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Release of Biologically Active Peptides from *Escherichia coli* at Subzero Temperatures[1]

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ABSTRACT

MOSS, C. WAYNE (North Carolina State University, Raleigh), AND M. L. SPECK. Release of biologically active peptides from *Escherichia coli* at subzero temperatures. J. Bacteriol. **91**:1105–1111. 1966.—Freezing and storage of *Escherichia coli* at −20 C in phosphate buffer resulted in loss of cell viability and a pronounced leakage of cellular material which had maximal absorption at 260 mμ. Greater loss in cell viability occurred when cells were frozen in distilled water, but only small amounts of 260 mμ absorbing material were detected. Unfrozen cells stored at 2 and 22 C in each menstruum showed little loss in viability, but cells in phosphate buffer released significant amounts of material during storage. Leakage material from cells in phosphate buffer contained greater amounts of ribonucleic acid and amino acids than did material from cells in distilled water. Leakage material from frozen cells contained protein in the form of peptides of relatively small molecular weight; this was not observed for unfrozen cells. These compounds protected a dilute cell suspension from the lethal effects of freezing, and also possessed biological activity for the recovery of cells which had been "injured" by freezing. Direct cell counts indicated that the material released was not a result of cell lysis.

The decrease in viability which occurs when various cell suspensions are frozen has been attributed to intracellular ice formation (13), concentration of solutes (15, 18), or a combination of both effects (14). Injury resulting from concentration of solutes may be due to an increased electrolyte concentration which would harm the cell membrane or interfere with the functioning of permeability control mechanisms (7). An indication of the alteration of the permeability barriers of various cell suspensions is the leakage of intracellular organic compounds from frozen-and-thawed cells into the surrounding medium. Hansen and Nossal (6) and Sakagami (17) found that the washings from frozen yeast cells contained coenzymes, carbohydrate, amino acids, inorganic salts, and protein. Lovelock (11) noted that cell membranes of red blood cells were damaged and lost lipid and phospholipids into the medium when frozen in a dilute saline suspension. Higuchi and Uemura (9) noted that the release of nucleotides from yeast cells suspended in citrate buffer was greatly enhanced by freezing and thawing. Lindeberge and Lode (10) found ultraviolet-absorbing material appearing in the extracellular fluid of frozen-and-thawed suspensions of *Escherichia coli* in amounts proportional to the loss in cell viability. The leakage material included organic phosphorus, ribose, and protein, and had a maximal absorption at 260 mμ, suggesting that it contained nucleotides or nucleic acids. Organic compounds with maximal absorption at 260 mμ are also released from freeze-dried *E. coli* (22) and from rapidly chilled *Aerobacter aerogenes* (19, 21).

In a previous study, Moss and Speck (16) noted that peptides from Trypticase were active in the recovery of *E. coli* which had been "metabolically" injured by exposure to subzero temperatures. The purpose of the present study was to evaluate the relationship between injury and death by freezing and release of ultraviolet-absorbing material. The nature of the leakage material and its biological activity for recovery of "metabolically" injured cells were also investigated.

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MATERIALS AND METHODS

Organism. E. coli Y, a laboratory stock culture previously isolated from raw milk, was used in all experiments.

Media. Trypticase Soy Broth was used as growth medium for the production of cells for freezing. Trypticase Soy Agar (TSA) and a minimal salts-glucose-agar (MA) were used as plating media to estimate numbers of injured and dead cells. A detailed description of the MA and the method of determining injury and death have been reported previously (16). Briefly stated, the number of dead cells was the difference between the plate count on TSA before and after freezing. The number of injured cells was determined by the difference in plate count on TSA and MA after freezing.

Freezing menstrua. Sterile distilled water and 0.038 M potassium phosphate buffer were used as freezing menstrua. The phosphate buffer was at *p*H 7.6 with an ionic strength of 0.1.

Freezing and storage. Volumes of 5 ml of the cell suspensions were frozen and stored in screw-capped tubes (20 by 120 mm) at −20 C in a deep-freeze cabinet for time intervals extending up to 7 days. The cooling rate was approximately 1.4 C per min to 0 C, and 0.5 C per min from 0 to −20 C.

Absorbancy measurements of leakage products from cells. Measurements of 260 mμ absorbing material in supernatant fluids were made in a Beckman model DU spectrophotometer with a 5-mm light path.

Preparation of cultures. Cells were grown into the stationary phase (15 hr) in Trypticase Soy Broth at 35 C. The cells were centrifuged at 3 C, washed 3 times with 0.85% NaCl, and resuspended in the desired freezing menstruum. The cells were thoroughly mixed, and 5-ml portions were transferred to a series of screw-capped tubes (20 by 120 mm). The contents of one vial served as a control. Several of the tubes were placed at 22 C, and the remainder of the tubes were rapidly cooled to 2 C in an ice bath. One-half of the samples were maintained at 2 C in a refrigerator, and the remainder were stored at −20 C in a freezer. A 1-ml amount of the control sample was used for plating onto TSA and for determining the total cell count, which was made microscopically by use of a Petroff-Hausser counting chamber and dark-field illumination. Cells from the remaining 4 ml were removed by centrifugation at 30,000 × *g* for 20 min. The optical density of the supernatant fluid from these cells was read at 260 mμ. After various intervals of frozen storage, cells were thawed for 5 min at 24 C and were then held at 2 C for 3 hr. Then the frozen-and-thawed cells which had been held at 2 C and the unfrozen cells stored originally at 2 C and at 22 C were plated. After plating, cells from the remaining 4 ml were removed by centrifugation, and the optical density of the supernatant fluid from each of the three samples was determined.

Analytical methods. In addition to optical-density measurements, the following analyses were made on concentrated portions of the supernatant fluid. Protein was estimated by a modification of the biuret reaction (23) and deoxyribonucleic acid (DNA) by the diphenylamine test (4). Ribonucleic acid (RNA) was determined by the orcinol reaction, with yeast RNA as the standard (2). Total carbohydrate was estimated by the indole reaction according to Ashwell (1). Inorganic and total phosphorus was determined by the method of Fiske and SubbaRow (5). Amino acids were identified by two-dimensional paper (Whatman no. 3 MM) chromatography with butanol-2-butanone-water (2:2:1) as first solvent and butanol-acetic acid-water (5:1:4, upper phase) as second solvent; ninhydrin (0.25% in acetone) served as the spray reagent. The constituent amino acids of peptides were determined by the same procedure after hydrolysis (6 hr at 121 C in 6 N HCl) and subsequent removal of excess HCl. Nucleosides and nucleotides were identified by the descending paper chromatographic procedure described by Markham (12). Qualitative detection of peptides was made by the biuret test or by heating chromatograms for 30 to 50 min at 70 C after staining with ninhydrin.

Concentration and fractionation of leakage products. Cells were grown in 6 liters of broth, harvested, and frozen as described above. After 7 days of frozen storage, cells were thawed at 24 C, and then held at 2 C for 3 hr. Cells were removed by centrifugation (7,000 × *g*, 20 min); the supernatant fraction was passed through Millipore filters (0.45-μ pore size). The supernatant fluid was concentrated to 30 ml at 45 C on a flash evaporator and was then lyophilized. A portion of the lyophilized material was removed for quantitative chemical analysis, and the remainder was reconstituted with sterile water and passed through Sephadex G-25 as described previously (16). The eluate was combined into fractions according to the absorption spectra (265 mμ), and each fraction was concentrated to 5 ml under reduced pressure. The fractions were passed through a Millipore filter and tested for ability to promote the growth of injured cells by addition to MA. Supernatant fluid from unfrozen cells (2 and 22 C) was concentrated and fractionated by the same procedures.

Components in the fraction(s) from Sephadex which were active for the recovery of injured cells were further separated by high-voltage paper electrophoresis, and biologically active zones were detected by bioautography (16).

RESULTS

Release of 260 mμ absorbing material. Figure 1 shows the absorption at 260 mμ of the supernatant fluid from *E. coli* suspended in 0.038 M phosphate buffer or in distilled water and stored at 2, 22, and −20 C. Compounds absorbing at 260 mμ were at the highest concentration in the supernatant fluid from cells frozen in phosphate buffer. The rate of leakage was greatest after freezing and during early storage (4 hr); after 4 days at −20 C, there was no further increase. At 2 C, small amounts of material were released from the cells; at 22 C, a gradual increase occurred during the 7 days, but with no abrupt rate changes. When cells were suspended in distilled

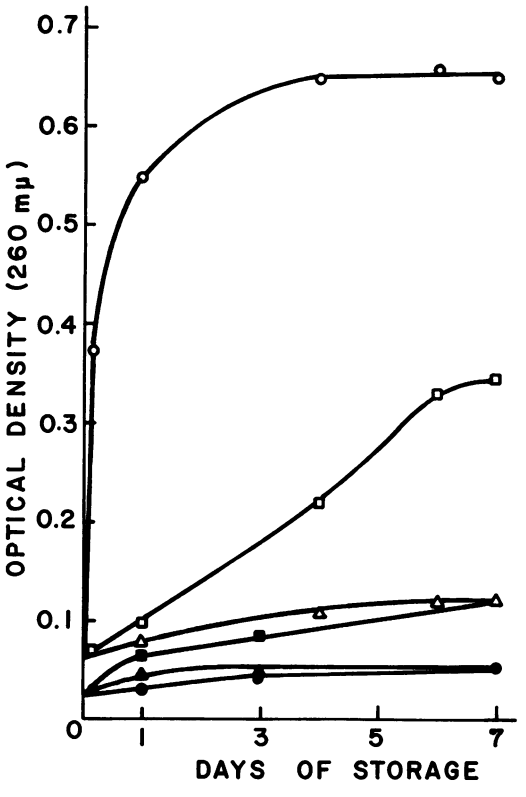

FIG. 1. *Release of 260 mμ absorbing material from Escherichia coli during storage in 0.038 M phosphate at 2 C (△), 22 C (□) and −20 C (○), and in distilled water at 2 C (▲), 22 C (■), and −20 C (●).*

water only, small amounts of material appeared to be released at any storage temperature.

Cell viability and release of 260 mμ absorbing material. A comparison of cell viability and release of 260 mμ absorbing material for cells in phosphate buffer is presented in Table 1. Initially, viability of the frozen cells decreased rapidly, and the decrease continued during storage. Concentrations of 260 mμ absorbing material released from this suspension of cells was initially high and increased during storage, suggesting a relationship between cell death and release of material. Cells stored at 22 C also released significant amounts of material during later periods of storage; however, this increase was not accompanied by cell death. Storage of cells at 2 C had no significant effect on viability. Direct counts of the total number of cells at each temperature did not change with storage, indicating that the material released was not a result of cell lysis.

The relationship between cell death and release of 260 mμ absorbing material, which was observed for cells frozen in buffer, was not apparent with cells frozen in distilled water (Table 2). After 4 hr at −20 C, 37% of the cells in water lost viability, compared with 20% for cells in buffer, but the optical density (260 mμ) of the supernatant fluid was 0.03 and 0.37, respectively. Moreover, the marked decrease in the number of viable cells in water at −20 C continued through 7 days without significant increases in the optical density of the supernatant fluid. At 2 and 22 C, only 10% of the cells lost viability after 3 days; after 7 days, the increased loss was not accompanied by a significant increase in 260 mμ absorbing material. The total number of cells at each temperature did not change during storage.

Examination of leakage products. The quantity of material released from frozen-and-thawed cells after storage 7 days in phosphate buffer ranged from 35 to 50 μg/ml, which represented 12 to 17% of cell dry weight. An analysis of the supernatant fluid revealed the following: carbohydrate, 8 to 11 μg/ml; protein, 12 to 16 μg/ml; RNA, 5 to 7 μg/ml; and organic phosphorus, 6 to 8 μg/ml. Amino acids were also present, and these accounted for most of the leakage material from cells at 2 C. The supernatant fluid from cells stored at 22 C contained both amino acids and RNA. Protein (14 to 18 μg/ml) and amino acids accounted for most of the material in the supernatant fluid from cells frozen in distilled water, whereas only amino acids were present in significant amounts in the unfrozen suspensions (2 and 22 C). No DNA was detected in any of the supernatant fluids.

Fractionation of leakage products through Sephadex. Filtration of the concentrated supernatant fluids from frozen-and-thawed cells through Sephadex gave a general separation into two zones absorbing at 265 mμ (Fig. 2) which were designated zones A (first from column) and C. Material between these zones (370 to 490 ml) was designated zone B. The percentage of material recovered in each zone from leakage material from cells frozen in phosphate buffer was 40, 30, and 30% for zones A, B, and C, respectively. The elution pattern of the material released from cells frozen in distilled water was similar to that from cells in phosphate buffer, with the exception of the markedly decreased absorbancy of zone C and the slight increased absorbancy of zone A. The elution pattern of material from unfrozen cells (2 and 22 C) in distilled water showed only one slight 265 mμ absorbing zone, which corresponded to zone C. Similarly, material from the unfrozen cells in buffer showed only one significant zone which also corresponded to zone C, but the intensity of absorption was much greater than that observed in distilled water. Subsequent qualitative

TABLE 1. *Effects of storage at −20, 22, and 2 C on cell viability and release of 260 mμ absorbing material from Escherichia coli Y suspended in 0.038 M phosphate buffer*

Treatment	Time of storage	Colony count (× 10^7/ml)	Survivors	Cell count (× 10^7/ml)	Optical density at 260 mμ of supernatant fluid
	days		%		
Frozen (−20 C)	Control	170		90	0.06
	0.17	130	80	180	0.37
	1	70	41		0.55
	4	40	24	190	0.65
	6	40	24		0.66
	7	30	18	200	0.65
Stored (22 C)	Control	170			0.06
	0.17	170	100	190	0.07
	1	160	94		0.10
	4	160	94		0.22
	6	160	94	190	0.33
	7	160	94	200	0.39
Stored (2 C)	Control	170			0.06
	0.17	170	98	200	0.06
	1	160	94		0.08
	4	160	94		0.11
	6	170	98	180	0.12
	7	150	90	190	0.12

TABLE 2. *Effects of storage at −20, 22, and 2 C on cell viability and release of 260 mμ absorbing material from Escherichia coli Y suspended in distilled water*

Treatment	Time of storage	Colony count (× 10^7/ml)	Survivors	Cell count (× 10^7 /ml)	Optical density at 260 mμ of supernatant fluid
	days		%		
Frozen (−20 C)	Control	190		210	0.02
	0.17	120	63	210	0.03
	1	58	30		0.03
	3	45	24	210	0.04
	7	20	10	210	0.05
Stored (22 C)	Control	190			0.02
	0.17	180	95	210	0.04
	1	170	90	200	0.07
	3	170	90	210	0.08
	7	130	70	210	0.10
Stored (2 C)	Control	190		210	0.02
	0.17	180	95	210	0.03
	1	170	90		0.04
	3	170	90	200	0.05
	7	160	84	210	0.05

tests of the eluted material present in each zone showed that zones A and B contained large amounts of protein and only traces of carbohydrate, amino acids, and organic phosphorus. Zone C contained carbohydrate, organic phosphorus, RNA, and amino acids, but only trace amounts of protein. Paper chromatographic examinations of zone C showed high concentrations of the aromatic amino acids and indicated that most of the RNA was in the form of nucleosides and nucleotides.

Recovery of injured bacteria by leakage material from frozen-and-thawed cells. The material present in each zone from Sephadex filtration of the supernatant fraction from cells frozen in phosphate buffer was tested for activity to promote

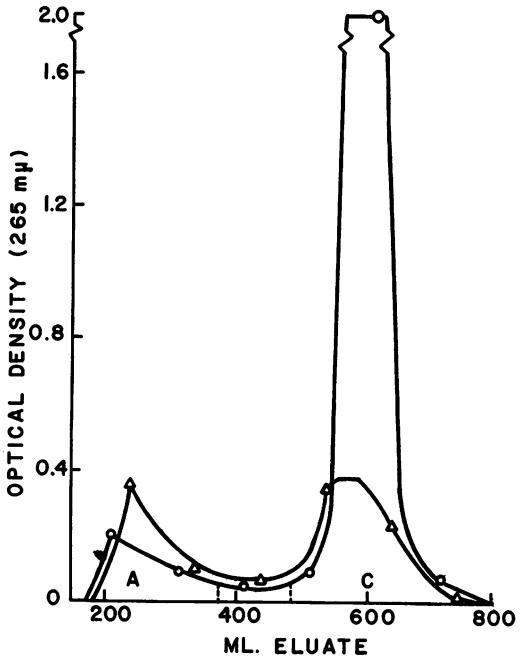

FIG. 2. *Elution pattern from Sephadex G-25 column of concentrated leakage material from cells frozen in 0.038 M phosphate buffer* (○) *and in distilled water* (△).

growth of injured cells by addition to MA medium (Table 3). The results show that zone A gave substantially more recovery (80%) than the other zones. Similarly, the leakage material in zone A from the supernatant fractions from cells frozen in distilled water gave substantially more recovery than the other two zones. The elution pattern through Sephadex indicated that zone A contained compounds of relatively small molecular weight, and these appeared to be peptides on the basis of their weak reaction with ninhydrin and strong biuret test. Separation of these compounds was unsuccessful by paper chromatography with butanol-acetic acid-water (5:1:4) or with butanol-2-butanone-water (2:2:1); however, after high-voltage paper electrophoresis, six well-separated zones which stained lightly with ninhydrin were detected. Two of these zones were active for the recovery of injured cells when tested by bioautography, and each gave a positive biuret test. Material corresponding to each of these active areas was eluted with water from several electrophoresis sheets, concentrated, and hydrolyzed with acid. Subsequent two-dimensional chromatography of the hydrolysates of each zone showed spots corresponding in position to alanine, aspartic acid, glycine, glutamic acid,

TABLE 3. *Recovery of cold-injured Escherichia coli Y by eluates[a] from fractions of cell leakage material[b] separated on a Sephadex G-25 column*

Plating medium	Colony count × 10^7/ml[c]	Injured survivors recovered
		%
TSA	80	
MA	30	(65)[d]
MA + zone A	70	80
MA + zone B	50	40
MA + zone C	38	16

[a] A 0.3-ml amount of eluate added per plate; solids per 0.3 ml were: A = 9 mg, B = 6 mg, C = 6 mg.

[b] Supernatant fraction from cells stored at −20 C for 7 days in 0.038 M phosphate buffer.

[c] Each figure represents the average of five trials, with each trial being the average of duplicate plates.

[d] Represents percentage of survivors injured; none was recovered.

isoleucine, leucine, proline, serine, threonine, valine, lysine, arginine, and histidine.

Protective effect of leakage materials. The material in zone A also provided protection against cell death during freezing. When a dilute cell suspension (2×10^6 per milliliter) was frozen in phosphate buffer with and without 1 mg/ml of zone A, survival after 4 hr at −20 C was 93 and 78%, respectively. Similarly, cell suspensions frozen in distilled water, with and without zone A, gave 86 and 65% survival, respectively.

DISCUSSION

The results obtained in the present investigation showed that during storage at various temperatures *E. coli* released organic compounds into the suspending medium. The quantity and nature of the material released was dependent upon the conditions of storage and the type of suspending medium. Cells stored in phosphate buffer released substantially more 260 mμ absorbing material than did cells in distilled water, and the quantity released was markedly increased by freezing. The enhanced leakage of material from cells frozen in distilled water was not evident from absorbancy measurements (Fig. 1), but was apparent from comparisons of subsequent protein analyses of the concentrated supernatant fluids.

The data of this study are similar to those of other workers (10, 22), who observed greater leakage of ultraviolet-absorbing material when cells were placed in suspending media of relatively high ionic strength. Wagman (22) found

that leakage of material from lyophilized *E. coli* in phosphate buffer of 0.1 ionic strength was accompanied by a dissociation of 25 and 40*S* ribonucleoprotein particles. He suggested that the disintegration was due to the removal by phosphate of magnesium or other divalent cations which are involved in the structural integrity of these particles (3). The applicability of this hypothesis in accounting for the leakage in phosphate buffer in the present study cannot be evaluated at this time, since the original site of the leakage material(s) is unknown. However, the finding that the cytoplasmic membrane of *E. coli* contains metabolically active ribosomes (8) makes this an attractive hypothesis, since an alteration of the membrane during freezing in phosphate buffer could presumably result in the release and subsequent dissociation of the membrane-bound ribosomes. The mechanism involved in the release of material in phosphate buffer must also be operative at 22 C. A portion of the material released at this temperature in the present study may be due to metabolism of endogenous RNA (20), but comparisons with cells stored at 22 C in distilled water indicate that this would account for only a small amount.

Viability of cells was related to the nature of the leakage material rather than to quantity, and peptides appeared to be the components released from frozen cells which were responsible for viability. No correlation was observed between loss in cell viability and loss of 260 mμ absorbing material; however, the appearance of peptide material in the supernatant fractions paralleled losses in cell viability. The beneficial effect of cellular leakage products on recovery of cells and the protection of cells against damage by freezing has been reported by Strange and Dark (19). These workers suggested that one or more components in the leakage products was necessary for survival or for reviving moribund bacteria and was resorbed by the bacteria from their external environment. This suggestion is supported by the observation that peptides from Trypticase added to minimal agar recovered *E. coli* which had been injured by freezing (16). However, leakage products from rapidly chilled bacteria may differ from leakage products from frozen cells in that the latter have been subjected to a phase change of water to ice.

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