

PROTECTIVE ACTION OF GLYCEROL IN THE  
FREEZING OF LEPTOSPIRAE

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Scientists have attempted to keep leptospirae viable for long periods of time by freezing, by drying, by drying after freezing, and by storing sealed cultures at room temperature. Freezing methods have received the most attention, but viability after prolonged storage is uncertain due to the destruction of a high percentage of leptospirae (Weinman and McAllister, *Am. J. Hyg.*, **45**, 102-121, 1947).

Glycerol protects cells of many kinds from the damage incurred in freezing and thawing, and rapid freezing is unnecessary with glycerol protected cells (Polge, *Proc. Roy. Soc. (London)*, **B**, **147**, 498-508, 1957). Slower rates of freezing thus may be used, avoiding the thermal shock suffered by many cells when frozen too rapidly. This study was undertaken to determine whether glycerol would reduce the proportion of leptospirae destroyed by the freezing and thawing process; long-term storage was not attempted.

Leptospirae were grown at room temperature in Ringen's modification of Gardner's medium (Ringen and Gillespie, *J. Bacteriol.*, **67**, 252, 1954). To a sterile tube containing 0.3 ml of culture, 50 per cent glycerol (sterilized by autoclaving at 121 C for 15 min) in saline was added to the required concentration. Glycerol treated cultures (and controls) were allowed to equilibrate osmotically at room temperature, then frozen. Tubes of frozen cultures were thawed in tap water; motility examinations were carried out within 15 min. Subcultures were made in 3 ml of medium, and incubated at 33 C for 8 weeks or until dark-field examination revealed leptospiral growth. The proportion of motile organisms in thawed cultures was approximated by counting 100 organisms (200 if none of the first 100 was motile) and recording the number showing true motility.

Glycerol was found to be toxic for many strains of leptospirae at room temperature, a 15 per cent concentration causing loss of motility in 30 to 60 per cent of the organisms within 30 hr. Five per cent glycerol was less toxic, but not without some

inhibitory effect. When cultures were frozen rapidly (by immersion in a bath of Dry Ice and ethyl alcohol), 5 per cent glycerol had no significant protective effect, but there was a marked protective action on many strains when freezing was carried out at a slower rate (by placing the

TABLE 1  
*Protective effect of glycerol in the  
freezing of leptospirae*

| Serotype                            | Motility after 2 Days<br>at -25 C |                |                         |
|-------------------------------------|-----------------------------------|----------------|-------------------------|
|                                     | No<br>glyc-<br>erol               | 1%<br>Glycerol | % 2.5%<br>Glyc-<br>erol |
|                                     | %                                 | %              | %                       |
| <i>L. autumnalis</i> #1.....        | 1                                 | 1              | 31                      |
| <i>L. autumnalis</i> #2.....        | 0                                 | 0              | 12                      |
| <i>L. autumnalis</i> #3.....        | 0.5                               | 1              | 33                      |
| <i>L. australis</i> A.....          | 0                                 | 0              | 55                      |
| <i>L. ballum</i> .....              | 0                                 | 0              | 1                       |
| <i>L. bataviae</i> .....            | 0                                 | 0.5            | 6                       |
| <i>L. biflexa</i> .....             | 0                                 | 0              | 4                       |
| <i>L. canicola</i> #1.....          | 0                                 | 0              | 0.5                     |
| <i>L. canicola</i> #2.....          | 0                                 | 5              | 11                      |
| <i>L. grippotyphosa</i> #1.....     | 9                                 | 3              | 3                       |
| <i>L. grippotyphosa</i> #2.....     | 3                                 | 3              | 14                      |
| <i>L. hebdomadis</i> .....          | 0                                 | 14             | 36                      |
| <i>L. hyos</i> .....                | 1                                 | 0              | 0                       |
| <i>L. icterohaemorrhagiae</i> #1... | 0                                 | 7              | 12                      |
| <i>L. icterohaemorrhagiae</i> #2... | 0.5                               | 16             | 19                      |
| <i>L. icterohaemorrhagiae</i> #3... | 0                                 | 0              | 2                       |
| <i>L. javanica</i> .....            | 10                                | 4              | 16                      |
| <i>L. pomona</i> .....              | 1                                 | 32             | 34                      |
| <i>L. pyrogenes</i> .....           | 0.5                               | 16             | 2                       |
| <i>L. sejroe</i> .....              | 1                                 | 10             | 9                       |

tubes on a shelf in a -25 C refrigerator). Since 2.5 per cent glycerol gave approximately the same degree of protection as 5 per cent glycerol, the lower concentration was selected for further work. Table 1 makes clear the marked increase in survival provided by glycerol for most of the strains of leptospirae tested. The cultures were equi-

brated 24 hr at room temperature with glycerol (controls without), then frozen at  $-25^{\circ}\text{C}$  and stored at this temperature for 2 days before thawing. A further reduction in the glycerol concentration to 1 per cent was undesirable for most strains.

Motile organisms showed all degrees of activity, from feeble twitching to normal spinning movements. Survival was equally good at  $-25^{\circ}\text{C}$  and at  $-50^{\circ}\text{C}$ , but use of a Dry Ice chest for the latter temperature necessitated sealing the cultures in glass to exclude carbon dioxide, which was found to be toxic. Although positive subcultures were sometimes obtained from materials which showed no motility after thawing, only

once was there failure to obtain growth when 0.5 per cent motility or greater was observed upon thawing. Subcultures from thawed, glycerol protected materials usually attained dense growth rapidly at  $33^{\circ}\text{C}$ , an indication of numerous surviving organisms.

Since it has frequently been observed that storage of glycerol treated microorganisms at temperatures above  $-70^{\circ}\text{C}$  is detrimental to long-term survival (Polge and Soltys, *Trans. Roy. Soc. Trop. Med. Hyg.*, **51**, 519-526, 1957; Hollander and Nell, *Appl. Microbiol.*, **2**, 164-170, 1954), it is suggested that glycerol protected leptospirae should be stored at  $-70^{\circ}\text{C}$  or lower, after initial freezing at a relatively slow rate.

### ADSORPTION METHOD FOR DETERMINING THE PERCENTAGE OF DEAD CELLS IN SUSPENSIONS OF *SARCINA LUTEA*

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In previous articles (Borzani and Vairo, *J. Bacteriol.*, **76**, 251, 1958; Borzani and Vairo, *Stain Technol.*, **35**, 77, 1960), adsorption methods were proposed for the measurement of total cell concentration and for determining the percentage of dead yeast cells.

The purpose of this note is to show that the adsorption of methylene blue can be used as a means for the measurement of the percentage of dead cells in suspensions of *Sarcina lutea*.

*S. lutea* was obtained from the collection of the Instituto Zimotécnico (University of São Paulo),

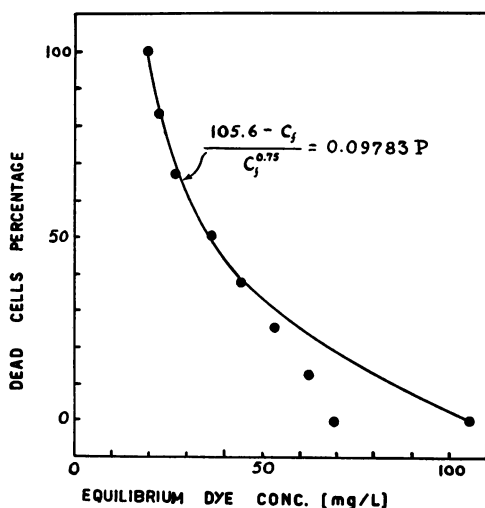


Figure 1. Relation between the percentage of dead cells of *Sarcina lutea* and the equilibrium dye concentration. Cell concentration = 1.19 g/liter; initial dye concentration = 105.6 mg/liter.

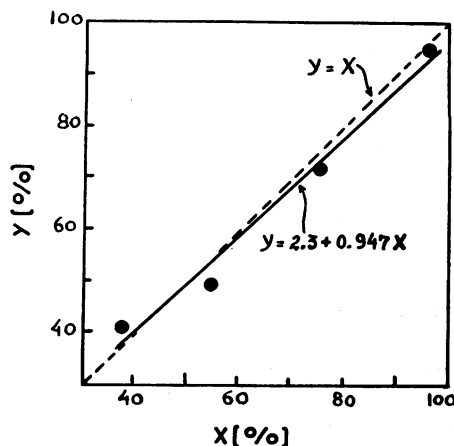


Figure 2. Relation between the percentage of dead cells of *Sarcina lutea* determined by the proposed method ( $Y$ ) and the real percentage of dead cells ( $X$ ). Experimental equation ( $Y = 2.3 + 0.947 X$ ) and theoretical equation ( $Y = X$ ) are represented.