

Pathogenesis of Alcohol-Induced Accumulation of Protein in the Liver

E. BARAONA, M. A. LEO, S. A. BOROWSKY, and C. S. LIEBER

From the Laboratory of Liver Disease, Nutrition, and Alcoholism, Bronx Veterans Administration Hospital, Bronx, New York 10468, and Mount Sinai School of Medicine, of the City University of New York, New York 10029

ABSTRACT Alcohol feeding to rats produced hepatomegaly, associated with enlargement of the hepatocytes. The increase in liver dry weight was accounted for not only by fat but also by protein accumulation, primarily in microsomes and cytosol, with a selective increase in export proteins: concentrations of both immunoreactive albumin and transferrin were augmented in liver microsomes and cytosol of ethanol-fed rats. To investigate the mechanism of this protein accumulation, [^{14}C]leucine was injected intravenously and its incorporation into both liver and serum proteins was measured after various time intervals. Rates of synthesis and export were assessed from protein labeling and specific activities of leucyl-tRNA. Synthesis of liver protein and proalbumin were enhanced by chronic ethanol feeding, but this was not associated with a corresponding rise in serum albumin output. Actually, there was a significant retention of the label in liver albumin and transferrin with delayed appearance in the serum of ethanol-fed rats. This indicated that, regardless of the changes in synthesis, the export of protein from the liver into the plasma was impaired. This alteration in export was associated with a decreased amount of polymerized tubulin in the liver of ethanol-treated animals. Thus, both enhanced protein synthesis and defective export contribute to the ethanol-induced accumulation of liver protein, and the decrease in liver microtubules represents a possible site for impairment of protein export.

INTRODUCTION

Enlargement of the liver is common in alcoholics. Hepatomegaly has been produced also in rats fed

nutritionally adequate diets containing 36% of calories as ethanol. We recently showed, in a preliminary report (1), that the increase in liver dry weight was not merely the result of fat accumulation, but also of increased liver protein, with a selective accumulation of some proteins which are primarily exported from the liver. The present study was undertaken to expand the observation of protein accumulation in the liver and to assess its mechanisms, particularly the possible roles of increased hepatic synthesis and decreased disposition of proteins.

METHODS

Materials. L-[U- ^{14}C]- (325 mCi/mmol) and L-[N-4,5- ^3H]- (5 Ci/mmol)-leucine and [ring C, methoxy- ^3H]colchicine (18.45 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). The immunoglobulin G fractions of rabbit antirat serum albumin and antirat transferrin as well as rat serum albumin and transferrin were purchased from Cappel Laboratories, Inc. (Downingtown, Pa.) and shown to react specifically with the corresponding serum proteins by agarose immunoelectrophoresis. Horse spleen ferritin and rabbit antihorse ferritin were obtained from Miles Laboratories Inc. (Elkhart, Ind.). Proteins with an immunoelectrophoretic purity 95% were used as standards. Rat serum albumin was labeled "in vitro" with ^{14}C by the method of Rice and Means (2), resulting in albumin-specific activities of 3×10^6 dpm/mg. Proalbumin was prepared from the immunoprecipitates of acetone powder extracts of liver microsomes by the procedure of Quinn et al. (3). The composition of the liquid diets used in this study has been previously described (4). They contain 18% of the total calories as protein, 35% as fat, 11% as carbohydrate, and 36% either as ethanol (alcohol diet) or as additional carbohydrate (control diet).

Animal procedures. Weanling CD male rat littermates, purchased from Charles River Breeding Laboratories (Wilmington, Mass.), were fed Purina chow (Ralston Purina Co., St. Louis, Mo.) until they reached a weight of 130–150 g. Then they were housed in individual cages and pair-fed the liquid diets described above for 4–6 wk. To minimize differences in the time of food intake preceding sacrifice, one-third of the amount of diet usually consumed was given at 9 a.m. and two-thirds at 5 p.m. the day before

Parts of this study were presented at the Annual Meetings of the American Federation for Clinical Research (Atlantic City, N. J., 4 May 1975) and the American Association for the Study of Liver Diseases (Chicago, Ill., 4 November 1975).

Received for publication 6 July 1976 and in revised form 21 April 1977.

sacrifice. On the following day (between 9–10 a.m.), rats were weighed and given 6 ml of diet/100 g body wt by gastric tube 90 or 150 (five pairs) min before sacrifice. This amount corresponds to one-fifth of the usual daily food intake of the pair-fed animals. In the case of the alcohol diet, this dose is equivalent to 3 g of ethanol/kg body wt and results in blood ethanol concentrations of 130–150 mg/100 ml at 90 min and 153 ± 9 mg/100 ml at 120 min (5, 6).

To assess contamination of the liver extracts with plasma, 1 mg of the ^{14}C -labeled rat serum albumin was injected in the dorsal vein of the penis, under ether anesthesia, 48 h before sacrifice. This interval was chosen to allow for equilibration with albumin of the extravascular space (7).

To study the time-course of labeling in liver and serum, [^{14}C]leucine was injected in the dorsal vein of the penis at various times (0.3, 1, 2.5, 5, 7.5, 10, 30, 45, 90 and 120 min) before sacrifice.

Rats were sacrificed by exsanguination from the aorta, under ether anesthesia. For determinations of protein content and radioactivity in serum and liver, blood was obtained from the aorta and this was followed by perfusion of the liver with 50–100 ml of ice-cold saline through the portal vein to minimize contamination of liver extracts with blood. Liver perfusion was also required for the measurement of cytochrome P-450 in the liver homogenates (8). For determinations of specific activities of serum and liver leucine and those of leucine bound to transfer ribonucleic acid (leucyl-tRNA), liver perfusion was omitted, blood was obtained from the aorta, and this was followed by freezing of the liver *in situ* between aluminum blocks previously cooled in liquid nitrogen. Data on liver weight, specific gravity, morphology, and microtubules were also obtained in rats in which the liver perfusion was omitted. Liver volume was determined by displacement of water at 20°C. Samples of the liver were dried in a dessicator containing Drierite (Drierite Co., Xenia, Ohio). Liver specimens were fixed in neutral formalin (10 g/100 ml) and stained with hematoxylin and eosin for morphological assessment. Hepatocyte area was calculated by counting the number of hepatocytes per standard area. Blind assessment was not possible because the ethanol-fed rats had visible accumulation of liver lipids.

Subcellular fractionation. Livers were homogenized in 3 vol of ice-cold 0.15 M KCl or 0.25 M sucrose by two up and down strokes in a Potter-Elvehjem homogenizer with a motor driven teflon pestle. Because the bulk of liver protein is contained in mitochondria, microsomes, and cytosol, these fractions were isolated by centrifugation. Nuclei and cell debris were sedimented at 700 g. The supernate was further centrifuged at 9,000 g for 30 min. The sediment was used as a source of mitochondria, whereas the supernate was again centrifuged at 104,000 g for 60 min to produce a microsomal pellet and a cytosolic supernate. To correct for the recovery of these fractions, glutamate dehydrogenase activity (9) was used as a marker of mitochondria (10, 11); cytochrome P-450 (12), as a marker of microsomes (8); and alcohol dehydrogenase (13), as a marker of cytosol (10). The protein content of each fraction was calculated by dividing the activity or content of the markers in the initial homogenate by their specific activities (per milligram of protein) in the subcellular fractions.

Analytical procedures. Total liver lipids were extracted according to the method of Folch et al. (14) and measured by the method of Amenta (15). Liver proteins were extracted according to Siekevitz (16), measured by the method of Lowry et al. (17), and their radioactivity determined by liquid scintillation counting in Aquasol (New England Nuclear, Boston, Mass.) DNA was measured in the liver by the method of Martin et al. (18). Albumin and transferrin

were measured in serum and liver by radial immunodiffusion (19), using the corresponding serum proteins as standards. These proteins were extracted from particulate liver fractions with both 0.5% desoxycholate (20) and ultrasonic vibration (21). Total liver immunoreactive albumin was also calculated from the isotopic dilution of labeled serum albumin in the acid ethanol extracts of the immunoprecipitates, as described by Gordon and Humphrey (7). Hepatic proalbumin was determined by isotopic dilution according to the method of Dorling et al. (22). Liver ferritin was measured by radial immunodiffusion using horse spleen ferritin as standard, according to the method of Bjørklid and Helgeland (23). Plasma volume was calculated from the dilution of T-1824 (Evans blue), as described by Gregersen (24), except that the dye was measured after clearing the serum of particulate fat by ultra-centrifugation at 1.3×10^6 g/min, because turbidity due to hyperlipemia was present in the ethanol-fed rats. Leucine concentrations and radioactivities were measured by ion exchange chromatography in an automated amino acid analyzer (model 119, Beckman Instruments, Inc., Cedar Grove, N. J.) provided with stream splitter device. Leucine-specific activities were determined in serum (25), protein-free supernates of the liver (26), and in hepatic leucyl-tRNA at various time intervals. The method used by Wallyn et al. (27) for determination of valyl-tRNA-specific activity was applied to leucyl-tRNA after demonstration that leucine, similarly to valine, originated solely from the amino acyl-tRNA pool. There was no contamination of the leucine bound to tRNA with differently labeled leucine added to the initial homogenates either in the free state or incorporated into serum albumin.

Rates of protein synthesis were calculated according to Morgan and Peters (26), except that the integral of the specific activity curve of leucyl-tRNA was used instead of that of free leucine (26, 28) because of the reported amino acid compartmentation during protein synthesis (29, 30). For calculation of the synthesis of total liver protein and proalbumin, the incorporation after 10 min of intravenous injection of [^{14}C]leucine was used. The values for leucine content of total liver protein (28) and proalbumin (3, 31) were obtained from the literature. Hepatic production of serum albumin was calculated from the data on leucine incorporation into serum albumin 120 min after injection, as described by Morgan and Peters (26).

Liver microtubules were assessed by the amount of polymerized tubulin, their major component. Free tubulin was measured by taking advantage of the ability of its dimer form to bind [^3H]colchicine in a 1:1 molar ratio (32). Unbound colchicine was adsorbed on activated charcoal (33). Polymerized tubulin was calculated from the difference in colchicine binding when microtubule depolymerization was allowed to occur (total tubulin) and when it was prevented (free tubulin), as recently reported by Patzelt et al. (34), except that the method to preserve the microtubules was that of Filner and Behnke (35). Subsequent depolymerization of the preserved microtubules produced an amount of tubulin equivalent to 84% of that calculated from the difference between total and free tubulin.

Statistics. The values obtained in the alcohol-fed animals were compared to those obtained in their pair-fed controls and the means of the individual differences were tested by the Student's *t* test (36). All values are expressed by their means \pm SEM.

RESULTS

Effects of ethanol feeding on body and liver weights. As previously reported (37), the ethanol-fed rats gained

less weight than their pair-fed controls. Thus, at the time of sacrifice, the body weights of ethanol-fed rats were 9.4% lower than those of pair-fed controls (229 ± 10 vs. 253 ± 11 g in controls; 24 pairs, $P < 0.001$). Despite the smaller body weight, the livers of the ethanol-fed rats were significantly heavier than those of controls (9.72 ± 0.47 vs. 8.47 ± 0.36 g; 24 pairs, $P < 0.01$). Expressed per 100 g of body wt, the liver increased in weight by 27% in the rats fed ethanol. This was associated with a parallel increase in dry liver weight (1.32 ± 0.39 vs. 1.01 ± 0.20 g/100 g body wt, in the controls; 24 pairs, $P < 0.01$). The wet weight/dry weight ratios were similar in both groups of animals. Ethanol feeding did not change the specific gravity of the liver (1.009 ± 0.010 vs. 1.016 ± 0.017 g/ml in controls; six pairs, NS). Thus, the changes in weight were accompanied by proportional changes in volume.

Effects of ethanol feeding on liver lipids, proteins, and DNA. As expected, ethanol feeding increased total liver lipids (from 145 ± 10 mg/100 g body wt in the controls to 302 ± 35 mg/100 g body wt, in the ethanol-fed rats; 12 pairs, $P < 0.01$). Total liver proteins also increased (from 573 ± 23 mg/100 g body wt in the controls to 706 ± 40 mg/100 g body wt in the ethanol-fed animals; 12 pairs, $P < 0.01$). In 12 additional pairs (Table I), in which livers were perfused with saline before subcellular fractionation, a similar increase in total protein was found. The absolute increases in lipid (151 ± 29 mg/100 g body wt) and in protein (142 ± 24 mg/100 g body wt) together accounted for almost the entire increase in dry liver weight (304 ± 40 mg/100 g body wt). However, the concentration of protein in the liver did not change (178 ± 9 vs. 173 ± 8 mg/g of liver in controls; 12 pairs, NS), indicating that the increase in protein was associated with a proportional increase in water. By contrast, total liver DNA in ethanol-fed rats was similar to that of controls (10.8 ± 0.5 vs. 10.3 ± 0.4 mg/100 g body wt in control; 10 pairs, NS),

with a corresponding decrease in concentration. Histologic sections of the liver showed that hepatocytes of ethanol-fed rats occupied a significantly larger area than those of pair-fed controls (602 ± 43 vs. 442 ± 36 μm^2 in controls; six pairs, $P < 0.01$), thus accounting for the hepatomegaly.

The recovery of mitochondria, microsomes, and cytosol in ethanol-fed rats (26.4 ± 2.4 , 60.0 ± 1.7 , and $56.0 \pm 5.2\%$, respectively) was similar to that of pair-fed controls (28.7 ± 1.6 , 58.1 ± 5.0 , and $57.6 \pm 5.1\%$, respectively). After correction for recovery, both microsomes and cytosol contributed significantly to the increase in liver protein induced by ethanol feeding (Table I). The greatest contribution to the protein increase was accounted for by the cytosol.

Effects of ethanol feeding on the composition of liver proteins. Liver protein not only increased in amount, but also changed in composition. Although total protein concentration did not change, the concentrations of albumin and transferrin increased significantly in the liver of ethanol-fed rats compared to their pair-fed controls. Liver extracts from alcohol and control rats were equally contaminated with plasma (28.5 ± 5.3 vs. 25.3 ± 5.6 μg of liver in controls; 10 pairs, NS). After subtraction of the contaminating plasma proteins, albumin and transferrin concentrations were found to be significantly increased both in microsomes and cytosol, as well as in the total liver extracts (Table II). Per 100 g body wt, the increases in total liver albumin (3.38 ± 0.32 vs. 2.08 ± 0.11 mg in controls; $P < 0.01$) and in transferrin (1.57 ± 0.09 vs. 1.04 ± 0.06 mg in controls; $P < 0.01$) were greater than per gram of liver, because of the hepatomegaly in the alcohol-treated animals. Though the values for albumin concentration calculated from the isotopic dilution of labeled serum albumin in liver extracts were higher than those measured by radial immunodiffusion, a similar increase was observed in alcohol-fed rats (1.021 ± 0.142 vs. 0.617 ± 0.135 mg/g liver in controls, seven pairs, $P < 0.05$). This rise was the result, at least in part, of increased concentration of proalbumin after ethanol treatment (0.453 ± 0.055 vs. 0.315 ± 0.031 mg/g liver in controls, five pairs; $P < 0.03$).

By contrast, the hepatic concentration of ferritin, a typical constituent protein, was significantly reduced (0.33 ± 0.03 vs. 0.40 ± 0.02 mg/g of liver in controls; seven pairs, $P < 0.02$).

Effects of ethanol feeding on the synthesis of liver proteins. Ethanol-fed rats had significantly higher concentration of leucine in the serum (235 ± 16 nmol/ml) than their pair-fed controls (133 ± 17 nmol/ml; 12 pairs, $P < 0.001$). Therefore, the radioactive leucine injected intravenously on a body weight basis, became significantly more diluted in the plasma leucine of alcohol-treated rats (Fig. 1). Radioactive

TABLE I
Effects of Ethanol Feeding on Protein Content
of Liver Cell Fractions

	Homoge- nate	Mito- chondria	Micro- somes	Cytosol
	mg of liver protein/100 g of body wt			
Ethanol-fed rats	778 ± 46	274 ± 31	154 ± 9	335 ± 15
Pair-fed controls	638 ± 19	233 ± 12	125 ± 11	280 ± 12
P^\dagger	< 0.01	NS	< 0.01	< 0.01

* Determinations were carried out in 12 male rat littermates pair-fed either alcohol or control diet for 24 days. Livers were perfused with saline. Degree of recovery of each fraction was assessed by specific markers (see text).

† Paired comparisons.

TABLE II
Effects of Ethanol Feeding on Albumin and Transferrin Concentrations in Rat Liver Cell Fractions*

Cytosol			Microsomes			Total liver extracts		
Ethanol-fed rats	Pair-fed controls	Difference	Ethanol-fed rats	Pair-fed controls	Difference	Ethanol-fed rats	Pair-fed controls	Difference
I. Albumin ($\mu\text{g/g}$ of liver)								
497	191	+306	270	206	+64	671	513	+158
506	249	+257	200	144	+56	550	455	+95
33	53	-20	232	192	+40	659	530	+129
382	124	+258	168	168	0	800	605	+195
415	467	-52	247	168	+79	480	600	-120
241	141	+100				957	695	+262
718	350	+368				1,183	803	+380
1,060	957	+103				988	707	+281
631	550	+81				838	705	+133
498 \pm 97	342 \pm 94	$t\ddagger = 3.1678$			$t = 3.5388$	792 \pm 76	624 \pm 37	$t = 3.5840$
Paired comparisons		$P < 0.02$			$P < 0.05$			$P < 0.01$
II. Transferrin ($\mu\text{g/g}$ of liver)								
168	110	+58	142	100	+42	440	375	+65
148	141	+7	140	130	+10	482	357	+125
138	122	+16	183	104	+79	386	289	+97
90	67	+23	155	65	+90	280	247	+33
154	105	+49	179	104	+75	388	312	+76
143	47	+96				319	289	+30
176	109	+67				359	338	+21
150	96	+54				385	380	+5
158	110	+48				268	229	+39
147 \pm 8	101 \pm 9	$t = 5.0416$			$t = 4.0340$	367 \pm 22	313 \pm 18	$t = 4.1869$
Paired comparisons		$P < 0.01$			$P < 0.02$			$P < 0.01$

* Values were obtained in rats pair-fed either ethanol-containing or control diets for 4–6 wk. Determinations in total liver extracts and cytosol were carried out in nine pairs and were corrected for protein derived from serum contamination. Microsomal determinations were performed in five additional pairs; there was no detectable contamination of this fraction with serum proteins.

$\ddagger t = (\text{mean difference})/(\text{standard error of the differences}) (36).$

leucine was taken up very rapidly by the liver: maximal specific activities of both free leucine and leucyl-tRNA were reached at about 1 min after injection and the pulse labeling of these pools was almost completed within 10 min. Liver concentration of free leucine was also greater in ethanol-fed rats (273 \pm 32 nmol/g of liver vs. 153 \pm 15, in controls; 12 pairs, $P < 0.02$). This increased concentration of liver leucine represents an even greater increase in total liver leucine because of the hepatomegaly of alcohol-fed rats. Thus, further differences in leucine-specific activity were observed both in the liver-free leucine and leucyl-tRNA pools (Fig. 1). Leucine incorporation into liver proteins reached maximal levels by 10 min after leucine injection, followed by progressive

decrease in protein radioactivity. At 10 min, leucine incorporation into total liver protein was similar in both groups of animals (1,343 \pm 129 dpm \times 10³/100 g body wt in ethanol-fed rats vs. 1,446 \pm 87, in controls; 12 pairs, NS), despite the lower specific activity of leucyl-tRNA in ethanol-fed rats. Thus, calculated rates of synthesis of total liver protein were significantly higher in ethanol-fed rats (22.4 \pm 2.2 vs. 14.5 \pm 1.2 mg/h per 100 g body wt in controls; 12 pairs, $P < 0.01$). Expressed per gram of liver, this difference was only barely significant (4.97 \pm 0.39 vs. 3.94 \pm 0.43 mg/h in controls; 12 pairs, $P < 0.05$).

During the first 10 min after intravenous injection, [¹⁴C]leucine was incorporated into liver albumin but not yet into serum albumin. At this time, albumin

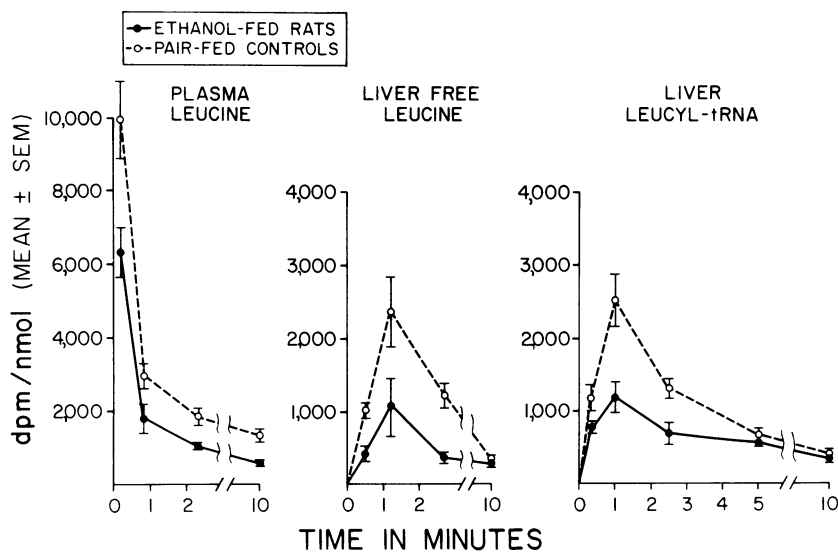


FIGURE 1 Specific activities of serum and liver leucine and leucyl-tRNA after intravenous injection of [^{14}C]leucine ($7\ \mu\text{Ci}/100\ \text{g body wt}$) to 23 pairs of rat littermates fed either ethanol-containing or control diet for 4–6 wk. Each point represents the average of two to five animals. Leucine-specific activities were lower in ethanol-fed rats compared to those in their pair-fed controls ($P < 0.02$; paired comparisons). The decreased specific activities were due to larger leucine pools in both serum and liver of ethanol-fed rats.

labeling was similar in ethanol-treated and control rats (Fig. 2). Over 90% of the radioactivity was recovered in proalbumin ($105 \pm 19\ \text{dpm} \times 10^3/100\ \text{g body wt}$ in ethanol-fed rats vs. 116 ± 19 , in controls; five pairs, NS). Most of the incorporation occurred in microsomes, where 82 and 69% of the total albumin labeling was recovered in alcohol and control rats, respectively. By contrast, only 1.2 and 1.3% of the total albumin labeling was recovered in the cytosol of alcohol and control rats, respectively. Because of greater isotopic dilution of the labeled leucine, calculated rates of proalbumin synthesis were greater in ethanol-fed rats than in controls (1.464 ± 0.276 vs. $0.907 \pm 0.169\ \text{mg/h per } 100\ \text{g body wt}$ in controls; five pairs, $P < 0.01$).

Effects of ethanol feeding on the export of protein from the liver. At 30 and 45 min after leucine injection, when serum albumin was labeled at maximal rates, total liver albumin labeling became significantly greater in ethanol-fed rats than in controls (Fig. 2). This increased incorporation of leucine into the liver albumin coincided with delayed appearance of radioactivity into serum albumin in the rats fed ethanol as compared to their controls. In controls, serum albumin reached near maximal labeling by 30 min, whereas it took 60–90 min for the ethanol-fed rats to reach comparable levels of serum albumin labeling. Similarly, labeling of serum transferrin was delayed, whereas incorporation into liver transferrin was increased in ethanol-fed rats (Fig. 2).

However, the feeding of ethanol for several weeks did not change the serum concentration of either albumin (33.2 ± 0.8 vs. $30.4 \pm 2.4\ \text{mg/ml}$ in controls; 12 pairs, NS) or transferrin (3.63 ± 0.13 vs. $3.42 \pm 0.01\ \text{mg/ml}$ in controls; 12 pairs, NS). Plasma volume was also similar in alcohol and control rats (3.66 ± 0.42 vs. $3.56 \pm 0.35\ \text{ml}/100\ \text{g body wt}$ in controls; six pairs, NS). Rates of serum albumin production calculated from serum albumin labeling at 120 min and the specific activity of leucyl-tRNA, were similar in both groups of animals (1.025 ± 0.129 vs. $0.817 \pm 0.049\ \text{mg/h per } 100\ \text{g body wt}$ in the pair-fed controls; six pairs, NS).

Effect of ethanol feeding on liver microtubules. The concentration of total tubulin was significantly decreased in the hepatic cytosol of ethanol-fed rats compared to their controls, whereas free tubulin remained unaffected (Table III). Thus, polymerized tubulin was significantly decreased, not only per gram of liver (Table III), but also per total liver (938 ± 158 vs. $1,168 \pm 108\ \mu\text{g}/100\ \text{g body wt}$ in controls; six pairs, $P < 0.02$).

DISCUSSION

In the present study we confirm and extend our preliminary report (1), which showed that alcohol-induced hepatomegaly in the rat is the result not merely of accumulation of fat, but also of a striking increase in liver protein. Protein accumulation after alcohol feed-

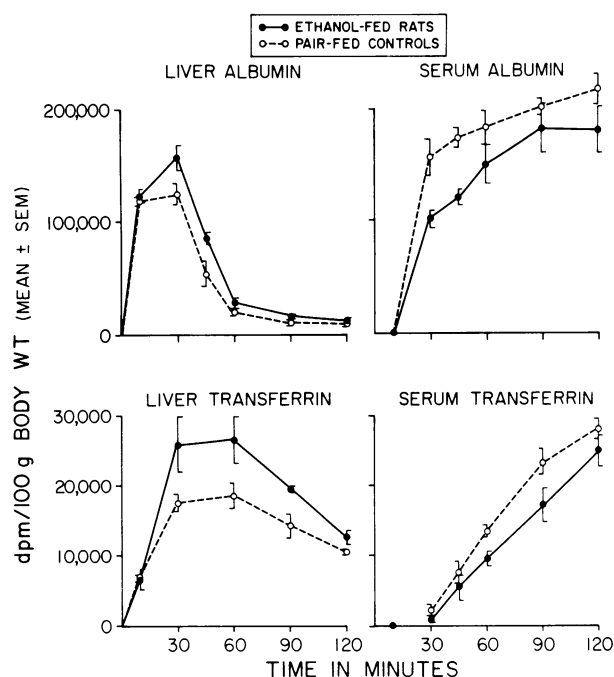


FIGURE 2 Incorporation of intravenously injected [^{14}C]-leucine into liver and serum albumin and transferrin at various time intervals in rats pair-fed either ethanol-containing or control diet for 4–6 wk. Each point represents the average of three to six animals. Labeling of liver albumin (30–45 min after injection) and of liver transferrin (30–90 min) was significantly greater in ethanol-fed rats, whereas labeling of serum albumin and transferrin (same time periods) was significantly reduced in these rats, compared to controls ($P < 0.01$; paired comparisons). After 60 min in the case of serum albumin, and at 120 min in the case of transferrin, the differences in labeling between both types of animals were not statistically significant.

ing is partially accounted for by proliferation of sub-cellular organelles which we described before (38, 39); we now find, however, that the greatest increase in cellular protein occurs in the soluble fraction or cytosol. Moreover, the increase in protein is associated with a change in its composition characterized by increased concentration of proteins normally exported into the plasma. Indeed, the hepatic concentration of albumin and transferrin was significantly increased in ethanol-fed rats when contamination with extracellular proteins was subtracted. It has been shown that liver proteins other than albumin are precipitated by antibodies against serum albumin (40, 41). The material present in the immunoprecipitates has properties compatible with those of an albumin precursor—proalbumin (31, 42, 43). The increase in immunoreactive albumin in the liver of ethanol-fed rats was due, in part, to a parallel increase in proalbumin. Because of the hepatomegaly in ethanol-fed rats, the differences in concentration of albumin and transferrin reflect an even larger in-

crease in their total hepatic content. Contrasting with the increased hepatic concentration of these proteins, total liver protein concentration did not change. The concentration of soluble constituent proteins (such as ferritin, organic anion-binding proteins [44], and alcohol dehydrogenase [45]) is unchanged or decreased in ethanol-fed rats.

Increased protein in the liver could result either from increased synthesis or from decreased export into the plasma or both. Acute ethanol administration to animals has been reported to inhibit hepatic synthesis of both constituent (46–48) and export (49–52) proteins *in vivo* and *in vitro*. Other investigators have reported that chronic ethanol administration increases protein synthesis in ribosomes (46) and microsomes (53), whereas acute administration produces opposite effects. In our study, synthesis of total liver protein was significantly enhanced in ethanol-fed rats compared to pair-fed controls. It must be pointed out, however, that the incorporation of [^{14}C]leucine into liver proteins was not affected, but the specific activity of leucine and leucyl-tRNA was markedly reduced because of the higher concentrations of both serum and liver leucine in ethanol-fed rats. Comparable increases in the serum concentration of branched chain amino acids has been reported in baboons chronically fed alcohol (54). Similarly, proalbumin synthesis was also increased in ethanol-fed rats. However, there is no evidence to suggest that albumin or transferrin was synthesized at a faster rate than other proteins, the concentration of which did not change. Furthermore, if increased albumin synthesis were the primary cause of the accumulation, one could expect this to be associated with a parallel increased output into the plasma. However, 30 and 45 min after [^{14}C]leucine injection, when secretion of newly labeled albumin was taking place at maximal rates, ethanol feeding produced opposite changes in liver and serum albumin labeling (Fig. 2). At 30 and 45 min after [^{14}C]leucine injection, incorporation into liver albumin was sig-

TABLE III
Effects of Ethanol Feeding on Hepatic Microtubular Protein*

	Total tubulin	Free tubulin	Polymerized tubulin
	$\mu\text{g/g liver}$		
Ethanol-fed rats	338 \pm 40	187 \pm 12	196 \pm 33
Pair-fed controls	423 \pm 33	184 \pm 10	289 \pm 21
P †	<0.01	NS	<0.01

* Determinations were carried out in 12 male rat littermates pair-fed either alcohol or control diet for 24 days.

† Paired comparisons.

nificantly increased, whereas incorporation into serum albumin was significantly decreased. Only after 60 and 90 min did serum albumin labeling reach levels comparable to those of controls. This delay cannot be attributed to an enlargement of the serum albumin pool, because both plasma volume and serum albumin concentrations were similar in alcohol-treated and control rats. Thus, the triad of increased albumin concentration in the liver, delayed transport of newly labeled albumin into the serum, and retention of albumin label in the liver, indicates that ethanol feeding interferes with the export of albumin, leading to its accumulation in the liver. These alterations are similar to those reported (22) after rats were given colchicine, an inhibitor of protein secretion (55–59). The modest increase in proalbumin after colchicine was attributed to concomitant inhibition of proalbumin synthesis (22). This was not the case in our rats chronically fed alcohol, in which the synthesis of proalbumin was increased rather than decreased. Nevertheless, the amount of albumin retained after several weeks of ethanol feeding appears much smaller than that expected from the difference between the calculated rates of proalbumin and albumin synthesis. This suggests that most of the retained albumin in these rats may be disposed by proteolysis. This possibility is consistent with the increased urea excretion previously reported in ethanol-fed rats (60) and with the increased concentrations of branched chain amino acids observed in this study. Because these amino acids are not significantly oxidized in the liver, their production has been used as an indicator of hepatic proteolysis (30). The effects of ethanol feeding on liver and serum transferrin labeling were similar to those on albumin (Fig. 2). Because the time required for transferrin to be secreted is longer than that for albumin (26), the differences in transferrin labeling were more persistent than those in albumin.

Export proteins are produced by ribosomes attached to the membranes of the rough endoplasmic reticulum (61, 62), from where they are thought to be released into the microsomal cisterna (20, 21), though the electron microscope evidence is conflicting (63, 64). It is therefore intriguing that, after alcohol feeding, albumin and transferrin concentrations increased not only in the microsomal fraction but also in the cytosol despite subtraction of the extracellular albumin or transferrin contaminating the liver extracts. Retention of albumin in the liver cytosol has also been reported in rats with chronic uremia (65). The possibility that microsomes from ethanol-fed rats could leak out more of their content during centrifugation is not likely because after administration of labeled leucine, the newly labeled albumin remained in the microsomal fraction, with only minimal labeling of the

cytosolic albumin. One must then wonder whether the cytosol becomes a storage site for retained export proteins.

The alteration of the export proteins from the liver produced by ethanol feeding could take place at many possible sites. Inasmuch as it appears to affect more than a single protein of the export class, a common site in their transport pathways should be looked for. One such possible site could be the microtubular system. This system has been postulated to play a role in the export of proteins in the liver as well as in other organs (55–59). The evidence is based mainly on the effects of colchicine and other related alkaloids which bind the microtubular protein (tubulin) and interfere with its assembly into microtubules (66) as well as inhibit the export of proteins from the liver (55–59). From the difference in colchicine binding before and after microtubule depolymerization, it was calculated that ethanol feeding markedly reduces the amount of polymerized tubulin as well as that of total tubulin. Microtubules decreased not merely in concentration, but also when expressed per total liver: thus, this decrease cannot be accounted for by dilution. The mechanism of this hitherto unrecognized effect of ethanol remains to be elucidated.

Thus, the alteration in microtubules induced by ethanol feeding may account, at least in part, for the delayed export of serum protein from the liver. This alteration in export leads to intrahepatic accumulation of protein. The contribution of this retention of export proteins to the total increase in liver protein is difficult to assess from the present data because only two proteins of the export class have been measured and the sum of both increases (1.83 mg/100 g body wt) accounts for only a small portion of the total increase in soluble proteins. The possible contribution of other secretory or constituent proteins awaits further assessment. In any event, at this stage of alcoholic liver damage, the delay in export is not sufficient to change serum protein pool or total output of albumin into the serum. Increased synthesis of nascent albumin in the liver may reflect a homeostatic compensation to maintain plasma levels. In the course of advancing alcoholic liver damage, decreased concentration of serum protein could result from either progressive deterioration of the secretory mechanism and/or concomitant failure in protein synthesis.

ACKNOWLEDGMENTS

The authors are grateful to Ms. Frances Finkelman and Mr. John Saeli for expert assistance.

This work was supported by grants from the U. S. Public Health Service (AA-00224, AM-12511), the National Council of Alcoholism, the Veterans Administration, a Research Scientist Development Award to Dr. E. Baraona, and a fellow-

REFERENCES

1. Baraona, E., M. A. Leo, S. A. Borowsky, and C. S. Lieber. 1975. Alcoholic hepatomegaly: accumulation of protein in the liver. *Science (Wash. D. C.)*. **190**: 794-795.
2. Rice, R. H., and G. E. Means. 1971. Radioactive labeling of proteins *in vitro*. *J. Biol. Chem.* **246**: 831-832.
3. Quinn, P. S., M. Gamble, and J. D. Judah. 1975. Biosynthesis of serum albumin in rat liver: isolation and probable structure of "proalbumin" from rat liver. *Biochem. J.* **146**: 389-393.
4. De Carli, L. M., and C. S. Lieber. 1967. Fatty liver in the rat after prolonged intake of ethanol with a nutritionally adequate new liquid diet. *J. Nutr.* **91**: 331-336.
5. Baraona, E., and C. S. Lieber. 1970. Effects of chronic ethanol feeding on serum lipoprotein metabolism in the rat. *J. Clin. Invest.* **49**: 769-778.
6. Baraona, E., R. C. Pirola, and C. S. Lieber. 1973. Pathogenesis of postprandial hyperlipemia in rats fed ethanol-containing diets. *J. Clin. Invest.* **52**: 296-303.
7. Gordon, A. H., and J. H. Humphrey. 1961. Measurement of intracellular albumin in rat liver. *Biochem. J.* **78**: 551-556.
8. Greim, H. 1970. Synthesesteigerung und Abbauehemmung bei der Vermehrung der mikrosomalen Cytochrome P-450 und b-5 durch Phenobarbital. *Naunyn Schmiedeb. Arch. Pharmacol. Exp. Pathol. (Jena)*. **266**: 261-275.
9. Schmidt, E. 1963. Glutamic dehydrogenase. In *Methods of Enzymatic Analysis*. H. U. Bergmeyer, editor. Academic Press Inc., New York. 752-756.
10. Totmar, S. O. C., H. Pettersson, and K.-H. Kiessling. 1973. The subcellular distribution and properties of aldehyde dehydrogenases in rat liver. *Biochem. J.* **135**: 577-586.
11. Hasumura, Y., R. Teschke, and C. S. Lieber. 1976. Characteristics of acetaldehyde oxidation in rat liver mitochondria. *J. Biol. Chem.* **251**: 4908-4913.
12. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**: 2370-2378.
13. Bonnichsen, R. K., and N. G. Brink. 1955. Liver alcohol dehydrogenase. *Methods Enzymol.* **1**: 495-500.
14. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1951. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497-509.
15. Amenta, J. S. 1964. A rapid chemical method for quantification of lipids separated by thin-layer chromatography. *J. Lipid Res.* **5**: 270-272.
16. Siekevitz, P. 1952. Uptake of radioactive alanine *in vitro* into the proteins of rat liver fractions. *J. Biol. Chem.* **195**: 549-565.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **93**: 265-275.
18. Martin, R. F., D. C. Donohue, and L. R. Finch. 1972. New analytical procedure for the estimation of DNA with p-nitrophenylhydrazine. *Anal. Biochem.* **47**: 562-574.
19. Mancini, G. W., O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*. **2**: 235-254.
20. Peters, T., Jr. 1962. The biosynthesis of rat serum albumin. II. Intracellular phenomena in the secretion of newly formed albumin. *J. Biol. Chem.* **237**: 1186-1189.
21. Glaumann, H. 1970. Studies on the synthesis and transport of albumin in microsomal subfractions from rat liver. *Biochim. Biophys. Acta*. **224**: 206-218.
22. Dorling, P. R., P. S. Quinn, and J. D. Judah. 1975. Evidence for the coupling of biosynthesis and secretion of serum albumin in the rat: the effect of colchicine on albumin production. *Biochem. J.* **152**: 341-348.
23. Bjørklid, E., and L. Helgeland. 1970. Sex differences in the ferritin content of rat liver. *Biochim. Biophys. Acta*. **221**: 583-592.
24. Gregersen, M. I. 1944. A practical method for the determination of blood volume with the dye T-1824. *J. Lab. Clin. Med.* **29**: 1266-1286.
25. Dickinson, J. C., H. Rosenblum, and P. B. Hamilton. 1965. Ion exchange chromatography of the free amino acids in the plasma of the newborn infant. *Pediatrics*. **36**: 2-13.
26. Morgan, E. H., and T. Peters, Jr. 1971. Intracellular aspects of transferrin synthesis and secretion in the rat. *J. Biol. Chem.* **246**: 3508-3511.
27. Wallyn, C. S., A. Vidrich, J. Airhart, and E. A. Khairallah. 1974. Analysis of the specific radioactivity of valine isolated from amino-acyl-transfer ribonucleic acid of rat liver. *Biochem. J.* **140**: 545-548.
28. Peters, T., Jr., and J. C. Peters. 1972. The biosynthesis of rat serum albumin. VI. Intracellular transport of albumin and rates of albumin and liver protein synthesis *in vivo* under various physiological conditions. *J. Biol. Chem.* **247**: 3858-3863.
29. Airhart, J., A. Vidrich, and E. A. Khairallah. 1974. Compartmentation of free amino acids for protein synthesis in rat liver. *Biochem. J.* **140**: 539-548.
30. Khairallah, E. A., and G. E. Mortimore. 1976. Assessment of protein turnover in perfused rat liver: evidence for amino acid compartmentation from differential labeling of free and tRNA-bound valine. *J. Biol. Chem.* **251**: 1375-1384.
31. Urban, J., A. S. Inglis, K. Edwards, and G. Schneiber. 1974. Chemical evidence for the difference between albumins from microsomes and serum and a possible precursor-product relationship. *Biochem. Biophys. Res. Commun.* **61**: 494-501.
32. Borisy, G. G. 1972. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. *Anal. Biochem.* **50**: 373-385.
33. Sherline, P., C. K. Bodwin, and D. M. Kipnis. 1974. A new colchicine binding assay for tubulin. *Anal. Biochem.* **62**: 400-407.
34. Patzelt, C., A. Singh, Y. Le Marchand, L. Orci, and B. Jeanrenaud. 1975. Colchicine-binding protein of the liver: its characterization and relation to microtubules. *J. Cell Biol.* **66**: 609-620.
35. Filner, P., and O. Behnke. 1973. Stabilization and isolation of brain microtubules with glycerol and dimethylsulfoxide. *J. Cell Biol.* **59**: 99.
36. Snedecor, G. W., and W. G. Cochran. 1967. *Statistical Methods*. The Iowa State University Press, Ames, Iowa. 6th edition. 91-97.
37. Pirola, R. C., and C. S. Lieber. 1972. The energy cost of the metabolism of drugs, including ethanol. *Pharmacology (Basel)*. **7**: 85-196.
38. Iseri, O. A., C. S. Lieber, and L. S. Gottlieb. 1966. The ultrastructure of fatty liver induced by prolonged ethanol ingestion. *Am. J. Pathol.* **48**: 535-555.
39. Ishii, H., J.-G. Joly, and C. S. Lieber. 1973. Effect of ethanol on the amount and enzyme activities of hepatic rough and smooth microsomal membranes. *Biochim. Biophys. Acta*. **291**: 411-420.
40. Schreiber, G., H.-M. Rotermund, H. Maeno, K. Weigand,

- and R. Lesch. 1969. The proportion of the incorporation of leucine into albumin to that into total protein in rat liver and hepatoma Morris 5123 TC. *Eur. J. Biochem.* **10**: 355-361.
41. Judah, J. D., and M. R. Nicholls. 1971. The separation of intracellular serum albumin from rat liver. *Biochem. J.* **123**: 643-648.
 42. Geller, D. M., J. D. Judah, and M. R. Nicholls. 1972. Intracellular distribution of serum albumin and its possible precursors in rat liver. *Biochem. J.* **127**: 865-874.
 43. Judah, J. D., M. Gamble, and J. H. Steadman. 1973. Biosynthesis of serum albumin in rat liver: evidence for the existence of "proalbumin." *Biochem. J.* **134**: 1083-1091.
 44. Reyes, H., A. J. Levi, Z. Gatmaitan, and I. M. Arias. 1971. Studies of Y and Z, two hepatic cytoplasmic organic anion-binding proteins: effect of drugs, chemicals, hormones, and cholestasis. *J. Clin. Invest.* **50**: 2242-2252.
 45. Lieber, C. S., and L. M. DeCarli. 1970. Hepatic microsomal ethanol-oxidizing system: *in vitro* characteristics and adaptive properties *in vivo*. *J. Biol. Chem.* **245**: 2505-2512.
 46. Kuriyama, K., P. Y. Sze, and G. E. Rauscher. 1971. Effects of acute and chronic ethanol administration on ribosomal protein synthesis in mouse brain and liver. *Life Sci.* **10** (Part II): 181-189.
 47. Perin, A., G. Scalabrino, A. Sessa, and A. Arnaboldi. 1974. *In vitro* inhibition of protein synthesis in rat liver as a consequence of ethanol metabolism. *Biochim. Biophys. Acta.* **366**: 101-108.
 48. Perin, A., and A. Sessa. 1975. *In vitro* effects of ethanol and acetaldehyde on tissue protein synthesis. In *The Role of Acetaldehyde in the Actions of Ethanol*. K. O. Lindros and C. J. P. Eriksson, editors. Satellite Symposium of the 6th International Congress of Pharmacology, Helsinki. The Finnish Foundation for Alcohol Studies. **23**: 105-122.
 49. Rothschild, M. A., M. Oratz, J. Mongelli, and S. S. Schreiber. 1971. Alcohol-induced depression of albumin synthesis: reversal by tryptophan. *J. Clin. Invest.* **50**: 1812-1818.
 50. Rothschild, M. A., M. Oratz, and S. S. Schreiber. 1974. Alcohol, amino acids, and albumin synthesis. *Gastroenterology.* **67**: 1200-1213.
 51. Jeejeebhoy, K. N., M. J. Phillips, A. Bruce-Robertson, J. Ho, and U. Sodtke. 1972. The acute effect of ethanol on albumin, fibrinogen, and transferrin synthesis in the rat. *Biochem. J.* **126**: 1111-1126.
 52. Kirsch, R. E., L. O. Frith, R. H. Stead, and S. J. Saunders. 1973. Effect of alcohol on albumin synthesis by the isolated perfused rat liver. *Am. J. Clin. Nutr.* **26**: 1191-1194.
 53. Renis, M., A. Giovine, and A. Bertolino. 1975. Protein synthesis in mitochondrial and microsomal fractions from rat brain and liver after acute and chronic ethanol administration. *Life Sci.* **16**: 1447-1458.
 54. Shaw, S., and C. S. Lieber. 1976. Characteristic plasma amino acid abnormalities in the alcoholic: respective roles of alcoholism, nutrition and liver injury. *Clin. Res.* **24**: 291A. (Abstr.).
 55. Le Marchand, Y., A. Singh, F. Assimacopoulos-Jeannet, L. Orci, C. Rouiller, and B. Jeanrenaud. 1973. A role for the microtubular system in the release of very low density lipoproteins by perfused mouse livers. *J. Biol. Chem.* **248**: 6862-6870.
 56. Le Marchand, Y., C. Patzelt, F. Assimacopoulos-Jeannet, E. G. Loten, and B. Jeanrenaud. 1974. Evidence for a role of the microtubular system in the secretion of newly synthesized albumin and other proteins by the liver. *J. Clin. Invest.* **53**: 1512-1517.
 57. Stein, O., and Y. Stein. 1973. Colchicine-induced inhibition of very low density lipoprotein release by rat liver *in vivo*. *Biochim. Biophys. Acta.* **306**: 142-147.
 58. Stein, O., L. Sanger, and Y. Stein. 1974. Colchicine-induced inhibition of lipoprotein and protein secretion into the serum and lack of interference with secretion of biliary phospholipids and cholesterol by rat liver *in vivo*. *J. Cell Biol.* **62**: 90-103.
 59. Redman, C. M., D. Banerjee, K. Howell, and G. E. Palade. 1975. Colchicine inhibition of plasma protein release from rat hepatocytes. *J. Cell Biol.* **66**: 42-59.
 60. Rodrigo, C., C. Antezana, and E. Baraona. 1971. Fat and nitrogen balances in rats with alcohol-induced fatty liver. *J. Nutr.* **101**: 1307-1310.
 61. Hicks, S. J., J. W. Drysdale, and H. N. Munro. 1969. Preferential synthesis of ferritin and albumin by different populations of liver polysomes. *Science (Wash. D. C.)*. **164**: 584-585.
 62. Redman, C. M. 1969. Biosynthesis of serum proteins and ferritin by free and attached ribosomes of rat liver. *J. Biol. Chem.* **244**: 4308-4315.
 63. Lin, C-T., and J. P. Chang. 1975. Electron microscopy of albumin synthesis. *Science (Wash. D. C.)*. **190**: 465-467.
 64. Guillouzo, A., G. Feldmann, M. Boissard, C. Sapin, and J. P. Benhamou. 1975. Ultrastructural location of albumin and fibrinogen in primary cultures of new-born rat liver tissue. *Exp. Cell Res.* **96**: 239-246.
 65. Shafritz, S., S. B. Grossman, and S. H. Yap. 1976. Influence of chronic uremia on hepatic protein synthesis. *Gastroenterology.* **71**: 929.
 66. Olmsted, J. B., and G. G. Borisy. 1973. Microtubules. *Annu. Rev. Biochem.* **42**: 507-540.