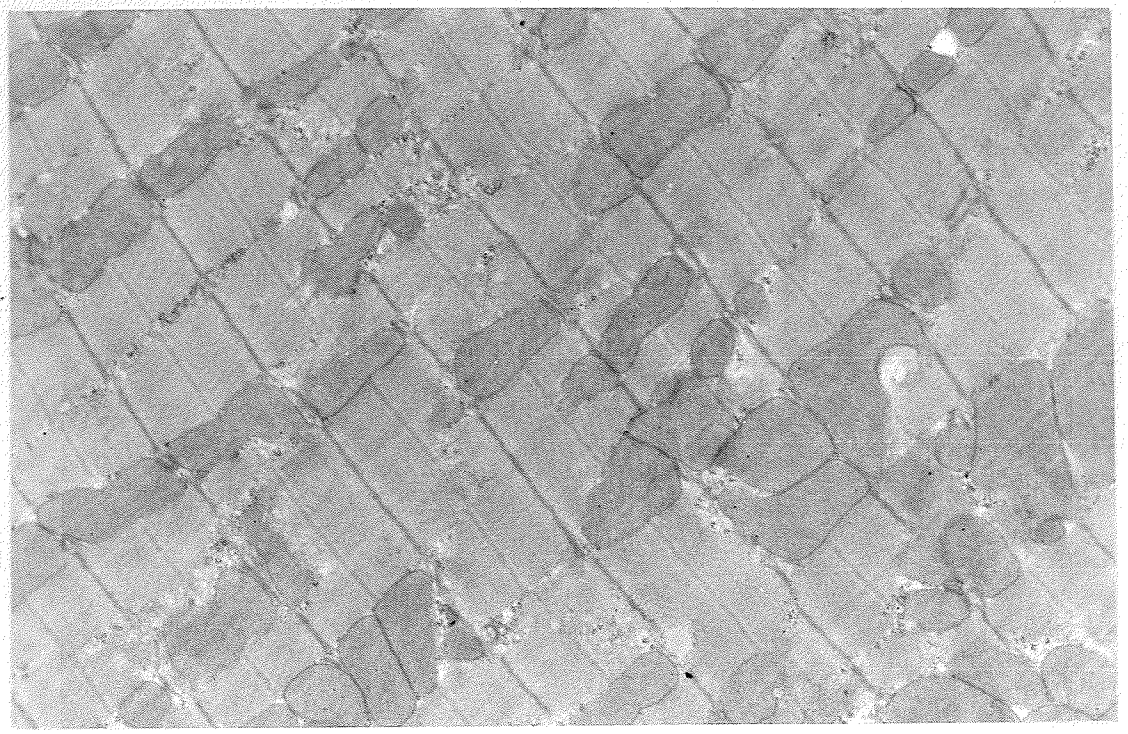
Statistical analysis of the data on the animal model was done by the Student's *t*-test. Analysis of the data on mitochondrial function and ion content was done by randomized analysis of variance using Duncan's multiple comparisons test. The data in tissue and plasma electrolyte concentrations were also analyzed with Duncan's multiple comparisons test. The data on the extracellular space measurements were analyzed by a one way analysis of covariance with one covariate, using a comparison of regression lines.

# III RESULTS

A. ANIMAL MODEL

After 12 weeks of drinking ethanol and abstaining from the ethanol for 24 hours prior to being killed, the rats appeared healthy, were not visibly different from the control animals, and showed no signs of alcohol withdrawal. Grossly the excised hearts appeared normal and the heart to body weight ratios of both control and alcohol-fed rats were equal: alcohol-fed = 2.13 $\pm$ 0.03 mg/g; control = 2.14 $\pm$ 0.03 mg/g. The blood alcohol levels obtained from the preliminary experiments were 67.4 $\pm$ 10.9 mg/dl after 6 weeks and 32.7 $\pm$ 15.8 mg/dl after 12 weeks. Electron microscopy of tissue taken from the left ventricle of both control and alcohol-fed rats revealed no structural damage. Both groups of rat hearts showed normal myofibrils, mitochondria, and intercalated discs (see Figures 5 and 6). Both groups of animals displayed good weight gain (Figure 7), but the alcohol-fed rats achieved only 90% of the weight of the control rats. It is interesting to note that once the alcohol-fed rats began drinking the 25% ethanol solution, while maintaining a fairly low blood alcohol concentration, their growth curve appeared parallel to the control rats for the last 8 weeks of the schedule.

Table 1 lists the average daily food and liquid intake for alcohol-fed and control rats. While the daily chow intake was fairly constant for the control rats, the alcohol-fed rats showed a decrease in daily chow intake over the first 4 weeks and a plateau over the last 8 weeks at approximately 16 grams per day, corresponding to the 4 week increase and plateau of alcohol consumption. Nevertheless, both groups of rats maintained a normal liquid to chow ratio (Warner and Breuer, 1972) indicating that the presence of ethanol in the drinking water was not a strong enough taste deterrent to prevent them from drinking the normal



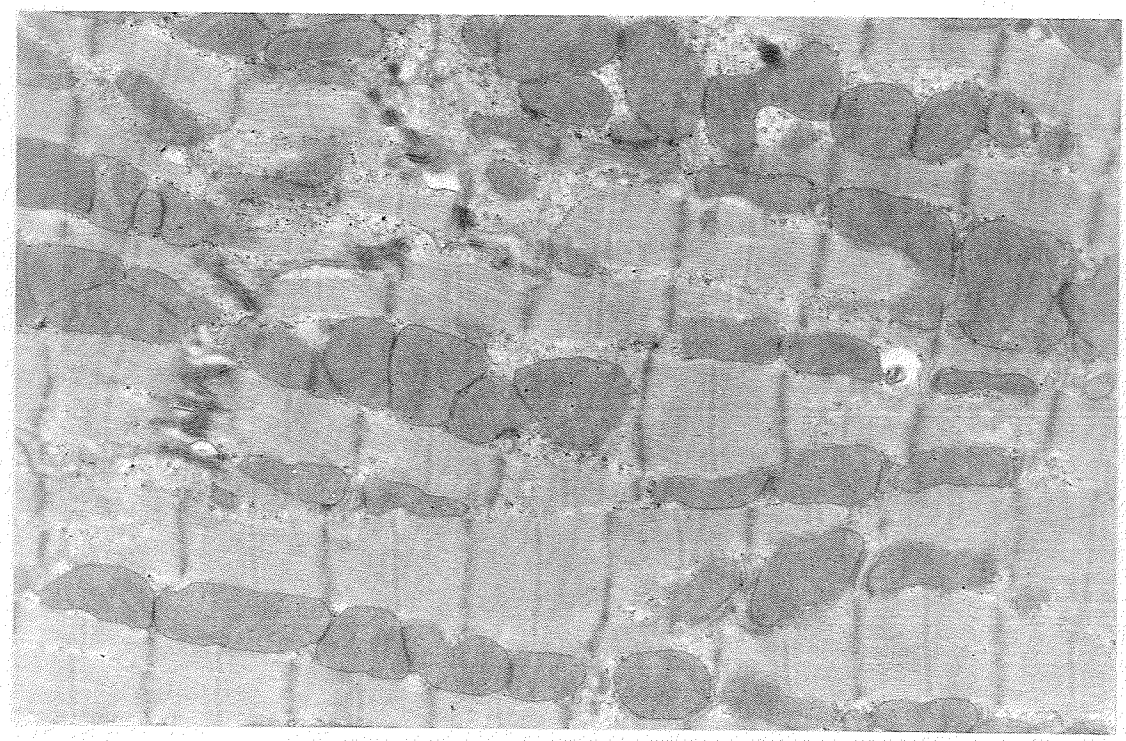


FIGURE 5. Electron micrograph of rat ventricle (Magnification 10,862 x).
Upper photo: Left ventricle from alcohol-fed rat.
Lower photo: Left ventricle from control rat.

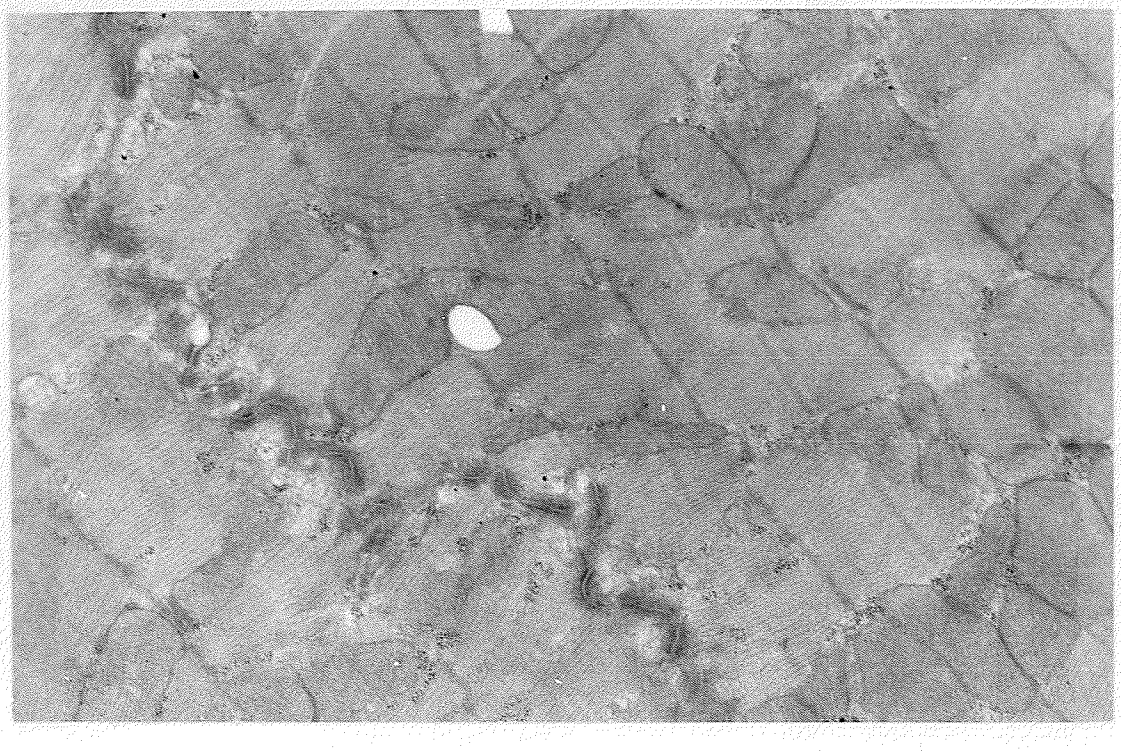
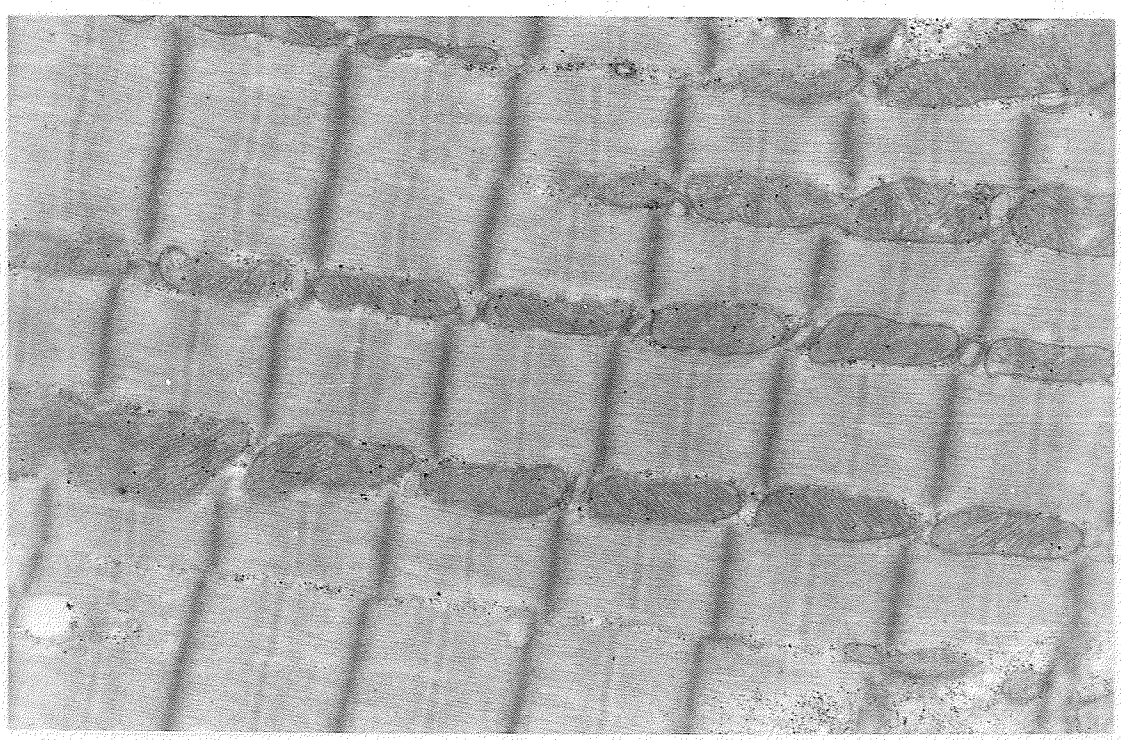


FIGURE 6. Electron micrograph of rat ventricle (Magnification 15,812 x).
Upper photo: Left ventricle from alcohol-fed rat.
Lower photo: Left ventricle from control rat.



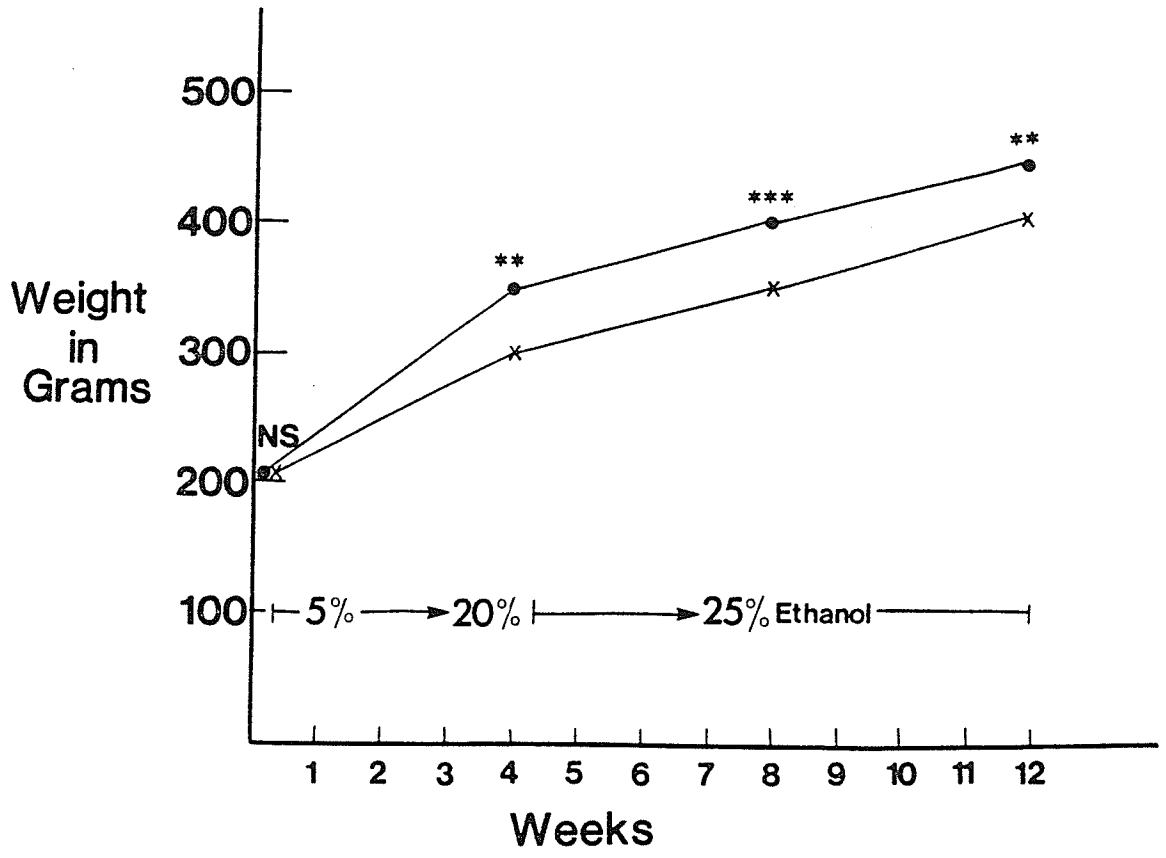



FIGURE 7. Growth of rats given ethanol in drinking water, increasing from 5% over 4 weeks and maintained at 25% for a further 8 weeks; x, ethanol-fed; ● , controls.
** $p < 0.01$
*** $p < 0.001$

TABLE 1

DAILY FOOD AND LIQUID INTAKE*

| | 1-2 WEEKS | | 3-4 WEEKS | | 5-6 WEEKS | | 8-9 WEEKS | | 11-12 WEEKS | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PER RAT | PER 100g | PER RAT | PER 100g | PER RAT | PER 100g | PER RAT | PER 100g | PER RAT | PER 100g |
| CONTROL | | | | | | | | | | |
| CHOW: (gms) | 24.7 | 10.5 | 26.6 | 8.9 | 26.0 | 7.5 | 25.6 | 6.5 | 25.0 | 5.8 |
| WATER: (mls) | 35.0 | 14.9 | 37.5 | 12.6 | 36.8 | 10.6 | 38.1 | 9.6 | 43.5 | 10.0 |
| WATER/ CHOW | 1.4 | | 1.4 | | 1.4 | | 1.5 | | 1.7 | |
| ALCOHOL | | | | | | | | | | |
| CHOW: (gms) | 22.6 | 9.9 | 18.5 | 6.7 | 15.1 | 4.9 | 16.8 | 5.0 | 16.8 | 4.4 |
| LIQUID: (mls) | 29.9 | 13.1 | 25.2 | 9.1 | 20.5 | 6.6 | 24.2 | 7.1 | 26.6 | 6.9 |
| LIQUID/ CHOW | 1.3 | | 1.4 | | 1.4 | | 1.4 | | 1.6 | |

*Calculated in 2-week blocks.

TABLE 2

AVERAGE DAILY CALORIC INTAKE+

| WEEKS | FAT A | FAT C | PROTEIN A | PROTEIN C | MIXED CARBOHYDRATE A | MIXED CARBOHYDRATE C | ALCOHOL/DEXTROSE A | ALCOHOL/DEXTROSE C | TOTAL A | TOTAL C |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-2 | 13.32 ± 0.42 | 13.26 ± 0.26 | 22.65 ± 0.71 | 22.59 ± 0.45 | 41.49 ± 1.31 | 41.35 ± 0.83 | 11.89 ± 0.37 | 8.93 ± 0.30 | 89.38 ± 2.78 | 86.12 ± 1.82 |
| | NS | | NS | | NS | | *** | | NS | |
| 3-4 | 10.89 ± 0.33 | 12.68 ± 0.27 | 18.50 ± 0.53 | 21.58 ± 0.46 | 34.01 ± 1.04 | 39.50 ± 0.82 | 22.90 ± 1.22 | 20.70 ± 0.44 | 86.29 ± 2.62 | 94.47 ± 1.98 |
| | *** | | *** | | ** | | NS | | * | |
| 5-6 | 8.89 ± 0.34 | 10.46 ± 0.12 | 15.17 ± 0.57 | 17.78 ± 0.20 | 27.64 ± 1.05 | 32.48 ± 0.38 | 29.18 ± 2.29 | 34.16 ± 0.40 | 80.88 ± 3.61 | 94.88 ± 1.09 |
| | ** | | ** | | ** | | NS | | ** | |
| 8-9 | 9.92 ± 0.36 | 10.24 ± 0.40 | 16.89 ± 0.61 | 17.44 ± 0.66 | 30.89 ± 1.12 | 31.91 ± 1.21 | 34.36 ± 1.04 | 33.51 ± 1.28 | 92.06 ± 2.47 | 93.10 ± 3.56 |
| | NS | | NS | | NS | | NS | | NS | |
| 11-12 | 9.94 ± 0.31 | 11.73 ± 1.20 | 16.90 ± 0.53 | 17.01 ± 0.43 | 30.91 ± 0.96 | 29.40 ± 1.26 | 36.38 ± 2.56 | 32.71 ± 0.83 | 94.14 ± 3.29 | 90.85 ± 2.30 |
| | NS | | NS | | NS | | NS | | NS | |

+ K Cal consumed daily per rat, calculated for 2 week periods.

A = alcohol-fed C = controls

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

amount of liquid in proportion to their chow. An analysis of the calorie content of the diet is shown in Table 2. The number of daily calories consumed as fat was the same for alcohol-fed and control rats for the first 2 weeks and the last 5 weeks, with a small but statistically significant difference from the third to sixth week. A similar trend occurred with the number of daily protein calories. The portion of the food pellet which is not fat, protein, nor substituted dextrose, is designated mixed carbohydrate and the same pattern occurred throughout the schedule as for fat and protein. The daily intake of alcohol calories for the alcohol-fed rats was the same as the daily intake of dextrose calories for the control rats for the last 10 of the 12 week schedule. The total daily caloric intake was different again only in the third to sixth week. However, both groups of animals consumed more than their total daily caloric requirements, including adequate protein and fat calories, and this is reflected in their growth curves (Figure 7).

Five rats were allowed to continue drinking 25% ethanol for an unspecified period of time to determine whether they would continue to drink and eat at the same rate and gain weight. There were no control rats. After 10 months the rats appeared healthy and their average weight was 529 $\pm$ 10 g. The average daily liquid intake was 35.9 ml/day and the average daily chow intake was 15.5 g/day. The liquid intake had increased from the end of the 12 week schedule, but calculated on a per weight basis was 6.8 ml/per 100 g rat per day which was unchanged from the end of the 12 week schedule. The daily chow intake was the same as at the end of 12 weeks, but when calculated on a per weight basis, was down to 2.9 g per 100 g rat per day. However, this value should be compared to a value of approximately 4.0 g per 100 g rat per day calculated from the data of Warner and Breuer

(1972), which is still 75% of the recommended allowance, as before. The total average daily caloric intake for these rats was 104.2, 9.1 calories of which were fat, 15.5 were protein, 28.5 were mixed carbohydrate, and 51.1 were alcohol. Thus there was little change in the caloric intake as derived from the chow, but the alcohol calories increased considerably over the 10 month period from 36 to 42% of the total daily calories.

B. MITOCHONDRIAL FUNCTION

At the end of the 12 week feeding schedule, the mitochondria were isolated from the hearts of the 4 groups of 8 animals each. The 4 groups consisted of: an alcohol-fed group, $A_1$; a dextrose control group, $C_1$; an alcohol-fed and magnesium supplemented group, $A_2$; a dextrose control and magnesium supplemented group, $C_2$. The mitochondria were isolated as described in the methods section, and samples were analyzed for protein yield, magnesium and calcium content and respiratory function. In addition, each mitochondrial sample was analyzed for respiratory function in the presence of 5 mM $MgCl_2$ and 100 mM KCl.

As already indicated, the protein assays were done at two different times. Groups $A_1$ and $C_1$ were done together, while $A_2$ and $C_2$ were analyzed at another time. Consequently the protein yields of $A_1$ ($36.3 \pm 2.3$ mg/ml) and $C_1$ ($33.3 \pm 2.5$ mg/ml) were not statistically different from each other, and there was no difference between the protein yields of $A_2$ ($26.8 \pm 3.7$ mg/ml) and $C_2$ ($26.4 \pm 5.0$ mg/ml) but there was a difference between groups $A_1$ and $C_1$ when compared to $A_2$ and $C_2$. However, this difference did not reach the level of statistical significance, but it may be important in the interpretation of the mitochondrial ion content data.

Table 3 shows the mitochondrial content of magnesium and calcium in alcohol-fed and control rats, with and without magnesium supplements. There is no difference in mitochondrial calcium levels in any of the groups. As was done with the protein assay, the calcium and magnesium assays were done at different times, $A_1$ and $C_1$ together, and $A_2$ and $C_2$ later. When the alcohol-fed rats ($A_1$) are compared to control rats ($C_1$) there is a significant decrease in the magnesium content of the mitochondria of $A_1$. When $A_2$ and $C_2$ are compared one finds no difference in the magnesium levels. The in vivo magnesium supplements did not appear to increase the mitochondrial levels of magnesium in either $C_2$ or especially $A_2$. However, because of the variability in the assay from one group ($A_1$ and $C_1$) to another ($A_2$ and $C_2$), one cannot compare $A_1$ and $A_2$ or $C_1$ and $C_2$ in a valid manner. Taking the two groups separately (i.e. $A_1$ and $C_1$ apart from $A_2$ and $C_2$) one can only say that the magnesium supplements prevented the alcohol-induced decrease in the mitochondrial magnesium content.

Tables 4 to 9 list the results of the analysis of respiratory function of the mitochondria. Chronic alcohol ingestion had no effect on ADP:O ratio or state 4 respiration (Table 4). There was a decrease in state 3 respiration in the alcohol-fed rats but this was not statistically significant ($A_1$ vs $C_1$; NS). The magnesium injections themselves appeared to lower state 3 oxygen consumption but this was not statistically significant. The most sensitive indicator of mitochondrial respiratory integrity is the respiratory control index (RCI) (Packer, 1960; Lehninger, 1975) and this parameter was affected by chronic alcohol ingestion. The RCI of $A_1$ was significantly lower than $C_1$ and this was reversed with magnesium injections ($A_2$). The control rats were not affected by the magnesium supplements ($C_2$).

TABLE 3

ION CONTENT (nmoles/mg)[+]

| EXPERIMENTAL GROUPS (N = 8) | CALCIUM | MAGNESIUM |
|---|---|---|
| $A_1$ | 8.07 ± .99 | 30.03 ± 3.71 |
| $C_1$ | 9.52 ± .64 | 37.71 ± 1.92 |
| $A_2$ | 11.28 ± 1.15 | 29.79 ± 1.38 |
| $C_2$ | 12.83 ± 1.66 | 32.13 ± 1.78 |

[+] Calculated as nanomoles of ion per mg of mitochondrial protein. $A_1$ = alcohol-fed, $C_1$ = control, $A_2$ = alcohol-fed, magnesium supplemented, $C_2$ = control, magnesium supplemented.

CALCIUM:
$A_1$ vs $C_1$, NS
$A_2$ vs $C_2$, NS
$A_1$ vs $A_2$, NS
$C_1$ vs $C_2$, NS

MAGNESIUM:
$A_1$ vs $C_1$, $P < .05$
$A_2$ vs $C_2$, NS
$A_1$ vs $A_2$, NS
$C_1$ vs $C_2$, NS

TABLE 4

MITOCHONDRIAL RESPIRATION+

| EXPERIMENTAL GROUPS (N = 8) | STATE 3 $O_2$ CONSUMPTION µmoles/g/min | STATE 4 $O_2$ CONSUMPTION µmoles/g/min | ADP:O | RESPIRATORY CONTROL INDEX |
|---|---|---|---|---|
| $C_1$ CONTROL | 109.6 ± 2.8 | 14.8 ± 0.4 | 2.90 ± 0.04 | 7.77 ± 0.17 |
| $A_1$ ALCOHOL | 100.8 ± 4.8 | 15.1 ± 0.6 | 2.92 ± 0.06 | 6.62 ± 0.24 |
| $C_2$ CONTROL + Mg | 93.5 ± 4.0 | 11.7 ± 0.5 | 3.04 ± 0.03 | 8.04 ± 0.23 |
| $A_2$ ALCOHOL + Mg | 90.8 ± 4.1 | 11.7 ± 0.6 | 2.93 ± 0.11 | 7.91 ± 0.07 |
| COMPARISONS | | | | |
| $A_1$ vs $C_1$ | NS | NS | NS | ** |
| $A_2$ vs $C_2$ | NS | NS | NS | NS |
| $A_1$ vs $A_2$ | NS | ** | NS | ** |
| $C_1$ vs $C_2$ | NS | * | NS | NS |

+ State 3 and state 4 oxygen consumption = micromoles of $O_2$ consumed per gram of mitochondrial protein per minute. Respiratory control index = ratio of state 3: state 4 respiraton. * $p < 0.05$; ** $p < 0.01$; NS, non significant

TABLE 5

MITOCHONDRIAL RESPIRATION[+]

WITH ADDITION OF 5mM $MgCl_2$ IN VITRO

| EXPERIMENTAL GROUPS (N = 8) | STATE 3 $O_2$ CONSUMPTION µmoles/g/min | STATE 4 $O_2$ CONSUMPTION µmoles/g/min | ADP:O | RESPIRATORY CONTROL INDEX |
|---|---|---|---|---|
| $C_1$ CONTROL | 147.1 ± 5.9 | 23.8 ± 1.2 | 2.37 ± 0.06 | 6.27 ± 0.19 |
| $A_1$ ALCOHOL | 138.4 ± 8.2 | 23.9 ± 0.9 | 2.45 ± 0.03 | 5.80 ± 0.22 |
| $C_2$ CONTROL + Mg | 121.0 ± 9.5 | 21.8 ± 1.0 | 2.38 ± 0.06 | 5.60 ± 0.47 |
| $A_2$ ALCOHOL + Mg | 118.0 ± 8.3 | 20.1 ± 1.1 | 2.39 ± 0.06 | 5.87 ± 0.33 |
| COMPARISONS | | | | |
| $A_1$ vs $C_1$ | NS | NS | NS | NS |
| $A_2$ vs $C_2$ | NS | NS | NS | NS |
| $A_1$ vs $A_2$ | * | ** | NS | NS |
| $C_1$ vs $C_2$ | ** | NS | NS | NS |

[+] State 3 and state 4 oxygen consumption = micromoles of $O_2$ consumed per gram of mitochondrial protein per minute. Respiratory control index = ratio of state 3: state 4 respiration. * $p < 0.05$; ** $p < 0.01$; NS, non significant

TABLE 6

MITOCHONDRIAL RESPIRATION[+]

WITH ADDITION OF 100 mM KCl IN VITRO

| EXPERIMENTAL GROUPS (N = 8) | STATE 3 $O_2$ CONSUMPTION µmoles/g/min | STATE 4 $O_2$ CONSUMPTION µmoles/g/min | ADP:O | RESPIRATORY CONTROL INDEX |
|---|---|---|---|---|
| $C_1$ CONTROL | 104.3 ± 5.1 | 18.8 ± 0.4 | 2.82 ± 0.05 | 5.49 ± 0.28 |
| $A_1$ ALCOHOL | 96.1 ± 3.4 | 16.3 ± 0.8 | 2.86 ± 0.10 | 6.03 ± 0.35 |
| $C_2$ CONTROL + Mg | 79.8 ± 7.8 | 16.1 ± 1.2 | 2.69 ± 0.06 | 5.14 ± 0.43 |
| $A_2$ ALCOHOL + Mg | 80.6 ± 7.5 | 13.4 ± 0.6 | 2.88 ± 0.05 | 6.06 ± 0.39 |
| COMPARISONS | | | | |
| $A_1$ vs $C_1$ | NS | * | NS | NS |
| $A_2$ vs $C_2$ | NS | * | NS | NS |
| $A_1$ vs $A_2$ | NS | * | NS | NS |
| $C_1$ vs $C_2$ | * | * | NS | NS |

[+] State 3 and state 4 oxygen consumption = micromoles of $O_2$ consumed per gram of mitochondrial protein per minute. Respiratory control index = ratio of state 3: state 4 respiration. * $p < 0.05$; NS, non significant

TABLE 7

CHANGE IN OXYGEN CONSUMPTION[+]

| EXPERIMENTAL GROUPS (N = 8) | IN VITRO 5 mM $MgCl_2$ | | IN VITRO 100 mM KCl | |
|---|---|---|---|---|
| | STATE 3 | STATE 4 | STATE 3 | STATE 4 |
| $C_1$ CONTROL | +37.9 ± 4.5 | + 9.2 ± 1.0 | - 4.9 ± 4.6 | +4.2 ± 0.6 |
| $A_1$ ALCOHOL | +33.8 ± 5.8 | + 8.4 ± 1.0 | - 8.6 ± 3.3 | +0.7 ± 0.8 |
| $C_2$ CONTROL + Mg | +27.4 ± 6.1 | +10.1 ± 0.8 | -13.8 ± 5.2 | +4.4 ± 0.9 |
| $A_2$ ALCOHOL + Mg | +27.2 ± 4.4 | + 8.4 ± 0.9 | -10.0 ± 4.4 | +1.7 ± 0.4 |
| COMPARISONS | | | | |
| $A_1$ vs $C_1$ | NS | NS | NS | ** |
| $A_2$ vs $C_2$ | NS | NS | NS | * |
| $A_1$ vs $A_2$ | NS | NS | NS | NS |
| $C_1$ vs $C_2$ | NS | NS | NS | NS |

[+] Oxygen consumption in state 3 and state 4 expressed as micromoles of $O_2$ consumed per gram of mitochondrial protein per minute. ** $p < 0.01$; * $p < 0.05$; NS, non significant

TABLE 8

RESPIRATORY CONTROL INDEX

| EXPERIMENTAL GROUPS (N = 8) | IN VITRO TREATMENTS | | | COMPARISONS | | |
|---|---|---|---|---|---|---|
| | NIL | 5 mM $MgCl_2$ | 100 mM KCl | NIL vs Mg | NIL vs K | Mg vs K |
| $C_1$ CONTROL | 7.77 ± 0.17 | 6.27 ± 0.19 | 5.49 ± 0.28 | ** | ** | NS |
| $A_1$ ALCOHOL | 6.62 ± 0.24 | 5.80 ± 0.22 | 6.03 ± 0.35 | ** | NS | NS |
| $C_2$ CONTROL + Mg | 8.04 ± 0.23 | 5.60 ± 0.47 | 5.14 ± 0.43 | ** | ** | NS |
| $A_2$ ALCOHOL + Mg | 7.91 ± 0.07 | 5.87 ± 0.33 | 6.06 ± 0.39 | ** | ** | NS |
| COMPARISONS | | | | | | |
| $A_1$ vs $C_1$ | * | NS | NS | | | |
| $A_2$ vs $C_2$ | NS | NS | NS | | | |
| $A_1$ vs $A_2$ | NS | NS | NS | | | |
| $C_1$ vs $C_2$ | NS | NS | NS | | | |

** $p < 0.01$; NS, non significant

TABLE 9

ADP:O

| EXPERIMENTAL GROUPS (N = 8) | IN VITRO TREATMENTS | | | COMPARISONS | | |
|---|---|---|---|---|---|---|
| | NIL | 5 mM $MgCl_2$ | 100 mM KCl | NIL vs Mg | NIL vs K | Mg vs K |
| $C_1$ CONTROL | 2.90 ± 0.04 | 2.37 ± 0.06 | 2.82 ± 0.05 | ** | NS | ** |
| $A_1$ ALCOHOL | 2.92 ± 0.06 | 2.45 ± 0.03 | 2.86 ± 0.10 | ** | NS | ** |
| $C_2$ CONTROL + Mg | 3.04 ± 0.03 | 2.38 ± 0.06 | 2.69 ± 0.06 | ** | ** | ** |
| $A_2$ ALCOHOL + Mg | 2.93 ± 0.11 | 2.39 ± 0.06 | 2.88 ± 0.05 | ** | NS | ** |
| COMPARISONS | | | | | | |
| $A_1$ vs $C_1$ | NS | NS | NS | | | |
| $A_2$ vs $C_2$ | NS | NS | NS | | | |
| $A_1$ vs $A_2$ | NS | NS | NS | | | |
| $C_1$ vs $C_2$ | NS | NS | NS | | | |

** $p < 0.01$; NS, non significant

Table 5 illustrates the changes in respiratory function induced by the *in vivo* addition of 5 mM $MgCl_2$. This was done in an attempt to prevent the decrease in RCI, which had been demonstrated previously by Dow (1970) and Wrogemann *et al.* (1970). The assumption in our experiments was that the decrease in RCI induced by alcohol may be due to a relative magnesium deficiency. State 3 and state 4 oxygen consumption (state 4 proportionally more) were increased, the RCI decreased, and the ADP:O ratio decreased. The effects of increased oxygen consumption with uncoupling has been demonstrated before (Chao and Davis, 1972; Brierley, 1974, 1976). However, there was no difference in the respiratory changes between the alcohol-fed rats and controls.

The *in vitro* addition of 100 mM KCl was also performed in an attempt to improve RCI, or to show a difference in oxygen consumption between alcohol-fed rats and controls. The rationale for this was that there is an intimate relationship between mitochondrial magnesium levels and respiration dependent potassium fluxes (Jung *et al.*, 1977; Chavez *et al.*, 1977). The effects of potassium on mitochondrial respiration are complex and incompletely understood (Gamble, 1957; Lehninger *et al.*,1967; Brierley, 1976; Gomez-Puyou and Gomez-Puyou, 1977) and the interpretation of these data is difficult. The *in vitro* addition of 100 mM KCl (Table 6) produced no change in state 3 respiration or ADP:O ratios and there was no differential effect of chronic alcohol ingestion on these parameters. However, the RCI was decreased in all groups with no statistically significant effect of chronic alcohol ingestion but a trend towards a greater decrease in the control groups ($C_1$ and $C_2$). Related to this change, the 100 mM KCl produced a statistically significant difference in state 4 respiration between alcohol-fed rats and control rats. The K-induced

stimulation of state 4 respiration was greater in the control rats ($C_1$ and $C_2$) than in the alcohol-fed rats ($A_1$ and $A_2$). Table 7 shows this more clearly, as the *in vitro* additions' effects on oxygen consumption are expressed as a change in oxygen consumption from a baseline value. 5 mM $MgCl_2$ stimulates both state 3 respiration and state 4 respiration (state 4 proportionally more) but there is no effect of chronic alcohol ingestion, nor an effect of *in vivo* magnesium supplementation. 100 mM KCl produces a slight but non significant decrease in state 3 respiration, with no effect of chronic alcohol ingestion or magnesium supplementation. However, the potassium induced stimulation of state 4 oxygen consumption is significantly less in the alcohol-fed rats, and the *in vivo* magnesium supplements did not reverse this difference. A comparison of the RCI changes induced by the various treatments is shown in Table 8. The *in vitro* 5 mM $MgCl_2$ addition has decreased the RCI by causing a stimulation of both state 3 and state 4 respiration, but a proportionally greater stimulation of state 4 (the denominator) thus reducing the RCI. There is no effect of chronic alcohol ingestion nor magnesium supplements *in vivo*. 100 mM KCl has also reduced the RCI, but the important change occurs in the denominator, i.e. state 4 respiration. Because of the alcohol-induced effect on state 4 respiration, one might expect a significant change in the RCI (i.e. $A_1$ vs $C_1$ and $A_2$ vs $C_2$) but the difference was not statistically significant. It is difficult to infer any important physiologic changes occurring here because the *in vitro* treatments have diminished the RCI's well below that of the alcohol induced effects seen in $C_1$. Table 9 shows a comparison of the ADP:O ratios. *In vitro* 5 mM $MgCl_2$ uncoupled the mitochondria, while 100 mM KCl did not, yet both treatments caused a decrease in RCI (Table 8). Again, chronic alcohol ingestion and the *in vivo* magnesium supplements had no effect.

C. TISSUE WATER AND ELECTROLYTE ANALYSIS

At the end of the 12 week feeding schedule, the rats were divided into 4 groups of 12 animals each: an alcohol-fed group was designated A and a control group was designated C. The other two groups which were placed back on standard lab chow and tap water for another 8 weeks, were designated $A_R$ (alcohol-recovery) and $C_R$ (control recovery). These animals were analyzed for plasma and intracellular electrolytes and tissue water distribution.

A comparison of the data from the extracellular space as measured by the tracer and morphometric methods is shown in Figure 8 for the alcohol-fed rats (A). The morphometric method indicates a constant value throughout the entire equilibration period of 6 hours, while the tracer method shows an ever increasing value over 6 hours, demonstrating the permeability changes induced by chronic alcohol ingestion. Figures 9 and 10 compare the extracellular space (ECS) as measured by tracer for all groups of animals, and shows that the alcohol-induced change in permeability has been reversed with abstention from alcohol ($A_R$ and $C_R$). Although the permeability change is well demonstrated, Figure 9 also shows that the intercept for A is lower than the other 3 groups. This is seen more clearly in Figure 11, where a decrease in the ECS is present from time zero, i.e. before the tracer is injected, as calculated by extrapolation. This decrease is present when measured by both the tracer and morphometric methods. However, the difference is not statistically significant. This decrease can perhaps partially be explained from the data in Figure 12, which shows a significant increase in intracellular water in the alcohol-fed group only (A), which again reverts to normal with abstention. These data therefore suggest a movement of water from outside the cell to the

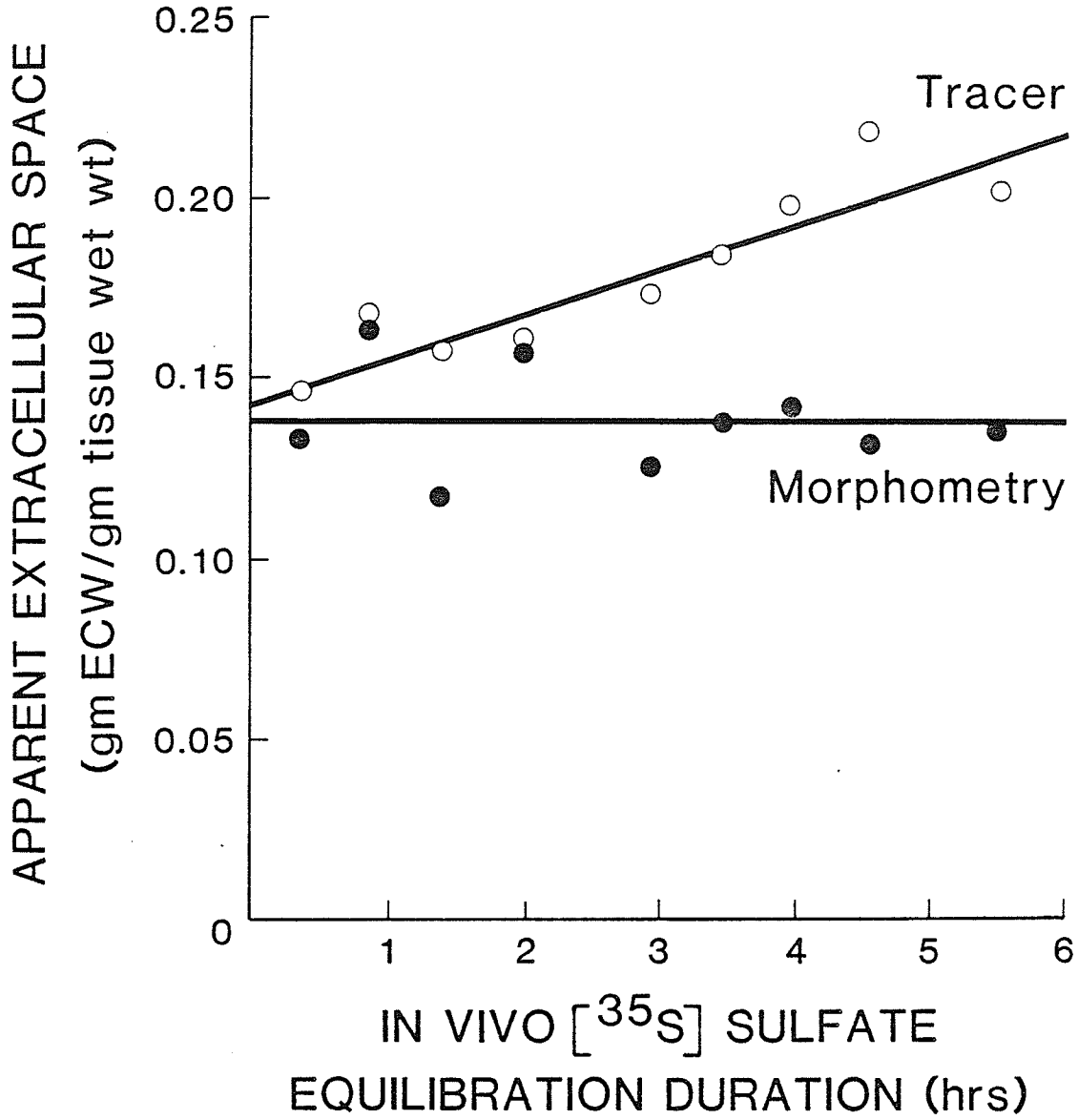



FIGURE 8. Apparent extracellular spaces determined by tracer and morphometric methods in left ventricle of alcoholic rat.
Slope: Tracer = $(+39.4 \pm 3.5)\ 10^{-7}\ hr^{-1}$
Morphometry = $(-3.0 \pm 3.4)\ 10^{-7}\ hr^{-1}$
$p < 0.05$
Intercept: Tracer = $0.139 \pm 0.006$
Morphometry = $0.137 \pm 0.006$
NS

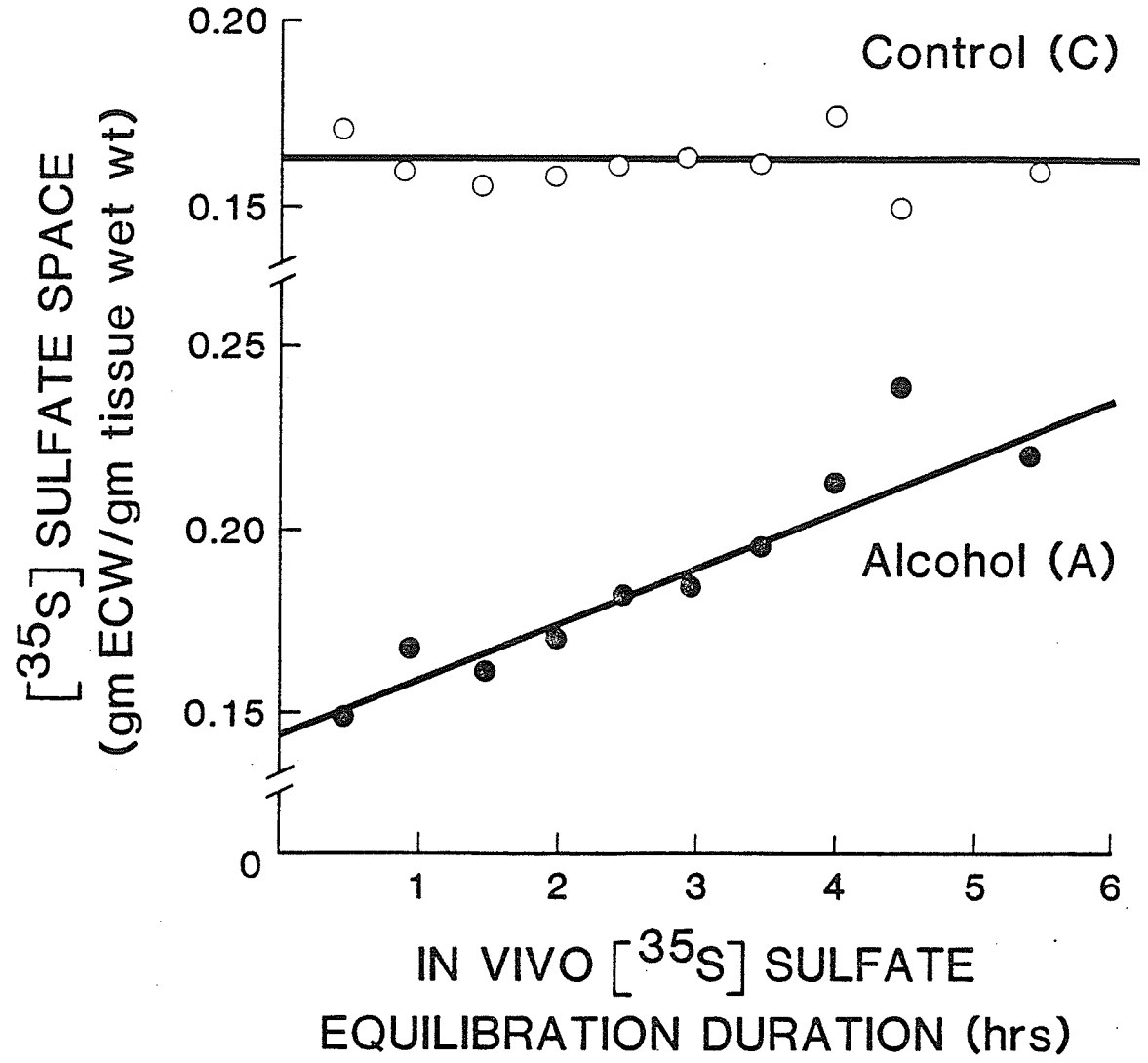



FIGURE 9. Left ventricular ($^{35}S$) sulfate space as a function of in vivo equilibration duration. Groups N = 12
Slope of A vs. C: $p < 0.05$

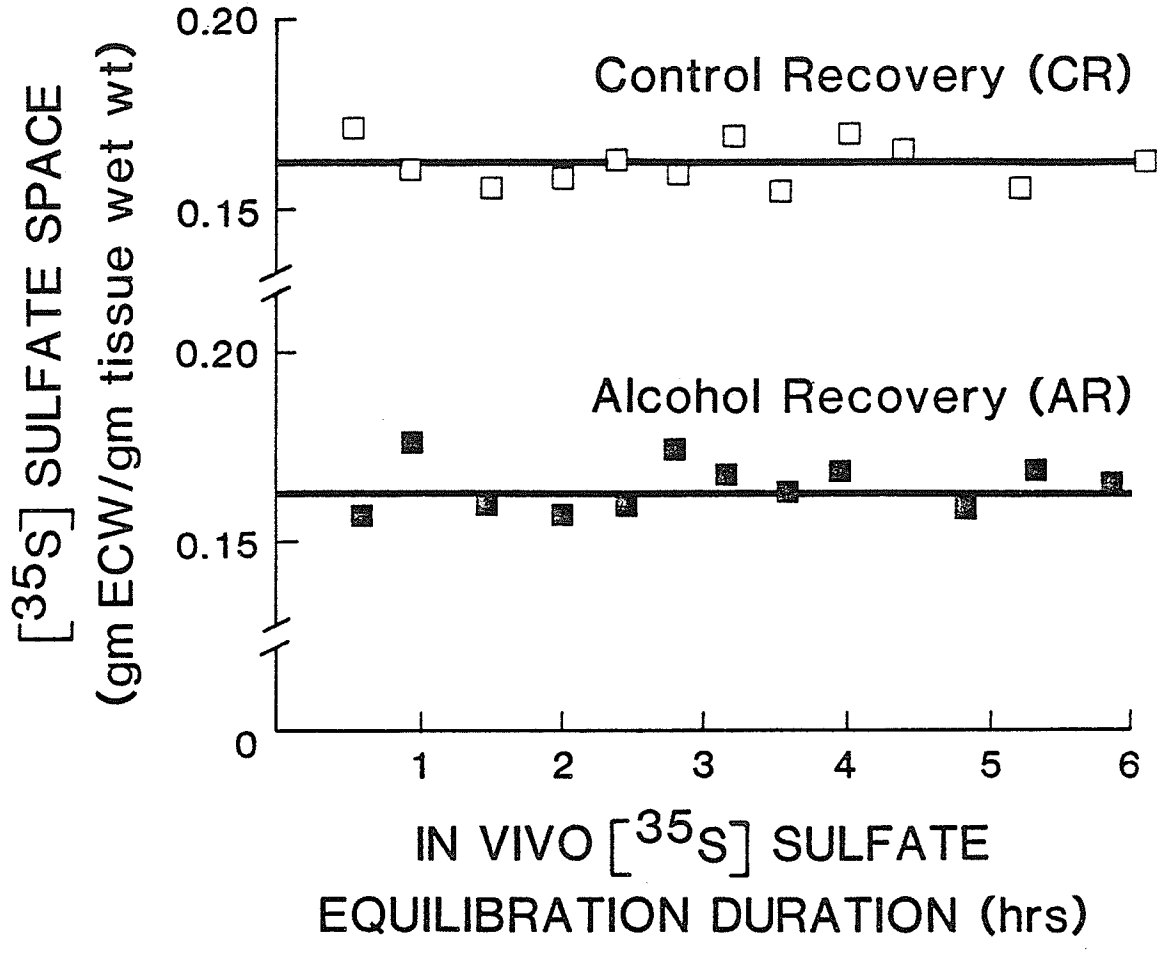



FIGURE 10. Left ventricular ($^{35}S$) sulfate space as a function of in vivo equilibration duration. Groups N = 12
Slope of $A_R$ vs. $C_R$: NS

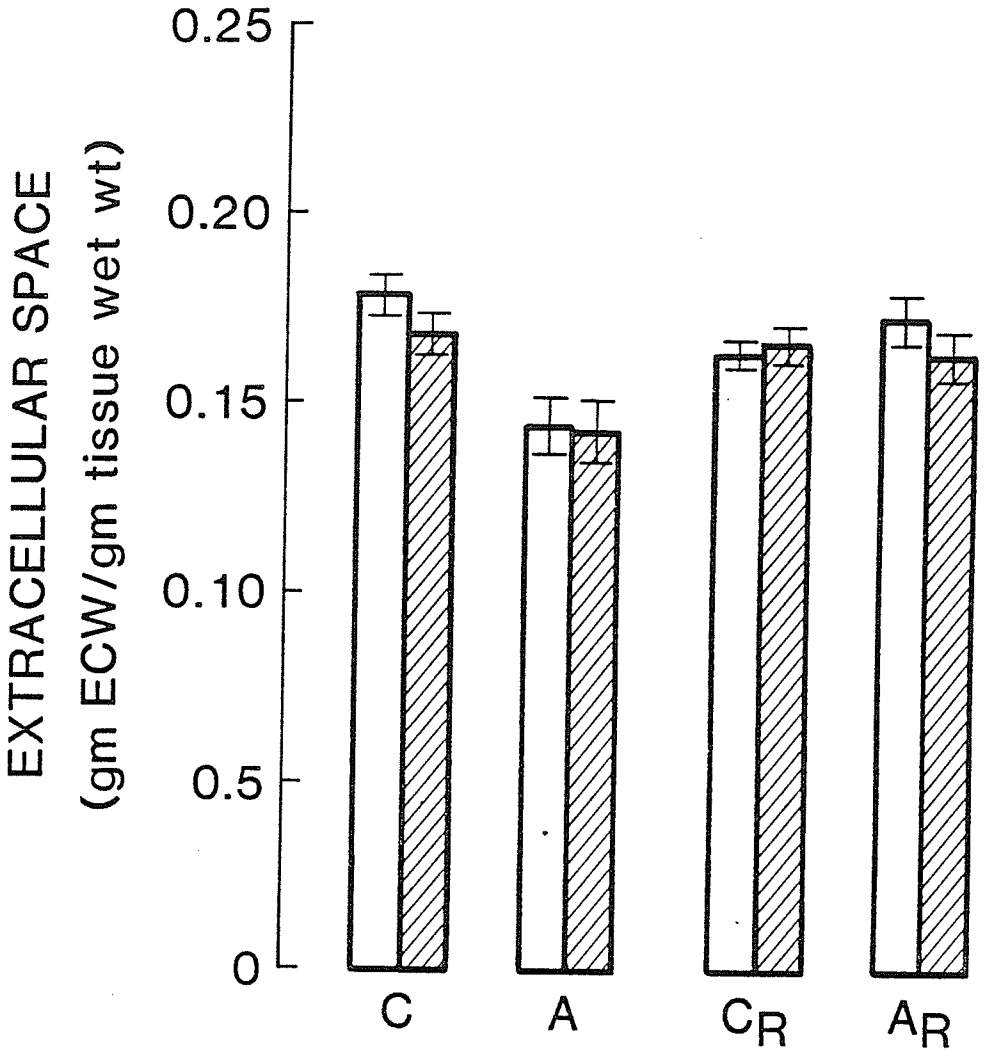



FIGURE 11. Left ventricular ECS determined from time zero regression valves of ($^{35}S$) sulfate. Groups N = 12
(□) Tracer; (▨) Morphometric; C = control; A = alcohol-fed; $C_R$ = control-recovery; $A_R$ = alcohol-recovery
A vs. C, $A_R$ vs. $C_R$, A vs. $A_R$, C vs. $C_R$; NS

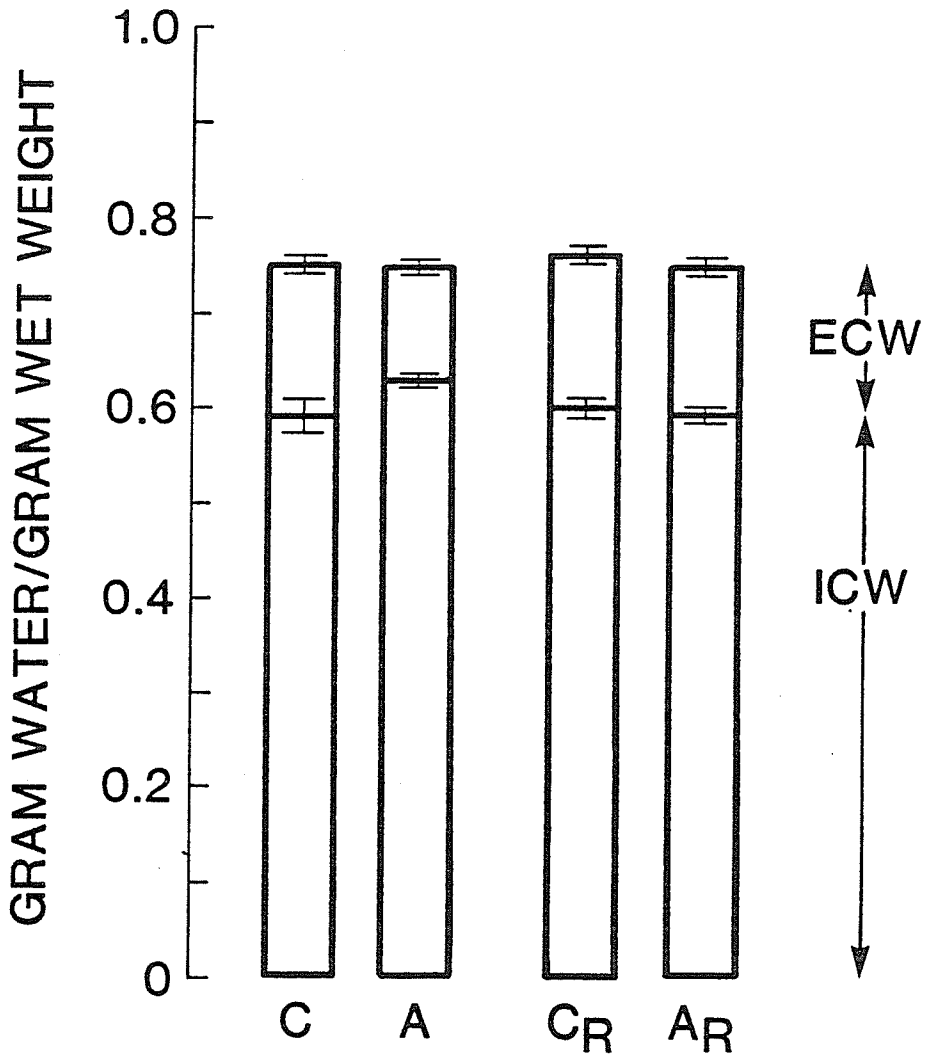



FIGURE 12. Distribution of tissue water. ECW = extracellular water; ICW = intracellular water; C = control; A = alcohol-fed; $C_R$ = control recovery; $A_R$ = alcohol-recovery. Groups N = 12 ICW: A vs. C, A vs. $A_R$, A vs. $C_R$; $p < 0.05$

TABLE 10

PLASMA ELECTROLYTE CONCENTRATIONS

millimoles/litre of plasma

| EXPERIMENTAL GROUPS (N = 12) | SODIUM | POTASSIUM | CALCIUM | MAGNESIUM |
|---|---|---|---|---|
| A ALCOHOL | 157.9 ± 1.1 | 4.2 ± 0.2 | 1.5 ± 0.1 | 1.13 ± 0.04 |
| C CONTROL | 158.1 ± 0.8 | 4.1 ± 0.1 | 2.0 ± 0.2 | 1.05 ± 0.07 |
| $A_R$ ALCOHOL-RECOVERY | 153.6 ± 0.7 | 4.4 ± 0.1 | 2.3 ± 0.1 | 1.10 ± 0.06 |
| $C_R$ CONTROL-RECOVERY | 149.4 ± 2.5 | 4.3 ± 0.2 | 2.4 ± 0.1 | 1.03 ± 0.04 |
| COMPARISONS | | | | |
| A vs C | NS | NS | * | NS |
| $A_R$ vs $C_R$ | NS | NS | NS | NS |
| A vs $A_R$ | NS | NS | * | NS |
| C vs $C_R$ | * | NS | * | NS |

* $p < 0.05$; NS, non significant

TABLE 11

NOMINAL INTRACELLULAR CONCENTRATIONS OF CATIONS

FROM RAT LEFT VENTRICLE

millimoles/litre of cell water

| EXPERIMENTAL GROUPS (N = 12) | SODIUM | POTASSIUM | CALCIUM | MAGNESIUM |
|---|---|---|---|---|
| A ALCOHOL | 18.9 ± 2.0 | 156.9 ± 5.0 | 0.67 ± 0.08 | 15.8 ± 1.1 |
| C CONTROL | 8.0 ± 0.8 | 165.7 ± 4.7 | 0.28 ± 0.05 | 16.9 ± 0.5 |
| $A_R$ ALCOHOL-RECOVERY | 10.5 ± 1.2 | 170.5 ± 2.6 | 0.32 ± 0.04 | 16.2 ± 0.8 |
| $C_R$ CONTROL-RECOVERY | 13.1 ± 0.9 | 167.2 ± 1.3 | 0.29 ± 0.08 | 16.8 ± 0.5 |
| COMPARISONS | | | | |
| A vs C | * | NS | * | NS |
| $A_R$ vs $C_R$ | NS | NS | NS | NS |
| A vs $A_R$ | * | * | * | NS |
| C vs $C_R$ | * | NS | NS | NS |

* $p < 0.05$; NS, non significant

inside. There is a small decrease in total tissue water in the recovery groups (significant only in the control group $C_R$); but this may be secondary to the ion changes which may be age related.

Plasma electrolyte concentrations are shown in Table 10. There was no change in the plasma concentrations of potassium or magnesium in any of the groups examined. There was no difference in plasma sodium between alcohol-fed (A) and control rats (C), but the recovery period produced a decrease in plasma sodium which was statistically significant only in the control ($C_R$) group. Because the recovery rats were 2 months older, the changes may be related to age. The plasma calcium concentration in the alcohol-fed rats (A) is significantly less than in the control rats (C) and the recovery period showed a rise in plasma calcium in both alcohol-fed ($A_R$) and control ($C_R$) rats. Again, this change may be age-related. Interpretation of plasma electrolyte concentrations is difficult because the correlation with tissue concentrations is poor (Jones et al., 1969).

Intracellular magnesium and calcium concentrations are shown in Table 11. There was no change in intracellular magnesium concentration in any of the groups examined, but the intracellular calcium concentration was more than doubled in the alcohol-fed rats (A). The recovery period again produced a return to control values of calcium. Chronic alcohol ingestion produced no change in intracellular potassium concentration (Table 11) and the recovery period elevated the levels in the controls ($C_R$) and alcohol-fed ($A_R$) rats, but only the difference between the two alcohol-fed groups (A vs $A_R$) was statistically significant. Again the rise in potassium may be age-related. A rise in intracellular sodium (Table 11) was produced by alcohol and the recovery period did not produce a complete return to the original control values but this may be age-related.

# IV DISCUSSION

A. ANIMAL MODEL

One of the purposes of this animal model was to study functional damage before structural damage was visible. The decision to examine the hearts after 12 weeks of alcohol ingestion was purely arbitrary, because there are no guidelines whatsoever to be taken from the literature on alcoholic cardiomyopathy. The goal was to allow the rats to drink long enough to produce biochemical changes but short enough so that no structural damage could be seen with electron microscopy. There are approximately 25 to 30 papers on the subject of alcohol-induced cardiac damage in rats which have been ingesting alcohol chronically. The variety of pathology reported, as well as the variety of rat strains, quantity and duration of alcohol ingestion and feeding methods have been mentioned above. The smallest dose of alcohol producing cardiac structural damage in rats was reported by Segel et al. (1975) who fed 5% ethanol to male Sprague-Dawley rats for 7 weeks. However, cardiac mitochondrial function was not depressed in these rats, but was depressed in rats drinking 25% ethanol for at least 19 weeks. Minimal structural changes have also been found in mice drinking 5% ethanol for 6 weeks (Burch et al., 1971). These two reports describe the most easily produced lesions seen in the literature. Most of the reports have examined structural damage only after longer drinking periods. Gvozdjak et al. (1973a), using intraperitoneal injections of alcohol every day for 10 weeks, were unable to produce any morphologic changes in rat heart. Hall and Rowlands (1970) produced minimal changes in cardiac structure in male Sprague-Dawley rats drinking 32% alcohol for 14 weeks, when using the electron microscope and found nothing with the light microscope. Fahimi et al. (1979) found no structural changes in the myocardium of male Sprague-Dawley rats drinking

ethanol as 36% of their daily calories for 18 weeks, but there was an increase in the catalase content. Mitochondrial function was not assessed in the latter two studies, but was found to be depressed in the studies by Gvozdjak et al. (1973a). Thus the evidence for structural damage even within the same strain (Sprague-Dawley) of rat is conflicting. In studies of mitochondrial respiration, significant depression of respiration has been found as early as 6 weeks (Williams and Li, 1977) and 8 weeks (Weishaar et al., 1977) in male Sprague-Dawley rats, but there was no mention of structural damage. Although sarcolemmal permeability changes in chronic alcoholism have been inferred (Wendt et al., 1965; Regan et al., 1969) the only study on rat myocardium showing loss of intracellular enzymes was by Wojcicki et al. (1975) who fed rats 15% ethanol for 8 months. However there were also gross structural changes. Segel et al. (1979) fed alcohol as 38% of the daily calories for 38-48 weeks to male Long-Evans rats (similar to the present study) and found no contractile deficiencies until the hearts were challenged with dobutamine or hypoxia. However, there was no assessment of structure or mitochondrial function.

After a review of the literature on the effect of chronic alcohol ingestion on rat myocardium, it was decided that a 12 week schedule would guarantee some biochemical alterations, especially mitochondrial depression, with a probable preservation of structure, and with the question of sarcolemmal permeability entirely open.

The lack of structural changes found in these experiments is consistent with the work of others (Hall and Rowlands, 1970; Fahimi et al., 1979) and is not surprising in view of the low blood alcohol levels produced. The decline in blood alcohol levels over the last 6 weeks may be due to the induction of liver enzymes responsible for the

metabolism of alcohol (Israel et al., 1975; Lieber et al., 1975), since there was actually an increase in alcohol consumption over the same time period. Also consistent with the low blood alcohol levels is the lack of withdrawal signs and the healthy growth curve achieved. The growth curve shown here for both the alcohol-fed and control rats exceeds the one described for the Long-Evans strain by Warner and Breuer (1972). Table 1 shows the average daily food and liquid intake. When the alcohol concentration is set at 25%, which is 36% of the daily caloric intake, the chow intake is reduced to approximately 16 grams per rat per day, which is 75% of the recommended daily chow intake of 20 grams (Warner and Breuer, 1972). This is not surprising because the predominant motivation for food is the need for calories. Indeed, this is the crux of the problem when trying to imitate the human alcoholic feeding pattern. However, even when the chow intake is reduced to 75%, all the nutrients contained in the chow are still consumed in excess (see contents of chow in methods section). In the specific case of magnesium, the concentration in the diet is 0.22% and if this were reduced by 25%, it would still be in excess of the recommended level of 0.04% (Warner and Breuer, 1972). This does not rule out a state of secondary malnutrition, i.e. loss of nutrients through malabsorption or hyperexcretion. Table 2 shows the average daily caloric intake from fat, protein, carbohydrate, alcohol and dextrose. There is a small but statistically significant decrease in the calories of fat, protein and carbohydrate consumed by the alcohol-fed rats during the third to sixth weeks. As mentioned earlier, the biologic significance of this small difference is not known, especially since all the animals received adequate nutrition. The substitution of dextrose for alcohol calories was successful in that there was no

difference between the two groups for ten of the twelve weeks. Although there are some statistically significant differences shown in Table 2 at various points in time, none of them show a consistent pattern throughout the 12 week schedule, and it would be extremely difficult to attribute any myocardial derangements to a difference in the number or type of calories consumed. These data show that all the rats were able to feed ad lib with food and liquid as separate sources and still maintain roughly iso-caloric intakes for 12 weeks.

This method of feeding the rats was chosen for several reasons. The laborious task of pair feeding, which requires daily measurements, was avoided. This technique is impractical for large numbers of animals, as Alexander et al. (1977a) have stated. They were feeding several hundred animals, however. Avoidance of liquid diets was also achieved, as liquid diets are expensive and do not provide the alcohol and water separate from the food. This concept has already been discussed and further support for avoidance of a totally liquid diet has been given by Porta and Gomez-Dumm (1968) and Moscatelli et al. (1978). Both these studies employed a sugar-ethanol solution to overcome the rat's natural aversion to alcohol. However, there was a 33% mortality rate in alcohol-fed rats with no deaths in the control group in the experiments of Porta and Gomez-Dumm. Half of the deaths were due to pneumonia, the precipitating cause of which was not explained. To overcome the rat's aversion to high alcohol concentrations and to avoid the possibility of an abrupt high alcohol load which may acutely dehydrate the rats (and predispose to various illnesses, such as pneumonia), the initial 4 week period of gradual increases in alcohol concentration was adopted. This was supposed to allow the rats a "conditioning" period. This same "conditioning" period occurs in the

young human as well. The vast majority of juveniles who first drink alcohol do not like the taste, but acquire a taste over several months or years. Extrapolation from human behavior to rat behavior is admittedly dangerous and the brevity of the conditioning period in the rat is likely not comparable. Nevertheless the data from the diets of the rats which drank for 10 months supports the idea that they can consume alcohol for prolonged periods without becoming dehydrated or otherwise unhealthy. However, blood alcohol levels have not been taken on these rats and their myocardial function has not yet been examined.

This animal model is not ideal, but it does fulfill certain nutritional requirements and closely simulates the human alcoholic's drinking and feeding habits. Obviously it is valid only for the specific strain of rat studied, namely Long-Evans and only for the duration of alcohol consumption used, namely 12 weeks. Thus there are some disadvantages. To apply this to a different animal or strain of rat would require a repetition of all the preliminary experiments in order to obtain the caloric intake of the various foodstuffs as well as alcohol and water before instilling dextrose in the control diet. Also, the experimentor has no control over the alcohol consumption of the rat. To obtain a dose-response curve, by either increasing the alcohol concentration or the duration of drinking, one would again need to do preliminary experiments before deciding on control diets. However, once a drinking and eating pattern was established at different concentrations or durations, the diet for the control rats could be made up in advance. This is no guarantee that each subsequent drinking schedule will produce iso-caloric intakes between alcohol-fed rats and controls, but the pattern so far has been fairly reproducible and is certainly more similar to the human real-life

situation. Judging from the data on the five rats drinking for 10 months, it would be easier to increase the duration of alcohol consumption than to increase the concentration of alcohol. Some advantages of this feeding schedule are that, once the regimen is established, it would require less labor and expense and the food and liquid are separately presented.

B. MITOCHONDRIAL ANALYSIS

Analysis of the mitochondrial magnesium and calcium content (see Table 3) highlights the importance of measuring both ions when assessing mitochondrial function. Defects in mitochondrial respiration have been associated with a loss of endogenous mitochondrial magnesium (Kun et al., 1969; Reed and Lardy, 1972) in rat liver mitochondria, which may involve influx of calcium ions (Reed and Lardy, 1972). In addition, magnesium deficiency states produced experimentally have resulted in cardiac mitochondrial respiratory depression (Nakamura et al., 1961; DiGiorgio et al., 1962) but in the former study mitochondrial magnesium levels were not depressed. It has been shown that extra-mitochondrial magnesium in vitro plays a regulatory role in mitochondrial respiration associated with calcium uptake in rabbit heart (Sordahl, 1974; Sordahl and Silver, 1975) and rat heart (Coelho and Vercesi, 1980). Therefore, the need to measure mitochondrial levels of both magnesium and calcium becomes apparent when searching for causes of mitochondrial dysfunction. In addition to the interaction of these two ions, the importance of measuring pH, potassium, and the levels of permeant anions such as phosphate (Lehninger et al., 1967) should be mentioned, but these were not measured in the present experiments.

The mitochondrial magnesium levels found in these experiments are in the same range as those found in other studies of cardiac mitochondria

(Wrogemann et al., 1973; Crompton et al., 1976; Wehrle et al., 1976; Wrogemann and Nylen, 1978) and liver mitochondria (Kun et al., 1969; Reed and Lardy, 1972). If the magnesium content from all groups of animals is within the normal range, one might question the biological significance of a drop from 37 nanomoles to 30 nanomoles. Because this value is a measure of the total mitochondrial magnesium, it does not take into account the intramitochondrial distribution. If the 7 nanomole loss occurred mostly or all from one compartment, such as the matrix, which contains 1/3 of the magnesium, or the intermembrane compartment which contains 43% (Bogucka and Wojtczak, 1971) then the difference would be more significant. Further support of the biologic significance comes from the work of Wehrle et al. (1976) who demonstrated that removal of as little as 1 nanomole of magnesium per milligram of mitochondrial protein could cause an increase in mitochondrial membrane permeability. This small loss was even less than the 2-3 nanomoles found earlier to be effective in changing permeability by Settlemire et al. (1968). Both of these studies were performed on beef heart mitochondria, so that caution must be used in the interpretation of these data when commenting on rat heart mitochondria, as magnesium metabolism shows considerable species variation (Shine, 1979). Nevertheless a difference of 7 nanomoles in rat heart mitochondria would appear to be biologically significant.

The magnesium supplements given in the last week of the feeding schedule abolished the alcohol-induced drop in mitochondrial magnesium. While the magnesium content in the supplemented groups were equal, their absolute values were similar to the original alcohol-fed animals. It appears that the magnesium supplements did not increase the mitochondrial content at all and the improvement in respiratory function must have a

different cause. However, as mentioned previously, the values obtained in the magnesium supplemented groups ($A_2$ and $C_2$) are really not comparable to the other two groups ($A_1$ and $C_1$) because the magnesium assays were done at different times. Thus, the variability of the assay from day to day, but not within the same day, becomes important. One can only compare $A_1$ to $C_1$ and separately compare $A_2$ to $C_2$. It is possible that the magnesium injections altered both the protein levels (which were also done in two stages) and the magnesium levels. If the protein yields were different, i.e. $A_2$ and $C_2$ being lower, then the magnesium contents of $A_2$ and $C_2$ would be lower because magnesium is highly protein bound. Indeed the protein yields of $A_2$ and $C_2$ (26 mg/ml) were lower than $A_1$ and $C_1$ (33 and 36 mg/ml) but not to a statistically significant degree.

The mitochondrial calcium levels of all groups were in the same range as reported by others (Ito and Chidsey, 1972, Wrogemann and Nylen, 1978) and were not statistically significantly different from each other. Defects in mitochondrial respiration have been associated with increased (Schwartz et al., 1973; Dhalla, 1978; Sordahl, 1979) unchanged (Sarma et al., 1976) and decreased (Bing et al., 1974) mitochondrial calcium content, as well as decreases in calcium binding and uptake (Lindenmayer et al., 1970; Bing et al., 1974; Dhalla, 1978; Sordahl, 1979). The method of mitochondrial preparation is important in determining the level of calcium and degree of binding and uptake (Dhalla, 1978; Sordahl, 1979). The presence of magnesium and its contribution to calcium fluxes has already been mentioned (Sordahl, 1974; Coelho and Vercesi, 1980) and other factors such as the presence of EDTA (Settlemire et al., 1968) and ruthenium red (Thakar et al., 1973;

Wrogemann and Nylen, 1978) are important. While the small concentration of EDTA used in the present experiments (<0.1 mM) compared to others (1-10 mM) would not prevent calcium influx (Wrogemann et al., 1973), the use of ruthenium red was specifically designed to prevent influxes. However, ruthenium red has been shown not to prevent efflux of calcium (Sordahl, 1974; Coelho and Vercesi, 1980). Therefore, if calcium levels had been high initially, leakage could have occurred in preparation. Alternatively the lack of high calcium levels may have been due to impairment of uptake in the already slightly defective mitochondria, as illustrated by the decrease in RCI.

The conclusions to be drawn from the ion content data are that chronic alcohol ingestion produces a relative mitochondrial magnesium deficiency in rat heart associated with a defect in respiration and that respiratory defect can be abolished by magnesium supplements.

The respiratory function of the mitochondria was depressed by chronic alcohol ingestion (see Table 4). While the ADP:O ratio is unchanged, there is a drop in the state 3 respiration, but not to a statistically significant degree. However, the respiratory control index is decreased and this is said to be the most sensitive indication of mitochondrial integrity (Packer, 1960; Lehninger, 1975). A decrease in the RCI from 7.77 to 6.62 might not appear to be biologically significant. This decrease could be viewed as an absolute decrease of 1.15 units or a 15% reduction. When compared to other studies, an absolute decrease of this magnitude may be significant. A decrease of 1.67 with succinate as substrate, was associated with changes in electron spin resonance, a sensitive indicator of the presence of free radicals produced by enzymatic oxidation-reduction activity in mitochondria

(Suzuki et al., 1978). In addition Lindenmayer et al. (1970) showed a decrease in RCI of 1.0 in the cardiomyopathic hamster associated with severe heart failure and a decrease in mitochondrial calcium uptake, again with succinate as substrate. Wrogemann et al. (1970) showed a decrease in RCI of 0.9 in skeletal muscle mitochondria of the dystrophic hamster associated with a defect in all indices of mitochondrial respiratory function, with pyruvate-malate as substrate, which is similar to our experiments. All these decreases in RCI were statistically significantly lower, but the control RCI's were in the range of 2.2 to 4.0. This illustrates the other way of viewing the decrease in RCI, i.e. as a percentage of the control value. The 15% decrease in RCI in our experiments, in addition to being statistically significant, was abolished by a specific treatment. An important purpose of these experiments was to produce early biochemical changes before any anatomic changes were seen. Therefore the goal of early reversible biochemical changes was achieved. To extrapolate to the condition of true alcoholic cardiomyopathy is not valid,and this is not the intent of these experiments. Indeed, as mentioned earlier, no true model of alcoholic cardiomyopathy has been developed. Perhaps examination of the hearts of the 5 rats drinking for 10 months would reveal such changes.

To further outline the futility of correlating early mitochondrial dysfunction with eventual cardiomyopathy and heart failure, one need only examine the earlier work of Sobel et al. (1967) who showed normal mitochondria with heart failure, while the work of Lindenmayer et al. (1968) showed severe mitochondrial damage with heart failure. It is not known which is cause and which is effect. The consensus today is that there is poor correlation of mitochondrial respiration with heart failure,

and it probably depends on the methods of mitochondrial isolation and the type of heart failure produced, i.e. pressure overload or volume overload (Dhalla, 1978; Sordahl, 1979).

In an attempt to discover the relationship between magnesium content, mitochondrial permeability to cations and respiratory function, two *in vitro* treatments were given, namely 5 mM $MgCl_2$ and 100 mM KCl. The relationship between these processes is very complex and poorly understood at this time (Lehninger *et al.*, 1967; Brierley, 1974, 1976; A. Gomez-Puyou and M.T. de Gomez-Puyou, 1977; Shi *et al.*, 1980). Based on earlier work by Wrogemann *et al.* (1970) who demonstrated an improvement in RCI in skeletal muscle mitochondria of dystrophic hamsters with the *in vitro* addition of 5 mM $MgCl_2$, the same treatment was used in the present experiments. Earlier reports had shown improvement in respiratory control in mitochondria when 5 mM $MgCl_2$ was added (Dow, 1970) but this response to magnesium may depend on the method of preparation (Schaller *et al.*, 1978). *In vitro* magnesium did not improve the respiratory control of the alcohol-fed rats and in fact decreased the RCI in all groups (see Table 5). In addition it decreased the ADP:O ratio in all groups and stimulated state 3 and state 4 oxygen consumption in all groups. This effect has also been reported by others (Chao and Davis, 1972; Brierley, 1976). Because the baseline state 3 oxygen consumption is different in each group, Table 7 was designed to show the change in oxygen consumption produced by magnesium. There is no significant difference in the increase in oxygen consumption in any of the groups treated with magnesium *in vitro*. One explanation of this is that whatever the mitochondrial damage produced by chronic alcohol and the decrease in magnesium, it could not be repaired in the 2-3 minute incubation time used in the assays. It has been shown

that the accumulation of magnesium is slow, especially when compared to calcium (Brierley, 1976). Another explanation for the effects of magnesium *in vitro* is the method of isolation, as suggested by Schaller *et al.* (1978), in their assays of rat heart mitochondria. In comparing two methods of isolation, one using trypsin, a proteolytic enzyme, with different durations of incubation, they found an optimum time of incubation with trypsin which produced good respiratory control independent of added free magnesium. They argued that the reason for the magnesium-induced stimulation of oxygen consumption and the decrease in RCI was the presence of a contaminating ATPase on the outside of the inner mitochondrial membrane, which responded to added magnesium. They also showed that the mitochondrial magnesium content was not affected by the various procedures. The use of a proteolytic enzyme for an optimum period of time to rid the mitochondria of the contaminating ATPase may be an explanation for the magnesium effects, or lack of them, but the present experiments used a proteinase with an incubation period of 16 minutes, which is close to the authors' optimum time of 20-30 minutes. Perhaps 16 minutes is not long enough, but the mitochondria had good respiratory control.

Mitochondrial ion uptake is associated with oxygen consumption (Brierley, 1976) and mitochondrial swelling (Dow, 1970; Munn, 1974). In particular, potassium influx and efflux are associated with oxygen consumption and are intimately related to mitochondrial magnesium content (Jung *et al.*, 1977; Chavez *et al.*, 1977; Duszynski and Wojtczak, 1977; Shi *et al.*, 1980). Therefore, the *in vitro* addition of 100 mM KCl, a concentration approaching that of the mitochondrial concentration (Brierley, 1976), was given to assess whether any damage to the

mitochondrial membrane induced by a magnesium depletion (via chronic alcohol) would result in changes in oxygen consumption induced by in vitro potassium. Inferred in this, but not proven, is a change in either potassium permeability or a change in mitochondrial potassium concentration which would influence potassium fluxes and oxygen consumption. Unfortunately mitochondrial potassium content was not measured. Table 6 shows the effects of 100 mM KCl on mitochondrial respiration. Potassium did not affect state 3 respiration or ADP:O ratios, which indicates that it does not interfere with phosphorylation. However the RCI was reduced in all groups, and the state 4 respiration was increased. The mitochondria isolated from the hearts of the alcohol-fed rats showed significantly less stimulation of state 4 oxygen consumption than the controls, and the magnesium injections had no effect. Table 7 shows the change in state 4 oxygen consumption more clearly. Because the role of potassium in oxidative phosphorylation is not clear (A. Gomez-Puyou and M.T. de Gomez-Puyou, 1977) and because mitochondrial potassium levels were not measured, one can only speculate on the reasons for the lack of increase in the state 4 respiration in the alcohol fed rats. State 4 respiration is characterized by a higher ATP/ADP ratio (Munn, 1974), mitochondrial swelling and a state of higher energy (Dow, 1970). Munn describes the mitochondria in state 4 as swollen, or in the orthodox form, and refers to this as an "energized" state. When the potassium is added, an increase in oxygen consumption occurs in state 4. If one assumes that this is a reflection of potassium uptake, as it is known that ion uptake increases oxygen consumption (Brierley, 1976) and does so in proportion to the amount of ion uptake (Brierley, 1974), then the lack of increase in state 4 may be due to a lack of uptake of potassium ion in the mitochondria of alcohol-fed rats. No measurement of pH or phosphate was

made, however. Perhaps the mitochondria are not "energized" enough i.e. do not have enough ATP, or there is a defect in the electron transport system such as an enzyme defect which requires magnesium, or the mitochondria have lost potassium initially and there is now not enough available for a potassium-potassium exchange (Lehninger *et al.*, 1967). Obviously more work needs to be done in this area, but these are truly interesting results and lend support to the other evidence of early mitochondrial respiratory dysfunction induced by chronic alcohol ingestion. While the *in vivo* magnesium supplements repaired the decrease in RCI in the mitochondria of the alcohol fed rats, the supplements had no effect on the potassium-induced changes. This suggests that the potassium-induced changes may be independent of magnesium and relate directly to alcohol effects on the mitochondria such as a change in membrane permeability. The decreased magnesium content may be an independent consequence of the chronic alcohol ingestion. Tables 8 and 9 illustrate that magnesium and potassium actually do affect different parts of the respiratory cycle. Magnesium increases both state 3 and state 4 respiration, while decreasing RCI and ADP:O ratios, which indicates that it affects both oxidation and phosphorylation. Potassium has no effect on state 3 respiration or ADP:O ratios, which suggests it does not interfere with phosphorylation at least.

The only firm conclusions to be drawn from the mitochondrial respiration data are that chronic alcohol ingestion produces a decrease in the respiratory control index of cardiac mitochondria in rats which is reversible with magnesium supplements, and that there is a loss of the potassium-induced increase in state 4 respiration which is not affected by the magnesium supplements.

C. TISSUE WATER AND ELECTROLYTE ANALYSIS

To our knowledge there have been no previous reports of the effect of chronic alcohol ingestion on the distribution of water and electrolytes in cardiac tissue. Analysis of total myocardial tissue water by Regan et al. (1974) showed a loss of potassium and gain of sodium but changes in fluid compartments were not taken into account. Studies on body fluid compartments after chronic alcohol ingestion have shown an increase in total body water in dogs (Beard et al., 1965) and an increase in all fluid compartments except the intravascular compartment in man (Beard and Knott, 1968). MacSweeney (1975) showed no change in body fluid compartments or potassium concentrations in chronic alcoholics, but these patients were studied a minimum of two weeks after cessation of drinking, which may have been too late. Recently Pierson et al. (1977) showed no change in total body water in man after chronic alcohol ingestion, but there was an increase in the extracellular to intracellular space ratio measured by radio-labelled sulfate, which indicates a leakage of sulfate into cells. A similar leakage of sulfate into the cells was shown in the present experiments (see Figure 8). In addition, their studies in the chronic alcoholic rat model produced similar fluid shifts, which returned to normal after 10 weeks of abstinence from alcohol.

The data shown here (see Figures 9 and 10) are consistent with the whole body studies in that we have shown an increase in the apparent extracellular space as measured by the tracer method, and a return to normal with abstinence from alcohol. Although there is an apparent increase in the extracellular space when measured by the tracer, there is a real increase in intracellular water, with total tissue water

being little changed, indicating a shift of water into the cells (see Figure 12).

Because plasma electrolytes do not correlate well with tissue levels (Jones et al., 1969), it is difficult to interpret any changes in plasma electrolyte concentrations with respect to the pathogenesis of alcoholic cardiomyopathy. Chronic alcohol ingestion has produced no change in plasma electrolytes in humans (Beard and Knott, 1968; MacSweeney, 1975) and dogs (Beard et al., 1965) as well as a decrease in sodium and potassium in humans (Barlow and Wooten, 1959). The most consistent change has been a decrease in magnesium (Jones et al., 1969; Sullivan et al., 1969; Mendelson et al., 1969) but this was not evident in our experiments (see Table 10).

There have been very few studies of the effect of chronic alcohol ingestion on the movement of electrolytes in and out of cells of any tissue. Jones et al. (1969) obtained deltoid muscle biopsies of chronic alcoholics and showed a decrease in total tissue magnesium, potassium, phosphorous and sodium, with no change in calcium. The decrease in sodium is at odds with the data shown here (see Table 11) and does not agree with the concept of increased permeability of the cell membrane. Lateral thigh muscle biopsies from chronic alcoholics (Anderson et al., 1980) revealed a decrease in phosphorous, magnesium and a small but nonsignificant decrease in potassium, while sodium and calcium were elevated. However, both the above studies made no distinction between intracellular and interstitial compartments. Total body analysis by Pierson et al. (1977) showed a shift of potassium out of cells and of sodium into cells in man and rats after chronic alcohol ingestion and these shifts were reversed with abstinence in the rats. The results of

our experiments are in agreement with the few studies in the literature and support the concept of increased sarcolemmal permeability with the attendant fluid and electrolyte shifts (see Table 11).

The movement of water, calcium and sodium into the cell and the movement of potassium, to a lesser extent, out of the cell provides an explanation for many of the observations made in alcoholic cardiomyopathy. The end-stage structural changes found in this disease are likely due to multiple factors, but certainly increased intracellular water might cause dilated T tubules, swollen mitochondria and sarcoplasmic reticula, and ruptured myofibrils, as described by several authors (Hibbs et al., 1965; Burch et al., 1971; Bulloch et al., 1972; Segel et al., 1975; Alexander et al., 1977b).

An increase in intracellular calcium, if allowed to enter the mitochondria in sufficient amounts, can cause swollen, defective mitochondria with a subsequent decrease in energy production (Dhalla, 1978; Sordahl, 1979). Decreased left ventricular function (Wendt et al., 1965; Regan et al., 1969) is one of many consequences. The increase in intracellular calcium produced in these experiments is from 0.3 mM to 0.7 mM, which is a measure of total intracellular calcium and does not consider the aspect of intracellular distribution. Some may be bound to the interior part of the sarcolemma, or bound to the sarcoplasmic reticulum, or other organelles. It is not known how much is actually free calcium, which is the type of calcium that the mitochondria would be taking up. The amount of free calcium in the cell normally ranges from $10^{-7}$ to $10^{-5}$ M (Langer, 1971; Endo, 1977) during relaxation and contraction phases, which is a range of 0.0001 mM to 0.01 mM. Therefore an increase of 0.4 mM would be significant, but how much is actually free is not known.

The reasons why an elevated calcium level might not be seen in the isolated mitochondria has already been discussed. However, dogs fed alcohol for 6 months showed a decrease (Bing et al., 1974) and dogs fed alcohol for 29 months (Sarma et al., 1976) showed no change in endogenous mitochondrial calcium content. Nevertheless, sequestration of calcium in other organelles can have deleterious effects (Dhalla, 1978).

Increases in intracellular sodium and decreases in potassium, with significant effects on resting membrane and action potentials, might explain the arrhythmias found in alcoholics (Leier et al., 1974; Ettinger et al., 1976, 1978; Luca, 1979). Banerjee and Sharma (1978) demonstrated an increase in Na-K-ATPase activity in cat myocardium after chronic alcohol ingestion. The changes in intracellular sodium and to a lesser extent potassium which are present in our results may be causing a compensatory stimulation of the enzyme (Dhalla, 1978).

In view of the known association of alcoholism and magnesium deficiency states (Jones et al., 1969; Sullivan et al., 1969; Mendelson et al., 1969; Belknap et al., 1978; Anderson et al., 1980), the resemblance of magnesium deficiency cardiomyopathy (Heggtveit et al., 1964; Heggtveit, 1965) to alcoholic cardiomyopathy and the reversal of EKG changes in alcoholic cardiomyopathy with magnesium supplements (Seelig, 1969; Luonmaki et al., 1975), it is surprising that there was no significant loss of intracellular magnesium in the present experiments, although a slight decrease was noted. As most of the intracellular magnesium in heart muscle is bound to protein or sequestered in organelles (Polimeni and Page, 1973) a small but significant amount might be lost or redistributed, with adverse effects on specific enzyme systems or membranes. The decrease of approximately 7 nanomoles per milligram of

protein in the mitochondria of the alcohol-fed rats would not be appreciated in a measurement of total intracellular magnesium, as only 12% of the magnesium is associated with the mitochondria (Polimeni and Page, 1974).

The data from the tissue water and electrolyte analysis can be summarized in this way. Chronic alcohol ingestion in the rat produced an increase in sarcolemmal permeability which resulted in a shift of water into the cells and a movement of ions down their electrochemical gradients and the entire process was reversed with abstinence from alcohol.

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