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Metabolic Injury to Bacteria

II. Metabolic Injury Induced by Distilled Water or Cu^{++} in the Plating Diluent[1]

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When distilled water from a tin-lined still served as the plating diluent, cells of *Aerobacter aerogenes* developed symptoms of metabolic injury as evidenced by increased counts on supplemented, as compared with minimal, plating medium. Cysteine was as effective as yeast extract as a supplement to the minimal medium in increasing the viable count. Mg^{++} and, to a lesser extent, phosphate buffer at the concentrations tested protected unfrozen cells, but not cells which had been frozen and stored, against the loss of capacity to grow on minimal medium. When the plating diluent consisted of distilled water redistilled in an all-glass still, the symptoms of metabolic injury did not appear. Spectrographic analysis revealed the presence of 10^{-7} M Cu^{++} in the distilled water, and Cu^{++} added to redistilled water serving as the plating diluent reproduced the metabolic injury effects induced by distilled water. It was concluded that freezing and storage damaged the cell membrane, rendering it more penetrable by toxic elements which were thereby enabled to act at sites in the cell where Mg^{++} and other solutes in the plating diluent could not serve as effective antagonists. Increased recovery of cells on supplemented medium could be ascribed to the capacity of the supplements to remove toxic elements which had become bound to the cells during suspension in the plating diluent.

Many bacterial species appear to develop increased nutritional requirements when exposed to various forms of sublethal stress. The phenomenon has been most extensively studied with bacteria subjected to freezing. Straka and Stokes (14), working with *Escherichia coli* and some species of *Pseudomonas*, observed that suspensions of the cells gave the same plate counts before freezing on both minimal and enriched agar media. After freezing and a period of storage, a reduction in the total numbers of cells was observed, but plate counts were always higher on the enriched medium. Those bacteria growing only on the enriched medium were referred to as metabolically injured cells. This conversion of a part of a population of cells by freezing, or by freezing and storage, to a dependence on an enriched medium for growth has been confirmed by a number of workers (1, 6, 8. 10, 13).

Straka and Stokes (14) and, more recently, Moss and Speck (8, 9) concluded that the factor in enriched media responsible for growth of metabolically injured cells was peptide in nature, since enzymatic but not acid hydrolysates of casein promoted growth of these cells when added as supplements to the minimal medium. MacLeod, Smith, and Gelinas (6), on the other hand, found that mixtures of amino acids or cysteine were as effective as more complex supplements to the minimal medium for increasing the bacterial count on suspensions of *Aerobacter aerogenes* or *E. coli* which had been frozen and stored.

In studies of metabolic injury, it has been customary to add a buffer or other electrolyte to the diluent used to dilute the suspensions of cells for plating. It has been found in the present study, with *A. aerogenes* as the test organism, that when the plating diluent consisted only of distilled water even unfrozen cells of the organism developed symptoms of metabolic injury.

The effect produced by distilled water has been traced to its content of toxic trace elements. One of the elements, Cu^{++}, when added to redistilled water reproduced the effects of the distilled water. The relation of these findings to the metabolic injury induced by freezing and storage is considered.

MATERIALS AND METHODS

Culture. The organism used was a strain of *A. aerogenes*, Mac no. 112 of the Macdonald College

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Culture Collection. The organism was maintained on slants of Trypticase Soy Agar (TSA; from BBL) at 2 C. The stock culture was transferred at monthly intervals.

Cell suspensions. Suspensions of the cells for freezing were prepared essentially according to the procedure of Straka and Stokes (14), as modified by MacLeod et al. (6). The cells from a 24-hr TSA slant culture of the organism were washed from the slant and diluted to 100 ml with a sterile solution containing 2×10^{-3} M $MgSO_4$ and 3×10^{-4} M phosphate buffer (K^+, *p*H 7.2). A 1-ml amount of the resulting suspension was diluted to 100 ml with sterile 0.5% beef extract. The suspension of cells in the beef extract solution was dispensed in 5-ml samples into screw-capped polypropylene tubes for freezing. Cell suspensions were frozen by immersing the tubes in liquid air. The frozen suspensions were stored in a commercial deep-freeze unit held at −20 C. To thaw a suspension, a tube was placed in a beaker of water at room temperature. As soon as the last trace of ice in the tube disappeared (after 2 to 3 min), dilutions for plating were begun. Preliminary serial dilutions were made with sterile redistilled water. The final dilution for plating was made with the plating diluent of choice, previously equilibrated to 25 C. Dilution bottles containing suspensions of cells in the final plating diluents were held in a constant-temperature water bath set at 25 C. Samples were removed for plating after various periods of incubation. Organisms in the plating diluents were plated by use of standard pour plate procedures. Plates were incubated for 48 hr at 30 C. Longer plating periods were tested and found not to increase the total count.

Experimental media. The minimal medium used contained (in grams per liter): K_2HPO_4, 1.75; KH_2PO_4, 0.75; sodium citrate, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; glucose, 4.0; agar, 15. To permit the addition of supplements to the minimal medium, the quantities of each of the components for 1 liter of the minimal medium were dissolved in 800 ml. The medium was dispensed in 8-ml volumes into a series of 18 by 150 mm culture tubes which were then capped and sterilized. To test the effect of supplements, tubes of the minimal medium were melted and cooled. Sterile supplement (and water where necessary) was added aseptically to each tube to make a final volume of 10 ml just before the plates were poured. Two supplemented media were used in these experiments. One was minimal medium containing 1% added yeast extract, and the other was minimal medium containing 10^{-3} M added cysteine.

Distilled and redistilled water. The distilled water used in these experiments was obtained from a commercial laboratory still described as being lined with block tin to protect against metal ion contamination. The redistilled water was prepared by distilling the water from the tin-lined still in a commercial laboratory all-glass still.

Trace element analyses. To bring the concentration of trace elements in distilled and redistilled water within the range of detection of the instruments used, 1,000:1 concentrations of the water samples were effected. Ten-liter volumes of the water samples to be analyzed were concentrated by evaporation to 10 ml. The concentrates were then analyzed qualitatively and quantitatively for metal ions by use of a Jarrell-Ash Ebert 3.4-m spectrograph. We are much indebted to Carlton Joyce of the Pulp and Paper Research Institute, Pointe Claire, Quebec, for performing these analyses.

Analyses for Cu^{++} were carried out on a few samples with a Perkin Elmer model 303 atomic absorption spectrophotometer.

Preparation of glassware. All glassware used to concentrate the distilled and redistilled water, to conduct the analyses, and to prepare dilutions of the cells for plating was held a minimum of 3 hr in a mixture of concentrated H_2SO_4 and HNO_3 acids (2:1). This treatment was followed by 3 rinses with tap water and finally by 10 rinses with redistilled water.

RESULTS

Development of metabolic injury in unfrozen and frozen cells. In the course of studying the nutritional factors in complex media responsible for the increases in plate counts when suspensions of cells which had been frozen and stored were plated (6), it became apparent that the composition of the plating diluent was intimately associated with the development of metabolic injury. When the plating diluent was distilled water, even unfrozen cells developed metabolic injury. Table 1 shows that, when unfrozen cells were suspended in distilled water for plating, the count on the suspension varied with the age of the suspension and the composition of the plating medium. When the cells were plated on the minimal salts-glucose medium, the count dropped rapidly to less than 4% of the initial count in 6 hr. When the plating medium was supplemented with yeast extract or cysteine, the count remained essentially constant for the first 2 hr, after which it also gradually declined. When the plating diluent consisted of distilled water supplemented with potassium phosphate buffer (*p*H 7.2) and Mg^{++} at the levels used in the previous study of metabolic injury induced by freezing and storage (6), no variation in plate counts with composition of the plating medium was obtained.

When a portion of the cells used to obtain the results shown in Table 1 was frozen and stored, the cells developed metabolic injury even when the plating diluent consisted of distilled water supplemented with Mg^{++} and buffer. After freezing and 4 weeks of cold storage, the cells were tested for their capacity to grow on minimal and supplemented media (Table 2). With the frozen and stored cells, differences in plate count between minimal and supplemented media were evident even after the shortest period of suspension in the plating diluent. As the time of suspension in-

TABLE 1. *Effect of composition of plating diluent, composition of the plating medium, and time of suspension in plating diluent on development of metabolic injury in unfrozen cells of Aerobacter aerogenes*

Plating diluent	Time of suspension in plating diluent	Plating medium		
		Minimal	Minimal + yeast extract	Minimal + cysteine
	min			
Distilled water	0[a]	267 ± 4[b]	267 ± 5	277 ± 4
	30	204 ± 6	273 ± 8	275 ± 9
	60	132 ± 4	241 ± 7	270 ± 10
	120	66 ± 5	233 ± 8	255 ± 4
	240	19 ± 1	174 ± 5	189 ± 3
	360	10 ± 1	124 ± 6	156 ± 3
Distilled water + PO_4^{-3} buffer + Mg^{++}[c]	0	289 ± 4	287 ± 5	288 ± 5
	30	286 ± 5	288 ± 3	288 ± 6
	60	288 ± 4	288 ± 5	288 ± 4
	120	290 ± 6	290 ± 6	289 ± 5
	240	238 ± 5	246 ± 4	248 ± 3
	360	217 ± 7	219 ± 6	219 ± 6

[a] Zero-time in suspension in the plating diluent means that a sample for plating was removed from the final dilution used for plating immediately after the cells had been evenly suspended in the plating diluent.

[b] Colonies appearing per milliliter of the dilution used for plating. Each result represents the average and the average deviation of the count on four plates.

[c] In all experiments, except where otherwise indicated, PO_4^{-3} buffer was 3×10^{-4} M (K^+ salt, *p*H 7.2); Mg as $MgCl_2$, 2×10^{-3} M.

creased, the differences increased. This was true whether distilled water alone or distilled water supplemented with phosphate buffer and Mg^{++} was the plating diluent, though in the later case there was an increased recovery of cells on both minimal and supplemented media.

It was of interest to know the relative capacity of the buffer and Mg^{++} to protect the unfrozen cells against the action of the distilled water in the plating diluent. The results in Table 3 show that Mg^{++} alone was as effective as the mixture in preventing the decrease in counts on the minimal medium which occurred after 2 hr of suspension of the cells in the plating diluent. The buffer was only partially effective in preventing the drop.

Metabolic injury in bacteria has been defined as the development in a proportion of a population of an increased requirement for nutrients (14). The results presented here show that the phenomenon developed when unfrozen cells were suspended in distilled water. When the cells were suspended in distilled water containing Mg^{++} and phosphate buffer, preliminary freezing and storage of the cells was required for the phenomenon to occur.

Relation of water quality to metabolic injury. The distilled water used in these experiments was obtained from a still lined with block tin to protect against metal-ion contamination. When the water from this still was redistilled by use of an all-glass still, the toxic action as measured by plating on a minimal medium was essentially eliminated (Table 4, experiment A). There was no loss in viable count on cells suspended in redistilled water for a 4-hr period, and the metabolic-injury effect as measured by the difference in count on minimal and supplemented media almost completely disappeared. That the toxic action of the distilled water was due to something introduced into the water by the distilled-water system employed was evident from the fact that a sample of tap water from the same source used to feed the still had no toxic action. To determine whether the piping used to distribute the distilled water to the laboratory could be introducing the toxic component, the toxicity of water from the storage tank associated with the still was tested. The results (Table 4, experiment B) showed that this water was as toxic as the water piped to the laboratory. In a further experiment not shown, water collected from the condenser was found to be toxic.

TABLE 2. *Effect of composition of plating diluent, composition of plating medium, and time of suspension in plating diluent on development of metabolic injury in cells of Aerobacter aerogenes which had been frozen and stored for 4 weeks*

Plating diluent	Time of suspension in plating diluent	Plating medium		
		Minimal	Minimal + yeast extract	Minimal + cysteine
	min			
Distilled water	0[a]	211 ± 2[b]	291 ± 4	292 ± 6
	30	141 ± 2	260 ± 3	261 ± 4
	60	99 ± 2	253 ± 5	253 ± 3
	120	54 ± 3	230 ± 5	232 ± 3
	240	11 ± 2	213 ± 3	213 ± 4
	360	0	188 ± 3	195 ± 7
Distilled water + PO_4^{-3} buffer + Mg^{++}	0	244 ± 4	330 ± 6	330 ± 2
	30	181 ± 3	328 ± 6	328 ± 4
	60	135 ± 3	308 ± 3	313 ± 5
	120	104 ± 5	301 ± 5	304 ± 4
	240	49 ± 3	274 ± 4	277 ± 4
	360	0	238 ± 4	245 ± 4

[a] For explanation of zero-time, see Table 1.

[b] Results expressed as in Table 1.

TABLE 3. *Relative capacity of phosphate buffer and Mg^{++} in the plating diluent to protect against the development of metabolic injury in unfrozen cells of Aerobacter aerogenes when distilled water was used in the plating diluent*

Solutes added to plating diluent		Time in suspension	Plating medium		
Mg^{++}	PO_4^{-3}		Minimal	Minimal + yeast extract	Minimal + cysteine
		hr			
0	0	0[a]	260 ± 3[b]	267 ± 3	266 ± 5
		2	88 ± 2	219 ± 3	220 ± 7
+	+	0	265 ± 3	268 ± 3	267 ± 3
		2	255 ± 4	261 ± 3	264 ± 4
+	0	0	262 ± 3	263 ± 3	264 ± 4
		2	255 ± 3	266 ± 5	267 ± 6
0	+	0	259 ± 3	263 ± 3	262 ± 3
		2	171 ± 5	246 ± 3	246 ± 5

[a] See Table 1.
[b] Results expressed as in Table 1.

TABLE 4. *Effect of different water sources used as plating diluent on the viability of cells of Aerobacter aerogenes in suspension*

Expt	Plating diluent	Time in diluent	Plating medium	
			Minimal	Minimal + yeast extract
		hr		
A	Distilled water	0[a]	155 ± 11[b]	189 ± 5
		4	19 ± 8	122 ± 7
	Redistilled water	0	174 ± 8	200 ± 10
		4	186 ± 2	203 ± 6
	Tap water	0	183 ± 5	194 ± 10
		4	184 ± 9	188 ± 7
B	Distilled water	0	144 ± 7	160 ± 5
		4	57 ± 6	133 ± 8
	Storage tank water	0	146 ± 6	161 ± 6
		4	43 ± 3	118 ± 5
	Distilled water concentrate	0	1 ± 1	18 ± 4
		4	0	1 ± 1
	Ash of concentrate in redistilled water	0	0	1 ± 1
		4	0	0

[a] See Table 1.
[b] Results expressed as in Table 1.

When the distilled water was redistilled in the all-glass still, the concentrate which accumulated in the boiling pot of the latter was found to have increased toxicity (Table 4, experiment B). A sample of this concentrate was taken to dryness; the residue was ashed in a platinum crucible, dissolved in redistilled water, and diluted with redistilled water to the volume of the original sample. The ashed residue exhibited undiminished toxicity, indicating that the toxic principle was inorganic in nature.

Trace elements in distilled water. Samples of distilled water and redistilled water were analyzed qualitatively and quantitatively for metal ions by use of a spectrograph. Table 5 shows the results of one of these analyses. Traces of B, Mg, Fe, Cu, and Ca were detected in both samples, with appreciably reduced amounts being found in the redistilled water.

When the elements detected as contaminants in the distilled water were added to redistilled water, only Cu was toxic at the level found in distilled water (Table 6). The pattern of its toxic action was essentially the same as that of the distilled water. When either distilled water or the redistilled water containing added Cu^{++} served as plating diluent, fewer cells grew on minimal as compared with supplemented medium and the difference in count increased as the time of exposure to the plating diluent increased. Thus, Cu^{++}, like distilled water, could produce in a suspension of unfrozen cells an effect indistinguishable from the metabolic injury induced by freezing and storage.

Characteristics of the Cu^{++} effect. Cysteine was found to be as effective as yeast extract as a supplement to the minimal medium in increasing the recovery of cells exposed to Cu^{++} in the plating diluent (Table 7). When phosphate buffer and Mg^{++} were included in the plating diluent, the reduction in count on the minimal medium due to the action of Cu^{++} were largely eliminated.

A portion of the same suspension of cells used to prepare the dilutions for the experiment reported in Table 7 was frozen and stored for 5 weeks and examined again (Table 8). The relative difference in counts between minimal and supplemental medium caused by the presence of Cu^{++} in the plating diluent was increased somewhat

TABLE 5. *Concentrations of trace-metal contaminants in distilled and redistilled water*

Element detected	Distilled water	Redistilled water
	μg/ml	*μg/ml*
B	0.003 (3×10^{-7})[a]	0.006 (1.5×10^{-7})
Mg	0.01 (4×10^{-7})	trace
Fe	0.04 (7×10^{-7})	0.004 (7×10^{-8})
Cu	0.006 (10^{-7})	0.0002 (3.3×10^{-9})
Ca	0.5 (1.3×10^{-5})	0.04 (10^{-6})

[a] Numbers in parentheses indicate the molar equivalents.

TABLE 6. *Effect of adding the trace metal contaminants detected in distilled water to the plating diluent on the capacity of unfrozen cells of Aerobacter aerogenes to grow on minimal and supplemented media*

Plating diluent[a]	Time in diluent	Plating medium	
		Minimal	Minimal + yeast extract
	hr		
Redistilled water	0	176 ± 6[b]	179 ± 4
	4	170 ± 3	177 ± 5
Distilled water	0	44 ± 8	150 ± 2
	4	6 ± 4	83 ± 6
Redistilled water plus			
B, 0.003 μg/ml	0	178 ± 6	182 ± 2
	4	176 ± 6	176 ± 7
Mg, 0.01 μg/ml	0	174 ± 9	175 ± 4
	4	174 ± 7	172 ± 6
Fe, 0.04 μg/ml	0	170 ± 4	173 ± 8
	4	166 ± 8	173 ± 4
Cu, 0.006 μg/ml	0	139 ± 9	156 ± 6
	4	71 ± 3	129 ± 6
Ca, 0.5 μg/ml	0	166 ± 8	171 ± 8
	4	161 ± 8	170 ± 6
B, Mg, Fe, Cu, Ca	0	144 ± 6	173 ± 5
	4	80 ± 9	153 ± 4

[a] B, Mg, Fe, Cu, and Ca were added as Na_3BO_3, $MgSO_4$, $FeCl_3$, $CuSO_4$, and $CaCl_2$, respectively.
[b] See Table 1.

TABLE 7. *Effect of a combination of phosphate buffer and Mg^{++} in the plating diluent on development of metabolic injury induced in unfrozen cells by adding Cu^{++} to the plating diluent*

Additions to redistilled water as the plating diluent		Time in plating diluent	Plating medium		
Cu^{++}	PO_4^{-3} + Mg^{++}		Minimal	Minimal + yeast extract	Minimal + cysteine
μg/ml		*hr*			
0	−	0[a]	227 ± 4[b]	228 ± 3	228 ± 2
0	−	2	227 ± 2	229 ± 2	229 ± 2
0.02	−	0	159 ± 4	226 ± 2	228 ± 2
0.02	−	2	50 ± 1	172 ± 5	172 ± 3
0	+	0	216 ± 2	216 ± 1	216 ± 4
0	+	2	216 ± 1	216 ± 2	216 ± 1
0.02	+	0	214 ± 3	214 ± 2	215 ± 1
0.02	+	2	167 ± 1	213 ± 3	212 ± 2

[a] See Table 1.
[b] Colonies appearing per milliliter of the dilution used for plating. Each result recorded in the experiment represents the average and the average deviation of the count on three plates.

with frozen and stored as compared with unfrozen cells. Also, phosphate buffer and Mg^{++} in the plating diluent were less effective in preventing the development of this difference in counts than they were with unfrozen cells.

Mg^{++} and the buffer were tested separately to determine which of these components in the plating diluent prevented the action of Cu^{++} in reducing the count on minimal medium. Each component was tested with unfrozen cells, and with a portion of the same cells subjected to freezing and a period of storage. When the buffer was tested with unfrozen cells (Table 9), the level of buffer ordinarily used in the plating diluent (3×10^{-4} M) had no capacity to prevent the action of Cu^{++} in reducing the count on the unsupplemented medium. However, 10 times the buffer level did have some protective action. After freezing and storage, even 10 times the buffer level showed little capacity to prevent the difference in count developing between minimal and supplemented medium. When Mg^{++} was present in the plating diluent at the level ordinarily used (2×10^{-3} M), it prevented the reduction in count on minimal medium caused by Cu^{++} in the plating diluent when unfrozen cells were plated immediately (Table 10). However, even 10 times this amount of Mg^{++} failed to prevent the development of metabolic injury induced by Cu^{++} in cells which had been frozen and stored.

Relation of the toxicity of distilled water to its Cu^{++} content. The relative capacity of Cu^{++} and distilled water in the plating diluent to induce

TABLE 8. *Effect of phosphate buffer and Mg^{++} in the plating diluent on development of metabolic injury induced in frozen and stored cells by adding Cu^{++} to the plating diluent*

Additions to redistilled water as the plating diluent		Time in plating diluent	Plating medium		
Cu^{++}	PO_4^{-3} + Mg^{++}		Minimal	Minimal + yeast extract	Minimal + cysteine
μg/ml		*hr*			
0	−	0[a]	122 ± 2[b]	123 ± 2	124 ± 2
0	−	2	102 ± 1	109 ± 1	109 ± 3
0.02	−	0	86 ± 3	114 ± 3	111 ± 2
0.02	−	2	13 ± 2	88 ± 2	88 ± 4
0	+	0	123 ± 4	140 ± 2	141 ± 2
0	+	2	112 ± 1	133 ± 3	131 ± 0
0.02	+	0	108 ± 2	137 ± 2	134 ± 2
0.02	+	2	32 ± 1	103 ± 1	107 ± 3

[a] See Table 1.
[b] See Table 7. Cells frozen and stored for 5 weeks.

TABLE 9. *Effect of the simultaneous presence of potassium phosphate buffer and Cu^{++} in the plating diluent on induction of metabolic injury in unfrozen cells and in cells which had been frozen and stored 5 weeks*

Additions to redistilled water as the plating diluent		Time in plating diluent	Plating medium			
			Unfrozen cells		Frozen and stored cells	
Cu^{++}	PO_4^{-3} buffer		Minimal	Minimal + cysteine	Minimal	Minimal + cysteine
μg/ml	*M*	*hr*				
0	0	0[a]	225 ± 4[b]	226 ± 2	105 ± 2	108 ± 2
		2	225 ± 2	227 ± 2	104 ± 2	105 ± 3
0	3×10^{-4}	0	237 ± 1	236 ± 3	103 ± 2	108 ± 3
		2	237 ± 3	239 ± 3	98 ± 3	104 ± 2
0	3×10^{-3}	0	208 ± 2	210 ± 3	130 ± 2	135 ± 3
		2	210 ± 2	212 ± 2	112 ± 2	118 ± 2
0.02	0	0	183 ± 2	226 ± 1	70 ± 1	91 ± 0
		2	13 ± 1	167 ± 3	6 ± 0	78 ± 4
0.02	3×10^{-4}	0	156 ± 2	234 ± 2	73 ± 2	103 ± 2
		2	4 ± 1	162 ± 1	31 ± 2	87 ± 0
0.02	3×10^{-3}	0	209 ± 2	213 ± 2	73 ± 3	124 ± 2
		2	155 ± 2	195 ± 3	48 ± 2	100 ± 1

[a] See Table 1.
[b] See Table 7.

TABLE 10. *Effect of the simultaneous presence of Mg^{++} and Cu^{++} in the plating diluent on induction of metabolic injury in unfrozen cells and in cells which had been frozen and stored 5 weeks*

Additions to redistilled water as the plating diluent		Time in plating diluent	Plating medium			
			Unfrozen cells		Frozen and stored cells	
Cu^{++}	Mg^{++}		Minimal	Minimal + cysteine	Minimal	Minimal + cysteine
μg/ml	*M*	*hr*				
0	0	0[a]	203 ± 2[b]	204 ± 4	139 ± 1	145 ± 3
		2	203 ± 0	207 ± 2	87 ± 3	89 ± 3
0	2×10^{-3}	0	216 ± 1	217 ± 2	141 ± 1	153 ± 2
		2	215 ± 2	218 ± 1	106 ± 4	146 ± 4
0	2×10^{-2}	0	208 ± 2	206 ± 3	143 ± 1	153 ± 3
		2	206 ± 3	207 ± 2	114 ± 2	138 ± 2
0.02	0	0	185 ± 2	204 ± 1	54 ± 2	104 ± 2
		2	10 ± 1	163 ± 1	10 ± 1	67 ± 2
0.02	2×10^{-3}	0	218 ± 1	218 ± 2	59 ± 2	120 ± 2
		2	113 ± 3	218 ± 3	32 ± 2	94 ± 3
0.02	2×10^{-2}	0	206 ± 3	206 ± 3	87 ± 4	133 ± 1
		2	205 ± 1	207 ± 4	36 ± 2	99 ± 2

[a] See Table 1.
[b] See Table 7.

metabolic injury in frozen, stored cells was compared. To simulate as closely as possible the conditions used for producing metabolic injury by freezing and storage, Mg^{++} and PO_4^{-3} were included in the plating diluent. The results (Table 11) show that the sample of distilled water produced a metabolic-injury effect slightly greater than that resulting from the presence of 0.01 μg of Cu^{++} per ml in redistilled water. This particular sample of distilled water contained 0.001 μg of Cu^{++} per ml as determined by atomic-absorption spectrophotometry. It is of interest that

the ashed residue of the distilled water was appreciably more toxic than the distilled water itself.

Since it appeared that the distilled water in these experiments was somewhat more toxic than could be accounted for by its Cu^{++} content, a number of other ions were tested for their capacity to induce metabolic injury. Ag^{+} and Hg^{++} produced effects similar to Cu^{++} in unfrozen cells of *A. aerogenes* (Table 12), with Ag^{+} being more and Hg^{++} somewhat less toxic than the Cu^{++}. Sn^{++}, Pb^{++}, Zn^{++}, and Cd^{++} produce no metabolic-injury effect when tested in the same concentration range.

TABLE 11. *Relative capacity of Cu^{++} and distilled water to induce metabolic injury when present in the plating diluent for cells which have been frozen and stored 3 weeks*

Plating diluent	Cu^{++} added	Time in diluent	Plating medium	
			Minimal	Minimal + yeast extract
	μg/ml	*hr*		
Redistilled water	0	0	261 ± 5[a]	284 ± 8
		4	254 ± 6	289 ± 4
	0.005	0	238 ± 4	276 ± 3
		4	213 ± 3	228 ± 6
	0.010	0	219 ± 4	278 ± 3
		4	161 ± 3	219 ± 3
	0.050	0	208 ± 4	280 ± 6
		4	0	124 ± 4
Distilled water	0	0	217 ± 4	271 ± 6
		4	135 ± 1	212 ± 2
Ashed residue of distilled water in redistilled water	0	0	178 ± 6	247 ± 4
		4	1.5 ± 0.5	132 ± 4

[a] See Table 1.

TABLE 12. *Relative capacity of Cu^{++}, Ag^{+}, and Hg^{++} to induce metabolic injury when added to the plating diluent for unfrozen cells of Aerobacter aerogenes*

Ion added to plating diluent[a]	Concn	Plating medium	
		Minimal	Minimal + cysteine
	M		
Cu^{++}	0	107 ± 5[b]	108 ± 4
	1.5×10^{-8}	99 ± 4	104 ± 4
	1.5×10^{-7}	14 ± 5	98 ± 6
	7.5×10^{-7}	0	8 ± 1
Ag^{+}	0	107 ± 5	108 ± 4
	1.5×10^{-8}	98 ± 6	100 ± 4
	1.5×10^{-7}	10 ± 4	64 ± 5
	7.5×10^{-7}	0	0
Hg^{++}	0	107 ± 5	108 ± 4
	1.5×10^{-8}	100 ± 7	102 ± 8
	1.5×10^{-7}	80 ± 7	93 ± 3
	7.5×10^{-7}	0	53 ± 4

[a] Cu^{++} was added as $CuSO_4$, Ag^{+} as $AgNO_3$, and Hg^{++} as $HgCl_2$.

[b] See Table 1. Platings were performed at zero-time.

TABLE 13. *Effect of distilled water from different still installations on development of metabolic injury in unfrozen cells of Aerobacter aerogenes*

Plating diluent	Time in diluent	Plating medium	
		Minimal	Minimal + cysteine
	hr		
Distilled water			
Sample A	0	116 ± 4[a]	123 ± 6
	4	59 ± 5	93 ± 5
Sample B	0	4 ± 2	119 ± 3
	4	0	46 ± 6
Sample C	0	117 ± 4	125 ± 7
	4	113 ± 3	118 ± 4
Sample D	0	112 ± 4	117 ± 4
	4	38 ± 3	121 ± 4
Redistilled D	0	117 ± 3	129 ± 2
	4	111 ± 3	122 ± 4

[a] See Table 1.

Other sources of distilled water. Since the distilled water in these experiments came from a single still installation, it was important to know whether the results were unique for water from this still. Accordingly, water from three other installations of the same type of still was tested (Table 13). Distilled water sample D was from the same source used in the previous experiments. This sample and the product obtained from it by redistillation in an all-glass still were used for comparison with water from the other stills. Water samples from stills A and B, when used as plating diluents, were as toxic or more toxic than water from still D when minimal medium was used for plating. Prolonged exposure to these waters reduced the count even on the medium supplemented with cysteine. Water from still C, however, proved to be nontoxic on either plating medium. Still C differed from the other stills in that it was new and recently installed.

DISCUSSION

The results reported here show that when steps were taken to reduce the content of toxic trace elements in the plating diluent, the metabolic injury produced by freezing and storage, as evidenced by the development of differences in plate

count on minimal and supplemented media in a suspension of cells of *A. aerogenes*, tended to disappear. It was evident, however, that freezing and storage produced a change in a proportion of the cells, because, before freezing, Mg^{++} and, to a lesser extent, phosphate buffer could protect the cells from the action of distilled water or Cu^{++} in the plating diluent, but after freezing and storage these additives were essentially ineffective.

Since supplements to the plating medium did not increase the plate count on suspensions of cells which had been frozen and stored unless toxic trace elements were present in sufficient concentration in the plating diluent, it may be concluded that the action of the supplements was to detoxify the toxic metals rather than to repair the damage to the cells caused by freezing and storage. The ability of cysteine when added to the plating medium to replace other more complex supplements in increasing the recovery of viable cells exposed to distilled water or Cu^{++} in the plating diluent is consistent with the well-known ability of this amino acid, as a sulfhydryl compound, to bind Cu^{++}, Hg^{++}, and other heavy metals (2). It may be concluded then that in the plating diluent a proportion of the cells bind toxic trace elements at sites which prevent cell multiplication. If the plating medium contains a component capable of removing the toxic element from the cell, and if the period of exposure of the cell to the toxic element has not been too prolonged, the cell will then be able to multiply and form a colony.

The fact that Mg^{++} in the plating diluent protected the cells from the toxic action of Cu^{++} and distilled water before freezing and storage but not after suggests that the locus of action of toxic trace elements in the two cases was different. Cu^{++}, Hg^{++}, and other heavy metals are known to act at the cell surface in some cells (4, 5, 11). It is not unlikely that in unfrozen cells of *A. aerogenes* Cu^{++} exerted its toxic action at the cell surface and that the ability of Mg^{++} to prevent its action was therefore due to its capacity to compete with Cu^{++} for critical sites on this surface.

Bacteria which have been frozen leak intracellular solutes (3, 9) and become more readily penetrable by solutes in the suspending medium (15), thus providing evidence that freezing causes damage to the cytoplasmic membrane of the cells. The fact that Mg^{++} could protect *A. aerogenes* from the toxic action of Cu^{++} before freezing but not after suggests that, after freezing, Cu^{++} may penetrate the cell membrane and inhibit growth by combining with metabolically important sites which are sufficiently different structurally from those at the membrane surface for Mg^{++} not to be effective as an antagonist. Thus, freezing and storage produce cellular injury which would seem to be damage to the membrane, leading to increased penetrability to such extracellular solutes as toxic trace elements. Failure of the cells to grow on minimal medium would then occur when the toxic elements acted at sites within the cell where Mg^{++} and other solutes in the plating diluent were unable to serve as effective antagonists. Metabolic injury as evidenced by increased counts on supplemented as compared with minimal medium would manifest itself if the supplement contained a component or components capable of detoxifying the toxic penetrating solute.

Other workers studying metabolic injury produced by freezing and storage have not found it necessary to include Mg^{++} in the plating diluent to prevent the development of differences in counts on minimal and complex media when unfrozen cells were used. It is therefore necessary to consider whether toxic trace elements in the plating diluent could have been a factor in the development of the metabolic injury reported in their studies. We have obtained evidence that *E. coli* 451-B, one of the cultures used by Straka and Stokes (14) and Moss and Speck (8), is appreciably less sensitive to Cu^{++} before freezing than our strain of *A. aerogenes*, though it also developed increased sensitivity to Cu^{++} after freezing and storage. Therefore, the lack of a need for Mg^{++} in the plating diluent before freezing and storage would not rule out the possible involvement of toxic trace elements in the development of metabolic injury in this organism after freezing and storage. Nakamura and Dawson (10) used distilled water further treated with a laboratory demineralizer in their studies of metabolic injury produced in *Shigella sonnei* by freezing and storage. Although this might at first seem to rule out toxic trace elements as a factor in their studies, Postgate and Hunter (12) reported that their laboratory-distilled *and -deionized* water contained 2×10^{-7} M Cu^{++}. This is twice the highest level of Cu^{++} detected in the distilled water in our experiments.

We have consistently found that our distilled water and its ashed residue were more toxic than could be accounted for by their content of Cu^{++}. It would appear that some other metal with properties similar to Cu^{++} was contributing to the inability of the cells to grow on minimal medium. Since the spectrographic method as applied here can detect only 70 of the known elements (7), it is quite possible that one or more other ions could be present in the distilled water in the same concentration range as Cu^{++} and still go undetected.

Since the basic lesion in cells injured by freezing

and storage seems to be membrane damage, leading to increased penetrability, changed sensitivity of the injured cells to medium components other than toxic trace elements might be expected. A supplement which is not toxic to cells before freezing and storage might become so to a proportion of them after. Differences in the relative capacity of supplements to promote the growth of unfrozen and of frozen and stored cells should therefore be interpreted with caution.

When suspensions of cells of gram-negative bacteria are frozen and stored, part of the population is killed in the sense that the cells will not grow under any of the cultural conditions devised, part is injured as indicated by the increased penetrability of a proportion of the cells to toxic materials, and part appears to be unchanged. Depending on the conditions and the method used for freezing, the number of bacteria in the population which are killed may far exceed the number which are injured. Straka and Stokes (14) suggested that nonlethal injury may represent the first stage in the development of a condition sufficiently critical to cause death of the cells. At the present time, it is not known whether this means that, in the part of the cell population which is killed by freezing, membrane damage is extensive enough to prevent the cells from growing and dividing.

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