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DIFFERENTIAL EFFECT OF CHRONIC ETHANOL ADMINISTRATION ON RATES OF PROTEIN SYNTHESIS ON FREE AND MEMBRANE-BOUND POLYSOMES *IN VIVO* IN RAT LIVER DURING DEPENDENCE DEVELOPMENT

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Abstract—Administration of ethanol thrice daily to rats in amounts sufficient to induce a high degree of physical dependence resulted in a 20% decrease in the rate of protein synthesis on liver membrane-bound polysomes *in vivo* after 3 days of treatment without affecting the rate on free polysomes. The inhibition was attributable to a decrease in the rate of polypeptide elongation as evidenced by comparable decreases in nascent chain synthesis and completed protein release without any change in leucine uptake by liver. Chronic ethanol treatment did not affect the quantity or distribution of free and membrane-bound polysomes, the DNA concentration, or the weight of liver. The inhibition of protein synthesis on membrane-bound polysomes cannot, therefore, be readily ascribed to ethanol-induced nutritional deficiencies or to some nonspecific toxic effect of ethanol.

Chronic ethanol administration has been shown to increase the content of cytoplasmic protein in rat liver [1, 2]. However, the role of protein synthesis in this accumulation is still in doubt in spite of much investigation [3–12]. Resolution of this question has been hampered because of the scarcity of suitable methods for producing ethanol dependence in adequately nourished rats and for studying protein synthesis *in vivo*. Furthermore, we are not aware of any previous attempts to study the effects of chronic ethanol administration on the rate of protein synthesis on free and membrane-bound polysomes *in vivo* in rat liver during the development of physical dependence on ethanol.

In the studies presented here, the intact rat was used to investigate the effects of chronic ethanol administration on the rate of protein synthesis on free and membrane-bound polysomes *in vivo* in liver during dependence development. Rats were rendered physically dependent on ethanol by forced administration of a vitamin-supplemented nutritionally complete liquid diet followed 1 hr later by ethanol, thrice daily, a regimen which produces relatively constant blood ethanol concentrations and maintains the body weight of ethanol-treated animals at the same level as that of the controls [13]. This approach minimized problems associated with inclusion of ethanol in the diet and provided a high degree of physical dependence in 3 days in well-nourished rats [13, 14]. Rates of protein synthesis were determined 10 min after administering a 500 μmoles/kg dose of low specific activity leucine, a technique which expands the intracellular pool of leucine and maintains it relatively constant during the period of measurement [15]. This approach not only minimized problems associated with uptake, compartmentation and reutilization of leucine, but it also provided a means of determining the actual rates of protein synthesis and of locating the step in protein synthesis blocked by inhibition. The results show that chronic ethanol administration selectively reduces the rate of protein synthesis on membrane-bound polysomes by decreasing the rate of polypeptide elongation.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley derived rats (Biolab, Minneapolis, MN), weighing 175–250 g, were housed individually in stainless steel cages with wire mesh floors and maintained in 12-hr light and dark cycles with free access to food and water for 3–5 days prior to use.

Induction of physical dependence. Rats (three to six per group) were rendered physically dependent on ethanol essentially as described by Baker *et al.* [13]. Both ethanol (or sucrose) and nutrients were administered by intragastric intubation using a 16-gauge pediatric feeding needle and a 20-ml plastic syringe. Ethanol (22.5%, v/v) or an equivalent volume of sucrose solution calorically comparable to the dose of ethanol (controls) was administered initially at a dose of 6 g/kg and, subsequently, 1 hr after each nutrient feeding at a dose of 0, 1.5, 3 or 5 g/kg as determined by behavioral criteria of intoxication [13] at 9:00 a.m., 5:00 p.m. and 1:00 a.m. for 1, 2 or 3 days (three, six or nine doses). Nutrients in the form of a nutritionally complete liquid diet (Sustacal, vanilla flavor, Mead Johnson Laboratories, Evansville, IN) supplemented with additional vitamins (0.3%, v/v, Homicebrin, Eli Lilly Co., Indianapolis, IN) were administered to both control and treated animals at 8-hr intervals (8:00 a.m., 4:00

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p.m. and 12:00 a.m.) for 1, 2 or 3 days (three, six or nine treatments). The amount of nutrients per feeding was determined by the difference between the initial body weight and the body weight just prior to each feeding converted to milliliters (1 ml of nutrients/g of weight loss) plus an additional 5 ml of nutrients. The mean doses of ethanol were 14.0 ± 1.0, 11.2 ± 1.0 and 14.7 ± 0.3 g/kg on days 1, 2 and 3 respectively. The mean volumes of nutrients per feeding were 13.3 ± 0.7, 15.7 ± 0.7 and 14.5 ± 0.3 ml on days 1, 2 and 3, respectively, for controls and 14 ± 1, 15.3 ± 1 and 16 ± 1 ml for ethanol-treated rats. The effect of this treatment regimen on withdrawal hyperexcitability, a measure of ethanol dependence, has been published [14].

Measurement of rates of protein synthesis in vivo. Twelve hours after the last dose of ethanol, when ethanol was undetectable in blood, each rat was lightly anesthetized with a circulating air–ether mixture and given an intravenous injection of low specific activity [4,5-^{3}H]leucine (2.5 mCi/kg of 5 Ci/mole in 3.3 ml of 0.9% NaCl/kg) via the jugular vein. Ten minutes after administering the isotope, the animal was perfused with ice-cold 0.25 M sucrose containing 1 mM $MgCl_2$ via the portal vein for 45 sec to rapidly terminate protein synthesis. The liver was excised and placed in cold perfusion medium; all subsequent steps were performed at 0–4°. A 20% (w/v) homogenate was prepared and centrifuged to separate free polysomes (nonsecretory protein-synthesizing compartment) from membrane-bound polysomes (secretory/membrane protein-synthesizing compartment) as described previously [16]. Aliquots (1 ml) of the two compartments were centrifuged at 1° for 25 min at 226,000 g_{max} to remove all ribosomes [17], and aliquots of that supernatant fraction were used to determine released protein radioactivity by trichloroacetic acid precipitation on filter paper discs [18] followed by digestion with 0.5 ml of NCS (Amersham/Searle, Arlington Heights, IL), acidification with glacial acetic acid, and counting in toluene fluor [16]. Another set of aliquots (3 ml) was layered over 4 ml of 1.38 M sucrose containing high salt medium [16] and centrifuged at 1° for 4 hr at 226,000 g_{max} to isolate and purify the polysomes. The polysomal pellets were dissolved in 0.5% (w/v) sodium dodecylsulfate, and aliquots of that solution were used to determine nascent chain radioactivity using Aquasol (Amersham/Searle) and ribosomal RNA recovery from the absorbance at 260 nm, assuming that 20 A_{260} units = 1 mg of RNA. Prior to separation of the two polysome fractions, four aliquots (0.1 ml) of the homogenate were placed on filter paper discs, two were digested with 1 ml of NCS, acidified, and counted as above to determine total intracellular radioactivity, and two were processed as described by Mans and Novelli [18] and then treated as above to determine total protein radioactivity; [^{3}H]leucine uptake was obtained by difference (total intracellular minus total protein radioactivity).

Display of polysomes. Equal portions of each polysome fraction and its corresponding ribosome-free supernatant fraction (prepared as described above) were layered over identical 19 ml 20–47% (w/w) linear sucrose gradients containing high salt medium and centrifuged in opposite buckets of the same SW 27 rotor (Beckman) at 1° for 2 hr at 131,000 g_{max}. After centrifugation, each set of gradients (sample and ribosome-free blank) was monitored simultaneously at 254 nm with a dual-beam analyzer (model UA-5, Instrumentation Specialities Co., Lincoln, NE) to obtain the difference profile [17].

Analytical determinations. The concentration of ethanol in blood (50 μl from the tail) was determined essentially as described by Roach and Creaven [19], using tertiary butanol as internal standard, and found to be between 3 and 4 mg/ml at various times from 1 to 8 hr after the last dose of ethanol throughout the entire treatment period as described previously [14].

Chemical analyses. DNA was determined by the method of Burton [20] using calf thymus DNA as standard. Protein was determined by the procedure of Lowry *et al.* [21], with bovine serum albumin serving as standard.

Statistical analysis. Statistical comparisons between two means were made using a two-tailed Student's *t*-test.

Table 1. Effect of chronic ethanol administration on body weight and on liver weight, DNA concentration, and free and membrane-bound polysome concentration*

Days	Treatment	Body wt (g)	Liver wt (g)	DNA concentration (mg/g liver)	Free polysome concentration (mg/g liver)	Bound polysome concentration (mg/g liver)
1	Control	234 ± 6	12.2 ± 0.2	2.74 ± 0.09	2.40 ± 0.05	5.64 ± 0.12
	Ethanol	234 ± 6	12.2 ± 0.2	2.60 ± 0.03	2.70 ± 0.18	5.41 ± 0.08
2	Control	231 ± 6	12.1 ± 0.1	2.48 ± 0.04	2.27 ± 0.06	5.94 ± 0.20
	Ethanol	232 ± 6	12.1 ± 0.2	2.56 ± 0.07	2.34 ± 0.02	5.38 ± 0.56
3	Control	232 ± 7	12.2 ± 0.1	2.62 ± 0.10	2.37 ± 0.08	5.40 ± 0.14
	Ethanol	230 ± 7	12.1 ± 0.2	2.48 ± 0.16	2.45 ± 0.10	5.29 ± 0.29

* Rats (six per group) were treated with ethanol or sucrose for the indicated times and weighed just prior to being killed. Livers were excised and weighed, and 20% homogenates were prepared and fractionated into free and membrane-bound polysome fractions as described under Materials and Methods. Aliquots of the homogenates and polysome fractions were used to measure DNA and polysomes respectively. Values are the means ± S.E. of determinations on six animals.

RESULTS

We have demonstrated previously that the treatment regimen used in this study produced a linear increase in ethanol dependence over the first 3 days of treatment without reducing body weight [14]. Here we examine the influence of this regimen on rates of protein synthesis on free and membrane-bound polysomes *in vivo* in rat liver.

Effect of chronic ethanol administration on DNA and ribosome concentration in liver. Table 1 shows that the concentration of DNA remained constant throughout the 3-day treatment period, suggesting that there were no large differences in the size or number of cells, since liver weights were also similar. The concentration of polysomes was determined by sedimenting the polysomes through a discontinuous sucrose gradient and measuring the absorbance at 260 nm. Previous studies have demonstrated nearly quantitative recovery of polysomes with this method [16]. Table 1 shows that there was no difference in the concentration of either free or membrane-bound polysomes throughout the 3-day treatment period. Moreover, the total concentration on polysomes (7.7 to 8.2 mg/g tissue) and the relative distribution of polysomes (30–33% free polysomes) were similar to those obtained in normal liver from fed rats, suggesting that ethanol probably had little effect on ribosome synthesis and degradation.

Effect of chronic ethanol administration on [^{3}H]leucine uptake and rates of protein synthesis in vivo *in liver.* Table 2 shows that there was no difference in [^{3}H]leucine uptake throughout the 3-day treatment period. In addition, Table 2 shows that there was no difference in the rate of protein synthesis (the rate of release of completed proteins) in either the free polysome compartment throughout the 3-day treatment period or in the membrane-bound polysome compartment during the first 2 days of treatment. There was, however, a 20% decrease in the rate on membrane-bound polysomes after 3 days of treatment, suggesting that ethanol-treated rats were synthesizing less secretory protein than control rats. This effect was not detectable at 24 hr after the last dose of ethanol (data not shown), indicating that it was not due to irreversible liver damage.

Site of ethanol inhibition of protein synthesis. Analysis of polysome size and of the flow of labeled leucine from nascent chains to released protein can be used to deduce the site of inhibition of protein synthesis. Figure 1 compares the distribution of the total complement of free and membrane-bound ribosomes on sucrose gradients after 3 days of chronic ethanol treatment with that of corresponding controls. In both compartments, ethanol increased the proportion of ribosomes which sediment faster than disomes, i.e. polysomes. As might be expected, a corresponding decrease in the proportion of ribosomes which sediment slower than disomes, i.e. ribosomal subunits and monosomes, was observed in the free compartment. (The difference in the subunit/monosome region between the two bound polysome compartments is an artifact due either to incomplete blank subtraction in this region of the profile [17] or, more likely, to cross-contamination of the bound

Table 2. Effect of chronic ethanol administration on the rates of protein synthesis in free and membrane-bound polysome compartments and on [^{3}H]leucine uptake of rat liver *in vivo**

		Free polysome compartment			Bound polysome compartment			Uptake
Days	Treatment	Released protein (dpm/mg RNA × 10^{-3})	Nascent chains (dpm/mg RNA × 10^{-3})	r/n	Released protein (dpm/mg RNA × 10^{-3})	Nascent chains (dpm/mg RNA × 10^{-3})	r/n	(dpm/g tissue × 10^{-4})
1	Control	326 ± 7	20.0 ± 0.3	16.3 ± 0.3	341 ± 26	26.5 ± 0.4	12.9 ± 1.1	156 ± 11
	Ethanol	357 ± 20	20.4 ± 0.8	17.5 ± 0.5	359 ± 20	26.0 ± 0.8	13.8 ± 1.0	154 ± 12
2	Control	318 ± 13	21.1 ± 0.3	15.1 ± 0.6	324 ± 21	27.6 ± 0.8	11.7 ± 0.6	154 ± 8
	Ethanol	302 ± 12	19.8 ± 0.3	15.3 ± 0.7	304 ± 14	26.5 ± 0.9	11.5 ± 0.3	163 ± 7
3	Control	327 ± 2	21.9 ± 0.8	15.0 ± 0.5	356 ± 11	28.8 ± 0.6	12.4 ± 0.4	147 ± 8
	Ethanol	317 ± 8	20.1 ± 0.6	15.8 ± 0.5	293 ± 15†	23.3 ± 0.7†	12.6 ± 0.4	155 ± 5

* Rats (six per group) were treated with ethanol or sucrose for the indicated times and then pulse-labeled with [^{3}H]leucine for 10 min. Livers were excised and processed for the isolation of polysomes and released proteins from free and membrane-bound polysome compartments as described under Materials and Methods. Values are the means ± S.E. of determinations on six rats.

† Treatment group different from respective control group, Student's *t*-test, $P < 0.05$.

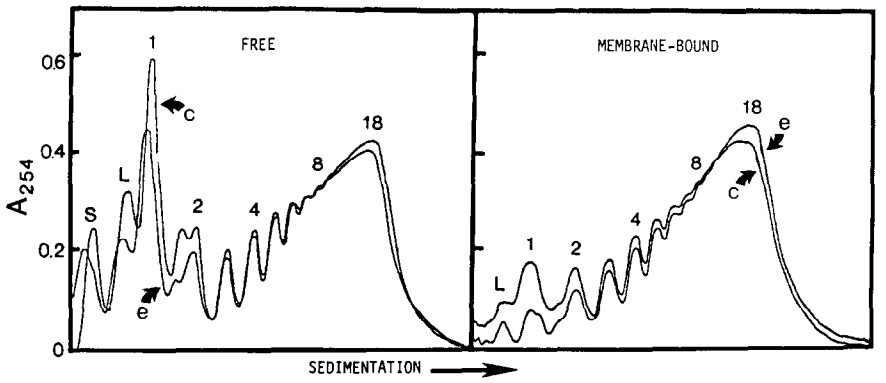

Fig. 1. Effect of chronic ethanol administration on the size and quantity of free and membrane-bound polysomes in rat liver. Rats were treated with ethanol or sucrose for 3 days. Livers were excised and fractionated into free and membrane-bound polysome compartments. Aliquots of the two polysome compartments and their corresponding ribosome-free blanks were displayed on sucrose gradients and monitored simultaneously at 254 nm to obtain a difference profile. Treatments are designated as follows: e = ethanol; and c = control. Ribosomal species are designated as follows: S = 40S ribosomal subunits; L = 60S ribosomal subunits; 1 = 80S monosomes; and 2, 4, 8 and 18 = di-, tetra-, octa- and octodecimosomes.

compartment with the free compartment [16, 17], since ribosomal subunits and monosomes are found only in the free state in rat liver.) In addition, Fig. 1 shows that the average size of membrane-bound polysomes was larger after ethanol treatment as indicated by a shift in the peak to denser regions of the gradient, whereas the size of free polysomes was virtually unchanged. The average size of membrane-bound polysomes increased from about 15-somes to about 18-somes as estimated by the extrapolation procedure of Morton [22], suggesting that either polypeptide elongation or termination was decreased relative to initiation. To choose between these alternatives, the flow of label from nascent chains to released protein was examined. Table 2 shows that there were comparable decreases in the rate of nascent chain synthesis and completed protein release (about 80% of control). This indicates that the rate of elongation was decreased, since if the major effect of ethanol were to decrease the rate of termination, the specific activity of nascent chains from ethanol-treated rats should be similar to that of the controls.

DISCUSSION

The present study shows that chronic administration of ethanol in amounts sufficient to induce a high degree of physical dependence in 3 days [14] inhibited protein synthesis on membrane-bound polysomes, but had little effect on protein synthesis on free polysomes. Our analysis of the potential mechanisms indicates that ethanol inhibited protein synthesis on membrane-bound polysomes by reducing the rate of mRNA translation, rather than by altering the amount or relative proportion of the two classes of polysomes, the uptake of amino acids, or the size of hepatocytes. Moreover, the decreased rate of translation on membrane-bound polysomes was not attended by a decrease in the average size of membrane-bound polysomes. On the contrary, the average size was increased slightly; hence, it is likely that mRNA is not rate-limiting for protein synthesis in the ethanol-treated rat. Furthermore, the differential effect of ethanol on protein synthesis on membrane-bound polysomes indicates that it was not inhibiting protein synthesis indiscriminately by inducing nutritional deficiencies, or by some non-specific toxic effect. Inhibitory effects of chronic ethanol administration on hepatic protein synthesis have also been observed by others [3, 5, 9, 10, 12]. However, in none of these cases has evidence been presented that chronic ethanol administration produced this effect by reducing the rate of mRNA translation. Indeed, all of the previous work in intact animals was performed with trace doses of high specific activity amino acids and long pulse-labeling times and, hence, of necessity disregarded possible differences in amino acid uptake, compartmentation, and reutilization. Differential effects of chronic ethanol administration on protein synthesis on free and membrane-bound polysomes have also been observed by others using cell-free protein-synthesizing systems as a measure of protein synthesis *in vivo* [12]. However, previous studies of protein synthesis *in vitro* have generally been difficult to interpret because of the notoriously low efficiency of liver cell-free systems (rates of elongation are usually 100–1000 times slower than *in vivo*) and because of insufficient evidence to show that the polysomes were representative and undergraded.

Since secretory proteins are synthesized predominantly on membrane-bound polysomes, it seems logical to conclude that chronic ethanol administration decreases the production of plasma proteins, the major secretory proteins of liver. This conclusion is not inconsistent with previous evidence demonstrating secretory protein accumulation in the liver [1], if one assumes that chronic ethanol administration interferes with the ability of the liver to export proteins as suggested by other workers [1]. However, in contrast to the previous work, the treatment regimen used in the present study did not cause liver

hypertrophy, a sign of fat and protein accumulation [1], presumably because of the protective effect of protein and choline in the liquid diet used here [23] and, more likely, because of the relatively short duration of treatment used here.

From the analysis of protein synthesis, we conclude that ethanol inhibits secretory protein synthesis by decreasing the rate of polypeptide elongation rather than by altering the rate of initiation or termination or by producing premature release of nascent chains. After chronic ethanol administration for 3 days, the average membrane-bound polysome was slightly larger than that of the controls in spite of a decrease in the rate of protein synthesis, thus ruling out effects at the level of initiation. The parallel decrease in nascent chain synthesis and completed protein release after ethanol treatment for 3 days shows that both the rate of ribosome movement along the mRNA and the rate of release of completed chains are decreased proportionately, indicating inhibition of elongation rather than termination or premature release of nascent chains. Inhibition of termination would preferentially depress the rate of completed protein release relative to that of nascent chain synthesis, whereas premature release would depress the rate of nascent chain synthesis relative to that of completed protein release. Furthermore, the differential effect of chronic ethanol administration on secretory protein synthesis indicates that it was not inhibiting elongation directly by reducing the concentration or activity of enzymes involved in peptide bond formation, the concentration of tRNAs, or the level of the energy charge, since these factors are common to both protein-synthesizing compartments. It may be that chronic ethanol administration causes some derangement of membrane structure that retards the rate on elongation on membrane-bound polysomes. These results are to be contrasted with the disaggregation of polysomes caused by acute ethanol exposure in perfused rabbit liver [24] and in reticulocytes incubated *in vitro* [25]. Since protein synthesis occurs only on polysomes, the maintenance of liver and erythrocyte function in the presence of ethanol appears inconsistent with the disaggregation of polysomes.

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