EVIDENCE FOR AN INFLUENCE OF MEMBRANE FLUIDITY ON THE LETHAL ACTIVITY OF THE ANTICOCCIDIAL IONOPHOROUS ANTIBIOTICS

CHARLES KENNETH SMITH II.
University of New Hampshire, Durham

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EVIDENCE FOR AN INFLUENCE OF MEMBRANE FLUIDITY ON THE LETHAL ACTIVITY OF THE ANTICOCIDIAL IONOPHOROUS ANTIBIOTICS

BY

CHARLES KENNETH SMITH II
B.S., University of Pittsburgh, 1973

A THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Zoology

September 1979
This thesis has been examined and approved.

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Ginny W. Daft, Assistant Professor of Animal Sciences

Edward K. Tillinghast, Associate Professor of Zoology

Date July 10, 1959
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ABSTRACT

EVIDENCE FOR AN INFLUENCE OF MEMBRANE FLUIDITY
ON THE LETHAL ACTIVITY OF THE ANTICOCCIDIAL
IONOPHOROUS ANTIBIOTICS

by

CHARLES KENNETH SMITH II

University of New Hampshire, September 1979

In vitro studies of the effect of the anticoccidial ionophorous antibiotics on the ultrastructure of intracellular Eimeria tenella sporozoites revealed that at appropriate concentrations of the drugs the parasite is destroyed while the host cell remains unharmed, demonstrating that these drugs can be selectively lethal for the parasite. Variable responses to the cytotoxic potential of these antibiotics has also been demonstrated for certain microorganisms. The basis of this phenomenon of differential sensitivities to the ionophorous antibiotics is unknown.

Radioactive ionophores and liquid scintillation spectroscopy showed that E. tenella sporozoites accumulate these antibiotics prior to invading their host cells and that the concentration retained is influenced by the ambient temperature.

The effects of the coccidioicidal ionophores against the intracellular sporozoite, determined by electron
microscopy, were characterized by gross swelling and vacuolization. The severity of the response was influenced by incubation temperature. The host cells demonstrated a decreased sensitivity to the activity of these compounds.

The influence of membrane fatty acid composition on the lethal activity of the carboxylic ionophorous antibiotics was also investigated using cultures of Acholeplasma laidlawii propagated in nutrients with different lipid compositions. The results showed that in an isothermal system the greater the degree of unsaturation of the membrane fatty acids the greater the sensitivity to the ionophorous antibiotics.

Considering the extracellular uptake of the ionophores by the sporozoites and the influence of temperature on this uptake, the influence of temperature on the effect of the antibiotics on the ultrastructure of the parasite, and the influence of membrane fatty acid composition on the toxicity of these drugs, I propose that the cytotoxic activity of the coccidiocidal ionophorous antibiotics is influenced by membrane viscosity.
INTRODUCTION

The subclass Coccidia of the class Sporozoa comprises a group of eukaryotic unicellular obligate intracellular parasites that are responsible for tremendous losses annually in animal protein production from cattle, sheep and chickens. Therefore considerable effort has been devoted to the discovery of compounds that demonstrate anticoccidial activity (Shumard and Callender, 1967). To date, a wide variety of drugs have been found only temporarily useful because of the eventual development of resistance by the parasites.

Recently, however, some members of a class of compounds known as the polyether monocarboxylic ionophorous antibiotics have been shown to be effective anticoccidial agents in chickens (Shumard and Callender, 1967; Mitrovic, et al., 1975; Berg and Hamill, 1978). No convincing evidence has confirmed the development of coccidial resistance to these antibiotics, adding to their importance. When used prophylactically at predetermined concentrations these drugs will prevent clinical coccidiosis while remaining non-injurious to the host. The specific mechanism of coccidiocidal activity is not understood, although it may involve the parasite limiting membrane because of the physical properties of these antibiotics.
The demonstration that certain species of coccidia, notably *Eimeria tenella*, invade cells grown in cultures and undergo subsequent asexual and sexual development (Patten, 1965; Strout and Ouellette, 1970) provided a unique system for studying the effect of these ionophorous antibiotics on the intracellular parasite. When sporozoites of *E. tenella* are inoculated into cultures of chick embryonic kidney cells in the presence of an appropriate level of an anticoccidial ionophore the subsequent intracellular parasite is irreversibly damaged but the host cells remain essentially unharmed (Pfeiffer, 1973; Boothby, 1975), indicating a differential response to the activity of these compounds by two different cell types. Variable cytotoxic responses to the ionophorous antibiotics has also been recorded for certain microorganisms (Ovchinnikov, et al., 1974). The basis of this phenomenon of differing sensitivities, while not understood, is important because of the potentially disastrous consequences of the development of lowered sensitivities by the coccidia themselves (i.e., resistance).

Since all cells possess limiting membranes and the ionophorous antibiotics are membrane-active compounds the phenomenon of variable sensitivities to the ionophorous may be related to differences in the physical and/or chemical nature of cell membranes. The work presented in this thesis was undertaken to explore the possible influence of the cell membranes on the activity of these antibiotics.
PART I
EIMERIA TENELLA: ACCUMULATION AND RETENTION OF ANTICOCIDIAL IONOPHORES BY EXTRACELLULAR SPOROZOITES
INTRODUCTION

The ionophorous antibiotics monensin (Shumard and Callender, 1967), lasalocid (Mitrovic, et al., 1975) and narasin (Berg, et al., 1976) are effective anticoccidial compounds. Used prophylactically at appropriate concentration the ionophores selectively destroy the intracellular parasite while remaining relatively noninjurious to the host cells. These drugs are not effective therapeutically in poultry.

These antibiotics possess unique physical properties making them capable of forming complexes with cations and transporting them into and through biological membranes (Pressman, 1976; Ovchinnikov, et al., 1974) and, as a consequence, uncoupling oxidative-phosphorylation in isolated mitochondrial preparations (Estrada-0, et al., 1967 and 1974; Wong, et al., 1977). Although the effect of each of these three ionophores on the ultrastructure of intracellular Eimeria tenella sporozoites in vitro is essentially identical (Pfeiffer, 1973; Boothby, 1975; Part II, this thesis), the parasite mitochondria do not appear to be involved in the destruction of the intracellular parasite and the specific mechanism of anticoccidial activity remains obscure.

McDougald and Galloway (1976) showed that treatment of extracellular E. tenella sporozoites with monensin had
no effect on the subsequent intracellular development of the parasites in vivo. Although their findings seem to preclude the possibility that extracellular sporozoites accumulate a measurable concentration of the ionophore, results of recent in vitro studies in our laboratory showed that pretreatment of extracellular *E. tenella* sporozoites with lasalocid severely curtailed subsequent intracellular development (Grimard, 1978). Thus a controversy exists.

This paper reports that *E. tenella* sporozoites accumulate and retain measurable quantities of $^{14}$C-labeled lasalocid (MW 591) and narasin (MW 764) prior to invasion of their host cells, and that the concentration incorporated is influenced by ambient temperature.
MATERIALS AND METHODS

$^{14}\text{C}\text{-lasalocid}$ (1.5 $\mu$Ci/mg) was obtained from Hoffman-LaRoche and $^{14}\text{C}\text{-narasin}$ (11.79 $\mu$Ci/mg) from Eli Lilly and Co. Both drugs were used without further purification. The incubation medium used throughout the experiment was Waymouth's MAB 87/3 (GIBCO) without serum. Aquasol 2 (New England Nuclear) was used as the liquid scintillation cocktail. The cpm for each sample were obtained with a Packard, Model 3320, Tri-Carb Scintillation Spectrometer.

Preparation of Sporozoites

Freshly sporulated oocysts of *Eimeria tenella* (Lilly 65 strain) were suspended in an excystation medium consisting of 5% avian bile and 0.25% trypsin in Hanks' balanced salt solution. After mechanical breakage of the oocysts in a tissue grinder the sporocyst suspension was incubated in a water bath at 40°C for 2 hr to induce release of the sporozoites.

The resultant sporozoite suspension was centrifuged at 1500 X g for 10 min and the supernatant fluid removed. The remaining pellet was resuspended in the incubation medium and centrifuged at 300 X g for 5 min to concentrate the heavier debris. The supernatant fluid containing the sporozoites was carefully removed by Pasteur pipet and the
purity of this sporozoite preparation confirmed by microscopic observation. This technique proved satisfactory in that very few unexcysted sporocysts were present as contaminants. The total number of sporozoites was determined by counting in a hemocytometer.

Preparation of Drug Solution

One mg each of crystalline $^{14}$C-lasalocid and $^{14}$C-narasin was carefully weighed on a Mettler M5 Microbalance and then dissolved in a drop or two of dimethylsulfoxide (DMSO). These solutions were then diluted in 10 ml of the incubation medium, giving a stock concentration of 100 g/ml for each drug. Prior to each trial these solutions were sonicated for 10 min to insure solvation of the drugs. The drug solutions were diluted 1:100 in the incubation medium giving a final concentration of 1 μg/ml for each of the ionophores. A 1 ml aliquot of each of these working solutions was dissolved in 10 ml of the liquid scintillation cocktail and the dpm determined as a check of the actual concentrations of each drug used in the trial.

Treatment of the Sporozoites

The purified sporozoite pool was divided into five equal volumes and treated as follows:

An aliquot of the sporozoite pool was suspended in each of the 10 ml working solutions of lasalocid and narasin for incubation at 40 C; a similar preparation was incubated at 25 C.
The final sporozoite population was incubated at 40 °C in medium containing no drug as a control. All sporozoite preparations were incubated for 4 hr.

Following incubation, the sporozoite suspensions were centrifuged at 1500 X g for 10 min and the supernatants containing the extraneous ionophore were carefully removed with a Pasteur pipet. A 1 ml aliquot of each supernatant was added to 10 ml of the scintillation cocktail. Each sporozoite pellet was then resuspended in 10 ml of medium without drug, recentrifuged and these supernatants removed and replaced with fresh nutrient. This procedure was repeated three times.

Just prior to the final centrifugation the number of sporozoites present in each preparation was determined with the aid of a hemocytometer.

After removal of the final wash, each sporozoite pellet was resuspended in 1 ml of unmedicated medium and dissolved in 10 ml of the scintillation cocktail.

The cpm for each sample were determined from ten min counts and converted to dpm from automatic external standard (AES) counts.

This entire procedure was repeated two additional times and the data are reported as trials 1, 2, and 3.

I also prepared working solutions of both lasalocid and narasin but without sporozoites. These were incubated and washed similar to the solutions with sporozoites. A final 1 ml was added to 10 ml of Aquasol 2 and the cpm were then recorded. This I identify as Control 2.
RESULTS

The total dpm determined for each drug treated sporozoite population, regardless of the incubation temperature, was 5-10 times greater than the dpm of the unmedicated control sporozoites (Table 1). Further, the dpm recorded from the final 1 ml wash of the tubes incubated with the labeled ionophores in the absence of sporozoites (control 2) were essentially identical to that recorded for the untreated controls (Table 1).

When the retention of the respective ionophores is considered in terms of µg/sporozoite, the uptake at 40 C is consistently twice that at 25 C for each trial with either drug (Table 2). This is especially evident if one compares the mean retention at the two incubation temperatures. This difference was shown to be significant with a probability of greater than 98%.

The data presented in Table 2 also illustrate the fact that the relative retention of lasalocid per sporozoite is consistently about one order of magnitude greater than that of narasin. The mean values particularly emphasize this point. This difference is also significantly with a probability of greater than 98%.
DISCUSSION

The dpm recorded for the sporozoite populations incubated in the presence of either lasalocid or narasin were 5-10 times greater than that recorded for the untreated sporozoite populations (Table 1). The magnitude of these differences must be viewed with the awareness that the specific activity of each of the labeled ionophores used was relatively small. Also, the dpm measured for the final 1 ml wash of the tubes incubated with the drug solution in the absence of sporozoites (control 2) was essentially identical to that of the untreated organisms (background). These results show conclusively that *Eimeria tenella* sporozoites accumulate measurable concentrations of the anticoccidial ionophores lasalocid and narasin prior to the invasion of their host cells. Support for the claim that the antibiotic incorporated by these extracellular sporozoites is retained comes from the results of Grimard (1978) who showed that sporozoites pretreated with lasalocid were destroyed after invasion of cells in culture in the absence of lasalocid. Therefore the effective concentration of drug accumulated in the membranes of the sporozoite must be independent of the concentration accumulated by the host cells, which may have important implications in terms of the selectivity of the drugs for the parasite.
The fact that the sporozoites incorporate these antibiotics prior to invading the host cells is important because it may be sufficient to explain the fact that the polyether ionophorous antibiotics are effective prophylactically but have little or no therapeutic value in chickens.

Preliminary trials showed that three washes of each treated sporozoite pellet with unmedicated nutrient were sufficient to reduce the radioactivity of the final supernatant fluids to background levels. This was necessary to insure that ionophore not incorporated, but merely loosely associated with or absorbed by the sporozoites was not being considered in the results. However, because of the physical properties of these antibiotics, with each successive wash a new equilibrium was probably being established for the aqueous-hydrocarbon distribution of the ionophores. If so, some ionophore that had been dissolved in the biomembranes in the original equilibrium distribution of these drugs may have been "washed out" making the final retention measurements artificially low. Therefore, the concentration of ionophore calculated per sporozoite (Table 2) may be a conservative value.

Both narasin and lasalocid form lipophilic complexes with cations and are therefore capable of transporting these bound ions into and through biological membranes. This activity can result in the uncoupling of oxidative-phosphorylation in isolated mitochondria causing a swelling of these organelles. In the host parasite model system
employed in this laboratory certain critical concentrations of these anticoccidial ionophores used in vitro result in destruction of the intracellular parasite (sporozoite) with no apparent cytotoxicity to the host cell (Pfieffer, 1973; Boothby, 1975). Initially it was assumed that the cocci-diocidal activity of these antibiotics involved the parasite mitochondria as the site of activity (Estrada-O, et al., 1967; Ryley and Betts, 1973), although this assumption did not readily explain the selective lethality.

Studies of the effect of these ionophores on the ultrastructure of the intracellular sporozoite showed no apparent morphological abnormalities of the parasite mitochondria (Pfeiffer, 1973; Boothby, 1975; Part II, this thesis). The most striking effect was a tremendous swelling of the sporozoite, usually at the anterior end, suggestive of extreme osmotic damage. This was true for lasalocid, narasin and monensin, indicating a common destructive mechanism. From these observations, the primary site of the lethal activity of these ionophores against the intracellular sporozoite appeared to be the plasmalemma rather than the sporozoite mitochondria.

The results of the study reported in this paper indicate that sporozoites incorporate the polyether ionophores prior to invading the host cell, and that the amount accumulated is influenced by temperature. Since membrane viscosity is inversely related to ambient temperature, these results suggest that the physical nature of a
biomembrane influences the amount of ionophore dissolved in the membrane. Thus the different sensitivities of the host cell and parasite to the cytotoxic potential of these antibiotics may be ascribed to differing chemical compositions of the host cell plasma membrane and the sporozoite plasma-lemma, as membrane chemistry also influences fluidity.

The data presented in Table 2 also show that the retention of lasalocid by the extracellular sporozoites of E. tenella is an order of magnitude greater than the retention of narasin given the same initial drug concentration and incubation conditions. Lasalocid forms a dimer to complex with H⁺ and divalent cations (Celis, et al., 1974) while narasin probably forms a one-to-one complex with monovalent cations based on its chemical similarity to the other monovalent binding monocarboxylic ionophores, e.g., monensin (Pinkerton and Steinreuf, 1970). Therefore, a greater number of lasalocid molecules would be required to achieve the same magnitude of ion transport across a given biomembrane as compared to narasin. A priori reasoning then suggests that beginning with equal concentrations of lasalocid and narasin in the cell environment the concentration of lasalocid accumulated in the biomembranes of the sporozoite would be greater than that of narasin to effect a similar degree of cytotoxicity. The results are consistent with this idea.

In conclusion, the results of this study show that sporozoites of E. tenella do incorporate the ionophorous
antibiotics prior to invading their host which may be sufficient to explain the prophylactic nature of the usefulness of these drugs. The amount of the antibiotic taken up by the parasites is influenced by temperature which suggest an influence of the physical state of the membrane in determining the aqueous-hydrocarbon distribution of these antibiotics.
Table 1. Uptake following 4 hr exposure to $^{14}$C-lasalocid and $^{14}$C-narasin by extracellular *Eimeria tenella* sporozoites.

<table>
<thead>
<tr>
<th>Trial Number</th>
<th>Number dpm Sporozoites</th>
<th>Number dpm Sporozoites</th>
<th>Number dpm Sporozoites</th>
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<tr>
<td></td>
<td>40 C</td>
<td>25 C</td>
<td>40 C</td>
<td>25 C</td>
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<tr>
<td>1</td>
<td>352 $2.5 \times 10^7$</td>
<td>320 $4.0 \times 10^7$</td>
<td>30 $3.0 \times 10^7$</td>
<td>270 $3 \times 10^7$</td>
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<tr>
<td>2</td>
<td>335 $1.2 \times 10^7$</td>
<td>226 $1.4 \times 10^7$</td>
<td>43 $1.3 \times 10^7$</td>
<td>209 $1.3 \times 10^7$</td>
<td>146 $1.5 \times 10^7$</td>
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<td>3</td>
<td>798 $1.6 \times 10^7$</td>
<td>500 $1.7 \times 10^7$</td>
<td>46 $1.7 \times 10^7$</td>
<td>644 $1.5 \times 10^7$</td>
<td>491 $1.9 \times 10^7$</td>
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<tr>
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<td>495</td>
<td>349</td>
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<td>374</td>
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<tr>
<td>Control 2</td>
<td>40 None</td>
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<td>36 None</td>
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Table 2. Retention of narasin and lasalocid by extracellular sporozoites of *Eimeria tenella* (µg/sporozoite)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Lasalocid Treated 25°C</th>
<th>Lasalocid Treated 40°C</th>
<th>Narasin Treated 25°C</th>
<th>Narasin Treated 40°C</th>
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<td>1</td>
<td>2.2 x 10^{-9}</td>
<td>3.8 x 10^{-9}</td>
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<td>3.1 x 10^{-10}</td>
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<tr>
<td>2</td>
<td>3.9 x 10^{-9}</td>
<td>7.3 x 10^{-9}</td>
<td>2.6 x 10^{-10}</td>
<td>4.9 x 10^{-10}</td>
</tr>
<tr>
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<td>7.6 x 10^{-9}</td>
<td>14.0 x 10^{-9}</td>
<td>8.9 x 10^{-10}</td>
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<td>8.4 x 10^{-9}</td>
<td>4.7 x 10^{-10}</td>
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PART II

EIMERIA TENELLA:  INFLUENCE OF INCUBATION TEMPERATURE
ON THE EFFECT OF THE IONOPHOROUS ANTIBIOTIC
NARASIN ON THE ULTRASTRUCTURE OF INTRACELLULAR SPOROZOITES
INTRODUCTION

Narasin is a member of the class of compounds known as the monocarboxylic acid ionophorous antibiotics. A unique property of this group is their ability to form lipophilic complexes with alkali metal cations and transport these cations into and through biological membranes, i.e., act as transmembrane carrier molecules (Ovchinnikov, et al., 1974; Pressman, 1976). Some of these compounds are effective anticoccidal agents, notably monensin (Shumard and Callender, 1967), lasalocid (Mitrovic, et al., 1975) and narasin (Berg, et al., 1976). In vitro, these drugs not only inhibit the development of the intracellular parasite, the sporozoites which initially invade degenerate and disappear by 24-48 hr in the presence of these ionophores (McDougald and Galloway, 1976; Boothby, 1975; Grimard, 1978).

Sporozoites of Eimeria tenella accumulate measurable concentrations of these ionophores prior to invading their host cells, although the amount of drug retained is influenced by ambient temperature (Part I, this thesis) the role of temperature on the lethal activity of the anticoccidal ionophores, manifested by ultrastructural changes of the parasite, is not known. This information is important because of the influence of temperature on certain physical properties of biological membranes and the
possible role of membrane structure in the selective activity of ionophores against sporozoites rather than host cells.

In this paper we describe the effect of narasin on the ultrastructure of the intracellular sporozoite of *E. tenella* and the influence of incubation temperature on the anticoccidial activity of this ionophore in vitro.
MATERIALS AND METHODS

All cultures were embryonic chick kidney cell primaries prepared from 18 day embryos. The narasin was provided by Eli Lilly and Company, Greenfield, Indiana.

Drug Challenge

In the first trial eight 25 cm$^2$ flasks (Corning) were prepared with $25 \times 10^5$ cells per flask in 0.1% lactalbumin hydrolysate (Difco) in Earle's BSS (GIBCO) supplemented with 5% fetal calf serum (GIBCO). After 24 hr incubation at 40 C two of these cultures were inoculated with $5 \times 10^6$ E. tenella sporozoites in 5 ml of medium containing 0.01 g/ml of narasin; two comparable cultures were prepared with sporozoites in the absence of narasin; two cultures without sporozoites were challenged with 0.01 µg/ml of narasin; and the remaining two cultures received neither drug nor sporozoites. One culture of each treatment was incubated at 40 C and the other at 30 C.

In a second trial, treatments were identical to those in the first trial except that four additional cell cultures were prepared; two were inoculated with $5 \times 10^6$ sporozoites each in medium containing 0.1 µg/ml of narasin while the other two received the same concentration of the ionophore but no sporozoites. One culture of each treatment was incubated at 40 C and the other at 30 C.
The concentration of the ionophore used was based on a preliminary dose-toxicity study which showed that at concentrations up to 0.1 μg/ml of narasin in the medium the host cells survive and continue to proliferate. At drug concentrations of 1.0 μg/ml and greater the host cell population declined steadily following drug challenge.

Additional cultures of chick embryonic kidney cells were prepared as described and inoculated with sporozoites in the presence of 0.01 μg/ml of monensin. These were incubated at 40 C for 24 hr.

Preparation for Electron Microscopy

Following 24 hr incubation all cultures were fixed with 3% cacodylate-buffered glutaraldehyde for 1 hr at room temperature. Each culture was then washed five times with cacodylate-buffered 0.25M sucrose and postfixed in 1% cacodylate-buffered osmium tetroxide for 30 min at 5 C. Dehydration was carried out in graded alcohols at room temperature as follows: 35% ethanol, 50% ethanol, 90% ethanol and 100% ethanol for 10 min each. The preparations were then subjected to three changes of pure resin (Epon 812) for 1 hr each, then embedded, sectioned and mounted on copper grids to be viewed on the electron microscope. Sectioning was done with a Porter-Blum MT-2 ultramicrotome and glass knives. The specimens were viewed in either a Phillips 200 or a JEOL JEM-100S electron microscope. All sections were stained with uranyl acetate and lead citrate prior to viewing.
RESULTS

Untreated Intracellular Sporozoites

The ultrastructure of untreated intracellular sporozoites from a culture incubated at 30 C (Fig. 1) appears identical to that of sporozoites from untreated cultures incubated at 40 C (Fig. 2). An abundance of micronemes and rhoptries, oriented generally parallel with the long axis of the organism, are evident in the anterior region of the sporozoites. The conoid apparatus is at the extreme anterior end of the parasites and the polar ring is suggested by subpellicular microtubules originating from the area of the conoid. The parasite mitochondria exhibit a rather densely staining matrix with the typical tubular cristae. The mitochondria of parasites incubated at 30 C are indistinguishable structurally from those of parasites incubated at 40 C. A characteristic clear globule is present, surrounded by densely staining ovoid (amylopectin) vesicles. Frequently two distinct clear globules are seen. Ribosomes are abundant and occasionally an area of endoplasmic reticulum can be identified. The sporozoite is limited by a three-membranous pellicle. The outer plasmalemma is continuous about the entire organism while the inner membranes are interrupted at the region of the anterior and posterior polar rings and in the area of micropore formation.
Micropores and undulations of the pellicle appear more frequently in sporozoites from the 40 °C culture.

Intracellular Sporozoites Treated with Narasin

The most noticeable effects of 0.01 μg/ml of narasin on intracellular sporozoites incubated for 24 hr at 40 °C are the appearance of large vacuoles (Fig. 3) and an obvious swelling, frequently resulting in ballooning of the anterior end (Fig. 4). When this occurs the bleb is contained only by the outer membrane of the pellicle, the plasmalemma; the inner membrane or membranes of the pellicle separate from the plasmalemma and are retained in a more posterior position of the sporozoite cytoplasm or disappear altogether (Fig. 4). The characteristic organization of the anterior region, obvious in normal untreated sporozoites, becomes chaotic. Often the nucleus of the sporozoite appears to have been pushed into juxtaposition to the outer pellicular membrane suggesting an increase in intracellular pressure (Fig. 4). When the sporozoite mitochondria are visible they appear structurally normal.

When cultures are treated with 0.01 μg/ml of narasin and incubated at 30 °C for 24 hr there is no evidence of cytotoxicity to the intracellular sporozoites. The parasites appear normal with no suggestion of swelling or vacuolization (Fig. 5).

When the concentration of the ionophore in the culture medium is increased to 0.1 μg/ml the effect on the intracellular sporozoite in cultures incubated at 40 °C
(Fig. 6) is similar to that observed from the cultures treated with 0.01 μg/ml and incubated at 40 C. A reduction of the incubation temperature by 10 C results in normal intracellular sporozoites, even at this higher drug concentration.

Treatment of cultures with 0.01 μg/ml of monensin (another anticoccidial ionophore) for 24 hr at 40 C has an effect on the intracellular parasite similar to that of narasin (Fig. 7), indicating a possible common destructive mechanism.

Effects of Narasin on Host Cell Ultrastructure

The ultrastructure of nonparasitized host cells was not altered by treatment with 0.01 μg/ml of narasin at either 30 C or 40 C (Fig. 8). A distinct effect, noted after 24 hr exposure to a higher drug level of 0.1 μg/ml when incubated at 40 C, was manifested in swollen, membrane-bound intracellular saccules (Fig. 9). These may represent elements of the smooth endoplasmic reticulum (ser) or the Golgi complex. Also the rough endoplasmic reticulum (rer) is distended, and the cisternae are filled with an amorphous electron-dense substance. Host cell mitochondri are frequently swollen and abnormal (Fig. 10). By comparison, when cultures treated with the higher drug level of 0.1 μg/ml of narasin were incubated at 30 C, no apparent effects on the host cells were detected.
DISCUSSION

Since it has been demonstrated repeatedly that the presence of the polyether ionophores in vitro causes the sporozoites that initially invade to be destroyed by 24-48 hr, it is likely that the ultrastructural effect on the intracellular sporozoite at 24 hr post drug challenge is a manifestation of the coccidiocidal effect. The results of this study show that this activity of narasin at 40 C causes ultrastructural changes in the intracellular sporozoite similar to that reported for monensin (Pfeiffer, 1973) and lasalocid (Boothby, 1975), but not at 30 C. This effect is seen as a gross swelling of the sporozoite, often evident as a bleb, limited only by the plasmalemma, at the anterior end of the organism and the formation of large vacuoles.

A possible explanation of the blebbing and vacuole formation is based on the unique physical properties of the ionophorous antibiotics, including their ability to act as transmembrane carriers of alkali metal cations (Ovchinnikov, et al., 1974; Pressman, 1976). If the primary site of drug activity against the sporozoite is the parasite plasmalemma, as seems reasonable, the swelling effect could be attributed to an increased intracellular osmotic pressure resulting from the ion-translocating capacity of these drugs. Since narasin demonstrates a high affinity for complexing with
sodium (Wong, et al., 1977), the act of transporting sodium across the membrane could be the basis of the lethal activity. The distinctive vacuoles that appear in the sporozoite as a result of ionophore treatment at 40 C may represent contractile vacuoles formed by the organism as a defense mechanism to sequester the excess sodium, water and additional solutes resulting from the ion-transporting properties of these drugs. The concept that the principle site of anticoccidial activity is the sporozoite plasmalemma and that the lethal results are attributable to the transmembrane ion carrying properties causing increased intracellular ion concentration and subsequent increased water and permeable solution concentration is entirely consistent with what is known of the physical properties of the carb­oxylic ionophores and the observations from this study.

When the incubation temperature is lowered by 10 C the swelling and vacuolization of the intracellular sporozoite, typical of narasin activity at 40 C, does not occur. Since the ionophores are membrane-active compounds this absence of effect may be explained as the result of increased viscosity of the sporozoite plasmalemma. Various physical techniques have demonstrated that the fluidity of a lipid bilayer is inversely proportional to the absolute temperature of the system (Schinitzky and Barenholz, 1974; Thompson and Huang, 1978). If the coccidiocidal activity of the ionophores is directly related to the rate of their transmembrane mobility, which seems likely because of their
physical characteristics, and the diffusion coefficient of a molecule is directly proportional to the viscosity of the environment (Tanford, 1961), then the viscosity or its inverse, the fluidity, of a biological membrane would influence the severity of the effect resulting from the ionophoric activity. In fact studies on lipid bilayers have shown that membrane structure does indeed influence the kinetics of carrier-mediated ion transport (Benz, et al., 1977). A priori reasoning then suggests that a lower ambient temperature would result in reduced sporozoite damage as the result of ionophore treatment. The observations made in this investigation support this reasoning.

There are other possible explanations for the observed phenomenon, but none that I feel is completely consistent with all the observations. Knowledge that the ionophores are known to inhibit mitochondrial ATPase (Estrada-O, et al., 1967, 1974; Wong, et al., 1977) in isolated rat liver mitochondria, with concomitant optical density changes, led to a tacit assumption that this was also the mechanism of anticoccidial activity. However, normal appearing mitochondria are frequently seen in drug-treated sporozoites. Although it is possible that the swelling and vacuolization of the intracellular sporozoites could result from ionophore activity against the mitochondria by creating some sort of energy debt without apparent ultrastructural changes to these organelles (Weinbach, et al., 1967), this is not in my opinion the best explanation. When
the drug level is sufficient to elicit a cytotoxic response from the host cells in vitro, the host mitochondria do show structural abnormalities. It would also be difficult to explain the different sensitivities displayed by the parasite and the host cell to the ionophores from this concept.

The observation that there are no apparent changes in the ultrastructure of the host cells as the result of 0.01 \( \mu \text{g/ml} \) of narasin at 40 C and that these cells survive and continue to proliferate as compared to the effect on the intracellular sporozoite, indicates that different cell types show differing responses to the ionophores in the same drug environment. The basis of this phenomenon is as yet unknown but the results of this study provide some insight. Part I of this thesis shows that extracellular sporozoites accumulate measurable concentrations of narasin and lasalocid but the amount incorporated is influenced by the incubation temperature. The results reported here indicate that incubation temperature, which influences the fluidity of a biological membrane, also influences the degree of destruction caused by the presence of an ionophorous antibiotic, narasin. Considered together, these results suggest that both the aqueous-hydrocarbon distribution and the rate of movement, i.e., the lethal activity, of these compounds are dictated by the physical state of a biomembrane. If so, then the different sensitivities of the host cell and the intracellular sporozoite may be attributed to differing physical properties of the limiting membranes as they influence the
activity of the drugs. These differing properties may well be a function of differences in chemical compositions, particularly sterol content or the nature of the fatty acyl chains.

There is no effect on the ultrastructure of the host cells by 0.01 μg/ml of narasin at either 30 C or 40 C, or by 0.1 μg/ml of narasin when the cells are incubated at 30 C. When the host cells are treated with 0.1 μg/ml of narasin for 24 hr at 40 C the drug has a definite effect on the ultrastructure. The large number of membrane-bound saccules or vesicles that result may possibly be attributed to a proliferation of the smooth endoplasmic reticulum or a swelling of elements of the Golgi complex. These two possibilities are not easily resolvable. A proliferation of the SER of kidney cells could represent a response of the cells to detoxify the drug. It is well known that the endoplasmic reticulum is the site of the cytochrome P450 system (Coon, 1978) which might be expected to respond to the presence of these monocarboxylic polyether polyalcohol ionophores. Support for this idea is that the cells ultimately survive and continue to proliferate, as if the drug were being detoxified. The possibility that these vesicles result from the accumulation of secretory substances in the Golgi and subsequent inhibition of release has been presented by Tartakoff and Vassalli (1977, 1978). Obviously more work is needed to resolve the true nature of these saccules.
A result of the ionophore induced distension of the intracellular sporozoite is often a separation of the plasma­lemmma and the inner membranes of the sporozoite pellicle. This response to the proposed osmotic change implies differences in permeability characteristics between these membranes. Two possible explanations for this phenomenon are available.

If both the outer plasmalemma and the inner membrane complex are typical bilayer membranes, differences in chemical compositions could account for these differing permeabilities. Perhaps the outer membrane has a higher protein-lipid ratio and is therefore not as readily permeable to cytoplasmic components as the inner membranous complex. A parallel may be drawn with mitochondrial membrane system where the outer membrane has a lower proportion of resident protein and is therefore more permeable than the inner (Tedeschi, 1976).

A second possible explanation is based upon observa­tions of the three-layered pellicle of sporozoan zoites. The outer membrane (plasmalemma) is typical of eukaryotic lipid­protein bilayers in appearance. The inner two membranes have not been well defined but are discontinuous in the area of the anterior and posterior polar ring and in the region of micropore formation. These two inner membranes are believed to originate from a flattening of cytoplasmic vesicles during zoite development (Porchet, 1972). A freeze-fracture study by Dubremetz and Torpier (1978) showed that the inner membranes supported this theory. Further, the inner membranes seem to be arranged in strips with distinct
transverse interruptions. There appears to be no membrane fusion between strips or across the transverse interruptions within the strips. Thus the idea of a series of plates formed from flattened cisternae is credible. Furthermore, no junction was seen between the plasmalemma and the inner complex. Since the inner membrane complex is discontinuous all but the most gross permeability capabilities of this complex would be negated. This may be sufficient to explain the separation of the inner complex and the plasmalemma as the sporozoite swells in response to the activity of the ionophores.
Figs. 1 to 7. Electromicrographs of intracellular (E. tenella) in cultures of chick embryonic kidney cells.

Figs. 8 to 10. Electromicrographs of chick embryonic kidney cells (host cells) from cultures exposed to various levels of narasin.

c - conoid apparatus
cg - clear globule
er - endoplasmic reticulum
g - Golgi complex
hm - host cell mitochondria
HN - host cell nucleus
m - sporozoite mitochondria
mi - micronemes
N - sporozoite nucleus
ov - ovoid vesicles
p - pellicle
r - rhoptry
Fig. 1. Sporozoite from an untreated culture incubated at 30°C for 24 hr. Magnification: 16700X

Fig 2. Sporozoite from an untreated culture incubated at 40°C for 24 hr. Magnification: 16700 X
Fig. 3. A sporozoite inoculated onto a culture treated with 0.01 μg/ml of narasin and then incubated at 40 C for 24 hr. The parasite is extremely swollen and large membrane-bound vacuoles (v) have appeared. There is also evidence of considerable cytoplasmic deterioration. Magnification: 22000X

Fig. 4. A sporozoite from a culture treated with 0.01 μg/ml of narasin and incubated at 40 C for 24 hr. This sporozoite shows the anterior ballooning effect that is commonly observed from exposure to narasin at 40 C. The sporozoite plasmalemma (pl) and the inner-membrane complex (im) have separated in the region of the bleb. The large membrane-bound vacuoles (v) characteristic of the ionophore effect are evident. Magnification: 21000X
Fig. 5. A typical sporozoite from a culture exposed to 0.01 μg/ml of narasin and incubated for 24 hr at 30°C. The ultrastructure of this organism is normal. Magnification: 15000X
Fig. 6. Sporozoite from a culture treated with 0.1 \( \mu g/ml \) of narasin and then incubated at 40 \( ^{\circ} \)C for 24 hr. The effect is similar to that resulting from treatment with 0.01 \( \mu g/ml \) at 40 \( ^{\circ} \)C (Fig. 3). v - membrane-bound vacuoles. Magnification: 18000X

Fig. 7. An intracellular sporozoite of E. tenella from a culture of chick embryonic kidney cells treated with 0.01 \( \mu g/ml \) of monensin and incubated at 40 \( ^{\circ} \)C for 24 hr. pl - plasmalemma; im - inner membrane complex. Magnification: 20000X
Fig. 8. Although incubated in the presence of 0.01 μg/ml of narasin for 24 hr at 40 C the host cells show no structural abnormalities. Magnification: 14000X

Fig. 9. Cells from a culture treated with 0.1 μg/ml of narasin and incubated for 24 hr at 40 C. The effect of the ionophore is apparent with the appearance of a multitude of swollen, membrane-bound intracellular saccules (s1). Magnification: 14000X
Fig. 10. Typical mitochondria of a host cell incubated in the presence of 0.1 μg/ml of narasin for 24 hr at 40 C. These organelles have become swollen and distorted. Magnification: 19000X
PART III

ACOLEPLASMA LAIDLAWII: THE INFLUENCE OF MEMBRANE
FATTY ACID COMPOSITION ON THE GROWTH-INHIBITORY
ACTIVITY OF NARASIN, A POLYETHER
IONOPHOROUS ANTIBIOTIC
INTRODUCTION

Narasin is a member of a class of compounds known as the polyether monocarboxylic ionophorous antibiotics. A general characteristic of these compounds is that they possess unique physical properties making them capable of acting as carriers of cations across biological membranes (Ovchinnikov, et al., 1974; Pressman, 1976). Because the kinetics of this ionophore-mediated cation transport have been shown to be influenced by membrane structure in artificial lipid bilayers (Benz, et al., 1977), it is important to determine whether the membrane composition of intact biological organisms may influence the growth inhibitory or lethal activity of these compounds.

The carboxylic ionophore narasin is active in vitro against a number of microorganisms, including the mycoplasma (Berg and Hamill, 1978). These organisms, in turn, provide a unique system to study the influence of membrane fatty acid composition on ionophore activity since the lipid composition of the plasma membrane (the only membrane) can be dictated to a large degree by regulating the lipid components of the growth medium (Razin, et al., 1966; McElhaney and Tourtellotte, 1969). The membrane can easily be isolated by osmotic lysis (Pollack, et al., 1965).
This paper describes the lethal activity of narasin on cultures of *Acholeplasma laidlawii* in nutrients with different lipid compositions, with the aim of demonstrating whether membrane composition may influence the activity of these compounds in intact biological systems.
MATERIALS AND METHODS

Stock cultures of Acholeplasma laidlawii, strain A, were maintained in a nutrient consisting of: 36.5 g of brain-heart infusion (BBL), 100 ml of a 25% solution (w/v) of yeast extract (BBL), 10⁵ units of penicillin G (Pfizer) and 20% inactivated horse serum (Flow); final volume adjusted to 1 liter with double distilled water and pH adjusted to 8.0-8.3. This nutrient will hereafter be referred to as the undefined nutrient.

Dose-Toxicity Curve Determination

Cultures of A. laidlawii were incubated in the undefined nutrient containing either 5.0, 1.0, 0.1, or 0.01 μg/ml of narasin. Two culture tubes were prepared for each treatment. Control cultures containing no drug were also prepared. Following incubation for 24 hr at 37 C two agar spread plates were prepared from each tube to determine the number of viable organisms. The agar, Barile, Yaguchi and Eveland (BYE) agar (BBL) with 20% heat inactivated horse serum (Flow), was poured into 60X15 mm plastic petri dishes (Corning) to form a continuous smooth layer. Each of the cultures was diluted serially 1:10 in the nutrient and two agar plates for each of the 10⁻⁴, 10⁻⁵ and 10⁻⁶ dilutions were prepared by spreading 0.2 ml of the cultures on the surface. Plates were incubated inverted at 37 C for 2 days.
The colonies per plate were then counted with the aid of a dissecting microscope to determine the number of surviving organisms per culture.

Preparation of Fatty Acid Defined Nutrient

A lipid-deficient nutrient was prepared according to McElhaney and Tourtellotte (1970). This nutrient was then supplemented with 100 µM of either linoleic acid or palmitic acid (Applied Science) as sterile ethanolic solutions. The alcohol content of the nutrient never exceeded 0.5%. The fatty acids had a stated purity of greater than 99%.

Drug Challenge

Cultures of *A. laidlawii* grown in either of the lipid-deficient, fatty acid supplemented nutrients, as well as in the undefined nutrient, were incubated in the presence of 0.1 µg/ml of narasin, based upon the results of the dose-toxicity study, for 24 hr at 37 C, and the number of surviving organisms was determined by the preparation of spread plates as described earlier. Two cultures were prepared for each treatment, and 2 spread plates for each culture. The procedure was repeated three times, approximately one month apart.

Membrane Isolation and Fatty Acid Analysis

Cultures of *A. laidlawii* were prepared in each of the different nutrients. Each of the cultures was harvested,
the cells lysed and the plasma membranes collected as described by Razin, et al. (1966). The membrane preparations were lyophilized and stored at -25 C until analyzed.

Transesterification was accomplished by bubbling BCl₃ (g) through the membrane samples dissolved in 5 ml CH₃OH:CH₂Cl (6:1 v/v) for 4 min (Koes, 1971). The samples were then refluxed for 10 min and the solvent evaporated under N₂ (g). The dried, transesterified membrane preparations were then extracted with CH₂Cl₂ and a sample subjected to gas-liquid chromatographic (GLC) analysis.

All GLC analyses were performed on a Barber-Coleman Model 10 chromatograph with a strontium-90 argon ionization detector. The 6 ft. X 0.25 in glass column was packed with ethylene glycol succinate (12% on 80/90 mesh Anakrom A) (Analabs) and maintained at 187 C with either 15 or 20 psi inlet pressure. The range of linear response was found to be one to fifty micrograms for each fatty acid methyl ester. Gas chromatographic peaks were identified by comparing retention times (Rₐ) with reference fatty acid methyl ester standards, Kₐ108 (Applied Science). Peaks were quantitated by triangulation.
RESULTS

Dose-Toxicity Relationships

Narasin concentrations of 5.0 and 1.0 μg/ml resulted in essentially total inhibition of growth of Acholeplasma laidlawii in maintenance nutrient; 0.1 μg/ml consistently resulted in approximately 20% survival of the organisms and the level of 0.01 μg/ml has no growth inhibitory effect. Based upon these findings I chose the concentration of 0.1 μg/ml as the challenge dose to study the influence of membrane fatty acid composition on drug lethality.

Influence of the Fatty Acid Composition of the Growth Nutrient

When incubated with narasin in an undefined nutrient with horse serum the percent survival of A. laidlawii averaged 21.3, range 18.0-25.0 (Table 1). When the organisms were exposed to the antibiotic in a nutrient containing 100 μM palmitic acid (16:0) as the only lipid, survival was 19.1%, range 5.5-25.0%. Organisms grown in a lipid-deficient nutrient containing 100 μM linoleic acid (18:2) were extremely sensitive to the ionophore, with a mean survival rate of only 0.5%, range 0.2-1.1%.

Analysis of variance shows that the percent survival of the narasin treated organisms in linoleate enriched nutrient is significantly less than those grown in either
the undefined nutrient or those grown in the palmitate supplemented nutrient at P < 0.01. On the other hand, the percent survival of the A. laidlawii grown in palmitate-supplemented, lipid-poor nutrient is not significantly different from that in undefined nutrient when exposed to 0.1 μg/ml of narasin.

Fatty Acid Composition of the Cell Membrane

The fatty acid composition of the membranes of organisms grown in undefined nutrient plus serum is largely palmitic acid (16:0), approximately 50%, with a high percentage of myristic acid (14:0), approximately 21% (Table 2). About 22% of the total fatty acids were unidentifiable, all with \( R_{18} \) values less than that for palmitate. A trace of oleic acid (18:1) was present, but no evidence of polyunsaturated fatty acids was seen.

The membrane fatty acid composition of the organisms grown in the palmitate supplemented nutrient is nearly 90% palmitic acid (Table 2). A trace of oleic acid is also present, but no evidence of polyunsaturated fatty acids was seen.

By comparison, when A. laidlawii is grown in nutrient supplemented only with linoleic acid (18:2) the fatty acid composition of the cell membranes has a high percentage, about 60%, of this diunsaturated acid (Table 2) at the expense of palmitate. The relative proportion of stearic acid (18:0) also increases.
DISCUSSION

The results of this study show that the presence of proportionately large amounts of the diunsaturated fatty acid linoleate in the cell membranes of Acholeplasma laidlawii increases their sensitivity to the growth inhibitory effect of the carboxylic ionophore, narasin. A. laidlawii grown in lipid-deficient nutrient supplemented only with linoleic acid incorporate this polyunsaturated fatty acid as large proportion (60.5%) of the total cell membrane fatty acid composition (Table 2). When challenged with 0.1 µg/ml of narasin at 37°C for 24 hr these organisms are 40 times more sensitive to the lethal effects of this drug, as determined by percent survival, than those grown in a lipid-rich undefined nutrient supplemented with horse serum, or those organisms propagated in a lipid-deficient nutrient supplemented with palmitic acid. The membranes of these cells have virtually no unsaturated fatty acids, but a high proportion of palmitate. The lipid composition of the membranes of the organisms grown in the undefined nutrient (Table 2) is similar to that reported elsewhere (Razin, 1967).

The fatty acyl composition of a biological membrane will largely dictate the viscosity of that membrane at physiological temperatures (in the liquid-crystalline state).
In general, shorter chain length and/or increased unsaturation lowers the viscosity at a given temperature (Lee, 1975; Lentz, et al., 1976a&b; Thompson and Huang, 1978). Therefore, the cell membranes of the \textit{A. laidlawii} grown in the linoleate-supplemented, lipid-deficient nutrient would be more fluid, due to the high percentage of the diunsaturated fatty acid, than the membranes of cells grown in either palmitate-supplemented, lipid-poor medium or in the undefined nutrient. The membranes of these cells contain essentially no long-chain unsaturated fatty acids. It appears that increased membrane fluidity makes the organism more susceptible to the growth inhibitory effect of narasin.

The carboxylic ionophores are capable of acting as mobile carriers of cations (Ovchinnikov, et al., 1974; Pressman, 1976). Since the diffusion rate of a molecule is directly proportional to the viscosity of its environment (Tanford, 1961), the rate at which these carrier molecules could move back and forth through a biological membrane would be largely determined by the physical state of that membrane. If the lethal activity of these compounds against \textit{A. laidlawii} is a result of this transmembrane motion, then the severity of the effect over time would be dependent upon the lipid composition of the membrane, as this dictates viscosity. The results of this study can be explained by this reasoning. The greater the degree of unsaturation (i.e., fluidity) of the cell membranes of \textit{A. laidlawii} the greater the susceptibility to the growth inhibitory effect of the ionophore.
However, the increase in unsaturation of membrane fatty acids (i.e., fluidity) is advantageous to the survival of the organism. The presence of unsaturated fatty acids in the membranes of A. laidlawii has been shown to increase the resistance of the organisms to osmotic lysis and to improve growth (Razin, et al., 1966). An increased amount of palmitate in the cell membranes, on the other hand, retarded the growth of these organisms.

Based upon these facts, increasing the degree of unsaturation of the cell membrane fatty acids of A. laidlawii increases the fluidity, enhancing both growth and the sensitivity to the ionophores. The reciprocal is also true.

It is at first perplexing that the organisms grown in the undefined nutrient should show a nearly identical response to the ionophore as those grown in palmitate supplemented nutrient. The membranes of the former group have a greater percentage of short chain saturated fatty acids that normally indicate a lower viscosity. Therefore, these cells should be more susceptible to the effects of the drug. However, the presence of serum in the growth medium of these organisms provides a supply of cholesterol which will be incorporated into the cell membranes (Razin, et al., 1966; De Kruyff, et al., 1972). The presence of cholesterol in biological membranes serves to stabilize the fluid nature of the membrane, generally producing a condensing effect at physiological temperature (Ladbrooke, et al., 1968; Lecuyer and Dervichian, 1969; Jain, 1975; Pang and Miller, 1978).
Cholesterol present in biological membranes and in lecithin liposomes lowered the permeability of the membrane for various substances (Bruckdorfer, et al., 1969; McElhaney, et al., 1970). In the case of A. laidlawii, cholesterol in the cell membranes results in a reduction in permeability to glycerol and erythritol (De Kruyff, et al., 1972) thus explaining the similar response to narasin by the cells grown in undefined nutrient plus serum and those grown in lipid-deficient medium supplemented with palmitate in the absence of serum and providing still further evidence that membrane composition can influence the activity of the ionophorous antibiotics.

Horse serum contains a variety of fatty acids, a high percentage of which is linoleate (18:2) (S. C. Smith, personal communication). The results of this work indicate that this fatty acid is not accumulated in the membranes of the A. laidlawii grown in undefined nutrient plus serum. However, the organisms propagated in the lipid-deficient nutrient supplemented with linoleic acid incorporate this fatty acid as a large percentage of their total membrane fatty acids. This paradox might be explained by the comparative overall fatty acyl composition of the membranes of the organisms from the two nutrients. The membranes of the cells grown in the undefined nutrient have a much higher proportion of short chain fatty acids than those grown in the linoleate supplemented nutrient. Whether synthesized or because of their availability in the nutrient, the
presence of these short chain fatty acids would have the effect of increasing membrane fluidity. Since microorganisms have the capacity to adapt their membrane fatty acid composition to that most favorable for survival (Silbert, 1974) it is possible that this high percentage of short chain fatty acids negates the need for unsaturated fatty acids and they simply do not incorporate them.

Alternatively the linoleate present in the serum may be esterified and the organism incapable of hydrolysing the esters. It is known, for example, that the saprophytic mycoplasmas do not incorporate cholesterol esters (Razin, 1967) and the parasitic varieties do incorporate cholesterol esters but cannot hydrolyze them.

The rather large increase in the stearic acid (18:0) percentage in the membranes of the organisms grown in the lipid-poor, linoleate supplemented nutrient may be a reflection of an attempt by the organism to modify the increasing fluidity brought about by the high degree of unsaturation. The long chain saturated fatty acid would have the effect of increasing viscosity. As to the origin of the stearate, the mycoplasma can synthesize long chain saturated fatty acids from acetate (Razin, 1967).
Table 1. Influence of Lipid Composition of Growth Nutrient on the Lethal Effect of 0.1 μg/ml Narasin Against *Acholeplasma laidlawii* A. (Organisms/ml as determined by plate counts; all counts are the mean of two plates.)

<table>
<thead>
<tr>
<th></th>
<th>Undefined Nutrient</th>
<th>Lipid-Poor Nutrient + 100 μM Palmitate</th>
<th>Lipid-Poor Nutrient + 100 μM Linoleate</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Surv</em></td>
<td>Untreated Treated</td>
<td>Untreated Treated</td>
<td>Untreated Treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.0</td>
<td>69 x 10⁶</td>
<td>12 x 10⁶</td>
<td>21 x 10⁴</td>
<td>19.5</td>
<td>57 x 10⁵</td>
<td>14 x 10³</td>
</tr>
<tr>
<td>19.5</td>
<td>82 x 10⁶</td>
<td>16 x 10⁶</td>
<td>35 x 10⁴</td>
<td>5.5</td>
<td>90 x 10⁵</td>
<td>27 x 10³</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.0</td>
<td>10 x 10⁸</td>
<td>22 x 10⁷</td>
<td>10 x 10⁶</td>
<td>24.0</td>
<td>66 x 10⁶</td>
<td>14 x 10⁴</td>
</tr>
<tr>
<td>19.2</td>
<td>12 x 10⁸</td>
<td>23 x 10⁷</td>
<td>10 x 10⁶</td>
<td>25.0</td>
<td>66 x 10⁶</td>
<td>22 x 10⁴</td>
</tr>
<tr>
<td><strong>Trial 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0</td>
<td>33 x 10⁶</td>
<td>82 x 10⁵</td>
<td>50 x 10⁴</td>
<td>16.0</td>
<td>22 x 10⁶</td>
<td>24 x 10⁴</td>
</tr>
<tr>
<td>24.0</td>
<td>31 x 10⁶</td>
<td>74 x 10⁵</td>
<td>31 x 10⁴</td>
<td>24.2</td>
<td>22 x 10⁶</td>
<td>24 x 10⁴</td>
</tr>
<tr>
<td><strong>Mean % Survival</strong></td>
<td></td>
<td></td>
<td></td>
<td>21.3</td>
<td>19.1</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td></td>
<td></td>
<td></td>
<td>18.0-25.0</td>
<td>5.5-25.0</td>
<td>0.2-1.1</td>
</tr>
</tbody>
</table>
Table 2. Fatty Acid Composition of the Cell Membranes of *Acholeplasma laidlawii* Grown in Nutrients with Different Lipid Compositions

<table>
<thead>
<tr>
<th>Carbon Number</th>
<th>Undefined Nutrient</th>
<th>Lipid-Poor Nutrient + 100 μM Palmitate</th>
<th>Lipid-Poor Nutrient + 100 μM Linoleate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Avg.</td>
</tr>
<tr>
<td>12:0</td>
<td>2.2±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>21.0±0</td>
<td>21.0±0</td>
<td>21.0±0</td>
</tr>
<tr>
<td>16:0</td>
<td>49.0±2.5</td>
<td>54.0±2.5</td>
<td>51.5±2.5</td>
</tr>
<tr>
<td>18:0</td>
<td>2.8±0.2</td>
<td>2.5</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>18:1</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>18:2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unidentified</td>
<td>24.8±2.2</td>
<td>20.4±2.2</td>
<td>22.6</td>
</tr>
</tbody>
</table>

*percent of total fatty acids*
PART IV
EVIDENCE FOR THE INFLUENCE OF MEMBRANE FLUIDITY
ON THE LETHAL EFFECT OF THE ANTICOCIDIAL
IONOPHOROUS ANTIBIOTICS: A SUMMARY
INTRODUCTION

The carboxylic ionophorous antibiotics monensin, lasalocid, and narasin are used commercially to prevent coccidiosis in chickens. One of the advantages of this group is that they do not promote a reduction in weight gain of the host animal (Shumard and Callender, 1967; Mitrovic, et al., 1975; Berg and Hamill, 1978), thus implying a selective cytotoxicity for the parasite. When sporozoites of *Eimeria tenella* are inoculated into cultures of chick kidney cells in the presence of an appropriate level of one of these antibiotics the subsequent intracellular parasite is irreversibly damaged, but the host cell remains unharmed (Pfeiffer, 1973; Boothby, 1975; Part II, this thesis), thereby confirming that these drugs are lethal for the parasite but not the host cell in the same drug environment. However, this phenomenon of differential response may pose a disadvantage to the usefulness of these compounds as anticoccidial agents in that strains of coccidia might develop that show significantly lowered sensitivity to these drugs. Although not confirmed, the existence of such "resistant" strains has been rumored. Therefore, a knowledge of the basis of decreased sensitivity to the anticoccidial ionophores would be important.

The group of compounds known as the carboxylic ionophores, including those with coccidiocidal activity, has
the capability of forming uncharged lipophilic complexes with alkali metal cations and transporting these into and through biological membranes (Ovchinnikov, et al., 1974; Pressman, 1976). Since these are membrane-active drugs the cell membranes of different cell types may provide an explanation of the phenomenon of differing sensitivities to the ionophores. This paper presents the collective evidence for a role of membrane fluidity in the lethal activity of the anticoccidial ionophores.
concomitant poor solubility in water. The complex thus formed is closed by the formation of hydrogen bonds between the carboxyl group on one end of the molecule and the hydroxyl groups on the other. This has been graphically described as "buttoning itself shut" (Pinkerton and Steinrauf, 1970).

The ion selectivity of the ionophorous antibiotics is a combined function of the energy required for desolvation of the ion and the liganding energy obtained on complexation (Pressman, 1968). A definite relationship exists between the size of the cavity formed and the ion selectivity. For example, nigericin, monensin and dianemycin complexes are similar in their ring structure, but differ considerably at the site of closure of the pseudocyclic ring, the largest number of atoms in the ring and consequently the biggest internal cavity being found for nigericin (Ovchinnikov, et al., 1974). Nigericin, as a result, prefers to complex potassium, whereas monensin and dianemycin preferentially complex sodium. Although the carboxyl may (e.g., nigericin) or may not (e.g., monensin) participate in cation liganding, the carboxylic ionophores form complexes with cations only in their deprotonated anionic form (Pressman, 1976).

The formation of lipophilic complexes with alkali metal cations makes these carboxylic ionophores capable of carrying these ions into and through biological membranes. The first report on the ability of ionophorous antibiotics
to increase permeability of lipid bilayer membranes was that of Mueller and Rudin (1967). Pressman, et al., (1967) subsequently showed that nigericin and valinomycin were able to transport ions across bulk phases too thick to accommodate intact channels and correctly inferred that these antibiotics function as mobile cation carriers. This mechanism was later found to extend to artificial lipid bilayers (Ciani, et al., 1969). Since then the action of the ionophorous antibiotics has been studied on bilayers prepared from highly varied lipids (Hilton and O'Brien, 1970; Szabo, et al., 1969; McLaughlin, et al., 1970; Stark and Benz, 1971; Stark, et al., 1972). Generally, the increase in cation permeability observed in these studies is independent of the lipid species, but the magnitude of the effect and its dependence on pH, ionic strength and on the transportable cation concentration is determined to a great extent by the molecular structures of the bilayer components. Ionophore activity on ion-transport in liposomes has also been studied (Henderson, et al., 1969). These compounds accelerate cation exchange between the liposomes and the environment, the selectivity sequence coinciding with that of complex formation. In preparations of isolated rat liver mitochondria the activity of the carboxylic ionophores has been shown to inhibit mitochondrial ATPase (Estrada-0, et al., 1967; Wong, et al., 1977) with concomitant optical density changes.

In order for ionophores to transport ions across membranes efficiently certain kinetic criteria must be met. At the
high-dielectric region of the membrane interface, complexation-decomplexation reactions must be rapid. The exchange of the ion solvation shell for the oxygen system of the ionophore must be a concerted reaction in order for the energy of activation of transport not to rise exceedingly. Once the complex leaves the interface and enters the low dielectric region of the hydrophobic membrane interior it attains high stability (Haynes, et al., 1969). Physical studies indicate that the complexation-decomplexation kinetics and diffusion rates of ionophores and their complexes across lipid barriers are so favorable that their transport turnover numbers across biological membranes attain values of thousands per second (Haynes, et al., 1974), exceeding the turnover numbers of most macromolecular enzymes.
Figure 11. Structure of monensin, lasalocid and narasin.
PHYSICAL PROPERTIES OF IONOPHORES

All of the anticoccidial ionophorous antibiotics currently used are carboxylic ionophores. Nigericin was the first of this class discovered. In 1950 it was isolated from the metabolites of an unknown streptomyces found in Nigerian soil (Harned, et al., 1951). Since then a number of similar compounds have been discovered, most with anticoccidial activity but varying degrees of host cell cytotoxicity (Shumard and Callender, 1967). All of the carboxylic ionophores contain tetrahydrofuran and tetrahydropyran rings and a carboxyl function (Fig. 11). All are capable of forming neutral complexes with alkali metal cations, the negative charge of the carboxyl group canceling the positive charge of the cation. In their complexed form all of the antibiotics of this class envelope the cation so that the ions are surrounded by strategically spaced oxygen atoms of the functional groups instead of by the solvate sheath of the solvent (i.e., replaces the hydration shell of water in aqueous solutions). The neutral oxygens ligand to cations via ion-dipole interactions analogous to the solvation of the ions in high-dielectric solvents (Gross and Witkop, 1965; Gromet-Elhanen, 1971; Grotens, et al., 1971; Groth, 1971; Gunn and Tosteson, 1971; Ovchinnikov, et al., 1974; Pressman, 1976). The exterior of the complex is overwhelmingly hydrophobic, explaining its high solubility in organic solvents and a
Monensin, lasalocid and narasin inhibit the development of intracellular Eimeria tenella sporozoites in cell culture systems (Pfeiffer, 1973; Boothby, 1975; Part II, this thesis), although activity against subsequent development stages has not been fully evaluated. The effect on the ultrastructure of the intracellular sporozoite in vitro is similar for all three antibiotics, suggesting a possible common destructive mechanism. This effect, characterized by the appearance of large vacuoles within the parasite and a general swelling of the organism, frequently appears as a ballooning at the anterior end of the sporozoite. When this occurs the bleb is limited only by the outer pellicular membrane, the plasmalemma; the inner-membrane complex is either retained in a more posterior position or disappears altogether. Because the sporozoite nucleus is often found in juxtaposition to the plasmalemma of the bleb (as though pushed aside) suggests an increased intracellular pressure.

By contrast, the host cells survive this drug treatment and continue to proliferate (Part II, this thesis). For each of the anticoccidial ionophores studied, at appropriate levels the effect of the drug on the ultrastructure of the host cells is negligible (Pfeiffer, 1973; Boothby,
1975, Part II, this thesis), although the higher drug levels can be lethal to the host cells as well (Part II, this thesis). This phenomenon of ionophore selectivity has been most dramatically documented for narasin.

A number of possible explanations for the effect of the coccidiocidal ionophorous antibiotics on the ultrastructure of the intracellular sporozoite exist. Based upon the physical properties of the carboxylic ionophores the swelling effect may reasonably be attributed to an increased intracellular osmotic pressure resulting from cation-translocation across the plasmalemma (i.e., increased cation permeability). The carboxylic ionophores cause rapid swelling of mitochondria when the incubation medium is a solution of the appropriate cation as a salt of permeant weak acids, or in the presence of a proton carrier (Ovchinnikov, et al., 1974). Such activity can be rationalized in terms of the ability of the carboxylic ionophores to stimulate transmembrane non-electrogenic $M^+\cdot H^+$ exchange. With both the carboxylic and valinomycin-type ionophores the cation selectivity sequences for the induced swelling are similar to the corresponding sequences revealed in experiments with model lipid membranes and also complexing studies in solution. Thus ionophores with different cation affinities could produce an equivalent effect, as occurs in the coccidia. The large vacuoles, also associated with coccidiocidal activity, may be contractile vacuoles formed by the injured organism as a defense in an
attempt to rid itself of the excess water and permeant solutes that would accompany increased cation permeability.

Alternatively, the observed effects may be ascribed to secondary metabolic effects of the altered ion permeability. Evidence for this possibility stems from the work of Harold (1970) who states that in ionophore inhibition of *S. faecalis* an important factor is impairment of ribosome function associated with the fall in potassium concentration. With carboxylic ionophores that can create an M⁺-H⁺ exchange, a decrease in intracellular pH could inhibit glycolysis and influence normal membrane transport activities.

Another consideration might be that the observed effect reflects an impairment of activity of the Na⁺-K⁺ pump. However, the ouabain-sensitive system of active potassium and sodium transport widespread in the plasma membrane of animal cells is by itself apparently resistant to ionophores (Gorden and De Hartog, 1968; Levinson, 1967), although these compounds may exert an indirect effect mainly by uncoupling oxidative-phosphorylation and thereby diminishing ATP pools.

Whatever the actual lethal mechanism the transmembrane cation carrier potential of these antibiotics appears to be involved.
THE DYNAMICS OF MEMBRANE LIPIDS

The basic hypothesis of generalized membrane structure most widely accepted today is the so-called fluid-mosaic model of Singer and Nicolson (1972). This model proposes that the lipid component of membranes is arranged in a bilayer and contributes the basic barrier properties to the membrane, an idea derived from the Davson-Danielli model (Danielli and Davson, 1935). Unlike the Davson model, the fluid-mosaic model describes the membrane proteins as being embedded in the lipid matrix to varying degrees.

Membrane lipids are generally regarded to be in a fluid or dynamic state at physiological temperature. Physical techniques such as electron-spin resonance (ESR), nuclear magnetic resonance (NMR), x-ray diffraction and calorimetry have detected a number of types of motion occurring in cell membranes (Lee, 1975; Nicolson, et al., 1977). Two types of motional modes have been described; intramolecular motions, involving rotations of groups about single bonds, segmental and long-range swinging motions of fatty acid chains, and polar headgroup conformational changes (Trauble, 1971; Lippert and Peticolas, 1972; Mendelsohn, et al., 1976; Seelig and Seelig, 1974; Yeagle, et al., 1975; Griffin, 1976); and, whole molecule motion which includes rotation (Cullis, et al., 1976; Lee, 1975) and translation, both lateral and transmembrane (Devaux and McConnell, 1972; Trauble and
Sackmann, 1972). The intramolecular motions are intimately related to the chemical structure of the lipid. The various motional modes exhibited by lipid molecules in a bilayer are expressed to varying degrees in system properties such as bilayer viscosity.

The viscosity of a phospholipid bilayer has been estimated using a number of physical techniques. From measurements of the depolarization of the fluorescent probe diphenyl hexatriene (DPH) incorporated into liposomes of dioleyl phosphatidylcholine a value of about 1 poise at 21°C was obtained (Lentz, et al., 1976a&b), approximately the same viscosity as mineral oil at room temperature and about 100 times that of water. The actual viscosity of a phospholipid bilayer or biological membrane is dependent on a number of variables including the type of lipid and the acyl chain composition, the nature and degree of hydration of the polar headgroups, the sterol content, and the temperature. As a rule, the lower the degree of unsaturation and the longer the chain length of the fatty acids, the greater the viscosity at a given temperature (Lentz, et al., 1976a; Kohler, et al., 1972; Barton and Gunstone, 1975). Likewise, membrane viscosity is directly related to the reciprocal of the temperature. Using a fluorescence depolarization method on normal lymphocytes and lymphoma cells from mice, Shinitzky and Inbar (1974) found the microviscosities to be 2.8 and 1.65 P at 25°C and 1.7 and 1.0 P at 37°C, respectively. Cholesterol, the most abundant sterol,
reduces the mean molecular area of the phospholipids in a membrane (Lecuyer and Dervichian, 1969; Jain, 1975) at physiological temperatures, increasing viscosity. This is called the condensing effect.

The physiological functions of biological membranes are influenced by lipid composition, primarily in terms of lipid fluidity. The fluidity can be described by defining a microviscosity that describes the resistance to motion by the bilayer on intrinsic bilayer components. Since the ionophorous antibiotics are membrane-active compounds, a priori reasoning suggests that membrane fluidity should influence the activity of these drugs. Evidence for such an influence is as follows.
EVIDENCE FOR A ROLE OF MEMBRANE FLUIDITY IN THE ACTIVITY OF THE ANTICOCCIDIAL IONOPHOROUS ANTIBIOTICS

Work reported to date on the effect of the anticoccidial ionophores has shown not only that these compounds result in irreversible damage to the intracellular parasite, but also that at appropriate coccidiocidal levels there is no apparent ultrastructural damage to the host cell (Pfeiffer, 1973; Boothby, 1975; Part II, this thesis). The host cells survive and continue to proliferate, indicating different sensitivities to the ionophores by different cell types. Unconfirmed reports that coccidial resistance (lowered sensitivity) to the ionophores has developed in rare instances also indicates differential sensitivities to the antibiotics. Resistance to these compounds exists in the microbial world. *Escherichia coli* are resistant to the neutral ionophore valinomycin, apparently due to the inability of the antibiotics to increase the $K^+$ permeability of the membrane of this organism, as is the yeast *Endomyces magnusii* (Ovchinnikov, *et al.*, 1974). The basis for this apparent differential sensitivity to the ionophores is unknown, but recent work, including that presented in this thesis, has pointed to membrane fluidity in influencing the effect of these drugs.

I showed (Part I, this thesis) that *E. tenella* sporozoites accumulate and retain measurable concentrations
of the ionophorous antibiotics lasalocid and narasin prior to invading their host cells. The amount retained, however, was influenced by the incubation temperature with lower temperature reducing the uptake of the antibiotics by the parasite. Because membrane viscosity would increase with decreasing ambient temperature (page 72) these results suggest that the aqueous-hydrocarbon distribution of these ionophorous antibiotics may be influenced by membrane fluidity.

In another study, I investigated the influence of incubation temperature on the effect of an anticoccidial ionophore on the ultrastructure of intracellular E. tenella sporozoites (Part II, this thesis). At 40 C the characteristic lethal effect of the drug was apparent; i.e., gross swelling and vacuolization of the parasite, while a 10 C reduction in incubation temperature resulted in none of the characteristic ionophore-induced damage to the parasite. When the challenge dose of the antibiotic was raised 10-fold the results were the same. Together with the results of the ionophore retention studies, these findings indicate that not only the aqueous-hydrocarbon distribution but also the severity of the coccidiocidal activity is influenced by temperature, which also influences membrane fluidity.

To study the influence of membrane structure on ionophore activity in an isothermal system, I investigated the influence of membrane fatty acid composition on the lethal effect of a carboxylic ionophore. The fatty acyl
composition of a biological membrane has a great influence on the physical state of that membrane (page 72). By regulating the fatty acid composition of the growth nutrient of *Acholeplasma laidlawii* I was able to vary the fatty acid composition of the membranes of the organism in different cultures. Those organisms whose membranes had a high degree of unsaturation were 40 times more sensitive to the lethal effect of an ionophore than those with membranes having virtually no unsaturated fatty acids. Since a higher degree of lipid unsaturation results in increased fluidity, these findings add further support to the premise that the lethal activity of the carboxylic ionophorous antibiotics is influenced by membrane viscosity.

The results of the work completed on intact biological systems is supported by experiments conducted earlier using lipid bilayers and liposomes. Szabo, et al. (1969), using lipid bilayers showed that for a lecithin-cholesterol ratio of 1:1, nonactin-induced conductivity is reduced by 1/20. In 1970 De Gier, et al., reported that the rate of valinomycin-promoted $^{86}$Rb$^+$ exchange in lecithin liposomes decreases significantly as the unsaturated fatty acid residue content diminishes. The significance of lipid molecule packing on the activity of the ionophores was further illustrated by the work of Krasne, et al. (1971), who showed that cooling of a lipid membrane to below the phase transition temperature (liquid crystalline to crystalline) of the corresponding lipid-water liquid crystals completely inhibited valinomycin-
or nonactin-induced K+ conductance. Under similar conditions gramicidin (a channel-former vs a mobile carrier) retains its activity. Thus the effect is hard to explain only by anti­biotic molecules being displaced from the membrane. A better interpretation is that it is an effect of decreased mobility of the ionophore-ion complex resulting from the phase transi­tion. The dependence of ionophore-induced conductivity on the hydrocarbon chain length of lipid molecules was studied by Stark, et al. (1972). The results of this investigation showed that the conductance of bilayers prepared from decane solutions of saturated lechithins falls by about two orders of magnitude as the number of the carbon atoms in the chain increases from 12 to 18. These authors interpreted their results to mean that the reduced conductivity is attributable to changes in the packing of the polar headgroups lowering the rate of the heterogeneous complexing reaction (the rate of ionophore-ion complex formation at the membrane-water interface) and not to decreased mobility of the complex within the hydrocarbon region.

Whether due to an influence on the rate of complexa­tion-decomplexation at the membrane-water interface or on the diffusion rate of the ionophore-ion complex in the hydro­carbon region of the membrane, the overriding conclusion to be drawn from the work with artificial lipid-bilayers and liposomes and the more recent work with intact biological systems is that the physical state (i.e., viscosity) of a biological membrane influences the activity of the
ionophorous antibiotics and thereby the lipid composition of the limiting membrane of a cell can directly influence the potentially lethal results.

In terms of the anticoccidial activity of the carboxylic ionophorous antibiotics this theory is important because it affords a plausible explanation for the selectivity of the drugs for the intracellular parasite and, even more importantly, it provides a means of explaining the potentially disastrous development of resistance to these compounds by coccidia.
REFERENCES


