PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **177**, 262–271 (1984)

Ethanol–Ascorbate Interrelationship in Acute and Chronic Alcoholism in the Guinea Pig (41941)

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Abstract. The effects of low (200 ppm) and of high (2000 ppm) ascorbic acid, in a nutritionally adequate diet, on blood ethanol levels have been studied in permanently carotid-cannulated, ethanol-infused, unanesthetized guinea pigs. In the acute study, the postinfusion rate of ethanol decline in the blood of animals treated with ascorbic acid was significantly higher when compared with animals treated with fructose, and the rate in the two treated groups was significantly higher than in untreated controls. In the chronic study, animals were infused with sublethal doses of ethanol (30% of the total caloric intake) for 8 weeks. Blood ethanol levels monitored throughout this period showed, at 3 hr postinfusion, a lower concentration in the group on a high ascorbic acid diet. Both experimental groups receiving ethanol lost significantly more body weight in the second week of dieting; but, while the group on high ascorbic acid regained weight steadily thereafter, the group on low ascorbic acid was still 50 g below the controls at the end of the experiment. Liver, kidney, and adrenal ascorbic acid concentrations were lower in the ethanol-treated groups compared to controls. Examination of the liver revealed more fatty metamorphosis or steatosis in the low ascorbic acid group, but there was no evidence of liver fibrosis or cirrhosis. These results demonstrate the feasibility of utilizing the guinea pig for the study of the biochemical and morphological sequelae of alcoholism. They further support the contention that a diet which is nutritionally adequate may no longer be so in the presence of high ethanol intake, and that supplemental vitamin C ingestion may afford protection against ethanol toxicity. © 1984 Society for Experimental Biology and Medicine.

There is now increasing evidence that chronic alcoholism is associated with vitamin deficiency, particularly the B vitamins and the two antioxidant vitamins, ascorbic acid (vitamin C) and α-tocopherol (vitamin E) (1–5). The B vitamins influence ethanol metabolism via the formation of nicotinamide adenine dinucleotide (NAD), the hydrogen acceptor during the conversion of ethanol to acetaldehyde, a reaction catalyzed by alcohol dehydrogenase (ADH) (6). Since ascorbic acid and α-tocopherol are involved in oxidation–reduction reactions, it is also possible that they could affect the oxidation of ethanol. The role of ascorbic acid in ethanol metabolism has been extensively studied in mice and rats (7–10). However, it has not been studied in animal models which lack the ability to synthesize ascorbic acid.

The guinea pig, in contrast to other animals, is incapable of synthesizing ascorbic acid. This is due to the fact that like a few other species (man, primates, red-vested bulbul, Indian fruit bat), it lacks the enzyme gulonolactone oxidase which converts glucose to ascorbic acid (11). Several authors have suggested that, unlike other rodents, the guinea pig, as an animal model, is more suitable for studies related to ethanol metabolism. This is not only due to its similarity to man with regard to ethanol absorption (12), but also due to metabolic similarities of the intermediates in the hepatic gluconeogenetic pathways, as well as similarities of liver morphology (13, 14). Admittedly, the guinea pig, as an animal model for alcoholism, has not been very popular due to the difficulty of blood sampling particularly in chronic studies.

We have previously reported on the effect of ascorbic acid on ethanol metabolism in mice and rats (7). Our studies demonstrated that ascorbic acid protected these rodents against acute and chronic ethanol toxicity. However, since these two species are capable of synthesizing ascorbic acid, our previous

0037-9727/84 $1.50
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findings could have been in part affected or altered by the animal's ascorbic acid synthesizing ability.

In the present study, we have chosen the guinea pig to study the interrelationship of ascorbate and ethanol in a setting in which dietary ascorbic acid intake can rigorously be controlled. Furthermore, ethanol was infused via a permanent carotid cannula to unanesthetized animals and ethanol blood levels were repeatedly monitored and quantitated. The results of these observations are reported here.

Materials and Methods. *Animals.* The animals used were male guinea pigs of the Hartley strain (Charles River Breeding Laboratory, Wilmington, Mass.). They were housed individually in stainless steel cages equipped with an automatic water system, and acclimatized for 1 week prior to experimentation. Careful adherence to the Principles of Care for Experimental Animals was exercised throughout the experimentation periods.

Diet. All animals were fed a basal diet supplemented in various groups with high or low levels of ascorbic acid, and some groups were pair-fed, as outlined under Experimental Design. The basal diet was an ascorbic acid-deficient diet purchased from Bioserve, Inc. (Frenchtown, N.J.). It contained casein, 21.96%; DL-methionine, 0.34%, corn oil, 10.09%; dextrose, corn starch, and sucrose, 22.53% each. To this basal diet, salt mix,[1] vitamin mix,[2] choline chloride, and fiber as cellulose were added at 5.0, 2.0, 1.3, and 14%, respectively.

Surgical procedure for permanent carotid cannulation. The animals were anesthetized by an intramuscular injection of a ketamin-xylazine mixture. The technique of exiting a carotid or jugular vein cannula in the neck region between the ears has been described previously in small animals, including the guinea pig (15–17). After the free end of the Silastin cannula was secured in place with a pocket suture, it was cut to a suitable length, filled with heparin, and closed with a plastic cap. Patency of the cannulation was checked and the cannula was heparinized on weekdays to prevent clotting. In order to ensure that the blood sample taken each time represented circulating blood, a volume of blood equivalent to four times the dead space was taken and then returned to the animal after blood sampling. Heparinized saline in equal volume to the blood sample was then slowly infused and the cannula was recapped.

Experimental Design. *Experiment 1 (acute study).* This experiment was designed to test the effect of ascorbic acid on the rate of ethanol elimination from the blood following a single infusion of ethanol via the carotid cannula. Because of the well-known enhancing effect of fructose on the rate of ethanol disappearance from the blood (18, 19), fructose was administered to one group of animals for comparative purposes.

Thirty animals, weighing an average of 500 g each, were permanently cannulated and allowed to recover for 1 week. They were then randomized into three groups of ten animals each. These groups will be referred to as saline–ethanol control, ascorbic acid–ethanol, and fructose–ethanol groups. Sodium ascorbate dissolved in saline (0.2 g/ml) and fructose (0.5 g/ml) dissolved in water were administered 1 hour before ethanol at rates of 0.04 and 0.10 g/kg body wt/min, respectively, for 10 min. Ethanol as 22% (v/v) in saline was infused with a pump at the rate of 0.25 g/kg/min for 10 min. Blood for ethanol determination was sampled from the cannula every hour for 10 hr.

Experiment 2 (chronic study). The objective of this experiment was to study the effect of high and of marginal dietary ascorbic acid on ethanol metabolism, and conversely the effect of ethanol on tissue ascorbic acid concentrations. Lipid peroxidation, as reflected

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[1] Salt mix. Ingredients are expressed as g/kg salt mix. Calcium carbonate, 167.54; calcium phosphate (tri), 156.37; cupric sulfate, 0.22; ferric citrate, 0.45; magnesium sulfate 55.84; manganese sulfate, 3.48; potassium iodide, 0.45; sodium chloride, 98.29; potassium phosphate dibasic, 100.52; sodium phosphate dibasic, 81.54; zinc carbonate, 0.22; magnesium oxide, 55.84; potassium acetate, 279.23.

[2] Vitamin mix. Ingredients are given as g/kg of vitamin mix. Fiber, QS to 1000; biotin, 0.0480; vitamin B12, 0.0032; calcium pantothenate, 3.2000; folic acid, 0.8000; inositol, 160.0000; menadione, 0.1600; niacin, 16.0000; pyridoxine, 1.2800; riboflavin, 1.2800; thiamine, 1.2800; vitamin A acetate, 1,380,000 IU; vitamin D3, 128,000 IU; vitamin E acetate 16,000 IU.

by malonylaldehyde metabolism, and tissue cations (Mg, Ca, Zn, Cu) concentrations were also studied.

Twenty-four male guinea pigs, weighing approximately 325 g each, were cannulated and randomized, after a week of postoperative recovery, into three groups of eight animals each. Group 1 (saline-high ascorbate-control group) received, intraarterially, a volume of saline equal to the volume of ethanol infused in the experimental groups. Its basal diet was supplemented with high ascorbate, viz., 2000 mg/kg diet. Group 2 (ethanol-marginal ascorbate group) received ethanol by infusion. Its diet contained marginal amounts of ascorbate, viz., 200 mg/kg diet. Group 3 (ethanol-high ascorbate group) received ethanol by infusion and its diet was similar to group 1, viz., 2000 mg of ascorbate/kg diet. Groups 1 and 3 were pair-fed the average food intake that group 2 had consumed the day before.

For every gram of food consumed by groups 2 and 3 which averaged 20 g/animal/day, 0.14 g of ethyl alcohol as 22% (v/v) in saline was infused with a pump over a period of 10 min to maintain the isocaloric design of the experiment. Thus ethanol substituted for 50% of the carbohydrate portion of the diet and 30% of the total caloric intake in groups 2 and 3. To maintain the isocaloric design of the diet, 0.899 g of sucrose/g of food was added to the diet of group 1 and 0.636 g/g food was added to group 2 and 3 diets. The amounts of fat and protein were the same in all groups.

Animals were maintained on this regimen for 8 weeks during which body weights were taken weekly, and ethanol infusions were performed five times a week. Random blood samples were obtained weekly in groups 2 and 3, 3 hr postethanol infusion for ethanol blood level determinations by our previously published modified Jain's method (7).

Organ and blood analysis. Animals were sacrificed by exsanguination, and blood, liver, heart, kidneys, adrenals, and brain were obtained. Plasma was separated for the analysis of total protein by Lowry's method (20) and malonylaldehyde by the thiobarbituric acid method of Mengel and Kahn (21). A piece of liver was fixed in 10% Formalin. Pieces of liver, kidney, and adrenal were frozen immediately and used for ascorbic acid analysis by the 2,4-dinitrophenylhydrazine method of Roe and Kuether (22) as modified by Schaffert and Kingsley (23). All other tissues were blotted of excess blood, weighed, and then frozen for trace metal analysis by atomic absorption spectrophotometry.

Histopathologic examination. Liver sections stained with hematoxylin and eosin were examined by the pathologist (A.F.) without knowledge of their identities. A scoring scale, modified from that used by Hartroft *et al.* (24), was used to assess the percentage of hepatocytes showing fatty infiltration: grade 0, 0% (nil); grade 1, up to 10% (minimal); grade 2, up to 20% (mild); grade 3, up to 50% (moderate); grade 4, up to 75% (marked); and grade 5, up to 100% (very marked). Blind scoring was performed at three different periods and the results were repeatedly consistent.

Statistical analysis. In Experiment 1, the rate of ethanol elimination from the blood was taken as the slope of the regression line calculated by the least square method relating ethanol concentration and time. In Experiment 2, data were statistically analyzed by utilizing unpaired Student's t test to determine means significantly different at less than 0.05.

Results. *Experiment 1.* The results of acute administration of ethanol and the comparative effects of ascorbic acid and fructose on the rate of ethanol disappearance from the blood are shown in Fig. 1. The decline in ethanol blood concentration with time is faster in both the fructose and the ascorbic acid-infused animals than in the saline controls. However, the temporal relationship differs in the two experimental groups. Fructose seems to have a greater efficacy in depressing ethanol blood levels during the first 4 hr, while ascorbic acid displays its greater effect in the last 4 hr of the experimental run. The curves also suggest that the rate of blood ethanol decline in the guinea pig is generally slower than in the small animals we have used previously, namely, mice and rats (7).

Experiment 2. Figure 2 shows the changes in body weight observed in the three groups. All groups lost weight during the first week of the experiment. Thereafter, the control group mostly maintained their body weights, while the two experimental groups receiving

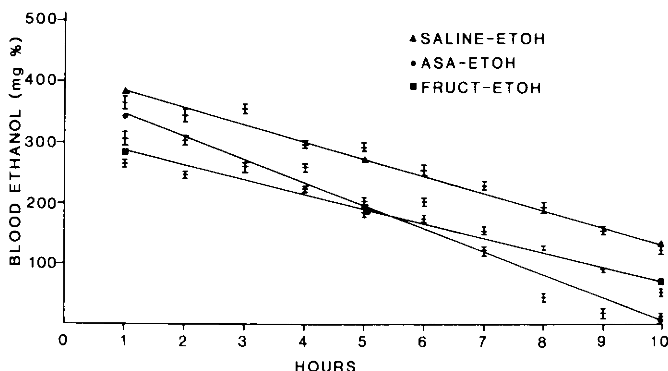

FIG. 1. Regression lines of blood ethanol concentration (mg%) in the three acute ethanol-treated groups: Saline–ethanol control, ▲, $y = -28x + 413$; ascorbic acid–ethanol ●, $y = -38x + 380$. $P < 0.001$; and fructose–ethanol, ■, $y = -23x + 304$, $P < 0.001$.

ethanol had significant weight losses during the second experimental week. They regained weight steadily thereafter. Group 3 reached, at 8 weeks, its level of initial body weight, but group 2 lost an average of 50 g/animal at the end of the experiment. Figure 3 demonstrates the effect of ascorbic acid on the rate of ethanol disappearance from the blood. Blood ethanol levels, at 3 hr postinfusion, were significantly lower in animals receiving high ascorbic acid ($P < 0.01$) as demonstrated by a mean blood ethanol concentration of 270 and 125 mg/100 ml in the groups receiving marginal and high ascorbic acid, respectively.

Table I shows ascorbic acid concentrations in liver, kidney, and adrenals. Highly significant decreases in groups 2 and 3 were seen in all tissues, particularly the adrenals which are known to be a good index of ascorbic acid status in the guinea pig. Even group 3, which received high ascorbic acid in the diet, had significantly depressed ascorbic acid in the liver, kidney, and adrenals. Urinary ascorbic acid excretion, however, was higher in the ethanol-treated groups as illustrated in Table II, particularly in group 2 which received marginal ascorbic acid; but the increased excretion did not reach statistical significance. Other variables in the table

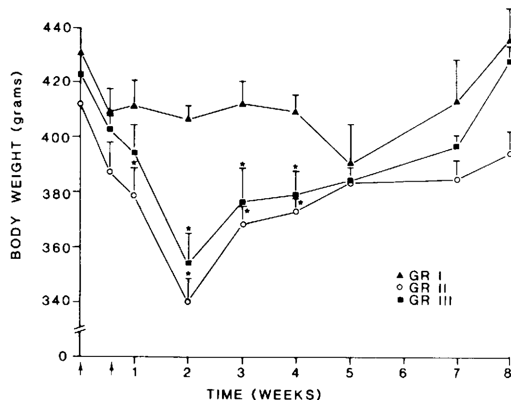

FIG. 2. Body weight vs time. Body weights of controls and of chronic ethanol-treated groups during the 8 week experimental period: Group 1 saline control ▲, group 2 ascorbic acid-deficient group ○, and group 3 ascorbic acid supplemented group ■. *$P < 0.05$.

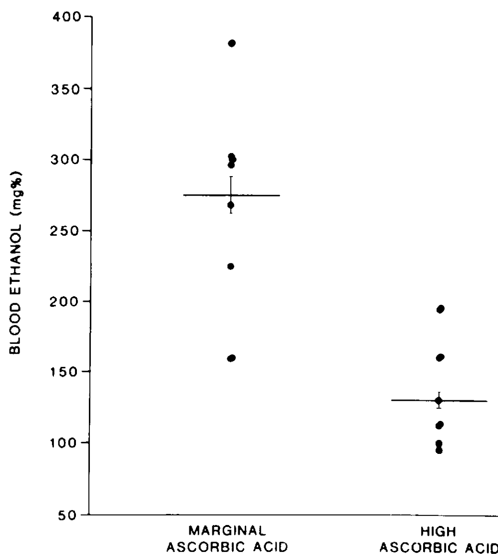

FIG. 3. Blood ethanol levels (mg%) at 3 hr postethanol infusion of marginal-ascorbic-acid and high-ascorbic-acid groups. ⟊ refers to means ± SEM.

TABLE I. CONCENTRATION OF ASCORBIC ACID IN LIVER, KIDNEYS, AND ADRENALS

Group	Ascorbic acid supplement to basal diet (mg/kg)	Ethanol infusion	Ascorbic acid concentration (μg/g of wet tissue)					
			Liver	t	Kidney	t	Adrenal	t
1 (6)[a]	2000	−	211 ± 30.2[b]		241 ± 17.96		1077 ± 11.5	
2 (5)	200	+	102 ± 17.9	3.12*	123 ± 6.70	5.81**	174 ± 56.3	8.41**
3 (4)	2000	+	116 ± 14.5	2.88*	190 ± 7.50	2.62*	150 ± 28.0	7.86**

[a] Number of usable animals.
[b] Mean ± SEM.
* $P < 0.05$.
** $P < 0.01$.

showed no significant differences, most likely due to the wide spread between the highest and lowest values of these cations (Ca, Mg) in urine. Plasma analysis for various parameters, as shown in Table III, did not show significant increase, except in malonylaldehyde and α-tocopherol with P values of <0.01 and <0.05, respectively.

Analysis of tissues for Ca, Mg, Zn, and Cu showed no significant differences except for diminished liver and skeletal muscle magnesium in the ethanol-treated group fed marginal ascorbic acid.

Histological examination of the liver revealed mild to moderate steatosis in some of the experimental animals. An example of moderate steatosis (grade 3) is shown in Fig. 4 and one of mild changes (grade 2) in Fig. 5. In the ascorbic acid-deficient ethanol-treated group, two of the animals had grade 2 and one had grade 3 steatosis. In the ethanol-treated high-ascorbic acid group three out of four animals showed minimal changes (grade 1). Fatty change was mostly encountered in the centrilobular zones.

Discussion. We have attempted in this study to test the suitability of the guinea pig as an animal model for chronic alcoholism; and since it lacks the ability to synthesize its own ascorbic acid, to study the interrelationship of ascorbic acid and ethanol under two experimental conditions, namely, in animals fed high or low levels of ascorbic acid.

Before utilizing the permanent cannulation technique, we have repeatedly attempted to administer ethanol to guinea pigs intraperitoneally and by stomach tube. A high mortality rate, caused by either infection, trauma, or stomach irritation, was, however, encountered. We had also incorporated ethanol in an alcohol-liquid-diet mixture or in a gelled-dried diet. These modes of administration, however, led to very limited ethanol consumption, and the blood ethanol levels attained were not of sufficient magnitude pharmacologically as to represent a good model for alcohol toxicity.

Although intraarterial alcohol administration through permanent cannulation is not ideal physiologically, we achieved both an adequate control of ethanol loading in pharmacological doses and a convenient and

TABLE II. TWENTY-FOUR HOUR URINARY EXCRETION (μg/24 hr) OF ASCORBIC ACID, CALCIUM, AND MAGNESIUM

Group	Ascorbic acid supplement to basal diet (mg/kg)	Ethanol infusion	Ascorbic acid	Calcium	Magnesium
1 (5)[a]	2000	−	121 ± 52[b]	569 ± 420	414 ± 320
2 (5)	200	+	340 ± 121	225 ± 139	15 ± 10
3 (4)	2000	+	195 ± 160	359 ± 48	95 ± 80

[a] Number of usable animals.
[b] Mean ± SEM.

TABLE III. PLASMA ANALYSIS

	No ethanol	Ethanol	
Ascorbic acid supplement to basal diet (mg/kg):	Group 1 (6)[a] 2000	Group 2 (5) 200	Group 3 (4) 2000
Hematocrit (whole blood)	42.40 ± 6.69[b]	33.25 ± 13.15	39.00 ± 1.73
Total protein (g/100 ml)	4.38 ± 0.82	4.18 ± 0.55	4.14 ± 0.58
Malonylaldehyde (n*M*/ml)	5.28 ± 3.19	8.82 ± 2.62**	10.33 ± 3.83**
α-tocopherol (μg/ml)	8.57 ± 2.54	13.6 ± 2.90*	9.14 ± 0.53
Ca (meq/l)	7.02 ± 1.08	7.13 ± 0.29	7.29 ± 0.20
Zn (μg%)	95.00 ± 13.40	90.00 ± 25.07	90.30 ± 25.50
Mg (meq/l)	1.07 ± 0.19	1.14 ± 0.17	1.14 ± 0.08

[a] Number of usable animals.
[b] Mean ± SEM.
* $P < 0.05$.
** $P < 0.01$.

speedy blood sampling for analysis of various parameters. Four percent of our animals died of infection, twelve percent of postsurgical complications, and twenty percent could not be used due to cannula blockage. The latter could have been avoided if we had heparinized the cannulae 7 days a week instead of 5.

A comparison of the observed effects of fructose and ascorbic acid in the acute study suggests that these compounds enhance the rate of ethanol disappearance from the blood by different mechanisms of action since the two regression lines are not parallel. Fructose is generally recognized as an agent which increases the rate of ethanol metabolism (18, 19), and is also known to act by regenerating

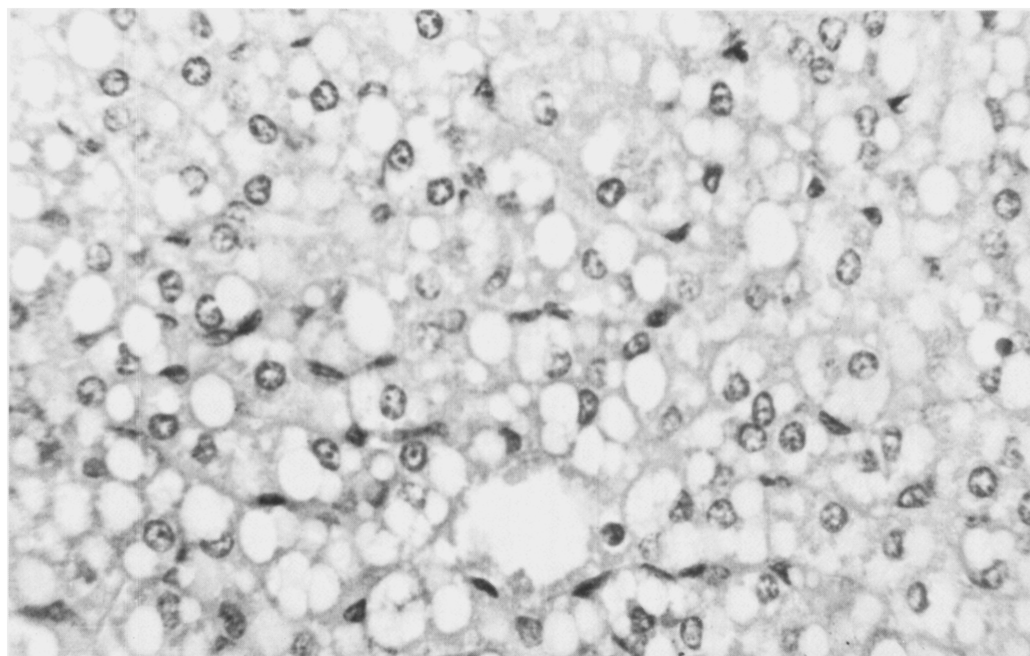

FIG. 4. Grade 3 (moderate) steatosis in ethanol-treated ascorbic acid-deficient animal.

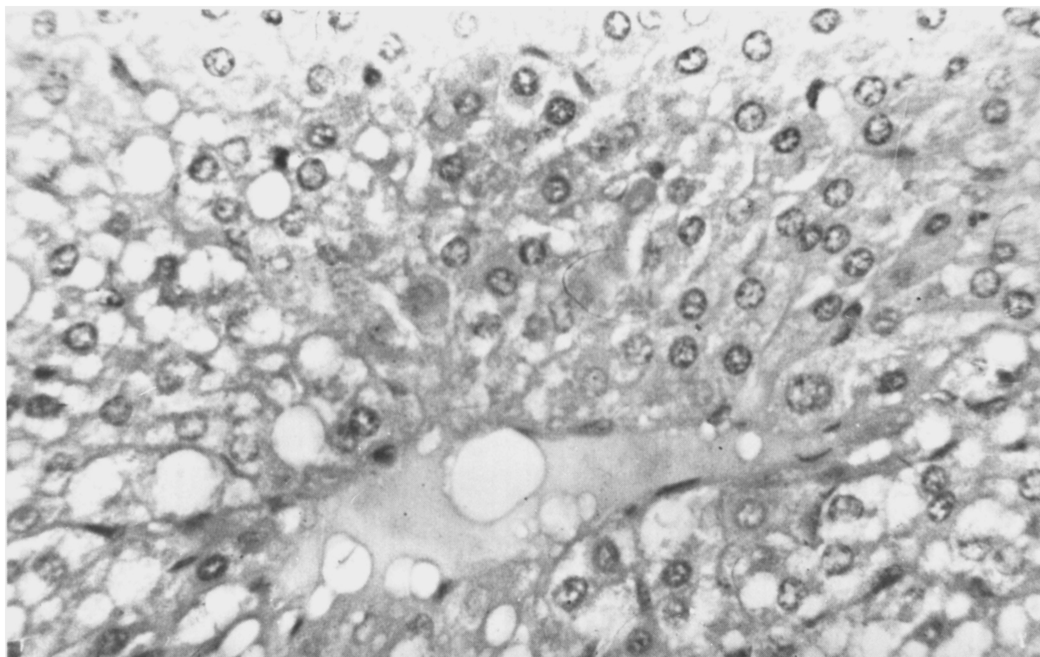

FIG. 5. Grade 2 (mild) steatosis in ethanol-treated ascorbic acid-supplemented animal.

the reduced NADH produced during alcohol oxidation to NAD (25). This is due to the fact that the V_{max} of ADH depends to some extent on the reoxidation rate of formed NADH (26). The mechanism of action of ascorbic acid remains undetermined.

In our chronic studies, the data suggest that alcohol intake depresses the body's ascorbic acid pool as demonstrated by the highly significant reduction of ascorbic acid content in the tissues examined. This is probably due, at least in part, to increased utilization of the vitamin. The urinary excretion of ascorbic acid was also increased in alcohol-treated animals, and this probably also contributed to depletion of the ascorbic acid body pool. Our findings in the adrenals confirm previous work in rats by Smith (27).

Recently, Hsu and Hsieh (9) demonstrated a high urinary excretion of ascorbic acid in rats receiving 20% ethanol in their drink. A high concentration of ascorbic acid was found in soft tissues. They also showed a significant increase of glucuronolactone and of gulonolactone conversion into ascorbic acid, which suggests an enhancement of ascorbic acid synthesis during ethanol intoxication. By contrast, in our experiment, a significant decrease of ascorbic acid content was observed in the liver, kidneys, and especially the adrenals of guinea pigs infused with ethanol.

In the two groups chronically infused with ethanol, there were significant increases ($P < 0.05$) of malonylaldehyde, a substance often taken as a measure of lipid peroxidation (21, 28, 29). Our findings thus support previous reports of increased lipid peroxidation in chronic alcoholism in man (30) and animals (31, 32). Although the mechanism is not clear, Reitz (32) suggests that it is probably induced through an NADH-catalyzed system.

The increase of α-tocopherol in the ethanol-treated ascorbic acid-deficient animals, but not in the ascorbic acid-supplemented animals, is difficult to explain. One consideration is that ethanol possibly increases intestinal absorption of α-tocopherol. This possibility, however, can be ruled out since both groups receiving ethanol did not demonstrate this increase. In addition, since intestinal absorption of α-tocopherol has been shown to be unaffected by dietary ascorbic acid supplementation (33), the difference in

plasma α-tocopherol levels between the two ethanol-treated groups canoot be explained on the basis of intestinal absorption alone. Another possibility is related to the high correlation of plasma α-tocopherol with total serum lipids as reported by Horwitt *et al.* (34). Although we did not measure serum lipids, the demonstration of liver steatosis particularly in ascorbic acid-deficient ethanol-treated animals could explain the increased α-tocopherol in these animals. It is interesting to note that this increase was not significant in the ascorbic acid-supplemented group which suggests that ascorbic acid might have an α-tocopherol sparing effect.

Our data on the body weight loss in alcohol-treated animals are supported by published findings (35, 36). Ethanol treatment is known to reduce body weight when animals are compared to pair-fed controls with equal caloric intake. At the end of 8 weeks, our control group maintained its body weight while alcohol-treated animals lost 50 g/animal in the low-ascorbic-acid group and a gain of 11 g/animal was observed in the high-ascorbic-acid group.

There is now increasing evidence indicating that ascorbic acid has a beneficial effect on ethanol metabolism in man (30, 31, 37, 38) and animals (8, 10, 39) and conversely that ethanol intake adversely affects the ascorbic acid status (37). Krasner *et al.* (38) reported that human volunteers receiving 1.0 g ascorbic acid for 2 weeks show a high correlation between the rate of blood ethanol disappearance and leucocytic ascorbic acid content ($r = 0.6$). This may point to a direct detoxification reaction between ascorbic acid and acetaldehyde or indirectly result from increased utilization of ascorbic acid due to increased catecholamine release. Although there was no strict control in these experiments, subsequent studies by the same authors (40) in alcoholic and nonalcoholic subjects again demonstrated a high correlation between low activities of ADH and low tissue levels of ascorbic acid. They also noted an enhanced rate of blood ethanol decline in healthy subjects after a period of ascorbic acid supplementation.

A dose-related protection by ascorbic acid against acetaldehyde, an intermediary of ethanol oxidation, has been documented by Sprince *et al.* (8), which again suggests a beneficial role of ascorbic acid in the intermediary pathways of ethanol metabolism. The central role of acetaldehyde in the chain of events which leads eventually to ethanol intoxication has recently been emphasized by von Wartburg (41) who suggests that its level in various target organs could serve as a biologic marker for high risk drinkers.

An interesting report by Busnel and Lehmann (10) demonstrated that an ascorbic acid dose of 500 mg/kg reversed the impaired swimming behavior of mice treated with ethanol. More recently, Moldowan and Acholoiu (39) studied the effect of ascorbic acid on mortality due to disulfiram–ethanol-induced acetaldehyde in mice by pretreating the animals with ascorbic acid. While a single dose had no effect on mortality rate, pretreatment of the animals for 3 days resulted in a reduction of disulfiram–ethanol mortality. In our chronic study, random sampling of blood ethanol, 3 hr postinfusion, showed a significant decrease of blood ethanol concentration ($P < 0.01$) in animals receiving high ascorbic acid in their diet. Whether this decrease is due to the effect of ascorbic acid on the enzymes responsible for ethanol degradation or on enzymes acting on the product(s) of its metabolism is not clear.

The demonstration of hepatic fatty metamorphosis after 8 weeks of ethanol treatment in the guinea pig lends further support to the desirability of using this animal for the study of alcoholism and its hepatotoxic sequelae, particularly in view of certain similarities between the guinea pig and man. In spite of the small number of animals used in these experiments, the demonstration of a reduction of fatty infiltration of the liver in animals receiving high ascorbic acid further suggests an association between ascorbic acid status and alcohol-induced hepatotoxicity. Whether a prolongation of chronic ethanol toxicity beyond these 8 weeks of experimentation can induce further aggravation of liver damage into a fibrotic or cirrhotic stage, or whether long term administration of ascorbic acid can halt or reverse the progression of the injury is not known. Further studies are needed to elucidate the answers to some of these fundamental questions.

This research was supported by Hoffmann-La Roche, Inc. and the Medical Research Service of the Veterans Administration. We are grateful to Dr. Hemmy N. Bhagavahn, Nutrition coordinator at Hoffmann-La Roche for reviewing the manuscript and to Pat Greene and Lois Sullivan of the Veterans Administration Pathology Department for their secretarial assistance. This work has been presented, in part, at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, April 10–15, 1983, Chicago, Illinois.

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Received September 29, 1983. P.S.E.B.M. 1984, Vol. 177.
Accepted June 20, 1984.