EFFECTS OF CHRONIC ALCOHOLISM

ON POLYADENYLATION IN

MOUSE LIVER

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PREFACE

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CHAPTER I

INTRODUCTION

The polyadenylation of mRNA precursors in eukaryotes
occurs by rapid addition of 150-250 AMP residues, possibly
by a chromatin-bound poly(A) polymerase (Sheiness et al.,
1975; Darnell et al., 1973; Rose and Jacob, 1980). En route
to the cytoplasm, a slow poly(A) extension occurs which may
be catalyzed by a soluble nuclear enzyme or one associated
with chromatin. Once in the cytoplasm, the poly(A) chain on
the mRNA is reduced in size (Sheiness et al., 1973). Con-
comitantly, additional AMP residues are added to the 3' OH
terminus (Diez and Brawerman, 1974). Thus, the length of
poly(A) on mRNA at any particular time represents a balance
between synthesis and degradation.

Previous experiments suggest that chronic alcoholism
may affect the length of the poly(A) tract (Bantle et al.,
1980). Changes in tract sizes may affect the processing of
poly(A^+)mRNA from nuclear RNA or the stability of the mRNA
which is in the cytoplasm (Brawerman, 1981).

This change may affect the stability of liver mRNA and
perhaps account for part of the reported decrease in protein
synthesis (Banks et al., 1970; Moreland and Smith-Keilland,
1979). The goal of this research was to measure the number

average poly(A) tract size of poly(A$^+$)mRNA by high resolution polyacrylamide gel electrophoresis. This would establish whether chronic alcohol treatment caused poly(A) tract size differences (Bantle et al., 1980). The RNA was extracted from the livers of mice that have been alcohol fed for three months as well as control mice fed isocaloric diets.

CHAPTER II

LITERATURE REVIEW

The Effects of Alcohol

<u>Alcoholism</u> <u>and</u> <u>Hepatic</u> <u>Disease</u>

Chronic alcohol consumption leads to steatosis, alco-
holic hepatitis and cirrhosis (French, 1976; Rubin, and
Lieber, 1967). The pathogenesis of these disorders at the
biochemical level is still unknown. Since the synthesis of
such proteins as albumin and fibrinogen is an important func-
tion of the liver, a change in their synthesis may serve as
an indication of alcoholic liver disease (Princen et al.,
1981). Although the effects of ethanol on hepatic protein
metabolism have been studied extensively, considerable con-
troversy exists as to the mechanism by which alcohol exerts
its influence on the metabolism of liver protein. There are
some indications that the endoplasmic reticulum network with-
in hepatic cells may be the initial target of alcohol follow-
ing its ingestion (Murty et al., 1980; Oratz and Rothchild,
1975). Since synthesis of albumin and fibrinogen occurs on
ribosomes attached to the rough endoplasmic reticulum
(Jeejeebhoy et al., 1975), changes in the structure of the
endoplasmic reticulum are likely to be associated with

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alterations in protein synthesis and in RNA metabolism. Understanding hepatotoxic effects of alcohol on RNA metabolism, the structure of endoplasmic reticulum, and the synthesis of plasma proteins may lead to the prevention of alcohol induced hepatic injury.

The cause of hepatic disease has been much debated. Opposing viewpoints suggest that it is due to either nutritional deficiency caused by ingestion of alcohol rather than foods containing proper nutrients or to a direct hepatotoxic effect of alcohol. Ethanol is metabolized in the liver by alcohol dehydrogenase (ADH) located in the cytosol. This metabolic process leads to the production of NADH and acetaldehyde (Isselbacher and Greenberger, 1964). The mechanism of how these metabolites may affect RNA or protein synthesis is unclear. Nonetheless, when 4-methylpyrazole (an inhibitor of ADH) is added to liver cells, the inhibitory effect of low concentrations of ethanol on amino acid incorporation into hepatic proteins was abolished (Moreland et al., 1980). This did not occur with higher alcohol concentrations (over 20 mM) suggesting that higher concentrations of alcohol exert their inhibitory effects by a mechanism independent of alcohol metabolism by ADH (Murty et al., 1980).

Many of the investigations on the effect of alcohol on protein and RNA synthesis (Banks et al., 1970; Moreland and Bessesen, 1977) have been performed by measuring the incorporation of ^{14}C and ^{3}H-amino acids into plasma and hepatic protein. Results (which will be covered later in

detail) are variable but generally favor the conclusion that alcohol deleteriously affects protein synthesis.

The Effects of Experimental Variables
on Liver Protein Synthesis

Conflicting results concerning the alcohol-induced changes in protein synthesis include those which report a decrease in synthetic rate in vivo (Banks et al., 1970; Moreland and Smith-Kielland, 1979) and in vitro (Moreland, 1974; Moreland and Bessesen, 1977; Rawat, 1976) as well as some studies which found little change in the rate of synthesis (Jarlested, 1972; Moreland and Smith-Kielland, 1979). These studies used a variety of diets, alcohol dosages, and strains of animals which may account for conflicting results. Variations in diets such as adding 5% w/v sucrose to a diet previously consisting of 10% v/v ethanol and laboratory chow resulted in fatty liver and regenerative nodules typical of cirrhosis (Tewari et al., 1975; Feinman and Lieber, 1973). The extra "empty" calories provided by sucrose in this diet lead to a decrease in consumption of the laboratory chow which supplies essential nutrients. In the past, considerable emphasis has been placed on impairment of nutrition as a major factor in liver disease affecting alcoholic human subjects. Many patients afflicted with alcoholism suffer from poor nutrition, and often their consumption of the important food items was inversely related to the magnitude of their alcohol intake

(Isselbacher and Greenberger, 1964). Studies by Lieber and Barona (1970) and Anderson et al. (1980) examined several diets on which laboratory animals could be maintained without causing changes in liver morphology. These diets insured that any changes in protein synthesis were attributed solely to alcohol treatment and not to alter cell populations.

The length of alcohol administration has also differed in the studies cited. Some investigators measured changes in protein synthesis after a single alcohol feeding (Moreland et al., 1980; Jeejeebhoy et al., 1975) while others studied chronic alcohol ingestion (Kuriyama, 1971; Jarlested, 1971). Both cases can be linked to human consumption in that the acute or "binge" drinker consumes a great deal of alcohol in a short period of time whereas a chronic alcoholic consistently drinks large amounts of alcohol. The results of these studies will be discussed in greater detail in the following sections.

Effects of Acute Alcohol Treatment
on Protein Synthesis In Vitro

Rat liver hepatocytes suspended in a synthetic medium in the presence or absence of ethanol were used to determine the effects of alcohol on protein synthesis (Moreland and Bessensen, 1977). This system was chosen because the authors felt that a long-term in vivo experiment investigating the interaction between alcohol and liver protein metabolism would give results which were difficult to

interpret based on nutritional imbalances caused by the inclusion of alcohol in the diet and effects mediated through hormones or other extrahepatic factors. Hepatocytes were therefore incubated at 37°C in the presence or absence of 50 mM ethanol. The incorporation of L-(U-^{14}C)-valine and L-(3,5-^{3}H$_2$)tryosine into both stationary cell protein and proteins released to the medium was reduced by ethanol. Later studies (Moreland et al., 1980) showed a dose-dependent reduction in ^{14}C-valine incorporation into medium and cell protein when an increasing amount of ethanol (10-105 mM) was added to suspensions. Since the effect of ethanol could also be repeated with 5 mM valine precursor concentration in the medium as well as physiological amino acid concentrations, the effects of ethanol on valine incorporation were thought not to be mediated through effects on valine pools, but on protein synthesis.

There is a positive correlation between the degree of depression of albumin synthesis and the concentration of ethanol in hepatocytes suspended in Hams' F10 medium containing 17.5% heat-inactivated horse serum (Jeejeebhoy et al., 1975). The synthesis of fibrinogens, a rapidly turning over protein was significantly depressed compared to albumin.

Albumin synthesis was also found to be lowered in studies using perfused livers (Rothchild et al., 1971; Chambers and Piccirillo, 1973). In the perfused liver, the acute administration of ethanol reduced the incorporation of labeled amino acids into circulating proteins (Jeejeebhoy et

al., 1975; Oratz and Rothchild, 1975) while the effect on stationary proteins was less pronounced (Moreland, 1975; Seakins and Robinson, 1964; Mookerjea, and Chow, 1969). The suggestion was made that perhaps some specific mechanism may be operating at a higher level of cellular organization which protects parenchymal liver cells against the more direct effect of ethanol on protein synthesis. In vivo studies also support the general conclusion that acute ethanol treatment reduces the uptake of certain amino acids into liver cell protein (Moreland, 1975) .

Effects of Acute Alcohol
Treatment in Vivo

Earlier investigators (Seakins and Robinson, 1964; observed that acute administration of ethanol had no effect on plasma and liver protein synthesis. On the other hand, later studies demonstrated that a single administration of ethanol in vivo caused a decrease in protein synthesis as measured in vitro using isolated liver ribosomes (Kuriyama et al., 1971), and specifically inhibited the hepatic production of exportable proteins, such as albumin and transferrin (Rothchild et al., 1974). Although the mechanism whereby alcohol or its metabolites produces hepatic injury is not understood, investigators have suggested the involvement of damage to the endoplasmic reticulum (Murty et al., 1980; Princen et al.,1981). An important function of the endoplasmic reticulum of the hepatocyte is the synthesis of plasma proteins which occurs

on ribosomes attached to endoplasmic reticulum (Jeejeehboy,
1975). Therefore, a hepatotoxic effect of alcohol on the
endoplasmic reticulum should result in a decrease in such
plasma proteins as albumin and fibrinogen (Rothchild et al.,
1974; Jeejeebhoy, 1975; Nadkarni, 1974). Electron micro-
graphs have shown loss of hepatic rough endoplasmic reti-
culum in alcohol-treated rats (Jeejeebhoy, 1975). The
effects of alcohol on the membranes of the endoplasmic reti-
culum were investigated by using radioactive choline and
measuring its incorporation into hepatic microsomal mem-
branes (Murty et al., 1980). It had been previously shown
(Nagley and Hallinan, 1968) that when radioactive choline is
administered in vivo, it becomes incorporated in rat
liver selectively into lecithin, the major constituent of
the microsomal membrane and therefore is a useful tool for
studying membranes. Ethanol (0.75 g/100 g body weight)
reduced the incorporation of ^{14}C-choline into total liver
microsomes by 43% and into membranes of the rough endo-
plasmic reticulum by 35% in comparison with control values.
Further membrane alterations were demonstrated by a decrease
in ^{125}I incorporation into proteins by microsomal mem-
branes. These investigations also showed that acute dosages
of ethanol inhibited the in vivo incorporation of ^{14}C-
leucine and ^{14}C-phenylalanine into liver protein and
plasma albumin and globulin. Further experiments (Murty et.
al., 1980) showed that acute alcohol administration reduced
the in vitro protein synthetic activity of hepatic

membrane-bound polyribosomes while free polyribosomes were relatively unaffected. It is still not clear whether the inhibitory effect of ethanol on protein synthesis via membrane-bound polyribosomes is due to a direct effect of ethanol on the ribosomes or to indirect effects of ethanol on the membranes or to cytosolic factors (elongation factor, tRNA, amino acid concentrations, etc.).

Rats given 4-8 g ethanol per kg body weight, 3-5 h before death were found to contain the same amount of equal sized free and membrane-bound polyribosomes (Princen et al., 1981). The ability to synthesize albumin and total protein were also equal for membrane-bound polyribosomes from both alcohol-fed and control rats. The albumin mRNA content was not changed in free and membrane-bound polyribosomal RNA fractions of ethanol-treated rats as compared to control rats. The cell sap prepared from rats treated with ethanol had a stimulating effect in cell-free protein synthesis. This again suggested that the cell sap of ethanol treated rats contains a factor or factors that stimulate in vitro translation of albumin mRNA proportional to the stimulation of total protein synthesis.

The discrepancies between these findings may be attributed to differences in experimental design. Most of the above studies were conducted on "fasted" rats or rats taken off their respective diets 14-24 hr prior to death or to ethanol administration. For those rats who were taken off all alcohol for 24 hr, some changes due to withdrawal may

have occurred. Other experimental protocols which involved removing the rats from all food prior to alcohol treatment obviously created a different nutritional status for the animal than those protocols that allowed the animal to be fed up to the time of death. Studies (Moreland and Smith-Kielland, 1979) have shown that no changes in protein, RNA or DNA content were found in non-fasted or "fed" rats while fasted rats showed a reduced liver RNA concentration along with a reduced RNA/DNA ratio in the cerebrum. Similar results were seen on the effects of ethanol on cerebral tissue in fed and fasted rats (Tewari and Nobel, 1977). Data showed that ethanol abstinence for 24 hr in physically dependent rats (14 days incubation with 10% v/v ethanol) resulted in a substantial inhibition of protein synthesis. They also found that there was complete recovery of protein synthetic capacity after 7 days withdrawal indicating that the observed withdrawal effects are temporary in nature. Rates of protein synthesis were based on ^{14}C-phenylalanine incorporation. These results suggest that ethanol with-drawal from physically dependent rats aggravates the exist-ing adverse effects of prolonged ethanol administration on protein synthesis. Therefore, the following review of chronic ethanol studies will be divided into those experi-ments involving "fasted" animals and those involving "fed" animals.

Effects of Chronic Alcohol

Treatment Using "Fasted"

Animals

Ethanol concentrations ranging from 10-15% v/v general-
ly caused an overall reduction in protein synthesis. After
a 2 week treatment period, an increase in ^{14}C-leucine
incorporation into hepatic proteins was reported in alcohol-
treated rats over controls (Kuriyama, et. al., 1971). Rats
fed for 4 weeks (Moreland, 1981) gave conflicting results in
that incorporation of labeled amino acids into proteins was
reduced in alcohol-fed rats to 55% that of control. In
earlier studies (Moreland, 1974), male rats on a 32% v/v
ethanol diet (controls given isocaloric sucrose) for 38 days
exhibited a tryptophan oxygenase activity that was 60% of
control activity. Additional studies (Jarlestedt, 1972;
Freinkel et al., 1965; Chambers et al., 1966; Albertini et
al., 1968) also reported depressed uptake of labeled amino
acids after chronic ingestion of 15-20% v/v ethanol.

Effects of Chronic Alcohol

Treatment Using "Fed" Animals

Rats given 5.7% v/v ethanol which corresponded to 40%
of the caloric content of the experimental diet were pair
fed with rats on a control diet made isocaloric by the
addition of sucrose to the experimental diet (Banks et al.,
1970). The control rats gained weight steadily over the 28
days of the experiment whereas the alcohol fed animals

stayed approximately the same size. Similar weight dif-
ferences were noted for alcohol-treated "fasted" rats
(Moreland and Bessesen, 1977; Moreland, 1981). There was a
40% reduction in the incorporation of ^{14}C-leucine into
protein in alcohol-treated rats as compared to control rats.

The above results (Banks et. al., 1975) also paralleled
other experiments on organs other than liver in regard to
de creased protein synthesis. Protein synthesis in kidney
experienced a similar decline while experiments on protein
synthesis in skeletal muscle showed no changes after 28 days
on a 5.7% v/v ethanol diet.

Extensive studies on the effects of alcohol on brain
protein synthesis (Tewari and Noble, 1975; 1977) also showed
a decrease in amino acid incorporation (^{14}C-leucine) in
C57BL/6J mice fed alcohol for 14 days. Their earlier
studies reported that brain ribosomes were resistant to dis-
sociation into subunits and chronic ethanol ingestion pro-
duced functional alterations in the properties of both
subunits resulting in the reduction of ribosomal biological
activity by affecting the reassociation process. The incor-
poration of amino acids was not limited to just leucine,
similar results were seen when ^{14}C-phenylalanine was used
as a precursor.

Other instances of alcohol affecting protein synthesis
were reflected in changes in testicular morphology and
decreased testosterone production in mice kept on a 5% v/v
alcohol diet for 34 days (Anderson et al., 1980).

Additionally, decreased total cardiac proteins were seen in fetal and neonatal hearts taken from mice suckling from alcohol-fed mothers (Rawat, 1979). When adult female rats were fed a liquid Metrical® diet with 6% v/v ethanol while the controls were given isocaloric sucrose, the offspring exhibited a 30% decrease in ^{14}C-leucine incorporation in cerebral proteins (Rawat et al., 1975). Although alcohol is not metabolized by the heart, acetaldehyde is and has been observed in the past to result in other metabolic derangements such as cardiac acceleration and septal defects (Chidsey et al., 1966). As with the chronic studies on the liver, the authors also point out certain changes in organ and body size with alcohol ingestion. The possibility of other factors such as diet and hormone interaction affecting those results was also suggested.

Other Variables Involved
In Alcohol Treatment

Moreland (1981) recently determined the effect of stress on protein synthesis in alcohol-treated rats. The results indicated that the stress of being injected was sufficient to influence the outcome of an experiment. The livers of injected rats showed a slightly increased amount of protein synthesis over the rats that were not handled. The untouched rats showed a lower level of liver protein synthesis over controls.

The possibility of catecholamines changing the rate of

protein synthesis was investigated by determining plasma
corticosterone levels. Plasma levels of corticosterone were
found to be higher in chronic alcohol-treated fed rats
(Kuriyama et al., 1971) and fasted rats (Moreland, 1974).
This hormone mobilizes fatty acids and enhances albumin
synthesis with chronic administration but inhibits it with
acute doses (Cain et al., 1970; Jeejeebhoy et. al., 1975).
Another effect of corticosterone is a stimulation of RNA and
protein synthesis as well as causing the aggregation of
ribosomes of the endoplasmic reticulum (Diez and Brawerman,
1967; Cox and Mathias, 1969).

Concluding Remarks on the Effects
of Alcohol on Protein Synthesis

The question still remains as to the mechanism by which
alcohol exerts its effects on protein synthesis. Several
possibilities include:

1. Ethanol affects carrier systems for the amino acids
    thus limiting the amounts of certain important
    amino acids for protein synthesis (Moreland, 1974).

2. Ethanol affects the endoplasmic reticulum which
    forms hyaline as a breakdown product. (Jeejeebhoy
    et. al., 1975).

3. Ethanol affects mitochondrial size and metabolism
    as seen with increased oxidation of a-glycero-
    phosphate and ß-hydroxybutyrate (Kiessling and
    Pilstrom, 1967).

4. Ethanol causes an increase in endogenous hormones which affect protein metabolism (Moreland, 1981).

5. Ethanol cuts down ATP production thereby decreasing energy levels of the liver cell. This decreases protein synthesis which is an energy requiring process (Banks et al., 1970).

6. Ethanol decreases aminoacyl-tRNA synthetase synthesis which results in decreased tRNA available. (Fleming et al., 1975; Tewari and Noble, 1971).

7. Ethanol causes a decreased affinity of ribosomes for mRNA (Tewari and Noble, 1975).

8. Ethanol may affect the synthesis of mRNA or the transport and stability of mRNA thereby affecting protein synthesis.

Although many of these possibilities include the involvement of RNA, less work has been completed on the effect of chronic alcoholism on RNA synthesis.

The Effects of Alcohol on
Liver RNA Synthesis

Banks et al. (1970) were among the few to determine the effects of chronic alcoholism on liver RNA. They determined changes in RNA to DNA ratios and RNA to protein ratios. The amount of liver RNA to liver weight (mg/g) did not change but RNA/DNA ratio was decreased in alcohol-treated mice. Recent studies (Bantle et al., 1980) with alcohol-treated female mice showed a twofold increase in the amount of

$5-^3$H-Uridine label incorporated into mRNA in liver poly-
somes over controls. Poly(A^+)mRNA content was estimated
to be 1.2 times greater. The proportion of newly labeled
poly(A^-)mRNA declined in ethanol-treated mice compared to
controls. Suggestions that the increase in mRNA might
include an increase in the size of poly(A) tracts have led
to further investigations.

The Effects of Alcohol on
RNA Synthesis in Other Organs

Kidney and skeletal muscle showed decreased levels of
those protein synthesis similar to that observed in liver
(Banks, 1970). RNA content was reduced in the kidney after
alcohol consumption and no clear distinction could be made
between control and experimental animals in skeletal muscle.
Clear distinctions were found in brain (Tewari and Noble,
1975). After 10 weeks on a 10% v/v ethanol diet, C57BL/6J
mice showed a decrease in incorporation of $5-^3$H-orotic
acid into tRNA, rRNA and polysomal RNA. The nuclear effects
of alcohol showed an initial increase followed by a depres-
sion in the incorporation of precursor label.

Just as the above studies had to contend with the same
variables as those involving alcohol and protein synthesis,
there have been some interesting studies on the effects of
other drugs on RNA synthesis. Female rats receiving ethio-
nine (10 mg/g body weight) intraperitoneally were compared
to controls given the same amount of saline. The animals

were then fasted overnight and livers homogenized to obtain nuclear and cytoplasmic RNA polymerase. RNA polymerase activities were determined _in vitro_. Ethionine caused a 20% and 33% decrease in the levels of poly(A) polymerase activity in the nucleus and cytoplasm respectively. However, a rise in _in vivo_ polyadenylation was seen. The main reason for this phenomenon was that ethionine also induced polysomal disaggregation and the mRNA was released as mRNP particles which resulted in an increased ability for the mRNA to serve as a primer (Dennis and Kisilevsky, 1979). This lead to increased poly(A) polymerase activity _in vivo_. Since longer poly(A) tracts may confer stability and prolong mRNA half-life, ethionine could be responsible for extending mRNA half-life (See: section on poly(A) function). These results parallel those reported by Bantle et. al. (1980) where an increase in poly(A^+)mRNA in alcohol treated mice was seen. This result suggested an increase in the rate of polyadenylation.

Possible Mechanisms of Alcohol-
Induced Effects on RNA Synthesis

Polyadenylation has already been suggested as a means of increasing mRNA half life (Bantle et al., 1980; Wilson et al., 1978; Karpetsky et al., 1979). Transport of mRNA from the nucleus to the cytoplasm has also been proposed as a site for inhibition by alcohol (Tewari and Noble, 1975). It may also be possible that an alcohol induced selective

degradation of poly(A) tracts would still leave an increased amount of poly(A^+)mRNA but with shorter tracts. Selective addition of adenylate nucleotides to poly(A^-)mRNA by increased poly(A) polymerase activities under the influence of alcohol could also be possible. Alcohol induced hormonal effects on protein synthesis have been reported and some mechanisms involving corticosteroids could also be associated with RNA synthesis. Since there are so many possible areas for further experimentation, it was necessary to limit the investigation to the study of the ability of alcohol to affect polyadenylation in mouse liver. There are many procedures available for pursuing the isolation of poly(A) and determination of tract size.

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Poly(A) Tracts - Functions

and Characterization

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Isolation and Characterization
of Poly(A) Tracts

Poly(A) tracts in RNA may be detected by utilizing the ability of poly(A) to hybridize with polyuridylate (poly(U)) or polydeoxythymidylate (poly(dT)). A number of approaches have been developed for the detection and quantitation of poly(A) which depend on annealing radioactive poly(U) or poly(dT) to the poly(A) tract (Gillespie and Gillespie, 1971; Fan and Baltimore, 1973; Kaufman and Gross, 1974; Sawin et al., 1977). The amount of poly(dT) or poly(U)

hybridized to RNA is then a measure of the poly(A) content.
A similar technique involved the hybridization of oligo-
deoxythymidylate (oligo(dT)) to RNA fractions. The resulting
oligo(dT)-poly(A) hybrids formed were detected by monitoring
the amount of cDNA synthesis by exogenously added reverse
transcriptase (Tracy and Kohne, 1980). Another method
involved hybridizing iodinated poly(U) to RNA and then treat-
ing the preparation with bisulfite which deiodinated non-
hybridized poly(U) but not poly(U) hybridized to poly(A)
(Sawin et al., 1979). The above methods were also used to
size the poly(A)-containing RNA by first sedimenting the RNA
into sucrose gradients and then detecting the poly(A) in
each fraction by individually hybridizing aliquots with
radioactive poly(U) or with radioactive poly(dT) (Sawin et
al., 1977; Scholem et al., 1973).

Sensitivity ranges for the above methods were from 0.7
ng of poly(A) detected by a reverse transcriptase (Tracy and
Kohne, 1980) to 1-10 ng of various RNAs as assayed by
hybridization with radioactive poly(U) or poly (dT) (Fan and
Baltimore, 1975; Gillespie and Gillispie, 1971).

A room temperature hydroxylapatite (HA) method was
developed to detect and quantify poly(A)-containing RNA
after hybridization to radioactive poly(dT) (Tracy and
Kuhne, 1980). This method along with a S-1 nuclease anal-
ysis can also be used to detect as little as 10^{-12}g of
polyadenylated RNA hybridized to ^{3}H-poly(dT). The hydroxy-
lapatite extended the uses of radioactive homopolymers to

detect, quantify and characterize RNAs containing homo-
polymer tracts.

Some preliminary studies on the characterization of
poly(A) tract sizes have been reported for rat liver (Savage
et al., 1978). The number average nucleotide length of the
poly(A) segment at the 3' terminal end of polysomal mRNA was
determined to be 85 adenylate residues by electrophoresis in
2.5% acrylamide gels containing 0.5% agarose. This con-
stituted about 8% of the total polysomal poly(A^+)mRNA. In
a recent study (Bantle et al, 1980), mouse liver polysomal
mRNA poly(A) tracts were sized in alcoholic and control
mice. The poly(A) tracts were estimated to be 116 and 75
nucleotides respectively although this figure was arrived at
by determining the amount of poly(U) hybridized and assuming
it represented 8% of the total poly(A^+)mRNA.

If, indeed, alcohol affects the poly(A) portion of
mRNA, then the effects of this action may be widespread
based on the various functions for the poly(A) tract that
have been proposed.

Possible Functions of the
Poly(A) Tract

The existence of poly(A) tracts has prompted a great
deal of speculation relating to the precise function of the
3'-polyadenylate sequences. Several observations have been
made concerning poly(A) tails. Carlin (1978) examined the
relationship between size of the poly(A) tracts and

phylogeny in order to infer the function of poly(A). Poly(A) tracts increase as the evolutionary level of the organism increases. Bacteriophages contain the smallest poly(A) tracts (1-5 AMP units) while mammalian cells contain the largest. However, it is known that the lifetime of mRNA in various types of eukaryotic cells varies within broad limits. mRNA is very stable in specialized cells; the lifetime of hemoglobin mRNA is 2-3 days (Karpetsky et al., 1980) while the lifetime of silk fibroin mRNA is several years (Suzuki and Brown, 1972).

It has also been shown that the dimensions of poly(A) decrease with the age of the RNA molecule containing it (Karpetsky, 1979). Newly formed mRNA contains variable poly(A) distribution. This distribution may be significant in determining the half-lives of these mRNA's.

An increase in the s half-life of poly(A^+)mRNA may be due to the observation that poly(A) regions have been shown to inhibit ribonuclease degradation of RNA in vitro (Rosenberg et al., 1975). The Poly(A^-)mRNA's are indeed degraded more rapidly than their adenylated counterparts. Greater than 20 adenine residues seems to be necessary for stabilization.

The simplest mechanism that explains the protecting role of poly(A) against nucleases would be that a 3' exonuclease would be the major degrading enzyme, and that the poly(A) provides a barrier against the action of the enzyme (Brawerman, 1981a). The actual mechanism of degradation may

be more complex. Since there are mRNA species lacking poly(A) tracts (histone mRNA's), it has been suggested that some stabilizing structure occurs at the 3' end of the RNA. Changes in stability may then be explained by a modification of such a structure by an appropriate cellular signal (Brawerman, 1981b).

No evidence (Perry et al., 1972; Woodland and Wilt, 1980) has been found for changes in mRNA stability in Xenopus oocytes after shortening the poly(A) tail. In addition, the mRNAs of 2 cell types were compared: (1) terminally differentiated cells such as lens epithelium and (2) multipotent cells such as hepatocytes which are capable of responding to sudden changes in biochemical alterations. There was no evidence showing that two messenger RNA populations differed in their 3' terminal poly(A) tracts.

Still another possible function of the poly(A) (Bina et al., 1980; Brawerman, 1981b) tail involves the splicing of mRNA precursors. Studies (Hruby, 1978) indicate that processing of adenovirus transcripts does not occur until after the addition of poly(A). The in vitro studies indicate that RNA transcripts that are not polyadenylated are not processed but are rapidly degraded in the nucleus suggesting that polyadenylation is needed for splicing of hnRNA to mRNA. Because polyadenylation appears to precede nucleocytoplasmic transport of mRNA in many cases, the primary function of poly(A) is considered by others to be at the level of transport of mRNA from the nucleus to the

cytoplasm (Schwartz and Darnell, 1974; Adesnik and Darnell, 1972). Possibly, the transport function of poly(A) is a step function of homopolynucleotide chain length and is therefore lost only below a certain poly(A) segment size. This appears to contradict evidence indicating that not all polyadenylated RNA in the nucleus is transported to the cytoplasm (Levy and McCarthy, 1976). A number of other objections have been raised to this proposal including: (1) poly(A) appears in organisms (prokaryotes) or organelles (mitochondria) wherein nucleocytoplasmic transport does not occur (Nakazato et al., 1975; Edmonds and Kopp, 1970); (2) mRNA lacking poly(A) is transported into the cytoplasm (Karpetsky et al., 1979); (3) cytoplasmic addition of poly(A) to mRNA is possible (Diez and Brawerman, 1974).

Another possible function of the 3' terminal poly(A) sequence is in the binding of messenger RNA to various intracellular membranes (Alam and Shires, 1975; Cardelli et al., 1976). It has been determined (Lande et al., 1975) that almost half of the poly(A) remains on membranes when the endoplasmic reticulum is treated with pancreatic ribonuclease. This view is consistent with the observation that mRNA, present as a component of the membrane-polysome complex, is less sensitive to exogenous ribonuclease digestion than mRNA in free polysomes (Aronson, 1965; Blobel and Potter, 1967). It has been suggested that poly(A) tracts may play a role in the formation of membrane-bound polysomes (Branes and Pogo, 1975).

Many questions remain unanswered regarding the function of poly(A). Although the bulk of the roles for poly(A) center around mRNA integrity, other functions for this polypurine sequence may exist that derive from the structural peculiarities of the polymer. The existence, for example, of regions of order in the single-stranded form of poly(A) stable at neutral pH results in the possibility of aromatic residue intercalation and selection of poly(A) binding proteins (Karpetsky et al., 1981). The importance of such mechanisms within the cell as well as the function of poly(A) remains to be elucidated.

Rationale for Continuing with Poly Adenylation as a Target for Alcohol Interruption of Normal Protein Synthesis

The effects of alcohol on RNA synthesis have already been discussed and the fact that poly(A$^+$)mRNA content is increased with alcohol treatment while protein production is not leads to the question of why this change exists. Other studies (Kuriyama et al., 1971; Cain et al., 1970; Dennis and Kisilevsky, 1979) have shown that hormones and drugs can affect mRNA metabolic activities such as synthesis of mRNA and mRNA binding properties with nucleoproteins (as previously described). It therefore seems feasible that alcohol may exert its specialized effect on polyadenylation. This may be a change in poly(A) polymerase activity or changes in the

degradation rates of poly(A) tracts. If increased tract sizes are seen after alcohol ingestion, then degradation of poly(A) tracts may be inhibited by alcohol consumption. Likewise, if shorter poly(A) tracts are seen in alcohol-fed mice, degradation of poly(A) tracts may be stimulated possibly by overcoming the protection offered by proteins associated with mRNA (if alcohol is reducing their synthesis or affecting them directly). Increased polyadenylation may also be a mechanism which the cell uses to offset the detrimental effects of alcohol. Several possibilities exist as to the actual mechanisms involved in understanding this complex question. The starting point in understanding polyadenylation in mouse liver and its interaction with alcohol will be to determine the poly(A) tract sizes of alcohol-fed and control mouse liver mRNA.

CHAPTER III

MATERIALS AND METHODS

Animals

Female Swiss Webster ICR strain mice (Timco®, Houston, Texas) of approximately 22 g weight were divided into four groups consisting of seven mice each. Purina® lab chow ad libitum and water were fed to half the mice. The water contained 5% v/v alcohol for the experimental group while the control group had water only. The remaining two groups were fed a Carnation® Slender liquid diet with one group receiving Slender and sucrose (77 g/l) and the experimental group receiving Slender and 5% v/v alcohol (Anderson, et. al., 1979). The Carnation diet was vitamin supplemented and all Carnation-fed animals were pair fed to insure that the amounts of food and liquid consumed by the alcohol and control groups were similar. Animals were weighed every month throughout the 90 day feeding period.

The mice were placed on diets lacking alcohol 48 hours prior to the analysis of poly(A) tract size. Body weights and rectal temperatures were recorded at the time of alcohol withdrawal and at 4, 7 and 24 hrs later. Following withdrawal, mice then received regular alcohol feedings for 48 hrs prior to isolation of nuclei and polysomes.

Isolation of Polysomal RNA

All glassware used in this study was washed in triple distilled water and heat-treated for 3 hours at 350°C to destroy RNAse activity. The mice were killed by cervical dislocation and the livers were excised, weighed and placed in beakers containing 5 ml of 0.01 M KCl, 0.04 M NaCl, 0.075 M $MgCl_2$, 0.05 M NH_4Cl_2, 0.025 M Tris-HCl (pH 7.5), 0.5 mg/ml heparin, 1.5 mg/ml yeast RNA and 0.006 M 2-mercapto-ethanol which was designated Buffer A (Sala-Trepat et al., 1978). Blood samples were obtained by cardiac puncture. The livers were homogenized using a Wheaton® Dounce Tissue Grinder (A pestle) (Markson®, Houston, Texas) kept cold in an ice bath. The homogenates were poured into Corex® test tubes, and centrifuged using a Sorvall® HB-4 rotor at 15,000 x g_{ave}, 10 min, 4°C in a Sorvall® refrigerated centrifuge. The resulting postmitochondrial supernatants containing the polysomes were drawn off the crude nuclear pellet and transferred to Erlenmeyer flasks to which 50 mg heparin and 3.0 mls 10% (v/v) Triton X-100-sodium deoxy-cholate were added with continuous stirring. The super-natants were then transferred to polyallomer centrifuge tubes (1" x 3 1/4") containing a 5 ml pad of 1.0 M sucrose, 0.05 M $MgCl_2$ and 2 mg/ml heparin. The tubes were centri-fuged using a Beckman SW 27 rotor (large buckets) at 110,000 x g_{ave}, 90 minutes, 4°C in a Beckman® L5-50 ultracentri-fuge. Polysomes were rinsed with 15 ml of NETS buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA) (Perry et al., 1972) and

homogenized with a glass Wheaton® tissue grinder with type A pestle. Polysomes were disrupted by the addition of 0.10% w/v SDS. RNA extraction was completed by a phenol-chloroform method (Perry et al., 1972). The redistilled phenol (Bethesda Research Laboratories® or Mallinckrodt®) was liquified in NETS and chloroform was then added to form phenol-chloroform solution (1:1 ratio). To inhibit RNAse activity, chelate metal ions and bind oxidation products, 8-hydroxyquinoline was added. Centrifugation of the extraction mixture was at 8000 x g_{ave}, 5 min, room temperature using a Sorvall® refrigerated centrifuge. The extraction procedure was repeated and the aqueous phases after the second extraction were added to the first and precipitated in 2.5 volumes of ethanol. The samples were placed at -20°C overnight to precipitate the RNA.

Extraction of Nuclear RNA

The nuclear pellets obtained from initial centrifugation of liver homogenate were resuspended in 2.2 M sucrose (RNAse-free), 0.1 M KCl, 0.001 M $MgCl_2$, 0.01 M Na acetate, pH 6.0, 0.5 mg/ml heparin. The pellets were resuspended using the Wheaton® glass homogenizer (Type A pestle) and the homogenates were layered over 5 ml pads of the same sucrose buffer, lacking heparin in 1" x 3 1/2" Beckman cellulose-nitrate centrifuge tubes. The samples were centrifuged in a Beckman SW 27 or SW 28 rotor at 110,000 x g_{ave}, 90 min, 4°C. The pellets were washed and

resuspended in 0.05 M Na acetate, 0.001 M EDTA, pH 5.2 using the homogenizer. An equal volume of phenol (containing 0.1% w/v 8-hydroxyquinoline) which had been liquified in homogenization buffer was added and the extraction carried out at 60°C for 5 min. Following extraction, the phases were separated by centrifugation at 8000 x g_{ave}, 10 min, 0°C. The first aqueous phase was removed and saved on ice. A second volume of buffer was added to the phenol and a re-extraction was carried out at room temperature for 10 min. Following phase separation by centrifugation, the aqueous phases of the extraction tubes were pooled and the RNA was precipitated overnight by the addition of 2.5 volumes of 100% ethanol at -20°C.

Purification of the RNA

The samples were pelleted by centrifugation and the alcohol poured off each pellet. The pellet was dried until all the alcohol was removed. Each pellet was resuspended in 1.5 ml of NETS buffer and this solution was layered over a 2.5 ml pad consisting of 20% w/v sucrose, 0.1 M NaCl, 0.01 M Tris, 0.001 M EDTA. The samples were centrifuged in a Beckman SW 41 rotor at 186,000 x g_{ave}, 3 hr at 4°C. Insoluble materials such as glycogen present in the preparation were pelleted and the purified RNA remained in the supernatant which was again ETOH precipitated at -20°C.

Determination of RNA Degradation

by Sucrose Gradient

Centrifugation

RNA stored as ethanol precipitates was pelleted by
centrifugation, dried and resuspended in 50 µl of 0.5 M
NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4. Approximately
45 µg of each preparation was layered over 5-20% w/v
sucrose gradients containing 0.1 M NaCl, 0.01 M Tris-HCl,
0.001 M EDTA, pH 7.4. Gradients were centrifuged in a
Beckman SW-41 rotor at 29,000 rpm for 15 hours at 4°C and
fractionated using an ISCO® density gradient fractionator
to record absorbance continuously at 254 nm.

Oligo(dT)-Cellulose Chromatography

The samples were pelleted by centrifugation at 15,000 x
g_{ave}, 10 min, 4°C and the supernatants (ETOH) were poured
off and the tubes allowed to dry. One ml of 0.01 M NaCl,
0.01 M Tris, 0.001 M EDTA buffer (pH 7.4) was added to each
sample along with 0.2% w/v SDS. The samples were incubated
at 55°C for 2 min to disaggregate the RNA. The final salt
concentration was then brought to 0.5 M by the addition of
4.0 M NaCl. Poly(A$^+$)mRNA from each of the four samples,
labeled AW (alcohol chow), CW (control chow), AC (alcohol
Carnation Slender®), CC (control Carnation Slender®), was
purified using oligo(dT)-cellulose (Sigma®) chromatography
(Bantle et al., 1976). The original procedure of Bantle et
al. (1976) was modified to entail only the initial heat

treatment of the RNA in order to minimize RNA aggregation. modified procedure minimized loss of poly(A^+)mRNA. Each sample was added to the column in high salt (0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA pH 7.5) after extensive washing with high salt buffer. The mRNA was eluted from the column with low salt (0.01 M Tris, 0.001 M EDTA pH 7.5). The flow rate was adjusted to insure that the mRNA eluted from the column in less than a 2 ml volume. A sample of each tube was placed in 500 µl of the low salt buffer and transferred to a sterile quartz cuvette where the amount of mRNA for the four groups was quantified. Enough sRNA was then added to each sample to bring the final concentration of RNA to 80 µg which was then precipitated in 2.5 volumes ETOH overnight at -20°C.

Isolation of Poly(A) Tracts

The alcohol and control poly(A^+)mRNA samples were centrifuged in a Beckman HB-4 rotor at 2500 x g_{ave}, 10 min, 4°C. The supernatants were poured off and the pellets were resuspended in 1.0 ml of 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.5. RNAse A (8.0 µg/ml) and 5 units/ml RNAse T_1 were added to each sample as well as to poly(A) size standards (Miles®) and samples incubated for 45 min at 37°C. The size standards were used to determine the effect of RNAse on poly(A) tract size. To each sample, 0.2% w/v SDS and 250 µg/ml proteinase K were added followed by incubation at 37°C for 2 hr. The proteinase K was preincubated at room temperature for 1 hr prior to use. The

total volume of the incubation mixture was kept under 1.5
ml. A micro phenol-chloroform extraction was performed for
each of the four samples (Perry et al., 1972). The phenol
(Mallinckrodt®) was liquified in Buffer B or NETS and con-
tained 8-hydroxyquinoline. The aqueous phase after the
first extraction was added to that of the second extraction
and the RNA was precipitated in 2.5 volumes of 100% ethanol
and stored at -20°C. The samples were pelleted by centri-
fugation at 2500 x g_{ave}, 45 min, 4°C. An additional
sample consisting of a poly(A) size standard was also sub-
jected to the enzyme treatment described above to determine
the effect of the RNAse and proteinase K on poly(A) size.
Each pellet was resuspended in 0.5 M NaCl, 0.01 M Tris,
0.001 M EDTA made up in 30% w/v sucrose. These samples
along with poly(A) size standards (Miles®) and yeast tRNA
(Sigma®) were analyzed using three different types of gels,
the first being on 4% w/v polyacrylamide gels (4% w/v
analytical grade acrylamide, 0.1% w/v bis-acrylamide (Miles
Co., Elkhart, Indiana, 0.019% w/v ammonium persulfate, 0.05%
v/v TEMED). The gels were prescanned using a Gilford®
spectrophotometer equipped with a gel scanner and then
placed in a Buchner® Polyanalyst electrophoresis unit. The
gels were electrophoresed for 2 hr at 4.5 mA/gel in a buffer
consisting of 0.1% w/v SDS, 0.02 M Na acetate, 0.002 M EDTA,
0.04 M Tris, pH 7.5. Following electrophoresis, the gels
containing the poly(A) standards were again scanned and the
location of the poly(A) standards determined. Samples were

also analyzed using 2.5% w/v polyacrylamide gels containing 0.05% w/v agarose (Biorad®), (2.5% w/v analytical grade acrylamide, 0.16% w/v bis acrylamide, 0.08% w/v ammonium per-sulfate, 0.08% w/v TEMED). Gels were pre-electrophoresed for 1 hr at 6.5 mA/gel in a buffer composed of 0.02 M sodium acetate, 0.001 M EDTA, 0.04 M Tris-HCl. Samples were layered on 2 gels and run for 4 hrs at 6.5 mA/gel. The third system was 10% w/v acrylamide gel (10% w/v analytical grade acrylamide, 0.25% w/v bisacrylamide, 0.04% w/v ammon-ium persulfate, 0.08% TMED).

<div align="center">Determination of Poly(A) Tract Sizes</div>

Poly(U) Hybridization

Gels containing samples AC, CC, AW, CW, and A_{95} were sliced with BioRad® 190 gel slicer into 1 mm slices. Each tube containing three of the slices also contained 150 µl of 0.5 M NaCl, 0.01 M Tris and 0.001 M EDTA, pH 7.5. These were incubated at 37°C overnight to allow the poly(A) to leach out. The buffer was drawn off each sample and the slices washed with another 150 µl aliquot of buffer which was added to the first. The 300 µl sample was brought up to 1.0 ml volume by the addition of 700 µl of 0.12 M phos-phate buffer. The following controls were also set up; 1.0 µg samples of poly(A) only, 1.0 µg poly(A) + 0.2 µCi (525 µCi/nmole P) of $5-^{3}H$ poly(U) (Miles® Biochemicals, Elkhart, Ind.) and 0.2 µCi poly(U) only. The samples were incubated for 3 hr at 37°C. Following incubation 8 µg/ml

RNAse A was added to each sample to a final concentration of 80µg/ml and incubated for an additional 20 min, 30°C. The samples were then layered over hydroxylapatite (HAP) columns to which 9 mls of 0.12 M phosphate buffer pH 6.8 was slowly added. The 9 mls were collected and saved and the column was resuspended in 2 mls of distilled water and added to a scintillation vial containing 17 mls of Biocount® cocktail (RPI). Radioactivity in each vial was determined using a Beckman® 3133P Liquid Scintillation Spectometer.

Nitrocellulose Filtration

Hybrids prepared as previously described were isolated by filtration on 0.45 µm Millipore® nitrocellulose fil-ters. The samples were diluted with 700 µl of NETS buffer rather than the phosphate buffer used for the hydroxylapa-tite procedure. To precipitate the poly(A)-5-^{3}H-poly(U) hybrids, 200 µg yeast tRNA was added to each sample, mixed and precipitated in equal volume of 10% w/v TCA. These sam-ples remained at 4°C for 20 minutes. Each sample was slowly filtered through a nitrocellulose filter (0.45 µ) and washed with 9 one-ml aliquots of 5% w/v TCA followed by 2 one ml rinses with isopropyl alcohol. The filters were dried and added to scintillation vials containing 5 mls of cocktail (toluene containing 4 g/l PPO and 0.1 g/l POPOP). The filters were counted at 18% efficiency with a Beckman® 3133P Liquid Scintillation Counter.

CHAPTER IV

RESULTS

Mice were fed different diets containing 5-10% v/v
alcohol. Since the consumption (mls/mouse/day) was dif-
ferent for each diet, the results will be given in relation
to the diet given to each group of mice.

Experiment 1: Fifteen mice were divided into three
groups. Group one was placed on a water and Purina Lab Chow
diet, group two a 10% v/v alcohol-water solution and Chow
diet and group three a diet of 10% v/v alcohol-5% sucrose-
water solution and Purina Chow. These mice (Timco®, fe-
male) remained on these diets for a period of six months.
The daily consumption of their respective diets was suf-
ficient to maintain body weights (Table I). No significant
difference was seen in the amount of weight gained on their
respective diets, nor were there significant differences in
liver weights for animals in each group. The polysomal RNA
was extracted as described in the methods section and anal-
yzed for degradation using sucrose density gradient centri-
fugation. A sedimentation profile typical of an undegraded
RNA preparation (Figure 1) shows the separation of unde-
graded 4-5S, 18S and 28S ribosomal RNA peaks. One broad
peak toward the lighter end of the gradient would be

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TABLE I

THE EFFECT OF CHRONIC ALCOHOL-SUCROSE TREATMENT
ON MOUSE BODY AND ORGAN WEIGHT (EXP 1)

	Control Diet	/ Alcohol Diet 10%v/v alcohol	Alcohol-sucrose Diet
Animals/Treatment	5	5	5
Initial Body Weight (g)	-*	-	-
Final Body Weight (g)	3.8+2.16	34.36+1.45*	32.84+1.23
Ave. Food Consumption (ml/mouse/day)	-	-	-
Ave. Alc. Consumed (ml/mouse/day)	-	-	-
Liver Weight (g)	1.93	1.62	1.84

*- Data not available.

Figure 1. A Typical $A_{(254)}$ Scan of Undegraded
Polysomal RNA. The Different RNA
Fractions Were Separated on a 5%-20%
Sucrose Gradient
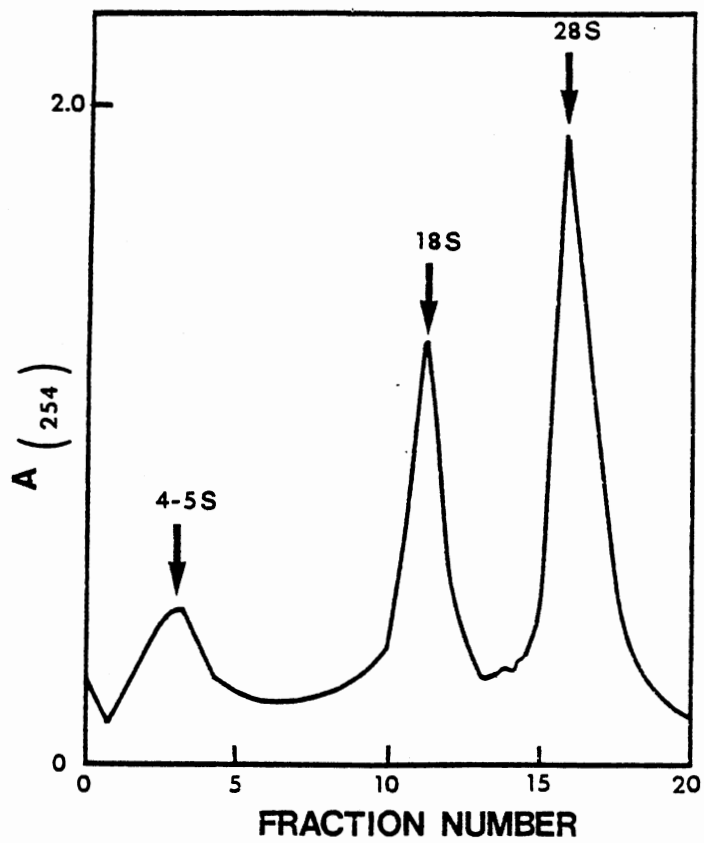
indicative of complete degradation. Since mRNA comprises
only 2-3% of the total polysomal RNA it is difficult to
detect alterations in the mRNA content of polysomes by com-
parison of absorbance scans of polysomal RNA gradients. The
yield of polysomal RNA/g liver weight (Table II) was 1.52
mg, 1.94 mg and 1.68 mg for control, alcohol and alcohol-
sucrose samples, respectively. Poly(A$^+$)mRNA was isolated
from the polysomal RNA by oligo (dT)-cellulose chromato-
graphy. Non-polyadenylated RNA was eluted from the
oligo(dT)-cellulose column using a high salt buffer (Figure
2) and the polyadenylated RNA was removed from the column in
low salt. Disaggregation of polysomal RNA by heat treatment
prior to application of the sample to the column was found
to improve the resolving power of this chromatographic tech-
nique. The amount of poly(A$^+$)mRNA/mg polysomal RNA was
quantitated spectrophotometrically for control, alcohol and
alcohol-sucrose samples (Table II). The accurate measure-
ment of the amount of poly(A$^+$) from the column by spectro-
photometry was not possible due to both low amounts of
poly(A$^+$)mRNA and the presence of oligo(dT)- cellulose
fines in the sample that contributed to absorbance. The
poly(A$^+$)mRNA could also contain variable amounts of aggre-
gated ribosomal RNA. Therefore, the poly(A$^+$)mRNA listed
gives only a general idea as to the amount present. The
260/280 ratios were 2.14 for controls, 2.09 for alcohol and
2.16 for the alcohol-sucrose sample, indicating RNA that was
free of protein for all samples. Poly(A$^+$)mRNA from each

TABLE II

CHARACTERIZATION OF RNA AND POLY(A) TRACTS
IN ALCOHOL, ALCOHOL-SUCROSE AND CONTROL
MICE (EXP 1)

	Control	Alcohol-Sucrose fed mice	Alcohol-fed mice
mg polysomal/RNA/g liver	1.52	1.68	1.94
μg poly(A^+)/mg polysomal RNA	10.93	4.65	9.20
μg poly(A^+)/mg polysomal RNA	0.24	0.29	0.24
260/280 ratios of poly(A)mRNA	2.14	2.16	2.09
Mass ave. poly(A) tract size	81.0	82.0	79.0
Number ave. poly(A) tract size	79.0	76.0	77.0

Figure 2. Isolation of Poly(A^+)mRNa by Oligo(dT)-
         Cellulose Chromatography. The Poly(A^+)
         mRNA was Eluted in the 0.02 m Tris
         Fraction
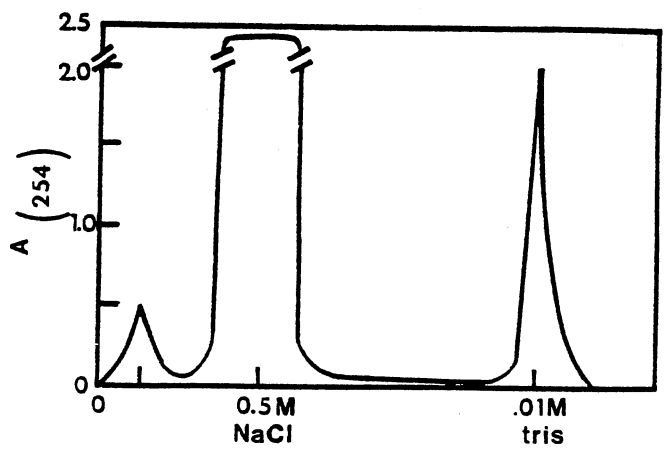
group was subjected to ribonuclease treatment as previously
described in Methods and the poly(A) tracts were isolated by
tract size using 10% w/v polyacrylamide gel electrophoresis.
Poly(A) standards of 74 and 95 nucleotides were used as mar-
kers. The gels were sliced and the poly(A) tracts in each
gel fraction(3 mm slices/fraction) subjected to $5-^3$H
poly(U) hybridization. Hybrids were isolated using hydroxy-
apatite columns. All HAP columns used in these experiments
bound greater than 95% double stranded molecules and less
than 3% single stranded molecules in 0.12 M phosphate
buffer. The plots of the distribution of $5-^3$H poly(U)
hybridized (Figure 3) for control, alcohol and alcohol-
sucrose fractions show a general range of poly(A) tract
sizes of 64 to 90 nucleotides with the majority of poly(A)
tracts between 80 and 90 nucleotides for the alcohol-sucrose
and control samples and 70 to 80 nucleotides for the alcohol
sample. Poly(A) tract sizes were determined using two dif-
ferent methods. First a Mass Average tract size was calcu-
lated by adding the cpm in each gel fraction to give a total
cpm for the entire gel. This total was divided in half and
starting with fraction one, each cpm fraction was added
until that number equaling half the total cpm of the gel was
reached. This fraction was correlated to the gel system.
The Mass Ave. tract size does not distinguish between cpm
obtained by one long hybrid or several short hybrids. It is
based soley on the ^{3}H-poly(U) bound to poly(A). There-
fore, the Number Ave. Nucleotide length was also used to

Figure 3. Polyacrylamide Gel Electrophoresis of
           Poly(A) Tracts Isolated From Poly(A)
           Containing Messenger RNA. Poly(A) Seg-
           ments Were Obtained by Nuclease Diges-
           tion of a Purified Messenger RNA Sample.
           The Gels (10% Polyacrylamide) Were Run
           at 7 mA/Tube for 5 Hrs. The Gels Were
           Sliced and 3 Adjacent 1 mm Slices Were
           Collected and the Poly(A) Eluted at 25°
           Overnight. Poly(A) Content was Determined
           by Hybridization with ^{3}H-poly(U).

The Arrows Give the Position of Poly(A) Standards of 95
and 74 Nucleotides Run on Separate Gels.
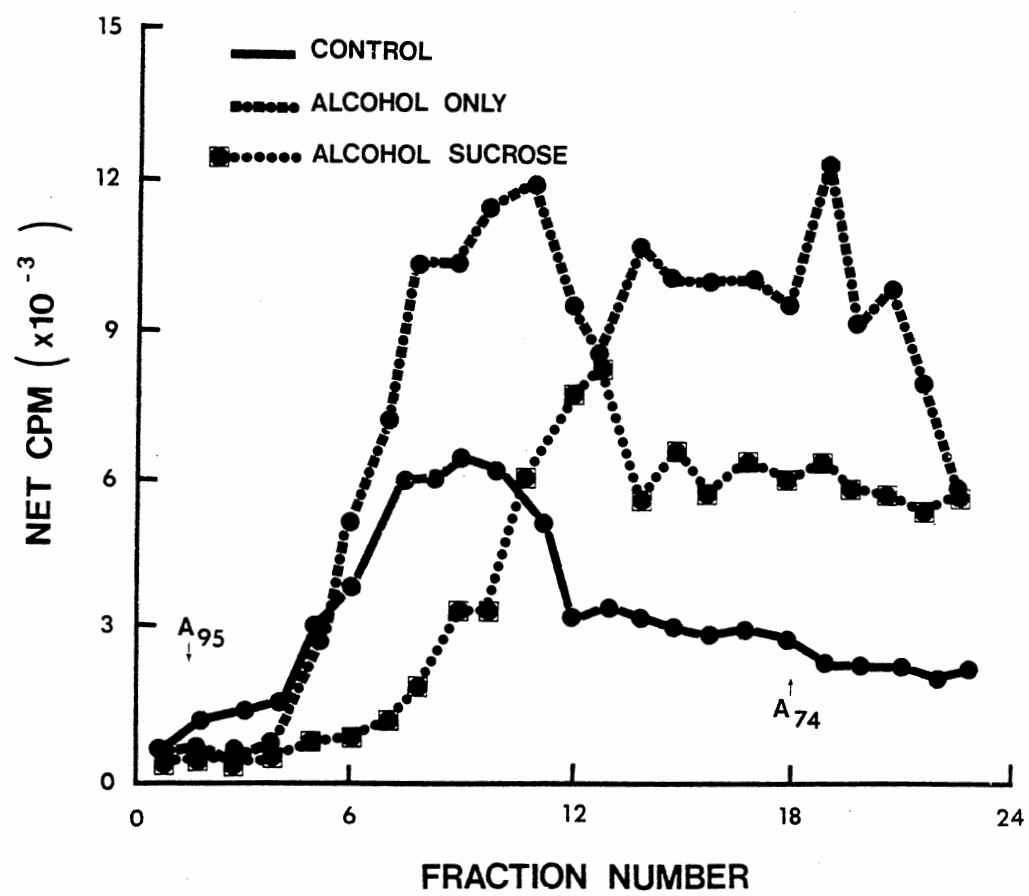
distinguish the size of hybrids containing ^{3}H-poly(U) which resulted in a certain cpm per fraction. The Number Ave. Nucleotide Length was calculated using the following formula:

$$\text{Number Ave. Nucleotide} = \frac{\Sigma_i (cpm_i)\, L_i}{\Sigma_i (cpm_i)}$$

where (cpm_i) is the radioactivity in fraction i and L_i is the nucleotide length of RNA sedimenting in fraction i. The mass average nucleotide lengths were calculated (Table II) as 79, 82 and 82 nucleotides for control, alcohol and alcohol-sucrose samples. The number average poly(A) tract sizes were similar being 79, 77 and 76 respectfully. The number of molecules per fraction (Figure 4) shows identical plots to that of the cpm/fraction. Increase in poly(A) quantities are seen in the alcohol fractions. The amount of poly(A)/mg polysomal RNA was determined for each sample and the control and alcohol-sucrose samples had similar concentrations of 0.28 µg and 0.29 µg/mg polysomal RNA while the alcohol sample had 0.48 µg which is 1.65 times more poly(A) than the other two samples. This suggests that perhaps even though the tract sizes are similar, the amount of poly(A) may be increased on the alcohol-water diet (Bantle et al., 1980).

Figure 4. Polyacrylamide Gel Electrophoresis of
          Poly(A) Segments Isolated From Poly(A)
          Containing Messenger RNA.

See Figure Legend 3 for Isolation Procedure.
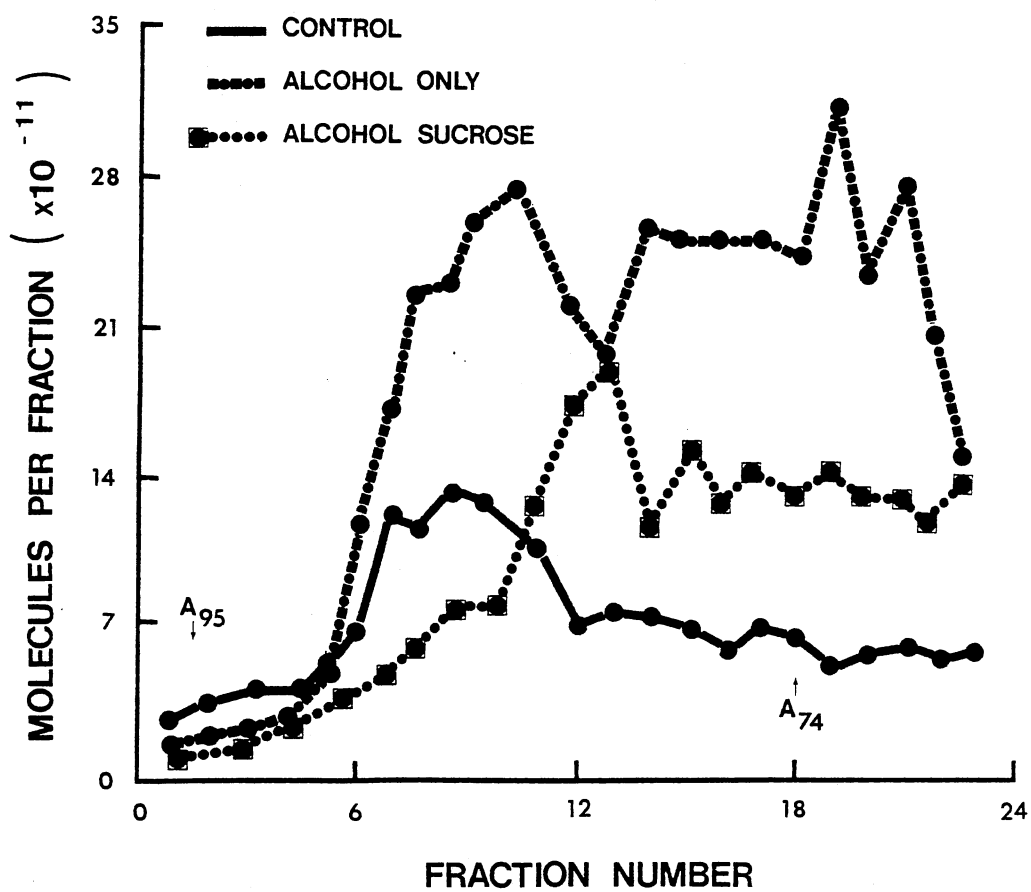
Experiment 2-Characterization of
RNA and poly(A)

Twenty-four mice were divided into groups of six. Two
of these groups were placed on a Purina® Chow diet ad
libitum with one receiving only water as a source of
liquid and the other a 5% v/v alcohol-water solution. The
remaining two groups were pair-fed Carnation Slender® and a
vitamin supplement. The group designated alcohol had 5% v/v
ethanol added to the Carnation Slender® and the Control
group of mice were given sucrose isocalorically. The amount
of alcohol consumed on these respective diets was 1.52
ml/mouse/day for the chow group and 2.51 ml/mouse/day for
the Carnation Slender® mice; or 6.3 g ethanol/kg body
weight for the Chow-fed mice and 11.3 g ethanol/kg body
weight for the Carnation fed mice (Table III). All four
groups of mice showed a weight gain for the 90 day study.
At about 30 days into the study the Carnation Control group
showed a slight decline in body weight (Figure 5) while the
alcohol group continued to gain weight but at a slower rate
than shown in the first month on the diet. The same pattern
is seen in the Chow-fed mice which showed a similar weight
gain for the first 30 days of the 90 day study and the
alcohol-fed mice maintained their weights for the remainder
of the study while the Control mice showed a slight decrease
in weight (Figure 6). This suggests that while the
Carnation Slender® diet served as a more effective vehicle
for alcohol ingestion, it did not allow continuous weight

TABLE III

THE EFFECTS OF CHRONIC ALCOHOL-SUCROSE TREATMENT
ON MOUSE BODY AND LIVER WEIGHT (EXP 2)

| | Chow-fed mice | | Carnation-fed mice | |
	Alcohol	Control	Alcohol	Control
Initial Body Weight (g)*	27.6+5.6	27.9+1.6	25.0+2.3	28.8+3.2
Final Body Weight (g)*	31.5+6.4	29.7+2.1	32.0+2.1	30.0+2.5
Ave. Alc. Consumed (ml/mouse/day)	1.52	-	2.51	-
Liver weight (g)	1.41	1.15	1.06	1.28

*Values are means $\pm$ the standard deviation. ** All livers in each group weighed together

Figure 5. Body Weights for the 90 Day Study. Carna-
            tion Control and Alcohol-fed Mice Were
            Weighed at Intervals of 0, 30 and 90 Days
            During the Study
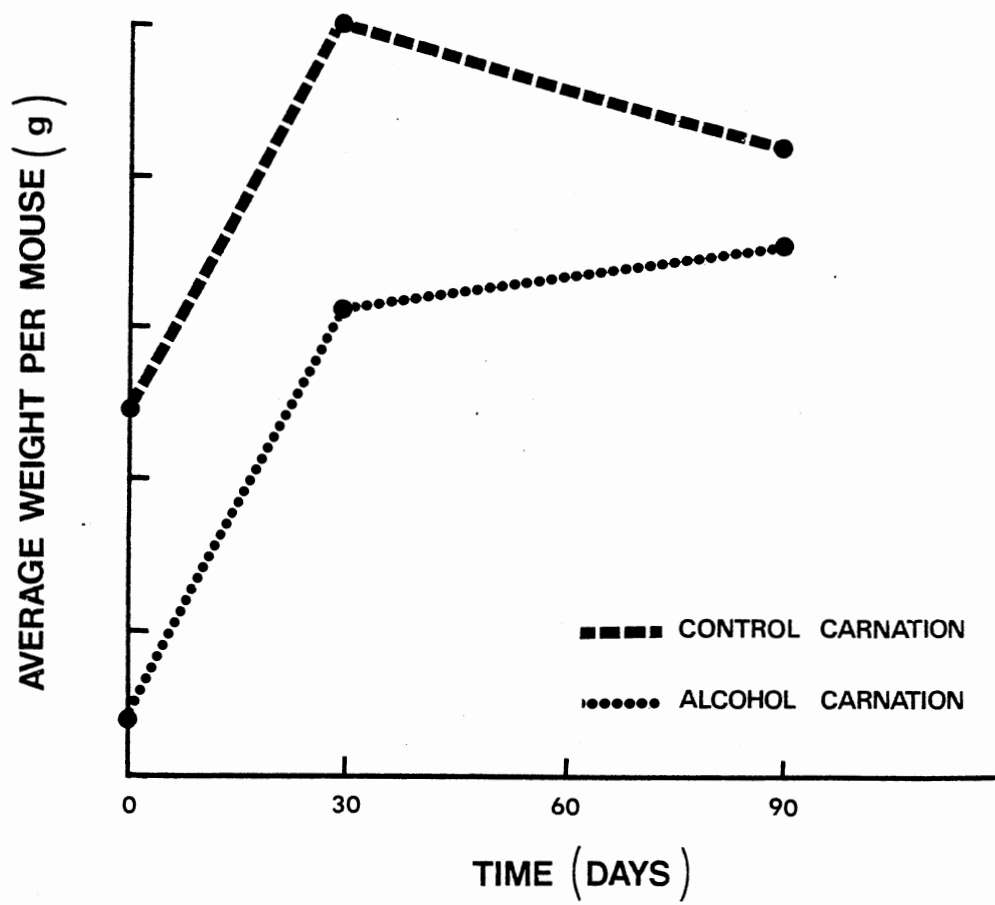
Figure 6.   Body Weights for the 90 Day Study. Chow-
            Fed and Alcohol-fed Mice Were Weighed at
            Intervals of 0, 30 and 90 Days
            During the Study
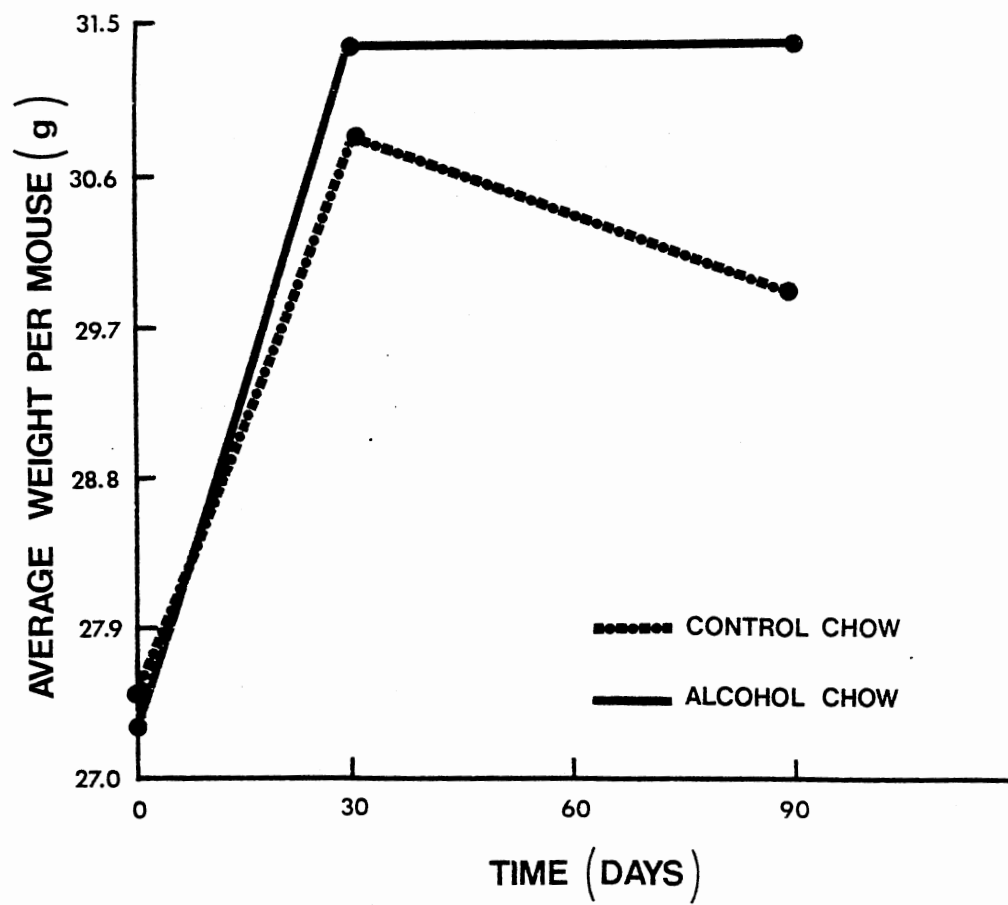
gain for control mice. Since livers from the mice were pooled rather than processed individually, it was not possible to compare yields of RNA or variations between procedures since one sample represented one type of diet. The quantity of polysomal RNA/g liver was 1.7 and 3.4 mg for Chow control and alcohol samples (Table IV). The Carnation samples showed 3.58 mg and 4.56 mg/given weight for control and alcohol preparations. These samples were subjected to oligo(dT)-cellulose chromatography and 6.49 µg and 4.19 µg of poly(A^+)mRNA/mg polysomal RNA were isolated for Carnation control and alcohol samples while larger amounts of 23.6 and 10.4 µg/mg polysomal RNA were isolated for Chow control and alcohol samples. The poly(A)mRNA from each sample was quantitated spectrophotometrically and the 260/280 ratios (Table IV) were indicative of pure RNA preparations. The four samples were then divided into two alliquots which were then run on different polyacrylamide gel systems to determine poly(A) tract sizes. The first samples following RNAse digestion, were layered on 10% polyacrylamide gels and electrophoresed for 4.5 hours at 6 mA/gel. The gels were sliced and the poly(A) eluted at 25°C overnight. The poly(A) from each gel fraction was then hybridized to 5-^{3}H poly(U) and the non-hybridized poly(U) digested away. The hybrids were isolated on HAP columns and the amount of radioactivity in each fraction was determined (Figures 7 and 8). From this information, the Mass Ave. poly(A) tract sizes were determined to be 82 and 87

TABLE IV

CHARACTERIZATION OF RNA AND POLY(A) TRACT IN
ALCOHOL AND CONTROL FED MICE (EXP 2)

| | Chow-fed mice | | Carnation-fed mice | |
	Alcohol	Control	Alcohol	Control
mg polysomal RNA/g liver	3.41	1.70	4.56	3.58
µg poly(A$^+$)mRNA/mg polysomal RNA	10.43	23.6	4.19	6.49
µg poly(A)/mg polysomal RNA	1.47	0.93	0.691	0.35
260/280 ratios of poly(A)mRNA	2.04	2.26	2.04	2.06
Mass Ave. poly(A) tract size	80	82	83	81
Number Ave. poly(A) tract size	86	84	83	83

Figure 7. Polyacrylamide Gel Electrophoresis of
           Poly(A) Segments Isolated From Poly(A)
           Containing Messenger RNA

Experimental Conditions Given in Figure Legend 3.

Figure 8.   Polyacrylamide Gel Electrophoresis of
            Poly(A) Tracts Isolated From Poly(A)
            Containing Messenger RNA

Experimental Conditions Given in Figure Legend 3
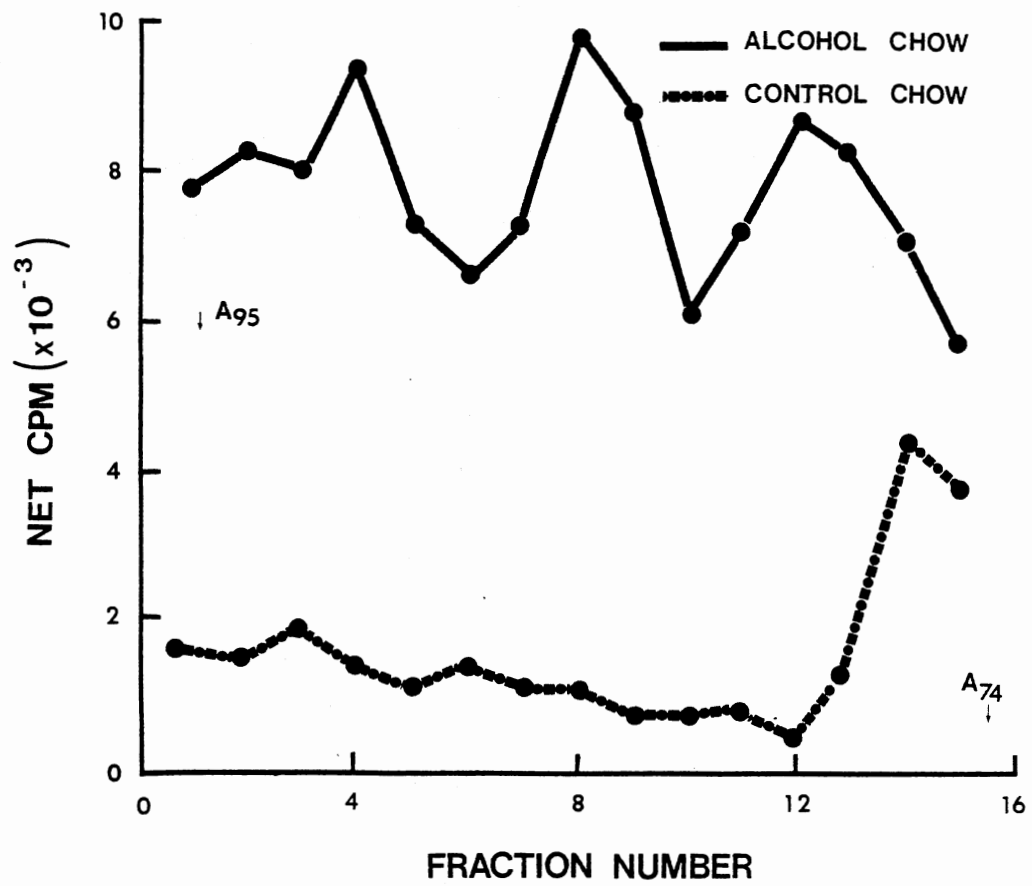
nucleotides for Chow control and alcohol and 83 nucleotides and 81 nucleotides for the Carnation control and alcohol samples. These figures were close to the Number ave. tract sizes which were 84, 86, 83 and 83 for these same samples. The number of molecules (not shown graphically) in each fraction was also determined. As seen in experiment one, the range of poly(A) tract sizes isolated on 10% w/v acrylamide gels for alcohol and control samples seem to be similar however the amount of poly(A) in both alcohol samples (Table IV) was 1.6 and 2.0 times greater than the controls regardless of diet. The samples when run for 1 hr 50' at 5 mA/gel on 2.5% w/v acrylamide-agarose gels gave much shorter values for the poly(A) tract sizes (Figures 9 and 10). Comparison of migration rates for poly(A) standards A_{51} and A_{95}, showed little resolution between the two. So although the entire range of poly(A) tracts from all four samples were contained on the gel, meaning that a single peak was seen with very little poly(A) at the top or bottom of the gel compared to the rest of the gel, it was not possible to separate the range of poly(A) tracts between 50 and 90 nucleotides on a 2.5% acrylamide gel as seen in a range of 74-95 nucleotides in 10% gels. Poly(A) content (μg/mg polysomal RNA) (Table IV) was greater for the alcohol group (0.69 and 1.47) than the content controls (0.35 and 0.93).

Figure 9.   Polyacrylamide Gel Electrophoresis of
            Poly(A) Segments Isolated From Poly(A)
            Containing Messenger RNA

Poly(A) Tracts Were Obtained by Nuclease Diges-
tion of a Purified Messenger RNA Sample. The Gels
(2.5% Polyacrylamide) Were Run at 5 mA/Tube for 1Hr 50
min. The Gels Were Sliced and 3 Adjacent 1 mm Slices
Were Collected and the Poly(A) Eluted at 25°
Overnight. Poly(A) Content was Determined by Molecular
Hybridization with ^{3}H-poly (U). The Arrows Give the
Position of Poly(A) Standards of 95 and 51 Nucleotides
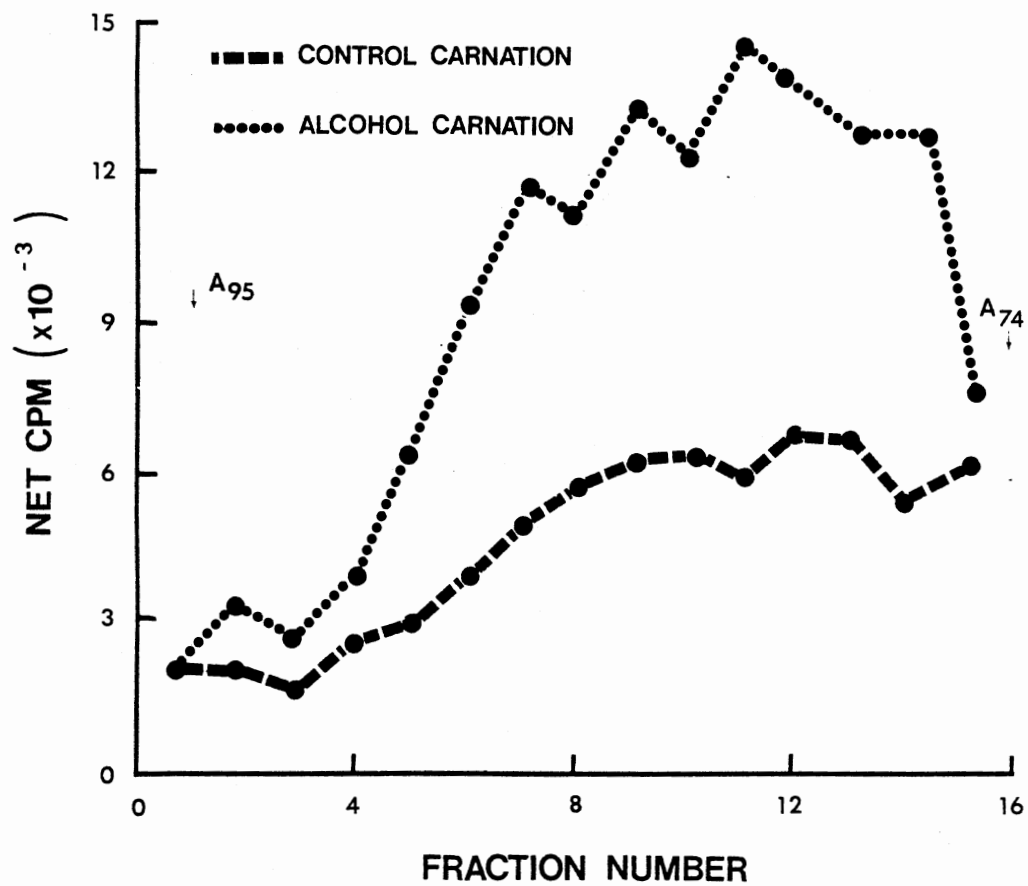Run on Separate Gels.
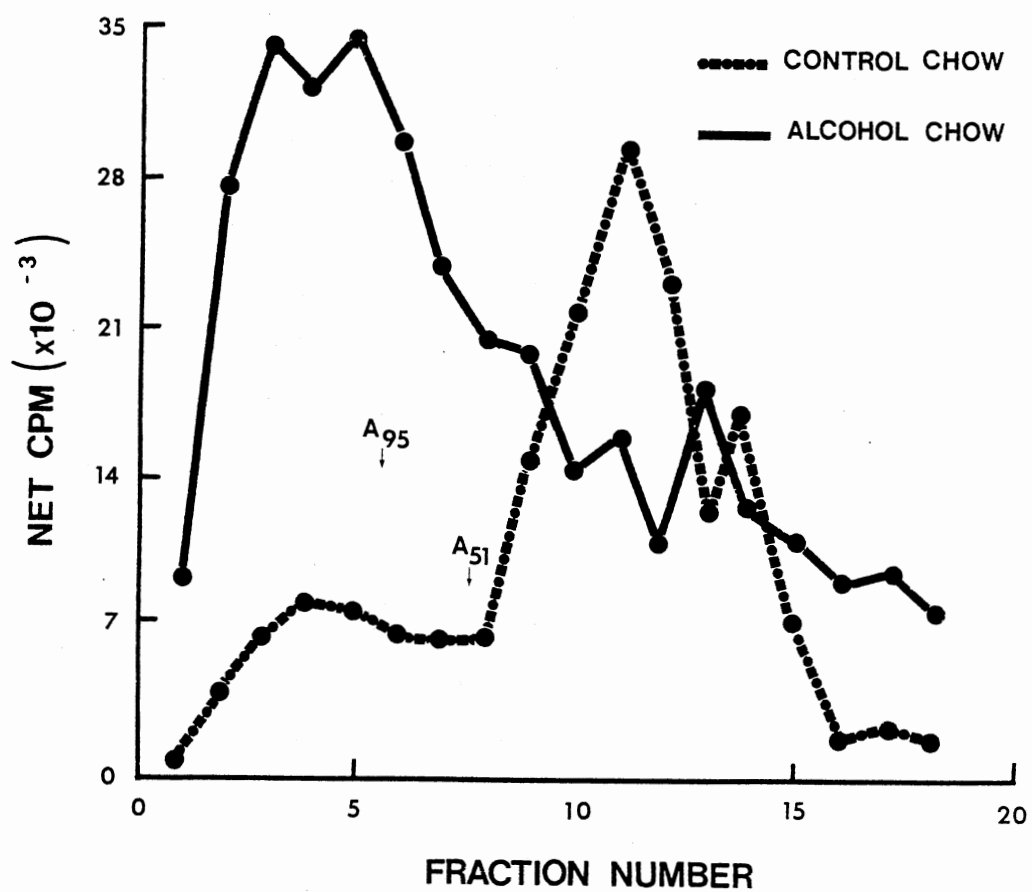
Figure 10. Polyacrylamide Gel Electrophoresis of
            Poly(A) Tracts Isolated From Poly(A)
            Containing Messenger RNA

Experimental Conditions Given in Figure Legend 9
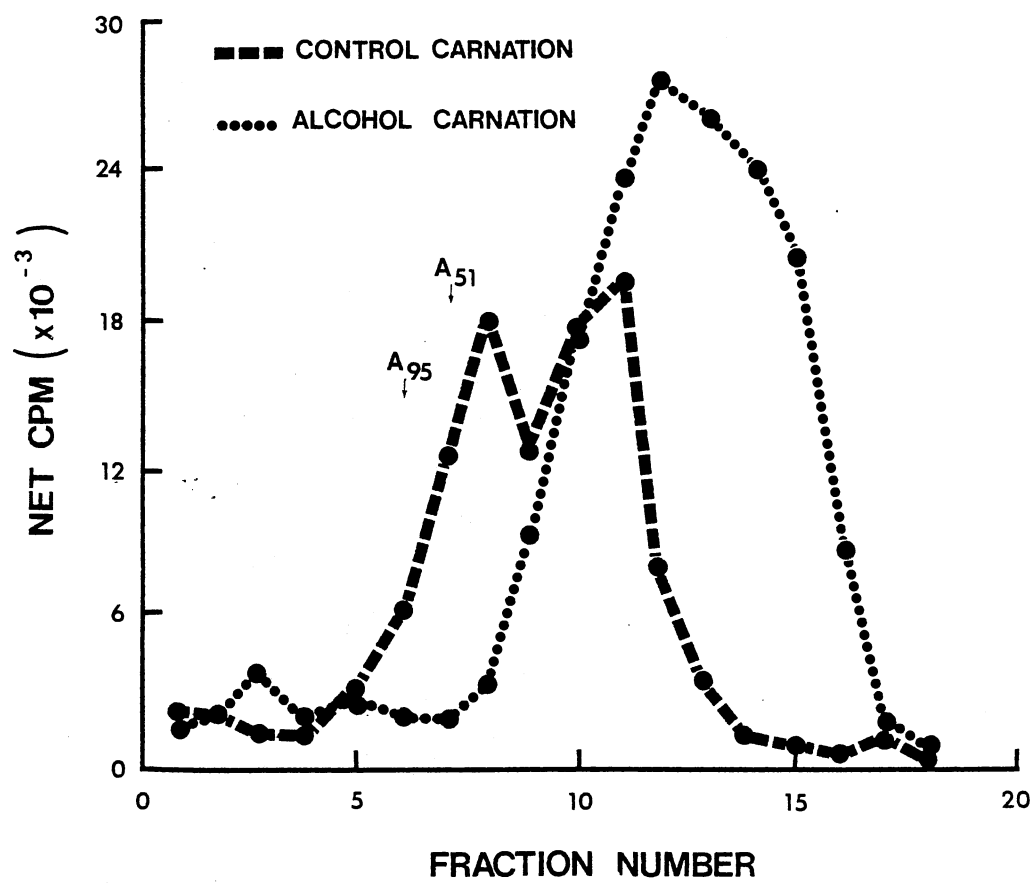
Experiment 3.   Use of 4% Polyacrylamide Gels

The mice (Timco®, females) in this experiment were also divided into Carnation®-fed and Purina® Chow-fed groups. Both alcohol groups were given 5% v/v ethanol. Only the mice on the Carnation Slender diets were given vitamin supplements and the Carnation controls were given sucrose isocalorically. Control mice were pair-fed with alcohol mice. Body weights for all mice were recorded at intervals of 30 and either 75 or 90 days (Figures 11 and 12). The change in body weights for the Carnation-fed mice are similar to that seen in Experiment 2 (Table V). A gain in weight was observed for the first 60 days on the Carnation diet which was small followed by a decline in body weight. The sharp increase in weight was seen for the first 30 days in the Chow fed mice after which the alcohol and control mice showed a minimul weight gain. These results are similar to other studies involving a 5% v/v alcohol diet (Banks et al., 1970). The fact that the Chow-fed mice were growing at the completion of the study while the Carnation-fed mice were showing a decline in body weight may be significant. None of the animals in Experiment 2 exhibited the growth seen in the Chow-fed animals in this experiment.

In order to better evaluate the level of alcohol addiction on the individual diets and to insure alcohol addiction, animals were taken off all alcohol for 24 hrs and then placed back on their diets until the time of death.

Figure 11. Body Weight for the 90 Day Study. Control
            and Alcohol-Fed Carnation Mice Were
            Weighed at Intervals of 0, 60 and 75 Days
            During the Study
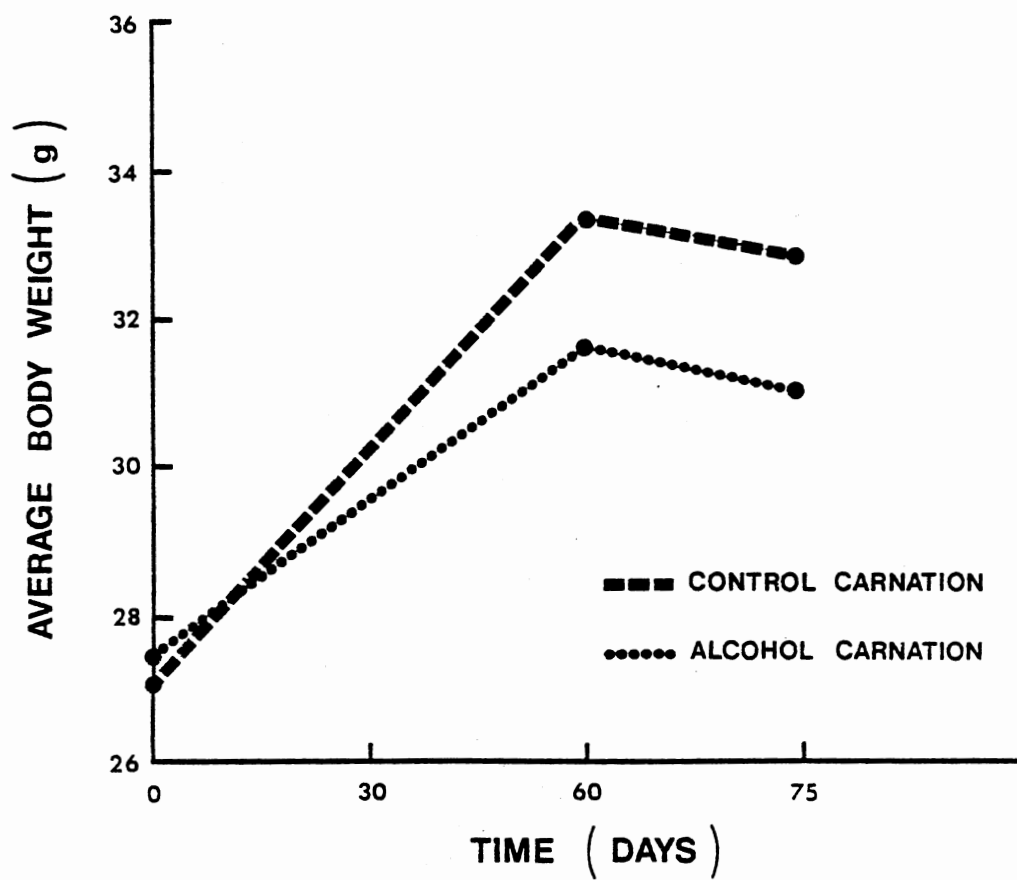
Figure 12. Body Weights for the 90 Day Study. Control
            and Alcohol-Fed Chow Mice were Weighed at
            Intervals of 0, 30 and 60 Days During the
            Study
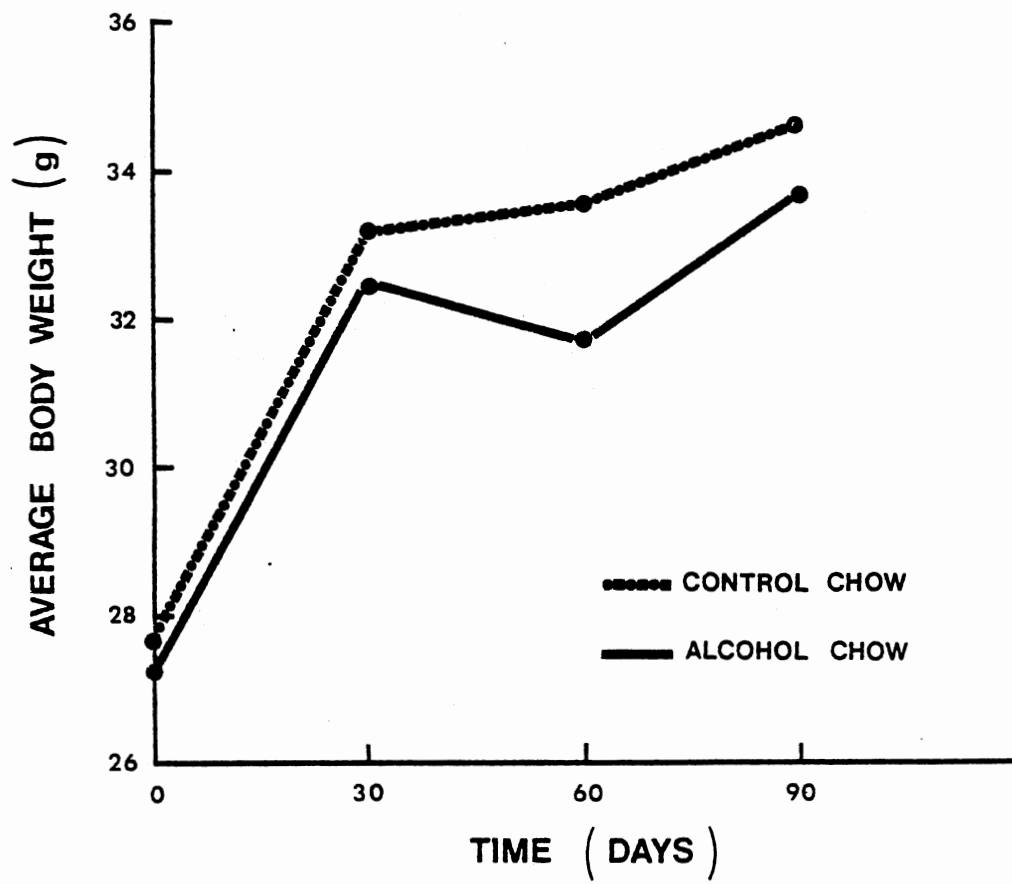
Their body weights and rectal temperatures were recorded several times during the 24 hr withdrawal period. Physically dependent mice should undergo hypothermia following withdrawal of ethanol as well as undergoing seizures. A 3-4g decrease in body weight 8 hrs after withdrawal would also be indicative of physical dependence (Reitzman and Tabakoff, 1976). The Chow, fed alcohol mice showed no decrease above normal diurnal variation (Figure 13) while a slight, but a significant (P=0.05) decrease at 5.2 hrs following withdrawal was seen in the alcohol-Carnation mice (Figure 14). No significant changes were seen in body weight during the 24 hr period for any mice (Tables V and VI). No behavioral signs of withdrawal were seen in the Chow-alcohol groups, however, approximately 25% of the alcohol Carnation mice displayed seizure activity at 8 hr following withdrawal. This seizure activity was absent when animals were checked at 24 hr. This activity in Carnation animals may be due to an increased alcohol consumption of 13.3 g ethanol/kg body weight for Carnation-fed alcohol mice as compared to 8.3 g/ethanol/kg body weight for Chow-fed alcoholic mice (Figure 15). The amount of liquid consumed in all four groups was less at the end of the study than what was seen in the first 8-10 weeks (Figures 16 and 17) and the average fluid intake was consistently less for the control mice regardless of the diet. The average fluid intake for the mice on the Carnation diet was 14 ml/mouse/day and 16 ml/mouse/day for control and alcohol mice respectively (Table VII). Less fluid

Figure 13. Changes in Rectal Temperatures Following
            Withdrawal From Alcohol. Rectal tempera-
            tures Were Taken From Control and Alco-
            hol-Fed Mice Following Removal of Alco-
            hol From the Chow Diet
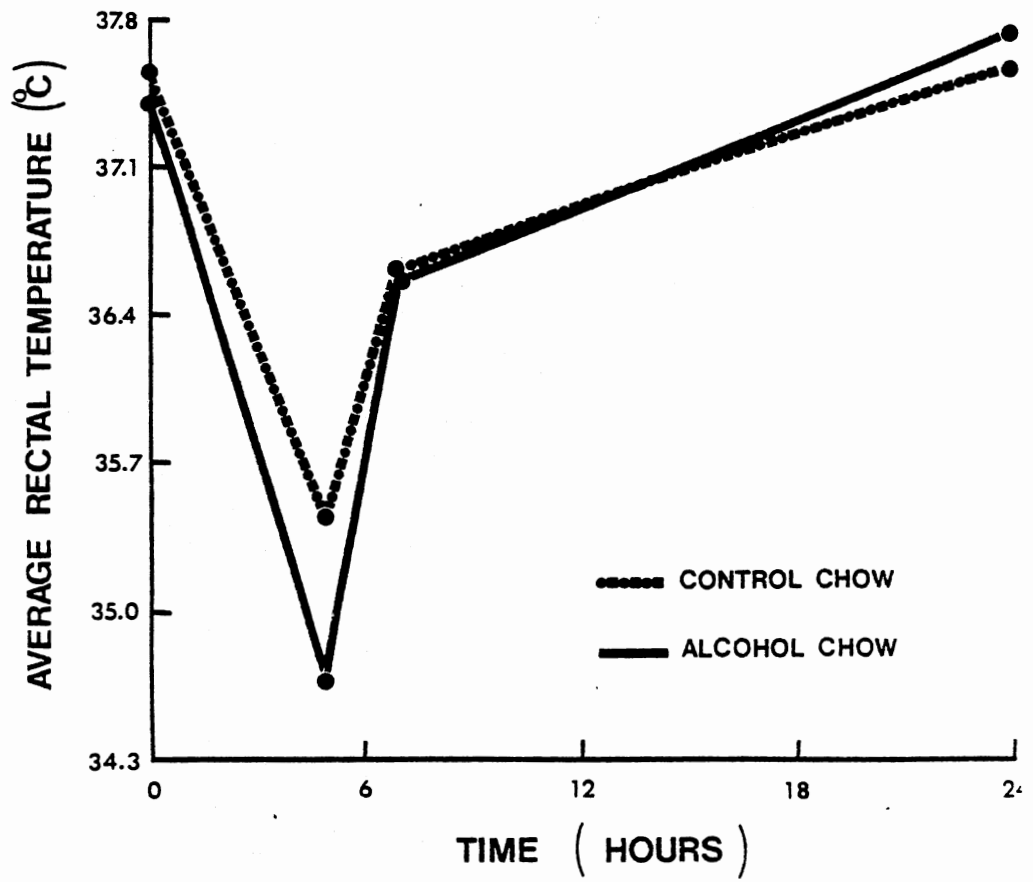
Figure 14.   Change in Rectal Temperatures Following
              Withdrawal From Alcohol. Rectal Tempera-
              tures Were Taken From Control and
              Alcohol-Fed Mice. Following Removal of
              Alcohol From the Carnation Diet
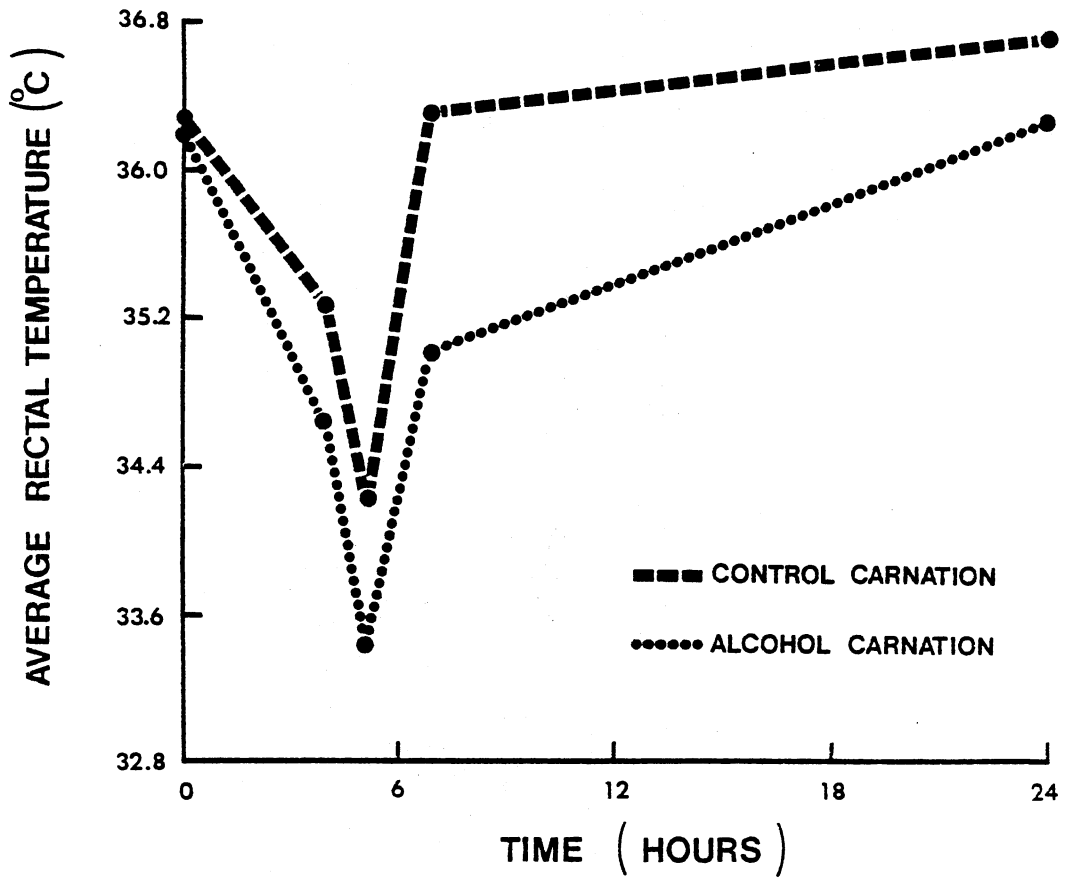
TABLE V

THE EFFECTS OF ALCOHOL WITHDRAWAL ON MOUSE WEIGHTS
AND RECTAL TEMPERATURES AT 0, 5, 7 AND 24 HOURS
AFTER WITHDRAWAL FROM THREE MONTHS OF
ALCOHOL FEEDING USING A DIET OF
CARNATION SLENDER (EXP. 3)

	# of Animals in group	Alcohol Carnation	Control Carnation	Ratio A:C
Body Weight (g)				
0 hr	6	39.4+3.0	40.4+6.5	0.97
5 hr	6	39.2+2.3	40.4+3.0	0.97
7 hr	6	38.4+2.5	40.6+6.0	0.95
24 hr	6	38.7+2.5	40.7+6.0	0.95
Rectal Temp.(°C)				
0 hr	5	36.2+0.65	36.2+0.4	1.00
5 hr	5	33.4+0.39	34.2+0.6	0.98
7 hr	5	35.0+0.62	36.3+1.2	0.96*
24 hr	5	36.2+0.88	36.7+0.59	0.98

Values are means $\pm$ S.D. Student's t test for grouped observations was used in determining the level of significance. * Values significant from the zero hour reading at the 0.05 level.

TABLE VI

THE EFFECTS OF ALCOHOL WITHDRAWAL ON MOUSE WEIGHTS
AND RECTAL TEMPERATURES AT 0, 5, 7 AND 24 HOURS
AFTER WITHDRAWAL FROM THREE MONTHS OF
ALCOHOL FEEDING USING A DIET OF
PURINA RAT CHOW (EXP 3)

	# of Animals in group	Alcohol Chow	Control Chow	Ratio A:C
Body Weight (g)				
0 hr	6	32.5+4.3	34.7+4.2	0.94
5 hr	6	35.1+2.1	34.9+3.8	1.00
7 hr	6	32.1+4.1	33.9+3.7	0.95
24 hr	6	33.2+2.6	34.4+4.1	0.96
Rectal Temp.(°C)				
0 hr	5	37.4+0.22	37.4+0.31	1.00
5 hr	5	34.7+0.87	35.5+0.17	0.98
7 hr	5	36.5+0.48	36.7+0.48	0.99
24 hr	5	37.7+0.35	37.6+0.70	1.00

Values are means $\pm$ S.D. Student's t test for grouped observations was used in determining the level of significance. * Values significant from the zero hour reading at the 0.05 level.

Figure 15. Daily Alcohol Intake for Carnation and
Chow-Fed Mice. The Average Amount of
Alcohol Consumed for Animals on Either
Diet was Determined Throughout Both
Studies
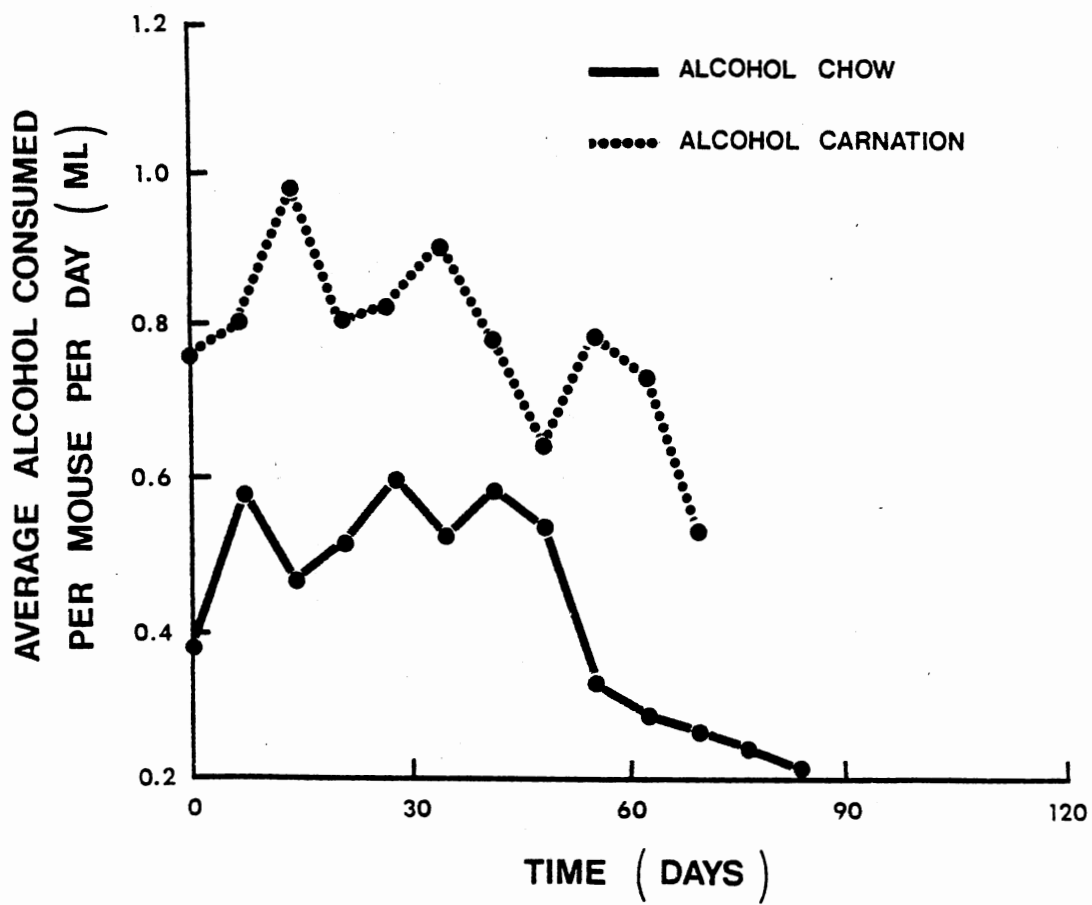
Figure 16. Daily Liquid Intake During the 75 Day
            Study.
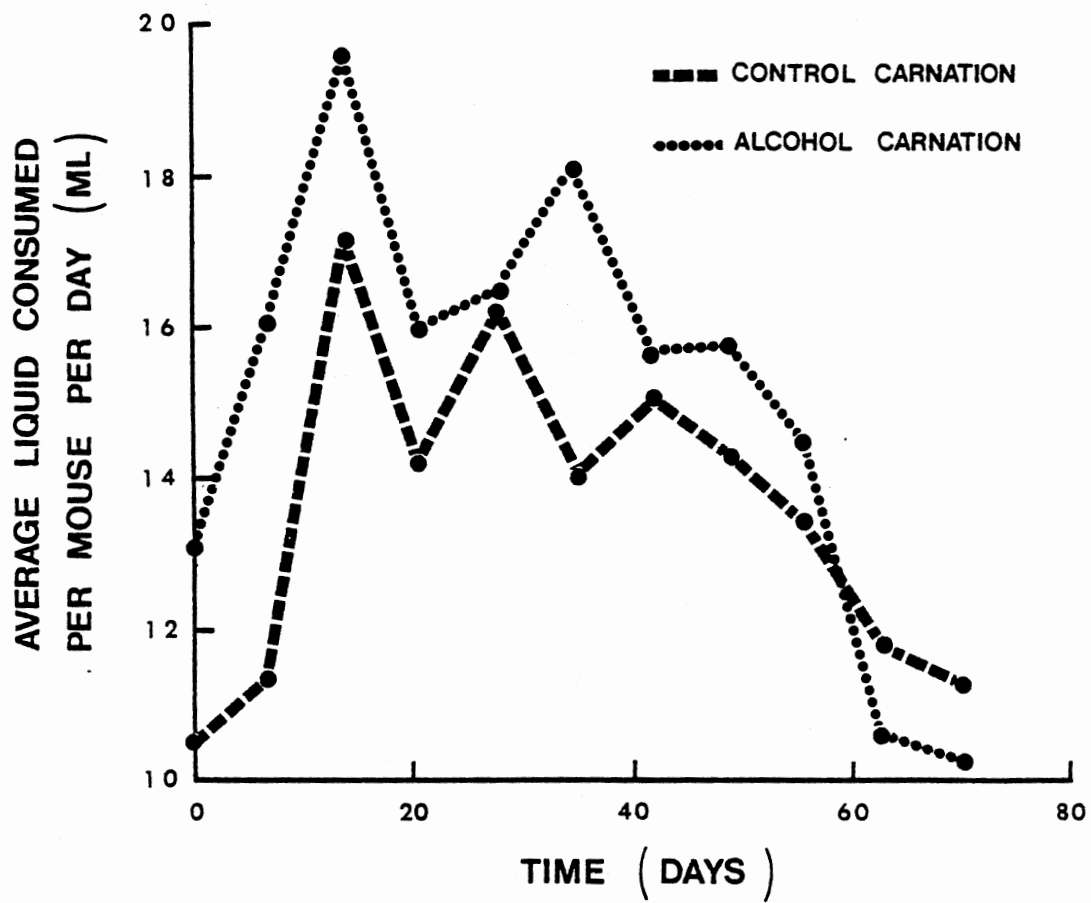
Figure 17.   Daily Liquid Intake During the 90 Day
             Study. The Amount of Water (for
             Controls) and Alcohol-Water Mixture
             (Experimentals) was Calculated for the
             Chow-Fed Mice
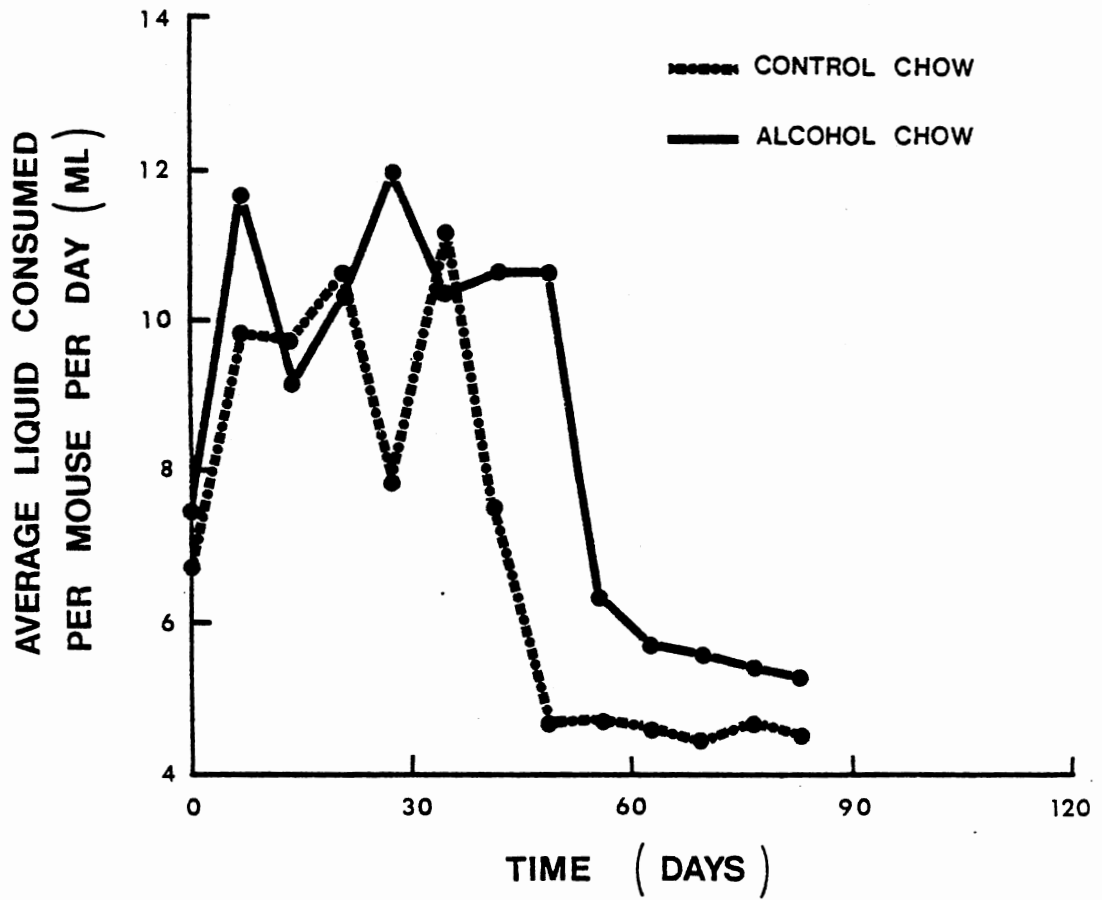
TABLE VII

THE EFFECT OF CHRONIC ALCOHOL-SUCROSE TREATMENT
ON MOUSE BODY AND ORGAN WEIGHT, CHOW OR
CARNATION SLENDER LIQUID DIET
(EXP 3)

	# of animals/ treatment	Alcohol Diet 50% Ethanol	Control Diet	Ratio A:C
Carnation Slender				
Initial Body Weight (g)	24	27.4+1.9	27.1+2.1	1.01
Final Body Weight (g)	24	30.9+2.1	32.8+2.1	0.94
Ave. Food Consumed (ml/day/mouse)	24	15.6+2.4	13.8+2.2	1.12
Ave. Alc. Consumed (ml/mouse/day)	12	0.78+0.24	--	--
Liver weight (g)	24	1.39+0.9	1.30+0.8	1.06
Purina Chow				
Initial Body Weight (g)	22	27.1+1.5	27.6+1.2	1.01
Final Body Weight (g)	22	33.7+3.1	34.6+4.7	0.97
Ave. Liq. Consumed (ml/mouse/day)	22	9.24+2.3	8.03+2.4	1.15*
Ave. Food Consumed (ml/day/mouse)	22	3.76+0.7	3.45+0.8	1.08*
Ave. Alc. Consumed (ml/mouse/day)	11	0.46+0.07	--	--
Liver weight (g)	22	1.45+0.9	1.53+0.8	0.95*

Values are means + S.D.. Student's t test for grouped observations was used in determining the level of signifi-cance. * values significant from the control diet at the 0.05 level.

was consumed for the Chow-fed groups; 8 ml/mouse/day for the control mice and 9 ml/mouse/day for mice drinking alcohol. It is not understood why the mice consumed less liquid during the latter weeks of the study but a severe reduction in intake is seen for all mice.

Purity of the RNA Fractions

The amount of polysomal RNA (mg)/g liver weight was 5.2 and 5.8 mg for Chow control animals (Table VIII). The mice were divided into groups of six at the time of death, and livers from 6 mice were removed and pooled to form one sample. Therefore the data will be given as duplicate experiments (1 and 2) for both Chow and Carnation mice. The mg polysomal RNA/g liver for Chow alcohol animals were 5.0 and 6.6 (Table IX). Fractions from each sample of polysomal RNA were analyzed for degradation by sucrose density gradient centrifugation. The Chow-fed samples showed no degradation but the Carnation samples were degraded and therefore not further investigated for poly(A) tract sizes (Table IX). The cause of the degradation is unknown. The amount of poly(A$^+$)mRNA/mg polysomal RNA for the four Chow groups after oligo(dT)-cellulose isolation was found to be 22.5 and 20.7 for the control samples and 25.4 and 17.3 for the alcohol samples. The poly(A$^+$)mRNA samples were subjected to nuclease degradation and then layered on 4% polyacrylamide gels for determination of poly(A) tract sizes.

TABLE VIII

CHARACTERIZATION OF RNA AND POLY(A) TRACTS IN
ALCOHOL AND CONTROL CHOW FED MICE
(EXP 3)

	Alcohol-fed Mice		Control Mice	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
mg polysomal RNA/g liver	6.55	4.96	5.21	5.80
µg poly(A$^+$)mRNA/mg polysomal RNA	23.4	17.3	22.5	20.7
µg poly(A)/mg polysomal RNA	0.59	1.09	0.60	0.5
260/280 ratio of poly(A) mRNA	2.23	2.23	2.16	2.52
Mass Ave. poly(A) tract size	1.30	1.17	1.30	1.35
Number Ave. poly(A) tract size	99.9	109	82.7	96.3

TABLE IX

CHARACTERIZATION OF RNA AND POLY(A) TRACTS IN
ALCOHOL AND CONTROL CARNATION FED MICE
(EXP 3)

	Alcohol-fed Mice		Control Mice	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
mg polysomal RNA/g liver	3.35	3.07	2.89	10.2
μg poly(A)mRNA/mg polysomal RNA	2.49	4.68	4.14	11.2
260/280 ratio of poly(A) mRNA	1.0	1.8	1.3	2.2

Determination of Poly(A) Tract Sizes

Along with the four Chow poly(A) samples, poly(A) standard of 95 nucleotides was also subjected to nuclease treatment. This was done to insure that the poly(A) tracts were resistent to the nuclease used to digest the nonpolyadenylated portion of the mRNA. The poly(A) standard was found to be resistant to nuclease digestion. The gels were scanned at 260 nm and the locations of four standards in the 4% gels after an electrophoresis time of 2 hr at 4.6 mA/gel were determined (Figure 18). The A_{115} standard with a M.W. of 41,045 moved into the gel at a rate of 2.2 cm/hr while the A_{94}, A_{51} and A_{23} standards with M.W.'s of 34,907, 18,951 and 7903 entered the gels with mobilities of 2.4 cm/hr, 3.0 cm/hr, and 3.5 cm/hr.

The standard and experimental poly(A) tracts were removed from the gels as previously described and hybridized to $5-^3$poly(U). Isolation of the hybrids was by filtration using nitrocellulose filters. The ability of the nitrocellulose filters to bind hybrids was determined by using known amounts of poly(A) to hybridize with poly(U) which was in excess. These hybrids were incubated with and without ribonuclease. Similar percentages (80%) of material bound to the filter suggest that ribonuclease treatment digested away excess poly(U) without affecting already formed poly(A-U) hybrids. The fact that less than 0.1% of enzyme-treated poly(U) bound to the filter indicated that the ability of

Figure 18. Relative Mobility of Poly(A) Standards in a
            4% Polyacrylamide Gel. Poly(A) Standards
            (Miles Co.) of 115, 95, 51, 23 Nucleo-
            tides in Length Were Electrophoresed for
            2 hr at 4.6 mA/Gels
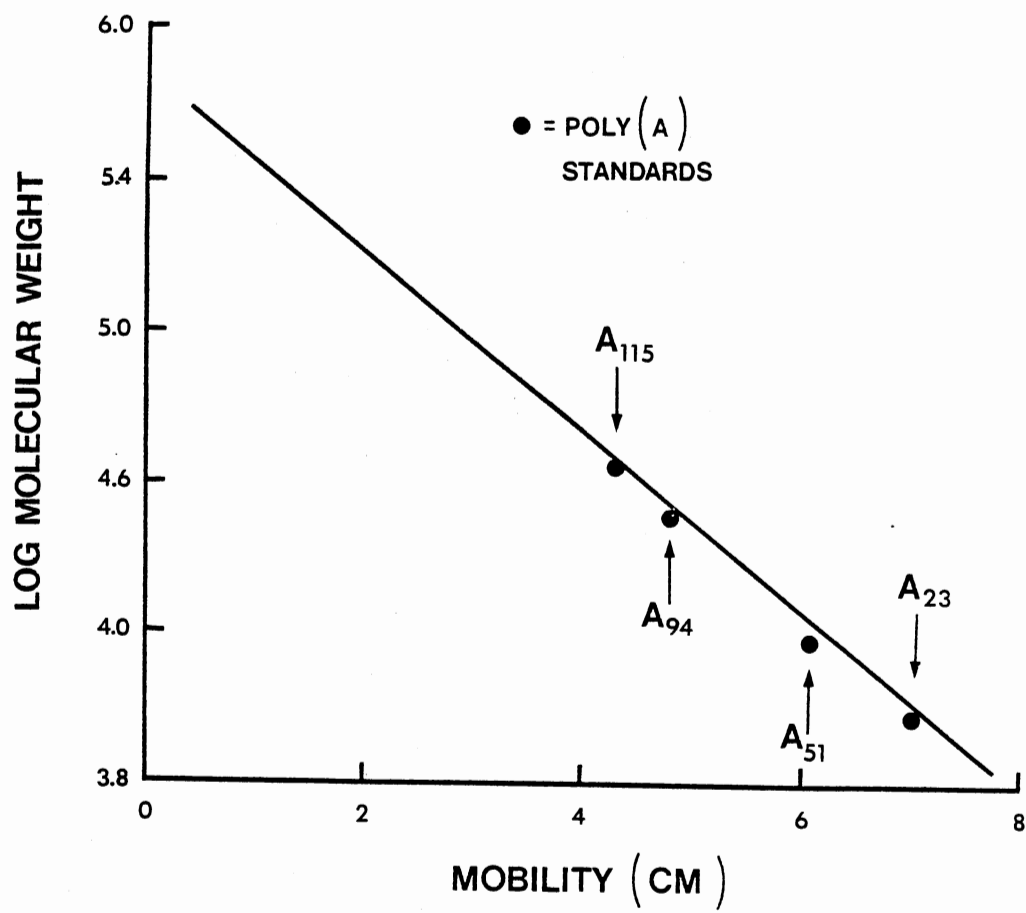
ribonuclease A to digest ^{3}H-poly(U) (Table X). In the
replicate experiments, the overall profiles of poly(A) from
alcohol-treated mice and control mice did not show any
marked differences in poly(A) tract sizes (Figures 19 and
20). The number average sizes of the poly(A) tracts were
found to be 105 for alcohol-treated samples and 89 from
control samples.

The radioactivity in each gel fraction presented in
Figures 19 and 20 was converted to the actual number of
number of poly(A) molecules present in that fraction and
then replotted in Figures 21 and 22. The majority of tract
sizes for all samples lie between 90 and 120 nucleotides.

Comparison of Diets and Resulting
Poly(A) Amounts and Sizes

The results from all experiments using 10% and 4% poly-
acrylamide gels reveal that the average poly(A) tract sizes
for both control and alcohol poly(A^+)mRNA lie between 77
and 109 nucleotides in length (Table XI). In four out of
six cases, the amount of poly(A) in alcohol samples was
greater than that seen in the control samples by at least a
factor of 1.6. The greatest increase in the amount of
poly(A) seems to be in mice fed Carnation Slender® and alco-
hol. Based on these results, the data suggests that alcohol
does not affect poly(A) tract size but increases the amount
of poly(A)/mg polysomal RNA.

TABLE X

EFFECTS OF RNAse TREATMENT ON POLY(A-U) HYBRIDS
AND POLY(U) ALONE

Sample	Counts cpn filter(cpm)	Total Counts added (cpm)	% Bound
0.1 µg poly(A)+ 0.16 µg poly(A) no RNAse A	44,768	56,200	79.6
0.1 µg poly(A)+ 0.16 µg poly(U)+ 8.0 µg/ml RNAse A	55,844	68,989	80.9
0.16 µg poly(U) no RNAse A	45,540	64,828	79.2
0.16 µg poly(U) 8.0 µg/ml RNAse A	672	87,902	0.76

Figure 19. Polyacrylamide Gel Electrophoresis of
            Poly(A) Segments Isolated From Poly(A)
            Containing Messenger RNA

Poly(A) Segments Were Obtained by Nuclease Diges-
tion of a Purified Messenger RNA Sample. The Gels
(4% Polyacrylamide) Were Run at 4.6 mA/Tube for 2 Hrs.
The Gels Were Sliced and 3 Adjacent 1 mm Slices Were
Collected and the Poly(A) Eluted at 25° Overnight.
Poly(A) Content was Determined by Hybridization with
^{3}H-poly(U). The Arrows Give the Position of Poly(A)
Standards of 115, 95, 51, and 23 Nucleotides Run on
Separate Gels
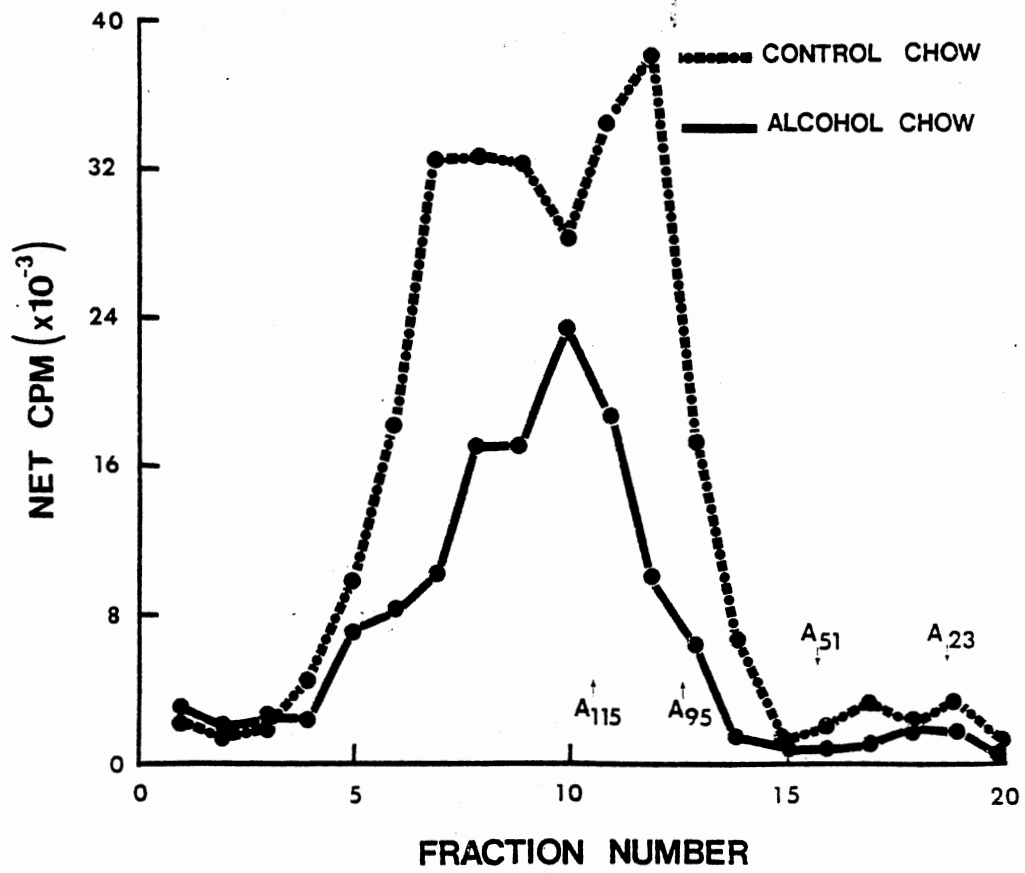
Figure 20.   Polyacrylamide Gel Electrophoresis of
             Poly(A) Segments Isolated From Poly(A)
             Containing Messenger RNA

Experimental Procedure Given in Figure Legend 19
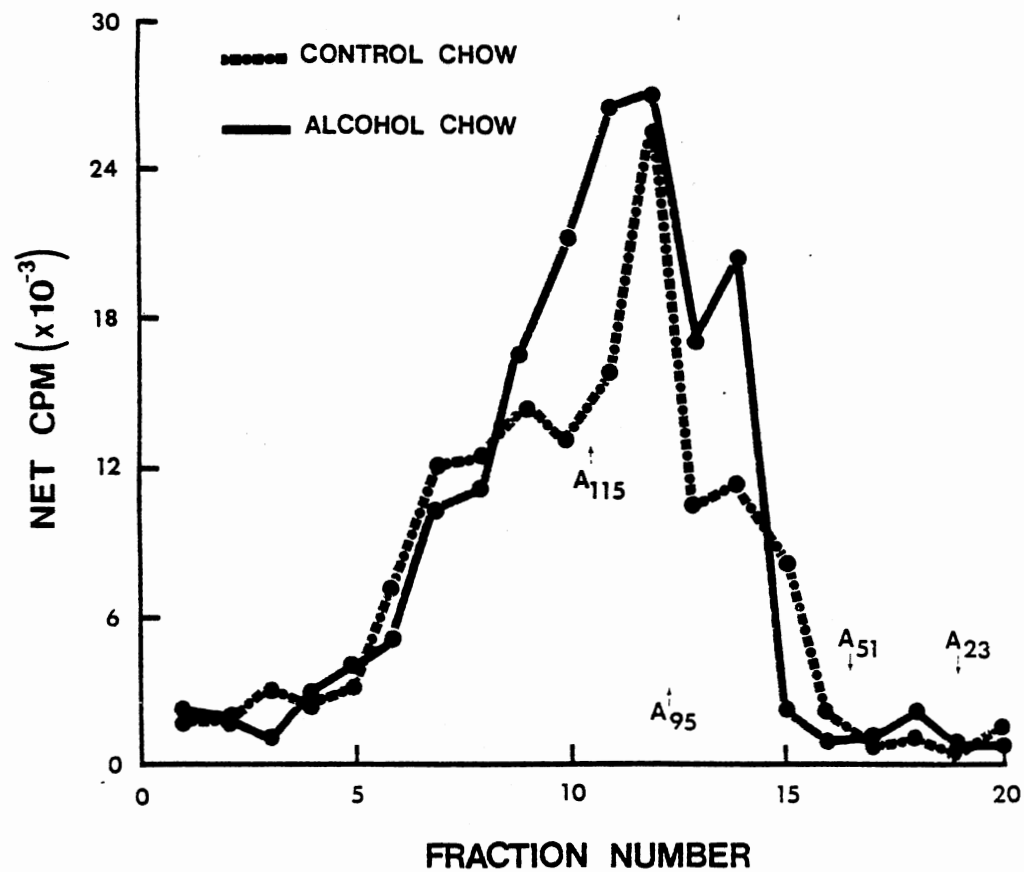
Figure 21. Polyacrylamide Gel Electrophoresis of
Poly(A) Segments Isolated From Poly(A)
Containing Messenger RNA

Experimental Procedure Given in Figure Legend 19. The
Number of Molecules per Fraction was Determined Using
the cpm/fraction and the Molecular Weights of Poly(A)
Tracts in that Fraction
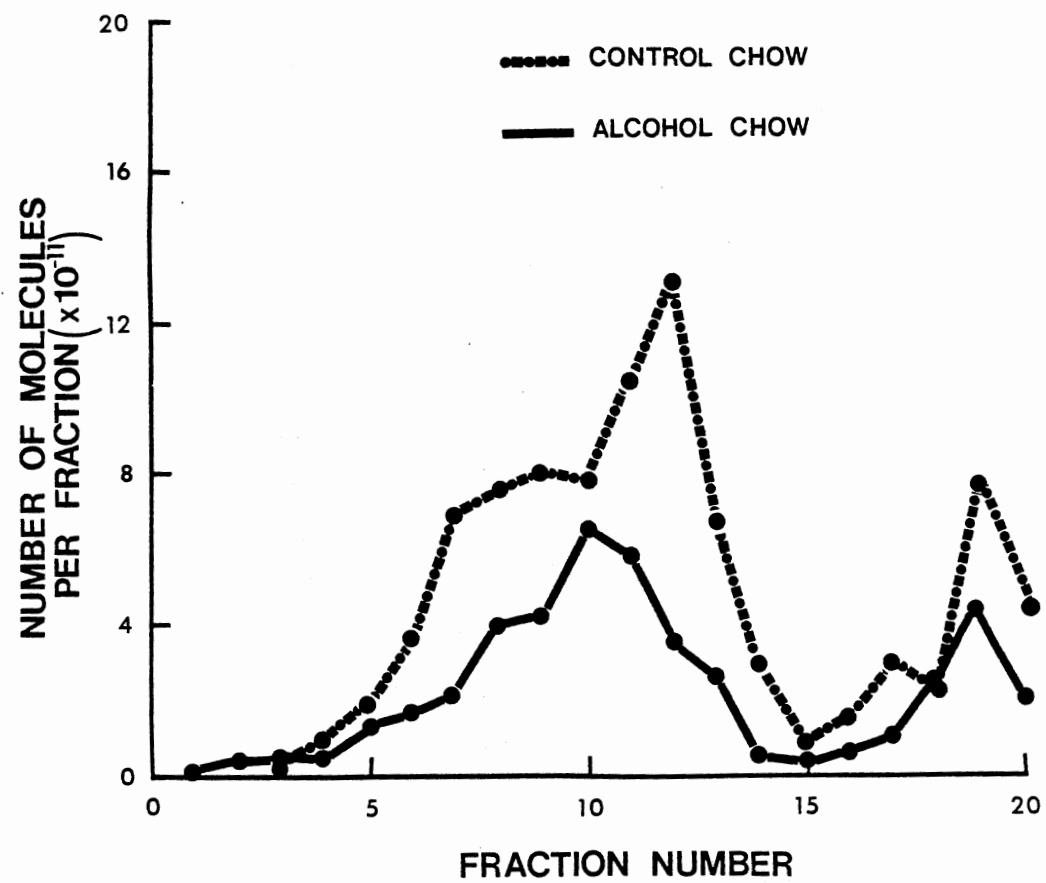
Figure 22. Polyacrylamide Gel Electrophoresis of
            Poly(A) Segments Isolated From Poly(A)
            Containing Messenger RNA

See Figure Legend 19 for Experimental Procedure
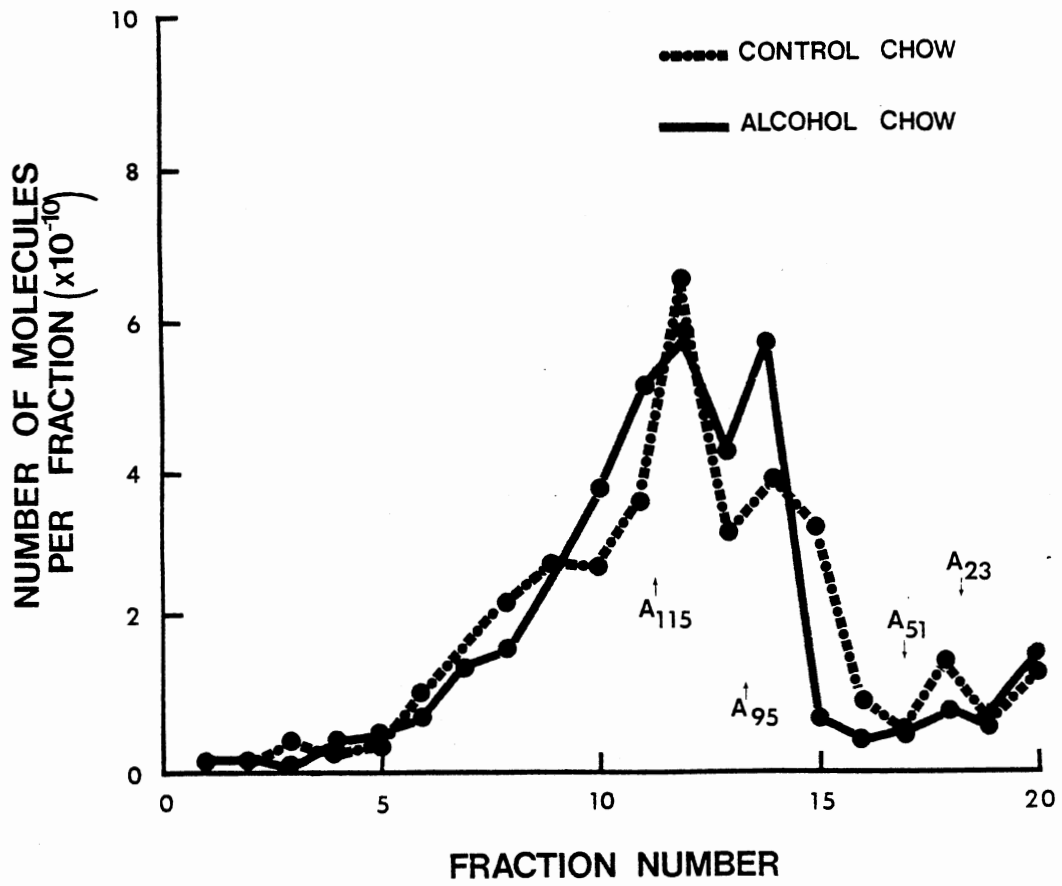
TABLE XI

SUMMARY: COMPARISON OF VARIOUS DIETS INVOLVING
ALCOHOL-FEEDING OF MICE AND THEIR EFFECTS
ON RNA CONTENT AND POLY(A) TRACT SIZES

	µg poly(A)/ mg polysomal RNA	Mass Ave. poly(A) tract size	Number Ave. poly(A) tract size
Exp 1 10% acrylamide gels			
Control-Chow	0.28	79.0	79.0
Alcohol (10% v/v)-chow and water	0.48	82.0	77.0
Alcohol (10%)-sucrose-chow (5% v/v)+ water	0.29	81.0	76.0
Exp 2 10% acrylamide gels			
Control Chow	0.93	82.0 40*	84.0 87*
Alcohol (5% v/v)-Chow	1.47	87.0 87*	86.0 30*
Control-Carnation	0.69	83.0 76*	83.0 43*
Alcohol (5% v/v)-Carnation	0.35	81.0 76*	83.0 81*
Exp 3 4% acrylamide gels			
Control Chow	0.60	130	83.0
" "	0.50	135	96.3
Alcohol Chow (5% v/v)	0.59	130	100
" "	1.09	117	109

* -refers to data using 2.5% w/v acrylamide gels

CHAPTER V

DISCUSSION

The purpose of this study was to determine the effects
of chronic alcohol ingestion on polyadenylation of mouse
liver mRNA. The initial working hypothesis was that chronic
alcohol ingestion resulted in longer poly(A) tract sizes.
This hypothesis was based on earlier studies (Bantle et al.,
1980) which showed an increased amount of poly(A) only in
alcohol-sucrose polysomal RNA after hybridization with
$5-^3H$ poly(U). The results of this current study did not
support this hypothesis since both alcohol-fed mice and con-
trol mice were found to have poly(A) tract sizes between 77
and 105 nucleotides in length. What is interesting is that
even though there was no difference in tract size, the
alcohol-fed mice did have a 1.6-2.0 fold increase in the
amount of poly(A)/mg polysomal RNA compared to controls in
most of the experiments. This does support previous results
and again suggests that chronic alcohol ingestion leads to
an increase in polyadenylation of mouse liver RNA. This
increase in poly(A^+)mRNA was also accompanied by a
decrease in poly(A^-)mRNA which resulted in no change in
total mRNA content in alcohol-sucrose fed mice (Bantle et
al., 1980). The question remains as to why there is a

decrease in protein synthesis (Banks et al., 1970; Moreland, 1974; Moreland et al., 1979) after alcohol ingestion.·

It was suggested that an increase in poly(A^+)mRNA degradation due to alcohol is responsible for the increase in polyadenylation seen in alcohol treated mice, perhaps as an effort by the cell to maintain poly(A^+)mRNA concentrations (Bantle et al., 1980). Since there are no current reports of increased ribonuclease activity in alcohol-treated animals, this may be an area which needs investigation. If the poly(A^+)mRNA in alcohol-fed mice is shorter due to degradation, this would result in a higher ratio of poly(A) to total mRNA in alcohol-fed mice compared to control mice having longer poly(A^+)mRNA molecules. An alcohol-induced increase in poly(A^+)mRNA degradation would also explain a decrease in protein synthesis if the shorter poly(A^+)mRNAs missing the 5'cap region when produced. Although the exact function of the 5' 7mG cap region is not fully understood, it is thought to be responsible for orienting the mRNA to the ribosome, and to promote binding between the 5' end of the mRNA and a complimentary end of rRNA (Avers, 1980). But decreased binding capacity of short poly(A^+)mRNAs at the ribosome would result in an increase in single ribosomes and a decrease in polyribosomes. This was not seen in liver polysome profiles from alcohol-sucrose fed mice and control mice (Bantle et al., 1980).

The amount of poly(A^+)mRNA degradation needs to be

determined to understand the results from this study which give increased poly(A)/mg polysomal RNA ratios for alcohol-fed mice. One way to determine this would be to measure the amount of ribonuclease activity from alcohol-fed and control mice by monitoring the destruction of labeled mRNA in the presence of cell homogenate.

It should now be possible to measure accurately the length of poly(A+)mRNA using high resolution polacrylamide gel electrophoresis under denaturing conditions. Poly(A+)-mRNA can be eluted from gel slices and hybridized to ^{3}H-poly(U) in order to identify the poly(A) tract. Since no change in tract size exists between alcohol-fed and control groups, it is possible to infer that any differences seen is due to changes in mRNA length. If a reduction in mRNA length was noted in the alcohol-fed groups, then it could be concluded that a higher amount of mRNA degradation occurred due to alcohol treatment.

If increased degradation of poly(A^+)mRNA is a result of alcohol influence then the ability of poly(A^+)mRNA to function in translation would be measured by injecting poly(A^+)mRNA extracted from alcohol-fed and control animals into Xenopus laevis oocytes. Translational efficiency and stability of the injected mRNA would be analyzed by monitoring the presence of the expressed mouse liver proteins such as serum albumin and ferritin.

If increased poly(A^+)mRNA degradation due to alcohol is not the reason for increased polyadenylation, then an

alcohol-induced influence similar to that of ethionine may be involved. In ethionine-intoxicted rats, in vivo poly-adenylation of liver mRNA increases even though mRNA synthesis and poly(A) polymerase activity decline (Dennis and Kisilevsky, 1979). Ethionine caused polysome dissagregation and as a result mRNA was released from the ribosome. It then served as a more efficient primer for poly(A) polymerase resulting in increased cytoplasmic polyadenylation. If poly(A$^-$)mRNA was preferentially adenylated, then this type of action would explain both increased poly(A) content and a decrease in protein synthesis. Alcohol may also increase mRNA synthesis which would explain the increase in 5-^{3}H Uridine incorporation seen in alcohol-sucrose treated mice (Bantle et al., 1980). This is unlike ethionine which inhibits mRNA synthesis. Further investigation of polysome profiles would be necessary since no evidence of decreased polysome aggregation was seen in earlier studies.

Increased polyadenylation in alcohol-fed mice could be due to an increase in poly(A) polymerase activity. This increase in activity may be an adaptive response of the hepatocyte to ethanol to maintain poly(A$^+$)mRNA concentration. Poly(A) polymerase levels have been altered in response to amino acid supply, glucocorticoid hormone and neoplasms (Rose et. al., 1976). It was also reported that increased poly(A) polymerase activity did not result in increased poly(A) lengths but only in the rate at which the tracts were added to poly(A$^-$)mRNA. Therefore, it is

reasonable to suggest that alcohol may also influence its activity.

In order to determine whether increased rates of poly-adenylation are directly responsible for the increase in poly(A) content reported in the 1980 studies, a further understanding of alcohol dosage effects is needed. Perhaps mice on a chronic 10% ethanol diet will experience changes in RNA synthetic rates not seen on a 5% alcohol diet. Variable results were seen comparing alcohol concentration and rates of protein synthesis in rats. On a 16% v/v eth-anol diet, no difference was seen in the amount of protein in alcohol and control liver samples (Moreland and Smith-Kielland, 1979). Even the controls gave variable results as far as protein and RNA content were concerned. Another diet(5.7% ethanol) was studied and a definite decrease in protein synthesis was reported in the alcohol-fed rats as compared to the control rats (Banks et. al., 1970). Increases in liver protein synthesis for rats on a 15% alcohol diet were reported while slight decreases in cerebellum protein synthesis was measured by ^{3}H-leucine incorporation (Jarlested, 1979). Again these variable results may be due to differences in the nutritional status of the animals at the time of death. It is hard to deter-mine just how much alcohol actually reaches the liver cells in these studies since blood alcohol levels were not deter-mined in most studies.

The problem of determining addiction to alcohol has

been addressed by several investigators. In the previous studies described in the Literature Review, there has been no definitive means of comparing the amount of alcohol consumed by the different experimental groups with the degree of dependency the animals achieved with their respective diets. One method of determining alcohol dependency is to measure blood alcohol levels at the termination of each study. Even this procedure gives variable results (Moreland and Smith-Kielland, 1979). Four animals were killed after chronic alcohol ingestion and while they were fed the same diet, only two of the four showed any appreciable blood alcohol levels. The authors suggested that perhaps a certain consumption level of alcohol is needed before marked changes in organ composition (of RNA, DNA and protein) occur. This particular study concluded that a moderate, but considerable consumption (25% of calories) of ethanol did not lead to observable changes in organ composition.

Since alterations in body temperature are one of the characteristic symptoms of alcohol withdrawal in humans (Gross et al., 1971), rectal temperatures were also determined for mice on a 7% alcohol diet which lasted 7 days (Ritzmann and Tabakoff, 1976). The results showed that the normal diurnal variation in rectal temperature was eliminated by ethanol consumption. Mice undergoing withdrawal after the 7 day alcohol diet (7%) were found to be hypothermic if kept at room temperature. If the mice were placed at 34°C, the hypothermia was reversed and the mice

became hyperthermic. The authors concluded that the temperature set point mechanism and the ability to regulate around this set point was disturbed in animals physically dependent on alcohol. This same study showed very little of these effects when mice were placed on a 6% alcohol diet rather than the 7% diet. While rectal temperatures were found to be consistently lower in the alcohol animals compared to the controls, the differences were not statistically significant. Drops in rectal temperature for the mice were the most profound at 6 and 8 hrs after withdrawal. A slight but significant drop in rectal temperature at 8 hr after withdrawal was also seen in mice fed 5% ethanol for 35 days (Anderson et al., 1980) however this was not accompanied by any physical signs of withdrawal such as seizure activity.

The alcohol-Carnation® mice in this study showed a significant decrease in rectal temperature at 5.2 hr following withdrawal. This was not seen in the Chow fed mice which consumed less alcohol/mouse/day. Other indicators of alcohol dependency are decreased body weight and seizure activity following alcohol withdrawal. Neither were seen in Anderson's study while some seizure activity was seen in the Carnation® group at 8 hr following withdrawal in this study. The Chow-fed alcohol mice showed neither changes in body weight nor seizure activity.

The mechanisms by which alcohol exerts its effects on thermoregulation are not understood. Single doses of ethanol have been found to increase the turnover of

norepinepinephrine in the CNS (Hunt and Majchrowiez, 1974).
Since serotonin injected into mice produces hypthermia, it
was suggested (Ritzmann and Tabakoff, 1976) that the hypo-
thermic effects of an acute dose of ethanol may be mediated
via a serotonergic mechanism. Although the above studies
(Anderson et al., 1980; Ritzmann and Tabakoff, 1976) agree
that 6-8 hr following withdrawal seems to be the time
period where changes in temperature and seizure increased in
activity is seen. The discrepancies between alcohol dosage
and withdrawal responses leave room for inquiry as to the
reliability of these various methods. If this study were to
be repeated, the most reliable indication of alcohol addic-
tion may be to measure the blood alcohol levels every two
hours on a larger number of mice from each experimental
group.

Some of the variations seen in the various studies
involving alcohol treatment and protein synthetic rates are
attributed to variations in diet. One study (Moreland and
Smith-Kielland, 1979) divided the alcohol and control groups
into further classifications of 'fed' (fed continually to
the time of death) and "fasted" mice (mice taken off diet 24
h prior to death). Both alcohol groups were fed 16% v/v
ethanol for 7-10 weeks. The 'fasted' rats showed no
differences in protein composition compared to control but
did show a significantly reduced RNA (mg RNA per g liver)
concentration for the alcohol-fed mice. No changes in
either protein or RNA composition were seen for alcohol and

control fed rats. The animals in the present study were fed up to the time of death except to a brief withdrawal period, as were the mice in previous studies (Bantle et al., 1980).

Diets chosen to determine the effect of alcohol on RNA synthesis included the addition of sucrose to the alcohol diet. If alcohol is given without sucrose, the liver does not show microscopic changes associated with cirrhotic livers (Porta et al., 1969). It was determined that without sucrose present, animals consumed enough of the laboratory chow to keep them on a nutritionally balanced diet, thereby preventing the onset of cirrhosis. When sucrose was given with the alcohol, rats ate less of the vitamin supplemented laboratory chow and such morphological changes as enlargement of hepatocytic mitochondria and flat cisternae of rough endoplasmic reticulum were seen. These changes are seen in almost all nutritional abberations. The mice in the last two experiments in this study were not given the alcohol-sucrose combination used in previous experiments (Bantle et al., 1980). This may indicate that alcohol alone is capable of causing increased polyadenylation, and that it is not a process mediated by the cell in times of nutritional imbalance.

Some explanation is needed to understand why the values for poly(A$^+$)mRNA/mg polysomal RNA do not follow the same pattern as those for poly(A). In all three experiments the control preparations were higher in poly(A$^+$)mRNA content than the alcohol samples. Several factors make it extremely

difficult to get an accurate measurement of the amount of poly(A)mRNA. The major contaminant is rRNA which aggregates with poly(A)RNA. Since the amount of poly(A^+)mRNA was determined by spectrophotmetric analysis following oligo-(dT)-cellulose chromatography it is possible that the amount of poly(A)mRNA reported is really a mixture of rRNA, oligo-(dT)-cellulose fines and poly(A) mRNA. Due to the possibility of decreasing the amount of poly(A^+)mRNA in each sample, the poly(A)mRNA fractions were not subjected to a DMSO diaggregation step followed by rebinding to another oligo)dT)-cellulose column. Therefore, values given for poly(A^+)mRNA lack the accuracy needed to express poly(A) yields as a percentage of poly(A^+)mRNA in alcohol-treated and control samples.

The effect of chronic alcohol treatment on messenger RNA content is important as a lowered quantity of mRNA could explain a decrease in protein synthesis. While polysome profile studies performed by Bantle et al. (1980) indicated no change in mRNA content, further studies may be needed to verify this observation. Since spectrophotometric methods are faulted as described above, this may require the use of a radioimmunoassay utilizing antibody binding to the 5' 7mG region of the mRNA as a means of accurately measuring mRNA content.

Another point which needs clarification is the poly(A) tracts sizes (35-48) which were reported for alcohol and control samples after separation in 2.5% polyacrylamide gels.

Another set of 2.5% gels were used with new poly(A) standards of 51 and 95 nucleotides to determine if the mobilities of the standards were correct as first reported (Exp 2). The relative mobililties for these two standards were the same in both electrophoretic runs. When compared to the 10% gels, the resolution of the 2.5% gels does not allow for separation of poly(A) tracts that are similar in size. Therefore the difference in tract sizes isolated from adjacent fractions in a 2.5% gel is approximately 13 nucleotides while the same two fractions in a 10% gel would differ in length by only 2 nucleotides. This may explain the differences in both the Mass Ave. nucleotide lengths reported and the Number Ave. nucleotide lengths from those given for other gel concentrations.

Another difficulty encountered in this study is the sharp decrease in food and/or liquid consumption in the last two experiments for all mice. This is in contrast to other studies (Banks et al., 1970; Anderson et al., 1980) in which food and/or liquid consumption remained relatively constant throughout the study. Since this lack of consumption was not reflected in large weight losses, the answer may lie in the manner in which food consumption was recorded or if any changes were made as to the number of animals per cage. If the lack of appetite is caused by addiction to alcohol, the decrease would have been limited to those mice receiving alcohol.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This study investigated the effect of chronic ethanol
ingestion on polyadenylation in mouse liver. Mice fed a
diet of 10% v/v ethanol-5% w/v sucrose were found to have
mRNA poly(A) tracts of 77 nucleotides long. Mice fed a diet
of 10% alcohol and water[*] had poly(A) tracts of 77
nucleotides while control groups had a poly(A) tract lengths
of 79 nucleotides long. These tract sizes were determined
using 10% polyacrylamide gels. A Carnation Slender® diet
combined with 5% v/v ethanol resulted in poly(A) tract
lengths of 83 nucleotides both , identical to the tract size
of the controls. The last diet used was a Purina laboratory
chow diet with 5% alcohol in water as the sole source of
liquid for the alcohol fed group and water for the control
groups. While the above studies used 10% polyacrylamide
gels, the Chow studies used both 10% and 4% polyacrylamide
gels. The 10% gels showed tract sizes of 83 nucleotides for
the control Chow group and 85 nucleotides for the Alcohol
group. Duplicate alcohol-fed groups had tract lengths of

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[*]- refers to source of liquid only. Chow added as
solid food.

100 and 110 when 4% gels were used and 83 and 96 nucleotides for the control groups. These results show no significant effect of alcohol on poly(A) tract sizes in mice kept on 5-10% alcohol diets for a period of 9-20 weeks.

Increases in the amount of poly(A)/mg polysomal RNA were repeatedly seen in all three experiments for the alcohol-fed mice. Therefore, it was determined that chronic alcohol ingestion (5-10%) results in a 1.6-2.0 increase in the amount of poly(A)/mg polysomal RNA for mice maintained on the diets described in this study.

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