Role of Suspending and Recovery Media in the Survival of Frozen *Shigella sonnei*[1]

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Received for publication July 24, 1961

ABSTRACT

NAKAMURA, MITSURU (Montana Stata University, Missoula), AND DIXIE ANN DAWSON. Role of suspending and recovery media in the survival of frozen *Shigella sonnei*. Appl. Microbiol. **10**:40–43. 1962.—*Shigella sonnei* was frozen at −20 C in saline, nutrient broth, and milk, and plated, after thawing, upon synthetic medium, nutrient agar, and blood heart infusion agar. There was a difference in the numbers of cells recovered when the frozen and thawed cells were grown on different media. The synthetic medium was unable to recover cells injured by freezing or did so only poorly compared to the complex media. The addition of meat extract, peptone, or Casamino acids to the synthetic medium improved its ability to recover injured cells as measured by bacterial colony counts. This is suggestive of metabolic injury caused by the freezing processes since the cells which survived freezing required an enriched medium for growth. In this paper the term metabolic injury is used to express a change in the nutritional requirements of the organisms which resulted in an increase in growth factor requirements. Freezing the cells in saline resulted in greater injury compared to cells frozen in nutrient broth or milk; this suggested that these suspending agents possessed some protective quality. The metabolic injury increased with an increase in the length of time the cells were held in the frozen state.

The survival of frozen and freeze-dried cells has been studied in a variety of microorganisms (Weiser and Osterud, 1945; Weiser and Hargiss, 1946; Hartsell, 1951; Fulton and Smith, 1953; Hollander and Nell, 1954; Eyles, Coleman, and Cavanaugh, 1956; Howard, 1956; Mazur, Rhian, and Mahlandt, 1957; Linscott and Boak, 1960; Polge and Soltys, 1960; Clement, 1961). Little is known about the effects of freezing on shigellae. Szturm-Rubinsten et al. (1960) found that *Shigella sonnei* isolated from ice used in a patient's food was responsible for dysentery. In the present study, we investigated the survival of frozen *S. sonnei*; the organism was frozen in several media and recovered upon different media with varying compositions. Straka and Stokes (1959) reported that some bacteria undergo metabolic injury at low temperatures. They found that cold injury produced an increase in nutritional requirements. Generally, the term metabolic injury has been used to indicate a loss of enzyme systems or a dependency upon additional growth factors for growth. Curran and Evans (1937) studied the importance of enrichments in the cultivation of bacterial spores exposed to lethal agents. Schlamm (1960) investigated the efficiency of two sampling media and of a living animal host in determining viability of injured *Pasteurella pestis*. It was pointed out that a critical evaluation is needed of the method for detecting viable organisms whenever experimental treatment produces apparent losses in bacterial viability. Stapleton and Engel (1960) also studied cultural conditions as determinants of sensitivity of bacteria to damaging agents.

MATERIALS AND METHODS

Three strains of *S. sonnei* were studied B-2569-2 was received from Commander T. M. Floyd, Naval Medical Center, Bethesda, Md.; strains F-6 and F-141 were received from Dr. Maxwell Finland, Boston City Hospital, Boston, Mass. The organisms were periodically checked morphologically, biochemically, and serologically (group D Shigella grouping sera)[3] for purity. Cultures grown at 37 C on nutrient agar[4] slants for 48 hr formed the stock strains and were stored at 4 C. These cultures were transferred to fresh media every 4 weeks. Cultures for experimental work were maintained in nutrient broth at 37 C and transferred daily. Twenty-four-hour cultures of *S. sonnei* were washed three times in buffered saline (pH 7.2) and resuspended in buffered saline to yield approximately 6×10^6 cells per ml. Bacterial counts of the initial cell suspension were obtained by decimally diluting the cell suspension in sterile distilled water (pH 7.2), pipetting 0.1 ml of the suspension onto nutrient agar plates, spreading

[1] This study was supported (in part) by a research grant (E-3734) from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

[2] Participant in the Undergraduate Research Participation Program supported by the National Science Foundation (G-12623).

[3] Lederle Laboratories Division, Pearl River, N. Y.

[4] Difco Laboratories, Inc., Detroit, Mich.

over the entire surface of the agar with a bent-glass "hockey stick," incubating aerobically for 24 hr at 37 C, and counting bacterial colonies on the Quebec darkfield colony counter.[5] Washed cells were suspended also in nutrient broth and in Bacto-skim milk (Difco) to produce about 6×10^6 cells per ml. Aliquots (10 ml) of the suspensions were dispensed into test tubes and frozen slowly at −20 C. Five tubes of each medium, i.e., saline, nutrient broth, and milk, containing the cells were thawed under running tap water. Tubes were thawed and examined periodically for a total of 50 days. The thawed tubes were diluted in distilled water and plate counts were made to determine the viable counts. The surviving populations were determined employing nutrient agar, blood heart infusion agar (Bacto-heart infusion agar (Difco) containing whole human or rabbit blood), and a chemically defined medium (Erlandson and Mackey, 1958). This medium contained 0.067 M dipotassium phosphate, 0.067 M monopotassium phosphate, 1% glucose, 0.05% magnesium sulfate, 0.03% thiamine hydrochloride, 0.85% sodium chloride, 0.25% aspartic acid, and 0.001% nicotinic acid. All media were prepared with distilled, deionized water (Crystalab Deeminizer[6]), adjusted to pH. 7.0 with concentrated sodium hydroxide or phosphoric acid, and autoclaved at 15 lb of pressure for 15 min. One and a half per cent agar was added to the synthetic medium for the preparation of solid synthetic medium for the plates. The highest grade chemicals commercially available were used throughout the study. Each experiment was repeated three or four times and the mean values were tabulated.

Results

The three strains of *S. sonnei* studied responded uniformly to the freezing treatments. The results of the effects of freezing the cells once in saline and recovering in synthetic medium, nutrient agar, and blood heart infusion agar are shown in Fig. 1. More viable cells were recovered from complex media than from the synthetic medium. As a matter of fact, bacterial colonies could not be detected on the synthetic medium after the cells had been kept frozen for more than 5 days. After an initial drop in the cell population, the numbers of cells that were recovered on blood heart infusion agar and nutrient agar remained constant.

[5] American Optical Company, Buffalo, N. Y.

[6] Crystal Research Laboratories, Inc., Hartford, Conn.

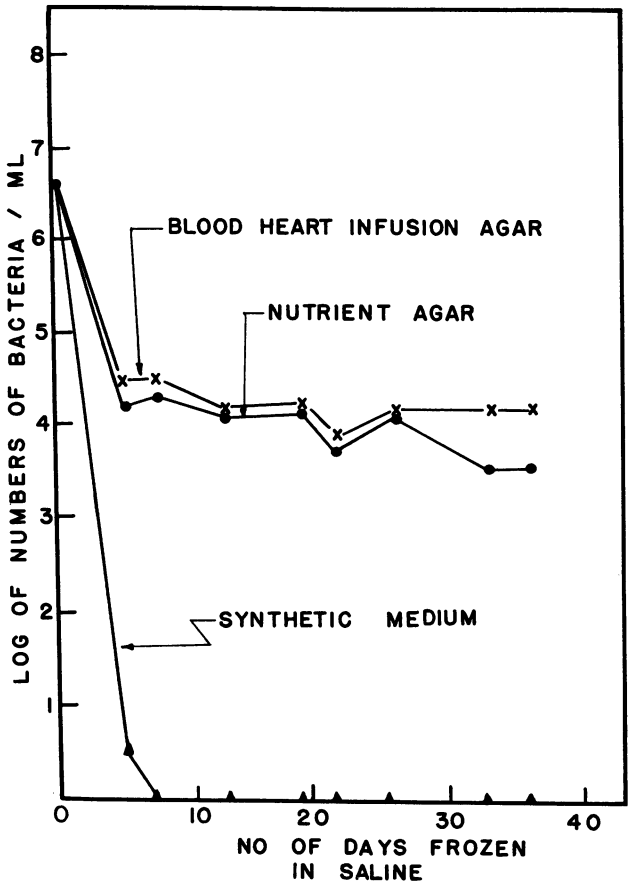

FIG. 1. *Recovery of Shigella sonnei frozen in saline employing blood heart infusion agar, nutrient agar, and synthetic medium as recovery media.*

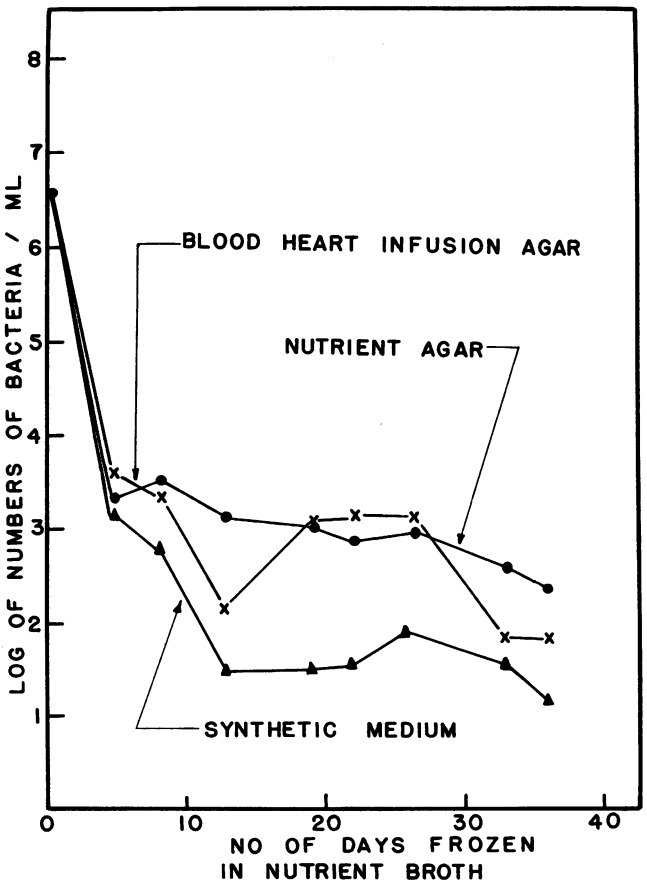

FIG. 2. *Recovery of Shigella sonnei frozen in nutrient broth employing blood heart infusion agar, nutrient agar, and synthetic medium as recovery media.*

Cells frozen in nutrient broth were recovered upon synthetic medium even after the cells had been kept frozen for 36 days, suggesting that there was less metabolic injury to these cells compared to cells frozen in saline (Fig. 2). More cells were recovered on blood heart infusion agar and nutrient agar than on synthetic medium when the cells were frozen in nutrient broth. The complex media recovered more cells than the synthetic medium when the cells were frozen in milk (Fig. 3). The largest numbers of cells could be recovered on synthetic medium when the cells were frozen in milk than when frozen in saline or nutrient broth. However, the number of organisms that could be recovered gradually declined with an increase in the length of time the cells were kept frozen. This did not appear to be the case when the cells were plated upon the complex media.

Synthetic media to which were added Bacto-meat extract (Difco), Bacto-peptone (Difco), and Bacto-Casamino acids (Difco) (added singly and in combinations) were able to recover as many cells as blood heart infusion agar or nutrient agar when the cells were frozen in nutrient broth or milk. However, this was not the case with cells frozen in saline suggesting that these additives were not protective for cells frozen in saline.

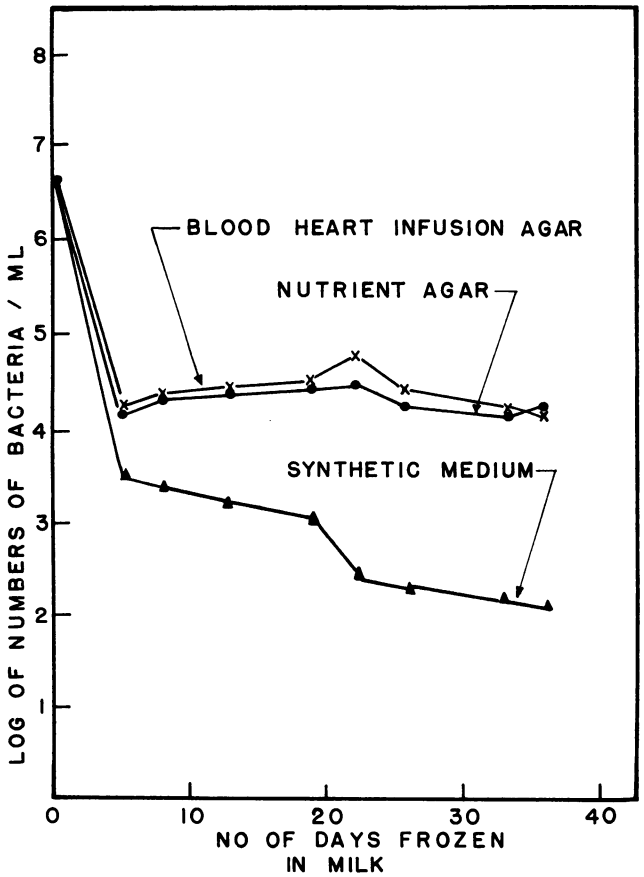

FIG. 3. *Recovery of Shigella sonnei frozen in milk employing blood heart infusion agar, nutrient agar, and synthetic medium as recovery media.*

DISCUSSION

The present investigation supports the view that freezing alters the nutritional requirements of microorganisms. Gunderson and Rose (1948), Hartsell (1951), and Straka and Stokes (1959) found that bacteria exposed to subzero temperatures became progressively less able to grow on minimal media. Curran and Evans (1937) reported that bacteria exposed to various physical and chemical agents were more exacting in their nutritive requirements than unexposed cells. We found that *S. sonnei* cells frozen and thawed once were differentially recovered depending upon the complexity of the medium. Synthetic medium was a poor recovery medium. However, when the synthetic medium was fortified with complex substances, the recovery was improved. This is highly suggestive evidence that the frozen and thawed cells were injured in such a way that their nutritional requirements were increased. Furthermore, it appears that meat extract, peptone, and Casamino acids contain factors which permitted recovery of metabolically injured cells. The extent of metabolic injury produced by freezing varied with the suspending medium in which the cells were frozen. Saline-frozen cells were subjected to more metabolic injury than cells frozen in nutrient broth or milk. Possibly this is related to the protective action of proteins or complex chemical substances upon cells which have undergone freezing and thawing. Straka and Stokes (1959) put frozen bacteria into three categories in terms of nutritional and viable states, namely, injured cells, dead cells, and unharmed cells. We agree with their belief that metabolic injury due to low temperatures might prevent the cells from synthesizing certain essential cellular components. Our data are in agreement with those of Straka and Stokes (1959) who found that there was an increase in the percentage of the killed cells as the time of storage in the frozen state was increased.

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