

Restoration of Phosphate Transport by the Phosphate-Binding Protein in Spheroplasts of *Escherichia coli*

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Reconstitution of phosphate transport in *Escherichia coli* was demonstrated. Conversion of *E. coli* K10 cells to spheroplasts decreased phosphate transport to about 2%. Addition of purified phosphate-binding protein at physiological levels to these spheroplasts caused a mean 14-fold increase in phosphate transport rate. Crude shock fluid fractions were also stimulatory but not if the shock fluid was obtained from mutants lacking phosphate-binding protein. The effect of the binding protein was abolished by its specific antibody. The phosphate was shown to have entered the cell, where it became esterified. Reconstitution was not possible with cold-shocked or osmotically shocked cells.

The role of osmotically shockable binding proteins in metabolite transport of *Escherichia coli*, and of other species as well, has often been questioned (6). Direct proof of the involvement of binding proteins in transport requires a demonstration of restoration, by the protein, of a specific transport function in cells that have lost it. Although several binding-protein preparations have been reported to stimulate specific transport systems (1, 2, 4, 13, 21), the evidence so far obtained seems to be unconvincing in some instances (6) and, in the case of previous work from this laboratory (13), cannot be reproduced.

The inability to reproduce this work (17) stems from at least two previously unrecognized factors. The first is the existence in *E. coli* of two major transport systems for phosphate, described by Willsky et al. (20). Only one system (PST) utilizes the phosphate-binding protein (PBP), which is coded by the gene *phoS*. Two other genes, *phoT* and *pst*, are also involved in this system. The second factor (discussed below) is the spontaneous recovery of phosphate transport in cells treated by the modified cold-shock procedure of Medveczky and Rosenberg (13), which tended to mimic the reconstitution process.

In the present work we avoided these complications by, first, using a strain that depended entirely on the PST system for phosphate transport and, second, by avoiding the use of cold-shocked cells in favor of spheroplasts. Substantial and reproducible reconstitution of phosphate uptake was demonstrated in this system and shown to be highly specific for PBP.

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MATERIALS AND METHODS

The chemicals used were of the highest purity available commercially. Triethanolamine-hydrochloride (TEA; Fluka, Purissimum) was further purified by crystallization of the hydrochloride from ethanol. Labeled orthophosphate (P; carrier free) was from the Australian Atomic Energy Commission, Lucas Heights, New South Wales. Lysozyme was from Fluka.

Media and buffers. Cells were grown in half-strength 56 medium (14) supplemented with glucose (20 mM) and thiamine (3 μ M).

"Uptake" medium (also used to deplete cells of phosphate) contained TEA (0.05 M), KCl (0.015 M), $(\text{NH}_4)_2\text{SO}_4$ (0.01 M), and MgSO_4 (0.001 M), all adjusted to pH 7.2.

Bacterial strains. The strains used were all derivatives of strain K10 of *E. coli* K-12 (8, 9) that lack the PIT system, a major phosphate transport system of *E. coli* (19, 20). All strains and their derivation are listed in Table 1.

Treatment of cells. Bacteria were grown with shaking at 37°C in 50-ml batches, in the medium described above. They were harvested from stationary-phase cultures, washed twice in uptake medium supplemented with glucose (20 mM) and thiamine (3 μ M), and resuspended in the same medium to a density (at 660 nm) of approximately 0.300, and the suspension was shaken in air at 37°C for 2 h to deplete the cells of phosphate.

Preparation of spheroplasts. Spheroplasts were prepared from P-depleted cells by the following procedure (P. Wookey, personal communication). The cells were centrifuged, washed twice in 30 mM TEA buffer (pH 7.0), and suspended at a density (at 660 nm) of 1.6 in 50 mM TEA-30% sucrose (pH 8.0) containing 200 μ M ethylenediaminetetraacetate (EDTA) (TEA-salt). The mixture was swirled gently at 24°C for 2 min, after which lysozyme solution (1 mg/ml in water) was added to a final concentration of 10 μ g/ml. Swirling was continued for 15 min, and

TABLE 1. *E. coli* strains used

Strain	Relevant genotype	Source/reference
K10	<i>pit</i>	Coli Genetics Center no. 5023
C72	<i>pit phoS</i>	Obtained from A. Garen; 9
C10la	<i>pit phoT</i>	Obtained from A. Garen; 9
AN518	<i>pit ilv</i>	Derived from K10 by ultraviolet irradiation mutagenesis and penicillin selection (7)
AN524	<i>pit phoS</i>	Derived from AN518 by cotransduction (16) with the <i>ilv</i> marker, using P1 λ c grown on C72
AN529	<i>pit phoT</i>	Derived from AN518 by cotransduction (16) with the <i>ilv</i> marker, using P1 λ c grown on C10la.

the resulting spheroplasts were recovered by centrifugation at $39,000 \times g$ for 3 min at 15°C, suspended in 50 mM TEA-30% sucrose (pH 8.0) in one-half of the volume of the digest, and stored on ice. The spheroplasts form a cohesive pellet that resuspends with difficulty. Treatment prior to centrifugation with a mixture of ribonuclease and deoxyribonuclease, by the method of Kaback (11), overcomes this and does not affect subsequent reconstitution.

The number of intact cells in the spheroplast suspension was determined by dilution into water, followed by plating onto solid nutrient medium containing glucose (20 mM) and sucrose (15%). Comparisons were made with a parallel series of dilutions into uptake medium containing 15% sucrose, followed by plating on the same solid medium.

Alkaline phosphatase was measured by the method of Willsky et al. (20). PBP and its antibody were prepared essentially as described by Gerdes and Rosenberg (10).

Measurement of phosphate transport. The procedure for measuring phosphate transport was essentially as described by Medveczky and Rosenberg (13), except that TEA buffer replaced tris(hydroxymethyl)aminomethane and the wash solution consisted of KCl (100 mM), TEA (10 mM), $(\text{NH}_4)_2\text{SO}_4$ (10 mM), and MgSO_4 (1 mM), adjusted to pH 7.2. The assay was started by the addition of $^{32}\text{P}_i$ to a final concentration of 50 μM (specific activity, 2 $\mu\text{Ci}/\mu\text{mol}$).

A second, "scaled-down," assay developed for working in small volumes is described in detail in the text.

Cold-shock and osmotic-shock procedures. Cold shock was essentially as described by Rae et al. (17), with P-depleted cells. In the experiments reported here, TEA replaced tris(hydroxymethyl)aminomethane or imidazole as the buffer, and the temperature change was from 30 to 2°C. Five minutes after cold shock, the cells were centrifuged and suspended in water at 2°C to give a density equivalent to 15 at 660 nm. Osmotically shocked cells were produced as described by Neu and Chou (15), using P-depleted cells. In the experiments in which a comparison with spheroplast preparations was made, both cold-shocked and osmotically shocked cells were suspended in the same medium (30 mM TEA [pH 7.0]-

30% sucrose-0.2 mM EDTA) and to the same density as the spheroplast preparations.

RESULTS

Restoration of phosphate transport in cold-shock cells. We initially attempted to improve PBP-stimulated transport of phosphate above the low value (26% maximum recently reported by Rae et al. [17]). For these experiments, we used strain K10, which contains only one phosphate transport system (PST), or strains AN524 and AN529, which were derived from K10 and carry mutations in components of this system. Under the various conditions tried with cold-shocked *E. coli* cells, PBP when added alone to the reconstitution system caused only a small stimulatory effect (Table 2), whereas the addition of KCl (1.13 or 2.25 M) or EDTA and KCl gave an uptake that was higher than with PBP added alone. No combination of additions could be found in which PBP aided the restoration process. The limited restoration observed is likely to represent spontaneous recovery observed when cold-shocked cells were shaken in the phosphate-free medium for 1 h at 37°C but not below 3°C. This recovery did not involve protein synthesis, since it took place in the presence of chloramphenicol (data not shown).

Restoration of phosphate transport in spheroplasts. Since cold-shocked cells were obviously unsatisfactory for restoration studies, we attempted to use spheroplasts prepared by

TABLE 2. Attempts to restore phosphate uptake in cold-shocked *E. coli* K10 cells by the addition of PBP

Additions ^a to restoration system (mM)		Phosphate uptake rate (nmol/mg [dry wt] per min)	
KCl	EDTA	Without PBP	With PBP
0	0	9.3	13.9
300	0	16.6	18.0
600	0	26.5	23.1
600	1.25	29.8	26.2
Nonshocked cells (no additions)		85.9	52.8

^a All additions were at 2°C. PBP (100 μg in 10 μl of water), or water alone (control), and other additions as shown above were made to samples (0.1 ml) of a suspension of phosphate-starved, cold-shocked cells (see text). After about 5 min, 20 μl of 0.2 M MgCl_2 was added, followed by 5 ml of uptake medium. The mixture was centrifuged, and the cells were suspended in 50 μl of fresh uptake medium, the entire procedure to this point being done at 2°C. The suspension was warmed to 37°C and diluted with 5 ml of uptake medium at 37°C preparatory to P_i uptake measurement (standard method).

EDTA and lysozyme treatment (see Materials and Methods) and stabilized in hypertonic medium. (It should be noted that when cold-shocked or osmotically shocked cells were suspended in the uptake medium containing 15% sucrose, little spontaneous recovery of phosphate uptake occurred.)

The spheroplast preparation was essentially cell free, since viability counts done after dilution into water gave values of 0.1% or fewer survivors, whereas dilution into hypertonic medium showed increased viability. Another criterion for the completeness of conversion to spheroplasts was the release of alkaline phosphatase into the medium. The EDTA-lysozyme treatment consistently gave values near 100%, whereas the cold-shock procedure (see above) released less than 1% of the total enzyme. Osmotically shocked cells released 60 to 90% of the phosphatase.

Electron micrographs of the spheroplasts appear similar to other published examples (5). In certain instances, fragments of the outer cell wall remained, whereas in others the alteration was more severe, resulting in vesicular structures. This is consistent with the observation that, during spheroplast preparation from cells induced for β -galactosidase, up to 20% of this enzyme was released in some cases.

In reconstitution experiments, additions of PBP to the spheroplasts were made to approximate the concentration of this protein in the periplasm of fully induced cells. We estimated this to be in the order of 1 to 5 mg/ml. In view of the limited supply of pure PBP, a final concentration of 1 mg/ml was used, and the procedure for measuring phosphate uptake was scaled down to a small volume ($\sim 200 \mu\text{l}$). Several variations of this procedure were successful, and the most effective one is described in some detail in the legend to Fig. 1 to permit accurate reproduction (see Introduction). When the rate of phosphate uptake of intact cells was measured with this technique, the results obtained closely approximated the values found with the standard method (see Materials and Methods).

In the above procedure, steady uptake of phosphate occurred when PBP was added to K10 spheroplasts (Fig. 1), the uptake rate reaching up to 30 times the rate in control experiments without added PBP (the average increase was 14-fold). Examination, by the same method, of the spheroplasts prepared from the other strains showed less stimulation of the *pho*S mutant (AN524) and, as anticipated, no effect with the *pho*T mutant (AN529) (Table 3).

In a variation of this procedure (data not shown), it was found that addition of PBP to

spheroplasts several minutes after $^{32}\text{P}_i$ addition caused an immediate increase of the uptake rate in strains K10 and AN524, but an actual decrease was observed in strain AN529.

The nature of the PBP-stimulated phosphate uptake seen in K10 spheroplasts was then ex-

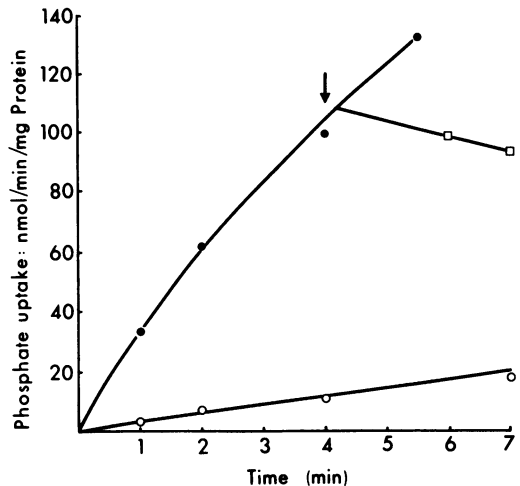


FIG. 1. Restoration of phosphate transport in spheroplasts of *E. coli* K10 by PBP. The spheroplast preparation (see text) was diluted with 2 volumes of uptake medium supplemented with 20 mM glucose and 15% sucrose, and with 1 volume of water to produce a density of about 0.8 at 660 nm. The suspension was warmed to 37°C, and at zero time 200- μl portions were transferred to vials containing 4 μl of 2.5 mM $^{32}\text{P}_i$ (50 mCi/mmol) and 50 μl of either water (\circ) or PBP (5 mg/ml) (\bullet). Samples (25 μl) of this suspension were withdrawn at intervals, immediately diluted into 1.0 ml of uptake medium containing 15% sucrose, filtered through a Sartorius membrane filter (SM 11106; pore size, 0.45 μm), and washed with two 1-ml lots of the same medium. The membranes were dried and counted as in the standard uptake procedure (13). At the point indicated by the arrow, 100 μl of the PBP-containing mixture was transferred to a prewarmed vial containing sufficient unlabeled P_i to raise its concentration to 10 mM (\square).

TABLE 3. Effect of PBP on phosphate uptake by spheroplasts of various *E. coli* strains

Strain	Relevant genotype	P uptake ^a : initial rate (nmol/min per mg of protein)	
		Without PBP	With PBP
K10	<i>pit</i>	1.7	43.6
AN524	<i>pit phoS</i>	3.1	9.0
AN529	<i>pit phoT</i>	0.8	1.1

^a Phosphate transport rates were measured as described in the legend to Fig. 1.

amined in some detail. As expected with spheroplast preparations, the $^{32}\text{P}_i$ that had accumulated in the presence of added PBP was not retained on filtration through membranes under lytic conditions, i.e., when the samples were diluted into water instead of hypertonic medium (Table 4). That genuine transport of phosphate into spheroplasts had occurred is demonstrated by the finding that after 2 min of uptake (in the presence of PBP) the phosphate was largely esterified (Table 5). Furthermore, only a small portion of the ^{32}P taken up by the spheroplasts in the presence of PBP was exchangeable (slowly) with added unlabeled P_i (Fig. 1). This feature is characteristic of phosphate uptake in whole cells of K10 (18).

The uptake rate increased to an optimum as the PBP concentration was raised (Fig. 2). With its concentration fixed at 40 μM , phosphate became limiting when PBP concentra-

tions exceeded 1 mg/ml (i.e., 25 μM , based on a molecular weight of 40,000). This probably accounts for the fall-off in uptake rate at higher PBP concentrations, since raising the concentration of P_i to 80 μM overcame this effect. In all of the experiments reported here, PBP was added to a final concentration of 1 mg/ml.

The stimulation of phosphate uptake in K10 spheroplasts by PBP was linear with increasing spheroplast concentration (data not shown).

Evidence that the stimulatory effect is specific for PBP was provided by the inhibitory effect of antibody raised against highly purified PBP (Table 6) and from studies on the effect of various protein fractions isolated during the fractionation of osmotic shock fluids from cells of strains K10 (parent) and AN524 (*phoS*).

In Fig. 3, the elution peaks marked "B" and "C" coincide with the activities of alkaline

TABLE 4. Osmotic sensitivity of the PBP-stimulated phosphate accumulation by spheroplasts from *E. coli* K10^a

Dilution condition	Phosphate accumulated by spheroplasts (nmol of P_i retained/mg of spheroplast protein after 3 min of uptake)	
	Without PBP	With PBP
(A) Diluted into 15% sucrose uptake medium	8.9	94.2
(B) Diluted into water	2.9	3.8

^a The experiment was as described in the legend to Fig. 2, except that, in addition to the usual procedure (A), some samples (B) were diluted into 1.0 ml of water before membrane filtration.

TABLE 5. Intracellular form of phosphate accumulated by spheroplasts of *E. coli* K10 in the presence of PBP^a

Expt no.	cpm in:		% esterified
	Inorganic P	Esterified P	
1	3,310	17,794	85.2
2	1,841	13,322	87.8

^a Experimental conditions were as described in the legend to Fig. 1. After 2-min incubation in the presence of PBP, the entire incubation mixture was diluted, filtered, and washed, and the filter membrane was extracted with 0.75 N HClO_4 on ice. The treatment of the extract was essentially by the method of Asada et al. (3) and consisted in the separation of P_i (as the butanol-benzene-soluble phosphomolybdate complex) from esterified phosphate (butanol-benzene insoluble) and the counting of each fraction.

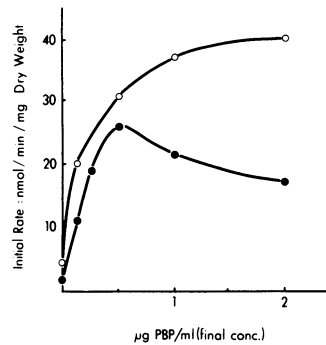


FIG. 2. Effect of PBP concentration on the rate of accumulation of phosphate by spheroplasts from *E. coli* K10. The conditions were as given in the legend of Fig. 1. PBP was added to give the final concentration shown. Phosphate was present at 40 μM (●) or 80 μM (○).

TABLE 6. Effect of antibody raised against PBP on reconstitution of phosphate transport, in *E. coli* spheroplasts

Uptake mixture ^a	Accumulated radioactivity (cpm $\times 10^3$)		
	1 min	2 min	4 min
Spheroplasts alone	0.77	1.60	2.75
Spheroplasts + PBP	5.25	11.0	15.8
Spheroplasts + PBP + antiserum (corrected for blank ^b)	0.52	0.65	2.06

^a The complete uptake mixture (125 μl) contained, where indicated, spheroplast suspension adjusted to produce $E_{600} = 0.750$, PBP (1 mg/ml), antiserum in slight excess of antigen requirement, or TEA-sucrose in place of spheroplasts. Uptake was commenced by addition of prewarmed spheroplasts (see text).

^b Blank determinations were done with PBP plus antiserum but with no spheroplasts, and represents the retention of a complex of antiserum, PBP, and ^{32}P on the filter membrane (12). The value of that blank was about 9,000 cpm and was constant with time.

phosphatase and PBP, respectively. Fractions A and B from K10 osmotic-shock fluid were expected to contain traces of the leading edge of the PBP peak, and each showed a small stimulatory effect on phosphate transport (Table 7). The same fractions derived from the *phoS* mu-

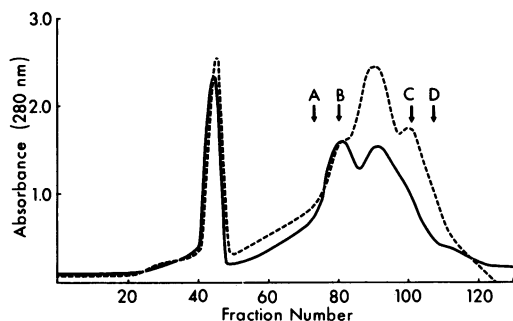


FIG. 3. Gel chromatography of shock fluid. The supernatant fractions from osmotically shocked cells of strain K10 (broken line) and strain AN524 (solid line) were concentrated 10-fold and fractionated with ammonium sulfate. The fraction precipitating between 60 and 90% saturation was recovered, dialyzed against tris(hydroxymethyl)aminomethane-hydrochloride buffer (100 mM, pH 8.4) containing $MgCl_2$ (10 mM) and KCl (1 mM) (TMK buffer), and applied to a Sephadex G-100 column. The column was eluted with TMK buffer, and the indicated fractions (A, B, C, and D) from each strain were concentrated and tested for their ability to stimulate phosphate transport (see Table 7).

TABLE 7. Stimulation of phosphate uptake in spheroplasts of *E. coli* K10 by shock fluid fractions from *E. coli* K10 or from AN524 (*phoS*)

Additions to assay mixture ^a	P _i uptake rate (nmol/mg of protein)	% of control
None (control)	1.26	100
Pure PBP	8.52	676
Fraction from K10 shock fluid:		
A	2.34	186
B	3.88	308
C	8.66	687
D	4.10	325
Fraction from AN524 shock fluid:		
A	0.76	60
B	0.92	73
C	1.29	102
D	1.39	110
Fraction C (AN524) + PBP	5.84	463

^a Each assay was carried out as described in the legend to Fig. 1. Fractions A, B, C, and D refer to the fractions shown in Fig. 3. The final concentration of each added fraction was 1.5 mg of protein/ml, and that of pure PBP was 1.0 mg of protein per ml.

tant (which is deficient in normal PBP) showed a small inhibition. Fraction C, which corresponds to the PBP peak, gave a stimulation comparable to that of PBP when isolated from K10 shock fluid and, as expected, no effect when isolated from AN524 shock fluid. In shock fluids from strain K10, fraction D, on the trailing edge of the PBP region, showed declining stimulation. The corresponding fraction from strain AN524 had no activity. When PBP was added along with fraction C from strain AN524, good stimulation of uptake was observed, indicating that the protein in fraction C was neither inhibitory nor stimulatory in respect to phosphate uptake by K10 spheroplasts.

In view of the questionable reproducibility of previous attempts to restore or reconstitute phosphate uptake with PBP, we thought it pertinent to present results showing the consistency of the restoration of phosphate uptake observed in our system. All of the data obtained over a 6-week period from several different spheroplast preparations have, therefore, been included in the summary presented in Table 8. As can be seen, PBP caused a mean 14-fold increase in uptake rates in K10 spheroplasts and better than a 2-fold increase in AN524 spheroplasts, with good reproducibility, whereas no effect was observed with AN529 spheroplasts.

DISCUSSION

The results leave little doubt about the success or failure of reconstitution of phosphate transport with the binding protein in whole cells as compared to that in spheroplasts. Even in strain K10, where the sole transport system for phosphate depends on the binding protein, reconstitution in cold-shocked or osmotically shocked whole cells proved impossible. Previous claims to that effect must be attributed to artifacts arising from spontaneous recovery of cold-shocked cells or from nonspecific stimulation of the type produced by KCl (Table 2). On the other hand, the reconstitution of the trans-

TABLE 8. Summary of data obtained on the restoration of phosphate uptake by PBP in spheroplasts from *E. coli* K10 and related mutants

<i>E. coli</i> strain	No. of expt	Phosphate uptake ^a (nmol/min per mg of protein)	
		Without PBP	With PBP
K10	22	1.6 ± 0.2	22.1 ± 1.8
AN524	13	1.56 ± 0.29	3.35 ± 0.61
AN529	5	0.63 ± 0.17	0.46 ± 0.18

^a Experiments were carried out as described in the legend to Fig. 1. Mean values are expressed ± standard error of the mean.

port in spheroplasts was both significant in extent and reproducible, and the results presented above are consistent with the entry of the phosphate into the spheroplasts.

Although there was some scatter of the data obtained from day to day, the statistical analysis of all data obtained by the method described points undeniably to a high degree of reconstitution. However, PBP has not been observed to restore transport rates to the level found in intact cells. The combination of PBP with crude shock fluid did not result in increased stimulation (data not presented). It is not likely, therefore, that a second periplasmic constituent is involved. However, evidence from electron microscopy and the observed release of β -galactosidase suggest that spheroplasts are highly fragile, and this factor must contribute to an underestimate of the extent of reconstitution. It is likely that within any population of spheroplasts those most extensively damaged, as well as the fraction mildly affected, and therefore resembling whole cells, will be refractory to PBP stimulation.

It is notable that the specific antiserum against the binding protein completely abolishes the reconstitution. This result and the reconstitution by the fractions from the crude shock fluid, and particularly the failure to reconstitute with the shock fluid from strain AN524, which lacks the binding protein, point to the specific effect of this protein in the reconstitution.

The need of specific interaction of the binding protein with another component of the PST system when reconstituting phosphate uptake is demonstrated by its failure to do so in spheroplasts of strain AN529 (*phoT*). This phenomenon also demonstrates that there is no nonspecific binding of phosphate through PBP to the filter membranes or cell components retained on them.

It was expected that spheroplasts from strain AN524 (*phoS*) would respond fully to the binding protein, but only a two- to threefold increase in uptake rate was observed. Spheroplasts from strains obtained by the transduction into strain K10 of the *phoS* allele from several strains (C31, C78, C86) described by Garen and Otsuji (9) behaved in the same manner as those from strain AN524. The reason for this behavior of the *phoS* strain is not clear.

The present results suggest that spheroplasts may prove the most effective preparation for the demonstration of reconstitution of transport by binding proteins in systems in which these proteins participate. It is notable that another successful reconstitution—that of glutamate transport (4)—was also demonstrated in spher-

oplasts. We believe that close attention to the method of spheroplast preparation and the use of conditions in which the binding protein can be added in high concentrations are essential to success. It is not likely that restoration can be successfully demonstrated in shocked cells.

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