Efficacy of salinomycin on infections of plasmodium berghei in male ICR mice

Anthony Stephen Tokarz

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EFFICACY OF SALINOMYCIN ON INFECTIONS OF PLASMODIUM BERGHEI IN MALE ICR MICE

by

Anthony Stephen Tokarz

B.S., Virginia Commonwealth University, 1976

Thesis

submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology
School of Basic Sciences
Medical College of Virginia
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Richmond, Virginia

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This thesis by Anthony Stephen Tokarz is accepted in its present form as satisfying the thesis requirement for the degree of Master of Science.
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The assistance of Drs. Joseph Formica and Susan Cross is also gratefully acknowledged.

The author expresses special thanks to Dr. David John for his technical assistance.

Finally, the typing skills of Mrs. Gayle Hylton are acknowledged and gratefully appreciated.
DEDICATION

This thesis is dedicated to the author's wife, Mary, whose love, understanding and support enabled the author to complete his research.

The author also dedicates this work to the memory of his mother.
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INTRODUCTION

Protozoan parasites of man constitute membership in all major groups of this phylum (Jawetz, et al, 1976; Burrows, 1973). The flagellated Trypanosome, a member of the group Mastigophora is a serious health problem in tropical climates, where the organism is the etiological agent of sleeping sickness and Chagas' disease. Members of the group Sarcodina are generally ameboid, and include the intestinal pathogen Entamoeba, and Nagleria, which causes a serious encephalitis.

Malaria, along with Toxoplasmosis, is one of the few Sporozoan diseases of man. Sporozoan are characterized by having a complete life cycle, often in two hosts. Distribution of the disease is confined primarily to the tropical and subtropical zones of the world. It is no longer a major health problem in areas of temperate climate. Malaria remains, however, the most common serious infectious disease in man, worldwide (Benenson, 1975). In the United States, a relatively large increase in the number of reported cases of malaria occurred during the late 1960's and early 1970's, as military personnel returned from Vietnam (CDC Annual Summary, 1978).
Malaria is caused by members of the genus Plasmodium. Four species of Plasmodium are the etiological agents of malaria in man; \textit{P. ovale}, \textit{P. malariae}, \textit{P. vivax}, and \textit{P. falciparum}, the last being the most serious and fatal (Markell and Voge, 1976).

The disease in man is primarily an infection of erythrocytes in the circulatory system. Malaria is transmitted to man almost exclusively by the feedings of female Anopheles mosquitoes. Clinical signs, including malaise, headache, fever and chills occur in a cyclic pattern, corresponding to the periodic release of new parasites from the infected erythrocytes. The timing of the fever and chill cycles will vary with the species of Plasmodium causing the disease. Complications such as blackwater fever, in which the host immune system attacks and lyses its' own infected and non-infected erythrocytes may occur, especially with infections by \textit{P. falciparum} (Beneson, 1975; Markell and Voge, 1976).

Relapses do occur with \textit{P. ovale}, \textit{P. malariae} and \textit{P. vivax}, but not with \textit{P. falciparum}. To be completely effective, a treatment must eliminate all parasites in the circulatory system and solid tissue phases of a natural infection. Latent infections may persist for thirty years or more (Burrows, 1973).

Quinine, and its synthetic derivative, chloroquine, have been the traditional drugs of choice in the treatment of prophylaxis of malaria. Both suppress nucleic acid metabolism in schizonts (parasites of the erythrocytic
cycle). Neither drug, however, has proven to be ideal because of the existence of Plasmodium strains resistant to these and other drugs. Following the first world war, a large number of synthetic antimalarial agents were developed (Thompson, 1972). With the use of antimalarial drugs came the development of Plasmodium strains resistant to the effects of one or more such drugs. Most recently, attention has been focused on chloroquine and multiple drug resistant strains of Plasmodium falciparum in South East Asia (Kinnamon and Roth, 1975). The appearance of such drug resistant strains has spurred the U.S. Army and private industry to search for new antimalarial agents and treatment regimens.

Since there has been little success in culturing Plasmodium in vitro (Peters and Howells, 1978), the traditional method of testing potential antimalarial agents has been in experimental animal systems infected with their own species of Plasmodium. One such model is the mouse-Plasmodium berghei system.

Plasmodium berghei is one of twelve rodent host-specific species of Plasmodium native to Central Africa (Killick-Kendrick, 1978). The protozoan is a natural parasite of the tree rat, Thamnomys surdaster (Vincke, 1954; cited by Landau and Boulard, 1978).

The parasite maintains a two host life cycle in natural infections, one vertebrate host, the other invertebrate. Gametocytes are ingested by the female Anopheles mosquito during a blood feeding on the vertebrate host. The macro-
gametocytes become female macrogametes in the lumen of the mosquito midgut. The microgametocytes undergo exflagellation to become male microgametes. The macrogamete is then fertilized by the microgamete to form a motile ookinete, which in turn migrates into the wall of the midgut. Here the ookinetes mature into oocysts within seven to fifteen days, depending on the ambient temperature. Upon maturation, the oocysts rupture and liberate sporozoites which migrate from the midgut wall to the salivary glands. This is the sporogenic, or sexual cycle.

With subsequent feeding by the mosquito, sporozoites are transferred from the mosquitoes' salivary glands to the blood stream of the vertebrate host. From here, sporozoites invade the liver and enter the hepatocytes (this portion of the vertebrate infection process is absent in animals inoculated with blood containing only erythrocytic stages). While in the hepatocytes, the sporozoites give rise to pre-erythrocytic schizonts. This is the primary tissue phase of infections. The schizonts mature in the hepatocytes and eventually rupture them, releasing merozoites into the circulatory system. In *P. berghei* infections, this initial release of merozoites occurs within forty-eight to seventy-two hours of inoculation with sporozoites. Once in the circulatory system, the merozoites enter erythrocytes, where they subsequently divide and form trophozoites or ring stages (Figures 1 and 2), schizonts (Figure 3), and finally new merozoites.
Figure 1

Geimsa's stain of early trophozoite (ring) stage in mouse erythrocytes, magnified 1000X. Early trophozoite (ring) stages in mouse erythrocytes stained with Geimsa's solution, magnified 1000X.

Figure 2

Late trophozoite stages in mouse erythrocytes stained with Geimsa's solution, magnified 1000X.
These merozoites then rupture the swollen remains of the erythrocyte and re-enter the circulatory system to infest other erythrocytes. Some merozoites change into gametocytes while in the erythrocyte. Hepatocytes are not infected by the circulating merozoites. This is the schizogenic or asexual cycle (Flynn, 1973; Killick-Kendrick, 1978). The ruptured erythrocytes release toxic waste products, accrued during schizogeny, which cause the fever and chills associated with malaria (Markell and Voge, 1976).

The various subspecies and strains of *P. berghei* exhibit certain characteristics which distinguish them from other non-murine *Plasmodium* species. The merozoites show a definite predilection for reticulocytes over mature erythrocytes (Figure 4). The primary tissue phase, when it occurs is relatively short, under fifty hours (Killick-Kendrick, 1974). Sporogeny is rapid, taking only nine days to complete. Rats, hamsters and mice are naturally receptive to infection by inoculation of the erythrocytic stages (Landau and Boulard, 1978).

The number of parasites detectable in the blood stream rises rapidly, with no decreases in parasitemia. When unchecked, the infection generally results in the death of the animal in seven to fourteen days (Voelii, 1965; Sengers et al., 1971). Increases in SGP-T and SGO-T transaminases have been detected in the blood of infected mice, along with a concomitant drop in fasting glucose levels in heavily infected mice (Sadun, et al., 1965). The latter effect is
Figure 3
Polymorphonucleocytes and early trophozoite stages within infected mouse erythrocytes stained with Geimsa's solution, magnified 1000X.

Figure 4
Erythrocytes and reticulocytes (purple) in the blood of normal mice stained with Geimsa's solution, magnified 1000X.
Probably due to an increased demand for glucose by Plasmodidia infected erythrocytes (Bryant et al., 1965).

Most drugs active against human malaria exhibit similar effectiveness against P. berghei (Thompson, 1972). With the development of multidrug resistant strains of P. berghei, it has been possible to develop massive drug testing programs utilizing animals relatively inexpensive and easy to manipulate (Peters and Howells, 1978).

Statement of Purpose

The objective of this study was to determine whether or not salinomycin, a drug with proven effectiveness against intestinal sporozoan parasites, has any effect on the survival time of mice infected with P. berghei.

Salinomycin is a monocarboxylic acid polyether antibiotic with a tricyclic spiroketal ring system and an unsaturated six-membered ring (Kinashi et al., 1973). It is a natural fermentation product of Streptomyces albus (Miyazaki et al., 1974). The drug is highly insoluble in distilled water and highly soluble in such organic compounds as methanol, acetone and ethyl ether at room temperature. Such selective solubility thus presents potential problems with dispersal in suitable solvents for in vivo experimental systems. Activity against Bacillus test organisms remains high at neutral to basic pH, while decreasing steadily as the pH of the diluent solution lowered below six. Salinomycin activity remained stable at a variety
of ambient temperatures, from 5 to 60°C.  

Salinomycin is a cationic ionophore (Mitani et al., 1975). The drug has the ability to complex with and cause the migration of select monovalent cationic alkalai metals, such as sodium, potassium, and cesium across phospholipid bilayer membranes. Experiments performed with rat mitochondria indicate that salinomycin causes the rapid release of potassium from the mitochondrial membranes, shutting down both coupled and uncoupled respiration within these membranes (Mituaki et al., 1976).

This selective transport activity of salinomycin seems to be deleterious to the growth of certain gram positive bacteria, mycobacteria and fillamentous fungi (Myazaki et al., 1974). Danforth et al., (1977) demonstrated the orally administered salinomycin is effective in eliminating the coccidial intestinal sporozoan Emeria from the digestive tracts of battery raised broiler chickens. Salinomycin has also been shown to eliminate coccidia from the intestines of cattle (Bens and Ernst, 1979).

The anticoccidial activity of salinomycin appears to be both coccidiocidal and coccidiostatic (Chappel, 1979). Drug activity is primarily directed against nuclear replication during the transition from sporozoite to trophozoite stage in the intestinal endothelium. Salinomycin when administered through the entire life cycle of coccidia in

---

1 Kaken Chemical Company, Salinomycin Data Sheet, 1974.
experimental chickens shuts down activity of all asexual stages.

The effects of salinomycin on normal hematologic parameters were first determined. Next, attempts were made to determine the optimal intraperitoneal (I.P.) dose and injection frequency for treatment of *P. berghei* in mice. The size of the initial inocula and parasitemia levels when treatment was begun were varied in order to determine the optimal time during the erythrocytic cycles to begin I.P. administration of salinomycin. Finally, the effects of orally administered salinomycin on *P. berghei* induced murine malaria were examined.
MATERIALS AND METHODS

Animals

20-25 gram male ICR mice were obtained from Flow Research Animals, Inc., Dublin, VA, and were maintained on Respond 3000 Rodent Chow and tap water ad libitum. Mice were generally allowed to increase their body weight to 25-39 grams before being used in experiments. All mice in experiments involving infectious materials were housed in special isolated workrooms. Mice were kept ten to a cage, unless otherwise indicated. Cage bedding was Beta-chip hardwood.

Plasmodium

The lethal NYU-2 strain of Plasmodium berghei was used in all experiments. The parasite was supplied by Dr. David John of the Department of Microbiology, Medical College of Virginia.

Plasmodium stocks were maintained in vivo by injecting I.P. male ICR mice with infected blood diluted in a 0.1 M solution of sodium citrate. The infected blood was obtained from previously injected mice by incisions in tail tips with straight razors. Plasmodium was passaged every seven or eight days. Two or three drops of blood from infected animals were diluted in 1 ml of sodium citrate solution, and 0.2 cc injected I.P. into each mouse. Injected mice lived
an average of 14 days and had parasitemias detectable by Geimsa's Stain within two days.

Innoca for experiments were prepared by obtaining the percent parasitemia and performing total erythrocyte counts with a hemacytometer (see below) for an infected donor animal. The number of infected erythrocytes per unit volume of whole blood were calculated. An appropriate volume of blood was then removed via cardiac puncture and diluted in sodium citrate solution to an appropriate concentration for subsequent injection into test mice.

Salinomycin (Figure 5) was obtained from Dr. Robert Tankersley, Chief Microbiologist, Research Department of A. H. Robins, Inc., in sodium salt form. Stock solutions of Salinomycin were made by suspending 100 mg of salinomycin in a solution of 2% absolute ethanol, 40% propylene glycol and 58% .16M phosphate buffered saline (PBS). Final concentrations of the stock solution was 2 mg/ml.

**Total Erythrocyte Counts**

Total erythrocyte counts were performed by 2 methods:

1) Hemacytometer counts. Mouse blood was collected from tail tips into 20 µl unopettes, which diluted the blood 1:100,000 in saline solution. Two Hemacytometer chambers (Clay-Adams) were then charged with diluted blood from the unopette. The total number of erythrocytes in the 4 corner squares and the center square of each chamber at 100X magnification were counted and averaged. The following
Figure 5

Salinomycin \((C_{42} H_{69} O_{11})\)
Salinomycin \( \text{C}_{42}^\text{H}_{69}^\text{O}_{11} \)
formula was used to determine the number of cells per mm$^3$:

\[
\text{Erythrocytes counted \times Dilution \times 4000}
\]

Number of Squares Counted

2) Electronic Cell Sorter Counts. Mouse blood was obtained as described above in 20 µl amounts. The blood was immediately dispersed in enough Isoton solution (Fisher Scientific Products) to yield a dilution of 1:100,000. Total erythrocyte counts were performed on a Coulter Counter (Coulter Electronics, Hialeah, FL) by sampling each specimen of diluted mouse blood twice, correcting each value for over-counts and averaging these two figures.

**Hematocrits**

The hematocrit or packed cell volume (PCV) was determined by filling 75 mm heparinized capillary tubes with mouse blood obtained as described previously. The tubes were then centrifuged for 8 minutes at 14,500 RPM in an International Microcapillary Centrifuge (International Equipment, Needham Heights, MA). Packed cell volume is defined as that percentage of the total blood volume consisting of blood cells.

**Hemoglobin**

Hemoglobin levels in the blood were determined by collecting 10 µl of mouse blood in 6 ml of cyanometh-
hemoglobin reagent (Stambio Products, San Antonio, TX), shaking, then reading absorbance at 540 nm within 45 minutes of dilution. Readings were made on a Coleman Junior II Spectrophotometer (Perkins Elmer, Oak Grove, IL).

Absorbance was converted to mg hemoglobin/dl blood by comparison to a previously established hemoglobin standard curve.

Post-Mortem Examination

Post mortem examinations were performed periodically to confirm deaths due to malaria, and to determine the effects of orally administered salinomycin. Moribund mice not already dead were sacrificed by cervical dislocations. The abdominal cavities of these animals were opened with scissors and forceps. When necessary, the liver and spleen were examined for darkening and enlargement. In oral administration experiments, the entire intestinal tract was removed and sectioned. Swabs from the intestinal lining of the jejunum and transverse colon were streaked on microscope slides with sterile phosphate buffered saline (PBS) and examined at 100X while still wet for the presence of Trichomonas, adult pinworms and bacteria. Contents from infected and treated animals were compared to samples from healthy, untreated mice.

Mean Survival Time (MST)

The MST value was calculated by multiplying the number
of mouse deaths in a group on a particular day by the number of days after infection the death occurred. These products were then summed and divided by the number of animals counted in a particular group. Deaths due to drug toxicity were excluded.

**Wintrobe Erythrocytic Indexes**

These were determined in order to provide objective quantitative standards for the hematologic values determined in the experiments described. These values are used to define anemias which may be discovered (Davidson and Henry, 1974). Values for the percentage of mouse erythrocytes infected by one or more malarial parasites were determined by direct microscopic examination of stained blood smears. One to two drops of mouse blood from tail snips were smeared on microscope slides, air dried and fixed in absolute methanol. The slides were then stained for five minutes in a 1:50 solution of Geimsa's stain. Smears were then examined at 1000X under an oil immersion objective. At least four fields and a minimum of 200 erythrocytes were counted. Percent parasitemia was deferred as that percentage of the total number of erythrocytes counted that contained Plasmodium.

**Mean Corpuscular Volume (MCV)**

This is the average volume of the erythrocytes of an organism. The index is expressed in fl (1 µl = 10⁹ fl) and is calculated by the formula:

\[
\frac{\text{Hematocrit Percentage (fl) \times 10}}{\text{Erythrocyte Count}}
\]
Mean Corpuscular Hemoglobin (MCH)

The weight of the hemoglobin (in picograms) in the average erythrocyte in a population of cells. It is determined by the formula:

\[
\text{Weight of Hemoglobin (pg) in 1 dl of Blood} = \frac{\text{Weight of Hemoglobin (pg) in 1 dl of Blood}}{\text{Volume of the Blood Sample (dl) \times Erythrocyte Count (dl)}}
\]

Mean Corpuscular Hemoglobin Concentration (MCHC)

The MCHC (the ratio of the weight of hemoglobin to the average volume of the erythrocyte expressed in g/dl.) was calculated by the formula:

\[
\text{MCHC} = \frac{\text{Hemoglobin (g/dl)} \times 100}{\text{PCV (ml/100 ml)}}
\]

Photomicrographs were taken by the author with a Zeiss Photomicroscope #3. Statistical analysis was by Student's T-Test.
RESULTS

Lethal Dose Range Determination for I.P. Administered Salinomycin in Mice

In preliminary experiments, seven groups of ten male ICR mice (average weight 23 grams) were used to determine lethal dose range for salinomycin suspended in ethanol-propylene glycol-PBS. Ten mice per group received one I.P. injection of salinomycin in 0.3 cc ethanol propylene glycol-PBS diluent. Each group was injected once with 1, 3, 6, 9, 12, or 15 mg/kg of salinomycin. The eleventh group was injected only with diluent. Mice were observed for fourteen days after injection for any signs of toxicity or death. As shown in Table 1, deaths occurred in groups of mice receiving 9 or more mg/kg of salinomycin. Signs of toxicity included hind leg paralysis, head rotation, panting and a hyperactive startle reflex. Mice died within three hours of drugs administration. No gross pathological changes were noted.

Effects of an Untreated Infection of P. berghei on Selected Hematologic Values of Mice

Five mice were each infected I.P. with approximately 4 x 10^3 Plasmodium bearing erythrocytes in 0.2 cc sodium citrate solution. Blood samples from tail snips were taken just prior to, and every twenty-four hours after inoculation, for twelve days. The hemoglobin, hematocrit and parasitemia levels were determined in order to ascertain the effects of
TABLE 1
LETHAL DOSE RANGE DETERMINATION FOR I.P. ADMINISTERED SALINOMYCIN IN MICE

<table>
<thead>
<tr>
<th>Dose</th>
<th>Number Deaths/Number Tested</th>
<th>% Killed</th>
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<tbody>
<tr>
<td>Diluent</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>0/10</td>
<td>0</td>
</tr>
<tr>
<td>9 mg/kg</td>
<td>3/10</td>
<td>30</td>
</tr>
<tr>
<td>12 mg/kg</td>
<td>5/10</td>
<td>50</td>
</tr>
<tr>
<td>15 mg/kg</td>
<td>10/10</td>
<td>100</td>
</tr>
</tbody>
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Plasmodium infection. As shown in Table 2, there was a slight overall decrease in the hemoglobin and hematocrit levels between day 0 and day 4. After day 4 the levels of these two parameters dropped sharply, while the parasitemia levels rose between days 4 and 8, peaking on day 8 (Figure 6). Between days 8 and 12, the day the last surviving mice died, the parasitemia level dropped somewhat, while the hematocrit and hemoglobin concentration leveled off. MST was 12.6 days.

**Effects of Salinomycin on Certain Hematologic Values in Mice**

The effects of I.P. injected salinomycin in mice were examined in two experiments. In the first experiment, two groups of ten mice each with an average weight of 33 grams were used. One group was injected with 4 mg/kg/day of salinomycin in 0.3 cc diluent three times. The other group was similarly injected, but only with diluent. Mouse blood was drawn from tail snips and samples were analyzed for hematocrit (HCRIT) and hemoglobin (HGB) levels just prior to the first injection, twenty-four hours after the last injection and five days after the last injection. As illustrated in Table 3 mean hemoglobin values for diluent controls and salinomycin administered animals did not vary significantly from each other during the course of treatment or up to five days afterward. The same lack of variance holds true for hematocrit levels of diluent controls and salinomycin groups, during treatment and afterwards.
TABLE 2

EFFECTS OF UNTREATED INFECTION OF P. BERGHEI ON SELECTED HEMATOLOGIC PARAMETERS OF MICE

<table>
<thead>
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<th>Mouse No.</th>
<th>HB (g/dl)</th>
<th>HCRIT (% PCV)</th>
<th>Parasitemia</th>
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<tr>
<td>Mean</td>
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</tbody>
</table>

1 Initial inoculum of 4 x 10³ infected erythrocytes/mouse in 0.2 cc sodium citrate solution

2 Mouse died.
Figure 6

Selected Mean Hematologic Parameters of Mice during the course of an infection by *Plasmodium berghei*. 

FIGURE 6

- △ = Hemoglobin (g/dl)
- ○ = Percent parasitemia
- ○ = Hematocrit (Percent PCV)

Days post infection

Hemoglobin (g/dl)
TABLE 3

EFFECTS OF I.P. ADMINISTERED SALINOMYCIN\(^1\) ON SELECTED HEMATOLOGIC PARAMETERS OF MICE

<table>
<thead>
<tr>
<th>Group</th>
<th>HGB (g/ml)</th>
<th>HCRIT (% PCV)</th>
<th>HGB (g/ml)</th>
<th>HCRIT (% PCV)</th>
<th>HGB (g/ml)</th>
<th>HCRIT (% PCV)</th>
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<td>53</td>
</tr>
</tbody>
</table>

Mean: 13.3 12.8 50.5 50.8 13.2 13.4 48.3 49.9 14.4 14.6 52 53

\(^1\) 4 mg/kg/day in 0.3 cc diluent 3 times, every 24 hours.

\(^2\) Animal died immediately after injection of salinomycin. Data not collected.

\(^3\) Mice in diluent control group accidently drowned after 4 days after last injection.
In the second experiment, mice were examined just prior to the first injection of salinomycin and twenty-four hours after the last injection, but not five days after the last injection. Erythrocyte counts, done with an electronic cell counter, were performed along with hematocrit and hemoglobin determinations so that Wintrobe Erythrocytic indexes could also be determined (Table 4). Mice in two groups of ten animals each (average weight 24 grams) were injected with 4 mg/kg/day of salinomycin or diluent, as previously described. The results (Table 4, Figure 7) indicate little or no change in any hematologic values after treatment with salinomycin or diluent.

Efficacy of Salinomycin Suspension on the MST of *P. berghei* Infected Mice

An attempt was made to determine what effect salinomycin might have on the course of malaria infection in mice. Two groups of 27 gram mice was each injected I.P. with $5 \times 10^6$ Plasmodium infected erythrocytes in 0.3 cc of sodium citrate diluent. One group of ten mice received no further treatment. The other group, containing twenty mice, received 3 subsequent I.P. injections of 8 mg/kg/day of salinomycin in 0.3 cc of ethanol-PBS-propylene glycol diluent, 24, 48 and 96 hours after infection. The second group contained 20 mice so that any possible deaths due to high drug toxicity would not reduce the number of animals in the group to statistically unmanageable numbers. As shown in
TABLE 4

EFFECTS OF I.P. ADMINISTERED SALINOMYCIN\(^1\) ON SELECTED HEMATOLOGIC VALUES AND WINTROBE ERYTHROCYTIC INDEXES OF MICE

<table>
<thead>
<tr>
<th>JUST PRIOR TO FIRST INJECTION</th>
<th>24 HRS. AFTER LAST INJECTION</th>
<th>ERYTHROCYTE (^2) COUNT</th>
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<tr>
<td><strong>HGB (g/dl)</strong></td>
<td><strong>HCRIT (% PCV)</strong></td>
<td><strong>Erythrocyte</strong></td>
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<td>Diluent Saline- Control mycin Group</td>
<td>Erythrocyte</td>
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<td>13.2</td>
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<tr>
<td>10</td>
<td>16.0</td>
<td>13.4</td>
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</table>

**MEAN**

| 14.3 | 14.0 | 53.4 | 52.8 | 13.5 | 14.5 | 13.4 | 15.6 | 49 | 57.1 | 11.8 | 15.5 |

\(^1\)4 mg/kg/day x 3 in 0.3 cc diluent 3 times every 24 hours.

\(^2\)Animal died soon after injection of salinomycin. Data not collected.


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<th></th>
<th></th>
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<td>37</td>
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</table>

**MEAN**

40 37 11 10 27 27 42 37 12 10 27 27

---

1 Animals died soon after injection of salinomycin. Data not collected.
Figure 7

Effects of I.P. administered salinomycin on Wintrobe Erythrocytic Indexes of Mice

Diluent Control Group (Ethanol-PBS-Propylene-Glycol Diluent, administered 3 times every 24 days.)

Salinomycin Group (4 mg/kg/day) administered every 24 hours)
Figure 7

Mean Corpuscular Volume (fL)

Mean Corpuscular Hemoglobin (pg)

Mean Corpuscular Hemoglobin Concentration (g/dL)

Just prior 24 hours after last injection

Just prior to first injection

24 hours after last injection

Just prior to first injection

24 hours after last injection

Just prior to first injection

24 hours after last injection
Table 5, untreated mice all died between days 6 and 12 post-infection while the group of treated mice lived almost twice as long and died between the twelfth and nineteenth day post-infection. This increase in MST was determined to be statistically significant by student's T-Test.

Wide-Range Dose Response of P. berghei Infected Mice to Salinomycin

Because the salinomycin suspension appeared to be effective in prolonging the MST of infected mice, an attempt was made to define the lower limits of dose effectiveness in Plasmodium infected mice. Two experiments were performed in order to determine the most efficacious dose for multiple administration of salinomycin in mice.

In the first experiment, six groups of eight to ten male ICR mice, with an average weight of 28 grams, were injected I.P. with 0.3 ml of a sodium citrate solution containing $5 \times 10^6$ infected erythrocytes. Subsequently, five of these mouse groups were injected I.P. with either 0.3 cc of diluent solution, or 2, 4, 6 or 8 mg/kg/day of salinomycin suspensions five times, every 24 hours. One group of eight mice received no further treatment after infection. Animals were observed daily for signs of disease or death. Periodic post mortems were performed and Geimsa stains of mouse blood were made to insure deaths were due to malaria. Efficacy of the drug was determined by comparison of the MST of each treated group with that of the infected untreated group. As shown in Table 6 and Figure 8, there was a slight, significant
Table 5

EFFICACY OF I.P. ADMINISTERED SALINOMYCIN SUSPENSION$^1$ ON THE MST OF \textit{P. BERGHEI} INFECTED$^2$ MICE

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<tr>
<td>20</td>
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</table>

| Total No. Mice     | 10        | 20          |
| MST                | 8.2       | 16.0        |

$^1$8 mg/kg/day, administered 24, 48 and 96 hours post infection.

$^2$5 x 10$^6$ infected erythrocytes/mouse in 0.3 cc sodium citrate solution.

$^3$Deaths probably due to drug toxicity. Not used in MST determination.

$^4$Drug administered this day.
### Table 6

**WIDE-RANGE DOSE RESPONSE OF P. BERGHEI\(^1\) INFECTED MICE TO I.P. ADMINISTERED SALINOMYCIN**

<table>
<thead>
<tr>
<th>Day Post Infection</th>
<th>Infected Untreated</th>
<th>Infected Diluent</th>
<th>2 mg/kg Day</th>
<th>4 mg/kg Day</th>
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<thead>
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<tr>
<td>MST</td>
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<td>10.5</td>
<td>13.0</td>
<td>11.7</td>
<td>13.8</td>
</tr>
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</table>

1. \(5 \times 10^6\) infected erythrocytes/mouse, injected I.P. in 0.3 cc sodium citrate solution.

2. 2-8 mg/kg/day, administered I.P. x 5, every 24 hours after infection.

3. Deaths probably due to drug toxicity. Not used in MST determination.

4. Drug administered this day.
increase in the MST of salinomycin treated animals which received 8 mg/kg/day of drug.

In a related experiment, five groups of 8 to 10 mice were similarly infected, but with \(9 \times 10^5\) infected erythrocytes. This time, three groups of animals received 2, 4, or 6 mg/kg/day of salinomycin suspended in 0.3 cc of diluent. Again, one group was injected only with diluent and one group of eight animals received no further treatment after infection. Salinomycin or diluent injections were administered five times, at 48 hour intervals after infection, in order to determine possible effects on MST in mice receiving a somewhat lower inoculum of Plasmodium, as well as an increase in the time interval between drug administrations. As shown in Table 7 and Figure 8, there was a steady increase in the MST of infected animals treated with salinomycin. Doses of 4 and 6 mg/kg/day caused significant increases in the MST of Plasmodium infected mice. As in the previous experiments, all animals eventually died of malaria.

Efficacy of Salinomycin on Low Initial Inoculum Infections of P. berghei in Mice

With the establishment of relatively effective dose ranges in the previous experiments, studies were designed to test the effectiveness of the drug when the number of parasites was severely limited. The effects of I.P. administered salinomycin on infections resulting from ultra-low initial inocula were studied in the next two experiments.
## TABLE 7
WIDE-RANGE DOSE RESPONSE OF *P. BERGHEI*¹ INFECTED MICE TO SALINOMYCIN²

### Number of Mouse Deaths by Day

<table>
<thead>
<tr>
<th>Day Post Infection</th>
<th>Infected, Untreated</th>
<th>Infected, Diluent</th>
<th>Infected 2 mg/kg Salinomycin</th>
<th>Infected 4 mg/kg Salinomycin</th>
<th>Infected 6 mg/kg Salinomycin</th>
</tr>
</thead>
<tbody>
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</table>

Total No. Mice 8 10 10 10 10

MST 5.1 8.3 9.1 18.5 19.6

¹9 x 10⁵ infected erythrocytes/mouse, injected I.P. in 0.3 cc sodium citrate solution.
²2-6 mg/kg/day administered I.P. 5 times every 48 hours after infection.
³Deaths probably due to drug toxicity. Not used in MST determination.
⁴Drug administered this day.
Figure 8

Wide-range dose response of *P. berghei* infected mice to I.P. administered Salinomycin.

- Infected, untreated control
- Infected, diluent control
- Infected, 2 mg/kg/day salinomycin
- Infected, 4 mg/kg/day salinomycin
- Infected, 6 mg/kg/day salinomycin
- Infected, 8 mg/kg/day salinomycin
Five I.P. injections, once every twenty-four hours after infection with $5 \times 10^6$ infected erythrocytes

Five I.P. injections, once every forty-eight hours after infection with $9 \times 10^5$ infected erythrocytes
In the first experiment, two groups of ten mice, with an average weight of 33 grams, were injected I.P. with 0.3 cc sodium citrate solution containing $1 \times 10^3$ infected erythrocytes. One group received no further treatment. The other group received on the same day 4 mg/kg/day of salinomycin suspension administered I.P. in 0.3 cc of diluent, and treatment was continued at 24 hour intervals for a total of five injections. As seen in Table 8, MST actually decreased slightly in animals receiving salinomycin suspension when compared to infected, untreated animals. Thus, salinomycin had no positive effect on the MST of animals infected with very low inocula of *P. berghei*.

A second experiment was performed in which mice received an I.P. injection of $10^2$ infected erythrocytes, as in the procedure previously described. The initial inoculum was apparently too low to cause a successful infection of the bloodstream from the peritoneal cavity, since no disease occurred.

**Efficacy of Salinomycin on Established Plasmodium Parasitemias in Mice**

In this set of experiments, detectable parasitemias were allowed to develop before treatment with salinomycin was started. Since salinomycin appeared to have no effect on MST when administered simultaneously with a low dose
### TABLE 8

**EFFICACY OF I.P. ADMINISTERED SALINOMYCIN\(^1\) ON LOW INITIAL INOCULATION INFECTIONS OF **P. BERGHEI\(^2\)** IN MICE

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Mouse Deaths</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Infected, Untreated Group</td>
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<tr>
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</tr>
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</table>

**Total No. Mice**

- (Survivors) \(8(2)^5\) \(7(3)^5\)
- MST \(20.6\) \(16.7\)

---

1. 4 mg/kg/day administered I.P. immediately after infection and every 24 hours five times thereafter.

2. \(1 \times 10^3\) infected erythrocytes, injected I.P. in 0.3 cc sodium citrate solution.

3. Death probably due to drug toxicity. Not used in MST determination.

4. Drug administered this day.

5. (No.) = Animals surviving infection.
inoculum of Plasmodium, the other extreme was examined. In this set of experiments, detectable parasitemias were allowed to develop before treatment with salinomycin was started. The number of parasites present in each animal at the start of treatment thus was far greater than in the previous experiments. One experiment started with a relatively high established parasitemia, the other with a low parasitemia.

The first experiment utilized three groups of ten mice each, with an average weight of 32 grams. The mice were inoculated I.P. with $5 \times 10^6$ infected erythrocytes in 0.3 cc sodium citrate solution.

The infections were allowed to become established and developed until the mice in all three groups had an average parasitemia of 45%, six days after inoculation. One group of animals was given five I.P. injections of 4 mg/kg/day of salinomycin in ethanol-PBS-propylene glycol suspension. Another group was similarly injected but with diluent only, a third group received no further treatment after infection. As can be seen in Table 9, there was no significant increase in the MST of salinomycin treated animals, when compared to the MST of the untreated and diluent control animals. In fact, most salinomycin treated animals died earlier than the control mice.

In the second experiment, drug administration began three days after infection with Plasmodium, when the average
### TABLE 9
EFFICACY OF I.P. ADMINISTERED SALINOMYCIN\(^1\) ON ESTABLISHED PLASMODIUM PARASITEMIAS\(^2\) IN MICE

<table>
<thead>
<tr>
<th>Day Post-infection</th>
<th>Number of Mouse Deaths by Day</th>
<th>Infected, Untreated</th>
<th>Infected, Diluent Group</th>
<th>Infected, Salinomycin (4 mg/kg/day Group)</th>
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<tbody>
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<td>10</td>
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<tr>
<td>MST</td>
<td></td>
<td>9.3</td>
<td>8.1</td>
<td>9.1</td>
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</table>

\(^1\) 4 mg/kg/day, administered on day five and every 24 hours after.

\(^2\) Average parasitemia of 45% of day six. Initial inoculum 5 x 10\(^6\) infected erythrocytes.

\(^3\) Salinomycin administered this day.

\(^4\) deaths this day probably due to drug toxicity, not used in MST determination.
parasitemia was 7%. The initial inoculum was $5 \times 10^4$ infected erythrocytes/mouse in 0.3 cc sodium citrate solution. One group of ten mice received no further treatment after infection, the other group of ten received five I.P. injections of 4 mg/kg/day salinomycin suspension. As shown in Table 10, there was no significant increase in the MST of salinomycin treated animals, when compared to infected untreated controls.

Efficacy of Orally Administered Salinomycin on Infections of P. berghei in Mice

In this experiment, the effects of multiple oral administrations of salinomycin in Plasmodium infections in mice, as well as the possible effects on normal murine intestinal flora. Five groups of eight to ten mice with an average weight of 39 grams were maintained. Three of these groups were injected I.P. with a $5 \times 10^6$ infected erythrocytes in 0.2 cc sodium citrate diluent. One of these groups received 30 mg/kg/day salinomycin in 0.2 cc diluent administered orally with an 18-gauge gavaging needle five times, once every 24 hours after infection. Another group was similarly administered the diluent only. A third group of mice received salinomycin without being infected, in order to determine the effects of orally administered salinomycin on normal, healthy mice. A fourth group was infected, but received no further treatment. The final group of mice was kept uninfected and untreated, to serve
### TABLE 10

**EFFICACY OF SALINOMYCIN$^1$ ON ESTABLISHED PARASITEMIAS$^2$ IN MICE**

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Mouse Deaths by Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected, Untreated Group</td>
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<tr>
<td>23</td>
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</tbody>
</table>

| Total No. Mice | 9 | 10 |
| MST | 10.6 | 9.8 |

\textsuperscript{1}4 mg/kg/day.

\textsuperscript{2}Average Parasitemia of 7% of day two.

\textsuperscript{3}Mouse death probably not due to Plasmodium infection. Not used in MST determination.

\textsuperscript{4}Drug administered this day.
as a control for the comparison of intestinal flora with total treated animals.

As infected mice became moribund or died, post mortems were performed. Spleens and livers exhibited darkening and enlargement, indicating infection by Plasmodium.

As shown in Table 11, there was no significant deviation in MST of salinomycin treated animals compared to untreated mouse groups. Uninfected mice receiving orally administered salinomycin exhibited no qualitative differences in intestinal flora when compared to swabs taken from uninfected, untreated animals (data not shown). Moribund animals infected with malaria demonstrated a marked absence of living Trichomonas, pinworms and even bacteria, when compared to intestinal specimens from healthy, untreated mice.
**TABLE 11**

EFFECTS OF ORALLY ADMINISTERED SALINOMYCIN$^1$ OF INFECTIONS OF *P. BERGHEI*$^2$ IN MICE

<table>
<thead>
<tr>
<th>Day</th>
<th>Number of Mouse Deaths by Day</th>
<th>Infected, Untreated Mice</th>
<th>Infected, Diluent Control</th>
<th>Infected, Salinomycin</th>
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</tbody>
</table>

**Total No. Mice**

<table>
<thead>
<tr>
<th>Infected, Untreated Mice</th>
<th>Infected, Diluent Control</th>
<th>Infected, Salinomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

**MST**

<table>
<thead>
<tr>
<th>Infected, Untreated Mice</th>
<th>Infected, Diluent Control</th>
<th>Infected, Salinomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5</td>
<td>11.8</td>
<td>14.6</td>
</tr>
</tbody>
</table>

* Mouse probably deaths not due to Plasmodium. Not used in MST determination.

$^1$ 30 mg/kg/day in 0.2 cc diluent.

$^2$ 5 x 10$^6$ infected erythrocytes in 0.2 cc sodium citrate solution.
DISCUSSION

The screening of substances for use as possible antimalarial agents has traditionally been a massive, low-yield undertaking. During World War II, more than 15,000 chemical substances were screened by the United States Government in order to find new effective antimalarial agents (Peters and Howells, 1970). The low success rate is typified by a more recent U.S. Army screening. Primary testing of 300 compounds was begun in experiments utilizing murine malaria similar to those described previously. Only 54 compounds of 300 were found to have any antimalarial activity. In similar screens with multiple drug resistant strains, fifteen more potential drugs were eliminated. Of the remaining 39 compounds, only one survived similar malaria screens, and this compound had yet to be tested for human toxicity (Kennamon et al., 1975).

Research with murine Plasmodium species, particularly P. berghei increased dramatically in the 1960's, along with the involvement of the U.S. Armed Forces in Southeast Asia (Peters and Howells, 1978). Most of this research was of a pharmacological nature, the testing of the efficacy of various substances on the chloroquine resistant strains of Plasmodium encountered by U.S. troops in this part of the world. The use of murine specific malaria has served to greatly facilitate the screening of large numbers of potential antimalarial compounds. Before the advent of wide-
spread use of murine Plasmodia for research in antimalarial chemotherapy, most work was done using avian and simian models. These systems proved to be both expensive and difficult to manipulate (Peters, 1970).

The NYU-2 strain of *Plasmodium berghei* has been shown to be particularly lethal in the experiments described here. In no instance did any mice infected with *P. berghei* and subsequently treated with salinomycin survive for more than 28 days after infection.

The appearance of Plasmodium strains naturally resistant to many popular antimalarial drugs has prompted researchers to examine new compounds for use as possible chemotherapeutic agents. Salinomycin was considered in this study because it has been shown effective in controlling intestinal infections by another sporozoan, *Coccidia*. Also, there are no known resistant strains of organisms sensitive to salinomycin or its related ionophores.²

In a preliminary study, mice were inoculated with Plasmodium in order to follow the effects on certain hematologic values during the course of infection. This was done in order to provide some baseline information on the potency of *P. berghei* in this system. The data also illustrates the effects of this portion of the life cycle on

²Statement by Dr. R.G. Stroud, Professor of Animal Science at the University of New Hampshire in an address ("Coccidiasis and Ionaphoric Coccidiastats") at the A.H. Robins Seminar Series of May 22, 1980.
the vertebrate host. The decline in hematocrit levels (Figure 6, Table 2) was inversely proportional to the rise in parasitemia counts with time and probably due to the destruction of infected erythrocytes during schizogenesis. The decreased blood hemoglobin levels were due to the breakdown of hemoglobin to hemazoin and other products by the increasing number of intraerythrocytic parasites.

Salinomycin has proven to be a difficult drug to use in the experimental model described. The drug is highly toxic to mice, having LD50 values of 10.8 mg/kg/day if administered I.P. and 80 mg/kg/day when given orally (Kaken data sheet). In contrast, concentrations of potential antimalarial agents used in other investigations have excelled 60 mg/kg/day, given subcutaneously (Adviado et al., 1969). Quinine has been administered orally to mice as a reference drug in concentrations exceeding 100 mg/kg/day (Thompson, 1972). Clearly, it may not be possible to administer salinomycin in high enough concentrations to eliminate the parasite from the blood stream. The lethal dose range (Table 1) was performed to obtain some estimate of the number of mouse deaths that could be expected at the relatively high concentrations used. An attempt was made to strike a delicate balance between concentrations of salinomycin high enough to have a positive effect on the course of infection and low enough to prevent the deaths of an inordinant number of animals due to drug toxicity. The six mouse deaths on day five (Table 9) in the infected
salinomycin treated group may be due to a lowered tolerance of the drug with a concurrent Plasmodium infection. Heavy adult mice were used in all experiments because they were more resistant to adverse effects of the drug than younger mice (data not shown).

A second problem with the use of salinomycin in vivo was the difficulty in finding a non-toxic solvent in which to dissolve the drug. Salinomycin is highly soluble in such solvents as methanol, ethanol and benzene, but these substances are poorly tolerated by laboratory animals. Salinomycin was initially dissolved in absolute ethanol and a Propylene-glycol-PBS suspension. Care had to be taken to ensure the drug was adequately dispersed when administered to animals.

Since malaria is a systemic disease of the blood, experiments were performed to determine what effects, if any, salinomycin might have on certain hematologic parameters. In the first experiment, the effects of multiple I.P. administration of salinomycin on hemoglobin and hematocrit levels were examined. No significant changes were detected 24 hours or five days after the last injection (Table 3). The experiment was repeated, this time with blood collected for total erythrocyte counts, so that the Winthrope Erythrocytic Indexes could be calculated for each mouse, before and after treatment. These indexes are useful in the detection and defining of specific anemias. Again, salinomycin appeared to have no effect on erythrocyte
number or size of hemoglobin concentration.

Salinomycin appears to be most effective against *P. berghei* when administered I.P. in relatively high concentrations over timed intervals greater than 24 hours. The MST of salinomycin suspension treated animals in the first experiment was almost double that of the infected untreated controls (Table 5). No mice were injected with diluent. The experiment was designed to see if the salinomycin suspension could produce any positive results with the concentration of the drug used. Injections were given at varied intervals so as to minimize any mouse deaths due to accumulated drug toxicity.

This initial experiment was followed up by two multi-dose studies in an attempt to define the most effective multi-administration regimen. This time, diluent controls were used. In the first experiment, animals were inoculated with $5 \times 10^6$ infected erythrocytes while in the second experiment, animals received only $8 \times 10^5$ infected erythrocytes. Mice were injected every 24 hours for five days with salinomycin or diluent in the former experiment, and every 48 hours for ten days in the latter. Again, MST was significantly higher in animals injected every 48 hours with 4 mb/kg/day or more of salinomycin when compared to infected untreated controls (Tables 6-7). Results indicate that for similar doses of salinomycin, the length of the treatment period may be more important than the frequency of treatment.
The extraordinarily low MST in the infected, untreated control second multidose experiment is worthy of note. This MST is almost half that of the infected control of the first experiment, yet animals in this group were given a substantially lower inoculum of infected erythrocytes. When the MST of treated animals in the second experiment are compared with those of the control animals of the second, there is no significant lengthening of MST. This low MST in the first control group would be due to the presence of a larger number of infected erythrocytes in the inoculum than was actually calculated.

Treatment with salinomycin produced no significant increases in MST when given to animals with established parasitemias or those infected with low numbers of parasitized erythrocytes (Tables 8-10). In the latter experiments, treatment with salinomycin began immediately after infection, instead of delaying at least 24 hours. Such negative results contrast with the results seen earlier, where animals receiving higher inoculum had higher MST upon treatment with salinomycin.

The oral administration experiment yielded no positive results (Table II). This was to be expected because of the insoluble nature of salinomycin, even in suspension. It was improbable that any significant amounts of salinomycin was absorbed by the intestinal tract and entered the bloodstream. No gross changes in normal intestinal flora were noted. Subtle changes in the microbial populations could
have occurred with salinomycin treatment, but these would have gone unnoticed since no quantitative assays were performed.

As previously mentioned, salinomycin has a demonstrated ability to shut down both coupled and uncoupled respiration in mitochondria. Other monocarboxylic polyether antibiotics have exhibited similar physiological effects on other cellular organelles and on such are considered valuable tools for the study of ion-carrier mechanisms in vivo (Mitani et al., 1976).

In murine-specific Plasmodium, the existence of two electron transports systems within the parasites mitochondria is strongly suspected, but unconfirmed. Antimycin A and rotenone, two electron transport inhibitors, have both proven effective in the inhibition of chloroquine induced hemazoin pigment clumping, an energy dependent process which occurs in the absence of oxygen (Homewood et al., 1972). The presence of \textit{P. berghei} specific co-enzyme Q has also been demonstrated (Skelton et al., 1970). Intra-erythrocytic malaria parasites are separated by three cellular membranes from the external circulating blood fluid. The problem with salinomycin may be in getting the drug into the site of respiration in quantities sufficient to shut down the process. Coccidial sporozoites, for example, are not destroyed by cationic ionophores unless they penetrate the external membranes of the host cells (McDougald and Galloway, 1976). Schizonts leaving the ruptured erythro-
cytes are devoid of these protective erythrocytic membranes still appear to be quite capable of continuing the disease cycle in animals treated with salinomycin.

As with the electron transport systems, little is known about the other Plasmodium membrane transport systems, especially the ion transport systems of mitochondria.

Salinomycin has a limited effect on the course of malaria in mice. Best results were obtained when salinomycin was administered I.P. in doses exceeding 4 mg/kg/day. Extending the period of drug treatment seemed to prolong survival time in infected animals. I.P. administration started at the same time as inoculation of low numbers of parasites had no positive effects on the MST of infected mice, nor did salinomycin have any effect when given I.P. to mice which already had established, detectable parasitemias. Likewise, oral administration had no positive effect on the MST of infected mice. Two serious problems affecting research with salinomycin are the high degree of toxicity to mice, and the drug's low solubility in most solvents suitable for insertion into biological systems.
LITERATURE CITED
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APPENDICES
I. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>HGB</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HCRIT</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean corpuscular hemoglobin concentration</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean corpuscular hemoglobin</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean corpuscular volume</td>
</tr>
<tr>
<td>MST</td>
<td>Mean survival time</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed cell volume</td>
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</tbody>
</table>
II. RECEIPES

A. Preparation of 0.16M Phosphate Buffered Saline

25X Stock Solution

ADD:

170 ml distilled H$_2$O
5.48 grams Na$_2$HPO$_4$
1.58 grams NaH$_2$PO$_4$
q.s. to 200 ml with distilled H$_2$O

1X Working Solution

ADD:

40 ml of 25X stock solution
8.5 g NaCl
q.s. to 1000 ml with distilled H$_2$O

pH 7.21 ± .05 adjust with 1 N HCl or 1N NaOH

B. Preparation of 0.14M Sodium Citrate Solution

ADD:

3 grams sodium citrate
.83 grams dextrose
100 ml distilled H$_2$O

Millipore filter into sterile bottle