Alcohols Protect *Escherichia coli* against Cold Shock (43240)

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Abstract. **Alcohols protect *Escherichia coli* against cold shock, and the concentration of alcohol which provided optimal protection declined with increasing hydrophobicity of the alcohol. The rate of loss of viability after the chilling transition was decreased by *n*-octanol, even when it was added after that chilling transition. Cold-shocked cells exhibited a sensitivity toward dioxygen, seen as greater enumeration on anaerobic, rather than on aerobic, trypticase-yeast extract agar plates, and addition of catalase or antioxidants, such as α-tocopherol or probucol, to the agar plates did not lessen this dioxygen sensitivity. Respiratory capacity was diminished by cold shock, and cyanide-sensitive respiration was more affected than was cyanide-resistant respiration. Discharging the proton gradient, with the uncoupler carbonyl cyanide trifluoromethoxyphenylhydrazone, did not change sensitivity to cold shock. There was no evidence for minimal medium recovery after cold shock. The data presented, as well as that already in the literature, are explained on the basis of membrane damage caused by patches of ordering transitions in one membrane leaflet, unmatched by comparable transitions in the mating leaflet.** [P.S.E.B.M. 1991, Vol 197]

Escherichia coli subjected to rapid chilling exhibit a marked decrease in viability (1, 2). This phenomenon, referred to as cold shock, is seen with logarithmic phase cells, but not with stationary phase cells (3–5). Damage to cell membranes appears to be a salient feature of cold shock. Thus, 0.3 *M* sucrose protects against cold shock (4), as do divalent cations (5). Moreover, cold shock is associated with increased permeability toward amino acids, nucleotides, and small proteins (6–13).

A weakening of the cell membranes, imposed by a phase transition, has often been identified as the cause of cold shock (6–8, 14, 15). There is support for this view. Thus, decreased temperature of growth is known to lower the melting point of *E. coli* membranes (16, 17), and growth at lower temperatures lowers the temperature at which cold shock is seen (18). The disordering of the fatty acid chains in *E. coli* membranes above the transition temperature has been observed through x-ray diffraction (18). The outer membrane of *E. coli* is potentially stressed by an electrostatic tension due to mutual repulsion of the anionic lipopolysaccharide, which is so abundant in the outer leaflet of this membrane (19, 20). Binding of metallic cations decrease this repulsion and so stabilizes the membrane (21, 22), and, as already mentioned, metal cations protect against cold shock (5).

Some of the properties of cold shock remain puzzling. One of these is the dependence upon the rate of chilling. Thus, rapid chilling imposes cold shock, but slower chilling does not (2, 4, 6). In addition, it is not clear why log phase cells are susceptible, whereas stationary phase cells are not (3–5). It appeared possible to rapidly increase the fluidity of the membranes of *E. coli* by addition of a series of homologous alcohols, which would partition into the membrane in proportion to their hydrophobicity. Alcohols have been used to lower the phase transition temperature of artificial phospholipid vesicles (23, 24) and should exert a similar action on natural membranes. Such alcohols might protect against cold shock and provide an additional tool for investigating its mechanisms. We now report such a protective effect of alcohols.

Materials and Methods

Compounds. α-Tocopherol, 2,4-dinitrophenol, and sterile bovine liver catalase were purchased from the Sigma Chemical Co. (St. Louis, MO), Matheson Co., Inc. (East Rutherford, NJ), and Worthington Bio-

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chemical Corp. (Freehold, NJ), respectively. Carbonyl cyanide trifluoromethoxyphenylhydrazone and 4,4′-(isopropylidenedithio) bis[2,6-di-*tert*-butylphenol] (probucol) were kindly supplied by E. I. DuPont De Nemours (Wilmington, DE) and by Merrell Dow Pharmaceuticals (Cincinnati, OH), respectively. All other compounds from commercial sources were of reagent grade.

Media. Trypticase-yeast extract (TSY) medium contained 30 g of tryptic soy broth and 5 g of yeast extract (both from Difco Laboratories, Detroit, MI)/liter, adjusted to pH 7.0 with HCl. Petri plates, for enumeration of viable cells, were prepared with TSY medium solidified with 1.5% Bacto-agar. Vogel Bonner (VB) salts contained 0.8 m*M* $MgSO_4$, 9.5 m*M* citric acid, 44 m*M* K_2HPO_4, and 17 m*M* $Na(NH_4)HPO_4$, at pH 7.0. VB medium was VB salts plus 0.5% glucose and 1 μg/ml of vitamin B12.

Bacteria. *E. coli* K-12 (ATCC 23716) was grown aerobically overnight (13–16 hr) in VB medium at 37°C on a rotary shaker at 200 rpm. The overnight cultures were diluted 100-fold, unless otherwise specified, into TSY medium plus 0.25% glucose in Delong flasks (flask vol:medium vol, 10:1), which were shaken aerobically at 200 rpm at 37°C for 2 hr. This provided the mid-log phase cells used for all experiments.

Cold Shock. Mid-log phase cultures, at a cell density of 1×10^5–3×10^7 colony-forming units (CFU)/ml, were chilled by dilution into ice-cold VB salts and were held in an ice bath for the specified interval before dilution with cold VB salts and plating onto TSY agar. Plates were incubated for 16–18 hr at 37°C either anaerobically in a Coy chamber under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2, or aerobically, before enumeration of colonies.

Results

Effects of Alcohols in Cold Shock. *E. coli*, in the mid-log phase of growth in TSY medium at 37°C, were diluted 30-fold into VB salts at a variety of temperatures ranging from 0 to 37°C. After 30 min at each temperature, the cells were plated onto TSY agar for enumeration of colonies. The data in Figure 1 demonstrate that cold shock became pronounced below 15°C and that the loss of viability plateaued in the 0–10°C range. Subsequent experiments were done by dilution into ice-cold VB salts in an ice bath.

Alcohols added to the cold diluent protected against the loss of viability associated with cold shock in a dose-dependent manner. Of course, the alcohols are also capable of exerting a toxic effect that increases with chain length (25), so that an optimum concentration for protection would be expected. The data in Figure 2 demonstrate that the concentration of alcohol needed for optimal protection decreased as the chain length of the alcohol increased. The optimal concentra-

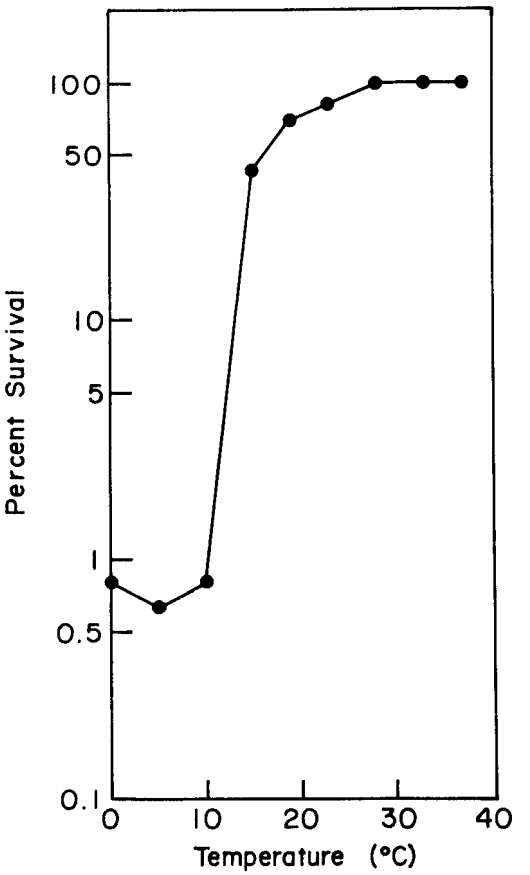

Figure 1. Effect of temperature of diluent on viability. Mid-log cultures of *E. coli* K12 at 1.0×10^5 CFU/ml were diluted 30-fold into VB salts maintained at the indicated temperature. After 30 min of incubation at each temperature, the cell suspensions were plated and counted.

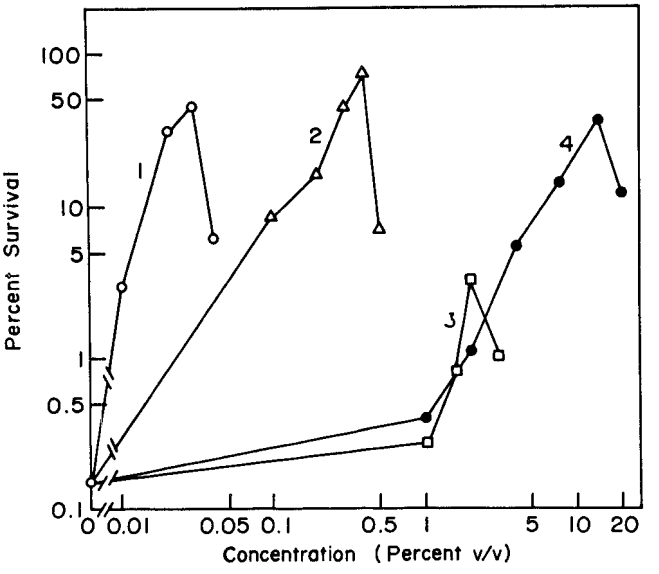

Figure 2. Alcohols protect against cold shock. Mid-log cultures at 3×10^6 CFU/ml were diluted 30-fold into VB salts containing the indicated concentrations of each alcohol at 0°C. After 40 min in the ice bath, the samples were diluted and plated. The alcohols used were Line 1, *n*-octanol; Line 2, *n*-hexanol; Line 3, *tert*-butanol; and Line 4, ethanol.

tions were *n*-octanol, 0.03%; *n*-hexanol, 0.4%; *tert*-butanol, 2%; and ethanol, 14%. Striking protections were seen at the optimal levels of ethanol, *n*-hexanol, and *n*-octanol, but not with *tert*-butanol, presumably due to the greater toxicity of the latter alcohol. Because the cells were not exposed to the alcohols until the moment of cold shock, protection could not have been due to effects of the alcohols on protein synthesis or on intermediary metabolism.

The longer the period of incubation of the cells in the cold diluent, the greater the loss of viability (Fig. 3). This is consistent with the creation of channels in the cell membranes, followed by loss of viability due to progressive leakage of cell components. This view is supported by the report that dense cell suspensions appeared to be less susceptible to cold shock than did dilute cell suspensions, and that the supernatant from a cold-shocked dense cell suspension protected a dilute cell suspension against cold shock (7). The data in Figure 3 also show that *n*-octanol, added at 0.03%, sharply decreased the rate of loss of viability, whether present in the cold diluent from the outset or added after the cold shock was underway, but the protective effect was greatest when the *n*-octanol was present from the outset.

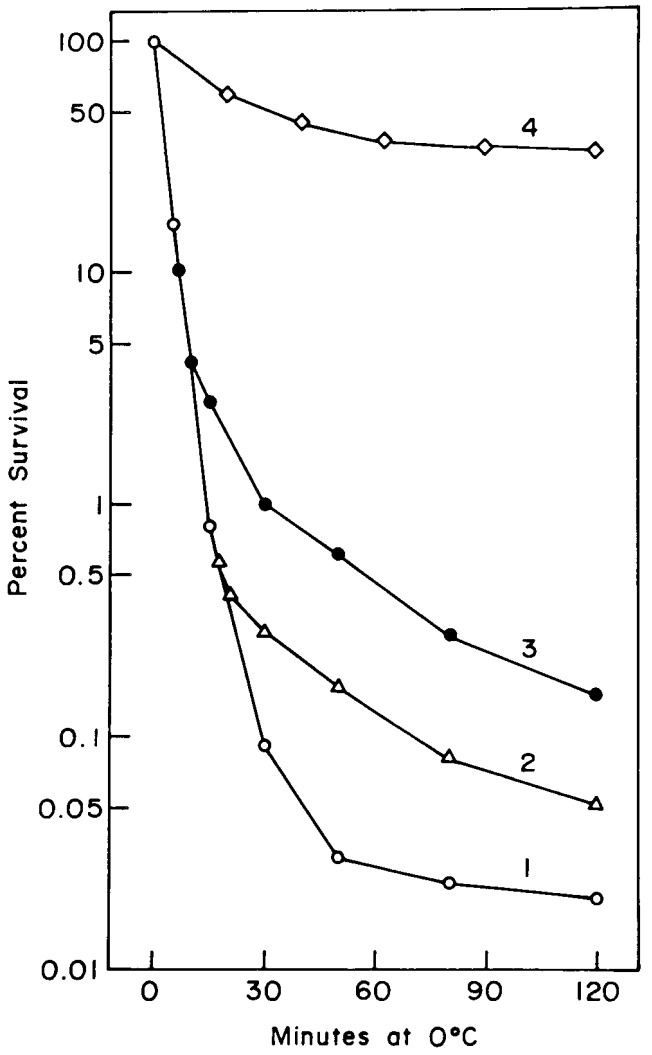

Figure 3. Effect of n-octanol on rate of loss of viability after cold shock. Mid-log cultures at 1 × 10^7 CFU/ml were diluted 30-fold into VB salts at 0°C. At intervals thereafter, aliquots were removed for dilution, plating, and counting. In each case, the diluent was identical to the medium in which the cells were already suspended; thus, dilution did not entail any change in composition or temperature of the suspending medium. Line 1, no additions; Line 2, *n*-octanol added to 0.03% 17 min after cold shock; Line 3, *n*-octanol added to 0.03% at 6.5 min after cold shock; and Line 4, *n*-octanol present at 0.03% at the time of cold shock.

Does Dioxygen Influence Cold Shock? Log phase *E. coli* in TSY medium were cold-shocked by 100-fold dilution into ice-cold VB salts. At intervals, the cold suspension of cells was sampled by diluting and plating, in duplicate, onto TSY agar. These plates were incubated overnight either aerobically or anaerobically at 37°C before enumeration. The results in Figure 4 show substantially higher counts of viable cells on the anaerobic plates. This could have been due to a deleterious effect of dioxygen exerted either during cold shock, or on the agar plates after cold shock. Cold shock was, therefore, performed with aerobic or anaerobic ice-cold diluent, followed by sampling at intervals and plating onto aerobic TSY plates. The Coy chamber was used to achieve truly anaerobic cold shock. The rate of loss of viability was the same with aerobic as with anaerobic cold diluent (data not shown). This indicates that dioxygen exerts some deleterious effects after the cold-shocked cells have been plated onto the TSY agar at 37°C.

MacKay and Derrick (26) noted greater viable counts when cold-shocked cells were plated on a glucose-minimal agar, as compared with a TSY agar, and concluded that the very low levels of H_2O_2 (12–13 n*M*) present in the TSY agar were the cause of this effect. We therefore tested the effect of plating 100 μl of a 0.1 mg/ml sterile solution of catalase onto the TSY agar, before plating the cold-shocked *E. coli*. Catalase on the TSY agar did not increase aerobic enumeration of cold-shocked cells (data not shown). The antioxidants α-tocopherol and probucol were dissolved to 5.0 m*M* in ethanol, and 50 μl of these solutions were similarly plated onto TSY agar 1 hr before the plating of the cold-shocked cells. These antioxidants also failed to increase aerobic enumeration of cold-shocked cells. The diminution of enumeration caused by aerobic incubation of the plates (Fig. 4) thus appears not to reflect auto-oxidation of components of the TSY agar.

If cold shock involves phase transitions in the cell membranes, which contain the electron transport assemblies of *E. coli*, it seemed possible that cold shock might influence both total respiration and the cyanide-resistant fraction of that respiration. Logarithmic phase *E. coli* were cold-shocked by 7.5-fold dilution into VB salts in an ice bath. At intervals thereafter, 25-ml ali-

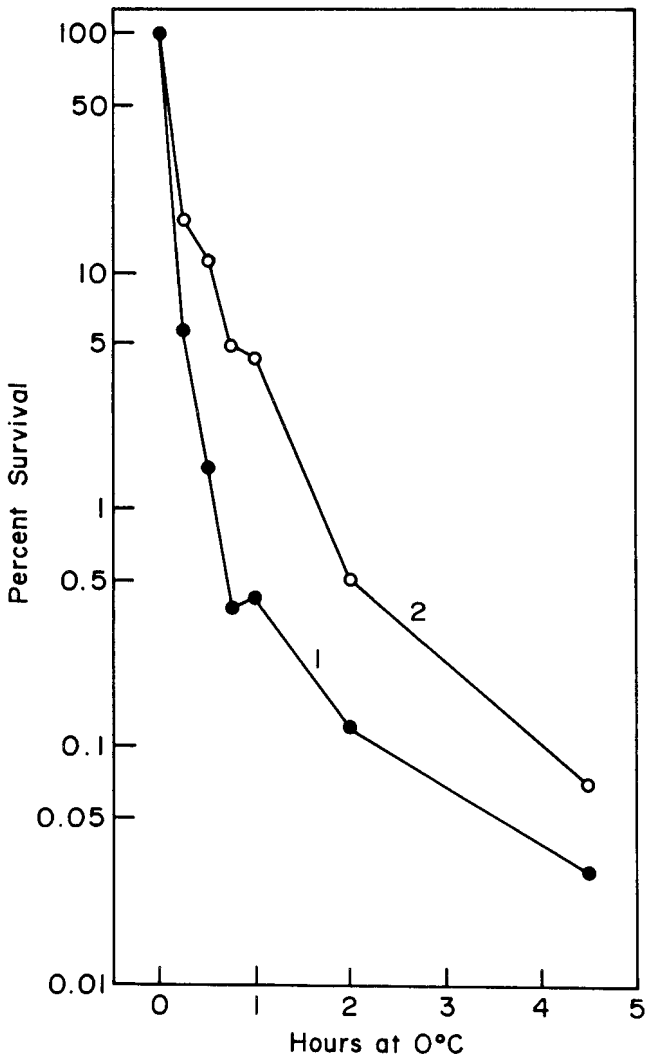

Figure 4. Effect of dioxygen on enumeration after cold shock. Mid-log cultures at 3×10^7 CFU/ml were diluted 100-fold into VB salts at 0°C. At intervals, samples were diluted and plated. The plates were incubated either aerobically (Line 1) or anaerobically (Line 2) before enumeration.

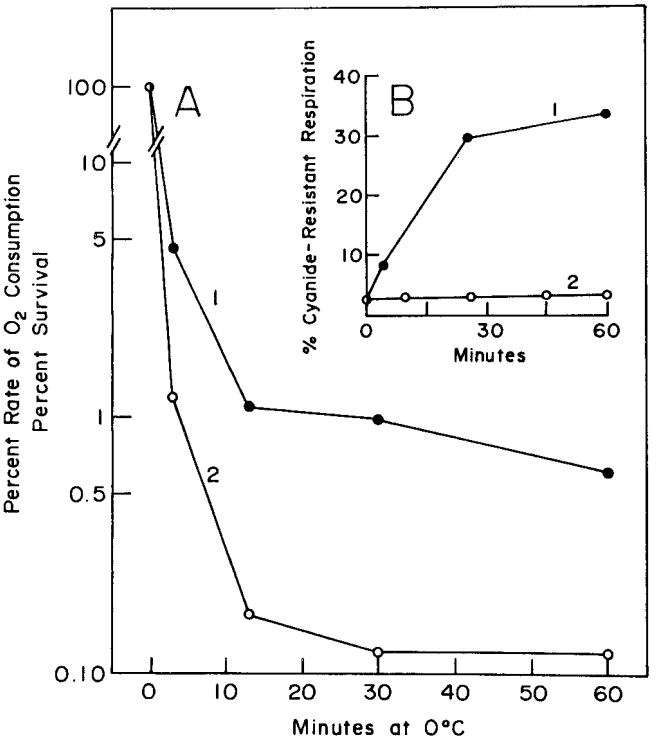

Figure 5. Effects of cold shock on survival and respiration. (A) In order to have enough cells for measurements of respiration, a 7% inoculum of an overnight culture was placed into TSY plus 0.25% glucose and, after 2 hr at 200 rpm and 37°C, cold shock was imposed by 7.5-fold dilution into ice-cold VB salts. At intervals, 25-ml aliquots were removed, centrifuged, and resuspended in 0.25 ml of VB salts at 0°C. This dense cell suspension was used for measurement of respiration by addition of 200 μl of the suspension into 1.7 ml of VB medium at 37°C under a Clark electrode (●) Line 1, and it was also used for dilution and plating (○) Line 2. (B) Since greater cell density was needed for measurement of cyanide-resistant respiration, mid-log cultures prepared as described in A were cold-shocked by only 5-fold dilution into VB salts at 0°C, and 250-ml aliquots of the cold-shocked cell suspension were collected by centrifugation and taken up in only 0.3 ml of VB salts. This dense cell suspension was used for measurement of respiration by dilution into VB medium at 37°C containing 1.0 m*M* cyanide. Line 1, minutes at 0°C after cold shock and Line 2, minutes at 37°C without cold shock.

quots were taken, and the cells were collected by centrifugation and were resuspended in 0.25 ml of VB salts. Measurements of A_{600nm} indicated no loss of cells during this procedure. The resultant, relatively dense suspensions were used for both measurement of rate of respiration (200 μl into 1.7 ml of VB medium at 37°C) and enumeration. The data in Figure 5A show that cold shock was accompanied by a rapid loss of respiratory capacity, which initially paralleled the loss of viability, but which plateaued after long periods of incubation in the cold diluent, reflecting the retention of respiratory activity by disrupted membranes. The capacity for cyanide-resistant respiration did not fall as rapidly as did the net respiration. Consequently, cyanide-resistant respiration, expressed as a percentage of total respiration, increased after cold shock, as shown in Figure 5B. Since CN^--resistant respiration is likely to be associated with production of O_2^- and H_2O_2, it appears possible that the increased dioxygen sensitivity of cold-shocked cells on agar plates may be due to increased intracellular production of O_2^- and H_2O_2, compounded by leakage of defensive molecules, such as reduced glutathione and superoxide dismutase.

Effects of EDTA. Divalent cations are thought to stabilize the membranes of *E. coli*, and treatment with EDTA increases the sensitivity of *E. coli* toward hydrophobic antibiotics (27). The strong binding of Ca^{2+} to lipopolysaccharide (28) and the ability of divalent cations to protect against cold shock (5) suggested that removal of bound divalent cations with EDTA should sensitize *E. coli* to cold shock. The data in Figure 6 affirm this expectation. Thus, 0.5 m*M* EDTA present in the cold diluent markedly increased both the rate and extent of loss of viability after cold shock (compare

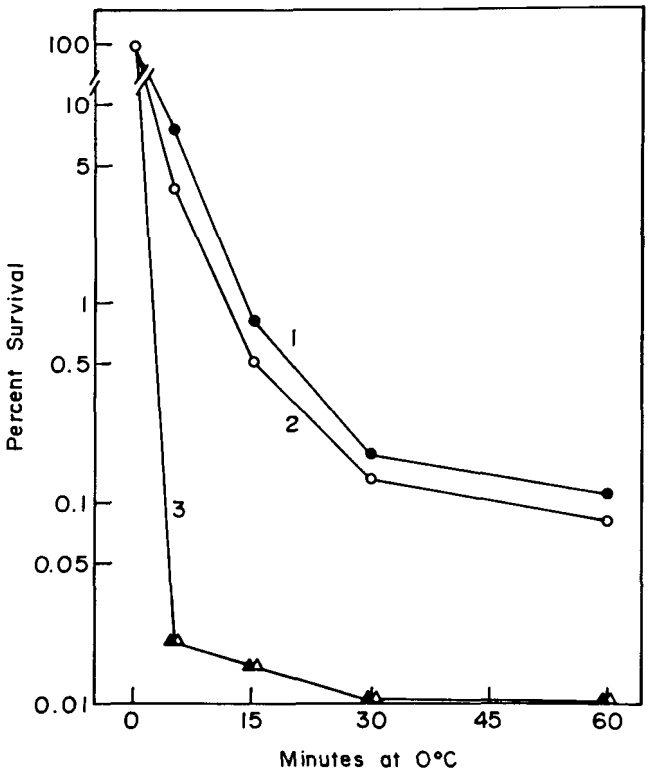

Figure 6. Effect of EDTA on cold shock. A mid-log culture at 3 × 10^6 CFU/ml was diluted into VB salts at 0°C without (Lines 1 and 2) or with (Line 3) 0.5 m*M* EDTA. At intervals after cold shock, aliquots were taken for dilution and plating onto TSY agar lacking (Lines 1 and 3) or containing (Lines 2 and 3) 0.5 m*M* EDTA. The solid points on Line 3 denote plates without EDTA, and the open points denote plates containing EDTA.

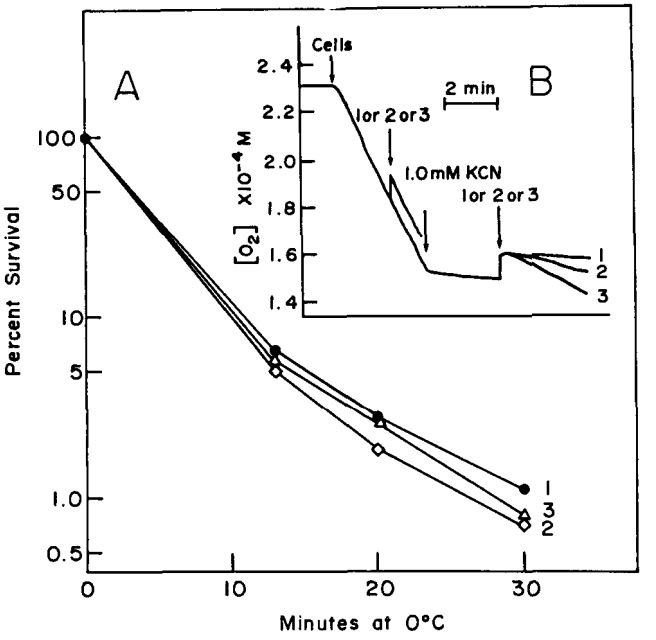

Figure 7. Effect of protonophores on cold shock and on cyanide-resistant respiration. (A) Mid-log cultures at 1 × 10^7 CFU/ml were diluted 90-fold into VB salts at 0°C and, at intervals thereafter, samples were taken for dilution and plating. Line 1, control culture; Line 2, FCCP added to 10 μM of the culture 1.5 min before cold shock; and Line 3, FCCP added to 10 μM 3.0 min before cold shock. (B) Mid-log cultures obtained as described in legend of Figure 5 were concentrated by centrifugation, followed by resuspension in 1/7 the original volume of VB salts at 25°C. Respiration was measured by diluting 70 μl of this dense cell suspension into 1.7 ml of VB medium at 37°C under a Clark electrode. At the second arrow, 2,4-dinitrophenol was added to 1.0 m*M*, or FCCP was added to 10 μM, or ethanol was added to 1%, as a control for the ethanol added as the solvent for the protonophores. At the third arrow, cyanide was added to 1.0 m*M*. At the fourth arrow, ethanol was added to 1% (Line 1), 2,4-dinitrophenol to 1.0 m*M* (Line 2), and FCCP to 10 μM (Line 3).

Lines 1 and 3, Fig. 6). The effect of EDTA was exerted during cold shock, rather than subsequently, because adding 0.5 m*M* EDTA to the TSY agar plates only slightly *decreased* enumeration (compare Lines 1 and 2, Fig. 6) Moreover adding EDTA to the TSY agar plates did not change survival of cells which had been cold-shocked in the presence of EDTA (compare open and filled data points on Line 3, Fig. 6).

Modification of Membrane Electrochemical Potential. The membrane potential should be greater in log phase cells, which are susceptible to cold shock, than in stationary phase cells, which are not susceptible. We examined the possible role of the membrane electrochemical potential in cold shock by using protonophores to discharge the proton gradients, before cold shock. Figure 7B demonstrated that 2,4-dinitrophenol or carbonyl cyanide trifluoromethoxyphenylhydrazone (FCCP) rapidly gained entry into *E. coli*. Thus, although these compounds did not affect respiration of log phase cells, they did increase cyanide-resistant respiration within 15 sec. FCCP at 10 μM was more effective in this regard than was 1.0 m*M* 2,4-dinitrophenol. The possibility that this effect of FCCP was due to its reacting with and thus decreasing the concentration of cyanide could be discounted, because the reaction mixtures contained a 100-fold excess of cyanide over FCCP. Figure 7A demonstrated that susceptibility toward cold shock was unaffected when FCCP was added to the cell suspensions 1.5 min or 3.0 min before cold shock. Similar results were obtained with 2,4-dinitrophenol (data not shown). We conclude that the greater susceptibility of log phase cells toward cold shock is not explicable on the basis of greater membrane electrochemical potential.

Is Minimal Medium Recovery Seen after Cold Shock? Damage to DNA, whether imposed by ultraviolet or ionizing radiation, can lead to cell death, unless it is repaired. This need for DNA repair is the basis for the much explored phenomena of minimal medium recovery and liquid holding recovery (29, 30). In the case of minimal medium recovery, irradiated cells show higher enumeration when plated on minimal, rather than on rich agar; whereas in the case of liquid holding recovery, holding irradiated cells in buffer before plating increases enumeration. In either case, the relative delay in growth caused by the minimal medium, or the temporary cessation of growth during incubation in

buffer, allows time for DNA repair before the cells are called upon to replicate their genome.

Because cold shock has been reported to cause single strand breaks in the DNA of *E. coli* (28), it seemed worthwhile to see whether cold-shocked cells exhibit liquid holding recovery. Mid-log cultures of *E. coli* in TSY at 37°C were cold-shocked by 30-fold dilution into ice-cold VB salts, followed by 30 min of incubation at 0°C. Aliquots of these cold-shocked cells were then diluted 10-fold into (i) VB salts at 0°C; (ii) VB salts at 30°C; or (iii) TSY at 30°C. At intervals thereafter, the cell suspensions were sampled for plating and enumeration onto TSY agar. The data in Figure 8 demonstrate that recovery was seen only in the rich medium (TSY) at 30°C (Line 1, Fig. 8), while there was actually more rapid loss of viability at 30°C in VB salts than there was at 0°C in this medium (Lines 3 and 2, Fig. 8). These results do not point to an important role for DNA repair in recovery from cold shock.

Discussion

Our data provide further support for the view (6–8, 14, 15) that cold shock is due to permeabilization of cell membranes imposed by an ordering phase transition. Thus, alcohols which, by partitioning into the membrane, can increase their fluidity (23, 24) and so prevent freezing, protected against cold shock when present at the instant of cold shock. As expected, from this view of their action, the more hydrophobic the alcohol the less of it was required for optimal protection. Alcohols were also able to partially reverse the effect of cold shock and to thus slow the rate of loss of viability when added after the cold shock. Of course, excessive fluidization of the membrane could itself have deleterious consequences, and for each alcohol examined, there was a range of concentration within which protection against cold shock was seen, and a higher range within which toxicity became evident.

The loss of viability after cold shock was time dependent at the ice bath temperature and was transiently accelerated by warming, as would be the case were it due to effusive loss of materials from the permeabilized cells. This effect of warming also implies that the permeabilization, imposed by cold shock, is only gradually repaired when the phase transition is reversed by warming.

Although cold shock itself was uninfluenced by the presence of dioxygen, the cold-shocked cells evidenced an increased sensitivity toward dioxygen. This was seen as a decrease in enumeration on aerobic, as compared with anaerobic, plates. This did not appear to be due to H_2O_2 produced by auto-oxidation of the rich TSY medium (26), inasmuch as addition of catalase to the TSY agar plates did not protect. It seems likely that cells permeabilized by cold shock will lose glutathione and reduced pyridino coenzymes, and possibly also enzymic defenses against oxygen toxicity, such as superoxide dismutases, hydroperoxidases, and alkyl hydroperoxide reductase. Such losses could account for the dioxygen sensitivity imposed by cold shock.

Why are cells growing rapidly in log phase uniquely susceptible to cold shock? This could be answered on the basis of changes in the structure and/or composition of the membrane during the transition from log to stationary phase, or due to a change in the transmembrane electrochemical potential. We eliminated the latter possibility by showing that protonophores, at concentrations which increase cyanide-resistant respiration, have no effect on susceptibility to cold shock. This leaves the former possibility.

One peculiar aspect of cold shock is the critical role of the rate of chilling (2, 4, 6). Thus, Leder (6) noted that chilling *E. coli* from 25 to 0°C caused at least 95% loss of permease-accumulated substrates, if chilling was accomplished within 1–2 sec, but caused no detectable loss when carried out over 60 sec. The inner and outer leaflets of cell membranes differ markedly in composition. Thus, by way of example, the outer leaflet of the outer membrane of *Salmonella typhimurium* is rich in lipopolysaccharide, but contains very little phospholipid, whereas the inner leaflet is rich in phospholipid (19). Patches of the inner and outer leaflets of the cell

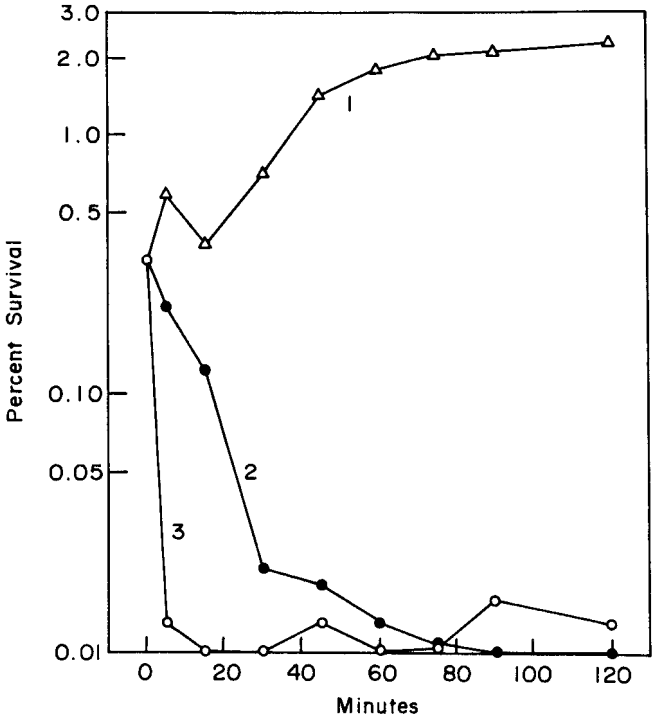

Figure 8. Effect of liquid holding recovery after cold shock. E. coli K-12 in mid-log phase and at 3×10^7 CFU/ml were cold-shocked by 30-fold dilution into VB salts at 0°C. After 30 min, aliquots of the ice-cold cell suspension were diluted 10-fold into TSY at 33°C (Line 1); VB salts at 33°C (Line 3); or into VB salts at 0°C (Line 2). At intervals, samples were taken and diluted, as described in Figure 3, for plating and counting.

membranes could, therefore, have different freezing points. Gradual freezing of a patch in one leaflet would favor freezing of the corresponding region in the other leaflet. This could be accomplished by migration within this leaflet of lipids capable of freezing at the imposed temperature. The result would be matched frozen patches in both leaflets. In contrast, sudden chilling would not allow time for this in-plane diffusion. The result would be frozen patches in one or the other leaflet, unmatched by mating frozen patches in the opposite leaflet. Because freezing surely occurs with change in volume, and therefore in area, unmatched freezing would cause buckling and perhaps rending of the membrane. These possibilities need to be further explored both theoretically and experimentally.

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