

## Localization of the dicarboxylate binding protein in the cell envelope of *Escherichia coli* K12

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Examination of the localization of the dicarboxylate binding protein (DBP) in the cell envelope of *Escherichia coli* K12 reveals that this protein is present on the cell surface, and also in the inner and outer regions of the periplasmic space. The cell surface DBP is released by treating the cells with EDTA. This protein can be surface labeled by lactoperoxidase radioiodination, and by diazo[<sup>125</sup>I]iodosulfanilic acid in whole cells. It also binds tightly, but not covalently, to lipopolysaccharide. The DBP located in the outer region of the periplasmic space is released when the outer membrane is dissociated by EDTA - osmotic shock treatment. The DBP located in the inner region of the periplasmic space is released only when the EDTA - osmotic shocked cells are subjected to lysozyme treatment. At the moment, it is not certain whether this protein is bound to or trapped by the peptidoglycan network. This protein cannot be surface labeled in whole cells or in EDTA - osmotic shock treated cells; and it is not associated with lipopolysaccharide. Analysis of transport mutants indicates that these DBP are coded by the same gene.

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L'examen de la localisation de la protéine liant le dicarboxylate (DBP) dans l'enveloppe cellulaire d'*Escherichia coli* K12 révèle que cette protéine est présente à la surface de la cellule et aussi dans les régions interne et externe de l'espace périplasmique. Le traitement des cellules avec l'EDTA libère la DBP logée à la surface de la cellule. Cette protéine peut être marquée en surface par radioiodation lactoperoxydasique et par l'acide diazo[<sup>125</sup>I]iodosulfanilique dans les cellules entières. Elle se lie aussi étroitement, mais non de façon covalente, au lipopolysaccharide. Le DBP située dans la région externe de l'espace périplasmique est libérée quand la membrane externe est dissociée par EDTA - choc osmotique. La DBP logée dans la région interne de l'espace périplasmique est libérée seulement lorsque les cellules soumises au EDTA - choc osmotique subissent un traitement au lysozyme. Actuellement, on ne sait pas de façon certaine si cette protéine est liée au ou trappée par le réseau peptidoglycane. Cette protéine ne peut être marquée en surface dans les cellules entières ou dans les cellules traitées par EDTA - choc osmotique et elle n'est pas associée au lipopolysaccharide. L'analyse de mutants de transport montrent que ces DBP sont codées par le même gène.

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At least one periplasmic binding protein and two cytoplasmic membrane transport components are involved in the translocation of dicarboxylic acids: succinate, fumarate, and malate, across the cell envelope of *Escherichia coli* (1, 2). Cells, from which the dicarboxylate binding protein has been removed or inactivated, are impaired in dicarboxylate transport (3, 4). Genetic analysis of the dicarboxylate transport mutants indicates that the *cbt* gene is responsible for the structure, or regulation of the dicarboxylate binding protein, and this gene maps at 16 min on the *E. coli* genetic map (3, 5, 6). Other than this genetic evidence concerning the role of the dicarboxylate binding protein in the whole cell transport process, no information is available

on the mechanism(s) by which this protein may carry out its function.

Ignorance concerning the mode of action of binding proteins is also perceptible in other transport systems. It has been postulated that binding proteins may function in the retention of substrates in the periplasmic space (7); alternatively, it has also been suggested that these proteins may function by transferring the substrates to specific cytoplasmic membrane transport components. In this case, specific physical interactions between the two types of transport components are thought to be involved (8). It is generally assumed that binding proteins function only in the periplasmic space (9). Not much is known on the localization of these proteins in the cell envelope and on their interactions with cell wall components.

We have recently demonstrated that some dicarboxylate binding proteins are present on the cell surface, and they play an important role in the outer membrane dicarboxylate transport system (10). Treat-

ABBREVIATIONS: DBP, dicarboxylate binding protein; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; ACS, aqueous counting scintillant; *p*-NPP, *p*-nitrophenyl phosphate; cpm, counts per minute.

ment of whole cells with nonpenetrating inhibitors, such as proteases or diazosulfanilic acid, not only inactivated the outer membrane dicarboxylate transport system, but also caused the release of this binding protein from the cell surface. One can also reconstitute this outer membrane transport system by the mere addition of purified dicarboxylate-binding protein to the inactivated cells or to transport-negative mutants.

The present communication reports our findings on the localization of the dicarboxylate binding protein in the cell envelope, and on the association of this protein with various cell wall components. This information should be useful in the elucidation of the overall role of the binding proteins in the mechanism of transport.

### Materials and methods

#### *Bacterial strains, culture media, and growth conditions*

All the strains used in this study are *Escherichia coli* K12. Strain CBT 43 is defective in succinate dehydrogenase (EC 1.3.99.1) and fumarate reductase; unless specified otherwise, most of the work reported in this communication was carried out with this strain. Strain BL 7 is a spontaneous *cbt* mutant isolated from CBT 43, and was selected by its ability to grow on pyruvate in the presence of 2 mM  $\beta$ -chlorolactate. Strain SK 11 is a mutant defective in UDPgalactose 4-epimerase (EC 5.1.3.2), and has the genotype, *galE, asd, leu, pps, thi, strA* (10).

Medium M9 (11) was used as a minimal medium. It was supplemented with amino acids and other growth factors, whenever required, at a concentration of 25  $\mu$ g/mL. Unless specified otherwise, the carbon source was added at a concentration of 1 mg/mL. LB medium was used as the complex medium (11).

Unless indicated otherwise, all bacterial strains were grown under vigorous agitation at 37°C in LB medium for 12 h. Ten millilitres of this culture was inoculated into 500 mL of M9 medium supplemented with succinate (to induce dicarboxylate transport), glycerol, and thiamine. After 10 h of incubation at 37°C with shaking, the culture was harvested. For  $^{35}\text{S}$  labeling, 1.25 mCi (1 Ci = 37 GBq) of  $\text{H}_2^{35}\text{SO}_4$  was added to the M9 medium.

Medium "121" was a low phosphate medium used to derepress the synthesis of alkaline phosphatase (EC 3.1.3.1) (12). One litre of this medium contained NaCl, 4.68 g; KCl, 1.5 g;  $\text{NH}_4\text{Cl}$ , 1.08 g;  $\text{MgCl}_2$ , 0.2 g;  $\text{Na}_2\text{SO}_4$ , 0.35 g;  $\text{CaCl}_2$ ,  $29 \times 10^{-3}$  g;  $\text{FeCl}_3$ ,  $0.5 \times 10^{-3}$  g;  $\text{ZnCl}_2$ ,  $0.27 \times 10^{-3}$  g, 8  $\mu\text{M}$  potassium phosphate, and Tris "121," 12 g. The pH was adjusted to 7.5 with HCl. Glycerol (1 mg/mL) was used as the carbon source and succinate (1 mg/mL) was used to induce the dicarboxylate transport system.

#### *Release of $^3\text{H}$ -labeled lipopolysaccharide by various treatments*

Strain SK 11, a mutant defective in UDPgalactose 4-epimerase, was used for this study. It was ascertained that this strain did not have the epimerase activity by assaying this enzyme using the methods described by Kalckar (13). Crude cell lysate from SK 11 had no detectable UDPgalactose 4-epimerase activity, whereas that from CBT 43 (*galE*<sup>-</sup>) had a specific activity of 0.066  $\mu\text{mol}/\text{mg}$  protein.

Strain SK 11 was grown overnight with shaking at 37°C in M9 medium containing 100  $\mu\text{Ci}$  of D-[1- $^3\text{H}$ ]galactose,

which had a specific activity of 9.7 Ci/nmol. The medium contained the following growth requirements: succinate, leucine, thiamine, lysine, and *meso*-diaminopimelic acid. Fucose (2.5 mM) was added to induce galactose transport. After harvesting, cells were suspended in 100 mL of unsupplemented M9 medium at a concentration of  $8 \times 10^7$  cells/mL. Following centrifugation, cells were suspended in 10 mL of 0.05 M phosphate buffer (pH 6.6) and subjected to various treatments described in Table 4. Following treatment, the cells were pelleted by centrifugation and the amount of radioactivity in the supernatant was determined.

#### *Selective disruption of various layers of the cell envelope and isolation of DBP*

Strain CBT 43 was grown as indicated in the appropriate sections. After harvesting, the cells were suspended at a density of  $8 \times 10^7$  cells/mL in 400 mL of unsupplemented M9 medium. Cells were then centrifuged and resuspended successively with stirring for 10 min in 400 mL of the following solutions: (i) cold double-distilled water, (ii) EDTA-sucrose solution which comprised 20% sucrose, 2 mM EDTA, and 0.03 M Tris-HCl (pH 7.2), (iii) cold double-distilled water (osmotic shock), and (iv) EDTA-sucrose solution containing 125  $\mu\text{g}/\text{mL}$  hen egg white lysozyme (EC 3.2.1.17). One hundred millilitres of the supernatants collected from each of the above steps was run through aspartate-coupled Sepharose 4B columns (2). After washing off unbound proteins with 0.01 M phosphate buffer (pH 6.6), the DBP was eluted from the column by 0.2 M sodium succinate in phosphate buffer. The elution profile was followed by absorbance measurements at 280 nm and by radioactivity measurements in each fraction.

We found that lysozyme could bind to the aspartate-Sepharose columns and could be eluted with 0.2 M succinate. This contaminating lysozyme would, therefore, affect the absorbance reading of DBP. To minimize the contribution of lysozyme, the supernatant from the lysozyme-treated cell was first passed through a chitin column which effectively removed lysozyme. The solution was then loaded onto the aspartate-Sepharose column. It should be mentioned that 100% of DBP applied was recovered after passage through the chitin column.

#### *Surface labeling by lactoperoxidase radioiodination*

Strain CBT 43 was grown as indicated earlier. After harvesting, the cells were suspended in 400 mL of unsupplemented M9 to a density of  $8 \times 10^7$  cells/mL. Following centrifugation cells were resuspended in 10 mL of 0.05 M phosphate buffer (pH 6.6). Surface labeling was carried out as described by Hubbard (14). Cells were incubated in the following reaction mixture for 30 min at 23°C: 20 mg glucose, 40 units lactoperoxidase, 1 mCi  $\text{Na}^{125}\text{I}$  (carrier free), 62.5 units glucose oxidase. After incubation, cells were centrifuged and washed with 400 mL of unsupplemented M9 medium. The cells were then subjected to various treatments described in the last section. DBP was isolated from the supernatants obtained by aspartate-Sepharose columns.

#### *Surface labeling by diazo[ $^{125}\text{I}$ ]iodosulfanilic acid*

[ $^{125}\text{I}$ ]iodosulfanilic acid (specific activity >1000 Ci/mmol) was diazotized according to the procedure outlined by New England Nuclear Co. Strain CBT 43 was grown as indicated earlier. After harvesting and washing with unsupplemented M9 medium, the cells were suspended

in 10 mL of 0.05 M phosphate buffer (pH 7.5) at a density of  $1.6 \times 10^9$  cells/mL. These cells were then treated with 0.5 mCi of diazo[ $^{125}$ I]iodosulfanilic acid ( $<50$  nM) for 1 h at 23°C. After washing with 200 mL of un-supplemented M9 medium, the cells were treated successively with 200 mL of (i) EDTA-sucrose solution, (ii) cold double-distilled water (osmotic shock), and (iii) EDTA-sucrose solution containing lysozyme as described earlier. The amount of DBP present in various supernatants was determined by affinity chromatography.

#### Thin-layer chromatography

The [ $^3$ H]galactose-labeled DBP was subjected to mild acid hydrolysis in 2 N HCl for 2 h at 100 to 110°C. The sample was washed several times with double-distilled water and was dried down in a desiccator over P<sub>2</sub>O<sub>5</sub> and KOH. Samples were spotted onto silica gel precoated thin-layer plates. The solvent system used for thin-layer chromatography was *n*-butanol-ethanol-water (104:66:30). Galactose, glucosamine, and *N*-acetylglucosamine were used as standards and were detected by spraying the plate with 5% silver nitrate in 25% ammonium hydroxide and heating to 100°C. The position of the radioactive material was determined by counting the silica gel.

#### SDS - polyacrylamide gel electrophoresis

SDS - polyacrylamide gels were prepared and used as described by Laemmli (15). Proteins from the DBP peak of the aspartate - Sepharose column were first precipitated by the addition of ice cold TCA (final concentration, 10%). After incubating for a minimum of 2 h at 4°C, the TCA-insoluble materials were pelleted by centrifugation. The pellet was dissolved in the sample buffer and dialysed against this buffer overnight. The sample was heated in the presence of 5% mercaptoethanol for 5 min at 100°C before loaded onto the gel: 12.5% SDS - polyacrylamide gel was used and bromophenol blue was used as the tracking dye. After running, the slab gel was dried under vacuum and 5-mm slices were cut. The slices were dissolved in 0.5 mL H<sub>2</sub>O<sub>2</sub> (30%) overnight at 60°C. Samples were then counted in 10 mL of ACS.

#### Two-dimensional gel electrophoresis

The EDTA - osmotic shock fluid from cells labeled with radioactive iodine was lyophilized and suspended in a small volume of sample buffer. The procedure used in two-dimensional gel electrophoresis was that described by O'Farrell (16), except that NP 40 was only used in the sample buffer, but not in the 4% nonequilibrium pH gradient gel. The first dimension consisted of 4% nonequilibrium pH gradient gel and the second dimension consisted of a 12.5% SDS - polyacrylamide gel.

#### Assay of alkaline phosphatase

The enzyme activity was measured spectrophotometrically at 23°C with *p*-NPP as substrate (12). The assay mixture (1 mL) contained 0.2 mg *p*-NPP in 0.5 M Tris buffer, pH 8.0. The liberation of *p*-nitrophenol was followed at 420 nm. The extinction coefficient of *p*-nitrophenol at 420 nm is  $13\,200\text{ M}^{-1}\text{ cm}^{-1}$  (pH 8.0). Calf intestine alkaline phosphatase obtained from Calbiochem Co. was used as the reference standard.

#### Materials

All chemicals used were of highest purity available. Na<sup>125</sup>I (carrier free), H<sub>2</sub><sup>35</sup>SO<sub>4</sub>, [ $^{125}$ I]iodosulfanilic acid labeling kits were purchased from New England Nuclear

Co. D-[ $^3$ H]Galactose and ACS were obtained from Amersham/Searle Co. Lactoperoxidase, lysozyme, glucose oxidase, D-fucose, and  $\beta$ -chlorolactate were obtained from Sigma. Silica gel precoated thin-layer plates (20 cm  $\times$  20 cm) were obtained from BDH Chemicals.

## Results

### Selective disruption of various layers of the cell envelope

The *Escherichia coli* cell envelope is composed of an outer membrane, a periplasmic space, and the cytoplasmic membrane. The outer membrane is characterized by the presence of lipopolysaccharide which occupies about 45% of the outer membrane surface and is found almost exclusively on the outer leaflet of the outer membrane (17). The lipopolysaccharide molecules are closely associated with each other and are stabilized by divalent cations, such as Mg<sup>2+</sup>. Treatment of cells with chelating reagents such as EDTA, therefore, disrupts the outer membrane, causing the release of lipopolysaccharide. The integrity of the outer membrane can thus be monitored by the release of lipopolysaccharide.

The periplasmic space is defined as the region between the outer and cytoplasmic membranes (18). The most prominent feature in this region is the peptidoglycan network, which is essentially a sacculus surrounding the cytoplasmic membrane. It consists of repeating units of *N*-acetylglucosamine and *N*-acetylmuramic acid. Tetrapeptide side chains are attached to the latter component. These side chains cross-link parallel polysaccharide chains (19). The mesh size of the peptidoglycan network is estimated to be  $12.5\text{ \AA} \times 10.3\text{ \AA}$  ( $1\text{ \AA} = 0.1\text{ nm}$ ) (20). Thus it is possible that this network may not be freely penetrated by large macromolecules, unless discontinuous areas exist in the network. Viewed in this way, the peptidoglycan may be regarded as a partition dividing the periplasmic space into outer and inner regions. It may, therefore, be necessary to disrupt different layers of the cell envelope selectively before one can determine the localization of the dicarboxylate binding protein.

It is well established that over 99% of the galactose incorporated into a UDPgalactose 4-epimerase (*galE*) mutant is found in the lipopolysaccharide (21, 22). Therefore, by subjecting [ $^3$ H]galactose-labeled *galE* mutant to various treatments, one can determine the amount of lipopolysaccharide released by simply measuring the radioactivity in the supernatants. In other words, this provides a simple method to monitor the integrity of the outer membrane. In the present study a *galE* mutant, SK 11, was first grown overnight in [ $^3$ H]galactose and was then subjected to various treatments described in Table 1. Table 1 shows that about 18 and 58% of the total releasable labeled lipopolysaccharide are released by lowering the ionic strength of the environment and by EDTA treatment, respectively. Further disruption of the outer membrane by osmotic shock treatment releases some more lipopolysaccharide. Thus in agreement with findings by other workers, the

TABLE 1. The amount of labeled materials released by treatments which selectively disrupt various layers of the cell envelope

Labeling procedure	Amount of incorporated counts released (% of total)	Amount of releasable labeled materials (% of total)			
		Cold water wash	EDTA-sucrose wash	Osmotic shock	Lysozyme-sucrose wash
1. [ <sup>3</sup> H]Galactose-labeled <i>galE</i> mutants	62	18	58	21	3
2. <sup>35</sup> S-labeled cells	46	7	9	54	30
3. Cells labeled by lactoperoxidase radiiodination	35	23	26	37	14
4. Cells labeled by diazo[ <sup>125</sup> I]-sulfanilic acid	34	—*	21	71	8

NOTE: Strain SK 11, a *galE* mutant, was used in the [<sup>3</sup>H]galactose-labeling experiment. Strain CBT 43 was used in the rest of the labeling studies. Cells were treated successively by cold water, EDTA-sucrose, osmotic shock, and by lysozyme-sucrose as described in the text. The amount of labeled materials released in various supernatants was expressed as a percentage of the total amount of labeled materials released by all treatments.

\*Denotes not determined.

TABLE 2. The release of alkaline phosphatase by selective disruption of the cell envelope

Treatment	Alkaline phosphatase activity*	% total activity
1. Medium wash	0.43	0.76
2. Cold water wash	0.32	0.57
3. EDTA-sucrose wash	1.88	3.33
4. First osmotic shock	42.23	75.00
5. Second osmotic shock	3.26	5.80
6. Lysozyme-sucrose wash	5.31	9.43
7. Spheroplast	2.88	5.11

NOTE: Strain CBT 43 was used in this study. Cells were grown in 1 L of LB medium for 8 h and harvested. Cells were then derepressed for alkaline phosphatase synthesis by growing overnight in the low phosphate medium, medium 121. Glycerol was the carbon source and succinate was added to induce the dicarboxylate transport system. After harvesting, cells were then subjected to various treatments to disrupt various layers of the cell envelope selectively, as described in Materials and methods. Alkaline phosphatase was precipitated from various washes by the addition of ammonium sulphate (80% saturation). The turbid suspension was stirred at 4°C for 2 h. After centrifugation, the pellet was suspended in 0.05 M Tris buffer, pH 8.0, and dialyzed overnight at 4°C. Alkaline phosphatase was then assayed according to the method described in the text.

\*Alkaline phosphatase activity is expressed as 10<sup>-8</sup> mol of *p*-NPP hydrolyzed per minute at 23°C, pH 8.0.

integrity of the outer membrane is disrupted by EDTA (17). When <sup>35</sup>S-labeled cells are subjected to similar treatments, both cold water wash and EDTA-sucrose wash release only small quantities of <sup>35</sup>S-labeled proteins. A large amount of <sup>35</sup>S-labeled proteins is released only when the outer membrane is dissociated further by osmotic shock treatment (Table 1). It is possible that these materials are bound to or by the outer membrane in the periplasmic space.

One of the specific enzymes used to cleave the peptidoglycan network is hen egg white lysozyme. This enzyme hydrolyses the β-1,4 linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid (19). Table 1 shows that cleaving the peptidoglycan does not cause further release of lipopolysaccharide. However a substantial amount of <sup>35</sup>S-labeled protein is released. It seems unlikely that the lysozyme treatment causes cell

lysis, as cytoplasmic enzymes such as glutamate dehydrogenase (EC 1.4.1.4) cannot be detected in the supernatant from lysozyme treatment. The specific activity of this enzyme, as assayed according to Sanwal (23), in the lysed spheroplasts was 0.037 μmol/mg protein. Based on the sensitivity of this enzyme assay, less than 1% of the glutamate dehydrogenase was released by lysozyme-sucrose treatment. It would thus, appear that substantial amounts of proteins are either bound to or trapped by the peptidoglycan network in the inner region of the periplasmic space.

It is well established that alkaline phosphatase (EC 3.1.3.1) is located in the periplasmic space of the envelope of gram-negative bacteria. Table 2 indicates the release of this enzyme by selective disruption of the cell envelope. About 85% of the enzyme was released by the osmotic shock treatment. Only a small percentage was released by medium wash, cold water wash, and by EDTA-sucrose wash. About 10% of the enzyme was released upon dissociation of the peptidoglycan network by lysozyme treatment. Thus in agreement with the findings by other workers (24), this study shows that alkaline phosphatase is located mainly between the outer membrane and the peptidoglycan network in the periplasmic space. More importantly, this study serves to demonstrate that the above-mentioned procedures can be used to disrupt various layers of the cell envelope selectively.

It may be apparent from the above studies that it is possible to disrupt various layers of the cell envelope selectively. Treatment of whole cells by cold water wash removes only proteins that are associated loosely to the cell surface, and this treatment does not cause significant damage to the outer membrane, as both lipopolysaccharide and alkaline phosphatase are not released to any significant extent by such treatment. Chelating reagents, such as EDTA, release a substantial amount of lipopolysaccharide, but only a small percentage of alkaline phosphatase and other proteins from the cells. This implies that although EDTA perturbs the outer membrane organization, it does not cause extensive dis-

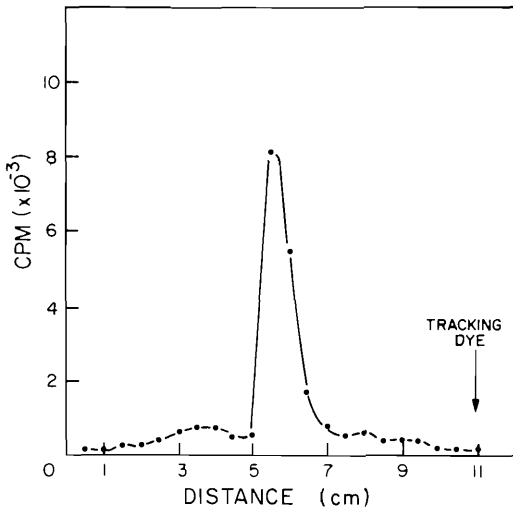


FIG. 1. SDS-polyacrylamide gel electrophoresis of the  $^{35}\text{S}$ -labeled DBP obtained by EDTA-osmotic shock treatment. Samples and gel electrophoresis were prepared as described in the text.

sociation of this membrane. Severe damage to this membrane occurs when these EDTA-treated cells are subjected to osmotic shock treatment. Substantial amounts of protein and lipopolysaccharide are released by the EDTA-osmotic shock treatment. The latter treatment does not seem to affect the peptidoglycan network. Macromolecules trapped by or bound to the peptidoglycan are released only when the sacculus is cleaved by lysozyme. The fact that cytoplasmic enzymes are not released by the lysozyme-sucrose treatment suggests that the integrity of the cytoplasmic membrane is maintained under such circumstances. The cytoplasmic membrane is disrupted only when the lysozyme treated cells (spheroplasts) are subjected to osmotic shock treatment.

#### Localization of the dicarboxylate binding protein in the cell envelope

We have previously reported that the dicarboxylate binding protein (DBP) contains sulfhydryl groups (4). It is, therefore, possible to label this protein with  $\text{H}_2^{35}\text{SO}_4$  in order to provide a more sensitive method to detect and to quantitate it. Column chromatography using aspartate-Sepharose 4B has been used successfully to isolate and to purify various dicarboxylate transport components (2, 4). Figure 1 shows that the DBP isolated by the aspartate-coupled Sepharose column is relatively pure; over 90% of the radioisotope is found in this protein peak. This is in agreement with our previous finding on the purity of this protein (4). It should be noted that the cytoplasmic membrane dicarboxylate transport components (SBP 1 and SBP 2) are not released from the membrane by the above mentioned treatments; they are released only when the cytoplasmic membrane is solubilized with detergents

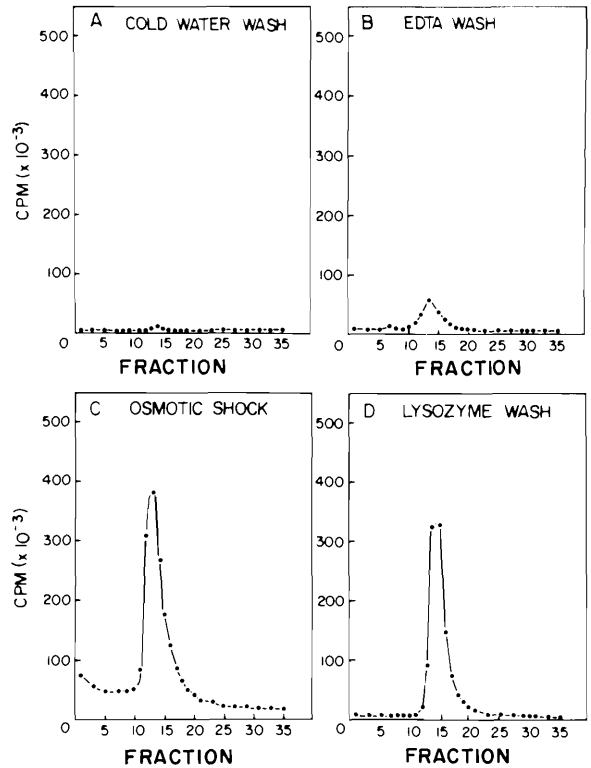


FIG. 2. Elution profiles of dicarboxylate binding protein from  $^{35}\text{S}$ -labeled cells. Strain CBT 43 was grown in M9 medium in the presence of  $\text{H}_2^{35}\text{SO}_4$  as indicated in Materials and methods. After harvesting, the cells were then subjected to various treatments as described in the text. Supernatants from various treatments were loaded onto the aspartate-Sepharose column. After washing off the unbound proteins, the dicarboxylate binding protein can then be eluted from the column by the addition of 0.2 M succinate. Fraction 1 indicates where succinate was added to the column. It should be noted that lysozyme was first removed from the supernatant from the lysozyme wash before loaded onto the aspartate-Sepharose column; lysozyme was removed by passage through a chitin column.

(2). Consequently, the amount of DBP released by various treatments can be determined by the size of the protein peak after elution of the column with succinate.

Using the above method to quantitate DBP, only negligible amount of DBP was found to be released by the cold water wash (Fig. 2). Thus, if this protein is present on the cell surface, it is tightly bound to the outer membrane. Perturbing the outer membrane with EDTA releases 7% of the total releasable DBP. This suggests that some DBP may be associated with the cell surface. A substantial amount of DBP (51%) is released if the outer membrane is disrupted further by osmotic shock treatment. This implies that a very large percentage of DBP is associated with the outer membrane, or is trapped by the outer membrane in the periplasmic space. Cleavage of the peptidoglycan network

TABLE 3. Surface labeling of dicarboxylate binding protein by lactoperoxidase radioiodination

Experiment No.	$^{125}\text{I}$ (mCi)	Lactoperoxidase (units)	Dicarboxylate binding protein released by EDTA - osmotic shock treatment	
			cpm	% of control
1	0.5	40	$113.8 \times 10^4$	100
2	0.5	—	$7.7 \times 10^4$	6.7
3	0.5	80	$94.5 \times 10^4$	83.0
4	1.0	40	$263.6 \times 10^4$	231.6

NOTE: Strain CBT 43 was grown for 10 h in M9 medium, harvested, and suspended in 400 mL of unsupplemented medium at a concentration of  $8 \times 10^7$  cells/mL. Following centrifugation, cells were suspended in 0.05 M phosphate buffer (pH 6.6).  $^{125}\text{I}$ , lactoperoxidase in the amounts indicated, 20 mg glucose, and 62.5 units glucose oxidase were added to the cells. The final volume of reaction mixture in each experiment was 10 mL. Labeling was allowed to proceed for 30 min at 23°C. The dicarboxylate binding protein was isolated from 400 mL of the EDTA - osmotic shock fluid as described in the text.

by lysozyme frees about 42% of the releasable labeled DBP. In the absence of lysozyme, a second wash of the shocked cells releases around 6% of the releasable DBP. As indicated in the last section, lysozyme-sucrose treatment does not cause cell lysis; therefore, this DBP is either bound to or trapped by the peptidoglycan network. The small amount of DBP released in the absence of lysozyme implies that either the DBP in the inner region of the periplasmic space cannot diffuse freely across the peptidoglycan, or the majority of this protein is bound to this network; consequently it is released only if the peptidoglycan is cleaved by lysozyme. Lysis of the spheroplasts (i.e., the lysozyme-treated cells) by osmotic shock treatment releases only minute quantities (around 6%) of DBP. This suggests that DBP is not present in the cytoplasm of the cells. This finding is in agreement with the hypothesis that non-cytoplasmic proteins are secreted across the cytoplasmic membrane as they are synthesized on the membrane-bound polysomes; consequently they are not found in the cytoplasm (25).

Results reported above suggest that at least two forms of DBP are present in the cell envelope: the free and the bound forms. The free form is defined as those proteins which are released from the cell by treatments which perturb the outer membrane, e.g., EDTA and EDTA - osmotic shock treatments. They are "free" in the sense that they are not bound to or by the peptidoglycan network in the periplasmic space. The bound form is defined as those proteins which are released only after the peptidoglycan network is cleaved, e.g., by lysozyme treatment. It is surmised that the bound form may be bound to or trapped by the peptidoglycan network to the inner region of the periplasmic space.

#### In situ labeling of DBP by lactoperoxidase radioiodination

So far our findings suggest that the free and bound forms may be associated with the outer membrane and

TABLE 4. Effect of various reagents on the integrity of the outer membrane of the cell envelope

Treatment	Amount of $^3\text{H}$ -labeled lipopoly-saccharide in intact cells (dpm)	Amount of $^3\text{H}$ -labeled lipopolysaccharide released from cells	
		dpm	% of total
<b>A</b>			
1. Control	$2.95 \times 10^6$	$2.77 \times 10^4$	0.94
2. Lactoperoxidase iodination	$2.95 \times 10^6$	$3.54 \times 10^4$	1.2
3. EDTA	$2.95 \times 10^6$	$1.1 \times 10^6$	37.3
4. EDTA-sucrose	$2.95 \times 10^6$	$1.3 \times 10^6$	44.1
<b>B</b>			
1. Control	$1.95 \times 10^6$	$1.2 \times 10^5$	6.2
2. Diazosulfanilic acid (0.8 mM)	$1.95 \times 10^6$	$8.2 \times 10^4$	4.2
3. EDTA	$1.95 \times 10^6$	$6.1 \times 10^5$	31.3

NOTE: Strain SK 11 (*galE*) was labeled for 10 h with D-[1- $^3\text{H}$ ]galactose in M9 medium as described. After washing, cells were suspended in 10 mL of buffer: 0.05 M phosphate buffer (pH 6.6) in A1, A2, B1, and B2; 0.03 M Tris-HCl (pH 7.2) in A3, A4, and B3. For A2, 40 units lactoperoxidase, 0.7  $\mu\text{g}$  sodium iodine, 20 mg glucose, and 62.5 units glucose oxidase were added. For B2, the final concentration of diazosulfanilic acid used was 0.8 mM. The final concentrations of EDTA and sucrose used in the relevant experiments were 2 mM and 20%, respectively. Cells were incubated in each treatment for 30 min at 23°C, centrifuged, and the radioactivity in the supernatants was determined.

the peptidoglycan network, respectively. In this and the following sections, nonpenetrating covalent labeling reagents are used to resolve the question of localization of these two forms of DBP. The first method used is lactoperoxidase radioiodination. Table 3 shows that lactoperoxidase is essential for the labeling procedure, and the amount of lactoperoxidase used in the present study is more than sufficient for the labeling reaction. It also shows that increasing the amount of  $^{125}\text{I}$  (carrier free) increases the amount of labeling. We also found that there was no further increase in labeling after 30 min of incubation. Table 4 shows that this labeling procedure does not release lipopolysaccharide from the cells. In other words, the integrity of the outer membrane is maintained in the presence of lactoperoxidase,  $\text{I}_2$ , glucose, and glucose oxidase. Since lactoperoxidase has a molecular weight of around 82 000, it is unlikely that this enzyme can penetrate an intact outer membrane. Consequently, only proteins exposed on the cell surface should be labeled by this procedure.

Table 1 shows that over 85% of the releasable [ $^{125}\text{I}$ ]-lactoperoxidase-labeled materials can be recovered from the cold water wash, EDTA-sucrose wash, and the osmotic shock supernatant. Lysozyme-sucrose treatment releases only minute quantities of the  $^{125}\text{I}$ -labeled materials. This is contrary to findings with the  $^{35}\text{S}$ -labeled cells. Thus the lysozyme releasable materials are not accessible to labeling by lactoperoxidase. Figure 3 is an autoradiogram of a two-dimensional gel of the EDTA - osmotic shock releasable materials from cells

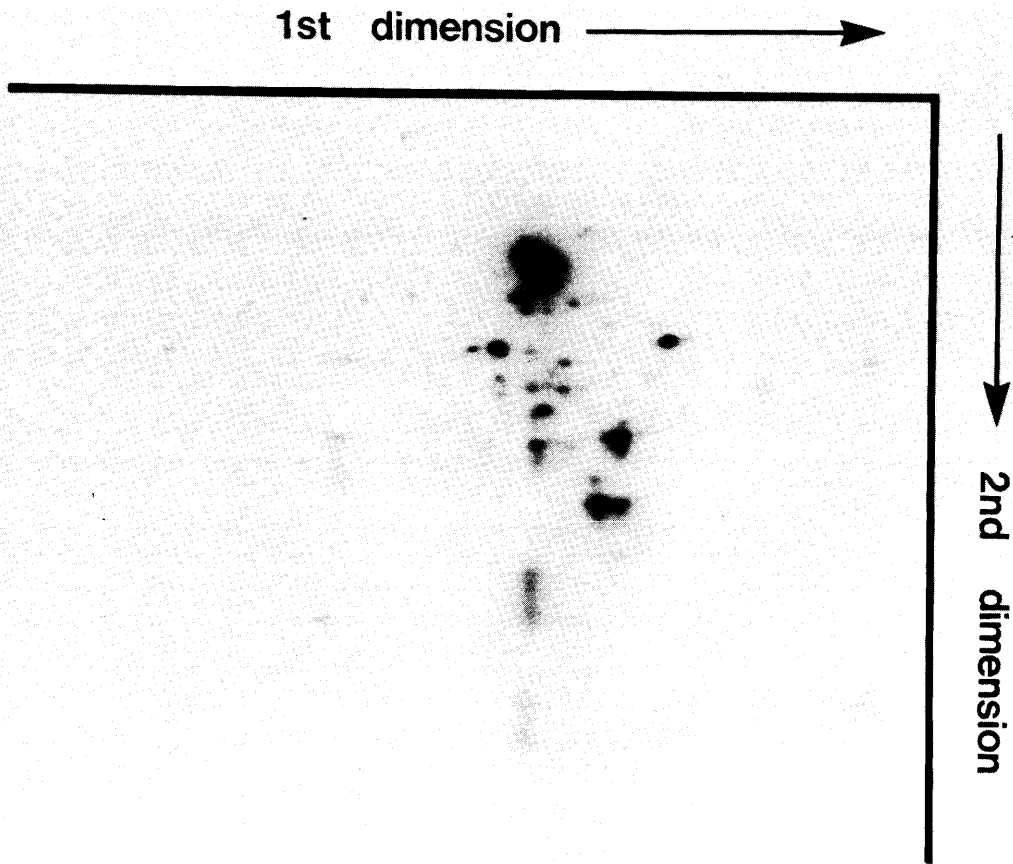


FIG. 3. Two-dimensional gel electrophoresis of the EDTA-osmotic shock fluid from cells labeled with lactoperoxidase radioiodination. The first dimension consists of 4% nonequilibrium pH gradient gel. The ampholine composition was pH 3.0–10.0. The second dimension consists of SDS electrophoresis in 12.5% acrylamide. At the completion of electrophoresis, the gel was dried and was exposed to Dupont Cronex X-ray film.

surface labeled by lactoperoxidase radioiodination. It shows that at least 40 EDTA – osmotic shock releasable proteins can be surface labeled by this method. A similar finding was made by Boos and co-workers (26).

Figure 4 shows that 5, 57, 33, and 5% of the surface labeled releasable DBP are recovered from the cold water wash, EDTA – sucrose wash, osmotic shock fluid, and the lysozyme–sucrose wash, respectively. It was observed that approximately the same amount of DBP was recovered by affinity chromatography after subjecting the cells to a particular treatment, regardless of the labeling procedure; and that the sulfur-35 counts reflected fairly closely the amount of protein present. Therefore, by assuming that 100% of the EDTA–sucrose releasable DBP is surface labeled, it may be calculated that about 100, 8, and 1% of the DBP released by cold water, osmotic shock, and lysozyme treatments, respectively, are surface labeled. This suggests that at least some of the free DBP are exposed on the cell surface, whereas the bound DBP is not. The fact that the majority of the DBP released by lysozyme and

by osmotic shock treatments are not labeled suggests that proteins located inside the outer membrane are not nonspecifically labeled by this technique under these conditions.

Attempts were made to label the bound DBP *in situ* by the same procedure, using EDTA – osmotic shock treated cells. Figure 5 shows that the bound DBP cannot be labeled even with cells which have been subjected to EDTA – osmotic shock treatment twice. On the other hand, the isolated bound DBP can be readily labeled by this procedure. The significance of this finding will be discussed later.

#### *In situ labeling of dicarboxylate binding protein by diazo[<sup>125</sup>I]iodosulfanilic acid*

The second labeling reagent employed is diazo[<sup>125</sup>I]-iodosulfanilic acid. Unlike lactoperoxidase, which reacts mainly with tyrosine and histidine, the diazonium salt of sulfanilic acid can react with a wide variety of amino acid residues, such as tyrosine, histidine, lysine, proline, tryptophan, phenylalanine, glycine, and cysteine in pro-

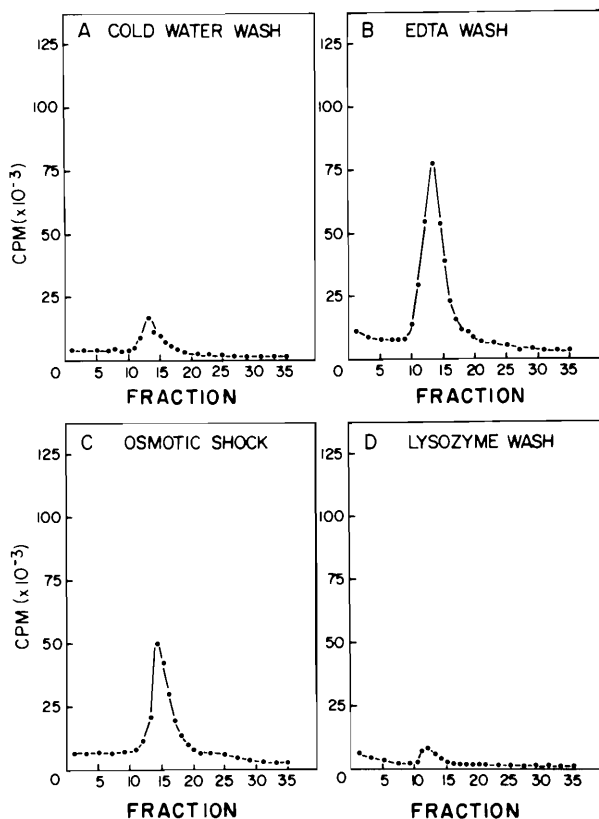


FIG. 4. Elution profiles of dicarboxylate binding protein from cells surface labeled by lactoperoxidase radioiodination. Strain CBT 43 was first labeled by lactoperoxidase radioiodination. Then it was subjected to various treatments as described in Fig. 2.

teins (27). This reagent is zwitterionic, and has been shown not to penetrate various cell membranes (28, 29). When present in high concentrations, it inactivates membrane functions by binding to the cell surface components (30). In fact, diazo[ $^{125}\text{I}$ ]iodosulfanilic acid has been used quite extensively to label cell surface components (28). Table 4 shows that 0.8 mM diazosulfanilic acid does not affect the integrity of the outer membrane. Recently, we have also observed that 0.8 mM diazosulfanilic acid inactivates the cell surface dicarboxylate binding protein, and the inactivated outer membrane dicarboxylate transport system can be restored by the mere addition of purified DBP to the treated cells (10). Transport studies carried out with membrane vesicles indicate that 0.8 mM diazosulfanilic acid inactivates the cytoplasmic membrane dicarboxylate transport system (unpublished observations). These facts taken together suggest that diazosulfanilic acid cannot penetrate the outer membrane, as it is unable to inactivate cytoplasmic membrane functions.

Table 1 shows that a large proportion of the proteins released by disintegrating the outer membrane is labeled

by diazo[ $^{125}\text{I}$ ]iodosulfanilic acid, whereas proteins, released by lysozyme treatment are hardly labeled. This again suggests that this reagent cannot penetrate the outer membrane; only proteins which reside on the cell surface can be labeled. Figure 6 shows that the DBP releasable by EDTA and osmotic shock treatments is extensively labeled by this reagent; whereas the bound DBP is not. By assuming that 100% of the EDTA-sucrose releasable DBP is surface labeled, it may be calculated that about 14 and 5% of the DBP released by osmotic shock and lysozyme treatments, respectively, are surface labeled. Therefore, in agreement with the lactoperoxidase labeling study, some of the free DBP are exposed on the cell surface, whereas the bound DBP is not.

#### Association with lipopolysaccharide

Lipopolysaccharide is found almost exclusively on the outer leaflet of the outer membrane, and is associated with quite a number of cell envelope components (17). The above surface labeling studies suggest that a significant amount of labeled free DBP is released on treating the cells with EDTA. Since lipopolysaccharide is also released by EDTA (Table 1), it would be interesting to determine whether the cell surface DBP has any affinity for lipopolysaccharide.

Strain SK 11, a *galE* mutant, was grown overnight in the presence of [ $^3\text{H}$ ]galactose. After subjecting these cells to treatments which selectively removed different layers of the cell envelope (as indicated in Fig. 2), the amount of DBP released was determined by affinity chromatography. Figure 7 shows that the DBP released by cold water wash, EDTA, and osmotic shock is labeled in these cells, whereas the bound DBP is not. By mild acid hydrolysis of these isolated DBP and analysis by thin-layer chromatography, it was found that at least 80% of the radioactivity in the hydrolysate was galactose. Amino acid analysis of the labeled protein showed that galactosamine, glucosamine, ammonia, and amino acids were not labeled. This suggests that the radioactivity associated with the isolated DBP remains as galactose. In the above experiments, about 6, 11, 7, and 7% of the radioactivity released by cold water, EDTA-sucrose, osmotic shock, and lysozyme treatments, respectively, are associated with DBP. Since a constant amount (around 7%) of the total [ $^3\text{H}$ ]galactose-labeled material is associated with DBP and that over 99% of the [ $^3\text{H}$ ]galactose incorporated into a *galE* mutant is found exclusively in lipopolysaccharide (17), it may be surmised that the galactose associated with DBP is likely to be in the form of lipopolysaccharide.

The next obvious question concerns the association of cell surface DBP with lipopolysaccharide. We approached this problem by testing if the lipopolysaccharide can be dissociated from DBP by altering the ionic strength of the buffer. The supernatant from EDTA-sucrose treatment of the [ $^3\text{H}$ ]galactose-labeled *galE* mutant was first fractionated by DEAE-cellulose chromatography. The radioactivity was eluted by 0.6 N NaCl.



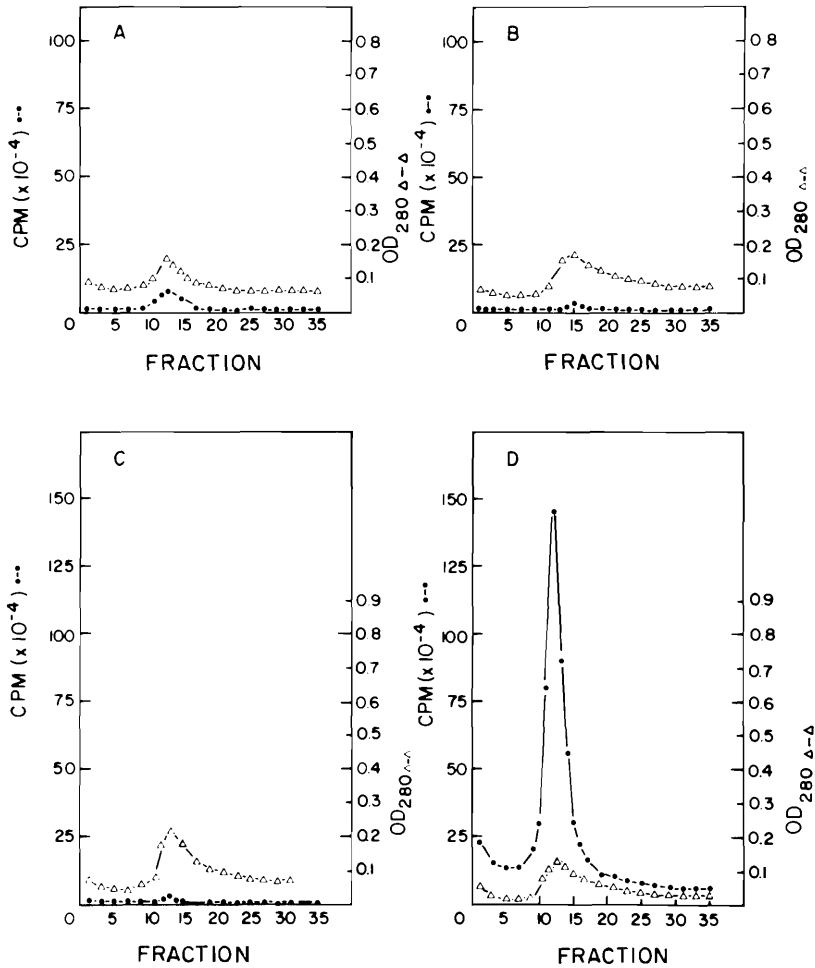


FIG. 5. Elution profiles of bound-dicarboxylate binding protein from selectively disrupted cells labeled by lactoperoxidase radioiodination. Strain CBT 43 was used in this study. (A) Intact cells were first labeled with lactoperoxidase radioiodination and then subjected to EDTA-lysozyme treatment as described in the text. After removing lysozyme by chitin column, the supernatant from the lysozyme treatment was loaded onto the affinity column. After washing, succinate was added to elute the dicarboxylate binding protein. (B) Cells were first subjected to EDTA-osmotic shock treatment and then they were labeled by lactoperoxidase radioiodination. After washing, they were subjected to lysozyme treatment. The amount of labeled bound DBP present in the supernatant was determined as described in (A). (C) Cells were subjected to EDTA-osmotic shock treatment twice before labeling by lactoperoxidase radioiodination. This second EDTA-osmotic shock treatment served to ensure the outer membrane was sufficiently dissociated. The amount of labeled bound DBP in the lysozyme supernatant was determined as described earlier. (D) Bound DBP was first isolated from unlabeled cells. Then it was labeled by lactoperoxidase radioiodination. After removing the iodine and glucose by passage through a Sephadex G25 column, the amount of labeled bound DBP was determined by affinity chromatography.

After desalting on Sephadex G25, this radioactive material was applied to an aspartate-Sepharose column. It was found not to bind to the affinity column. Similar studies carried out with  $^{35}\text{S}$ -labeled cells indicated that [ $^{35}\text{S}$ ]DBP was eluted from the DEAE-cellulose column by 0.2 N NaCl. Thus it seems that  $^3\text{H}$ -labeled lipopolysaccharide is dissociated from DBP by altering the ionic strength of the buffer and is then eluted from the DEAE-cellulose column by 0.6 N NaCl. Since DBP is not associated with the recovered lipopolysaccharide, the

latter can, naturally, no longer bind to the affinity column. The finding that isolated DBP contains lipopolysaccharide implies that the two molecules bind tightly to one another.

Assuming 100% of the EDTA-sucrose releasable DBP is associated with lipopolysaccharide, it may be calculated that about 74, 3, and 0.4% of the DBP released by cold water, osmotic shock, and lysozyme-sucrose treatments, respectively, bind with lipopolysaccharide. Compared with the surface labeled DBP,

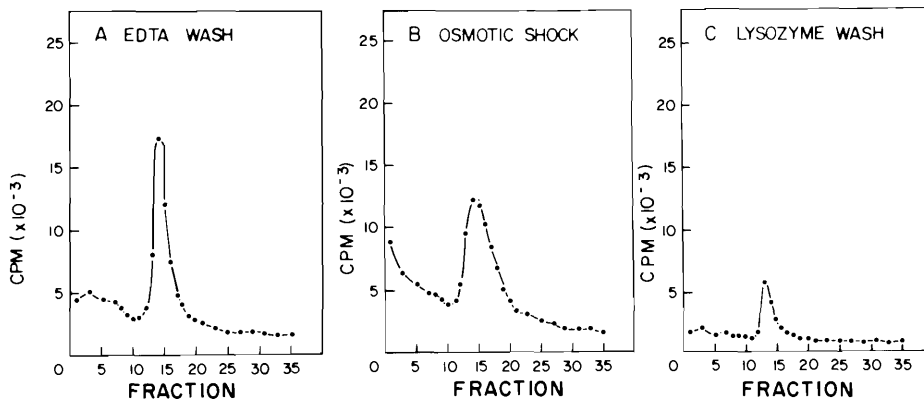


FIG. 6. Elution profiles of dicarboxylate binding protein from cells surface labeled by diazo[ $^{125}\text{I}$ ]iodosulfanilic acid. Strain CBT 43 was first labeled by diazo[ $^{125}\text{I}$ ]iodosulfanilic acid ( $< 50 \text{ nM}$ ) as described in the text. After labeling, the cells were subjected to various treatments as described in Fig. 2.

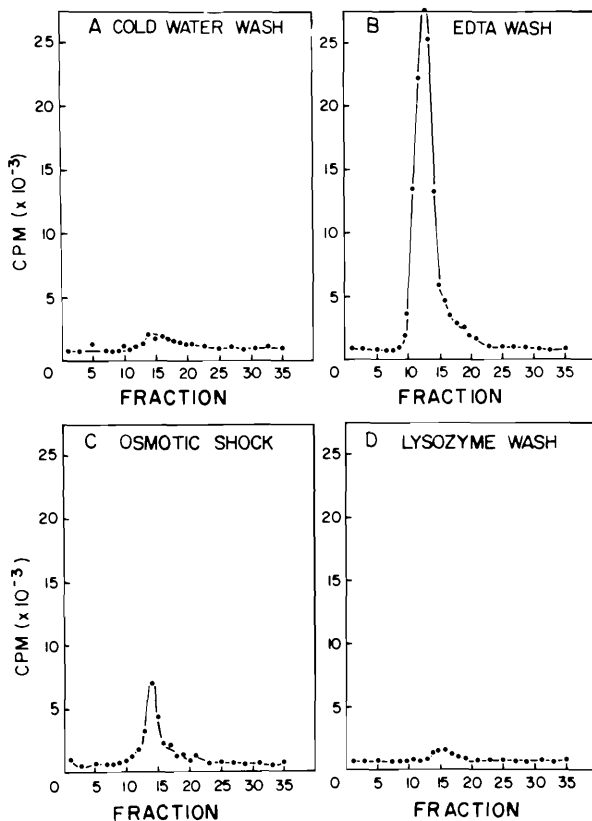


FIG. 7. Elution profiles of dicarboxylate binding protein from a *galE* mutant grown in the presence of D-[ $^3\text{H}$ ]galactose. Strain SK 11 was grown overnight in the presence of  $100 \mu\text{Ci}$  of D-[ $^3\text{H}$ ]galactose as described in the text. After harvesting and washing, the cells were subjected to treatments as described in Fig. 2. The amount of dicarboxylate binding protein in various supernatants was determined by affinity chromatography.

the slightly lower percentages of lipopolysaccharide-associated DBP recovered in various supernatants are probably due to the dissociation of lipopolysaccharide from DBP in very low ionic strength surroundings. The important implication from this study is that the cell surface DBP is associated with the lipopolysaccharide, while the other forms are not.

#### *The cbt gene is responsible for both free DBP and bound DBP*

The question that arises from the above study is whether both forms of DBP are coded by the same gene. In other words, one would like to know whether these are two different proteins or two different forms of the same protein. We have previously reported that DBP is coded by the *cbt* gene (4). Unfortunately, the *cbt* mutant we had was not isogenic with the strain (CBT 43) used in the present investigation. A spontaneous *cbt* mutant (BL 7) was isolated from strain CBT 43. This mutant was selected by its ability to grow on pyruvate in the presence of  $\beta$ -chlorolactate. It is unable to transport succinate, malate, and D-lactate, characteristics of the *cbt* mutant (5). The frequency of occurrence of this mutant was around  $10^{-6}$ , which suggests that it is likely to be a single gene mutation. Exponentially grown cells were used to compare the levels of DBP in both the mutant and the parental strains. Figure 8 shows that the *cbt* mutant has lower amounts of both free and bound forms of DBP, as compared with the parental strain. We also found that spontaneous revertant of this *cbt* mutant contained the same amount of both free and bound DBP as compared with the parental strain (data not shown). This suggests that the *cbt* gene may be responsible for both free and bound forms of DBP. We are currently studying the possibility that the mutation may be due to alterations in a regulatory site for two separate *cbt* genes.

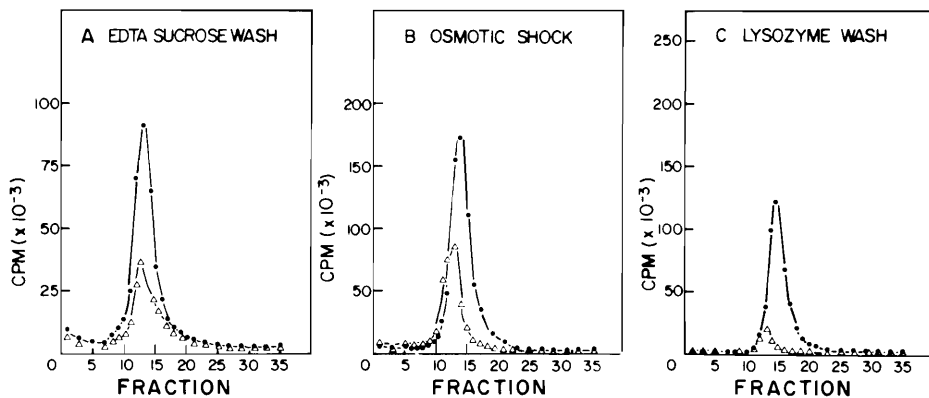


FIG. 8. The amount of dicarboxylate binding protein released by various treatments from strains CBT 43 and BL 7.  $^{35}\text{S}$ -labeled cells in the exponential phase were used in this study. Strain BL 7 is a spontaneous *cbt* mutant isolated from strain CBT 43. The same amount of cells from both strains was subjected to various treatments as indicated. ●, Elution profile from strain CBT 43; △, elution profile from strain BL 7.

### Discussion

In the past decade, quite a number of binding proteins have been isolated from the cell envelope of gram-negative bacteria (9). These proteins are characterized by their ability to bind with specific substrates, without catalyzing any chemical changes. Both biochemical and genetic studies indicate that they play an essential role in whole cell transport processes. Although quite a number of the binding proteins have been purified, crystallized, and even sequenced (9, 13), not much is known about their mode of action. Our ignorance in this area may be attributed to the lack of information on the localization of the binding proteins in the cell envelope. Based on the finding that these proteins are released from the cell envelope after disrupting the outer membrane by EDTA-osmotic shock treatment, it is generally concluded that they reside in the periplasmic space. Recently, Boos and co-workers (26) have shown that quite a number of EDTA-osmotic shock releasable proteins can be surface labeled by lactoperoxidase radiiodination (26); this suggests that some periplasmic proteins are accessible to the external environment.

Three transport components have been shown to be involved in dicarboxylate transport in *Escherichia coli* K12 (1). Two transport components (SBP 1 and SBP 2) reside in the cytoplasmic membrane; whereas the third component, the dicarboxylate binding protein (DBP), is located outside the cytoplasmic membrane. Both genetic and biochemical studies indicate that DBP plays an essential role in dicarboxylate transport in whole cells. The present investigation represents our initial attempt to understand the mode of action of the dicarboxylate binding protein. We approached this problem by selectively removing various layers of the cell envelope and by using surface labeling reagents so as to define the location of the dicarboxylate binding protein.

Table 1 shows that one can release some of the loosely

bound components on the cell surface by lowering the ionic strength of the surrounding environment. Substantial amounts of lipopolysaccharide and surface labeled materials are released when the outer membrane is disrupted by EDTA in the presence of sucrose. It should be noted that these two treatments do not release alkaline phosphatase, a marker enzyme for periplasmic proteins (Table 2). Osmotic shock treatment of the EDTA-treated cells causes the release of a significant amount of periplasmic proteins, including alkaline phosphatase. Table 1 also shows that further treatment of the shocked cells with lysozyme in the presence of sucrose releases a substantial quantity of  $^{35}\text{S}$ -labeled materials. Since cytoplasmic enzymes, such as glutamate dehydrogenase, are not detected in the supernatant obtained after lysozyme-sucrose treatment, it is unlikely that the  $^{35}\text{S}$ -labeled materials come from the cytoplasm. The peptidoglycan network has a mesh size of  $10.3 \text{ \AA} \times 12.5 \text{ \AA}$  (20) and is cleaved specifically by hen egg white lysozyme (19); it may, therefore, be inferred that the  $^{35}\text{S}$ -labeled materials released by lysozyme are either trapped by the peptidoglycan network towards the inner region of the periplasmic space, or they are bound to the peptidoglycan layer itself. It may be of interest to note that alkaline phosphatase is not found in this inner region of the periplasmic space. The above studies indicate that sequential treatment of the cells by cold water wash, EDTA-sucrose, osmotic shock, and lysozyme-sucrose may, therefore, enable one to determine whether a particular component is located on the cell surface, associated with the outer membrane, bound to or confined by peptidoglycan network in the cell envelope.

Fractionation of the  $^{35}\text{S}$ -labeled proteins released by cold water wash indicates presence of only negligible amounts (0.6%) of DBP. This suggests that DBP is not loosely bound to the cell surface. In fact, we have shown in a separate study that DBP is released from the surface

only by treating the cells with papain, or pronase, which does not affect the integrity of the outer membrane (10). From this, it would appear that DBP binds fairly tightly to the cell surface. About 7 and 51% of the total releasable  $^{35}\text{S}$ -labeled DBP are released by disrupting the outer membrane by EDTA-sucrose and osmotic shock treatments, respectively. DBP released by the above treatments are referred to as "free DBP."

Surface labeling reagents were used to determine the disposition of free DBP on the cell surface. Lactoperoxidase radioiodination and treatment with diazo[ $^{125}\text{I}$ ]-iodosulfanilic acid were used for this purpose. By assuming that 100% of the EDTA-sucrose releasable DBP can be surface labeled, it can be calculated from the above labeling studies that around 100, 11, and 3% of the DBP released by cold water, osmotic shock, and lysozyme-sucrose treatments, respectively, are surface labeled. The labeled DBP released by the latter two treatments may just be due to contamination by the cell surface DBP. Again, this finding suggests that the proteins located inside the outer membrane are not labeled by the above surface labeling techniques.

Figure 2 shows that about 0.6, 7, and 51% of the total releasable  $^{35}\text{S}$ -labeled DBP are released by cold water, EDTA-sucrose, and osmotic shock treatments, respectively. In other words, about 59% of the total releasable DBP is free DBP (according to our definition of free DBP). Since 100% of the cold water releasable DBP and about 11% of the osmotic shock releasable DBP are surface labeled, it may be calculated that about 13% ( $7.6\% + (51 \times 0.11\%)$ ) of the total releasable DBP or about 23% of the releasable free DBP are exposed on the cell surface. It should be noted that these figures do not represent the total amount of cell surface DBP present, since not all cell surface DBP is released from the outer membrane. This is based on our recent observation that at least threefold more  $^{125}\text{I}$ -labeled DBP are released on treating the cells with pronase or papain (10). Thus the cell surface DBP seems to bind very tightly to the outer membrane components.

Studies with the *galE* mutant suggest that lipopolysaccharide is noncovalently bound to DBP. Assuming all the EDTA-sucrose releasable DBP contain lipopolysaccharide, it may be calculated that about 75 and 3% of the DBP releasable by cold water and osmotic shock treatments, respectively, are associated with lipopolysaccharide. Since lipopolysaccharide is found exclusively on the outer membrane, it seems likely that only the cell surface DBP is associated with these molecules. The slightly lower percentage of cell surface DBP in the two preparations as calculated from the amount of lipopolysaccharide-associated DBP present are probably due to the dissociation of lipopolysaccharide from DBP in the presence of cold water.

In agreement with our previous findings (10), the present investigation indicates that some DBP are present on the cell surface. It is thought the cell surface DBP may function by interacting with the matrix protein (also referred to as porin) on the cell surface,

thereby conferring substrate specificity to the otherwise nonspecific porin channels. A discussion of this "porin—outer membrane binding protein" model is presented elsewhere (32).

It may be noted that not all the free DBP can be surface labeled. These proteins may reside in the outer region of the periplasmic space; therefore, they are released only when the outer membrane is dissociated. They may exist in a free state, or bound to the inner surface of the outer membrane. It is also possible, though not likely, that the binding of these proteins to the cell surface may render them inaccessible for labeling by lactoperoxidase or by diazosulfanilic acid. At the moment, evidence is not available to distinguish these possibilities.

Figure 2 shows that when  $^{35}\text{S}$ -labeled cells are treated further with lysozyme after osmotic shock treatment, a substantial amount of  $^{35}\text{S}$ -labeled DBP is released. Since there is no cell lysis in the lysozyme treated cells and  $^{35}\text{S}$ -labeled DBP cannot be detected in the cytoplasm, the lysozyme-releasable DBP is likely to be located in the periplasmic space between the peptidoglycan and the cytoplasmic membrane. At the moment, it is not clear whether this DBP is bound by the peptidoglycan network in the inner region of the periplasmic space, or it is bound directly to this network. This form is tentatively referred to as "bound DBP." Analysis of a spontaneous *cbt* mutant (BL 7) indicates that both free and bound DBP are defective in this mutant (Fig. 8). In other words, the *cbt* gene seems to be responsible for both forms of DBP.

Surface labeling studies with intact cells suggest that the bound DBP is not accessible for labeling (Figs. 4 and 6), and Fig. 7 indicates that this protein is not associated with lipopolysaccharide. To gain more information on the localization of this protein, we attempted to label this protein using EDTA-osmotic shocked cells by lactoperoxidase radioiodination. Although the purified bound DBP can be readily labeled, *in situ* labeling of this protein in EDTA-osmotic shocked cells has been unsuccessful (Fig. 5). Several implications can be drawn from the above observations. (i) The peptidoglycan network is not permeable to lactoperoxidase. (ii) It is unlikely that the bound DBP can diffuse across the peptidoglycan network, as this protein is not labeled in EDTA-osmotic shocked cells even after 30 min of incubation. In other words, the bound DBP is probably confined to the inner region of the periplasmic space by the peptidoglycan network. (iii) If the bound DBP associates tightly with the peptidoglycan, then it is not likely to be located on the outer surface of this network, unless the binding of this protein to the network renders it inaccessible for labeling. (iv) More importantly, it shows that the DBP releasable by lysozyme is not the residual free DBP found outside the peptidoglycan; the latter form should be labeled in the EDTA-osmotic shock treated cells by lactoperoxidase radioiodination. Thus the bound DBP differs from the free DBP in that it can be released from

the cell envelope and labeled by lactoperoxidase radioiodination only if the peptidoglycan network has been cleaved. It is possible that this protein may play an important role in transferring the substrate to the membrane bound transport components.

### Acknowledgements

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