# THE EFFECT OF CHRONIC ETHANOL AND SUCROSE INGESTION ON MESSENGER RNA IN MOUSE LIVER AND BRAIN

By

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THE EFFECT OF CHRONIC ETHANOL AND SUCROSE INGESTION ON MESSENGER RNA IN MOUSE LIVER AND BRAIN

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# CHAPTER I

## INTRODUCTION

In view of the possible role that drugs play in modifying macromolecular metabolism, this study was undertaken to investigate the effect of chronic ethanol and sucrose ingestion on the amount of liver polysomal poly(A)mRNA and the incorporation of newly-made mRNA into polysomes in mouse brain and liver. Sucrose was given to the mice in the ethanol solution as Porta et al.(1969) have shown that ethanol alone is incapable of inducing liver cirrhosis in laboratory animals fed normal solid diets. They could however induce cirrhosis in animals given a sucrose supplemented ethanol solution as sole source of liquid in addition to a normal solid diet. Little work has been done in this field and only Tewari et al.(1975) have conducted a detailed investigation on alterations in brain RNA metabolism following chronic ethanol ingestion in rats and mice. No work has been done on the effect of chronic ethanol ingestion on liver RNA metabolism.

The rate of ethanol metabolism is known to be increased by chronic ethanol administration (Tobon et al., 1971). Three principal mechanisms, which are capable of oxidizing ethanol to acetaldehyde have been described. These are the catalase, alcohol dehydrogenase and microsomal ethanol oxidizing systems. Only the latter two operate in vivo. Although alcohol is metabolized mostly by the liver alcohol dehydrogenase system (Isselbacher, 1964), studies on the effects of

ethanol administration on alcohol dehydrogenase activity have yielded varying results with some studies showing increases (Hawkins et al, 1966), and others no change (Lieber et al, 1968; Tobon et al, 1971) and even decreases in alcohol dehydrogenase activity (Morrison et al, 1967). Chronic alcoholic patients have faster rates of disappearance of ethanol and certain drugs from the blood (Kater et al, 1969). Tobon et al. (1971) point out that the increased rate of ethanol disappearance after ethanol administration cannot solely be explained by increases in the microsomal ethanol oxidizing activity. They speculate that unknown factors present in vivo such as differences in the intracellular distribution of ethanol or in the availability of cofactors may therefore be even more important determinants of the rate of ethanol degradation than the microsomal activity of the ethanol oxidizing enzymes.

Ethanol exerts many different types of action in the body. The metabolic effects of ethanol may be of at least three different types; firstly those resulting from alterations in the metabolic pools and and cofactors produced by the metabolism of ethanol itself (Banks et al, 1970; Kalant et al, 1970), secondly, those resulting from neuroendochrine disturbances secondary to the state of intoxication (Kuriyama et al, 1971) and thirdly those produced directly by the pharmacological action of ethanol on specific cells or processes (no confirmed reports). It is often difficult to determine to which of these catagories or combinations of them a particular metabolic consequence of ethanol ingestion may belong. In addition the metabolic effects of ethanol may vary with dose given, the composition and amount of the rest of the diet, the animal species used and the

duration of exposure to ethanol (Porta et al., 1969; Kuriyama et al., 1971). Chronic exposure may lead to adaptive changes both in rate and pathways of ethanol metabolism and in physiological effects of the drug itself. Many of the apparent contradictions and conflicting interpretations found in the literature may be the result of failure to consider sufficiently the effects of dose, duration and manner of exposure to ethanol.

An example of the difference in results obtained using chronic and acute ethanol ingestion is furnished by a report of Rothschild et al. (1971), which indicates that in isolated perfused liver, albumin synthesis is depressed by 65-75% in the presence of 50mM ethanol. On the other hand, livers from animals receiving chronic ethanol treatment synthesized albumin at the same rate as those from sucrose fed controls where the perfusion liquid did not contain added ethanol. The most likely cause for this is the difference in the blood ethanol levels in vivo and in vitro.

Another example of this type of difference appears in the work of Kuriyama et al. (1971). After a single administration of ethanol (4g/kg), by intraperitoneal injection, the measurement of $^{14}$C-leucine incorporation into protein by brain ribosomal system, prepared from mice 1.5 and 3 hours post injection, indicated a slight but significant decrease over controls. Liver ribosomes prepared from the same mice were more markedly inhibited in the protein synthesizing capacity due to ethanol treatment. Contrary to the acute effect of alcohol, they found a stimulation in protein synthesis in the ribosomal preparation after chronic ethanol administration for both brain and liver.

Whereas Kuriyama et al. (1971) find a stimulation in protein

synthesis due to chronic ethanol ingestion in mouse brain and liver, Jarlstedt (1972) reports an overall depression in protein synthesis in the brain and a slight stimulation of $^{14}$C-leucine incorporation into hepatic proteins. Tewari et al.(1971) have also measured the rate of protein synthesis following chronic ethanol ingestion and found it to be decreased as measured by $^{14}$C-leucine incorporation in vivo. The difference in results in brain between Kuriyama et al.(1971), on one hand, and Jarlstedt (1972) and Tewari et al.(1971) on the other may well be due to the length of ethanol treatment. Kuriyama et al.(1971) only treated their mice for two weeks whereas the other mice were treated for a period greater than two months. Several other factors may also contribute to the discrepancy in results; for example Tewari et al.(1971) and Jarlstedt (1972) gave their controls water to drink, whereas Kuriyama et al.(1971) fed theirs a sucrose solution, equivalent in calories to the alcohol received by the alcoholics. In all cases a different concentration of ethanol was used; Tewari et al.(1971), 10% v/v; Jarlstedt (1972), 15% v/v; and Kuriyama et al.(1971), 6% v/v. Tewari et al.(1971) and Jarlstedt (1972) used in vivo incorporation of $^{14}$C-leucine as a measure of protein synthesis, whereas Kuriyama et al. (1971) used an in vitro assay. In addition different strains of mice were used in each study.

Porta et al.(1969) were the first group to successfully reproduce cirrhosis in laboratory animals (rats). Although enlargement of hepatocytic mitochondria and the presence of large Mallory bodies were seen in animals consuming alcohol (Porta, 1969) it had not been possible to produce cirrhosis in alcohol drinking animals fed nutritionally adequate diets. They postulate that the effect of

alcohol on the liver is only potentiated by an inadequate diet particularily lacking in lipotrops, that is substances capable of curing or preventing fatty liver formation. Supplementation of the diet by a high proportion of calories derived from sucrose and alcohol disrupts, by simple dilution, the balance between calories and essential food factors present in a diet, the latter being obtained from the solid food. Alcohol itself seems not to be consumed in sufficient quantities to produce a deficiency of essential food factors in the animal. One of the earliest detectable changes seen in rats consuming alcohol as part of a nutritionally inadequate regimen is the dilation of the usually flat cisternae of the rough endoplasmic reticulum (Porta, 1969). This is a change that is observed in almost all nutritional aberrations, in addition however Porta et al. (1969) observed the known morphological changes leading to cirrhosis; the development of fatty liver and the onset of fibrosis, caused by the stimulation of collagen proline hydroxylase activity seen after chronic ethanol ingestion (Feinman et al.,1973). They further observed the appearance of regenerative nodules and the architectural distortions characteristic of cirrhosis.

As seen above, Tewari et al.(1971, 1975) have shown that chronic ethanol ingestion by mice leads to a measurable decrease in brain protein synthesis. As it was not clear whether the changes observed were related to the direct action of ethanol on the system, or were a consequence of induced changes in RNA metabolism, they decided to compare the incorporation of labeled precursor into various species of brain RNA in control (water drinking) and ethanol (10% v/v)drinking mice. Either $^{3}$H-uridine or $^{3}$H-orotic acid was injected intraventric-

ularily under ether anesthesia. In ethanol drinking mice they found an initial increase in incorporation of label into nuclear RNA compared with the controls. After 3 hours incorporation however the situation was reversed. They postulate that chronic ethanol treatment may cause a defect in the nuclear membrane and/or the transport mechanisms involved in the transport of RNA from the nucleus, leading to an initial accumulation of nuclear RNA. Then with time,and in the face of decreased nuclear and/or nucleolar RNA synthesis, in the ethanol drinking mice this pattern is reversed. In a similar study using rats they obtained a different result. In this case they looked at labeled precursor incorporation at 10, 20 and 120 minutes after the initial pulse. In the ethanol drinking rats a pronounced inhibition of nuclear RNA synthesis was observed over the entire labeling period. Indeed the maximum inhibition was found to be present at 10 and 20 minutes. Using mice they also looked at in vivo labeling of brain polysomal RNA over a two hour period after injection of radioactive precursor, Incorporation of $^{3}$H-orotic acid into polysomal RNA was inhibited in the alcohol drinking mice at all times as compared to the controls (over 70% at 1 and 2 hour points). No control experiments were performed to detect possible nuclear leakage, during the isolation of the polysomes from the cytoplasm, which would result in the contamination of the polysomes with rapidly labeled nuclear RNA. They point out that it is possible that the inhibition observed in the polysomal fraction at earlier times, up to one hour, represents messenger RNA synthesis, while the later time points reflect inhibition of ribosomal RNA synthesis. Using rats they have shown that chronic ethanol ingestion drastically inhibited incorporation of label into ribosomal RNA

in free ribosomes from alcoholics compared to controls whereas only a mild inhibition was observed with membrane bound ribosomes from alcoholic rats. In these experiments they did not look at messenger RNA.

Using mice they also found an inhibition of the incorporation of $^{3}$H-uridine into mitochondrial RNA from ethanol drinkers compared to controls. They suggest that reduced permeability of the mitochondrial membrane to nucleic acid precursors as a result of exposure to ethanol may be responsible. Similar results were found using rats. The inhibition of mitochondrial RNA synthesis due to ethanol is of interest since most of the intramitochondrial RNA is not nuclear in origin and, therefore, the effects observed in this organelle are independent of the effects demonstrated in the nuclear fraction. Decreased incorporation of uridine into tRNA due to ethanol ingestion was also observed using rats and mice. This suggests another site at which ethanol may exert its effect on brain tissue. In later studies on mice Fleming et al.(1975) observed that chronic ethanol ingestion caused a pronounced inhibition in the formation of $^{14}$C-phenylalanyl tRNA. Transfer RNAs and synthetases were isolated from the brain and characterized. It was found that the major effect of ethanol ingestion on aminoacylation is exerted on the aminoacyl tRNA synthetase. They postulate that ethanol induced hormonal changes may affect tRNA methylase activity and consequently lead to alterations in the regulating function of the tRNA acceptor activity.

Experiments were also carried out to determine if the above results might have been the results of the effects exerted by ethanol directly on the nucleotide pool. In separate experiments $^{3}$H-uridine

and $^{3}$H-orotic acid were injected into the brains of alcoholic and control mice. At one and twenty four hours post-injection the incorporation of $^{3}$H into the acid-soluble nucleotide pool was determined. The experiments carried out did not show appreciable differences in the conversion of the labeled precursor to acid soluble nucleotides. However, they only looked at uridine, adenine, guanine and cytosine pools as a whole. They did not look at individual UTP, UDP and UMP pools separately.

Jarlstedt et al.(1972) have observed the effect of long term ethanol administration on the RNA content of purkinje cells from rat brain. Premature degradation of these cells has been shown to be associated with chronic alcoholism. Rats were killed under ether anesthesia and purkinje cells isolated by micromanipulation. The RNA was extracted from one or two cells using the method of Edstrom (1964); the amount of RNA present was determined photometrically. After the rats had ingested ethanol for two months they found a decrease (7-20% depending on the region the cells came from) in the purkinje cell RNA content in the ethanol drinking mice compared to the controls. After eight months this difference had disappeared. They postulate that the nervous system which was still maturing during the first two months of ethanol administration (the cerebellum matures late in the rat) was more sensitive to the effects of ethanol.

The only mention in the literature of the effect of chronic ethanol ingestion on liver RNA is in the form of changes in RNA to DNA and RNA to protein ratios (Banks et al., 1970). They found that ethanol consumption by rats did not alter liver RNA content on a concentration basis (mg/g liver weight) but when expressed on a DNA or cellular basis

it was reduced significantly. The protein to RNA ratio (w/w) was elevated in the livers from the experimental group as compared to the controls.

The studies cited above indicate that chronic ethanol ingestion by rodents induces changes in RNA metabolism which may in turn affect the protein synthesizing capacity of neural cells. This is of particular interest as changes in protein and RNA metabolism are known to affect learning processes in experimental animals (Hyden et al., 1963; Koenig, 1958). No definitive study on the effect of chronic ethanol ingestion on messenger RNA in the brain has included the necessary control experiments for nuclear leakage during the preparation of the polysomes, which would result in the contamination of the messenger RNA by rapidly labeled nuclear RNA.

The purpose of this study was to carry out such experiments, not only for the brain, but also for the liver. One might expect changes in RNA metabolism in the liver due to chronic ethanol ingestion firstly, as protein synthesis is affected and secondly because the liver is the major detoxification organ of the body. Untoward effects of ethanol and nutrient deficiency are inseparable in most human alcoholics. The addition of sucrose to the ethanol solution, given as sole source of liquid, made it possible to mimic more closely the clinical situation where the chronic alcoholic may not consume a nutritionally adequate diet.

# CHAPTER II

# MATERIALS AND METHODS

## Animals

Adult Swiss Webster ICR strain mice obtained from Timco (Houston, Texas) were given a 10% v/v ethanol, 5% w/v sucrose solution for 4-12 months as sole source of liquid. The control animals drank water. Both groups were fed laboratory chow (Purina) ad libitum and kept in the same 12 hour alternating light and dark cycle. All experiments were begun between the first and third hour of the light cycle. The animals were not withdrawn from alcohol prior to experimentation.

## Labeling of Polysomes

Liver polysomes were labeled by intraperitoneal injection of each mouse with 500uCi of $^{3}$H-uridine, 29 Ci/mMole (Research Products International). Brain polysomes were labeled by intercranial injection of 5uCi/mouse of $^{3}$H-uridine. The mice were first anesthesized with ether (Squibb), or by intramuscular injection of 2.5 ul of Vetalar (Parke-Davis) per mouse. After a 60 minute incorporation time the mice were killed by cervical dislocation and the polysomes extracted.

## Extraction of Polysomes

Polysomes were extracted at 0-4$^{o}$C by a modification of the method

of Lee and Brawerman (1971). 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. Mouse brains or livers were homogenized, after weighing, in a Dounce homogenizer in homogenization buffer (5% w/v sucrose, 0.1 M KCl, 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.5) and 0.1% v/v Triton X-100 for the brains and 0.05% v/v for the livers), 1.25 mls of buffer was used per brain and 5 mls per liver. Bentonite (Sigma), an RNAse inhibitor, was added before homogenization, 125 ul per brain or 500 ul per liver (Poulson,1977). A teflon pestle was used for the livers whereas a glass 'A' pestle was used for the brains. The homogenate was centrifuged at 755g average for 5 minutes and then at 14,000 g average for 10 minutes, at 4°C. The resulting postmitochondrial supernatants were then equalized on the basis of protein concentration by adding buffer to the more concentrated sample.

## Polysome Gradients

Equal volumes of the postmitochondrial supernatants from alcoholic and control mice were layered onto 10-40% w/v sucrose gradients in homogenization buffer lacking Triton X-100. As a control for nuclear RNA contamination, EDTA was added to a second equal volume of control and experimental postmitochondrial supernatants to a final concentration of 0.01 M and layered onto 10-40% w/v sucrose gradients, as above except that 0.025 M EDTA was substituted for 0.01 M $MgCl_2$. The gradients were spun in an SW 27 rotor at 185,000g maximum for 2 hours 10 minutes at 4°C. The gradients were then fractionated using an ISCO density fractionator. The relative amounts of polysomes in each gradient was determined by planimetry of the absorbance scans.

## Estimation of the $^{3}$H-uridine Incorporation into Polysome Associated mRNA

Incorporated $^{3}$H-uridine in the fractions from the polysome gradients was precipitated by the addition of 2.5 volumes of ethanol (carrier RNA or DNA was added to a final concentration of 40 ug/ml). During fractionation, using an ISCO density fractionator, wall friction drags unincorporated $^{3}$H-uridine down through the gradient making the precipitation step necessary. After sitting overnight at -20°C, the precipitates were spun down at 10,000g average for 10 minutes at 4°C and resuspended in one ml of water. Four mls of Quantafluor (Mallinkrodt) were added to each sample in a minivial prior to counting. Samples were counted for 50 minutes. Counting efficiency was estimated at 38%.

## Extraction of Polysomal RNA

RNA was extracted from polysomes that had been cut from polysome gradients and pelleted at 185,000g maximum for 15 hours in an SW 27 rotor at 4°C. The pellets were resuspended in 0.1 M NaCl, 0.001 M EDTA and 0.01 M Tris-HCl (pH 7.5), (2 mls for the brain polysomes and 5 mls for the liver polysomes). The RNA was extracted by the pH 7.5 phenol-chloroform technique of Perry et al.(1972), (phenol, nucleic acid grade Bethesda Research Laboratories; Chloroform, Mallinkrodt). The RNA was stored as an ethanol precipitate at -20°C.

## RNA Gradients

Equal quantities, usually 200 ug, of the polysomal RNA from both alcoholic and control mouse polysomes were layered over 5-20% w/v

sucrose gradients in 0.1 M NaCl, 0.001 M EDTA and 0.01 M Tris-HCl (pH 7.5) and spun in an SW 41 rotor at 286,000g maximum for 5.5 hours at 4°C. The gradients were fractionated using an ISCO density fractionation system. The relative amounts of RNA in each gradient was determined by planimetry of the absorbance scans.

When mice had been injected with $^{3}$H-uridine prior to killing the fractions were divided. Half was counted for pulse labeled radioactivity, while the other half was reserved for poly(U)-hybridization.

## Quantitation of mRNA by $^{3}$H-Poly(U)-Hybridization to Poly(A) mRNA Tracts

Separate or pooled fractions from each RNA gradient were incubated with $^{3}$H-poly(U), (Miles Laboratories, 35 uCi/umole phosphate), at 40°C for 15 hours. The amount of poly(U) added depended on the amount of RNA present in the samples. Ribosomal RNA constitutes 98% of polysomal RNA the rest being mRNA. Poly(A) tracts make up 10% of the mass of the mRNA (Bantle et al, 1976). Ribosomal and transfer RNA do not have poly (A) tracts (Aviv et al., 1972). Tritiated poly(U) was added to give a 1.5 fold excess of $^{3}$H-poly(U) over poly(A). Under these conditions triplex formation does not involve more than 7% of the added poly(U) (Bantle et al., 1976). Two methods were used to digest away unhybridized $^{3}$H-poly(U) and labeled polysomal RNA.

In the first method RNAse A (Worthington Biochemical Corp.) was added to a final concentration of 33 ug/ml and the samples incubated at 37°C for 20 minutes in 0.1 M NaCl, 0.001 M EDTA and 0.01 M Tris-HCl (pH 7.5). In the second method S1 (Miles Laboratories) was used instead of RNAse. Since S1 requires zinc ions for activity no EDTA

could be present in the buffers used. After poly(U)-hybridization 1/10 volume of 10x S1 buffer, (3 M NaCl, 10 mM Zn acetate, 0.3 M Na acetate, pH 4.5), was added to each sample followed by S1 to a final concentration of 10,000 units/ml (Maxwell et al, 1978). After a 40 minute incubation at 37°C a further equal aliquot of S1 was added to each sample followed by an equal incubation.

After either digestion by RNAse A or S1, $^{3}$H-poly(U) bound to the poly(A) tracts of mRNA was precipitated by the addition of an equal volume of 10% w/v trichloroacetic acid after the addition of carrier RNA to a final concentration of 40 ug/ml. The precipitates were caught on Whatman GF/C filters and after washing in 5% w/v trichloroacetic acid, were counted at 18% efficiency using nonaqueous cocktail (toluene containing 4 g/1 PPO and POPOP 0.1 g/1).

A third method of quantitating $^{3}$H-poly(U) bound to poly(A) tracts of mRNA could be used when mice were not injected with $^{3}$H-uridine prior to killing. After $^{3}$H-poly(U)-hybridization the samples were passed over 0.5 ml hydroxylapatite columns (Biorad) at 45°C. Single stranded RNA was eluted with 15 one ml applications of 0.08 M sodium phosphate buffer, pH 6.8, and collected into scintillation vials as 5 three ml fractions. The temperature was then raised to 60°C and molecules that had a double stranded region were eluted using 5 one ml applications of 0.4 M sodium phosphate buffer, pH 6.8, collected as one ml fractions. Two mls of water was added to each 0.4 M fraction and then 17 mls of Beckman Ready-Solv GP was added to all the fractions prior to counting at 38% efficiency.

## Measurement of Uridine Nucleotide Availability in Alcoholic and Control Mouse Livers

Alcoholic and control mice were killed one hour after intraperitoneal injection with $^{3}$H-uridine (500 uCi/mouse). After removal of the gall bladder, the livers were dissected out, cut up and frozen in liquid nitrogen within 2 minutes of death. The frozen liver tissue was weighed and transferred rapidly to a tube containing 6 mls of 0.6 N perchloric acid and homogenized thoroughly using a Sorval Omnimix. The homogenates were centrifuged at 14,000g average for 10 minutes at 4°C. The pH of the supernatants was adjusted to pH 6-7 by dropwise addition of 5 M KOH, after buffering with 0.05 M Tris-HCl, pH 6.5. The supernatants were then chilled rapidly to -20°C. The precipitated potassium perchlorate was removed by centrifugation at 14,000g average for 5 minutes at 4°C. The resulting supernatant was stored at -20°C.

Ion-exchange chromatography on poly-(ethyleneimine)-cellulose (PEI cellulose) thin layer plates (Brinkman) was used to separate the uridine nucleotides (Randerath et al, 1964). Samples (10 ul) were applied on a starting line one cm from the lower edge of the plate. ATP, GTP, UMP and UTP were used as standards ($10^{-2}$M solutions in water). Interfering salts present in the samples were removed by prerunning the plates with water as a solvent. The nucleotides remain bound to their points of origin while the salts move up the plate and into the paper wick attached to the top of the plate. The plate was then developed in a closed tank using 4 M formic acid-sodium formate buffer pH 3.4. The chromatography took 60 minutes and the distance travelled by the solvent front from the point of origin was 13 cms. The nucleo-

tides were visualized under UV light to check that a good separation had been obtained. PEI cellulose plates contain a fluorescence indicator. The incorporation of tritium into the uridine nucleotides, as well as their positions on the plate, were determined by counting the radioactivity on the plate. Each lane of the plate was marked off into 1 cm sections starting at the points of origin and ending at the solvent front. Each of these sections was scraped off into a scintillation vial and, after the addition of 10 mls of cocktail (toluene containing 4 g/l PPO and 0.1 g/l POPOP), was counted at 21% efficiency.

# CHAPTER III

## RESULTS

### The effect of Chronic Ethanol and Sucrose Ingestion on Polysome Associated mRNA in Mouse Liver

This study investigated the effect of chronic ethanol and sucrose ingestion on the amount of liver polysomal poly(A)mRNA and the incorporation of newly-made mRNA into polysomes. One hour after injection of $^{3}$H-uridine polysomes were extracted from livers of alcoholic and control mice and centrifuged in density gradients as previously described (Materials and Methods). Figure 1 shows the typical polysome profiles obtained. Three repetitive experiments showed no significant difference in liver weight due to ethanol and sucrose treatment and no repetitive qualitative or quantitative differences between alcoholic and control polysome profiles. The EDTA treatment successfully disrupted the polysomes as the EDTA gradient O.D. profile reveals little high molecular weight nuclear RNA contamination (Figure 1). This was also supported by the very low level of $^{3}$H-uridine incorporated counts in these EDTA gradients. Since mRNA comprises only 2-3% of the total polysomal RNA it is impossible to detect alterations in the mRNA content of polysomes by comparison of polysome O.D. scans.

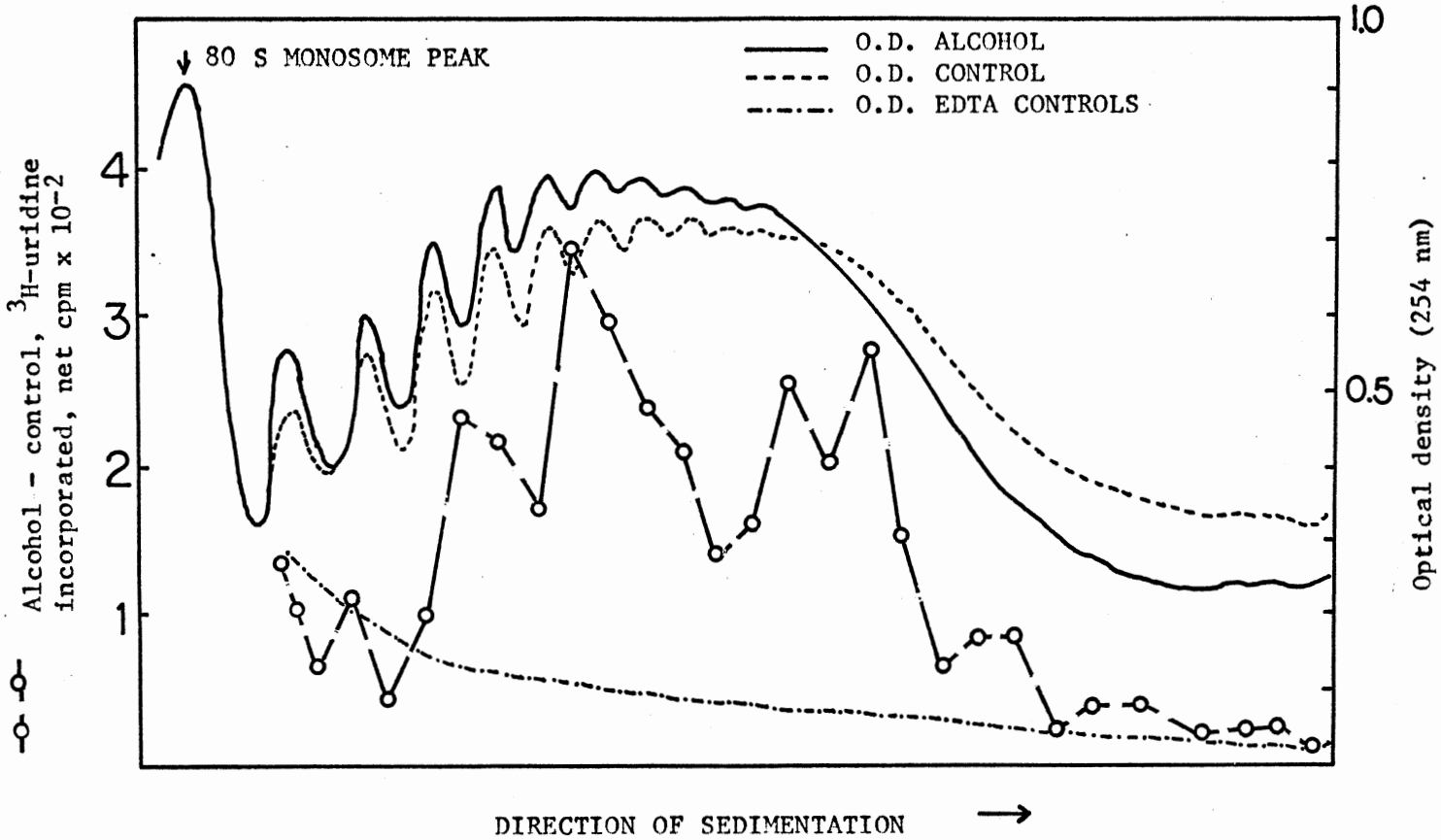


Figure 1. O.D. Profiles of Polysomes Isolated from Control and Alcoholic Mouse Livers. O.D. Scan of a Typical EDTA Control Gradient. Plot of the Distribution of the Difference in $^{3}H$- Uridine Incorporation into mRNA in Polysomes Isolated from Control and Alcoholic Mouse Livers.

Table I shows a comparison of $^{3}$H-uridine incorporation into polysome associated mRNA in alcoholic and control mouse livers. Three repetitive experiments showed a significantly greater incorporation of $^{3}$H-uridine into polysomal mRNA isolated from alcoholic than control mouse livers (see Appendix A for t test). From Figure 1 it can be seen that the distribution of the difference in $^{3}$H-uridine incorporation extends throughout the polysome profile.

This increased incorporation of $^{3}$H-uridine into polysome associated mRNA isolated from alcoholic mouse livers is also seen when RNA is purified from polysomes, sedimented in density gradients and radioactivity in fractions from these gradients is quantitated. Figure 2 shows the typical O.D. scan obtained, showing 4-5 S, 18 S and 28 S ribosomal RNA peaks. As can be seen in experiment 2, Table I an alcohol:control ratio of 2.51 was calculated for net cpm $^{3}$H-uridine incorporated/200 ug purified polysomal RNA. Figure 2 shows the distribution of the difference in $^{3}$H-uridine incorporated counts between corresponding fractions from alcohol and control gradients. This difference curve is the same shape as each of the individual component curves (not shown). It should be noted that ribosomal 18 S and 28 S O.D. peaks have no corresponding label directly under them indicating that the pulse label was specific for mRNA.

These experiments show a significant increase in $^{3}$H-uridine incorporation into polysome associated mRNA in liver due to alcohol and sucrose treatment. A large fraction of mRNAs in eukaryotic cells contain a 3' terminal covalently linked poly(A) sequence, 150-200 nucleotides in length (Edmonds et al, 1971). The $^{3}$H-uridine incorporation experiments measure incorporation of isotope into poly(A)$^{+}$ and

TABLE I

COMPARISON OF $^{3}$H-URIDINE INCORPORATION INTO LIVER mRNA AND $^{3}$H-POLY(U)-HYBRIDIZATION TO POLY(A) TRACTS OF LIVER mRNA ISOLATED FROM ALCOHOLIC AND CONTROL MICE

| | Experiment | mg PMS* applied to polysome gradient | net cpm $^{3}$H-uridine incorporated into mRNA/ mg PMS* | net cpm incorporated /200 ug purified polysomal RNA | net cpm $^{3}$H-poly(U)-hybridized/ 200 ug purified polysomal RNA |
|---|---|---|---|---|---|
| 1 | Alcohol | 20 | 1,025 | -- | 11,132 |
| | Control | 20 | 731 | -- | 5,996 |
| | A:C | | 1.40 | | 1.86 |
| 2 | Alcohol | 58 | 1,290 | 9,990 | 19,390 |
| | Control | 58 | 557 | 3,980 | 8,935 |
| | A:C | | 2.32 | 2.51 | 2.17 |
| 3 | Alcohol | 104 | 1,342 | -- | 12,486 |
| | Control | 104 | 622 | -- | 6,900 |
| | A:C | | 2.16 | | 1.81 |

PMS* - Postmitochondrial supernatant protein.

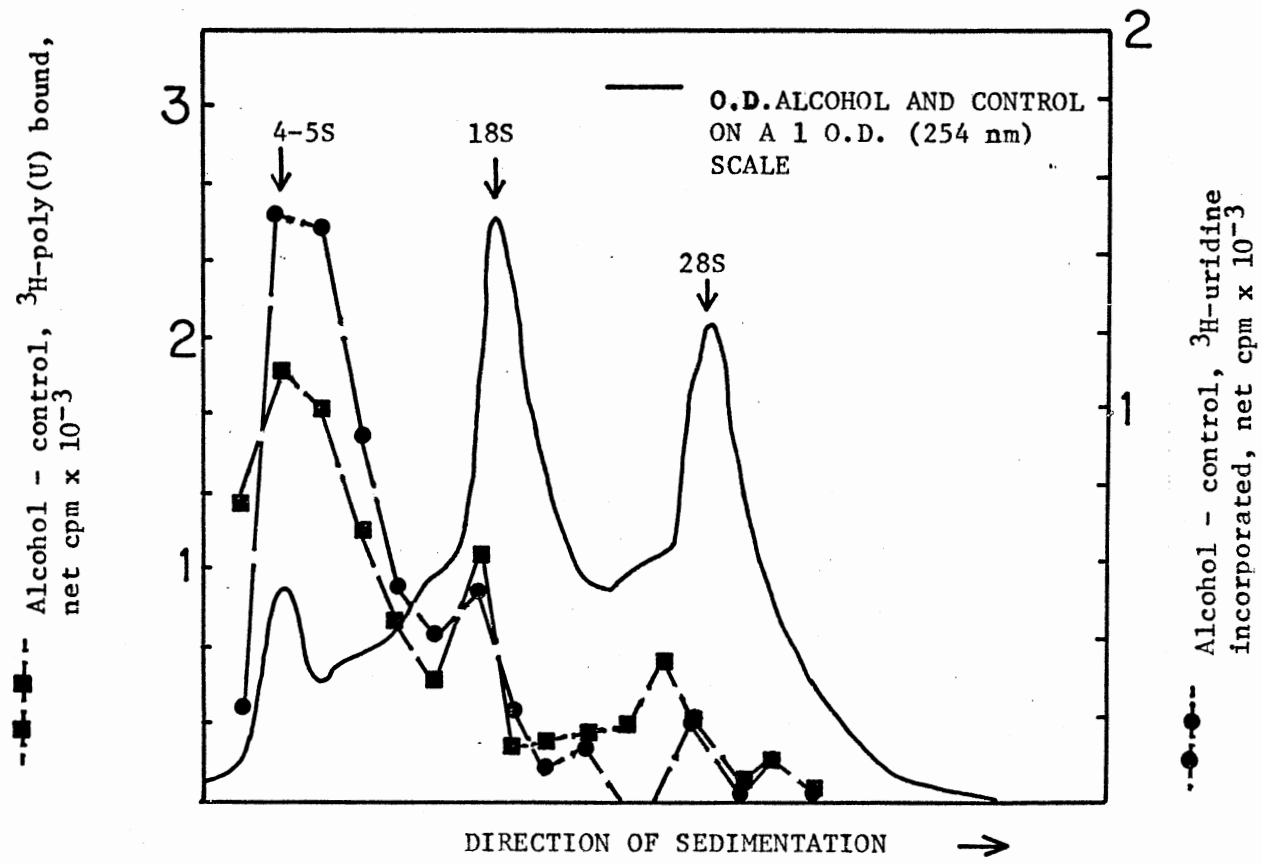


Figure 2. O.D. Profile of Isolated RNA from Mouse Liver. Plots Showing the Difference in $^{3}$H-Uridine Incorporation into mRNA and $^{3}$H-Poly(U)-Hybridization to the Poly(A) Tails of mRNA in Alcoholic and Control Mouse Purified Liver Polysomal RNA.

poly(A)$^-$ populations of mRNA associated with polysomes. A second test for the presence of mRNA used in this study only detects poly(A)$^+$mRNA. Hybridization of $^3$H-poly(U) with the poly(A) tracts of mRNA and subsequent digestion of unhybridized $^3$H-poly(U) and labeled RNA (single stranded) allows the measurement of the quantity of polyadenylated mRNA present in polysomes. Ribosomal RNA does not have poly(A) tracts (Aviv et al., 1972). Table I shows the results of $^3$H-poly(U)-hybridization to the purified polysomal RNA from alcoholic and control mouse liver polysomes. These data show that in each of 3 experiments more $^3$H-poly(U) hybridized to the polysomal RNA from the alcoholic than the control mouse liver polysomes, with alcohol:control ratios of 1.86, 2.17 and 1.81. The t test was used to demonstrate that this was a statistically significant difference (see Appendix A). Assuming that the poly(A) tract represents 10% (by weight) of the poly(A)$^+$mRNA molecules the average number of micrograms of poly(A)$^+$mRNA/ 200 ug total polysomal RNA in the alcoholic liver was calculated to be 3.31 ug as compared to 1.67 ug in the control liver (the specific activity of the poly(U) was 0.11 uCi/ug polymer and the counting efficiency was 18%). Figure 2 shows a plot of the distribution of the difference in $^3$H-poly(U)-hybridization in corresponding alcohol and control gradient fractions. It can be seen that this plot, which is the same shape as each of the individual plots for $^3$H-poly(U)-hybridized in alcohol and control gradient fractions, follows very closely the $^3$H-uridine incorporation plot, again indicating that it represents $^3$H-uridine incorporation into mRNA.

The estimation of the relative amounts of poly(A)$^+$mRNA in polysomal RNA isolated from alcoholic and control mouse livers by $^3$H-poly

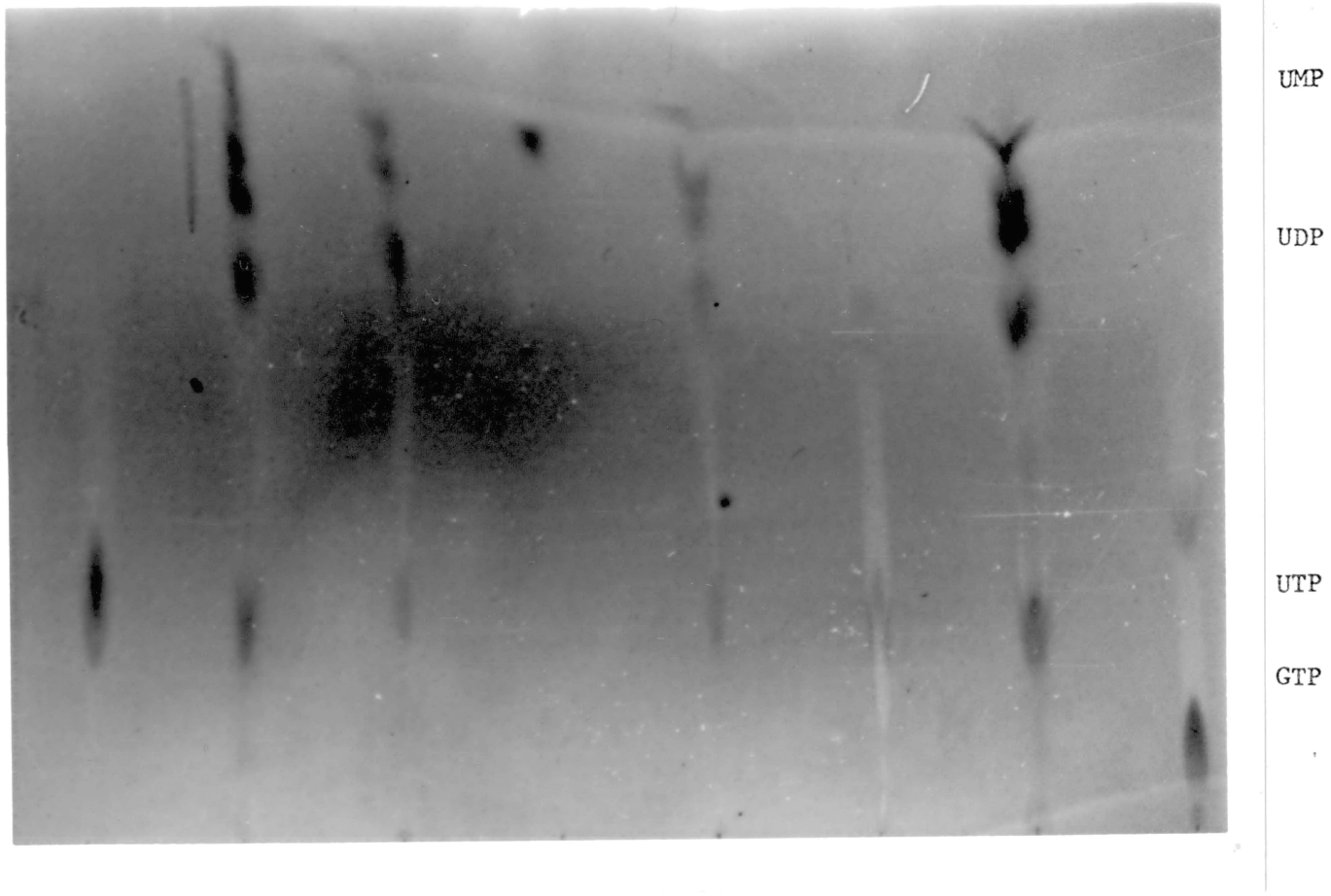


Figure 2. PEI Cellulose Thin Layer Chromatography of Nucleotides from Alcoholic and Control Mouse Livers and Standard Nucleotide Solutions.

TABLE II

DISTRIBUTION OF $^{3}H$ BETWEEN UTP, UDP AND UMP IN ALCOHOLIC AND CONTROL MOUSE LIVERS ONE HOUR POST INTRAPERITONEAL INJECTION WITH $^{3}H$-URIDINE

| | cpm | |
|---|---|---|
| | Alcohol | Control |
| UTP | 28,234 | 28,474 |
| UDP | 1,953 | 1,928 |
| UMP | 2,213 | 1,949 |

(U)-hybridization is independent of any pool size change that alcohol and sucrose ingestion may have induced. However, the results from the $^{3}$H-uridine incorporation experiments could have been influenced by changes in uridine metabolism in the liver. To check on such a possibility the distribution of isotope between UTP, UDP and UMP was compared in alcoholic and control mouse livers one hour post injection with $^{3}$H-uridine. Figure 3 shows the separation of uridine nucleotides isolated from alcoholic and control mouse livers on PEI cellulose thin layer plates, together with the locations of the standards used. The results shown in Table II indicate that chronic ethanol and sucrose ingestion had no effect on the availability of uridine nucleotides in mouse liver.

## The Effect of Chronic Ethanol and Sucrose Ingestion on Polysome Associated mRNA in Mouse Brain

This study set out to investigate the effect of chronic ethanol and sucrose ingestion on the amount of brain polysomal poly(A)$^{+}$mRNA and the incorporation of newly-made mRNA into polysomes. The results obtained were found to be influenced by the type of anesthetic used prior to injection of the brains with $^{3}$H-uridine.

One hour after injection with $^{3}$H-uridine polysomes were extracted from brains of alcoholic and control mice, which had been anesthesized with either vetalar (ketamine-HCl) or ether, and centrifuged in density gradients as described in Materials and Methods. The difference in the O.D. scans between brain polysomes from mice injected with vetalar (Figure 4) or anesthesized with ether (Figure 5) are due to 2 factors.

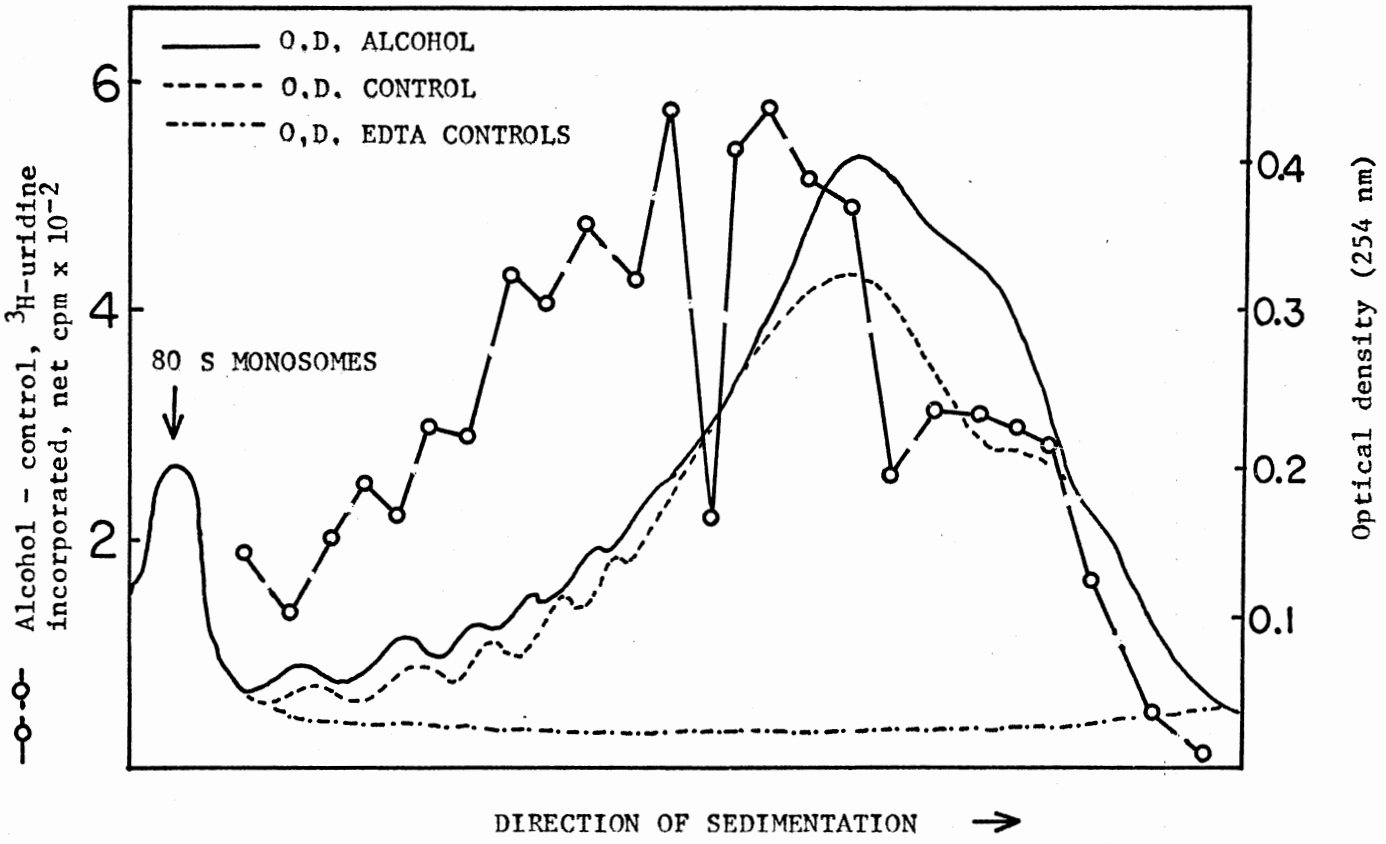


Figure 4. O.D. Profiles of Polysomes Isolated from Control and Alcoholic Mouse Brains After Vetalar Anesthesia. O.D. Scan of a Typical EDTA Control Gradient. Plot of the Difference in $^{3}$H-Uridine Incorporation into mRNA in Polysomes Isolated from Control and Alcoholic Mouse Brains After Vetalar Anesthesia.

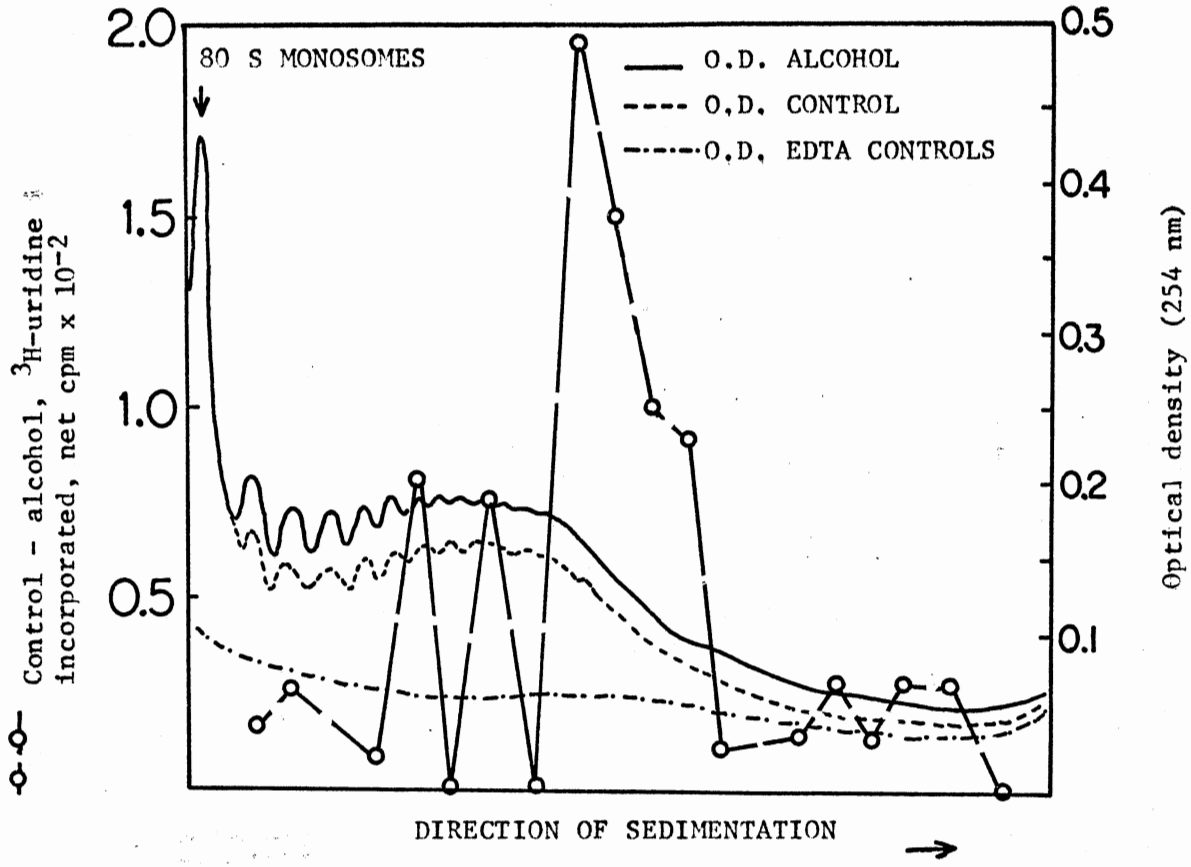


Figure 5. O.D. Profiles of Polysomes Isolated from Control and Alcoholic Mouse Brains After Ether Anesthesia. O.D. Scan of Typical EDTA Control Gradient. Plot of the Distribution of the Difference in $^{3}H$-Uridine Incorporation into mRNA in Polysomes Isolated from Alcoholic and Control Mouse Brains After Ether Anesthesia.

The enlarged hump in the O.D. profiles from the vetalar treated mice may be due to either contamination of the polysomes with glycogen or polysome aggregation. This feature was frequently seen in the polysome profiles and was not restricted to vetalar-treated mice. It did not interfere with subsequent processing of the polysomes but did make quantitation of the polysomes by planimetry of the O.D. scans less reliable. The vetalar injected mouse polysomes were run in an SW 27 rotor while those from the ether anesthesized mice were run in an SW 41 rotor. The EDTA treatment successfully disrupted the polysomes as the EDTA control gradient profiles reveal little high molecular weight nuclear RNA contamination (Figures 4 and 5). This was also supported by the very low level of $^{3}$H-uridine incorporated counts in these EDTA gradients (not shown).

When vetalar is used as the anesthetic greater incorporation of $^{3}$H-uridine into polysome associated mRNA is seen in the polysomes isolated from alcoholic as compared to control mouse brains (Table III). Campagnoni et al. (1971) have shown that a 60 minute pulse labeling period is specific for mRNA and that there is no significant incorporation of label into ribosomal RNA during this time period in rodent brains. When ether anesthesia is used prior to injection of $^{3}$H-uridine into the mouse brains a decreased incorporation of isotope into polysome associated mRNA is seen in alcoholic mouse brains as compared to control mouse brains. This is the opposite result to that obtained with vetalar anesthesia. Plots of the distribution of the difference in $^{3}$H-uridine incorporation into polysome associated mRNA from alcoholic and control mouse brains are shown in figures 4 and 5 and it can be seen that the differences extend throughout the polysome profiles in

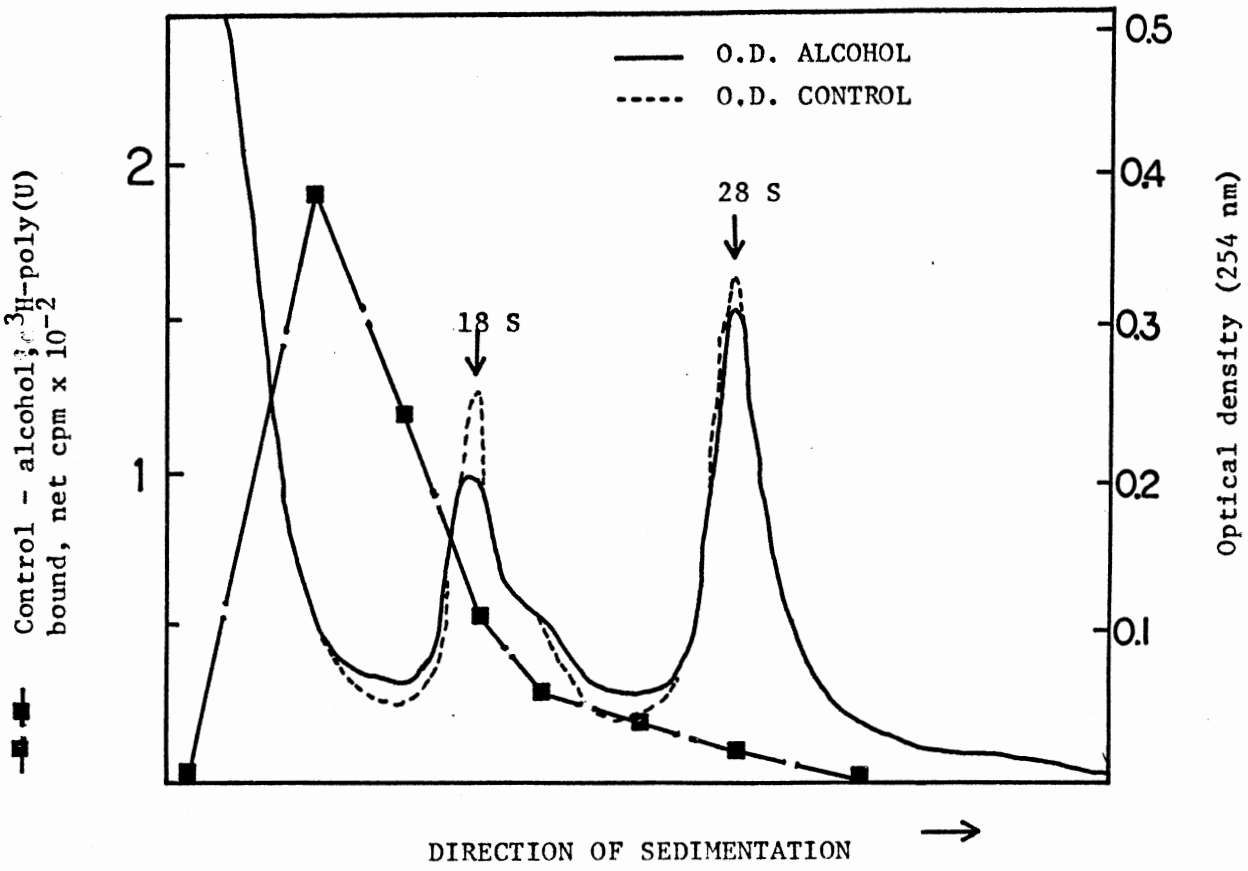


Figure 6. O.D. Profiles of Isolated Polysomal RNA from Control and Alcoholic Mouse Brains After Ether Anesthesia. Plot Showing the Difference in $^{3}H$-Poly(U)-Hybridization to the Poly(A) Tails of mRNA in Ether Anesthesized Control and Alcoholic Mouse Brain Polysomal RNA.

each case. The t test was used to demonstrate that these differences in $^{3}$H-uridine incorporation into polysome associated mRNA in alcoholic and control mouse brains are significant, although alcohol and sucrose treatment appeared to have the opposite effect in each case.

Polysomal RNA was purified from the brain polysomes of etherized alcoholic and control mice and centrifuged in density gradients as described in Materials and Methods. The 18 S and 28 S ribosomal RNA peaks are well separated and show no signs of degradation (Figure 6). The location and quantitation of the polyadenylated mRNA present was achieved by hybridization of $^{3}$H-poly(U) to any poly(A)$^{+}$mRNA in the fractions from control and alcoholic gradients as described in Materials and Methods. A significantly greater amount of $^{3}$H-poly(U) hybridized in the fractions from the control gradients as compared to the alcohol gradients (Table III and Appendix A for t test). This method of estimation of the poly(A)$^{+}$mRNA content of polysomal RNA is independent of any changes that ether or alcohol and sucrose may induce in the brain. Assuming that poly(A) represents 10% of the mass of poly(A)$^{+}$ mRNA molecules the amount of poly(A)$^{+}$mRNA in 200 ug of polysomal RNA was calculated to be 1.34 ug in the case of the etherized alcoholic mouse brains and 2.65 ug in the etherized control mouse brains (the specific activity of the $^{3}$H-poly(U) was 0.11 uCi/ ug polymer and the counting efficiency was 18%). The plot of the distribution of the difference in $^{3}$H-poly(U)-hybridized between corresponding fractions from alcohol and control gradients is given in Figure 6, and is similar in shape to each of its component curves. The ultra violet absorbing material at the top of the gradients was frequently seen in polysome profiles and is probably due to SDS (SDS is added to polysomes prior

TABLE III

COMPARISON OF $^{3}$H-URIDINE INCORPORATION INTO BRAIN mRNA AND $^{3}$H-POLY(U) HYBRIDIZATION TO POLY(A) TRACTS OF BRAIN mRNA ISOLATED FROM ALCOHOLIC AND CONTROL MICE ANESTHESIZED WITH EITHER VETALAR OR ETHER

| Experiment | | mg PMS* protein applied to polysome gradient | net cpm $^{3}$H-uridine incorporated into mRNA/mg PMS* | net cpm $^{3}$H-poly(U)-hybridized/200 ug purified polysomal RNA |
|---|---|---|---|---|
| VETALAR | | | | |
| 1 | Alcohol | 13.1 | 862 | -- |
| | Control | 13.1 | 574 | -- |
| | A:C | | 1.05 | |
| 2 | Alcohol | 4.1 | 935 | -- |
| | Control | 4.1 | 508 | -- |
| | A:C | | 1.84 | |
| ETHER | | | | |
| 1 | Alcohol | 6.8 | 874 | -- |
| | Control | 6.8 | 1,136 | -- |
| | A:C | | 0.77 | |
| 2 | Alcohol | 40.2 | 447 | 6,015 |
| | Control | 40.2 | 1,218 | 10,711 |
| | A:C | | 0.37 | 0.56 |
| 3 | Alcohol | 5.6 | 516 | 9,548 |
| | Control | 5.6 | 756 | 12,380 |
| | A:C | | 0.68 | 0.77 |

*PMS- postmitochondrial supernatant

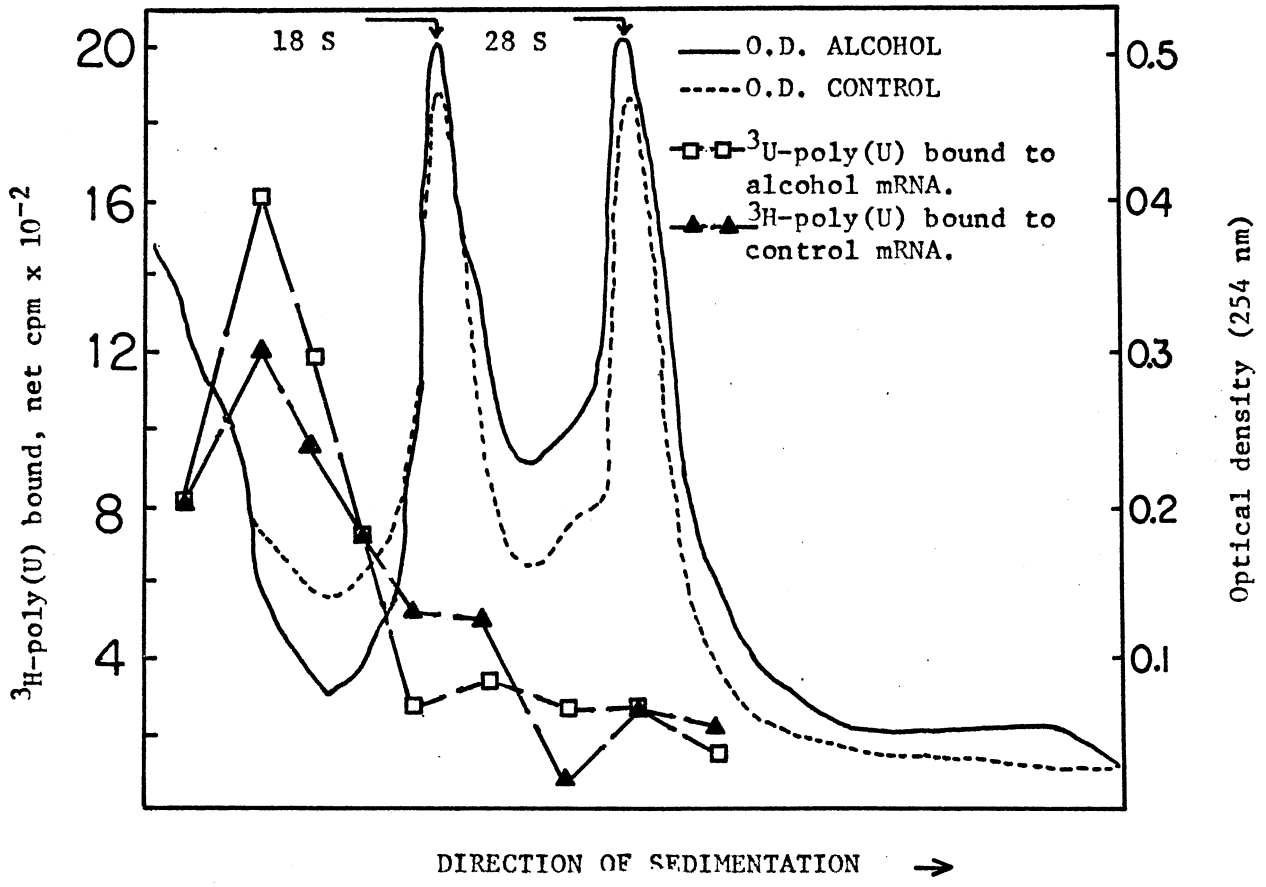


Figure 7. O.D. Profiles of Isolated Polysomal RNA from Control and Alcoholic Mouse Brains (no Anesthetic Given). Plots of $^3$H-Poly(U)-Hybridization to the Poly(A) Tails of mRNA in Control and Alcoholic Mouse Brain Purified Polysomal RNA.

to phenol extraction). It does not represent degraded poly(A)$^+$mRNA as no gross degradation is seen in the RNA in the gradients and $^3$H-poly(U) did not hybridize to the top fraction of either the control or alcohol gradients. The results from the $^3$H-poly(U)-hybridization experiments are in good agreement with the $^3$H-uridine incorporation experiments using etherized mice.

To circumvent the influence of the type of anesthetic used on the results obtained, unlabeled polysomes were prepared from the brains of alcoholic and control mice. Polysomal RNA was extracted and sedimented in density gradients (Materials and Methods). $^3$H-poly(U)-hybridization was performed in the fractions from these gradients. Figure 7 shows the O.D. profiles of the polysomal RNA and the distribution of the $^3$H-poly(U)-hybridized counts for both alcoholic and control gradients. Three repetitive experiments showed no significant difference in the amount of $^3$H-poly(U)-hybridized /200 ug purified polysomal RNA in alcohol and control gradients (Table IV and Appendix A for t test). This implies that the amount of poly(A)$^+$mRNA associated with polysomes is not altered by chronic ethanol and sucrose treatment. The estimation of the relative amounts of poly(A)$^+$mRNA by poly(U)-hybridization is independent of any pool size changes that ethanol and sucrose ingestion may have produced.

TABLE IV

COMPARISON OF $^{3}$H-POLY(U)-HYBRIDIZATION TO THE POLY(A) TRACTS OF BRAIN mRNA ISOLATED FROM UNANESTHESIZED ALCOHOLIC AND CONTROL MICE

| | Experiment | net cpm $^{3}$H-poly(U)-hybridized / 200 ug purified polysomal RNA |
|---|---|---|
| 1 | Alcohol | 11,468 |
| | Control | 8,418 |
| | A:C | 1.36 |
| 2 | Alcohol | 9,287 |
| | Control | 9,630 |
| | A:C | 0.96 |
| 3 | Alcohol | 7,540 |
| | Control | 11,063 |
| | A:C | 0.68 |

# CHAPTER IV

## DISCUSSION

The aim of this study was to examine the effect of chronic ethanol and sucrose ingestion on polysome associated mRNA in mouse brain and liver. To date, no reports have appeared in the literature on the effect of chronic ethanol and/or sucrose ingestion on any liver RNA population. Tewari et al.(1971, 1975) have investigated the effect of chronic ethanol ingestion on mouse and rat brain RNA populations. In their work they used ether anesthesia on their animals prior to experimentation. This study found that ether anesthesia influences the results obtained when comparing $^{3}$H-uridine incorporation into polysome associated mRNA in alcoholic and control mouse brain. In addition, they did not perform control experiments to detect and correct for nuclear RNA leakage.

The mice used in this study were given a 10% v/v ethanol, 5% w/v sucrose solution as sole source of liquid for 4-12 months prior to experimentation. This regimen was chosen as opposed to that used by Tewari et al.(1971, 1975) who gave their animals ethanol unsupplemented with sucrose as Porta et al (1969) have shown that the latter regimen is incapable of inducing liver cirrhosis in laboratory animals fed a normal solid diet. They could however, induce cirrhosis in laboratory animals given a 10% v/v ethanol, 5% w/v sucrose solution as sole source of liquid in addition to a normal solid diet. Reduced intake of essen-

tial nutrients due to anorexia or unavailability of food is characteristic of chronic human alcoholics. Nutrient deficiencies may occur because of ethanol induced malabsorption or malutilization of nutrients (Leevy et al., 1975). Estimates of the relative importance of these possible causes of malnutrition have not been determined. Untoward effects of ethanol and nutrient deficiency are inseparable in most human alcoholics. The addition of ethanol to the sucrose solution made it possible to mimic more closely the clinical situation where the chronic alcoholic may not consume a nutritionally adequate diet. As the mice were obtaining added calories in the form of sucrose from their drinking water they did not consume as much solid food, which contains all their required nutrients.

In this study chronic ethanol and sucrose ingestion was found to affect the incorporation of $^{3}$H-uridine into polysome associated mRNA and the amount of polysome associated poly(A)$^{+}$mRNA in mouse liver. Alcoholic mice showed an increased incorporation of $^{3}$H-uridine into polysome associated mRNA. The difference in $^{3}$H-uridine incorporated counts were spread throughout the polysome profile indicating that many different types of mRNA may be affected. The position of a particular mRNA molecule in a polysome profile is determined by its length as it is usual for all mRNA molecules in the profile to be completely charged with ribosomes (Krall et al., 1975). No significant incorporation of $^{3}$H-uridine into ribosomal RNA was observed indicating that the pulse was specific for mRNA. Control experiments were performed to detect and correct for possible nuclear RNA contamination. In three separate experiments $^{3}$H-uridine incorporation into polysome associated liver mRNA in the alcoholic exceeded the control mouse by ratios of 1.4, 2.32

and 2.16. The chronic ingestion of alcohol and sucrose produced no change in liver weight or polysome yield. No significant difference in the availability of UTP, UDP and UMP was found in alcoholic and control mouse livers. These results do not distinguish between there being 1.4-2.32 times more mRNA from alcoholic mouse liver present in the polysomes than there being the same amount of mRNA present but labeled to higher specific activity with $^{3}$H-uridine. $^{3}$H-poly(U)-hybridization experiments showed that there is 1.18-2.27 times more poly(A)$^{+}$mRNA in polysomes isolated from alcoholic mouse livers assuming that there is no change in length of the poly(A) tract due to ethanol and sucrose ingestion. Clegg et al.(1978) found a decrease in poly(A) tract size distribution in the 2-cell mouse embryo as compared to the fertilized or unfertilized egg (90-170 and 120-200 nucleotides respectively). They conclude however, that the drop in poly(A) content which is found in the 2-cell stage cannot be accounted for solely by a reduction in poly(A) tract length and may, therefore, represent a turnover of maternally inherited poly(A)$^{+}$mRNA.

It is possible that the increased $^{3}$H-uridine incorporation into polysome associated mRNA in alcoholic mouse livers represents a 1.4-2.32 fold increase in the amount of poly(A)$^{+}$ and poly(A)$^{-}$mRNA associated with polysomes. The $^{3}$H-poly(U)-hybridization experiments show that there is 1.18-2.17 times as much poly(A)$^{+}$mRNA present, assuming that there is no change in poly(A) tract length. Since no quantitative difference in the amount of polysomes in the alcohol and control gradients was observed one could postulate that the ribosome spacing is more open in the polysomes isolated from alcoholic mouse liver. If this were the case one would expect to see repetitive qualitative

differences between alcohol and control polysome profiles. The alcohol gradient would have significantly greater quantities of light polysomes and fewer heavy ones. This was not observed but this theory can not be excluded on this basis as the polysome gradients, as preparative gradients, contained larger quantities of polysomes than one would run on analytical gradients geared towards maximum resolution.

If the ribosome spacing is unaltered by ethanol and sucrose ingestion in mouse liver, given that there is no quantitative difference in the polysomes, then the amount of mRNA in each gradient must be the same. Yet the $^{3}$H-poly(U)-hybridization experiments show that there is 1.81-2.17 times as much poly(A)$^{+}$mRNA in alcoholic mouse liver polysomes. It is therefore postulated that the increase in poly(A)$^{+}$ mRNA is accompanied by a corresponding decrease in the amount of poly-(A)$^{-}$mRNA in the alcoholic mouse liver polysomes. If this is so then the $^{3}$H-uridine incorporation data indicates a faster rate of turnover of newly-synthesized mRNA in these polysomes. This theory assumes that there is no change in poly(A) tract length due to ethanol and sucrose ingestion.

The effect of chronic ethanol and sucrose ingestion on polysome associated mRNA in mouse brain was also investigated. The results obtained were found to be influenced by the type of anesthesia used on the mice prior to injection of their brains with $^{3}$H-uridine. If vetalar was used as the anesthetic an increased incorporation of $^{3}$H-uridine into polysome associated mRNA was observed in the alcohol as compared to the control brain polysomes (alcohol:control ratios of 1.5 and 1.8). On the other hand if ether was used, the situation was reversed with 1.3-1.5 fold greater incorporation of $^{3}$H-uridine into polysome assoc-

iated mRNA in brains from control mice as compared to alcoholics. When purified polysomal RNA from etherized control and alcoholic mice was used in $^{3}$H-poly(U)-hybridization experiments it was found that there was 1.3-1.78 times as much poly(A)$^{+}$mRNA associated with polysomes in the control as compared to the alcoholic mouse brain (assuming that there is no change in poly(A) tract size).

Two possible theories could be used to explain these results, based on whether or not chronic ethanol and sucrose ingestion together with ether anesthesia cause the total amount of polysome associated mRNA to alter. $^{3}$H-poly(U)-hybridization experiments show that there is 1.3-1.75 times as much poly(A)$^{+}$mRNA associated with control as compared to alcoholic etherized mouse brain polysomes. The decrease in the amount of poly(A)$^{+}$mRNA could be accompanied by a corresponding increase in poly(A)$^{-}$mRNA, thus keeping the total amount of polysome associated unchanged. The decreased $^{3}$H-uridine incorporation seen in the etherized alcoholic mouse brain would then represent decreased turnover of polysome associated mRNA. It is possible that chronic ethanol and sucrose ingestion together with ether anesthesia does alter the amount of polysome associated mRNA. For instance, both poly(A)$^{+}$ and poly(A)$^{-}$mRNAs may be decreased or just poly(A)$^{+}$mRNA alone may be affected, leading to a decrease in the total amount of polysome associated mRNA. As the quantity of polysomes, as measured by planimetry of the O.D. scans, was the same in alcohol and control gradients any change in the amount of polysome associated mRNA should be reflected in qualitative changes in the alcohol polysome profiles (with decreased polysome associated mRNA a shift towards heavier polysomes, caused by increased ribosome stacking). No such qualitative changes were seen in alcohol gradients.

This theory can not be dismissed however, as the resolution in these preparative gradients may not have been sufficient to enable the detection of such qualitative changes.

As it is not known what effect ether alone has on polysome associated mRNA, nor the antagonistic or synergistic effects it may have with alcohol it is impossible to separate the effect of ether from any effect of chronic ethanol and sucrose ingestion may have on polysome associated mRNA. It should also be noted that $^{3}$H-uridine incorporation experiments on anesthesized mice could be influenced by unknown changes that chronic ethanol and sucrose ingestion and/or anesthesia could have on the availability of uridine nucleotides. Tewari et al.(1975) have found that chronic ethanol treatment of mice did not affect the availability of uridine in etherized alcoholic mouse brains. The results obtained in this study on the incorporation of $^{3}$H-uridine into polysome associated mRNA in alcoholic and control etherized mice are in good agreement with their results.

Tewari et al.(1975) found that incorporation of $^{3}$H-orotic acid, after a 60 minute pulse, into alcoholic mouse polysomes is only 53% of the incorporation into control mouse polysomes. Since they found an initial increase in the incorporation of label in to nuclear RNA in alcoholic mouse brains as compared to controls (0-3 hours post injection) they postulate that chronic ethanol ingestion may cause a defect in the nuclear membrane and/or the transport mechanisms involved in the transport of RNA from the nucleus. They only perform pulse label experiments, a pulse chase experiment would have allowed them to follow the label incorporated into nuclear RNA, over a particular time period, into the cytoplasm and thus check the validity of their theory.

To circumvent the effect that anesthesia appeared to be having on the results obtained, unlabeled polysomal RNA was prepared from unanesthesized alcoholic and control mice. This RNA was then used in $^{3}$H-poly(U)-hybridization experiments. No significant difference was found in the amount of polysome associated poly(A)$^{+}$mRNA isolated from the brains of alcoholic and control mice. It should be noted that the estimation of the amount of poly(A)$^{+}$mRNA present by poly(U)-hybridization is independent of any pool size change that ethanol and sucrose ingestion may induce in mouse brain. No quantitative or qualitative changes were observed between the alcohol and control polysome profiles and thus the total amount of mRNA in the polysomes may be unaffected by the ethanol and sucrose treatment. It is possible however, that the resolution in these preparative gradients may not have been sufficient to detect some qualitative change, brought about by an increase or decrease in the amount of polysome associated poly(A)$^{-}$mRNA in mouse brain due to chronic ethanol and sucrose ingestion.

The interactions of ethanol with other drugs have been recognised for many years. Amorosi (1931) exposed alcoholized guinea pigs to chloroform and ether. He found that the excitatory phase of anesthesia was prolonged and more violent. However, the total amount of anesthesia required to cause death was about a third that required with the control animals. Schuppel (1970) reported interactions between barbiturates and ethanol and postulated that inhibition of hepatic hydroxylase activity by large doses of ethanol may account for the increased sensitivity of intoxicated individuals to barbiturates while induction of the barbiturate hydroxylation system by prolonged exposure to

alcohol might partially account for the resistance of sober alcoholics to barbiturates. The molecular mechanism by which vetalar or ether interact with ethanol is unknown.

In conclusion, this study has shown that chronic ethanol and sucrose ingestion causes an increase in the amount of poly(A)$^+$mRNA associated with polysomes in mouse liver. This may or may not be accompanied by a change in the amount of poly(A)$^-$mRNA. If the total amount of polysome associated mRNA remains constant, that is, if there is a corresponding decline in the amount of poly(A)$^-$mRNA in the alcoholic mouse liver, the increased $^3$H-uridine incorporation into polysomes isolated from alcoholic mouse liver represents increased turnover of polysome associated mRNA. This theory could be tested by isolating and quantitating poly(A)$^+$ and poly(A)$^-$mRNA populations from total polysomal RNA isolated from alcoholic and control mouse livers. Kuriyama et al. (1971) and Jarlstedt (1972) find that chronic ethanol ingestion increases hepatic protein synthesis, this could be a reflection of the increased turnover of newly-synthesized mRNA in polysomes isolated from alcoholic mice. However it could also be due to a greater amount of mRNA in alcoholic mouse liver polysomes. Increased protein synthesis is not unexpected in the liver due to chronic ethanol ingestion as it is the major detoxification organ of the body. Activities of hepatic microsomal enzyme systems, which metabolize ethanol and other drugs, have been shown to increase after chronic ethanol ingestion (Lieber et al, 1968). Accelerated blood clearance of drugs including ethanol is also seen following ethanol ingestion (Misra et al., 1971).

Interactions of ethanol with anesthetics were observed while exam-

ining the effect of chronic ethanol and sucrose ingestion on polysome associated mRNA in mouse brain. In the absence of anesthesia alcohol and sucrose treatment had no effect on the amount of polysome associated poly(A)$^+$mRNA in mouse brain. These results are interesting in that they demonstrate that a drug can affect mammalian gene expression in one tissue with no apparent effect in the other and that the action of one drug may be modified by the presence of another drug in the system.

Ethanol and sucrose ingestion may not affect the amount of polysome associated mRNA in unanesthesized mouse brain or the ratio of poly(A)$^+$ to poly(A)$^-$mRNA in the brain polysomes. It is possible however, that gene expression is affected qualitatively by the induction of alterations in the composition of the mRNA being translated due to ethanol and sucrose ingestion. A comparative study of gene products in alcoholic and control mouse brains would need to be performed to test this hypothesis. Tewari et al.(1971, 1977) find a decrease in protein synthesis in vivo and invitro due to ethanol ingestion. They found that ribosomes isolated from ethanol treated rats (administered by gastric intubation twice daily for 14 days) exhibited a lower activity than control rat ribosomes (given sucrose solution isocalorically balanced to ethanol) in a cell free system synthesizing polyphenylalanine using a poly(U) template (Tewari et al., 1977). They also found an increase in the protein content of the ribosomal fraction following ethanol administration and postulate that ethanol induced changes at the ribosomal level may result in the defective association of mRNA with the ribosomes leading to an overall depression in brain protein synthesis. Another site at which chronic ethanol ingestion

could affect protein synthesis is shown by the work of Fleming et al (1975) who showed a decrease in tRNA aminoacyl synthetases due to chronic ethanol ingestion. Spirin et al. (1976) have shown that ethanol (0.1- 3 M) had a greater inhibitory effect on poly(U) directed translation by Escherichia coli ribosomes in the absence of elongation factors than in factor dependent systems. It is suggested that the factor free system is energitically more weak and hence more susceptible to inhibition than the more powerful ribosomal machinery involving elongation factors with GTP. It is possible that ethanol ingestion could affect the availability and/or action of elongation factors thus making the translation process more sensitive to the drug.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

This exploratory study investigated the effect of chronic ethanol and sucrose ingestion on the amount of liver and brain polysome associated poly(A)$^+$mRNA and the incorporation of newly-made mRNA into polysomes. Mice were injected, intraperitoneally for the liver or intercranially for the brain, with $^3$H-uridine and after 60 minutes incorporation the organs were weighed and polysomes extracted. Samples applied to density gradients were equalized on the basis of protein content, EDTA dissociation of polysomes was used to detect and correct for possible contamination of the polysomes with nuclear RNA. After fractionation, planimetry of the polysome profiles was used to compare the relative amounts of polysomes in each gradient. $^3$H-poly(U)-hybridization with purified polysomal RNA sedimented into density gradients was used to quantitate and locate the poly(A)$^+$mRNA present. All experiments were analyzed using the t test for paired observations.

Chronic ethanol and sucrose ingestion was found to increase the incorporation of $^3$H-uridine into liver polysomal RNA and the amount of poly(A)$^+$mRNA associated with liver polysomes (assuming that ethanol does not affect poly(A) tract length). Ethanol and sucrose treatment did not affect the yield of liver polysomes. It was not possible to positively ascertain whether or not the treatment affected the poly(A)$^-$ mRNA population associated with liver polysomes. Two possible models

are put forward which could explain these data. In the first model, which assumes that the poly(A)$^-$mRNA population associated with liver polysomes is increased or remains unchanged by ethanol and sucrose ingestion, postulates that the ribosomal spacing is more open in the alcoholic mouse liver polysomes. The second model, which assumes that the increase in poly(A)$^+$mRNA content of the polysomes is accompanied by a corresponding decrease in the amount of poly(A)$^-$mRNA thus keeping the total amount of polysome associated mRNA the same, postulates that the increased incorporation of $^3$H-uridine odserved in the alcoholic mouse liver is due to increased turn over of polysome associated mRNA. Chronic ethanol and sucrose ingestion had no effect on the availability of uridine nucleotides in the liver.

When anesthesized mouse brains were used in similar experiments it was found that the results obtained depended on the type of anesthesia used. However, unanesthesized mice showed no difference in the amount of poly(A)$^+$mRNA due to chronic ethanol and sucrose ingestion (assuming no alteration in poly(A) tract length). If the poly(A)$^-$mRNA population is unaffected by this treatment it is still possible that chronic ethanol and sucrose ingestion cause qualitative changes in gene expression.

In conclusion, this study has shown that chronic ethanol and sucrose ingestion does affect mRNA associated with liver polysomes in the mouse by increasing $^3$H-uridine incorporation and the amount of poly(A)$^+$mRNA associated with liver polysomes. No effect of chronic ethanol and sucrose ingestion on poly(A)$^+$mRNA associated with brain polysomes could be demonstrated in unanesthesized mice. If anesthesized mice were used, differences in $^3$H-uridine incorporation into

brain polysome associated mRNA could be demonstrated indicating an ethanol:anesthetic interaction. The molecular nature of this interaction is unknown.

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APPENDIX A

## PAIRED t STATISTIC

Given a set of paired observations from two normal populations with means $\mu_1, \mu_2$ (unknown)

| $x_i$ | $x_1$ | $x_2$ | ... | $x_n$ |
|---|---|---|---|---|
| $y_i$ | $y_1$ | $y_2$ | ... | $y_n$ |

let

$$D_i = x_i - y_i$$

$$\overline{D} = \frac{1}{n} \sum_{i=1}^{n} D_i$$

$$s_D = \sqrt{\frac{\Sigma D_i^2 - \frac{1}{n}(\Sigma D_i)^2}{n-1}}$$

$$s_{\overline{D}} = \frac{s_D}{\sqrt{n}}$$

The test statistic

$$t = \frac{\overline{D}}{s_{\overline{D}}} ,$$

which has n - 1 degrees of freedom (df), can be used to test the null hypothesis

$$H_0: \mu_1 = \mu_2 .$$

This test was performed on a Hewlet Packard HP-25 calculator using the following program and programming instructions:

PROGRAM

| DISPLAY LINE | DISPLAY CODE | KEY ENTRY |
|---|---|---|
| 00 | | |
| 01 | 41 | - |
| 02 | 25 | $\Sigma+$ |
| 03 | 13 00 | GTO 00 |
| 04 | 14 22 | f s |
| 05 | 24 03 | RCL 3 |
| 06 | 14 02 | f $\sqrt{x}$ |
| 07 | 71 | ÷ |
| 08 | 14 21 | f $\bar{x}$ |
| 09 | 21 | $x \rightleftarrows y$ |
| 10 | 71 | ÷ |
| 11 | 74 | R/S |
| 12 | 24 03 | RCL 3 |
| 13 | 01 | 1 |
| 14 | 41 | - |
| 15 | 13 00 | GTO 00 |
| 16 | | |
| 17 | | |
| 18 | | |
| 19 | | |
| 20 | | |
| 21 | | |
| 22 | | |
| 23 | | |
| 24 | | |

PROGRAMMING INSTRUCTIONS

| STEP | INSTRUCTIONS | INPUT DATA/UNITS | KEYS | | | | OUTPUT DATA/UNITS |
|---|---|---|---|---|---|---|---|
| 1 | Key in program | | | | | | |
| 2 | Initialize | | f | REG | f | PRGM | |
| 3 | Perform for i = 1,..., n: | | | | | | |
| | Input one pair of observations | $x_i$ | ↑ | | | | |
| | | $y_i$ | R/S | | | | i |
| 4 | Delete erroneous data | $x_k$ | ↑ | | | | |
| | | $y_k$ | - | f | $\Sigma-$ | | |
| 5 | Compute t and df | | GTO | 04 | R/S | | t |
| | | | R/S | | | | df |
| 6 | For new case, go to step 2 | | | | | | |

The results of this statistical test, performed on the experimental data obtained in this study, are given in Table V in this Appendix.

TABLE V

ANALYSIS OF RESULTS USING THE T TEST
FOR PAIRED OBSERVATIONS

| Parameters measured in Alcoholic and Control Mice | Degrees of Freedom | t | Confidence Level at which Difference is significant ($\alpha$)+ |
|---|---|---|---|
| LIVER | | | |
| $^3$H-uridine incorporation into mRNA /mg PMS* protein | 2 | 4.04 | 0.1 |
| $^3$H-poly(U)-hybridized/ 200ug purified polysomal RNA | 2 | 4.15 | 0.1 |
| BRAIN - VETALAR | | | |
| $^3$H-uridine incorporation into mRNA /mg PMS* protein | 1 | 5.14 | 0.1 |
| BRAIN - ETHER | | | |
| $^3$H-uridine incorporation into mRNA /mg PMS* protein | 2 | 2.45 | 0.1 |
| $^3$H-poly(U)-hybridized/ 200ug purified polysomal RNA | 1 | 4.15 | 0.1 |
| BRAIN - NO ANESTHETIC | | | |
| $^3$H-poly(U)-hybridized/ 200ug purified polysomal RNA | 2 | 0.14 | No significant** difference |

+ ($\alpha$) is the probability of rejecting $H_o$ if it is in fact true.
* postmitochondrial supernatant.
** null hypothesis accepted, = 0.005.

## APPENDIX B

## GLOSSARY OF TERMS

| | |
|---|---|
| ATP | Adenosine-5'-triphosphate. |
| Alcohol | Synonymous with ethanol. |
| Chronic | Greater than 4 months duration. |
| Contaminant Nuclear RNA | High molecular weight rapidly labeled ribonucleoprotein of nuclear origin that is coisolated with polysomes. |
| EDTA | Ethylenediaminetetraacetic acid. Preferential chelator of divalent cations. Disodium salt was used in this study. |
| Ethanol | Synonymous with alcohol. |
| GTP | Guanosine-5'-triphosphate. |
| Messenger RNA (mRNA) | Single stranded RNA molecule that is synthesized during transcription in the nucleus, is complementary to one strand of DNA and serves to transmit the genetic information in the DNA to the ribosomes for protein synthesis. It includes both poly(A)$^+$ and poly(A)$^-$ messenger RNA. |
| Monosome | Complex that consists of a single ribosome attached to a single strand of messenger RNA. |
| PEI cellulose | Polyethyleneimine ion-exchange cellulose. |
| Poly(A) | Polyadenylic acid, a homopolymer of adenine-containing nucleotides. |
| Poly(A) Tract/Tail | Tail of poly(A), 150-250 nucleotides in length, that is covalently linked to the 3' end of many eukaryotic messenger RNA molecules. |

| | |
|---|---|
| Poly(A)$^+$ or Poly(A) mRNA . . | Messenger RNA that has a poly(A) tail covalently linked to its 3' end. |
| Poly(A)$^-$mRNA . . . . . | Messenger RNA molecules lacking a poly (A) tail. Does not distinguish between those molecules that never had a poly (A) tail and those that lost it through cleavage. |
| Poly(U) . . . . . | Polyuridylic acid. A homopolymer of uracil containing nucleotides. |
| Polysome . . . . . | A strand of messenger RNA with two or more ribosomes attached to it. |
| POPOP . . . . . | 1,4-bis(2-(5-phenyloxazolyl))-benzene. A scintillant used in liquid scintillation counting. |
| PPO . . . . . | 2,5-diphenyloxazole. A secondary scintillant used in liquid scintillation counting. |
| SDS . . . . . | Sodium dodecyl sulphate. A detergent. |
| Ribosomal RNA (rRNA) . . . . | RNA that is linked, non covalently, to the ribosomal proteins in the 2 ribosomal subunits and that constitutes about 80% of the total cellular RNA. Usually designated by sedimentation coefficients of 5, 18 and 28 S. |
| Transfer RNA (tRNA) . . . . | A low molecular weight molecule, 4-5S, that binds an amino acid and transfers it to the ribosomes for incorporation into a polypeptide chain during translation. |
| UDP . . . . . | Uridine-5'-diphosphate. |
| UMP . . . . . | Uridine-5'-monophosphate. |
| UTP . . . . . | Uridine-5'-triphosphate. |

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