JOURNAL OF BACTERIOLOGY, Mar., 1965
Vol. 89, No. 3

Factors Affecting the Rate of Killing of *Escherichia coli* by Repeated Freezing and Thawing

ELLIOT L. PACKER, JOHN L. INGRAHAM, AND STANLEY SCHER

Department of Bacteriology, University of California, Davis, and Space Sciences Laboratories, University of California, Berkeley, California

Received for publication 24 October 1964

ABSTRACT

PACKER, ELLIOT L. (University of California, Davis), JOHN L. INGRAHAM, AND STANLEY SCHER. Factors affecting the rate of killing of *Escherichia coli* by repeated freezing and thawing. J. Bacteriol. **89**:718–724. 1965.—Repeated freezing and thawing of cultures of *Escherichia coli* grown in a minimal medium and frozen in the same medium without carbon source resulted in a linear decrease in the log of the number of surviving cells as a function of the number of freeze-thaw cycles. The slope of this curve, which can be determined accurately, is an index of susceptibility of a culture to death by freezing and thawing. The effect of the physiological state of the culture on the killing rate was determined. Contrary to previous reports, the phase of growth, the state of aerobiosis, and the density of the culture had no effect on the degree of susceptibility to death by freezing and thawing. However, presence of spent growth medium (a filtrate of a stationary culture) in the freezing medium protected cells against death by freezing and thawing. Protection by spent growth medium is effective at high dilutions ($1:10^5$), and is lost if spent growth medium is heated in the presence of alkali. It is suggested that the protection afforded by spent growth medium accounts for differences between our results and those reported in the literature.

The effect of the physiological state of a culture of *Escherichia coli* on its susceptibility to killing by repeated freezing and thawing was first studied by Harrison (1955, 1956) and Harrison and Cerroni (1956), who reported that aerobically grown cultures were more resistant than anaerobically grown cultures. Toyokawa and Hollander (1956) reported that exponentially growing cultures of *E. coli* were more resistant than cultures in the stationary phase. Also, it has been reported that *E. coli* is more resistant to death by repeated freezing and thawing as the concentration of cells frozen is increased (Harrison et al., 1952; Record and Taylor, 1953; McDougal, 1954; Pelczar, 1961; Major, 1953; Major, McDougal, and Harrison, 1955). In all the above studies, cultures were grown in complex media.

In this study, the effect of cell concentration, aeration, and phase of growth on the killing rate of *E. coli* subjected to repeated freezing and thawing was re-evaluated with the use of cultures grown in a glucose-salts medium.

MATERIALS AND METHODS

Organism. E. coli ML30, obtained from Jacques Monod, was used in all experiments.

Media. Glucose basal salts medium (BSG) contained: KH_2PO_4 (J. T. Baker Chemical Co., Phillipsburg, N.J.), 13.6 g; $(NH_4)_2SO_4$ (Allied Chemical Division, New York, N.Y.), 2.0 g; $CaCl_2$ (Baker), 0.01 g; $FeSO_4 \cdot 7H_2O$ (Baker), 0.0005 g; $MgSO_4 \cdot 7H_2O$ (Baker), 0.02 g; glucose (Baker), 1.0 g; and deionized distilled water, 1,000 ml; adjusted to pH 7.4 with saturated NaOH (Baker). Glucose and $MgSO_4 \cdot 7H_2O$ were each autoclaved and added separately. Freezing, thawing, and diluting was done in the same medium without glucose (BS).

Trypticase Soy Agar (BBL) was used as plating medium to estimate numbers of viable cells.

Culture technique. Cultures grown overnight at 32 C were inoculated into tubes containing BSG in a water bath at 37 C; the cultures were sparged with air or with a mixture of 95% N_2 and 5% CO_2. Samples were withdrawn in the late exponential phase and at 6 to 40 hr after the stationary phase had been reached. Spent growth medium (SGM) was prepared from each culture by centrifuging suspensions of stationary-phase cells and filtering the supernatant liquid through a Millipore filter (0.45 μ).

Freezing and thawing. Samples were either frozen and thawed directly or serially diluted in BS in 18-mm screw-capped tubes and frozen in a Dry Ice-acetone bath to −78 C and thawed to 11 C (Fig. 1). The cell suspensions were kept at −78 C for 20 min and at 11 C for 20 min. The freezing and thawing process was repeated up to 16 times; 1.0-ml samples were removed after each cycle for measuring optical density (420 mμ) and for plating.

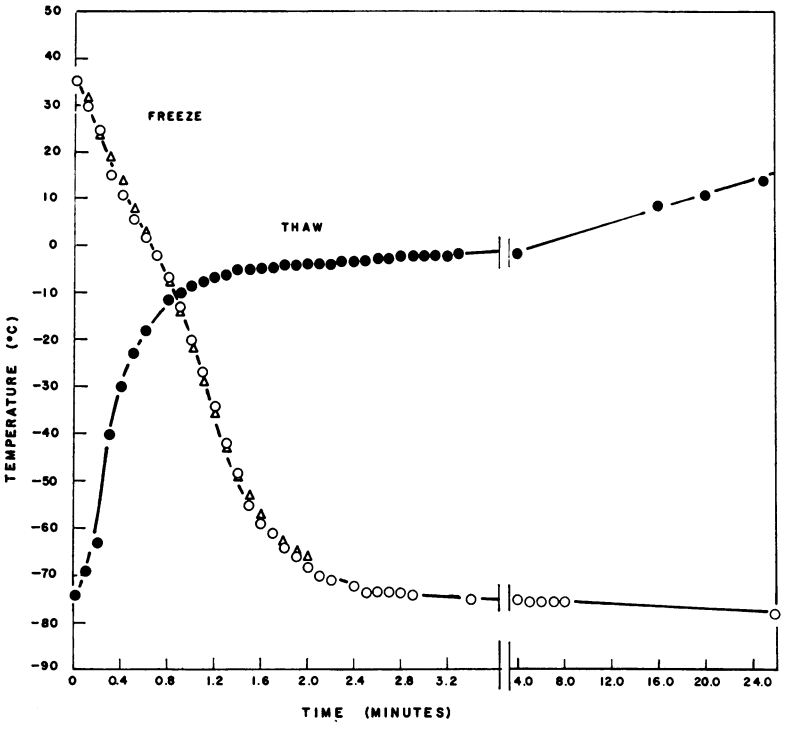

FIG. 1. *Rate of freezing and thawing of freezing medium. The freezing medium was frozen in a Dry Ice-acetone bath at −78 C (○), thawed in an 11 C water bath (●) and refrozen to −78 C (△).*

The plates were incubated at 37 C or at room temperature (22 to 24 C) and counted after 15 or 20 hr, respectively.

RESULTS

Effect of the physiological state of the culture: aeration and phase of growth. Cultures grown aerobically and anaerobically were sampled during the late exponential phase and 11 hr after the stationary phase had been reached (Fig. 2). Samples were diluted 1:100 in BS and were frozen and thawed 16 times. The number of surviving cells was determined after various numbers of freeze-thaw cycles.

The logarithm of the number of survivors is a linear function of the number of freeze-thaw cycles, and similar killing rates (K_{FT}, defined as the decimal decrease of viable count per freeze-thaw cycle) were found for the four cultures; i.e., the killing rate was found to be independent of the phase of growth and the state of aerobiosis during growth (Fig. 3).

In an experiment with a higher concentration of cells, the K_{FT} values of aerobically grown and anaerobically grown cells in the exponential phase of growth were also similar (lower curves, Fig. 4).

The logarithm of the decrease in optical density is also a linear function of the number of freeze-thaw cycles. The rate of decrease in optical density is less than one-third as great as the decrease in the number of viable cells. A typical experiment is shown in Fig. 4 (upper curves).

Effect of cell concentration. In preliminary experiments, killing rates appeared to increase as the concentration of cells frozen was decreased (Fig. 4). However, dilution of cultures in BS, prior to freezing, also dilutes the constituents of SGM.

The following experiments were done to determine whether cell concentration, SGM concentration, or both affected the killing rates. A culture containing 10^9 cells per milliliter was diluted to 10^8, 10^7, 10^6, 10^5, and 10^4 cells per milliliter with SGM and with BS as the diluents; i.e., in the first set, with SGM as a diluent, cells alone were diluted, and in the second, with BS as a diluent, both cells and SGM were diluted. These suspensions were repetitively frozen and thawed. The results show that killing rates were independent of cell concentration but dependent on the concentration of SGM (Fig. 5a and b).

For each experiment, we calculated the ratio: K_{FT} of cells frozen in 100% SGM to K_{FT} of cells

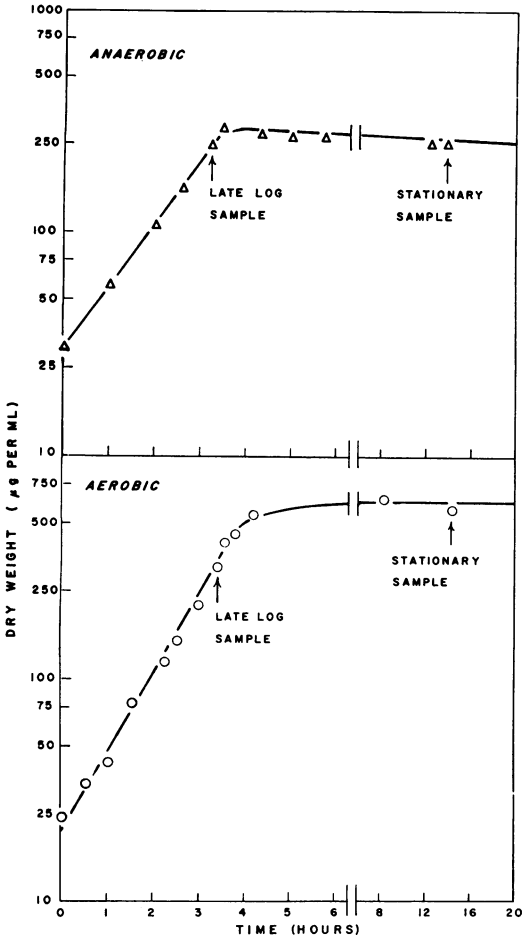

FIG. 2. *Growth of Escherichia coli in BSG at 37 C under aerobic (○) and anaerobic (△) conditions. Times of sampling for freezing and thawing are indicated.*

frozen in each SGM concentration. The K_{FT} of cells frozen in 100% SGM is the condition giving maximal protection. In this way, we could compensate for the different K_{FT} values of cells frozen in 100% SGM observed in different experiments (Table 1). A plot of this ratio multiplied by 100 (percentage of maximal protection) against the logarithm of concentration of SGM in the freezing medium yields a linear relationship (Fig. 6).

Stability of SGM. The stability of the protective factor(s) in SGM, was tested by heating to 121 C for 20 min three 1.0-ml samples of SGM, one with no additions, another containing 0.05 ml of 8 N HCl (to *p*H 1.0), and the third containing 0.05 ml of saturated NaOH (to *p*H 12.0); 0.3 ml of the three treated samples of SGM was added to 9.7 ml of BS containing about 10^5 cells per milliliter and 0.01% untreated SGM to make a 3% solution of treated SGM. K_{FT} values were determined for the above samples and compared with K_{FT} values obtained with cells frozen in media with and without 3% untreated SGM. As a control, a parallel series of experiments were done with BS. BS is highly buffered at *p*H 7.4; thus, addition of 0.3-ml acidic or basic samples to 9.7 ml of BS did not alter the *p*H of BS. Figure 7 summarizes the results of these experiments.

The protective factor(s) in SGM appears to be stable to heat in acidic or neutral solution but unstable to heat in basic solution. Protective factors appeared to be formed in BS if heated in acidic solution; in contrast, BS heated in alkali exerts no protection.

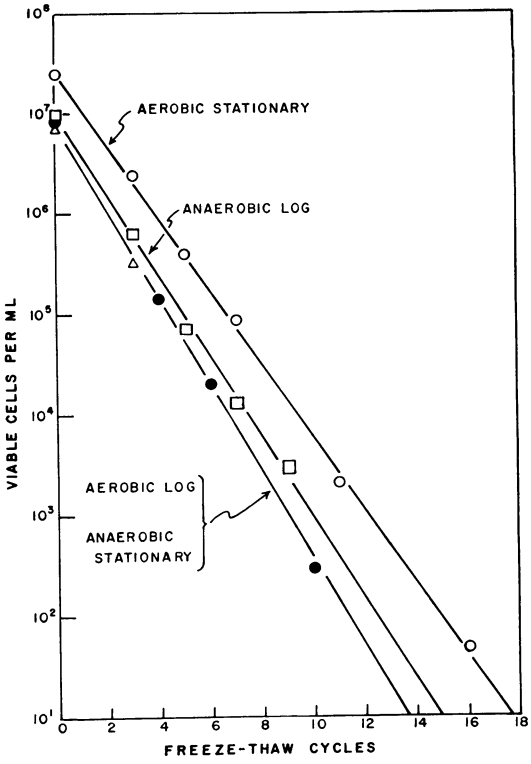

FIG. 3. *Effect of phase of growth and state of aeration during growth on the survival of Escherichia coli after repeated freezing and thawing. Symbols: ○, aerobically grown stationary cultures (K_{FT} = 0.384); □, anaerobically grown log cultures (K_{FT} = 0.400); △, aerobically grown log cultures (K_{FT} = 0.417); ●, anaerobically grown stationary cultures (K_{FT} = 0.417). Statistical analysis shows that the slopes of the above curves do not differ significantly.*

Heating SGM in alkali appears to eliminate the protective effect of SGM, because the K_{FT} of cells frozen in 3.0% base-treated SGM containing 0.01% untreated SGM is not significantly different from the K_{FT} of cells frozen in only 0.01% untreated SGM (Fig. 6).

DISCUSSION

The methods employed in this investigation differ from those used in previous investigations of repeated freezing and thawing of *E. coli*. In the past, *E. coli* was grown in nutrient broth and was usually frozen to −22 C in the same medium and thawed to 37 C. In our experiments, *E. coli* grown in a glucose-salts medium, was frozen in the same medium without glucose and was thawed at a temperature that precludes rapid growth (Ng, Ingraham, and Marr, 1962) or increase of cell numbers, as multinucleate (log)

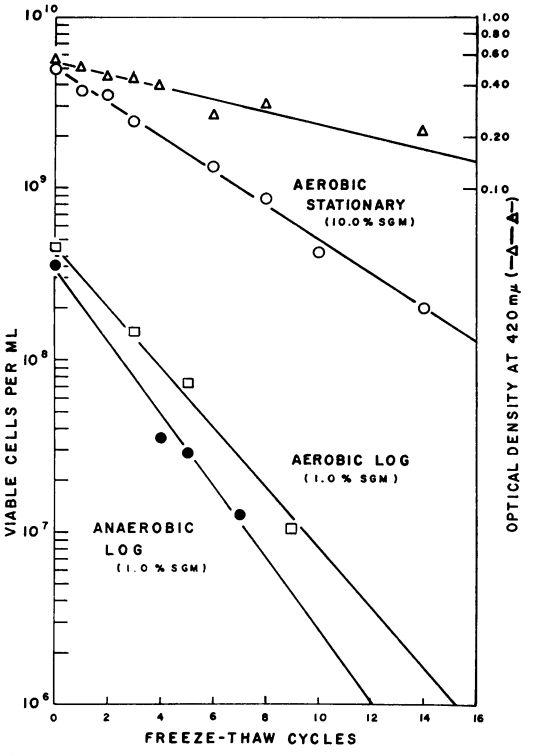

FIG. 4. *Effect of phase of growth and state of aeration during growth on survival and optical density of Escherichia coli after repeated freezing and thawing. Symbols: ○, viable cells per milliliter of aerobically grown stationary cells frozen in 10.0% SGM (K_{FT} = 0.100); □, viable cells per milliliter of aerobically grown log cells frozen in 1.0% SGM (K_{FT} = 0.182); ●, viable cells per milliliter of anaerobically grown log cells frozen in 1.0% SGM (K_{FT} = 0.207); △, optical density of aerobically grown stationary cells frozen in 10.0% SGM in the freezing medium. Statistical analysis shows that the slopes of the lower curves do not differ significantly.*

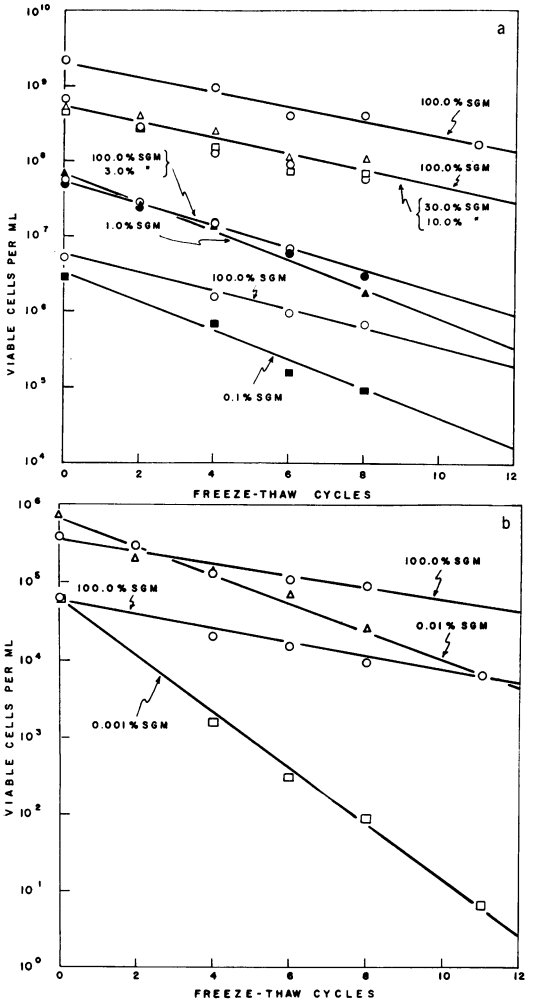

FIG. 5. *Viability of Escherichia coli after repeated freezing and thawing. The intercept of each curve at the ordinate indicates the initial concentration of cells frozen. Concentrations of SGM are indicated on each curve. Different concentrations of cells frozen in 100% SGM were obtained by diluting a stationary culture with a Millipore filtrate of its spent growth medium. Different concentrations of cells frozen in different concentrations of SGM were obtained by diluting a stationary culture with fresh BS. The K_{FT} values for these curves are listed in Table 1. (a) SGM concentrations: ○, 100.0%; □, 30.0%; △, 10.0%; ●, 3.0%; ▲, 1.0%; ■, 0.1%. (b) SGM concentrations: ○, 100.0%; △, 0.01%; □, 0.001%.*

TABLE 1. *K_{FT} values obtained by freezing and thawing varied concentrations of Escherichia coli in varied concentrations of SGM (Fig. 5)*

Per cent SGM in freezing medium	Initial concn of cells (cells/ml) frozen					
	10^9	10^8	10^7	10^6	10^5	10^4
100.0	0.108	0.108	0.148	0.123	0.091	0.090
30.0		0.108				
10.0		0.108				
3.0			0.173			
1.0			0.200			
0.1				0.194		
0.01					0.187	
0.001						0.358

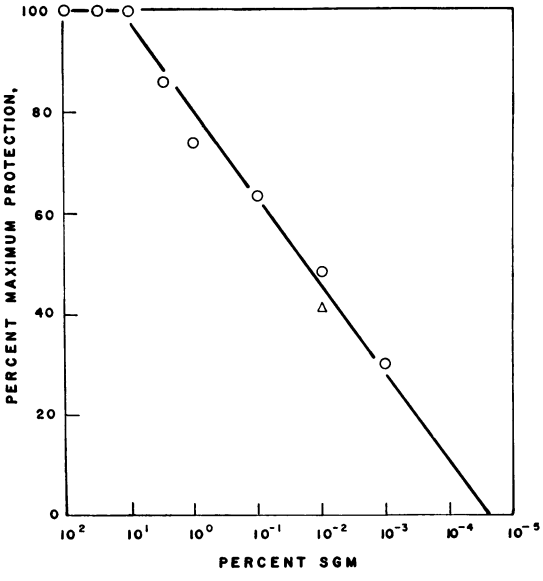

FIG. 6. *Effect of SGM concentration on the protection of Escherichia coli subjected to repeated freezing and thawing. Symbols: ○, untreated SGM; △, base-treated SGM containing 10^{-2}% untreated SGM.*

cells divide into uninucleate cells during starvation (Schaechter, Maaløe, and Kjeldgaard, 1958). A rich medium was used for plating to permit growth of cells rendered incapable of growth on minimal medium by freezing and thawing (Straka and Stokes, 1959).

Weiser and Hargiss (1946) reported that mortality of *E. coli* was greatest with very rapid cooling and slow thawing. For this reason, we kept the rate of freezing and thawing constant.

In our experiments, the time that the cells were held at −78 C and at 11 C was 20 min, but this period of time is not critical. When we stored cells at −78 C for 10 min to 12 hr and at 11 C for 20 min to 1 week, the linearity or slope of killing curves of comparable experiments was not altered. These results are consistent with those of Haines (1938), who showed that cells die during prolonged storage at temperatures near 0 C but remain viable if stored at −78 C.

In certain respects, our results differ from those reported in the literature. Ambrosini and Bretz (1963) reported that the killing of *E. coli* is diminished if the cells are frozen in lysates of *E. coli*. In our experiments, however, both the optical density and the number of survivors decrease linearly with the number of freeze-thaw cycles, thus clearly establishing that the level of the products of lysis released does not affect the killing rate. The decrease in optical density is less than the decrease in number of survivors, thus eliminating lysis as the sole cause of death.

Our results indicate that the previously reported differences of susceptibility to repeated freezing and thawing between log- and stationary-phase cultures, between cultures grown aerobically and anaerobically, and between dense and dilute cell suspensions are not significant under the conditions we employed. Dilution of a culture with fresh medium without carbon source does, indeed, increase the killing rate of a culture subjected to repeated freezing and thawing, but it was found that dilution of the culture medium rather than dilution of the cells was responsible for increasing the killing rate; i.e., protective factor(s) appeared to be present in the culture medium. Presumably, the protective factor(s) is produced during exponential growth because the killing rates of cells held in the stationary phase at 37 C for 6 to 40 hr in comparable experiments were similar. This factor(s) is heat-labile in alkali. However, fresh salts medium, without a carbon source, which has been heated with acid also protects, thus establishing that protection in this case is not dependent on the production of an organic compound. It appears to be a consequence of an alteration of the ionic composition of the medium. The protective effect of SGM, however, could result from organic compounds produced during growth which might alter the ionic composition of the medium by chelation (Neilands, 1957).

The profound effect of a few per cent of either acid-treated fresh salts medium or untreated culture medium on the killing rate of *E. coli* may explain why one obtains varied killing rates of cells frozen in apparently identical media.

The literature contains numerous reports of the effects of solute concentration on death by freez-

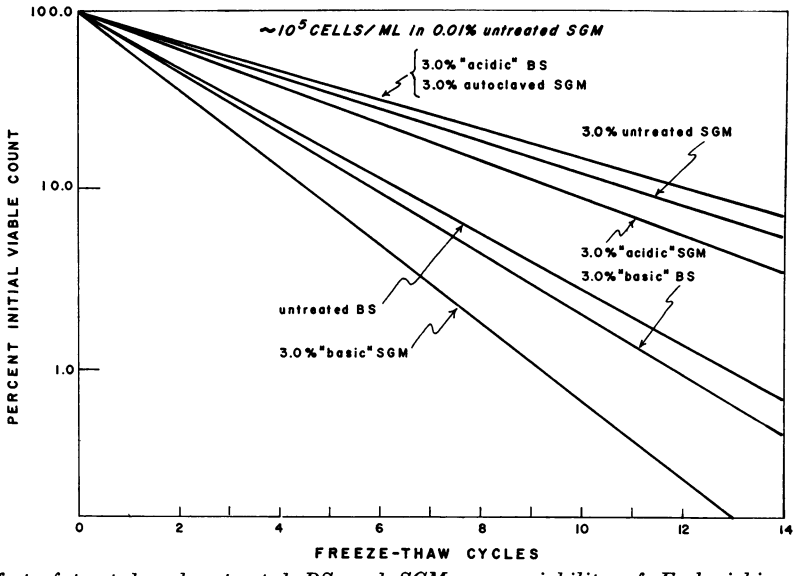

FIG. 7. *Effect of treated and untreated BS and SGM upon viability of Escherichia coli after repeated freezing and thawing. Treated and untreated SGM and BS were added to 0.01% untreated SGM freezing mixture containing ~10⁵ cells per milliliter. "Acidic" and "basic" media were autoclaved prior to addition into the freezing medium. The above curves were normalized to facilitate comparison.*

ing. During freezing, cells are exposed to high concentrations of solute. Depending on the solute and the organism, high solute concentration can be injurious by impairing normal metabolism or permeability, or they can be protective (Bogen, 1948; Borgstrom, 1961). Concentrated solutes are most injurious while in solution, but they also do damage in the frozen state (McFarlane, 1938). Electrolytes are known to influence lipoprotein dissociation (Lovelock, 1957).

With these facts in mind the nature of the protective effects reported here cannot be quantitatively studied until the precise ionic composition of the freezing medium is known.

ACKNOWLEDGMENTS

We are grateful to A. G. Marr and F. C. Neidhardt for reading the manuscript. This investigation was supported in part by National Aeronautics and Space Administration grant NSG-126-61 and by Public Health Service grant AI-05526 from the National Institute of Allergy and Infectious Diseases.

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