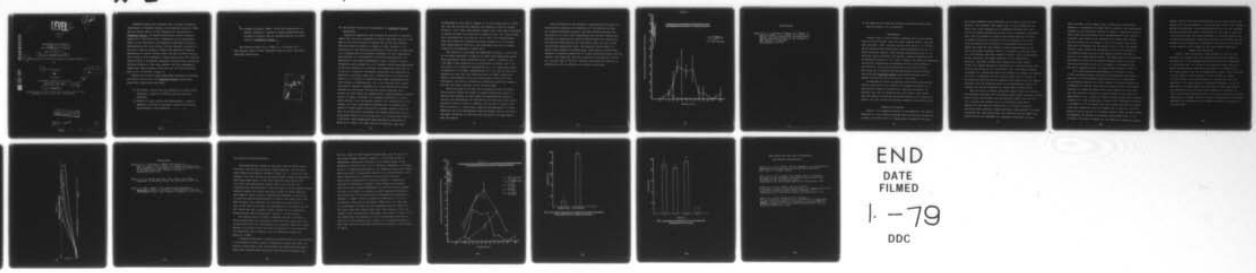
AD-A060 399 BIOMEDICAL RESEARCH INST ROCKVILLE MD F/G 6/13
PRESERVATION OF INFECTIVE STAGES OF RODENT MALARIA.(U)
JUL 78 C P STROME, J L LEEF N00014-76-C-0724
UNCLASSIFIED A-2 NL

1 OF 1
AD
AO 60 399



END
DATE FILMED
1-79
DDC

LEVEL III

12

SC

OFFICE OF NAVAL RESEARCH

(15) Contract #N0014-76-C-0724

Task No. 204-043

(9) Annual Report, Number (14) A-2

(6) Preservation of Infective Stages of Rodent Malaria.

by

(10) C.P.A. Strome

and

J.L. Leef

Biomedical Research Institute
12111 Parklawn Drive
Rockville, Md. 20852

(12) 22p.

(11) 7 July 1978

Freezing studies were initiated with a series of baseline experiments to determine whether or not dimethyl sulfoxide (DMSO) had any adverse effect on the infectivity of sporozoites of Plasmodium berghei. We reported preliminary results (progress report #A-1) indicating that it apparently had no deleterious effect on the sporozoites and subsequent experiments (Figure 1) substantiated this early finding. We also reported (progress report #A-1) that we planned to examine the effects of freezing sporozoites at various cooling rates, and that we would initiate this series by first examining a rapid cooling rate. This rate was chosen because a preliminary experiment indicated that sporozoites survived freezing at that rate. However, our work subsequently showed that rapid freezing (400°C/min) was far from the optimal rate of 20° to 60°C/min (Figure 1).

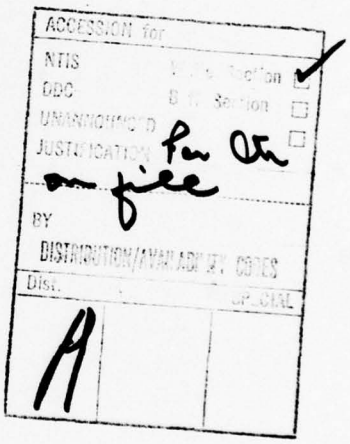
During the course of these experiments designed to determine the optimal cooling rate for Plasmodium berghei sporozoites, three basic observations were made:

(1) The optimal cooling rate for sporozoites in whole mouse serum was in excess of 30°C/min, but was less than 100°C/min.

(2) Because of these cooling rate requirements, a special apparatus would have to be built, capable of obtaining rates between 30 and 100°C/min. → over

(3) Dimethyl sulfoxide (DMSO), is not the preservative of choice; therefore, a survey of other preservatives must be made to evaluate them as cryopreservatives for sporozoites of Plasmodium berghei.

The following report will, therefore, be divided into 3 main chapters each of which addresses itself to one of the above mentioned observations.



(1) The Optimum Cooling Rate Requirements of Plasmodium berghei Sporozoites.

Sporozoite suspensions were prepared as described in progress report #A-1 and were resuspended in either 100% mouse serum or 7.5% DMSO in 45% serum contained in Medium 199 (M-199) for freezing. The mouse serum was chosen to compare to DMSO, because it is a constituent of the sporozoite purification process and its qualities as a cryopreservative are general knowledge. All cell suspensions were adjusted to a concentration of 400,000 sporozoites/ml, as determined by duplicate hemacytometer counts. Unfrozen, control suspensions were injected in 0.1 ml volumes (40,000 sporozoites/dose) at the time freezing was initiated; all preparations were kept at 0°C prior to injection. The percent infectivity was determined as described in progress report #A-1. Preparations to be frozen were frozen in 1 ml volumes in 15x100 mm test tubes. The samples were first brought to approximately -3°C in an insulated bath containing ethanol (absolute) equipped with a coil through which liquid nitrogen was circulated. The bath was further equipped with a stirrer and each sample was supplied with a copper-constantin thermocouple (22 gauge) connected to a Honeywell Electronik 112 multipoint recorder. When the samples reached -3°C, crystallization was induced (to avoid irreproducible supercooling) by seeding with a column of frozen medium contained in a pasteur pipette. After the heat of the fusion had dissipated, the samples were ready to continue being cooled in one of three ways: (1) cooling rates from 0.2 to 30°C/min. were accomplished using apparatus as described by Leibo et al (1970); (2) rates from 30 to 100°C/min. were done

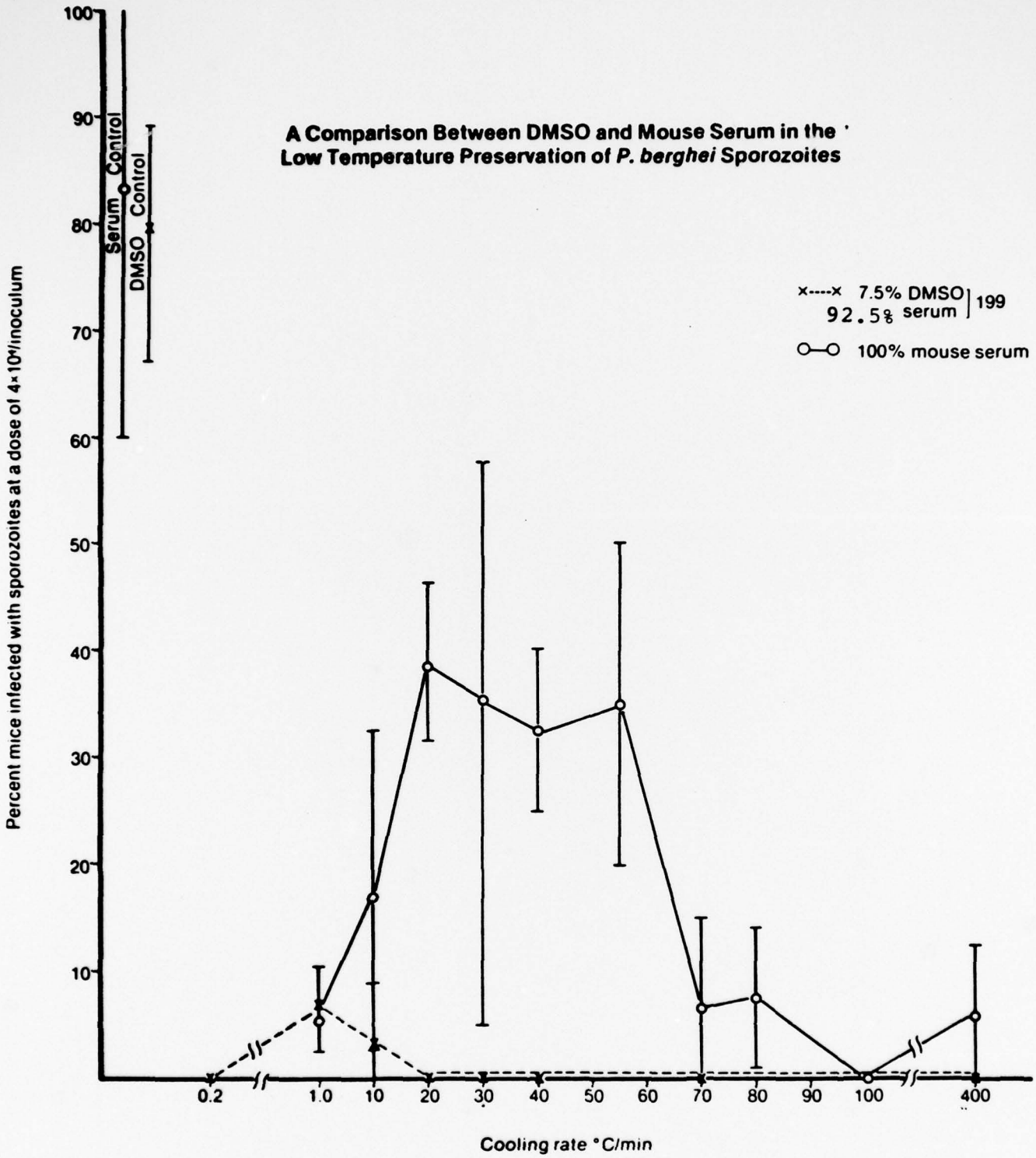
as described in this report, Chapter 2; (3) the cooling rate of 400°C/min. was done by directly immersing the sample in a dry-ice ethanol slurry at -79°C. When these samples reached -79°C, they were transferred to liquid nitrogen and stored for at least one hour. All cooling rates were calculated over the range of -10 to -65°C. All frozen preparations were thawed by immersion and rapid agitation in a 37°C water bath (approximately 300°C/min.) and inoculated into mice to assess infectivity as described in report #A-1.

The results of this study are summarized in figure 1. Each point on the graph represents the mean of at least three experiments where each experimental group contained 10 mice. Figure 1 indicates that 7.5% DMSO is most effective at a cooling rate of 1°C/min. but it is not nearly as an effective cryopreservative, as is whole mouse serum. In addition to 7.5% DMSO, concentrations of 5, 10, and 15% were also examined but they were less effective than 7.5% DMSO. Whole mouse serum is most effective over the cooling rates of 20 to 60°C/min. Furthermore, the peak in retention of infectivity following freezing was 40% with serum, whereas, it was 10% using DMSO.

The first phase of our work was to demonstrate that the sporozoite could be frozen and stored in liquid nitrogen and remain infective after thawing. We also wanted to demonstrate the specific cooling rate requirements for sporozoites and these goals were accomplished as shown in Figure 1. However, this survey of cooling rates had to be done at a single dose, otherwise the magnitude of a given experiment would be unmanageable. Once the optimal area on the curve was known titrations in that zone were initiated to further quantitate the results.

We are currently in the process of quantitating the study with mouse serum by assaying the infectivity of the material by means of a standard dilution titration. The dose schedule selected was 64,32,16,8,4,2,1 and 0.5×10^3 sporozoites per inoculum respectively. The data is analyzed by the Reed and Muench method for determining the effective dose causing 50% infectivity (ED_{50}) for both frozen and unfrozen sporozoites. Thus, the comparison can be made in a direct manner between two populations, which yields a quantitative estimate of absolute infectivity of frozen sporozoites versus unfrozen control. This study has just been initiated and preliminary results at a cooling rate of 20°C/min. indicate that preserved material is approximately 25% as infective as unfrozen sporozoites.

Figure 1


A Comparison Between DMSO and Mouse Serum in the Low Temperature Preservation of P. berghei Sporozoites
Serum Control
DMSO Control
×----× 7.5% DMSO 92.5% serum] 199
O—O 100% mouse serum
Percent mice infected with sporozoites at a dose of 4×10⁴/inoculum
100
90
80
70
60
50
40
30
20
10
0.2
1.0
10
20
30
40
50
60
70
80
90
100
400
Cooling rate °C/min

Bibliography

Leibo, S.P., J. Farrant, P. Mazur, M.G. Hanna, Jr., and L.H. Smith. 1970. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. Cryobiology 6:315-332.

(2) An Apparatus for Obtaining Variable Controlled Cooling Rates Over the Range of 30 to 100°C/min.

Introduction

Various types of cells require different cooling and warming rates to retain optimal viability after freezing (Mazur, 1972 and Leef and Mazur, 1978). Variable cooling rates from 0.1 to 30°C/min. can be obtained using conventional cooling apparatus such as the Linde biological freezer; fixed cooling rates ranging from 0.1 to 500°C/min. may be obtained with manually regulated apparatus such as described by Leibo et. al. (1970). However, we found that obtaining variable but reproducible intermediate cooling rates between 30 and 100°C/min. with existing apparatus to be quite difficult. This range was of interest because preliminary results obtained with freezing Plasmodium berghei sporozoites indicated that the optimal cooling rate was on the order of 50°C/min.

We designed and built an apparatus which yields reproducible cooling rates from 10 to 100°C/min. The apparatus consists of a cold alcohol reservoir connected to a delivery tube where the delivery flow rate is controlled by a ball valve. Cold alcohol from the reservoir displaces the warmer alcohol in the sample container; the rate of this displacement determines the cooling rate.

Material and Methods

Figure 1 is a schematic drawing of the apparatus. The alcohol reservoir is a five gallon stainless steel utility pail mounted on a stand. A one half inch i.d. copper pipe is secured to the pail

by a brass bulkhead fitting modified to fit nearly flush with the bottom of the reservoir. The copper pipe is also connected to an Apollo ball valve equipped with a graduated indicator dial. The tip of the handle controlling the ball valve is actually a moveable disc. When the handle is turned to the desired setting, the disc is pushed forward into a notch cut on the indicator dial. Thus, given settings must be identical or the moveable disc will not engage the notch for that setting. A pipe continues down to the lip of the sample container where it is joined to a one half inch i.d. polyethylene tube that extends to within 1 inch of the bottom of the container. The sample container (P.G.C. Scientifics, Rockville, Maryland) is made from standard wall pyrex tubing (8.5x3 inch inside dimensions) equipped with a overflow arm one inch from the top having an i.d. of one half inch. The sample container is secured to a laboratory stand with standard lab clamps; its capacity is 600 ml. A laboratory stirrer (T-line, model 101) is mounted on the frame and the stirring rod propeller positioned within the sample container near the discharge point of the polyethylene tubing. The contents are stirred vigorously to insure fast, uniform mixing.

When the valve is opened, precooled alcohol flows into the sample container and mixes with the warmer alcohol while the excess alcohol flows through the overflow arm. Polyethylene tubing (one half inch i.d.) connects the overflow arm to a circulatory pump which empties into the overflow holding tank, a 25 gallon insulated stainless steel container. Copper-constantin thermocouples (22 gauge) are placed into each sample tube, the reservoir and the sample container and are all connected to a Honeywell Electronik 112 multi-

point recorder. In one sample tube, an additional thermocouple leading to a Honeywell Visicorder 906C is joined to the thermocouple attached to the multipoint recorder to obtain a continuous cooling curve record during freezing. Both thermocouples are run through a stainless steel needle which penetrates the sample tube stopper and are situated at the center of the 1.0 ml sample. The cooling rates were calculated over the range of -10 to -65°C. The sample freezing tubes (100x15 mm) containing 1.0 ml of cell suspension are mounted around the lip of the sample container and held in place by wire under spring tension. The sample tubes are mounted one inch above the mouth of the sample container vessel to prevent alcohol from contacting the lip or stopper of the sample tubes. The entire apparatus rests in a plastic pan in case of overflow.

In a given experiment approximately 2.5 gallons of cold (-79°C) denatured ethanol is manually transferred to the alcohol reservoir from the alcohol holding tank where it is maintained at equilibrium with dry ice. This alcohol is filtered through two thicknesses of medical gauze mounted across the top of the alcohol reservoir to remove any debris that could occlude the valve opening. The reservoir contents are further cooled by the addition of approximately 2 to 3 liters of liquid nitrogen bringing the reservoir contents to a final temperature of about -90°C. The sample container is brought to -3°C by opening the valve briefly and when the samples reach that temperature they are seeded with a crystal of ice from medium frozen in the tip of a Pasteur pipette. After the phase change is complete, the samples are allowed to equilibrate back to -3°C (4-5 min). The valve is opened and the samples are cooled to approx-

imately -85°C at the flow rate determined by the valve setting. The samples are then transferred to liquid nitrogen (-196°C) for storage. During the experiment, the overflow alcohol returns to the insulated overflow holding tank which is maintained at -79°C and is ready for a new cooling cycle. The sample container is partially emptied with a conventional laboratory aspirator, filled to the overflow arm with alcohol at room temperature, adjusted to -3°C with the cold alcohol in the reservoir and is ready for the next freezing experiment.

Results and Discussion

Figure 2 shows the cooling rates obtained at various valve settings; the cooling rates are quite reproducible as judged by the magnitude of the standard deviations. The initial temperature of the alcohol and its volume in the reservoir should always be about the same before experiments are begun or the cooling rate at a given setting will not be reproducible. Figure 2 shows tracings of four typical cooling curves at a slow (11°C/min.), medium (32 and 43°C/min.) and rapid (80°C/min.) cooling rate for this apparatus. If a higher degree of linearity is desired, it can be achieved at all rates except the most rapid by first determining the temperature where deviation from linearity is observed in a trial run and then opening the valve to a faster flow rate when that temperature is reached. However, when the temperature of the samples approaches the temperature of the sample container, non-linearity is unavoidable.

Figure 1 Schematic Drawing of the Cooling Apparatus

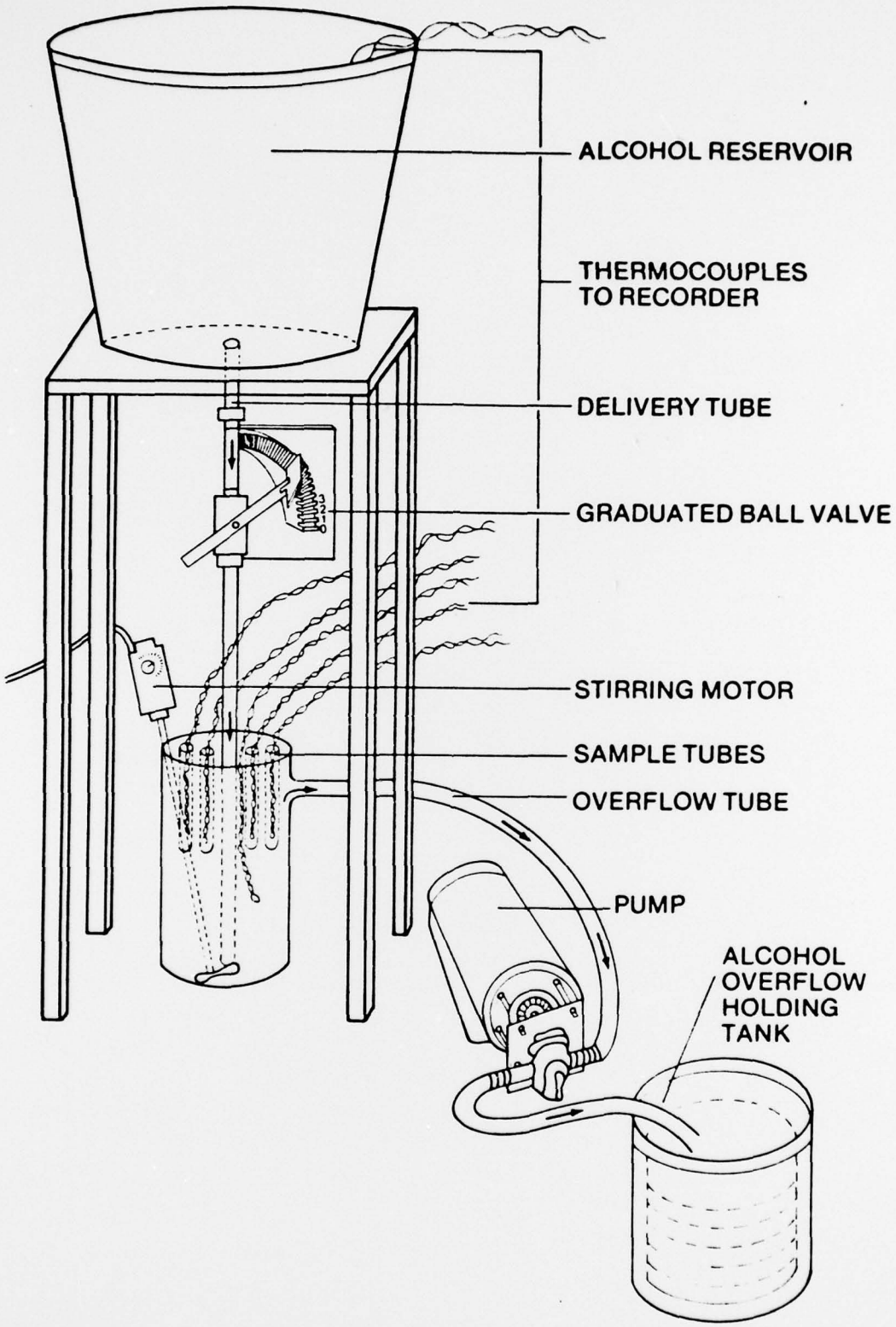


Figure 2

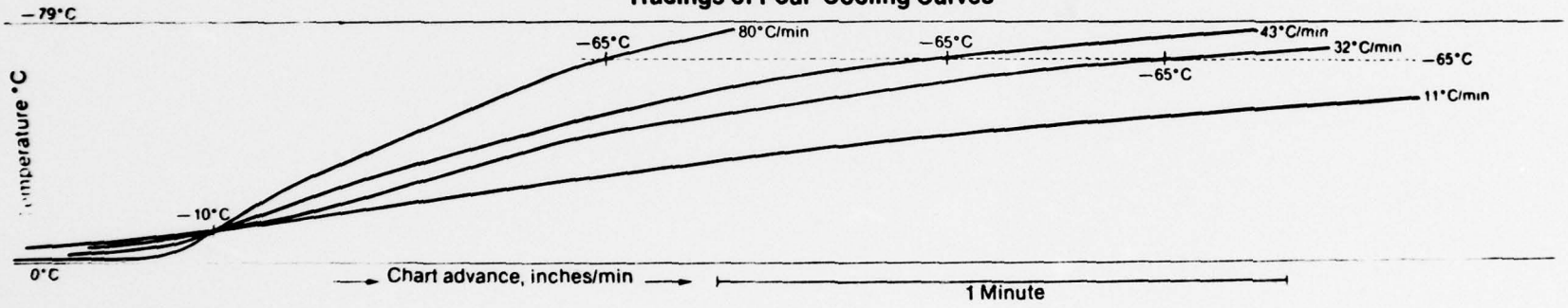
Cooling Rates Obtained at Various Valve Settings of the Cooling Apparatus

Valve Setting	Revervoir Temp. °C		Cooling Rate °C/min
	Before Run	After Run	
1.0	−89 ± 4.2	−87 ± 4.0	4.6 ± 1.1
1.5	−93 ± 2.8	−92 ± 0.7	11.3 ± 2.1
2.0	−91 ± 2.1	−89 ± 1.0	29.8 ± 3.5
3.0	−89 ± 2.6	−88 ± 3.5	38.4 ± 1.3
3.5	−91 ± 1.5	−89 ± 1.9	45.3 ± 2.2
4.0	−94 ± 0.7	−91 ± 2.1	47.0 ± 3.6
4.5	−90 ± 2.8	−89 ± 4.2	52.3 ± 3.1
4.75	−92 ± 1.0	−90 ± 3.0	78.0 ± 2.1
5.0	−92 ± 0.6	−91 ± 2.3	90.7 ± 2.3
5.5	−91 ± 1.9	−90 ± 2.4	94.8 ± 1.3
6.0	−93 ± 2.5	−91 ± 2.3	112.5 ± 12.8

The above cooling rates were calculated over the range of 10 to 65° C. The reservoir is routinely filled to the same volume before an experimental run.

Figure 3

Tracings of Four Cooling Curves



Bibliography

Leibo, S.P., J. Farrant, P. Mazur, M.G. Hanna, Jr., and L.H. Smith. 1970. Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose or glycerol. Cryobiology 6:315-332.

Mazur, P., S.P. Leibo, and E.H.Y. Chu. 1972. A two-factor hypothesis of freezing injury. Exp. Cell Res. 71:345-355.

Leef, J.L. and P. Mazur. 1978. Physiological Response of Neurospora Conidia to freezing in the dehyrated, hydrated, or germinated state. Appl. Environ. Microbiol. 35 (1): 72-83.

(3) Survey of the Preservatives.

The preservatives studied to date fall into two broad classifications, low and high molecular weight compounds. The low molecular weight were dimethyl sulfoxide (DMSO) (5,7.5, 10 and 15%) and glycerol (5,10 and 15%) while the high molecular weight preservatives were whole mouse serum (45 and 100%) polyvinyl pyrrolidone (PVP) (5,10,15%) and hydroxy ethyl starch (HES) (5,10 and 15%). All solutions with the exception of DMSO were made at 2x the concentrations needed in medium 199 (M-199) and equal volumes of those solutions were added to equal volumes of sporozoites suspended in 100% serum to yield the desired concentration in M-199 in 50% mouse serum. The DMSO solutions were prepared, V/V with DMSO and mouse serum, so that in the case of 10% DMSO, the concentration of the serum was 90%. All sporozoites were isolated, frozen, thawed and the infectivity determinations made as described in chapter 1 of this report.

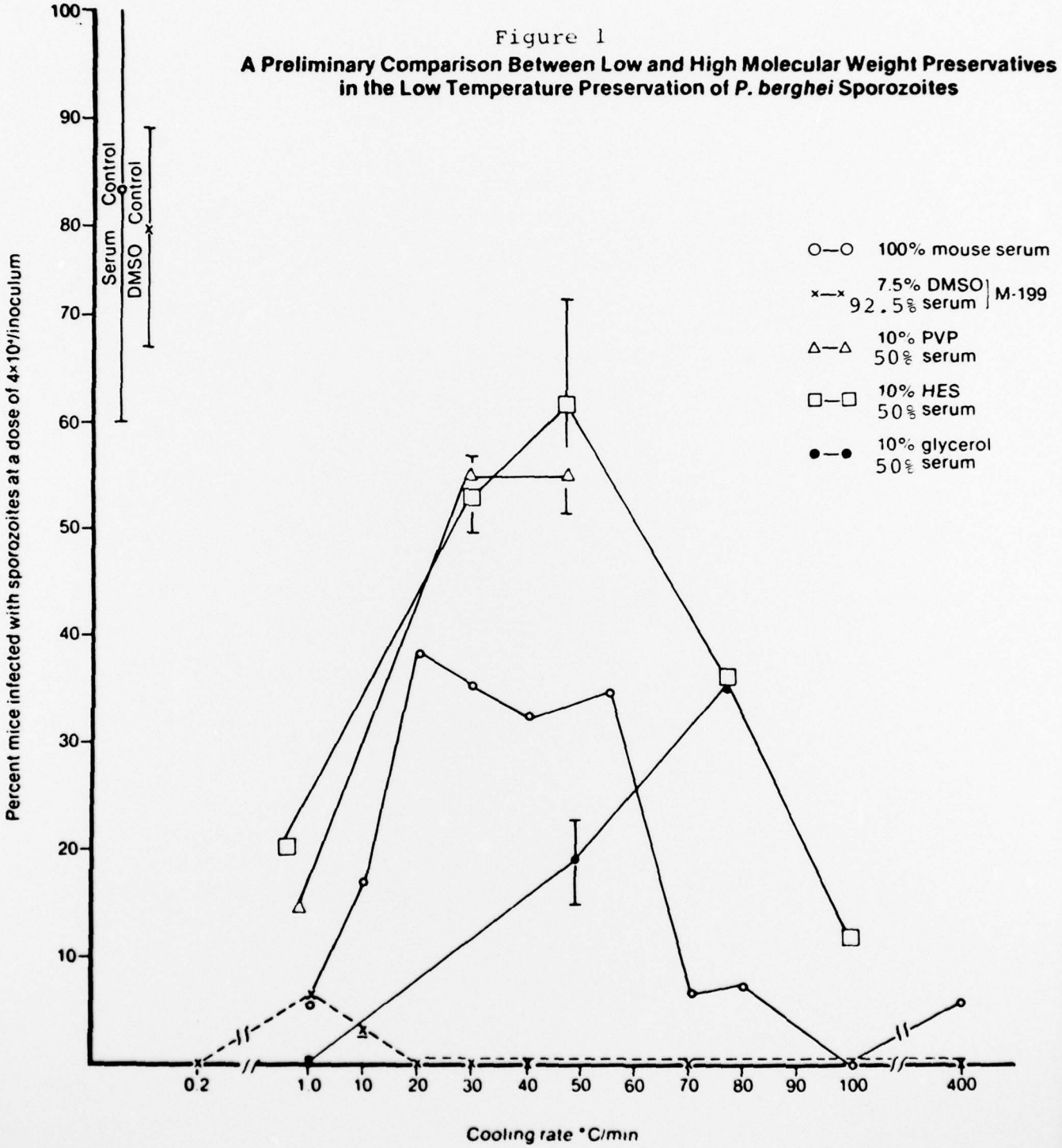
The results shown in figure 1 indicate that HES is clearly a better preservative for sporozoites (peak 65%) than serum (peak 40%) or DMSO (peak 10%); PVP also appears to be superior (peak 55%) though perhaps not as good as HES. The data for glycerol is too incomplete for comparison, but it appears to be as effective as serum and superior to DMSO.

It would be desirable to examine the effectiveness of preservatives in the absence of serum; however, sporozoites rapidly lose their infectivity when serum is not in the medium. We found that when sporozoites were isolated under serum free conditions and injected into

mice at a dose of $4x10^4$ sporozoites/inoculum, only 10% ($\pm$ 14) of the animals became infected (figure 2). In the same series of experiments, sporozoites isolated in our routine manner in the presence of serum were 96% ($\pm$ 6.3) infective. Therefore, at present, serum is a required constituent of all suspending media used. Experiments were done to demonstrate whether or not the sporozoites lose their infectivity as a function of time outside the host during their preparation for freezing (figure 3). Sporozoites isolated as described in chapter 1 were divided into aliquots that were either injected immediately or held for various lengths of time at 0°C prior to injection of $4x10^4$ sporozoites/inoculum. The infectivity of the sporozoites was determined as described in chapter 1. Figure 3 shows the effect of incubation at 0°C prior to freezing. There was no measurable effect up to 3.5 hours and it is extremely unlikely that the processing time for samples to be frozen would ever extend beyond that time. However, after that length of time deterioration does begin, since after 24 hours at 0°C, the infectivity has declined to 11.1%. We concluded time outside the host in the unfrozen state, should not be prolonged beyond 3.5 hours and that the sporozoites should not be handled in the absence of serum.

Figure 1

A Preliminary Comparison Between Low and High Molecular Weight Preservatives in the Low Temperature Preservation of *P. berghei* Sporozoites



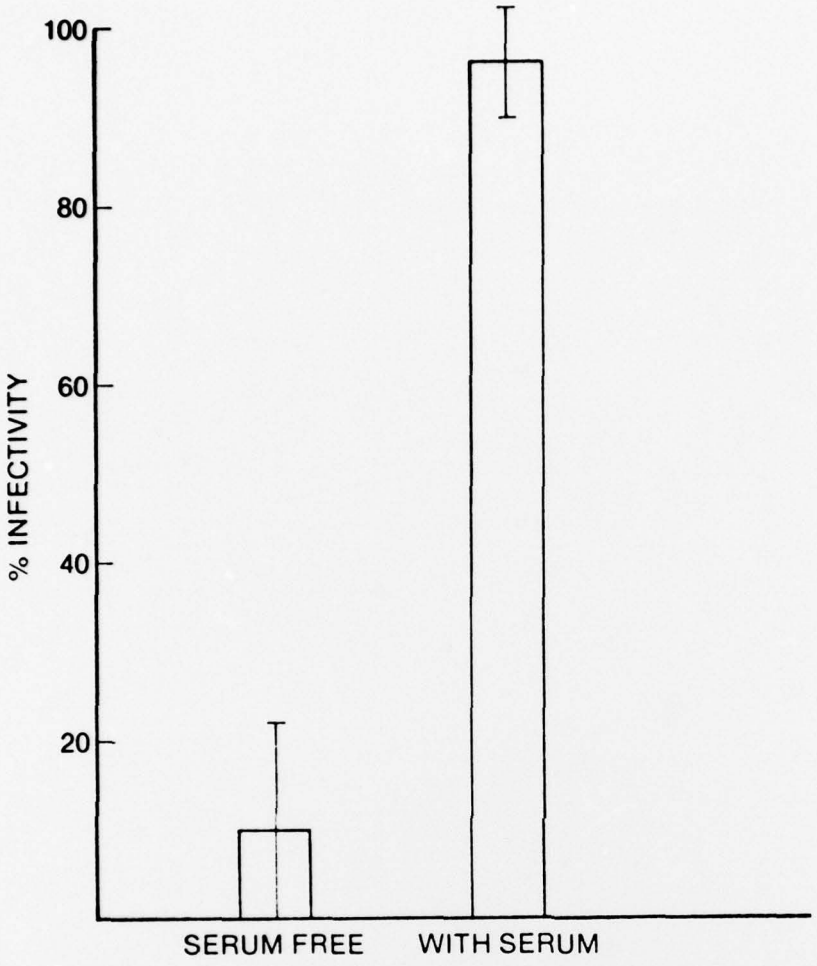


FIG. 2. The effect of the presence or absence of whole mouse serum on the infectivity of *P. berghei* sporozoites.

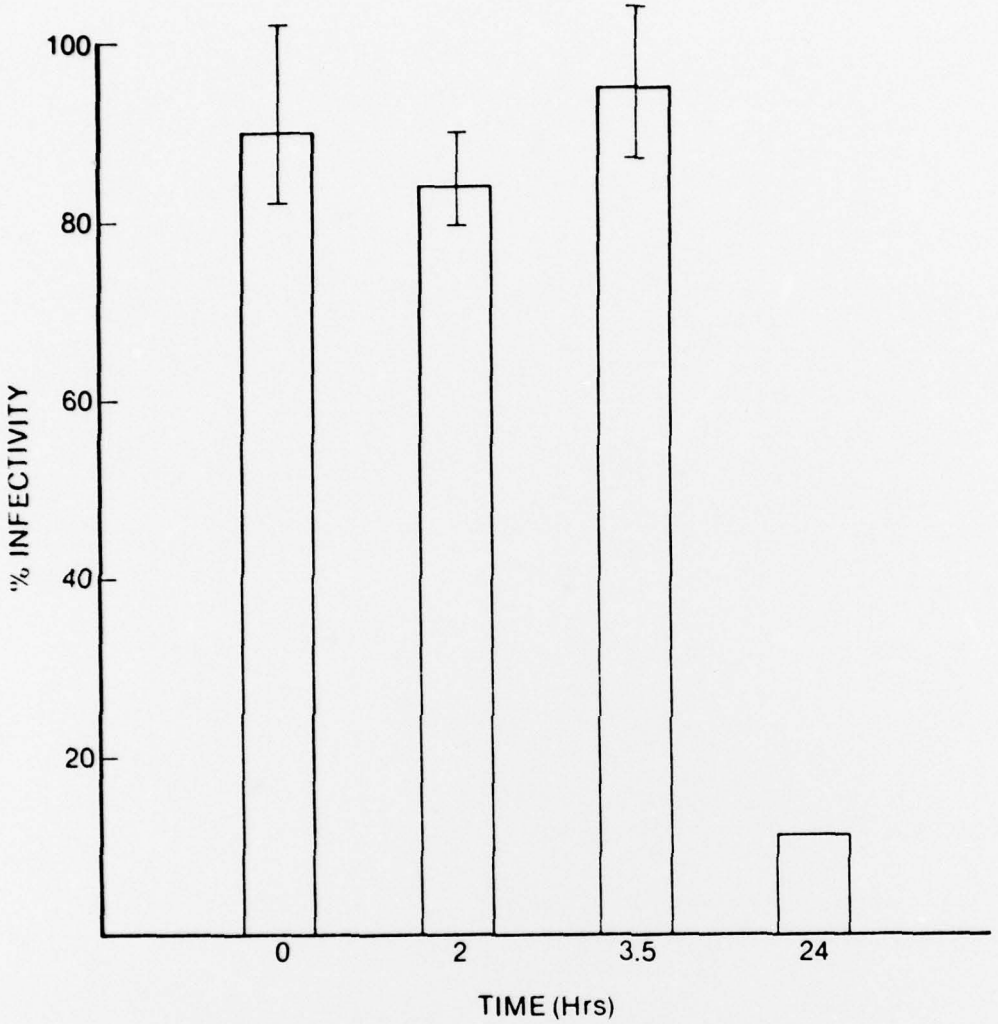


FIG. 3. The effect of incubation at 0° C on the infectivity of sporozoites of *P. berghei*.

Work during the last year has generated

the following publications:

Leef, J.L., C.P.A. Strome, and R.L. Beaudoin. The cryobiological preservation of sporozoites using DMSO and mouse serum. Fed. Proc. 37 (3):682. (1978).

Leef, J.L., C.P.A. Strome, C.A. Kroener and R.L. Beaudoin. An apparatus for obtaining controlled cooling rates between the range of 10° to 100°C per minute. Presented at the 15th Annual Meeting for Cryobiology. 1978.

Leef, J.L., C.P.A. Strome, and R.L. Beaudoin. A comparative study of the preservation of P. berghei sporozoites using high and low molecular weight compounds. Presented at the 15th Annual Meeting for Cryobiology. 1978.

Leef, J.L., C.P.A. Strome and R.L. Beaudoin. The low temperature preservation of sporozoites of Plasmodium berghei. To be presented at a workshop on the immunology of Malaria sponsered by the World Health Organization, Bethesda Maryland, October 2-5, 1978.