Apparent High Degree of Asymmetry of Protein Arrangement in the *Escherichia coli* Outer Cell Envelope Membrane†

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ABSTRACT: Ghosts from *Escherichia coli* have been oxidized with $CuSO_4$–*o*-phenanthroline or ferricyanide–ferrocene. Upon oxidation they became resistant to boiling dodecyl sulfate. The resulting rod-shaped "oxidation containers," apparently held together by disulfide bridges, are practically pure protein. They are soluble in dodecyl sulfate when reduced and they contain a set of about 30 different polypeptide chains. The four major ghost membrane proteins are not represented among the "oxidation proteins." Comparison of data obtained from digestion of ghosts with trypsin or particle-bound trypsin showed that most of the "oxidation proteins" appear to be located at the outer surface of the ghost membrane which is derived from the outer cell envelope membrane. One of the major ghost membrane proteins, II*, is partially digested by trypsin, and it is shown that its trypsin sensitive part is also exposed only at the outer surface of the ghost membrane. Native cells could be oxidized only with low yields of "oxidation containers." However, cell envelopes prepared without detergents or chelating agents, as well as cells depleted of phospholipid or treated with sucrose–Triton X-100, are completely accessible to oxidation. In each case, the same set of proteins as that present in "oxidation containers" from ghosts was found to be covalently linked. Treatment of cells with trypsin caused the loss of about five "oxidation proteins" and a complete loss of oxidizability of the ghosts derived from these cells. It therefore appears that arrangement and localization of the "oxidation proteins" are not greatly different in cells and in ghosts, *i.e.*, that these proteins are also situated asymmetrically at the outer cell envelope membrane.

During studies on the determination of shape in bacteria, we have shown that rod-shaped "ghosts" (structures which are surrounded by a derivative of the outer membrane[1] of the cell envelope, devoid of murein, and free from all cytoplasmic material except for remaining fragments of the cytoplasmic membrane) can be isolated from *Escherichia coli* cells (Henning *et al.*, 1973a,b). Proteins contained in these ghosts can be separated electrophoretically into four bands (I, II, III, and IV; see Figure 2).

One step in the purification of these ghosts is a trypsin treatment which removes a rather heterogeneous set of proteins (*cf.* Figure 2). Most of these proteins can be covalently linked to each other *in situ* by oxidation, resulting in ghosts which are able to retain the size and shape of the cell when treated with hot SDS.[2] In this paper, we describe the oxidation phenomenon, some of the properties of the relevant proteins, and their localization in the outer cell envelope membrane. These proteins appear to be distributed asymmetrically with respect to the inner and outer surfaces of this membrane.

Materials and Methods

Cells. The *E. coli* K12 strain W945-T3282 (Henning *et al.*, 1972) was grown at 30° in Antibiotic Medium No. 3 (Difco) with forced aeration. The medium was supplemented with diaminopimelate (20 μg/ml) and thymine (50 μg/ml). Ghosts were prepared as described before (Henning *et al.*, 1973; procedure II).

Treatment of Cells for Oxidation. "Native" cells were harvested near the end of the logarithmic growth phase, washed once with 0.85% NaCl, and used directly for oxidation. Cells without phospholipid were obtained by extracting lyophilized (from aqueous suspension) cells four times with chloroform–methanol (2:1). Cell envelopes were prepared by breaking cells with glass beads, followed by differential centrifugation (Braun *et al.*, 1973a). Treatment of cells with sucrose and Triton X-100 was performed as described (Henning *et al.*, 1973b).

Oxidation. Cells, ghosts, or envelopes were suspended in 70 mM Tris-HCl at about 10 mg dry weight/ml (pH 8.7); *o*-phenanthroline plus $CuSO_4$ were added at final concentrations of 0.2 and 0.072 mM, respectively (Kobashi and Horecker, 1967). The suspension was incubated at room temperature for 3 hr with aeration. For oxidation with potassium ferricyanide and ferrocene, 10 mM ferricyanide was brought to 5 mM in ferrocene by rapidly adding a solution of ferrocene in acetone to an aqueous ferricyanide solution. When desired, sodium tetraphenylborate was added at 5 mM. The resulting suspension was vigorously shaken; aqueous suspensions of cells, ghosts, or envelopes were added to yield final concentrations of 5 mM in ferricyanide, 2.5 mM in ferrocene, and about 10 mg dry weight of substrates/ml. The mixtures were incubated at room temperature with shaking for at least 4 hr. In all cases, cellular material was recovered by centrifugation and washed four times with water.

Oxidation Proteins. Oxidized ghosts were freed from murein by suspending them at about 1 mg dry weight/ml in 25 mM ammonium acetate containing egg-white lysozyme at 50 μg/ml. (Oxidation of ghosts can be performed equally well before or after removal of murein with lysozyme.) After 1 hr at room temperature, ghosts were centrifuged (10 min, 12,000*g*), suspended in 2% SDS, and kept for 5

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[1] The term "membrane" is used in this communication as a morphological designation, *i.e.*, it is only meant to describe the unit membrane profile visible by electron microscopy.

[2] Abbreviation used is: SDS, sodium dodecyl sulfate.

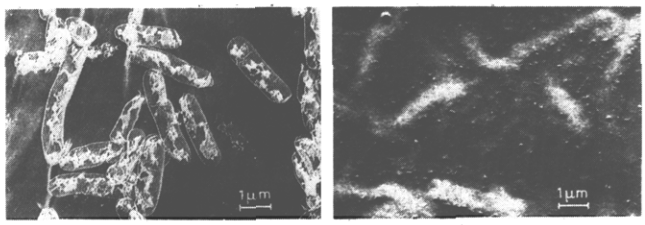

FIGURE 1: Shadowed ghosts. Left: Normal ghosts that are soluble in SDS and that yield electrophoretic patterns as shown in Figure 2A. Right: "Oxidation containers" obtained by oxidation of ghosts with $CuSO_4$-*o*-phenanthroline and subsequent extraction with hot SDS. They are completely soluble in SDS upon reduction.

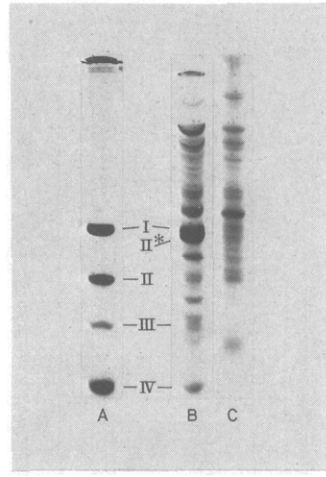

FIGURE 2: SDS polyacrylamide gel electrophoresis of: (A) ghosts after trypsin treatment; (B) ghosts before trypsin treatment; (C) proteins present in "oxidation containers" such as those shown in Figure 1. It is very difficult to obtain good photographs of C-type electrophoreses; when larger amounts of protein are applied to a gel in order to make minority bands visible others begin to smear. The number of about 30 such proteins mentioned in the text can therefore not be taken from this photograph alone, and this number was derived by inspection of many different gels.

min in boiling water. The resulting "oxidation containers" (see Figure 1) were recovered by centrifugation (6 hr, 100,000*g*) and extracted one more time with hot SDS. They were then precipitated with acetone (final concentration 90%), washed three times with acetone, and lyophilized from aqueous suspension. Oxidized envelopes were processed identically. Oxidized cells were placed in 25 mM ammonium acetate containing either 30% sucrose (w/v) or 30% sucrose and 1% Triton X-100 (v/v). Lysozyme was added, and they were left at room temperature for at least 4 hr. Subsequent treatments with SDS were performed as described above.

Trypsin Treatments. Ghosts or cells to be digested with trypsin were suspended in 20 mM Tris-HCl (pH 9) at about 10 mg dry weight/ml. Trypsin was added at 500 μg/ml, and the suspension was incubated at room temperature for 10 hr. Murein sacculi were isolated (Braun and Rehn, 1969) from such samples, lyophilized, and subjected to acid hydrolysis in 4 N HCl for 14 hr at 104°. For experiments with particle-bound trypsin, a commercially available preparation (Trypsin-30 Enzygel, Boehringer, Mannheim) was used. Before use, the gel was washed six times with 20 mM Tris-HCl (pH 9). The insoluble trypsin was added at 2 mg of gel (dry weight)/ml, and the mixture was stirred during incubation under the same conditions as specified above. Substrates and gel could be separated easily, because the gel settled much faster than any of the substrates. For digestions at 4°, the concentrations of solubilized and particle-bound trypsin were increased two-fold. As controls in all these experiments substrates were incubated without trypsin, and spontaneous degradation of proteins was not observed (exception: see Discussion, subsection Artefacts).

Analytical Procedures. Protein was measured by amino acid analyses of acid hydrolysates (6 N HCl, 24 hr at 110°) with a Beckman Multichrom B analyzer; oxidation with performic acid was performed at 0° according to Hirs (1956). For electrophoresis, method II of the procedure described (Henning *et al.*, 1973a) was used. Ketodeoxyoctonate was determined according to Osborn (1963). Inactivation of phage T5 was determined according to Braun *et al.* (1973b).

Electron microscopy was performed with a Philips EM 201 microscope following standard methods detailed earlier (Henning *et al.*, 1969).

Results

Oxidation and Proteins Involved ("Oxidation Proteins"). Purification of ghosts normally involves treatment of cells with Triton X-100 in 40% sucrose, 4 M urea, trypsin, and, finally, lysozyme. Ghosts not treated with trypsin were subjected to oxidation with $CuSO_4$ and *o*-phenanthroline (Kobashi and Horecker, 1967) and then digested with lysozyme. Nonoxidized ghosts are readily soluble in 1% SDS. In contrast, oxidized shosts can be kept in boiling 4% SDS without disappearing, but the addition of a reducing agent causes immediate solubilization. Treatment of ghosts, prior to oxidation, with iodoacetamide completely prevents the oxidative formation of ghosts resistant to hot SDS. The appearances of oxidized ghosts and normal ghosts in the electron microscope are shown in Figure 1. It can be seen that the detergent treatment causes a certain degree of fragmentation; we estimate that approximately 50–70% of the oxidized ghosts remain morphologically intact.

Ghosts treated with trypsin before oxidation can no longer be successfully oxidized. Similarily, trypsin treatment of oxidized ghosts causes loss of morphological resistance to hot SDS. The protein compositions of ghosts before trypsin treatment, after trypsin treatment, and after oxidation and subsequent extraction with hot SDS are shown in Figure 2. Most proteins which are susceptible to trypsin digestion are tied into the oxidized ghosts; however, the major proteins (I–IV) of the normal ghost membrane are not represented in the oxidized ghosts. After the oxidized ghosts were extracted with hot SDS, they were washed with acetone in order to remove the detergent. The dry weight of the ghosts was found to be 3–4% of the dry weight of whole cells.

Oxidized ghosts extracted with SDS and oxidized with performic acid were subjected to amino acid analyses. It was found that 80–90% of their dry weight (lyophilized) can be accounted for by protein; the values for two different preparations were 81 and 89%. There is 1 mol of cysteine per ~8000 g of protein. The proteins do not contain amino sugars or ketodeoxyoctonate and thus no lipopolysaccharide or residual murein. In view of the high protein content, we have not attempted to elucidate the nature of the 10–20% nonprotein material, *e.g.*, residual water and non-amino acid protein substituents.

Localization of Oxidation Proteins. Figure 3, provided as an aid to understanding the results presented in this section, illustrates certain relevant features of the *E. coli* cell

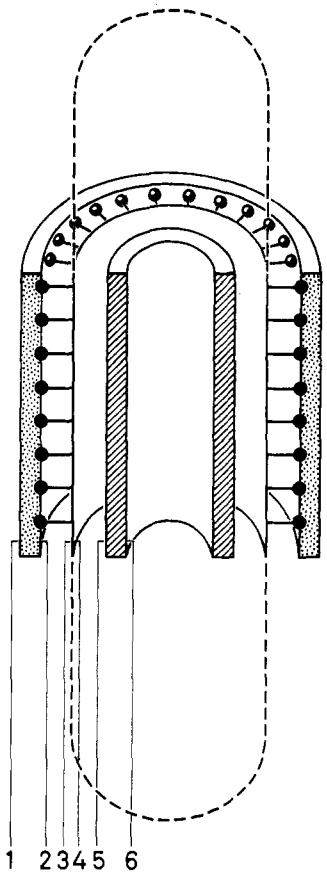

FIGURE 3: Scheme of the *E. coli* cell envelope: (1) outer surface of the outer membrane; (2) inner surface of the outer membrane; (3) outer surface of the murein sacculus; (4) inner surface of the sacculus; (5) outer surface of the cytoplasmic membrane; (6) inner surface of this membrane. The dotted line indicates the covalently closed structure of the sacculus;(●–) lipoprotein covalently attached to murein; it has been shown (Bosch and Braun, 1973) that the murein-bound lipoprotein extends only into the outer membrane.

envelope. This figure is schematic and thus somewhat oversimplified (*cf.* dePetris, 1967).

Ghosts not treated with trypsin possess a largely intact (*i.e.*, morphologically intact) cytoplasmic membrane; this membrane is observed to be fragmented after digestion by trypsin (Henning *et al.*, 1973a). Since only ghosts which have not been treated with trypsin can be oxidized successfully, the oxidation proteins could be situated in this cytoplasmic membrane, in the outer membrane, or in both.

The oxidation proteins were found to be located at the outer membrane through the following experiment. Oxidized ghosts which had not been subjected to lysozyme were treated with soluble trypsin or with particle-bound trypsin. Soluble trypsin had three effects on the ghosts: disappearance of the oxidation proteins, cleavage of protein II* of the outer membrane to yield its tryptic fragment, protein II (Henning *et al.*, 1973a; see Figure 2), and fragmentation of the cytoplasmic membrane. In contrast, particle-bound trypsin also caused the disappearance of the oxidation proteins, and converted protein II* to fragment II, but did not fragment the cytoplasmic membrane. In support of this observation, microscopic examination of the carrier particles showed that even the smallest particles are much larger than a ghost; the carrier particles with the bound trypsin could not possibly have penetrated the intact sacculi present in the ghosts. Therefore, since the cytoplasmic membrane appears to be inaccessible to the particle-bound enzyme, it certainly seems that the oxidation proteins belong to the outer cell envelope membrane.

If the cytoplasmic membrane is not accessible to particle-bound trypsin, one may suspect that it also cannot penetrate the outer ghost membrane. Thus, if the immobilized enzyme should act only on the outer surface of the outer membrane, the oxidation proteins could be localized at only that surface. The following experiment showed that the particle-bound trypsin in fact does not penetrate the outer ghost membrane. Both isolated murein sacculi and ghosts (as long as they were not subjected to proteolytic digestion) contain a lipoprotein covalently bound to murein (Braun and Bosch, 1972). Soluble trypsin (Braun and Rehn, 1969) and particle-bound trypsin cleave the lipoprotein from sacculi equally well. Such is not the case with ghosts. We treated ghosts with soluble trypsin or particle-bound trypsin and used both preparations to prepare murein sacculi. These were acid hydrolyzed and analyzed for their amino acid composition. The soluble trypsin had completely removed the covalently bound lipoprotein from the murein, and the particle-bound trypsin had not (see Table I). Therefore, this experiment provides quantitative evidence that the insoluble enzyme cannot penetrate the outer ghost membrane.

The oxidation proteins therefore appear to be located at the outer surface of the outer ghost membrane. It could be, however, that this statement is not correct concerning its exclusiveness. A protein may penetrate the membrane and only part of it may be exposed at the outer surface. Action of trypsin on the exposed part may cause a conformational change which in turn may cause the protein to be expelled from the membrane. We cannot completely exclude such a possibility but believe it to be rather unlikely for two reasons. At temperatures around 0°, one would expect the ghost membrane to be frozen (if there is fluidity at room temperature) and, therefore, presumably much less able to expel a protein in the way discussed. Removal of the oxidation proteins with soluble trypsin, or particle-bound trypsin, however, was found to be possible at 4° as well as at room temperature. Second, there are about 30 different oxidation proteins and it appears fairly improbable that all of them would, upon trypsin action, behave the way discussed above. We think that the experiments described provide a very reasonable basis for the assumption that at least the majority of the oxidation proteins are located only at the outer surface of the ghost membrane.

In view of this localization, one may suspect that phage receptors and similar types of proteins belong to the oxidation proteins. The receptor for phage T5 has been shown to be a protein (Braun *et al.*, 1973b), and we have tried to detect its presence among the oxidation proteins. As determined by inactivation of T5, oxidized ghosts contain this phage receptor, and it disappears upon trypsin treatment. However, it also disappears upon extraction of oxidized ghosts with hot SDS. The effect of SDS may simply be due to inactivation, and the question of whether the T5 receptor is actually an oxidation protein could not be answered.

Action of Trypsin on and Oxidation of Whole Cells. Whole cells could not be oxidized in the same way as ghosts. That is, whole cells treated with $CuSO_4$ and *o*-phenanthroline yielded ghosts that, after lysozyme removal of murein, were soluble in SDS. The question thus arose as to whether and to what extent the results obtained with ghosts are artefacts of the ghost purification procedure and, if so, whether they may bear no relevance to intact cells. Although the problem could not be definitely solved, the sum

Table I: Amino Acid and Amino Sugar Composition of Sacculi from Ghosts.[a]

	Sacculi from Ghosts			Theoretical Values for	
	No Trypsin	Treated with Particle-Bound Trypsin	Treated with Soluble Trypsin	Murein	Murein with Lipoprotein
Aspartic acid	15.8	16.2			14
Threonine	2.6	2.7			2
Serine	6.3	6.7			6
Glutamic acid	13.2	14.0	11.7	10	15
Alanine	24.2	27.2	19.6	20	29
Valine	4.0	4.0			4
Methionine	2.3	2.2			2
Isoleucine	1.3	0.9			1
Leucine	5.2	4.7			4
Tyrosine	1.1	1.0			1
Lysine	5.3	5.2	1.2	1	5
Arginine	4.4	4.6			4
Diaminopimelic acid	10.3	10.0	12.1	10	10
Muramic acid	6.4	6.6	7.0	10	10
Glucosamine	9.7	9.6	10.0	10	10

[a] The experimental values are expressed as numbers of residues per mole of lipoprotein, and they are referred to valine = 4.0 (Braun and Bosch, 1972); in the case of murein obtained from trypsinized ghosts, they are referred to glucosamine = 10.0 (Braun and Rehn, 1969). The theoretical values show the composition expected if one lipoprotein is attached to every tenth repeating disaccharide unit of murein (Braun and Rehn, 1969). The theoretical murein composition is that for sacculi treated with trypsin, which leaves one lysine residue on the murein. Hydrolysis was performed on performic acid oxidized samples for 24 hr at 105° in 6 N HCl; under this condition muramic acid is partially destroyed (Braun and Rehn, 1969).

of the following observations would appear to strongly argue that we are not looking at a grossly artefactual situation.

It has been found that ghosts cannot be oxidized with ferricyanide, but that they can be oxidized easily with the cupric–phenanthroline complex. The ineffectiveness of ferricyanide is most probably due to the inavailability to this oxidant of all or some of the required sulfhydryl groups. We therefore made use of a more lipophilic redox agent, the water-insoluble compound ferrocene (Hinkle, 1970). At least part of the ferrous ion in ferrocene can be oxidized with ferricyanide in aqueous suspension when ferrocene dissolved in acetone is mixed rapidly with an aqueous ferricyanide solution. Whole cells were incubated with ferricyanide, ferrocene, or a mixture of both. They were then transferred to 30% sucrose either with or without Triton X-100 in order to make them sensitive to lysozyme, and the lysozyme was added. Successful oxidation occurred only with cells that had been incubated in the ferricyanide–ferrocene mixture. However, the presence or absence of detergent influenced the quantity of ghost production markedly. Without Triton X-100, only about 10% of the cells yielded ghosts resistant to hot SDS; with the detergent, the normal fraction of the cells (80–90%) produced oxidized ghosts. Electrophoretic analysis of these oxidized ghosts after extraction with hot SDS revealed the same band pattern as that shown in Figure 2.

It is difficult to eliminate the possibility that the cells may not have been oxidized at all before the sucrose or sucrose plus Triton treatments, but that, instead, successful oxidation occurred only when these treatments had created, partially or completely, the "right" artefacts of protein arrangement. (Ferrocene or ferricene present in the cell membrane certainly cannot be removed by washing.) The yield of oxidized ghosts from cells treated with lysozyme in sucrose without detergent could not be increased by adding tetraphenylborate to the ferricyanide–ferrocene mixture, even though this increases the solubility of ferrocene in a lipid phase (Hinkle, 1970). Treating the cells with $CuSO_4$–*o*-phenanthroline and then with ferricyanide–ferrocene with or without tetraphenylborate was also ineffective.

However, two quite different procedures not involving the use of sucrose or detergent allow the preparation of "oxidation containers." We prepared cell envelopes according to a procedure (Braun *et al.*, 1973a) which involves only mechanical treatment of cells (without detergents or chelating agents), and which allows the isolation of perfectly rod-shaped envelopes. The murein in these envelopes is sensitive to lysozyme. They were subjected to treatments with ferricyanide, $CuSO_4$–*o*-phenanthroline, and the ferricyanide–ferrocene mixture. Lysozyme was added to all samples without any further treatment of the envelopes. Only those envelopes that had been oxidized with the ferricyanide–ferrocene mixture became insoluble in boiling SDS. Again, the electrophoretic band pattern obtained from these oxidized envelopes was the same as that shown in Figure 2. Finally, it was possible to show that still another treatment of cells makes them oxidizable. Lyophilized cells were extracted with chloroform–methanol. Such phospholipid-depleted cells could be completely oxidized with the cupric–*o*-phenanthroline complex, while lyophilization alone did not yield cells that could be oxidized in this way.

The results presented so far in this section are summarized in Table II. While it has not been proven that intact cells can be oxidized, it is clear that a variety of very different treatments causes oxidizability and that in each case the

Table II: Oxidation of Ghosts, Cell Envelopes, and Cells.[a]

Oxidant	Ghosts	Envelopes	Whole Cells in Sucrose	Whole Cells in Sucrose plus Triton X-100	Cells Lyophilized	Cells Lyophilized without Phospholipid
Ferricyanide	−	−	−	−	n.t.	n.t.
$CuSO_4$–*o*-phenanthroline	+	−	−	−	−	+
Ferricyanide–ferrocene	+	+	+/−	+	n.t.	n.t.

[a] + or − means covalently stabilized "oxidation containers" have or have not been obtained; +/− means containers were obtained at low yield (see text); n.t. means not tested.

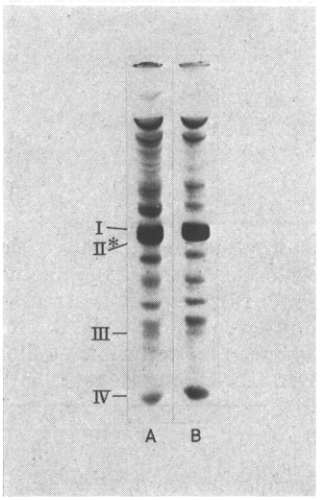

FIGURE 4: SDS polyacrylamide gel electrophoresis of: (A) ghosts not treated with trypsin; (B) ghosts prepared from cells that had been treated with trypsin for 12 hr. Localization of the missing proteins is mainly above the major proteins I and II*.

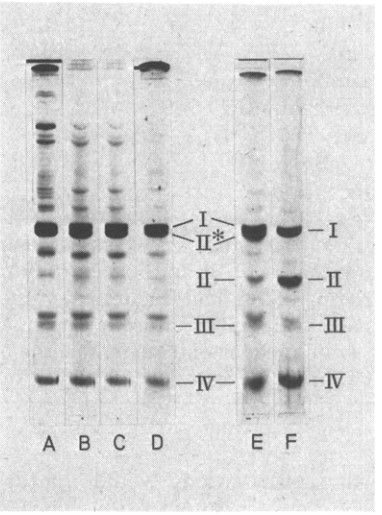

FIGURE 5: SDS polyacrylamide gel electrophoresis of: (A) ghosts not treated with trypsin. This preparation was subjected to trypsin digestion for: (B) 5 min; (C) 10 min; (D) 30 min; (E) 60 min; (F) 8 hr. Ghosts A could and ghosts B–F could not be oxidized to yield "oxidation containers." Ghosts A–D could and ghosts F could not be cross-linked with dimethyl suberimidate. Ghosts E could be cross-linked with reduced yield (see text).

same set of proteins has been linked covalently. This fact, together with the result to follow, makes it fairly unlikely that the arrangement of the oxidation proteins in the ghost membrane represents a gross artefact.

It was possible to demonstrate a complete loss of oxidizability in ghosts prepared from whole cells which had been depleted of only a few oxidation proteins. Ghosts prepared without further trypsin treatment from trypsin-treated, whole cells could not be oxidized. Electrophoretic analyses of these ghosts showed that some, but by no means all, of the oxidation proteins were missing (Figure 4). As one might suspect, it was also found that murein isolated from trypsinized cells had not lost the covalently bound lipoprotein.

Chemical Cross-Linking and Oxidation Proteins. We have recently shown that ghosts not treated with trypsin can be cross-linked with dimethyl diimido esters to yield covalently stabilized ghosts possessing the shape and size of the cell (Haller and Henning, 1974). Upon extraction with hot SDS, these containers are almost pure protein and contain most of the major ghost membrane proteins (I–IV). Such cross-linking can no longer be achieved after trypsin digestion. We had argued that the reason for this effect of trypsin is the partial digestion of one of the major outer membrane proteins, protein II*. In view of the findings reported in this communication, one might also assume that, for successful chemical cross-linking, the major outer membrane proteins *plus* the oxidation proteins are required. The following experiment shows that this is not the case, *i.e.*, that our previous interpretation of the trypsin effect is correct.

Ghosts were incubated with trypsin for various time periods, and the action of trypsin was interrupted by the addition of trypsin inhibitor. Different samples, either cross-linked with dimethyl suberimidate (method: Haller and Henning, 1974) or oxidized as described in this paper, were analyzed electrophoretically after various times of incubation. After only 5 min of trypsin digestion, ghosts which were oxidized and then treated with lysozyme were no longer resistant to hot SDS. In contrast, ghosts could still be successfully chemically cross-linked after 30 min of trypsin treatment. If the trypsin treatment was prolonged to 1 hr, the yield of cross-linked ghosts remained significant, although reduced (80% at zero time *vs.* 30% after 1 hr). However, as shown in Figure 5, the oxidation proteins had been almost completely removed after this hour of proteolytic digestion, while protein II, the tryptic fragment of protein II*, was just beginning to appear in greater amounts. As shown before, complete cleavage of protein II* is paralleled by complete failure to cross-link trypsin-treated ghosts. Thus, there is little doubt that tryptic cleavage of

protein II* and failure to cross-link ghosts are causally related. It is also clear that the oxidation proteins are not required for cross-linking of ghosts.

Discussion

Artefacts. It could not be shown convincingly that native cells can be oxidized. Nevertheless, it is unlikely that we are looking at a grossly artefactual situation. Three major types of artefacts are possible: localization, arrangement, and number of oxidation proteins may not be the same in cells as in ghosts.

It was determined that, in this protein mixture, there is one cysteine residue per about 8000 daltons of protein. This concentration of cysteines clearly is much too low to allow *any* arrangement of the oxidation proteins leading to the covalently stabilized containers described. Removal of a few (about five) oxidation proteins is possible by trypsin treatment of whole cells, and ghosts derived from such cells can no longer be oxidized. Thus, it actually appears that the arrangement of these proteins may be rather ordered. A number of different treatments of cells (extraction of phospholipids, treatment with sucrose–Triton X-100, preparation of cell envelopes) make the proteins in question accessible to successful oxidation. The resulting containers consist of practically pure protein and in each case consist of the *same* set of polypeptide chains. We have also found that the situation is not unique to *E. coli;* the same type of oxidation containers can be obtained from the Pseudomonad *Spirillum serpens* (Schweizer, Sonntag, and Henning, *J. Mol. Biol.*, in press). Thus, the sum of these observations makes one fairly confident that the localization and arrangement of the oxidation proteins in cells do not differ considerably from those in ghosts. It must be pointed out, nevertheless, that there is no proof at all that the contact between all these proteins, apparent in ghosts, cell envelopes, and cells subjected to various treatments, also exists in native cells.

Finally, we cannot be sure if the number of about 30 oxidation proteins is correct. There may be fewer, since cellular proteases may become active during the various treatments and cause fragmentation. It is indeed likely that proteases are among the oxidation proteins. With several preparations of oxidation containers, we have microscopically observed slow breakdown of these rods during several days of storage in water at room temperature. Electrophoretic analysis of such preparations revealed an extensive degradation of the polypeptide chains, and most of the material which stained with Coomassie Brilliant Blue moved as a heavy smear at the front. Yet, the excellent reproducibility of the electrophoretic profile of oxidation proteins, and its independence of the type of treatment of cells, makes it rather unlikely that the number is vastly overestimated. We would also not expect, in view of the average molar concentration of cysteine residues, that covalently stabilized containers could still be obtained when considerable proteolytic cleavage had occurred.

Oxidation Proteins and Proteins of the Outer Cell Envelope Membrane. As far as one can tell in view of the present knowledge on proteins of the outer membrane, none of the often so-called major proteins (molecular weight range from about 8000 to about 40,000) of this membrane is among the oxidation proteins. Ghost protein IV is identical with the lipoprotein described by Braun and collaborators (Braun and Bosch, 1972; Hantke and Braun, 1973), does not contain a sulfhydryl residue, and cannot, therefore, become linked to the oxidation proteins. Ghost protein I (quite probably identical with protein A_1 of Bragg and Hou (1972), with protein 1 of Schnaitman (1974a), with protein B of Koplow and Goldfine (1974), and with the *Salmonella* ~35K protein of Ames (1974)) has been isolated and partially characterized chemically (Garten and Henning, 1974). It is identical with an envelope protein isolated from *E. coli* B (Rosenbusch, 1975) and consists of a single or at most two very similar polypeptide chains. The protein from both sources has been shown to possess only one cysteine residue. The situation as yet is less clear for ghost protein II*. In all likelihood it is identical with protein B* recently purified by Reithmeier and Bragg (1974; see this reference for probable identity of this protein with proteins observed by other workers), and they reported the absence of cysteine from it. We have isolated the tryptic fragment of II*, protein II (Garten and Henning, 1974), and have shown that it appears to consist of a single polypeptide chain that contains one cysteine residue (it is not yet known if a free sulfhydryl group is present). Proteins II* and B* certainly are identical with protein 3 of Schnaitman (1974a); however, his data suggest that protein 3 consists of two polypeptide chains. At any event, it appears that the cysteine content of protein II* is also very low, and the absence of proteins I and II* (and, of course, IV) from the oxidation proteins need not have any implications about their possible association with the former. Although somewhat trivial, it should also be pointed out that the failure of trypsin to attack protein I or to further degrade protein II (both are degraded in the isolated state) does not necessarily mean anything about their localization, *i.e.,* they need not be protected by their host membrane against proteolytic attack but may simply be folded such that no trypsin-sensitive bonds are exposed.

A final point of great interest concerning the role of proteins of the outer cell envelope membrane regards the isolation of otherwise fairly healthy mutants exhibiting various types of abnormal protein composition of this membrane (Wu, 1972; Koplow and Goldfine, 1974; Ames *et al.,* 1974; Schnaitman, 1974b; Chai and Foulds, 1974). In particular, massive deficiencies of many of these proteins (probably including oxidation proteins) have been found in strains of *Salmonella* (Ames *et al.,* 1974) and *E. coli* (Koplow and Golfine, 1974) defective in lipopolysaccharide synthesis. These results have no direct bearing on the interpretation of the data reported in this communication as far as they concern the localization of the proteins in question. We have suspected, however, that the arrangement of the oxidation proteins may be a rather ordered one. If so, it appears that such an order is not a requirement for viability or the formation of an outer membrane.

Arrangement, Asymmetry, and Models of the Outer Membrane. We have mentioned above the possibility of a rather ordered arrangement of the approximately 30 oxidation proteins and the other, major membrane proteins in (or at) the outer cell envelope membrane. One is tempted to visualize such an arrangement as repeating "supersubunits," *i.e.,* a number of different polypeptides forming a large unit similar to a multienzyme complex. Different complexes could be repeated over and over across the surface of the membrane. Such a possibility obviously has attractive features, such as possibly facilitating the proper placement of different polypeptide chains.

The majority of the oxidation proteins are degraded by particle-bound trypsin acting only on the outer surface of the ghost membrane. Also, protein II* is completely converted to its tryptic fragment under the same conditions.

The same effects are also observed at low temperatures. Therefore, it appears that the protein distribution in the ghost membrane is asymmetric in two respects. First, the largest parts of most of the oxidation proteins are exposed to proteolytic attack at the outer surface of the ghost membrane, and there is no symmetric counterpart exposed at the inner surface of this membrane, since otherwise particle-bound trypsin would be expected to remove only 50% of these proteins. Second, and by the same argument, the trypsin-sensitive part of protein II*, one of the major ghost membrane proteins (see preceding subsection), also appears to be exposed only at the outer surface of the ghost membrane.

Asymmetry of membrane architecture, particularly in the localization of proteins and phospholipids, has been shown or suspected to exist in a number of systems (*e.g.*, Fukui *et al.*, 1971; Bretscher, 1972; Blaurock and Stoeckenius, 1971; Zwaal *et al.*, 1973; Altendorf and Staehelin, 1974; Johansson and Hjertén, 1974); evidence suggestive of asymmetry has been obtained by Bragg and Hou (1972) for proteins of the outer *E. coli* cell envelope membrane. The data described in this communication constitute, as far as we know, one of the most extreme cases of apparent asymmetric protein distribution. We believe, however, that it would be rather premature to draw a model of the outer cell envelope membrane, since major parameters are unknown: the arrangements of phospholipid and lipopolysaccharide, for example, whether symmetric, asymmetric, continuous, discontinuous, or entirely dynamic. It is also obvious that this study cannot be taken as a final proof for the arrangement of the oxidation proteins in the native outer cell envelope membrane.

The following final point may be worth mentioning. The function of the oxidation polypeptide chains so far remains unknown. It has been suggested above that one or more proteases might be among them, and, in view of their localization, one may suspect that phage receptors and similar types of proteins are also present. In any case, it is rather safe to assume that the oxidation proteins do have functions other than those possibly related to structure. If the close contact between all these proteins apparent in ghosts should also exist in cells, then such a protein net may imply the possibility of an extensive communication system between proteins of different functions over the whole cell envelope.[3]

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[3] It has now been found that *Salmonella typhimurium* can be oxidized the same way as *E. coli*, and it was observed that deep rough mutants of *Salmonella* (Ames *et al.*, 1974) do not miss the oxidation proteins. In contrast to wild type *Salmonella*, with the deep rough mutants (lipopolysaccharide defective), *native cells can be oxidized* with ferrocene. It therefore is very likely that native cells can normally not be oxidized because lipopolysaccharide protects the outer membrane from the required lipophilic oxidant. In any case these results clearly rule out that artificial protein rearrangement and/or denaturation is required for successful oxidation.

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