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Effects of Dietary Ethanol upon Experimental Trypanosomal (*T. cruzi*) Myocarditis.* (32399)

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Ever since Bollinger in 1884(1) attributed chronic cardiac hypertrophy to the excessive intake of beer, the association of primary myocardial disease with chronic alcoholism has led to periodic postulation of an etiologic role of alcohol in the pathogenesis of primary myocardial disease(2-6).

Although acute administration of alcohol had been found to have cardiac effects, both in experimental animals(7,8) and in man(9), we could find no reports of definite heart disease produced experimentally by chronic ingestion of ethanol under controlled conditions when the present studies were being carried out.

Previous attempts in this laboratory to assure chronic intake of ethanol in mice by adding ethanol to drinking water failed because of the animals' failure to drink any but the most dilute solution of ethanol. The success reported by Lieber and associates in feeding rats a liquid diet containing ethanol (10) for other purposes prompted the present investigation, which was designed to study possible effects of chronic ingestion of ethanol on the heart of the healthy mouse, as well as upon acute experimental trypanosomal myocarditis, a model studied previously in this laboratory(11,12).

Material and methods. Animals. Forty-eight weanling, male C3H mice‡ were housed 6 per cage and kept at a temperature of approximately 22°C. They were fed Purina Rat Chow until 21 weeks old and weighing 22 to 28 g. At this time, they were divided into 4 groups (Table II), equal in number and in mean weight (25.1 g), transferred to individual cages and given liquid diets in graduated drinking tubes as the only source of food and water. The mice were inspected daily and weighed weekly.

TABLE I. Composition of Control Diet.

	g/l	% of total cal.
Amino acids	47.35	19
Fats	43.00	42
Sucrose	95.00	38
Salts	10.00	
Choline	.00025	
Methionine	.00150	
Vitamin	.05	
Sod. carrageenate	4.00	

Diets. The diets were adapted from those of Lieber and associates(10). The composition of the control diet, used in Groups 1 and 2, is given in Table I. The ingredients were blended with distilled water to give a final concentration of approximately 1 calorie per ml of diet. The ethanol diet, used in Groups 3 and 4, was identical, except that it contained only

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60 g/l of sucrose, the remainder of the sucrose having been replaced isocalorically with 20 g/l of ethanol, accounting for 14.3% of total calories. Ethanol was introduced into the diet gradually. During the first 2 days, the animals were given the liquid diets with 10 g/l of ethanol, which was increased to its final concentration of 20 g/l on the third day. Animals in Groups 1 and 2 finished their diet more rapidly than those in Groups 3 and 4. Caloric intake was kept equal for all animals by allowing the alcohol groups to determine the amount to be fed to the sucrose groups. The average intake of mice in Groups 3 and 4 was 15 ml of diet, or about 300 mg ethanol per day.

A preliminary study had shown that young mice fed the control liquid diet or Purina Rat Chow had similar growth rates. This was confirmed by a group of 11 C3H mice of the same age as the experimental mice, with an initial mean weight of 25.2 g, fed simultaneously with Purina Rat Chow *ad libitum*, whose mean final weight aws 29.5 g, not significantly different from the final weight of the uninfected control group 1 (Table II). Another preliminary study had established that adult mice would not maintain normal body weight when the concentration of ethanol in the diet exceeded 20 g/l.

Infection. After 65 days on the special diets, at the approximate age of 30 weeks, animals in Groups 2 and 4 were given an intraperitoneal injection of 9,000 *Trypanosoma cruzi*. The same strain of *T. cruzi* (Colombian) was used in previous experiments to produce acute myocarditis in susceptible C3H mice in this laboratory(11,12). After the 72nd passage in Harvard Swiss mice, the parasite was passed once through weanling C3H mice and then used in the present experiment. At weekly intervals, the degree of parasitemia was estimated semiquantitatively in all inoculated animals(11).

Pathology. All mice were sacrificed with ether 49 days after inoculation, and 114 days after the special diets were started. The heart was severed 2 mm above the origin of the great vessels and weighed§ to the nearest

§ Precision-Balance, Roller-Smith, Bethlehem, Pa.

TABLE II. Mortality, Heart and Body Weights, and Histopathology in the 4 Experimental Groups.

Group	Diet	Infection	No. of animals	No. deaths	Final body wt, g	Heart wt mean ± SD, mg§	Heart wt / Body wt × 10^3 mean ± SD‖	Histopathology: Degree of inflammation*	Histopathology: Degree of necrosis*	Histopathology: Parasitic aggregates, No./section
1	Control	0	11	0	30.5	110.1 ± 3.2	3.63 ± .18	0	0	0
2†	"	+	12	8	23.9	133.4 ± 17.7	5.63 ± .64	1.6	0.6	1.5
3	Ethanol	0	12	3	29.0	114.6 ± 5.1	3.95 ± .13	0	0	0
4‡	"	+	12	9	24.6	145.6 ± 19.8	6.43 ± 1.34	2.5	1.2	5.0

* Scale 1 to 3.
† Weights and histopathology based on 4 survivors plus 4 animals which died at height of disease.
‡ Weights and histopathology based on 3 survivors plus 7 animals which died at height of disease.
§ Significance of difference between groups: group 1 *vs* 2, $P < .01$; group 1 *vs* 3, $P < .05$; group 1 *vs* 4, $P < .01$; group 3 *vs* 4, $P < .01$.
‖ Significance of difference between groups: group 1 *vs* 2, $P < .01$; group 1 *vs* 3, $P < .01$; group 1 *vs* 4, $P < .01$; group 3 *vs* 4, $P < .01$.

0.2 mg. The viscera were examined grossly. Portions of the heart, lung and liver were taken for microscopic study. Tissues were fixed in 10% Formalin, embedded in paraffin, sectioned at 8 μ, and stained with hematoxylin and eosin. Approximately 5 sections per heart were examined. The severity of cellular and parasitic infiltration was graded by both authors independently and without knowledge of the identity of the slides. Each heart was graded 1^+, 2^+ or 3^+, indicating mild, moderate or severe cellular infiltration. Degree of necrosis was similarly graded. The number of parasitized cells in one complete section was counted.

Results (Table II). Uninfected animals (Groups 1 and 3). There was no significant difference in appearance among animals on control or ethanol diets. The ethanol-fed mice occasionally appeared hyperactive with peculiar jumping movements. All uninfected animals followed similar growth curves. One mouse in the control group (Group 1) escaped from its cage at the beginning of the experiment and was eliminated from the group. Three animals in Group 3 died during the study, one each on the 11th, 58th, and 84th day of the special diet. The animals of Group 1 had a mean heart weight of 110.1 $\pm$ 3.2 mg and a heart weight/body weight ratio of 3.63 $\pm$ 0.18 $\times$ 10^{-3}. The 9 surviving animals of Group 3 had a mean heart weight of 114.6 $\pm$ 5.1 mg and a heart weight/body weight ratio of 3.95 $\pm$ 0.13 $\times$ 10^{-3}. The animals fed ethanol had significantly heavier hearts ($p<0.05$). Their heart weight/body weight ratios were also significantly above those of the control animals ($p<0.01$). Gross and microscopic examination of the hearts was unrevealing and did not differentiate between the groups.

Infected animals (Groups 2 and 4). There was no significant difference in appearance between the infected animals on the control diet (Group 2) and those on the ethanol diet (Group 4). Compared to the uninfected animals, however, all infected animals appeared ill. They were less active, their fur was shaggy, and they weighed less at termination of the study. The curve of body weight after infection followed the pattern noted previously(12). In the first 16 days of the infection the body weight of the infected animals rose above that of the uninfected mice, but fell below it as the parasitemia reached its peak. This loss of weight persisted, with some recovery, until the animals were sacrificed at the conclusion of the experiment.

The level of trypanosomes in the blood reached its peak in the 4th week at about 12 trypanosomes per high power field, and was approaching zero at termination of the experiment. There was no significant difference in trypanosome counts between animals in Groups 2 and 4.

Of the 12 infected animals in Group 2, 4 survived to the end of the study. Eight mice died as follows: 1 each on the first, fourth and fifth day after inoculation, 2 each on the 25th and 27 days, and one on the 32nd day. Immediate postmortem examination did not reveal a cause of death in the animals dying early after inoculation. None of the animals in Group 2 were noted to be in congestive heart failure. Only 3 of the 12 infected animals in the group given ethanol (Group 4) survived to the end of the study. One died of overdosage of ether on the day of inoculation, and one each died on the 13th, 32nd, 33rd, 40th and 42nd day after inoculation. Three mice died on the 43rd day. Two of these latter had definite ascites and pleural effusions, associated with a large, dilated heart and, in one case, with pulmonary edema. These animals are thought to have died in congestive heart failure.

The mean heart weight of the 4 survivors of Group 2 was 146.9 $\pm$ 5.9 mg, and the heart weight/body weight ratio was 5.36 $\pm$ 0.38 $\times$ 10^{-3}. Inclusion of the 4 animals who died at the height of the disease resulted in a mean heart weight of 133.4 $\pm$ 17.7, and in a heart weight/body weight ratio of 5.63 $\pm$ 0.64 $\times$ 10^{-3}. The mean heart weight of the 3 survivors in group 4 was 135.7 $\pm$ 12.5 mg, and the heart weight/body weight ratio was 4.91 $\pm$ 0.52 $\times$ 10^{-3}. Inclusion of 7 animals who died at the height of the infection resulted in a mean heart weight of 145.6 $\pm$ 19.8 mg, and in a heart weight/body weight ratio of 6.43 $\pm$ 1.34 $\times$ 10^{-3}. There was no significant difference in mean heart weight or

heart weight/body weight ratio between the two groups, irrespective of whether or not the earlier deaths were included.

Gross pathologic examination revealed enlargement of the heart, especially of the right atrium and right ventricle, in the infected animals. The spleens of all infected mice were enlarged. On microscopic examination, there was focal and diffuse mononuclear infiltration of the myocardium, associated with patchy necrosis. There was some tendency for the infiltrates to involve especially the subepicardial myocardium of the right ventricle. Scattered intracellular leishmanial forms of *T. cruzi* were seen. There was a tendency to more extensive myocardial involvement in group 4, and some hearts stood out for the severity of infiltration and parasitic involvement (Fig. 1). Yet, the differences in

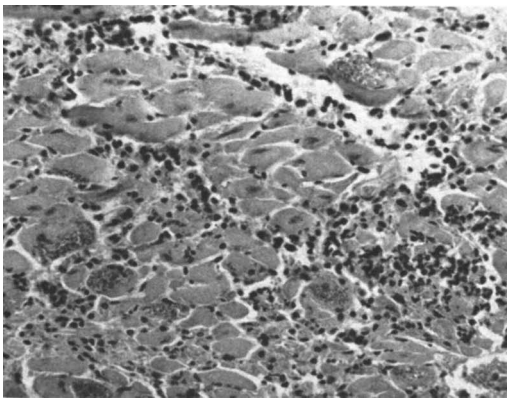

FIG. 1. Right ventricular myocardium (× 160), H & E stain, of an animal in Group 4, which had died 43 days after inoculation. There is infiltration with predominantly mononuclear cells, and there are many intracellular aggregates of leishmanial forms of *Trypanosoma cruzi.*

myocardial infiltration, necrosis, or parasite counts between the two groups of infected animals were not significant statistically.

Discussion. The present study has demonstrated that the incorporation of ethanol into a liquid diet, introduced by Lieber and associates(10), can be used successfully to overcome the natural aversion of mice to alcohol, previously observed in this laboratory. In the manner described, it was possible to administer enough ethanol to account for 1/7 of the total caloric intake for a period of 16 weeks. The mortality of 25% in the uninfected ethanol group (Group 3) remains unexplained.

The small increases in heart weight (+4.1%) and in heart weight/body weight ratio (8.8%) found in Group 3 compared to Group 1 are significant because of the small scatter of values in each group, the smallest we have encountered(11,12). These small standard deviations suggest that a measured liquid diet, adjusted in amount so that most animals ingested their full daily portion, may reduce variability of heart weight and permit the detection of small differences between groups. These differences in heart weight between Groups 3 and 1 must be associated with the only known difference between these two groups, namely the intake of alcohol. Theoretically, this difference could be due to an increased water content of heart tissue in the ethanol-fed animals. This possibility cannot be ruled out on the basis of the present data. Alternatively, the increased heart weight could represent cardiac hypertrophy. It was not associated with any histopathologic lesions demonstrable by the light microscope.

The mortality of 67% during the first 8 weeks after infection with *T. cruzi* in the group on control diet indicates that after the 72nd mouse passage the Colombian strain of *T. cruzi,* originally characterized by a very low mortality, has become more virulent. This is in keeping with an earlier experience(11).

The most striking result of the administration of ethanol to mice preceding and following inoculation with *T. cruzi* was the death of two animals on the 43rd day of infection in florid congestive heart failure with ascites. This had not been previously observed in this laboratory in over 600 mice inoculated and observed during the acute phase of the disease, not even after forced exercise.

Although the heart weight of the infected, ethanol-fed animals exceeded that of the infected controls by 9.1%, and the heart weight/body weight ratio of Group 4 exceeded that of Group 2 by 14.2%, (differences greater than those between Groups 1 and 3), these differences are associated with large intra-group variability and are not statistically significant. Similarly, although the ethanol-fed animals tended to show more evidence of myocarditis, this difference was

not significant. Nevertheless, although a relationship has not been established, the data may be considered to suggest an enhancing effect of ethanol upon acute Chagasic myocarditis. Alcoholic intoxication repeatedly has been found associated with an increased morbidity and mortality in both clinical and experimental bacterial infections(13). Thus in rabbits alcoholic intoxication lowered resistance to pneumococcal infections after active or passive immunization, and was associated with a reduction of local inflammatory responses(14). We are not aware, however, of any previous studies of the effect of alcohol upon protozoan infections.

The present study was prompted by the question: does alcoholic heart disease exist? After acute administration of ethanol in large doses, significant depression of myocardial contractility as well as changes in myocardial metabolism have been demonstrated both in animals(7,8) and in man(9). Such acute cardiac toxicity, however, does not permit conclusions as to effects of chronic ingestion of ethanol. Nor is an etiological role of ethanol established for the primary myocardial disease seen in chronic alcoholic patients, who are usually exposed to intermittent malnutrition and not infrequently to infection.

Most recently, Maines and Aldinger(15) have administered ethanol orally to rats and, after 4 months, have observed consistent decreases in ventricular contractile force compared to pair-fed control animals. These physiologic effects were at times associated with cardiac enlargement and with a tendency to arrhythmias after administration of epinephrine. Whereas the rats studied by Maines and Aldinger were described as chronically sedated and lethargic, and were also characterized by lower fluid intake and body weight than their controls, the mice of the present study did not exhibit evidence of decreased bodily activity and in body weight followed their controls closely. These two studies would seem to complement each other, establish that a chronic myocardopathy can be induced experimentally by ingestion of ethanol, and lend support to the hypothesis that ethanol may play an etiologic role in the primary myocardiopathy of the alcoholic(2-6).

The evidence of an enhancing effect of ethanol upon Chagasic myocarditis raises the question whether some cases of alcoholic myocardiopathy may represent the late stages of a modified form of myocarditis.

Summary and conclusions. The effects of prolonged ingestion of alcohol upon the course and severity of acute experimental trypanosomal myocarditis in young male C3H mice were studied. Twenty-four mice were fed a liquid control diet. After 65 days, 12 of these animals were inoculated with 9,000 *Trypanosoma cruzi* (Colombian). Twenty-four mice were fed a similar diet except that 1/7 of its calories were derived from ethanol, isocalorically substituted for sucrose. Half of these animals were infected after 65 days. Mice were weighed, and parasites in blood smears were counted once weekly. After 114 days on the diets, the surviving animals were sacrificed. The uninfected animals maintained their weight normally. The ethanol-fed animals differed from their controls by having slightly but significantly heavier hearts (4% of heart weight and 9% of heart weight/body weight ratio). Three of 12 ethanol-fed animals died early. The infected animals lost weight on either diet and showed a mortality of 17/24 irrespective of diet. Parasitemia was not affected by ethanol. Two of 9 animals of the ethanol-fed group who died early, died on day 43 of the infection in congestive heart failure with markedly dilated hearts and ascites, a picture not seen in other mice of this series. The heart weight of the infected, ethanol-fed mice exceeded that of the infected controls by 9%, and the heart weight/body weight ratio by 14%; neither difference was statistically significant. Histopathologic examination suggested more inflammation, necrosis and parasites in the myocardium of ethanol-fed animals. It is concluded that chronically administered dietary alcohol may increase heart weight and may, at least in some animals, enhance the effects of acute Chagasic myocarditis.

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Incorporation of C^{14}-Orotic Acid and C^{14}-Amino Acid into Pigeon Pancreas Slices Following Cholinergic Stimulation.* (32400)

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Hokin and Hokin(1) have reported that stimulation of enzyme secretion by carbamylcholine and acetylcholine is not accompanied by any increase in rate of incorporation of P^{32} into RNA in slices of pigeon pancreas incubated *in vitro*. Schucher and Hokin(2) also reported that cholinergic stimulation of enzyme secretion is not accompanied by increased enzyme synthesis. These views on the lack of an obligatory link between protein synthesis and RNA turn-over, and the lack of an effect of secretion on the rate of synthesis have been re-presented in a more recent symposium(3). In view of current theories of regulation of protein synthesis it seemed improbable that the synthesis of pancreatic enzymes proceeds at a constant rate which is independent of secretory activity. Therefore, experiments were performed on the effect of altered secretory rate on incorporation of labeled orotic acid into RNA and of labeled protein hydrolysate into protein.

Materials and methods. Preparation and incubation of tissues. Male White King pigeons (*c* 600 g) were fasted for 24 hours before sacrifice. The pancreas was removed under ether anesthesia, trimmed free of connective tissue and fat, chilled in ice and sliced with a Stadie-Riggs slicer. The thickness of the slices was approximately 0.5 mm. The entire pancreas was sliced and alternate slices were used for the experimental and control conditions. The slices were placed in 5 ml of bicarbonate-saline buffer(4) containing glucose (200 mg/100 ml), amino acid mixture (20 mg/100 ml), sodium pyruvate (0.02 M final concentration) and 20 μc orotic acid-6-C^{14} (Nuclear-Chicago, specific activity 44.5 mc/mM). These conditions are essentially similar to those of Hokin and Hokin(1,5). Acetylcholine (to a final concentration of 1 μg/ml) was added to one vessel. The incubation vessels were gassed with 95% O_2, 5% CO_2 for 90 minutes at 40°. The slices were then washed with buffer for an hour with 6 changes in a Dubnoff shaker. Some experiments were carried out with carbamylcholine given intramuscularly (0.15 mg/kg) an hour before killing of the birds. In this case a bird given an injection of 1 ml of saline served as the control. In experiments to study the incorporation of labeled amino acids, 20 μc of labeled protein hydrolysate

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