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STUDIES OF PHOSPHATE TRANSPORT IN *ESCHERICHIA COLI*

I. REEXAMINATION OF THE EFFECT OF OSMOTIC AND COLD SHOCK ON PHOSPHATE UPTAKE AND SOME ATTEMPTS TO RESTORE UPTAKE WITH PHOSPHATE BINDING PROTEIN

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SUMMARY

1. The first stage of osmotic shock caused a slight reduction in the primary uptake of inorganic orthophosphate (P_i) in AB3311 cells of *Escherichia coli* which normally exhibit a biphasic type of phosphate uptake. The second stage of osmotic shock resulted in a marked reduction (a total of 80–85 %) in the primary uptake phase and a lesser reduction (50 %) in the secondary uptake. When osmotically shocked cells are allowed to recover in a phosphate-free, but otherwise complete medium sufficient repair occurs in the shocked cells to overcome growth lags and to restore the above losses in phosphate uptake almost to normal after 90–180 min of recovery.

2. Extensive investigation was made of the more mild cold shock procedure which involves the rapid disperion of *Escherichia coli* cells into 80 vol. of water at 2 °C. The most consistent cold shock effects, as evidenced by reductions in phosphate uptake, were obtained in cells after they were washed in appropriate buffered salts media, suspended in a minimal volume of water and shocked from 37 °C rather than 24 °C. Less severe shocks were obtained after washing in imidazole/salts/glucose or Tris/salts/glucose media than in NaCl/Tris.

3. A number of attempts were made to restore by the addition of phosphate binding protein the reduction in phosphate uptake of *E. coli* AB3311 cells caused by a variety of cold shocks. In no instance was good restoration of phosphate uptake achieved. Qualitatively, it appeared that a better restoration of uptake occurred in unstarved and starved cells washed in imidazole/salts/glucose where the cold shock effect was less severe.

INTRODUCTION

When gram-negative bacteria are subjected to osmotic shock (i.e. sucrose/Tris/EDTA treatment followed by sudden injection into cold dilute $MgCl_2$ or water),

several hydrolytic and smaller proteins [1, 2] and some lipid and carbohydrate components are released (ref. 3 and unpublished data). Amongst the proteins shocked out are a number of binding proteins (see refs. 4–7) which are implicated as possible recognition proteins in the transport of the respective solutes that they bind. Included in this group [4–9] is the phosphate binding protein first described by Medveczky and Rosenberg [10, 11] and more recently shown by Gerdes and Rosenberg [12] to be a product of the *phoS* gene, a regulator gene of alkaline phosphatase in *Escherichia coli* [14]. This phosphate binding protein was shown to possess properties and characteristics appropriate with its involvement in phosphate transport [11]. Osmotic shock caused release of this binding protein and greatly inhibited phosphate transport. Even though the loss of uptake by osmotic shock could not be restored by the addition of phosphate binding protein, it was found with a milder cold shock (rapid dispersion of cells into 80 vol. of water at 3 °C) where loss of phosphate binding protein and uptake were less that about 50 % restoration of uptake could be achieved upon the addition of phosphate binding protein.

Subsequent examination of this phenomenon by other workers (Pardee, A. B., personal communication) and by ourselves showed that cold shock effects and reconstitution of phosphate uptake in shocked cells could neither be reproduced consistently, nor to the extent previously reported [11]. In view of these difficulties a detailed study was commenced of the effects of various shock procedures and modifications of these procedures on phosphate uptake. Initially examination was made of the osmotic shock procedure introduced by Neu and Heppel [1, 15, 16]. Then the cold shock procedure originally described by Medveczky and Rosenberg [11] was investigated and modifications were made which gave more reproducible effects on phosphate uptake. Finally, using these modifications attempts were made to achieve restoration of phosphate uptake by the addition of phosphate binding protein. The results of these studies are now reported.

MATERIALS AND METHODS

Chemicals. Whenever possible, chemicals of analytical reagent grade were used. Radioactive orthophosphate ($^{32}P_i$) was obtained as carrier-free $H_3{}^{32}PO_4$ either from the Australian Atomic Energy Commission or the Atomic Energy of Canada Ltd. Each shipment was diluted with carrier and pretreated as previously described [11]. Phosphate binding protein was kindly provided by Dr. R. G. Gerdes (for preparation see ref. 12).

Bacterial culture. The Reeves *met*$^-$ strain of *E. coli* K12 (AB3311), used in previous studies [10–12], was grown overnight with shaking at 37 °C in one of two phosphate media: Medium I, containing 50 mM Tris · HCl buffer (pH 7.4), 10 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 0.1 % yeast extract, 20 mM glucose and 2 mM methionine [10], or Medium II, containing 2 mM NaH_2PO_4/K_2HPO_4 buffer (pH 7.0), 35 mM sodium citrate (pH 7.0), 10 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 60 μM $Ca(NO_3)_2$, 2 mM methionine and 3 μM vitamin B_1. Phosphate-free media were obtained by deletion of either yeast extract from Medium I (i.e. Tris/salts/glucose medium) or phosphate from Medium II. For some experiments imidazole buffer (50 mM, pH 7.4) replaced Tris buffer in the Tris/salts/glucose medium. Cells were harvested by centrifugation and then were either washed in phosphate-free media by resuspension and

centrifugation, or deprived of phosphate for 2–4 h at 37 °C in phosphate-free media.

Shock procedures. (a) *Osmotic shock*: As described by Neu and Chou [16] the above cells were washed once in 0.01 M Tris · HCl (pH 7.3)/0.03 M NaCl at 3 °C and then subjected to osmotic shock. The latter involved the suspension of cells in 20 % sucrose/0.03 M Tris · HCl (pH 7.3) at room temperature in a ratio of 1 g (wet wt.) to 80 ml. EDTA was added to give a final concentration of 1 mM and the suspension was stirred 10 min (i.e. stage I). The cells were centrifuged, suspended in a small volume of water at 20 °C and then rapidly dispersed into 80 vol. water at 2 °C (i.e. stage II). The final density of cells aimed at was equivalent to 40 Klett. After 5 min the cells were collected for use by centrifugation at 0 °C.

In the recovery experiments carried out, AB3311 cells were grown overnight in Medium II, harvested and then deprived of phosphate for 2 h at 37 °C in phosphate-free Medium II. After collection by centrifugation the cells were subjected to osmotic shock. The shocked cells were then resuspended in phosphate-free Medium II and incubated at 37 °C with shaking for 3 h. Samples of cells were taken at intervals for growth studies in Medium II and for phosphate uptake.

(b) *Cold shock*: Investigation was made of the effects of a number of factors on the normal cold shock procedures [11]. The latter involved the suspension of cells in a minimal volume of distilled water at room temperature (24 °C) and their rapid dispersal into 80 vol. of water at 2 °C. After 5 min the cells were centrifuged in the cold and then used for uptake measurements. The effect of pH and differences in temperature drop, suspending media and washing procedures for the cells were all examined. Comments on the findings are given in Results.

Phosphate uptake. Membrane filters (Gelman Metricel; pore size, 0.20 μm; diameter, 25 mm) were soaked in 140 mM NaCl/7 mM KH_2PO_4 prior to use in phosphate uptakes which were measured essentially as described by Medveczky and Rosenberg [17]. The final concentration of $^{32}P_i$ used in the uptake medium was 100 μM.

Restoration of phosphate uptake after cold shock. Attempts were made to restore phosphate uptake in several different preparations of cold-shocked cells. The usual procedure was to suspend the shocked cells in ice-cold medium (imidazole/salts/glucose) to 1000 Klett and then transfer 1 ml into each of two 50-ml conical flasks precooled on ice and containing: (a) 10 μl 1 M $MgSO_4$ plus 20 μl water (i.e. the control) and (b) 10 μl 1 M $MgSO_4$ plus 20 μl of solution containing 50 or 100 μg phosphate binding protein. After mixing the flasks were allowed to sit on ice for 2–4 min before dilution in 5 ml of the above ice-cold medium. In a few instances the cells were warmed and diluted to 10 ml and used directly. Normally the cells were briefly centrifuged (Sorvall SSl rotor brought to 10 000 rev./min and then shut off) at room temperature and the loosely packed pellet was suspended in 10 ml imidazole/salts/glucose medium for phosphate uptake measurements. The cell density was measured in Klett units at the end of the uptake.

RESULTS

Osmotic shock and phosphate uptake

Fig. 1 shows the effect of stages I and II of osmotic shock on the phosphate uptake of *E. coli* AB3311 cells. Non-shocked cells show the typical biphasic phosphate

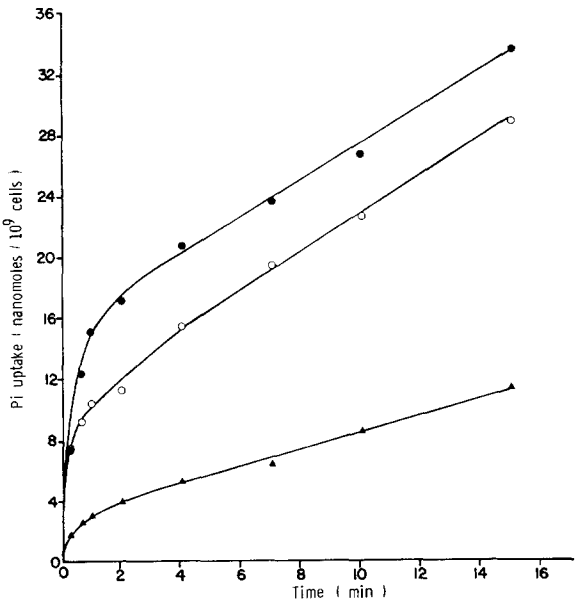

Fig. 1. The effect of stage I (sucrose/Tris/EDTA treatment) and II (rapid dispersion of cells into water at 2 °C) of osmotic shock on phosphate uptake by cells of *E. coli* strain AB3311. Cells were subjected to osmotic shock following the procedure of Neu and Chou [16] as detailed in Materials and Methods. Phosphate uptakes expressed as nmol/10^9 cells were measured essentially as outlined by Medveczky and Rosenberg [17]. (1) ● — ●, cells grown in Medium I and deprived of phosphate in Tris/salts/glucose medium at 37 °C for 2 h; (2) ○ — ○, cells described in (1) after stage I of osmotic shock and (3) ▲ — ▲, cells described in (1) after stages I and II of osmotic shock.

uptake curve described previously [11, 17]. As already commented on elsewhere, a major portion of this uptake reflects genuine active transport [17, 18]. The sucrose/Tris/EDTA treatment stage causes sufficient disruption to reduce the primary uptake by about one-third with little effect on the secondary uptake. Stage II (the cold shock step) profoundly reduces the primary uptake further (up to a total reduction of 80 %) and causes a significant reduction in the secondary uptake (approx. 50 %).

Since cells remain viable after osmotic shock despite profound inhibitory effects on many transport systems [5, 6], including phosphate, it was of interest to examine whether recovery of uptake could take place in phosphate-free media. Under these circumstances growth is prevented due to the absence of phosphate, but all other growth requirements are met and considerable repair is possible within the shocked cell. It can be seen in Fig. 2 that when cells are allowed to recover over 0–180 min at 37 °C both phases of phosphate uptake, and in particular the primary uptake phase, are gradually restored. In parallel with the restoration of phosphate uptake separate growth studies showed that there is a progressive reduction in growth lag from 30 to 0 min after 90 min of recovery.

Cold shock and phosphate uptake

A number of parameters were examined in attempting to establish a procedure that gave reproducible effects of cold shock on phosphate uptake. In all of the studies

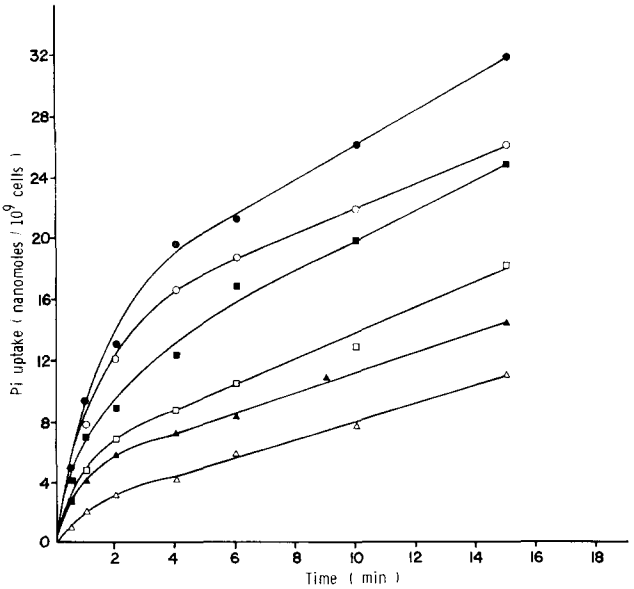

Fig. 2. The recovery of phosphate uptake observed in *E. coli* AB3311 cells subjected to osmotic shock and allowed to recover by incubation in phosphate-free Medium II. Osmotic shock and phosphate uptakes carried out as noted in Fig. 1. (1) ● — ●, cells grown in Medium II and deprived of phosphate in phosphate-free Medium II at 37 °C for 2 h; (2) cells allowed to recover at 37 °C for: 0 min, △ — △; 15 min, ▲ — ▲; 45 min, □ — □, 90 min, ■ — ■, and 180 min, ○ — ○.

TABLE I

SUMMARY OF EFFECTS OBTAINED BY THE ADDITION OF PHOSPHATE BINDING PROTEIN TO WASHED AND STARVED AB3311 CELLS FOLLOWING COLD SHOCK

Details on treatment of AB3311 cells and on cold shock procedure from 24 → 2 °C and 37 → 2 °C are described in Materials and Methods. Numbers of experiments are shown in parentheses below shock procedure. The average degree of shock achieved is calculated as the percentage reduction in P_i uptake observed after 20 min with the range shown below in parentheses. Reconstitution is calculated as the percentage restoration of P_i uptake ultimately achieved after 20 min upon the addition of phosphate binding protein (50 or 100 μg) as described in Materials and Methods. The range is shown below in parentheses

Treatment prior to cold shock	Cold shock procedure	Degree of shock (%)	Reconstitution achieved (%)
I Unstarved "washed" cells			
(a) Washed in imidazole/salts/glucose medium	24 → 2 °C	37	25.6
	(3)	(29–41)	(17–32)
	37 → 2 °C	43	12.2
	(5)	(27–56)	(0–27)
(b) Washed in NaCl (30 mM)/Tris (10 mM)	37 → 2 °C	59	9.7
	(4)	(45–74)	(0–23)
II Cells starved 2 h in imidazole/salts/glucose medium			
(a) Washed in imidazole/salts/glucose medium	37 → 2 °C	39	15.9
	(3)	(33–46)	(0–26)
(b) Washed in NaCl (30 mM)/Tris	37 → 2 °C	45	2.8
	(4)	(33–56)	(0–11)

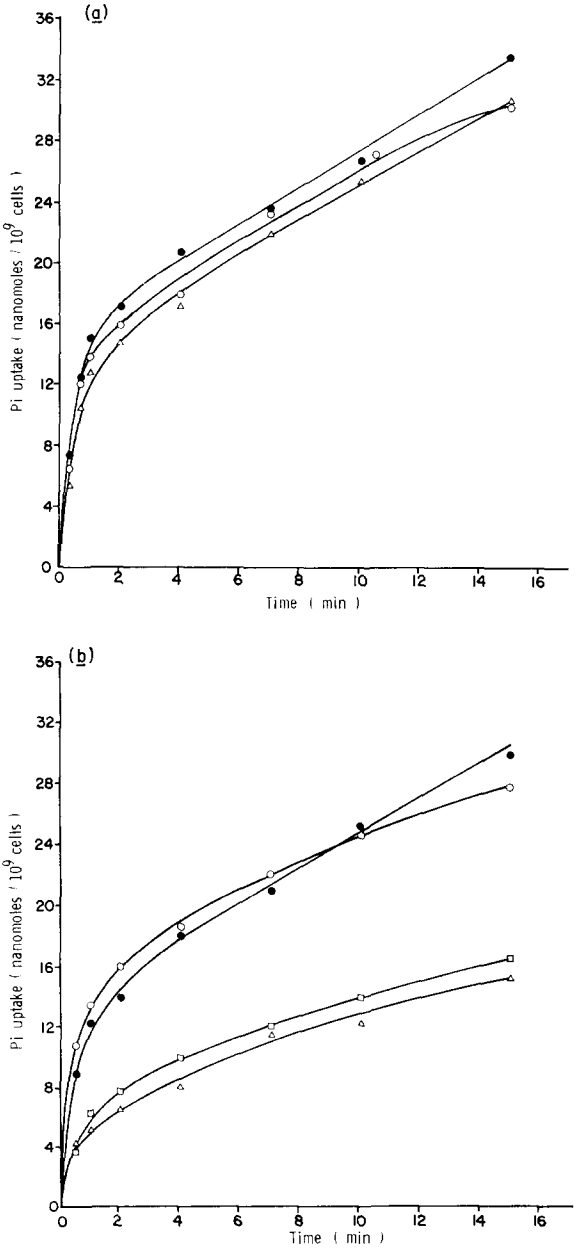

Fig. 3. The effect of cold shock on phosphate uptake of *E. coli* AB3311 cells under various conditions. Phosphate uptakes carried out as for Fig. 1. (a) Cold shock effects on unwashed cells. (1) ●—●, cells grown on Medium I and used directly; (2) ○—○, cells from (1) cold shocked from 25 → 2 °C and (3) △—△, cells from (1) cold shocked from 37 → 2 °C. (b) (1) ●—●, cells grown on Medium I and deprived of phosphate in Tris/salts/glucose medium at 37 °C for 2 h; (2) ○—○, cells from (1) washed once in 30 mM NaCl/10 mM Tris (pH 7.3); (3) △—△, cells from (2) cold shocked from 25 → 2 °C and (4) □—□, cells from a separate experiment were prepared as in (2) and cold shocked from 37 → 2 °C.

the manipulative procedure (i.e. suspension of cells in about 1 ml of appropriate medium and shocking from 24 or 37 °C into 80 vol. water at 2 °C) used for cold shock was not changed. Studies on pH effects showed the best range to be 7.0–7.3. Some results of the above studies are shown in Fig. 3a and b, and Table I. As a minimum it was necessary to wash the cells by suspension and centrifugation from appropriate media since, in general, no appreciable effect of cold shock was observed on unwashed cells (Fig. 3a). Of three media examined, it was observed that cold shocking after washing with Tris/salts/glucose and imidazole/salts/glucose gave about the same effect, i.e. an average 38 % reduction in overall phosphate uptake for cold shock from 24 → 2 °C and 43 % for 37 → 2 °C (Table I), whereas preliminary washing in NaCl/Tris resulted in a more severe shock (a 59 % reduction in phosphate uptake, Table I). This same pattern was observed in similar studies carried out on cells deprived of phosphate (i.e. starved) for 2–4 h (Table I). With all wash media, and in particular NaCl/Tris, the main effect of raising the temperature of the cells from 24 to 37 °C is to ensure a more consistent shock effect since, in general, this effect was about equal for shocking from 24 → 2 °C or 37 → 2 °C (Fig. 3b). Thus, it may be concluded that cells should be first washed in one of the above media and then cold shocked from 37 °C for the most consistent effects on phosphate uptake. Examination of where the cold shock affects phosphate uptake indicates that with few exceptions, the primary uptake is

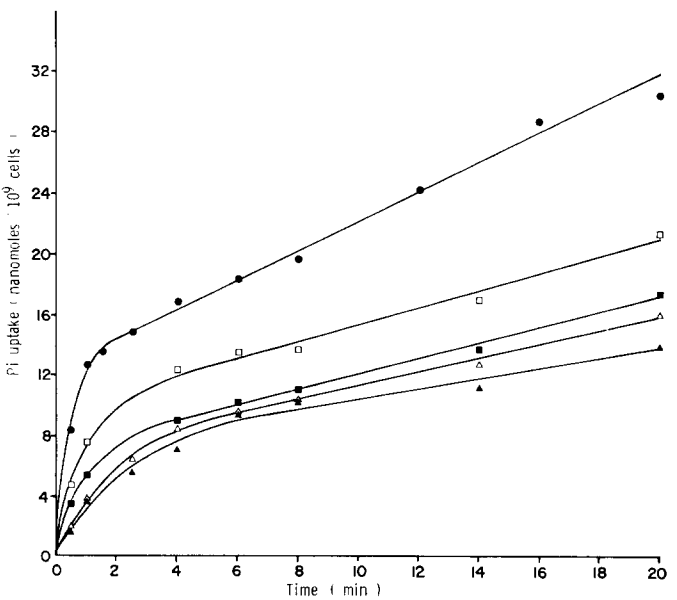

Fig. 4. Attempts to restore phosphate uptake by the addition of phosphate binding protein to phosphate-starved cells of *E. coli* AB3311 previously washed either in imidazole/salts/glucose or in NaCl/Tris and then cold shocked from 37 → 2 °C. Phosphate uptakes carried out as for Fig. 1. (1) ●—●, cells grown in Medium I and starved in Tris/salts/glucose medium at 37 °C for 2 h; (2) ■—■, cells from (1) washed in imidazole/salts/glucose, cold shocked from 37 → 2 °C and treated as for the control described in the section on restoration in Materials and Methods; (3) □—□, cells from (2) plus phosphate binding protein (100 μg); (4) ▲—▲, cells from (1) washed in 30 mM NaCl/10 mM Tris (pH 7.3) and then cold shocked and treated as in (2) and (5) △—△, cells from (4) plus phosphate binding protein (100 μg).

affected much more strongly than the secondary uptake as was observed for osmotic shock.

Attempts to restore phosphate uptake after cold shock

As initially described by Medveczky and Rosenberg [11] and in parallel with the above experiments on cold shock, a number of attempts were made to restore phosphate uptake after cold shock by the addition of phosphate binding protein. The results from representative experiments done on starved cells washed in imidazole/salts/glucose medium or NaCl/Tris and shocked from 37 → 2 °C are depicted in Fig. 4. A summary of the results obtained for all experiments on unstarved and starved cells appear as part of Table I. In none of the experiments reported was there a particularly good restoration of phosphate uptake (maximum of 32 %). The trend evident amongst all the data obtained was that of an inverse relationship between restoration of phosphate uptake and degree of shock. Thus with the milder shocks evident, particularly with cells (unstarved or starved) washed in imidazole/salts/glucose medium, there was a relatively better restoration of uptake.

DISCUSSION

Since the available evidence from our laboratories [10–12, 17–19] strongly indicates that the phosphate uptake or transport of AB3311 cells falls largely into what are now called the "shock-sensitive permeases" [6], it is of some interest to examine the effect of the various stages of osmotic shock and of cold shock (which is less severe) on phosphate uptake in this strain of *E. coli*. The small reduction in uptake for stage I and the large reduction in uptake for stage II which occur during osmotic shock agree well with the movement of proteins from the periplasmic space observed in the early studies on osmotic shock [1, 2, 15]. Amongst the periplasmic proteins released, Gerdes and Rosenberg [12] have found only one with phosphate binding properties which they have isolated in highly purified form. The evidence would, therefore, strongly implicate this phosphate binding protein in phosphate transport and it seems likely that the losses in uptake observed with stages I and II of osmotic shock and with cold shock are directly related to the loss of phosphate binding protein [11]. Study of recovery of osmotically shocked cells has provided good evidence that these cells are capable of restoring the component(s) of phosphate transport disrupted by osmotic shock even under conditions where growth cannot occur. This would mean that if phosphate binding protein is required, it must be resynthesized during the recovery phase. Extension is required of these experiments on recovery to include examination of whether this protein is synthesized in proportion to phosphate uptake restoration.

The more detailed investigation of cold shock undertaken than that originally described [11] could prove to be quite helpful for future studies that might be carried out, particularly, in relation to the reconstitution of transport systems such as phosphate. It has been shown that this milder procedure of shock is somewhat dependent for its effect on a preliminary wash of cells in a salts medium containing imidazole or Tris. Without this treatment and the precaution of shocking from 37 °C instead of room temperature inconsistent shock effects result. The data obtained are consistent with the view that some alteration of the outer membrane of *E. coli* has occurred due

to the wash step which allows the cold shock to be more effective.

All efforts to achieve the restoration of uptake originally reported by Medveczky and Rosenberg [11, 17], were unsuccessful. In fact, in all of the different conditions studied, our best average restoration reached no more than 26 %. Thus, we are unable, at present, to report good restoration of phosphate uptake upon the simple addition of phosphate binding protein. Either the shock has caused too severe a disruption for easy reconstitution, or some additional factor that is removed during shock is required.

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