The Release of Enzymes by Osmotic Shock from 
*Escherichia coli* in Exponential Phase

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SUMMARY

A number of degradative enzymes are specifically released from exponentially growing *Escherichia coli* by osmotic shock. They include alkaline phosphatase, cyclic phosphodiesterase, 5'-nucleotidase, acid phosphatase, and the ribonucleic acid-inhibited endonuclease that is active with deoxyribonucleic acid. Small amounts of RNase are also set free, but some 16 control enzymes are found to remain within the cell. Only about 4% of the cellular protein is released into the shock medium.

The procedure for osmotic shock has been modified in order to obtain about 80% viability with exponential cells, with good release of enzymes. In the first stage, cells are exposed to 20% sucrose-1 × 10⁻⁴ M EDTA. In the second stage, the pellet of sucrose-treated cells is rapidly dispersed in cold 5 × 10⁻⁴ M MgCl₂, which causes the enzymes to be liberated.

At least 90% of the DNA endonuclease and 50% of the latent ribonuclease are released from exponentially growing *E. coli* when as much as 2 × 10⁻³ M MgCl₂ is used in the procedure for osmotic shock.

In the present investigation we have carried out a more detailed examination of the procedure as applied to cells in exponential phase. Growing cells were found to be more susceptible to osmotic shock than was generally true for stationary cells. Accordingly, the procedure has been modified so that good release of enzymes is consistently obtained with high viability. The kinetics of release has been studied, and the process was found to be completed within about 1 min. The method has been applied to several other gram-negative organisms, and additional properties of the released enzymes are reported.

EXPERIMENTAL PROCEDURE

Materials

*E. coli* soluble RNA was purchased from General Biochemicals. Bis(p-nitrophenyl)phosphate, o-nitrophenyl phosphate, isopropyl β-D-thiogalactopyranoside, o-nitrophenyl galactoside, and various nucleotides were also obtained from commercial sources. Highly polymerized sperm DNA, used as carrier, was purchased from Calbiochem. Tritium-labeled DNA from *E. coli* K-12 (λ) was the generous gift of Dr. Arthur Weissbach. Crystalline lysozyme (muramidase) was purchased from Worthington.

Strains of *E. coli*—Strain K-17, kindly supplied by Dr. N. D. Zinder (12), had been derived, by an indirect route, from *E. coli* K-10. It showed an especially high content of cyclic phosphodiesterase and acid phosphatase. Strain C90, a mutant constitutive for alkaline phosphatase, and U7 (13), a mutant with a deletion for alkaline phosphatase, were gifts from Dr. A. Garen. Strains B, W, and K-12 were also used in this work.

Methods

Growth of Cells—Stock cultures of *E. coli* were maintained on nutrient agar slants.

The low phosphate medium (Medium A) contained 0.12 m Tris, 0.08 m NaCl, 0.02 m KCl, 0.02 m NH₄Cl, 0.003 m Na₂SO₄, 0.001 m MgCl₂, 2 × 10⁻⁴ m CaCl₂, 2 × 10⁻⁴ m ZnCl₂, and 0.5% Difco Bacto-peptone, adjusted to pH 7.5 (14). Medium B consisted of 0.04 m K₂HPO₄, 0.022 m KH₂PO₄, 0.08 m NaCl, 0.02 m NH₄Cl, 0.003 m Na₂SO₄, 0.001 m MgCl₂, 2 × 10⁻⁴ m CaCl₂, 2 × 10⁻⁴ m ZnCl₂, and 0.5% Difco Bacto-peptone, adjusted to pH 7. The media were supplemented with 0.6% glycerol, 0.6% glucose, or 1.1% sodium succinate. Isopropyl β-D-thiogalactopyranoside was used to induce β-galactosidase in the inducible strains. Organisms were incubated at 37°C on a rapid rotary shaker.
Procedure for Osmotic Shock of Cells in Exponential Phase—The method involved suspending bacterial cells, first in sucrose-EDTA solution, and then in $5 \times 10^{-4}$ M MgCl$_2$. A set of enzymes were selectively released into the MgCl$_2$ solution. In the usual procedure, *E. coli* were harvested at a cell density of about $5 \times 10^8$ per ml, and the sedimented cells were washed twice with about 40 volumes of cold 0.01 M Tris-HCl (pH 7.1) and 0.03 M NaCl. One gram (wet weight) of cells was suspended in 40 ml of 0.033 M Tris, pH 7.1, at 24°C. A small sample was removed for the preparation of enzyme assays, before the suspending medium was made up to 20% in sucrose.

Stage I of the procedure involved the addition, with rapid stirring, of 40 ml of 40% sucrose-0.033 M Tris-HCl, pH 7.1, followed by sufficient 0.1 M Na$_2$EDTA, pH 7.1, to give a concentration of $1 \times 10^{-4}$ M. The mixture was stirred on a rotator shaker for 10 min at 24°C (about 180 rpm) and centrifuged for 10 min at 13,000 × g in a cold room. The supernatant fluid was removed.

In Stage II, the well drained pellet was rapidly dispersed in 80 ml of ice-cold, $5 \times 10^{-4}$ M MgCl$_2$ solution. A glass stirring rod with a rubber policeman was convenient for this purpose. The suspension was gently stirred in an ice bath for 10 min and centrifuged, and the supernatant fluid, containing the released enzymes, was removed.

A sample was taken for measurement of viability immediately before centrifugation. Viability was determined on serial dilutions of cells in tryptone broth, which were plated on tryptone agar containing 0.5% NaCl.

Sonic extracts were made by treatment for 90 sec with a Branson Sonifier, model 1TS5. Extracts were also prepared by successively treating cell suspensions ($8 \times 10^8$ cells per ml) in 0.03 M Tris, pH 7.1, for 10 min at 37°C with CHCl$_3$, 20 μg per ml of lysozyme, and 2 μmoles per ml of EDTA. These extracts were made from washed but unshocked cells.

Enzyme Assays—Previously published methods were used for measuring RNase (15, 16), β-galactosidase (17), alkaline phosphatase (18), cyclic phosphodiesterase (1), and acid phosphatase (1).

The assay for 5'-nucleotidase (1) was modified so that the reaction mixture (0.1 ml) contained 10 μg of bovine serum albumin, 0.4 μmole of 5'-AMP, 0.5 μmole of CoCl$_2$, 1.6 μmoles of CaCl$_2$, and 8 μmoles of sodium acetate buffer, pH 5.8. Enzyme dilutions were made in 0.05 μ acetate buffer containing 0.1 mg of albumin per ml. Activities, except for those of the nucleases, are expressed as micromoles hydrolyzed per hour. The DNA endonuclease (11, 19) was assayed by a procedure similar to that of Weissbach and Korn (19), but with a high level of RNase as recommended by Shortman and Lehman (20). The reaction mixture contained [H]-labeled *E. coli* K-12 λ DNA (0.1 absorbance unit, 106 cpm); 30 μmoles of Tris-HCl buffer, pH 7.5; 1 μmole of MgCl$_2$; 1 μmole of β-mercaptoethanol; and 50 μg of pancreatic RNAse (previously heated for 10 min at 90°C) in a volume of 0.2 ml. It was important to destroy endogenous RNA, which inhibits the endonuclease. After an incubation period of 30 min at 37°C, carrier sperm DNA (0.2 ml, 2.5 mg per ml) and 0.1 ml of cold 6% HClO$_4$ were added. An aliquot (0.4 ml) of the supernatant solution was mixed with 10 ml of Bray's solution (21) and 0.04 ml of 2 N NH$_4$OH and counted in a Packard Tri-Carb liquid scintillation spectrometer. This assay was linear with enzyme concentration when less than 10% of the substrate had been solubilized. A unit was taken as that amount of enzyme catalyzing the solubilization of 1000 cpm in 30 min.

For DNA exonuclease I (22), the reaction mixture (0.3 ml) contained 20 μmoles of glycine buffer, pH 9.5; 2 μmoles of MgCl$_2$; 8 μmoles of *E. coli* soluble-RNA; and heated *E. coli* K-12 (μg DNA (0.1 absorbance unit, 106 cpm). Incubation and further treatment were as described for the endonuclease. A unit was that amount of enzyme catalyzing the solubilization of 1000 cpm in 30 min. Addition of soluble-RNA increased the specificity of the assay for exonuclease I because it inhibited endonuclease activity.

In assays for adenosine deaminase activity, the reaction products were separated by paper chromatography and quantitatively eluted. The assay for UDP-glucose pyrophosphorylase was carried out according to Kornfeld, Kornfeld, and Ginsburg (23). Protein was determined according to Lowry et al. (24).

RESULTS

Conditions for Osmotic Shock of Cells in Exponential Phase

Effect of Variations in Composition of Shock Medium—In previous work (1) *E. coli* in stationary phase were treated with sucrose-EDTA-Tris solution (Stage I), after which the pellet of cells was quickly dispersed in cold water to cause the specific release of certain enzymes (Stage II). Further work indicated that distilled water was as satisfactory as other media for cells in stationary phase but was poorly tolerated by exponentially growing cells. Much better results were obtained with dilute MgCl$_2$ solutions as the shock medium in Stage II, as shown by greater viability of cells, a shorter lag period before resumption of growth, and a smaller loss of material absorbing at 260 mμ (Tables I and II and Fig. 1). Lysis of cells, as judged by escape of β-galactosidase, was usually about 1 to 2% for cells treated with MgCl$_2$ and substantially higher after cold water shock. The MgCl$_2$ could not be replaced by 2 × 10^{-3} M NaCl. A concentration of $5 \times 10^{-4}$ M MgCl$_2$ was sufficient for good results and no advantage was gained by the further addition of Ca$^{2+}$, Zn$^{2+}$, or Co$^{2+}$ (Tables I and II). When the concentration of MgCl$_2$ was as high as $5 \times 10^{-3}$ M, the release of cyclic phosphodiesterase and of 5'-nucleotidase was occasionally reduced.

Effect of Different Concentrations of EDTA—In Stage I of the procedure for osmotic shock, *E. coli* were treated with a solution containing EDTA, sucrose, and Tris buffer. The specific release of enzymes was as great with $1 \times 10^{-4}$ M EDTA as when higher concentrations were used; in fact, with strain K-37 the yield of enzymes was reduced by only 30% in the absence of EDTA. Cells in exponential phase appeared to be unusually sensitive to this agent; the lag period before recovery was definitely prolonged with $3 \times 10^{-4}$ and $1 \times 10^{-3}$ M EDTA (Table II). Even greater differences were obtained with strain K-12; the lag period was 55 min with $1 \times 10^{-4}$ M EDTA, 80 min with $3 \times 10^{-4}$ M EDTA, and in excess of 3 hours with $1 \times 10^{-3}$ M EDTA.

Effect of Other Conditions on Release of Enzymes from Exponential Cells by Osmotic Shock—The specific release of cyclic phosphodiesterase and 5'-nucleotidase was almost as great when 51 mle sucrose was used in Stage I as with higher concentrations (Table I). In other experiments this was confirmed even in the presence of as little as $1 \times 10^{-4}$ M EDTA, and similar results were obtained when acid phosphatase was measured. However, in the absence of sucrose at Stage I there was little or no release of these enzymes during subsequent treatment with cold water or MgCl$_2$ solution.
Effect of different conditions on release of enzymes by osmotic shock and on recovery of shocked cells

_E. coli_ K-12 were grown in Medium B, with 0.6% glycerol, to an optical density of 0.34 at 600 nm (approximately 5 x 10^8 cells per ml). The cells were quickly chilled, harvested, and washed twice with 40 to 50 parts of cold 0.01 M Tris (pH 7.1)-0.03 M NaCl.

For Stage I of the procedure, the pellet of cells was suspended in parts of a solution composed of 0.083 M Tris (pH 7.1), 1 x 10^-4 M EDTA, and sucrose, as indicated in Column 2. After being stirred for 10 min at 24°, the mixture was centrifuged, and the supernatant medium was removed and saved for enzyme assays.

For Stage II, the pellet was rapidly dispersed in 80 parts of cold shock medium (composition in Column 2), and the suspension was stirred at 2° for 10 min. A sample was removed to measure viability or rate of recovery of growth in tryptone broth (see "Methods" and the legend to Fig. 1). The remaining suspension was centrifuged, and enzymes released into the supernatant shock medium were measured. Assays are presented as units of enzyme released per g (wet weight) of cells. Blank spaces in the tables mean that no assay was carried out. The tables show release of material in the supernatant fluid when cells were centrifuged in Stage I or Stage II; also shown are data on equivalent extracts of unshocked cells.

<table>
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<th>Suspension medium</th>
<th>Cyclic phosphodiesterase</th>
<th>5'-Nucleotidase</th>
<th>Acid phosphatase</th>
<th>Total A260 release</th>
<th>Protein release</th>
<th>β-Galactosidase</th>
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- Medium A contained 1 x 10^-3 M MgCl_2, 2 x 10^-4 M CaCl_2, 1 x 10^-4 M ZnCl_2, and 1 x 10^-4 M CoCl_2.

See "Methods."

This sonic extract was incubated for 60 min before addition of substrate (see the text).

Experiments of shocked cells showed 135 units remaining.

Experiments of shocked cells showed 50 units remaining.

Replacement of sucrose by 0.4 M NaCl in Stage I resulted in maximal release of 5'-nucleotidase during subsequent osmotic shock in strain U7 and also proved effective in promoting release of alkaline phosphatase from strain C90.

The viability of exponential cells was reduced by 40% when Tris-HCl buffer, pH 8 (measured at 24°), rather than buffer at pH 7.1, was used in the procedure for osmotic shock.

Viability Studies—Viability of cells shocked with cold water was only about 50% when compared with washed cells that had not been shocked (Table 1). With dilute MgCl_2 as the shock medium, viability was consistently 70 to 80% and occasionally as high as 90%. It is possible that these figures are low because the cells have a tendency to stick together and may not separate perfectly on dilution.

Results with Other Strains—Quite similar observations were noted with other strains of _E. coli_, including K-12, B, and W.

Effect of Spermine—In view of the beneficial effect of MgCl_2 when present in the shock medium, it was of interest to try spermine. In a number of experimental situations, such as the stabilization of spheroplasts (25, 26), polyamines and Mg++ have similar effects. However, when Stage II of osmotic shock was carried out with cold 1 x 10^-3 M spermine tetrahydrochloride and cells were subsequently diluted 25-fold in tryptone broth, the lag period before resumption of growth was much longer than that
Release of Enzymes by Osmotic Shock after cold water shock (Fig. 2). In the experiment shown here the lag period after cold water shock was unusually short, but the effect of spermine was repeatedly demonstrated under the more usual conditions where water itself delayed recovery. The effect of spermine was partially overcome by 1 X 10^{-3} M MgCl₂. Control, unshocked cultures in tryptone broth, to which as high a concentration of spermine as 1 X 10^{-3} M was added, showed no inhibition in rate of growth. A suspension of cells in 40% sucrose-0.033 M Tris, pH 7.1, was treated with 1 X 10^{-3} M spermine and directly diluted into tryptone broth; here again spermine had no deleterious effect on rate of growth. It is presumed that shocked cells may take up unusually large amounts of spermine, a compound which has been shown to be toxic for bacterial cells (27).

Release of DNase—A substantial fraction of the RNA-inhibited endonuclease of E. coli B was released both into the sucrose-Tris-EDTA solution (Stage I) and into the shock medium (Stage II) when exponentially growing cells were subjected to the standard procedure for osmotic shock (Table III). This behavior was unusual because no more than a small fraction of the other released enzymes was found in the sucrose solution; almost all of the activities were liberated during subsequent osmotic shock (Stage II). The release of DNase into the sucrose solution was stimulated by EDTA. Under the same conditions less than 2% DNA exonuclease I (22) was liberated. It should be mentioned that the RNA-inhibited endonuclease was also set free in biomass.

| TABLE II  |
| Effect of concentration of EDTA on behavior of exponential cells subjected to osmotic shock |

E. coli B were grown in Medium B, with 0.6% glycerol. Experimental conditions were as in Table I, with the following exceptions. In Stage I cells were suspended in a medium composed of 20% sucrose, 0.033 M Tris (pH 7.1), and 1 X 10^{-4} M EDTA (Experiment 1); 3 X 10^{-4} M EDTA (Experiment 2); or 1 X 10^{-3} M EDTA (Experiment 3). In Stage II cells were quickly dispersed in different shock media, whose composition is given below. In Stage I, release of enzymes, in units per g (wet weight) of cells, did not exceed 300 for cyclic phosphodiesterase, 200 for 5'-nucleotidase, and 20 for acid phosphatase.

<table>
<thead>
<tr>
<th>Shock medium</th>
<th>Cyclic phosphodiesterase</th>
<th>5'-Nucleotidase</th>
<th>Acid phosphatase</th>
<th>Total Ave</th>
<th>Total protein</th>
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<td>MgCl₂</td>
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<td>Extract, intact cells</td>
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<td>1900</td>
<td>220</td>
<td>20</td>
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</table>

* Leakage of β-galactosidase was 10% after water shock and 2% after MgCl₂ shock.
* Only 1% of this amount of activity remained in shocked cells.
* 5'-Nucleotidase in extracts of shocked cells varied from 5 to 10% of these amounts.
* Made by treatment with CHCl₃, lysozyme, and EDTA (see "Methods").
...old when *E. coli* were converted into spheroplasts by treatment
with lysozyme and EDTA (28).4

To our surprise, the release of DNase in Stage II rose with in-
creasing concentrations of MgCl2 (Table IV); as a shock medium,
9 × 10−2 m MgCl2 was seven times as effective as water. This
was in contrast to experience with cyclic phosphodiesterase, 5'-
nucleotidase, and acid phosphatase. The total release of DNase
for the entire procedure was 90% when osmotic shock was carried
out with 2 × 10−2 m MgCl2.

The endonuclease released by osmotic shock was in an inactive
state (Table III). Thus, in the absence of pancreatic RNAse
very little activity could be measured, either in the sucrose solu-
tion or in the shock fluid. Presumably the enzyme was inhibited
by endogenous RNA that was also set free by the treatment.
Although evidence of RNA could not be found in these fractions
by chemical methods,1 the amount of RNA required to give this
inhibition would be very small, since the *K* for soluble RNA is
9 × 10−9 m (expressed as moles of RNA nucleotide) (11).

*E. coli* K-37, a derivative of K-10, was found to have only 8 to
15% of the endonuclease activity of *E. coli* B, in agreement with
results reported by Stockman and Lehman (19). From 3 to
30% of this low level of enzyme was released under conditions
described in Table III; high concentrations of MgCl2 were not
tried.

**Release of RNAse—**During Stage I of the osmotic shock pro-
cedure about 7% of the “latent” RNAse (8–10) was released into
the sucrose medium (Table IV). As with the DNA endonu-
clease, release during Stage II was favored by relatively high
concentrations of MgCl2 (1 to 2 × 10−2 m). Total release was
as high as 53%. When the shock medium contained as much as
1 × 10−2 m MgCl2, the total release of material absorbing at 260
from the liberation of protein, and the leakage of β-galactosidase
was sharply reduced, compared with results with water (Table
IV). In fact, the losses of ultraviolet-absorbing material and
β-galactosidase were even smaller than those observed with 1 × 10−4 m MgCl2, which was used for the selective release of
phosphatases and cyclic phosphodiesterase.

**Further Studies on Cells in Stationary Phase**

Cells in stationary phase were reinvestigated to determine the
effect of different shock media on the release of enzymes. In
this work the previously published procedure (I) was varied in
two respects. All growth media (see “Methods”) were relatively
high in their content of Ca++ and Mg++, and all Tris buffers were
at pH 7.1 (when measured at 23°). Satisfactory release of cyclic
phosphodiesterase, 5'-nucleotidase, and acid phosphatase was
achieved on each of five occasions with cold water as the shock
medium and concentrations of EDTA in Stage I that varied
from 1 × 10−4 to 3 × 10−4 m. Part of the data is shown in Table
V. Viability varied from 70 to 80%. In contrast to the experi-
ence with exponentially growing cells, the use of 5 × 10−4 m MgCl2
as shock medium greatly reduced the yield of enzymes and did
not shorten the lag period before growth was resumed in tryptone
broth. Similar results were obtained when the liberation of
alkaline phosphatase was measured.

**Release of 5'-nucleotidase was reduced to 3 to 10% of the usual
level in the cold water wash when EDTA was omitted from the

4D. Dussoix, and I. R. Lehman, personal communication, and
K. J. Hilmo, unpublished observations. Also, T. Fukasawa,
personal communication.

5R. W. Broekman, unpublished observations.

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**Table III**

Release of DNA endonuclease and exonuclease I during osmotic shock

| Stage | Suspension medium | Endo-
|-------|------------------|--------|
|       |                  | nuclease | Exo-
|       |                  |         | nuclease | β-Galactosidase |
| I     | Omit EDTA        | 34      | 536  |
|       | Water            | 2,350   |      |
| II    | 1 × 10−4 m EDTA  | 2,800*  | 2.4  | 0.8  |
|       | Water            | 1,050b  | 63   | 112  |
|       | 5 × 10−4 m MgCl2 | 2,000b  | 67   | 77   |
|       | 2 × 10−4 m MgCl2 | 5,700   | 24   | 34   |
| I     | 3 × 10−4 m EDTA  | 4,600b  |      |      |
|       | 5 × 10−4 m MgCl2 | 1,800b  |      |      |
| II    | 1 × 10−4 m EDTA  | 4,450   | 18   | 48   |
|       | 5 × 10−4 m MgCl2 | 1,450   | 16   | 23   |
| Sonic extract of intact cells | 14,300* | 1,750c | 2,260 |

* This gross leakage of β-galactosidase is due to the absence of
  EDTA in Stage I. Osmotic shock is better tolerated by exponen-
  tally growing cells if EDTA is used. This is currently being in-
  vestigated.

* When RNAse was omitted, the following levels were found (in
  units per g): Experiment 2, Stage I, 4.7; Experiment 2, Stage IIa,
  24; and Experiment 2, Stage IIb 0.7. When RNAse was omitted and
  0.66 μ mole of *E. coli* soluble RNA was added, levels were: Ex-
  periment 2, Stage I, 11; Experiment 2, Stage IIa, 0; Experiment 2,
  Stage IIb, 0.1; and sonic extract, 11.

* When soluble RNA was omitted, 3,020 units per g were found.

---

procedure (Table V). This more pronounced requirement for
EDTA than noted previously (1) is probably due to the increased
content of Ca++ and Mg++ in Medium B, used in the present
work. When Medium B was modified by including only 1 ×
10−4 m MgCl2 and 1.5 × 10−7 m CaCl2, and no ZnCl2, the yield
of cells in stationary phase and viability after osmotic shock were
considerably reduced. However, the specific release of 5'-nu-
cleotidase, cyclic phosphodiesterase, and acid phosphatase was
nearly 40% when osmotic shock was carried out in the absence
of EDTA.

In contrast to results with exponential cells, the release of en-
zymes was reduced by 50% when *E. coli* in stationary phase were
tried with as little as 5% sucrose in Stage I of the procedure.

**Rate of Release of Alkaline Phosphatase and of Cyclic Phospho-
diesterase during Osmotic Shock—** *E. coli* C90 were grown to sta-
tionary phase in Medium A supplemented with 0.6% glycerol.
Cells were washed three times with 0.6 m Tris (pH 7.1)-0.03 m
NaCl and suspended in 20% sucrose-0.033 m Tris (pH 7.1)-1 ×
10−3 M EDTA (80 ml per g wet weight, of cells). After 10 min
of gentle agitation at 23° the mixture was centrifuged, the super-
natant solution was decanted, and the pellet of cells was spread
in a thin layer on the inner surface of a centrifuge tube. An equal
Release of Enzymes by Osmotic Shock

**Table IV**

Effect of increasing concentration of MgCl₂ in shock medium on release of enzymes

<table>
<thead>
<tr>
<th>E. coli B were grown in Medium B with 0.6% glycerol. Experimental conditions were as in Table I except that the concentration of MgCl₂ in the shock fluid was varied as indicated below. Assays are presented, as units per g (wet weight) of cells, first for a sonic extract of intact E. coli, then for the 20% sucrose-0.053 M Tris-1×10⁻⁴ M EDTA supernatant fluid after centrifuging cells in Stage I. Finally, the release of enzymes into five different shock media is shown.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DNA Nucleoside Dioxygenase</strong></td>
</tr>
<tr>
<td>Extract, cells</td>
</tr>
<tr>
<td>Stage I, sucrose</td>
</tr>
<tr>
<td>Stage II, shock fluid</td>
</tr>
<tr>
<td>a. H₂O</td>
</tr>
<tr>
<td>b. 5×10⁻⁴ M MgCl₂</td>
</tr>
<tr>
<td>c. 1×10⁻³ M MgCl₂</td>
</tr>
<tr>
<td>d. 2×10⁻³ M MgCl₂</td>
</tr>
<tr>
<td>e. 5×10⁻³ M MgCl₂</td>
</tr>
</tbody>
</table>

* Total release of A₂₆₀ in units per g for shock fluids a to e, respectively, was 86, 47, 25, 12, and 15.

**Table V**

Effect of different shock media on release of enzymes from cells in stationary phase

| Conditions were as described in Table I, except as noted here. E. coli K-12 were grown to stationary phase in Medium B, with 0.6% glycerol, and washed three times with 40 parts cold 0.01 M Tris (pH 7.1) - 0.03 M NaCl. Experiment 3 differs in that Medium E was used. The pellet of cells was suspended in parts of 20% sucrose-0.053 M Tris, pH 7.1, containing 1×10⁻⁴ M EDTA (Experiments 1 and 2) or 3×10⁻⁴ M EDTA (Experiment 3). There was no significant release of enzymes at this stage. In Stage II, osmotic shock was carried out under conditions shown in Column I. Column 4 represents acid phosphatase except for Experiment 3, in which induced alkaline phosphatase was measured. Extracts of cells were made with CHCl₃, lysozyme, and EDTA (see “Methods”). Viability of shocked cells was found to be 80% in Experiments 1 and 2. |

<table>
<thead>
<tr>
<th>Shock medium</th>
<th>Cyclic phosphodiesterase</th>
<th>5'-Nucleotidase</th>
<th>Phosphatase</th>
<th>Total A₂₆₀</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water</strong></td>
<td>2550</td>
<td>5200</td>
<td>570</td>
<td>26</td>
<td>2.8</td>
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<tr>
<td>1×10⁻³ M MgCl₂</td>
<td>1580</td>
<td>3760</td>
<td>285</td>
<td>18</td>
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<tr>
<td>5×10⁻⁴ M MgCl₂</td>
<td>1220</td>
<td>3860</td>
<td>250</td>
<td>20</td>
<td>2.0</td>
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<tr>
<td>Intact cells, extract</td>
<td>2800</td>
<td>630</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1600</td>
<td>3600</td>
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<td></td>
<td></td>
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<tr>
<td>Intact cells, extract</td>
<td>1750</td>
<td>1800</td>
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<td></td>
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<tr>
<td><strong>Experiment 3</strong></td>
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<td></td>
</tr>
<tr>
<td>Water</td>
<td>1900</td>
<td>550</td>
<td>48</td>
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<td></td>
</tr>
<tr>
<td>Intact cells, extract</td>
<td>2050</td>
<td>560</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In another experiment the concentration of EDTA in Stage II was 3×10⁻⁴ M, and the results were almost exactly the same. Extracts of shocked cells contained 40 units per g after shock and 620 units per g after shock treatment with 1×10⁻³ M MgCl₂.

**Further Studies on Two Enzymes Released by Osmotic Shock**

**Observations on 5'-Nucleotidase**—The 5'-nucleotidase activity released by osmotic shock greatly exceeded the amount that was measured in extracts (Table I, Experiments 1 and 3). The activity of a sonic extract could be increased 2- to 4-fold if the extract was diluted in water and incubated for 60 min at 37°C before the addition of salts, buffer, and substrate (Table I, Experiment 2). More activity was liberated at greater dilutions. Thus, a sonic extract (in 0.03 M Tris, pH 7.1, 0.5 mg protein per ml) was found to contain 740 units per g (wet weight) of cells if assayed without prior incubation. If incubated undiluted or 5- or 15-fold diluted, the values were 600, 940, and 1,340 units per g, respectively. The activation was partially decreased by the presence of Ca²⁺ and Mg²⁺ at levels which were optimal for enzymatic activity, and it was substantially decreased by EDTA at levels which did not inhibit the enzyme. Other experiments demonstrated that activation occurred with the 100,000×g supernatant fraction; ribosomes contained no significant amounts of enzyme and did not contribute to the activation. The 5'-nucleotidase activity measured in the shock fluid was not increased by a prior incubation. Unfortunately, only 60% of the activity found in a fully activated sonic extract could be accounted for by the sum of Σ

* In another experiment the concentration of EDTA in Stage II was 3×10⁻⁴ M, and the results were almost exactly the same. Extracts of shocked cells contained 40 units per g after shock and 620 units per g after shock treatment with 1×10⁻³ M MgCl₂.

Another fraction of the cells was treated similarly except omission of EDTA. In Stage II, treatment with cold was released only 100 units per g of 5'-nucleotidase and 290 units of g of cyclic phosphodiesterase.
nucleotidase set free plus that found in the shocked cells. With each of the other enzymes a more satisfactory balance was obtained, with 85 to 110% of the activity accounted for.

The 5'-nucleotidase in a fraction obtained by DEAE-cellulose chromatography (1) was inhibited 100% by 0.01 m ZnCl₂ and 40% by 1 × 10⁻³ m ZnCl₂. The stimulation by Co⁺⁺ reported previously (1) was found to be considerably increased by dialyzing the fraction obtained by osmotic shock against 10⁻³ m Na₂EDTA and then against water. When tested with 0.01 m MgCl₂, 0.01 m CaCl₂, and 5 × 10⁻⁴ m CoCl₂, the relative activities were: no supplement, 1; Mg⁺⁺ alone, 30; Ca⁺⁺ alone, 23; Co⁺⁺ alone, 214; Co⁺⁺ plus Mg⁺⁺, 269; and Co⁺⁺ plus Ca⁺⁺, 364.

Properties of Acid Phosphatase—This enzyme was assayed by measuring the rate of hydrolysis of glucose 6-phosphate. In strain K-37 its activity varied from 1200 units per g for cells grown to stationary phase in succinate to 140 units per g for glucose-grown cells. It thus corresponds to an enzyme described in the literature (4, 5), and it is the only one of the released enzymes that is subject to repression by glucose.

Application of Osmotic Shock to Other Species of Bacteria

The procedure for osmotic shock released most of the 5'-nucleotidase and acid phosphatase activity of Aerobacter aerogenes that had been grown to stationary phase. As was true for E. coli, the acid phosphatase which appeared in the shock medium was glucose-repressible. With Salmonella typhimurium, release of cyclic phosphodiesterase amounted to only 30% of the activity present in a sonic extract, and acid phosphatase was not set free by osmotic shock. High levels of 5'-nucleotidase and of cyclic phosphodiesterase were present in Proteus mirabilis, but measurable amounts were not found in the shock medium.

Infection of Shocked Cells with DNA of λ Phage—Shocked cells were more efficient than untreated cells as recipients of λ-DNA in the absence of helper phage, in a system otherwise similar to that described by Kaiser and Hoggness (29). E. coli K-12S was grown to early exponential phase in a synthetic medium (30) containing 0.5%, glucose. Osmotic shock was carried out as described in "Methods" except that twice the volume of shock medium (usually 5 × 10⁻⁴ m MgCl₂) was used, and EDTA was not required. Shocked cells (2 × 10⁸) and λ-DNA prepared by phenol extraction (29) (2 × 10⁻⁴ to 5 × 10⁻¹ absorbance unit at 260 μm) were incubated for 60 min at 30° in a reaction mixture containing 0.005 m Tris-HCl (pH 7.1), 0.055 m CaCl₂, and 0.030 m MgCl₂ in a total volume of 0.2 ml. Suitable aliquots were plated on tryptose agar plates, and plaques were scored after 24 hours at 37°. These conditions differ from those described by Kaiser (29) in that the bacteria were grown in a more complex medium and were not infected with helper phage, and the concentrations of MgCl₂ and CaCl₂ were increased from 0.01 m.

Under the present conditions, in one experiment 3.0 × 10⁹ plaques per absorbance unit of DNA were obtained with shocked cells and 0.12 × 10⁹ plaques with untreated cells. This should be compared with 1 to 2 × 10⁶ plaques per unit of DNA with helper phage. Unfortunately, the results with shocked cells were variable. Thus, in successive experiments the plaques per DNA unit were 2.4, 0.4, 0.8, and 3.4 × 10⁹ for shocked cells and 0.25, 0.04, 0.06, and 0.22 × 10⁹ for untreated cells in the same experiments. The plaque-forming ability of the DNA was)

* Experiments done in collaboration with Dr. A. Weissbach.
* A. D. Kaiser, personal communication.
The RNA-inhibited DNA endonuclease is unique among the specifically released enzymes because it is set free to a substantial extent simply by exposing cells to sucrose and EDTA. An effort was made to demonstrate this activity with intact cells and with tritium-labeled DNA as substrate. The results were complicated by the fact that detectable DNase activity was released into the medium by washed exponentially growing cells no matter how carefully they were handled, and with every suspension medium that we happened to use.

It has been found that treatment of exponentially growing E. coli cells with EDTA renders them temporarily permeable to actinomycin D (33), and similar observations have been made with respect to other agents that ordinarily do not penetrate normal cells (33-35). We have found that cells subjected to osmotic shock also show uptake of actinomycin D. Attempts to show uptake of biologically active DNA were disappointing. However, the results on infection by X-DNA offered sufficient encouragement to warrant brief presentation. In future efforts to improve the system the use of osmotic shock with relatively large concentrations of MgCl₂ should be explored (Table IV). This modification was found quite recently to cause almost complete release of the DNA endonuclease, an enzyme that might possibly interfere with the uptake of λ-DNA.

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The Release of Enzymes by Osmotic Shock from *Escherichia coli* in Exponential Phase
Nancy G. Nossal and Leon A. Heppel


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