treponema-like organism isolated from *I. dammini* may be involved in the etiology of Lyme disease. It is interesting that organisms presenting the morphological characteristics of spirochetes were said to be associated with ECM in Europe as early as 1948 (*15*). Although this was never confirmed, a recent study (*16*) showing that resolution of lesions and concurrent symptoms occurs faster in patients treated with penicillin suggests a penicillin-susceptible bacterium as an etiologic agent of Lyme disease.

Our results establish the susceptibility of the domestic rabbit to the *I. dammini* spirochete and demonstrate the possible value of the indirect immunofluorescence test as a diagnostic tool for Lyme disease. They also suggest the need for additional investigations not only into the epidemiology and ecology of Lyme disease and related disorders, such as ECM of Europe (*17*), but also into the relations between the spirochete and its vector *I. dammini*.

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8. The ticks were first examined by the hemolymph test [W. Burgdorfer, *Am. J. Trop. Med. Hyg.* **19**, 1010 (1970)]. Subsequently they were dissected for the preparation of multiple smears from gut, malpighian tubules, salivary glands, central ganglion, and testes or ovary. Smears were stained according to the Giménez method [D. F. Giménez, *Stain Technol.* **39**, 135 (1964)] or with Giemsa. Once spirochetes were detected, wet preparations of tissues were examined also under dark field.
9. For electron microscopy, diverticula of midgut were removed by dissection and were processed according to S. F. Hayes and W. Burgdorfer [*J. Bacteriol.* **137**, 605 (1979)].
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11. Kelly's medium [R. Kelly, *Science* **173**, 443 (1971)] modified by addition of CMRL medium 1066 (Gibco No. 330-1540) and Yeastolate (Difco) for final concentrations of 5 and 0.2 percent, respectively (H. G. Stoenner, in preparation).
12. Fifteen to twenty *I. dammini* females and equal numbers of males for mating (males may ingest small amounts of blood) were placed on each of eight rabbits. The ticks were contained in metal capsules attached by adhesive tape to the shaved abdomen of each rabbit.
13. In accordance with the data of R. N. Philip, E. A. Casper, R. A. Ormsbee, M. G. Peacock, and W. Burgdorfer [*J. Clin. Microbiol.* **3**, 51 (1976)] midgut smears of infected ticks or cultured spirochetes were used as antigen. Fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobulin (Chappel Laboratories) was used at a 1:50 dilution in phosphate-buffered saline with 1 percent bovine serum albumin.
14. Fluorescein isothiocyanate–conjugated goat antibody to human immunoglobulin (BBL, Cockeysville, Md.) was used at 1:100 dilution in phosphate-buffered saline with 1 percent bovine serum albumin.
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17. Since submission of this manuscript, microscopic examination by one of us (W.B.) of midgut smears from *Ixodes pacificus* from Oregon and of *I. ricinus* from Switzerland also revealed, in some instances, the presence of spirochetes.
18. We thank the Nature Conservancy Incorporation for permission to collect ticks in their Shelter Island Preserve. We also thank E. Bosler, S. Guirgis, D. Massey, and J. Coleman for their assistance in collecting ticks. Special thanks also to W. H. Hadlow, Epidemiology Branch, Rocky Mountain Laboratories, for the histologic characterization of the rabbit lesions.

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Lack of Correlation Between Hepatic Mitochondrial Membrane Structure and Functions in Ethanol-Fed Rats

Abstract. *A current hypothesis suggests that alterations in the chemical composition and the subsequent changes in the structure of the membrane could account for the functional derangements observed in the hepatic mitochondria of animals fed ethanol for extended periods. An examination of this hypothesis reveals that the liver mitochondria of ethanol-fed rats show a dissociation between the respiratory functions and the lipid composition and microviscosity of the membranes.*

One of the most characteristic features of alcoholic liver disease is the striking alteration in mitochondrial morphology and function. This condition has now been reproduced in an animal model (*1*). Hepatic mitochondrial preparations from ethanol-fed rats, which develop a fatty liver, and ethanol-fed baboons, which develop cirrhosis, exhibit a decreased ability to oxidize substrates and to form high-energy phosphate metabolites (*1*, *2*). The mechanisms underlying these changes are still being debated. Recently it was proposed that these functional changes may be directly related to alterations in the structural properties of cellular membranes produced as a consequence of prolonged ethanol consumption (*3*). Since the functions of the membrane are governed in part by their physical state, the hypothesis has been formulated that a correlation exists between changes in the structural properties of the membrane phospholipids and the functional alterations observed after long-term ethanol intake (*3*). However, our data indicate that such a simple correlation does not explain these functional changes. Hepatic mitochondrial preparations from ethanol-fed rats displayed severe functional impairment, yet the fluidity and lipid composition of the mitochondrial membranes were remarkably similar to those of mitochondrial membranes from pair-fed control animals. Conversely, mitochondria from the pair-fed control group and Purina Chow–fed animals were functionally similar, in spite of the differences in the lipid composition and fluid state of their membranes.

In this study we measured the fatty acid composition of the phospholipids and the microviscosity of mitochondrial membranes and the membrane-dependent functions of liver mitochondria from ethanol-fed rats and their respective pair-fed controls and then compared these findings to similar data obtained from Chow-fed rats. For that purpose, 16 male Sprague-Dawley rats (weighing 125 to 150 g each) were pair-fed a liquid diet containing 36 percent of its energy as ethanol, or alternatively in the control, as carbohydrates. The constituents of this diet have been described in (*4*). After 3 to 5 weeks on this diet, the animals were treated in the following manner. At 9:00 a.m. on the day before the experiments they were given one-third of their daily ration, and all food, except water, was removed at 4:00 p.m. The animals were killed on the next day between 9:00 and 10:00 a.m. At the same time, ten male Sprague-Dawley rats, maintained on Purina Chow (the Chow-fed controls) were fasted for a similar period of time before being killed. Standard procedures (*5*) were used to prepare the liver mitochondria, to analyze the fatty acid com-

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position, and to measure the ability of the mitochondria to oxidize substrates. We also assessed the microviscosity (fluidity) of the membranes by loading the mitochondrial preparations, which had been frozen and thawed with 12(9-anthroyloxy)stearic acid (12 AS) (Molecular Probes, Plano, Texas), in a molar ratio of 200:1 phospholipid phosphorus to probe. The final solution contained 60 to 100 μg of membrane protein in 2.0 ml of phosphate-buffered saline (*p*H 6.6). Steady-state fluorescence polarization measurements were made in a polarization spectrofluorometer (SLM Instruments, Champaign, Illinois) and expressed in terms of fluorescent anisotropy

$$r_s = \frac{2P}{3 - P}$$

where P is the degree of fluorescent polarization, which is assumed to provide an index of the fluidity of the lipid portion of the membrane (*6*). Control samples containing only tissue, or probe, were tested routinely and the appropriate corrections made.

Table 1. Fatty acid (FA) composition (percentage by weight) of the phospholipids of hepatic mitochondrial preparations from ethanol-fed rats, their pair-fed controls, and Purina Chow–fed rats after 18 hours of starvation. Values are the mean ± the standard error of the mean of relative amounts of FA, expressed as a percentage of the total FA by weight.

FA*	Ethanol-fed rats (N = 8)	Pair-fed controls (N = 8)	Purina Chow–fed rats (N = 10)
16:0	15.0 ± 0.3	18.2 ± 0.7†	19.5 ± 1.0
16:1	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.2
16:2	0.3 ± 0.1	0.3 ± 0.1	
18:0	21.2 ± 0.7	17.9 ± 0.5†	20.6 ± 0.5
18:1	15.6 ± 0.8	12.5 ± 0.3	9.4 ± 0.4‡
18:2	10.9 ± 1.1	12.7 ± 0.5	18.4 ± 0.5‡
20:4ω6	26.3 ± 0.8	26.8 ± 0.7	20.3 ± 1.3‡
22:5ω6	2.5 ± 0.4	2.4 ± 0.3	1.1 ± 0.1
22:5ω3		0.3 ± 0.1	
22:6ω3	4.4 ± 0.5	4.9 ± 0.3	5.6 ± 0.3
Total unsaturated FA	59.9 ± 0.9	60.3 ± 0.9	55.6 ± 1.1‡
Total saturated FA	36.4 ± 0.8	36.5 ± 0.7	40.2 ± 0.8‡
Total polyunsaturated FA	44.3 ± 1.1	47.0 ± 1.0	45.0 ± 1.3
Phospholipid phosphorus (micromoles per milligram of protein)			
	0.32 ± 0.04	0.30 ± 0.04	0.19 ± 0.02‡

*The first number signifies the number of carbon atoms in the chain; the second number indicates the number of double bonds in the FA. The number after ω indicates the position of the first double bond counting from the terminal methyl group. †$P < .01$ when values in the ethanol-fed group are compared to values for the pair-fed control group. ‡$P < .001$ when values in the Chow-fed group are compared to values for either of the other two groups.

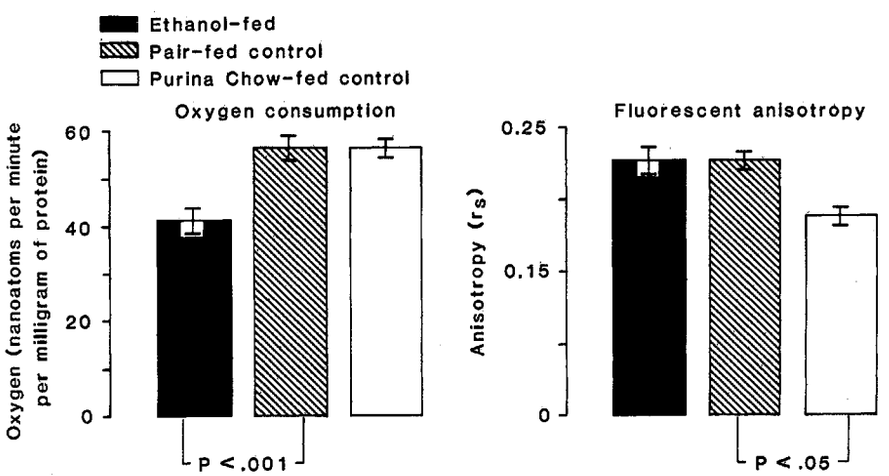

Fig. 1. Dissociation between the ethanol-induced depression of respiration and the microviscosity of the membranes of rat liver mitochondria. The rate of oxygen uptake during state 3 respiration induced by adenosine 5′-diphosphate (150 μ*M*) in the presence of glutamate (3.3 m*M*) is given in the left panel. Oxygen consumption was measured polarographically. The results are expressed as means ± standard errors of the means (N = 6). The assessment of the microviscosity of the membrane is presented in the right panel and is expressed as fluorescent anisotropy (r_s) (see text). The values are expressed as means ± standard deviations of the means (N = 7). All these experiments were carried out at 28°C.

The fatty acid composition of the phospholipids of mitochondrial preparations obtained from the different groups is presented in Table 1. The ratios of phospholipid to protein were similar in both the ethanol-fed animals and their pair-fed controls (0.32 ± 0.04 μmole of phospholipid phosphorus per milligram of protein in the ethanol-fed rats versus 0.30 ± 0.04 in the control rats). On the other hand, the ratio of phospholipid to protein in the preparations from Chow-fed controls were found to be significantly lower (0.19 ± 0.02 μmole of phospholipid phosphorus per milligram of protein) ($P < .001$). No significant differences were observed in either the relative or the absolute amounts of total saturated, unsaturated, or polyunsaturated fatty acids between ethanol-fed rats and their pair-fed controls. Indeed, the only differences displayed between these two experimental groups were the relative amounts of the individual saturated fatty acids; there was a greater proportion of stearic acid and less of palmitic acid in the preparation from ethanol-fed animals. By contrast, significant differences were noted between the above-mentioned groups and that of the Chow-fed animals in the amounts and proportions of the fatty acids of the membrane phospholipids. The relative amount of unsaturated fatty acids was significantly lower, and that of saturated fatty acids significantly higher, in the membranes from Chow-fed rats as compared to the other preparations.

However, no correlation was found between these changes and the ability of the mitochondria to oxidize substrates. Compared to the controls, the mitochondria of ethanol-fed animals exhibited a significant decrease in the oxidation rates for substrates entering sites I, II, and III of the respiratory chain. State 3 respiration was lower by 27.2 percent ($P < .001$) when glutamate was used as substrate, and 18.3 percent ($P < .001$) and 21.8 percent ($P < .02$) when succinate and ascorbate, respectively, were used as substrates in the ethanol-fed group. Mitochondria isolated from the pair-fed and Chow-fed groups showed no differences in their capacity to oxidize these substrates. The results obtained with the use of glutamate as a substrate are presented in Fig. 1.

The fluorescence polarization measurements of these preparations are also presented in Fig. 1. The microviscosity of the membranes from ethanol-fed rats and their pair-fed controls were not significantly different; the r_s values were 0.23 ± 0.05 and 0.23 ± 0.03, respective-

ly. We tested the fluidizing effect of ethanol on these preparations by increasing the concentration of ethanol in vitro from 8 to 130 m*M*. This caused a progressive decrease in the microviscosity (increased fluidity) of the membranes, which was similar in both groups. The decrement in the r_s value from 8 to 130 m*M* ethanol was highly significant ($\Delta r_s = 0.09$; $P < .01$). By contrast, the r_s values obtained in membranes from the Chow-fed group were initially lower than those of the other experimental groups ($r_s = 0.19 \pm 0.03$ versus 0.23 ± 0.05; $P < .05$), and increasing the concentrations of ethanol from 8 to 130 m*M* affected their microviscosity to a lesser degree (0.19 ± 0.030 to 0.17 ± 0.01). The data indicate that mitochondrial membranes from the Chow-fed rats fasted for 18 hours are more resistant to the fluiding effects of ethanol in vitro than membrane preparations from ethanol-fed rats. Thus, the addition of ethanol at concentrations comparable to those present in man and animals consuming ethanol altered the structural properties of the mitochondrial membranes from both the ethanol-fed and the pair-fed control rats to the same extent, decreasing their microviscosity (increasing the fluid state of the lipid portion of the membrane).

That the phospholipids are required for membrane integrity and for the optimal activity of the respiratory chain is not disputed here. However, the data do not support the hypothesis that functional changes in the activity of the respiratory chain are necessarily altered by changes in the properties of the membrane phospholipids. Our findings, and those of others (*1*), have demonstrated that long-term ethanol consumption reduces the state 3 respiration rate in mitochondria, especially for substrates linked to the reduced form of nicotinamide adenine dinucleotide (site I). We have detected no differences in the capacity of mitochondrial preparations from Chow-fed rats and those from rats fed the control liquid diet to oxidize substrates entering the respiratory chain at different levels. However, there were striking differences between these two groups not only in the proportions of the fatty acids of the phospholipids but also in their relative amounts. The microviscosity of these membranes also differed, as did their response upon the addition of physiological concentrations of ethanol. The initial r_s values for the preparations from the Chow-fed animals were lower than for the other experimental groups; preliminary data from our laboratory indicate a lower cholesterol content in these preparations, which is consistent with the observed results. Moreover, the membrane preparations from the Chow-fed animals were more resistant to the fluidizing effects of ethanol than the preparations from the other groups, a fact that may in part be due to the greater proportion of saturated fatty acids in the membrane phospholipids of these preparations. The relative proportions of saturated and unsaturated fatty acids were not significantly altered by the long-term consumption of ethanol; thus we lack evidence of a structural adaptation of the membrane under these circumstances.

The available data on changes in the lipid composition of mitochondrial membranes from ethanol-fed animals are conflicting (*7*). The fatty acid content of the diet, the duration of the ethanol treatment, or the fasting of the animals prior to the experiments could account for these discrepancies. Our findings are in agreement with those of some investigators (*8*) but in disagreement with those of others, who noted a decrease in polyunsaturated fatty acids, especially arachidonic (*7*). However, the differences described in these experiments are less than that observed between preparations from our two control groups, which exhibited similar functional activities. At this stage it would be premature to ascribe the severe functional derangements to relatively minor alterations in the lipid composition of the membranes. Moreover, the degree of saturation of the fatty acids is only one of many factors regulating the structural state of the membranes. Other factors, such as the cholesterol and calcium content and the protein composition of these preparations, remain to be defined. Thus, although our results confirm the effect of long-term ethanol intake on the mitochondrial function, a direct causal relationship could not be established between these changes and those in the structural properties of the membranes.

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Role of Calcium in Trypanocidal Drug Action

Abstract. *The synergistic effect of serum on the drug combination of salicylhydroxamic acid plus glycerol, which is active against* Trypanosoma brucei, *is due to diffusible calcium ions. The synergistic activity can be removed by dialysis of the serum or by addition of calcium chelating agents. A buffer containing calcium can mimic the synergistic activity of serum. This finding may have important implications in the clinical management of African trypanosomiasis in humans. Calcium also has a synergistic effect on melarsoprol, the only drug available for treating sleeping sickness patients with central nervous system involvement, and the concentration of calcium has been reported to be depressed in the serum of experimentally infected animals.*

The protozoan parasites *Trypanosoma brucei gambiense* and *T. b. rhodesiense* cause African sleeping sickness in humans, and the closely related *T. b. brucei* is one of several trypanosomes that cause a similar disease in animals. There has been little advance in the treatment of these diseases in the last 25 years, and the drugs in current use are inadequate (*1*). The therapeutic potential of a combination of salicylhydroxamic acid (SHAM) and glycerol, which blocks the carbohydrate catabolic pathways essential to these parasites, was previously reported by our laboratory (*2*). Subsequently, we found that this drug combination caused a more rapid and complete destruction of parasites when the infected rats were treated in vivo than when the parasites were treated in vitro suspended in a phosphate-saline-glucose (PSG) buffer (*3*). We attributed this dif-

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