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Immunocytological Investigation of Protein Synthesis in *Escherichia coli*

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Ferritin-conjugated specific antibodies have been used to localize β-galactosidase and both the monomer and active dimer of alkaline phosphatase in frozen thin sections of cells of *Escherichia coli* O8 strain F515. The even distribution of the ferritin marker throughout cells that had been induced for β-galactosidase synthesis, frozen, sectioned, and exposed to ferritin-anti-β-galactosidase conjugate showed that this enzyme was present throughout the cytoplasm of these cells. Frozen thin sections of cells that had been derepressed for the synthesis of alkaline phosphatase were exposed to both ferritin-anti-alkaline phosphatase monomer and ferritin-anti-alkaline phosphatase dimer conjugates, and the ferritin markers showed a peripheral distribution of both the monomer and the dimer of this enzyme. This indicates that alkaline phosphatase is present only in the peripheral regions of the cell and argues against the existence of a cytoplasmic pool of inactive monomers of this enzyme. This peripheral location of both the monomers and dimers of alkaline phosphatase supports the developing concensus that this enzyme is, like other wall-associated enzymes, synthesized in association with the cytoplasmic membrane and vectorially transported to the periplasmic area, where it assumes its tertiary and quaternary structure and acquires its enzymatic activity.

Protein synthesis has not yet been unequivocally localized in the procaryotic cell. Many workers have suggested that protein synthesis occurs in association with the cytoplasmic membrane (6, 25, 29), but others have presented evidence that it occurs in direct association with chromatin (19, 33), which is widely distributed throughout the cytoplasm. This question assumes a special importance in relation to the synthesis of proteins that must cross the cytoplasmic membrane en route to their protected functional "nitches" (3) in the bacterial cell envelope (4). A parallel system has been well defined for eucaryotic cells in which partly folded proteins are passed from polysomes on the cytoplasmic aspect of the rough endoplasmic reticulum into the lumen of this structure where they assume their functional forms (22-24).

Several investigators have recently presented physiological evidence (12, 26, 34) in support of our suggestion (4) that the cell wall-associated proteins of the procaryotic cell are synthesized on the inner aspect of the bacterial cytoplasmic membrane and extruded into the periplasmic area by a similar vectorial mechanism. We have reasoned that such enzymes should be found, by immunocytological means, only at the periphery of the cell, whereas cytoplasmic enzymes should be found throughout the cell whether they are formed in direct association with the chromatin or in association with the cytoplasmic membrane.

This paper tests these hypotheses by immunocytological methods, which also serve to test currently used enzyme localization methods (5).

MATERIALS AND METHODS

Escherichia coli O8 strain F515 is a deep, rough mutant (heptose-deficient lipopolysaccharide; 30). Growth media, growth conditions, preparation of the ferritin antibody conjugates, and electron microscopy were as previously described unless otherwise indicated (17).

Cells to be induced for the synthesis of β-galactosidase were grown in basal salts medium (18) with 0.25% (wt/vol) lactose added as a carbon source and 50 μg each of L-methionine, L-proline, and L-histidine per ml added according to the requirements of this strain.

Anti-β-galactosidase was produced by the subcutaneous injection of purified enzyme (Worthington Biochemical Corp.) into the back of a mature sheep at weekly intervals for a period of 4 weeks followed a week later by a test bleed of the animal. The antiserum obtained was fractionated as previously described (17). Antibodies against both the monomer and the dimer of alkaline phosphatase were pro-

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duced and fractionated as previously described (17). The activity and specificity of these antibodies were assessed by their formation of precipitin lines by the Ouchterlony method.

Cells to be frozen and thin sectioned were fixed for 1 h at 4°C in 1.0% glutaraldehyde in 0.1 M veronal acetate buffer at pH 7.4. Pretreatment of cells and sectioning was as previously described (31), and the resulting sections were exposed to the ferritin-antibody conjugates as described by Painter et al. (21). These preparations were then examined without further staining, using an AEI EM 801 electron microscope at an accelerating voltage of 60 kV.

Alkaline phosphatase was assayed at room temperature as described by Garen and Levinthal (8) using whole cells, β-galactosidase was assayed with toluenated cells (1% Toluene at 37°C for 1 h) at room temperature by the method of Lederberg (13), and protein concentrations were determined by the method of Lowry et al. (14). All assays were monitored with a Perkin-Elmer Coleman 124 spectrophotometer equipped with a recorder.

RESULTS

The effects of the fixation procedure, used to prepare cells for freezing and thin-sectioning, on the activity of both alkaline phosphatase and β-galactosidase were assessed by assaying cells of *E. coli* O8 strain 515, before and after fixation, at various stages of growth in batch culture. The assumption that the retention of enzymatic activity would be related to the retention of the antigenicity of these enzyme molecules was made. Figure 1 shows that fixation in 1% glutaraldehyde in 0.1 M veronal acetate buffer (pH 7.4) for 1 h at 4°C reduced alkaline phosphatase activity to 5 to 10% of the level of unfixed cells. Figure 2 shows that this fixation also reduced β-galactosidase activity to about 10% of the unfixed level in stationary-phase cells and to lower levels in exponentially growing cells. Thus, the fixation schedule required for freezing and thin sectioning resulted in a retention of about 10% of the normal enzymatic activity of both alkaline phosphatase and β-galactosidase and at least 10% retention of antigenicity.

To ensure that the antisera produced against the commercial enzymes used in this study would cross-react with the respective enzymes produced by the cells under examination, they were reacted with the cells in agar double-diffusion (Ouchterlony) tests. Figure 3 shows that the antiserum produced against commercially available β-galactosidase (center well) reacted with both the commercial enzyme (wells A and C) and with a sonic extract of cells induced, with respect to β-galactosidase, by growth on lactose (well D). The failure of the antiserum to react with a sonic extract of cells repressed, with respect to β-galactosidase, by growth on

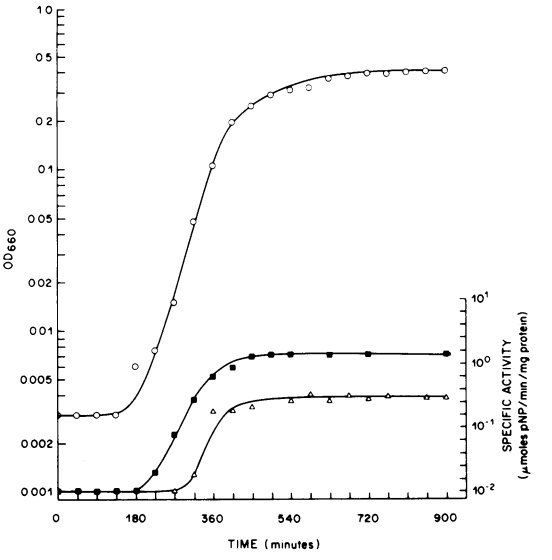

FIG. 1. *Fixation effects on the whole cell activity of alkaline phosphatase in E. coli O8 strain F515. Symbols: ○, Growth as determined by measuring the optical density at 660 nm; ■, specific activity of cell-associated alkaline phosphatase before fixation; △, specific activity (micromoles of p-nitrophenol per minute per milligram of protein) of cell-associated alkaline phosphatase after fixation.*

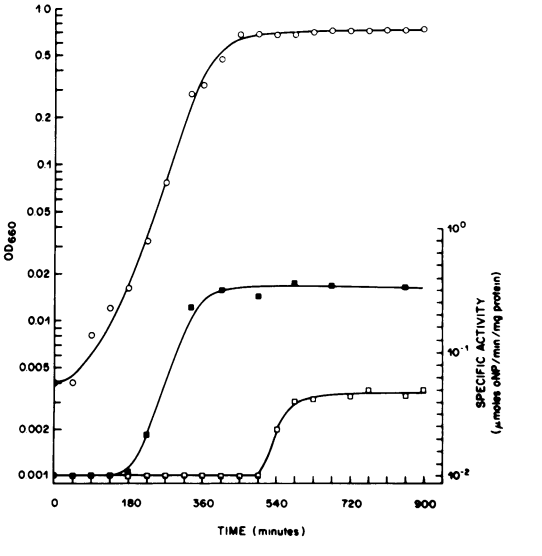

FIG. 2. *Fixation effects on the enzymatic activity of β-galactosidase in E. coli O8 strain F515. Symbols: ○, growth as determined by measuring optical density at 660 nm; ■, specific activity (micromoles of o-nitrophenol per minute per milligram of protein) of β-galactosidase prior to fixation; □, specific activity (micromoles of o-nitrophenol per minute per milligram of phosphate) of β-galactosidase after fixation.*

glucose (well B) attests to the absence of antibodies to cellular components other than the enzyme. Similar tests of the anti-alkaline phosphatase monomer and the anti-alkaline phosphatase dimer sera (17) also established both the specificity of the antibodies and the immunological cross-reactivity of the commercial enzyme with the enzyme produced by the cells used in this study.

β-Galactosidase was localized by fixing, freezing, and sectioning cells in which the enzyme had been induced and by reacting the resultant thin sections with the ferritin-anti-β-galactosidase conjugate. This enzyme was chosen to visualize the distribution of a cytoplasmic enzyme and to test this basic immunocytological procedure. The ferritin marker was heavily concentrated on the sectioned cells (Fig. 4), and the background levels of ferritin produced by nonspecific association of the conjugate with the Formvar substrate were very low. The darker peripheral areas of these unstained sections of cells are discernible in contrast to the less electron-dense cytoplasmic areas, and the ferritin marker was observed to be evenly distributed over both of these areas of the cells. Control preparations, in which thin-sectioned cells that had been induced for β-galactosidase but had not been derepressed for alkaline phosphatase synthesis were reacted with the ferritin-anti-alkaline phosphatase dimer conjugate, showed very few ferritin molecules in association with the cells, and the extent of this labeling was equivalent to the background level seen on the formvar substrate. Similarly, control preparations, in which cells that had not been induced for β-galactosidase synthesis were reacted with ferritin-anti-β-galactosidase conjugate, showed ferritin labeling that was equivalent to the background level seen on the formvar substrate (Fig. 5).

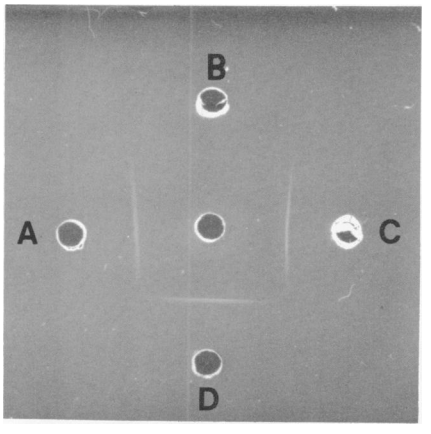

FIG. 3. *Ouchterlony double-diffusion plate of anti-serum to β-galactosidase. Center well contains the anti-serum. Wells A and C contain commercial β-galactosidase (Worthington). Well B contains a sonic extract of strain F515 grown in basal salts medium with glucose as a carbon source (no detectable β-galactosidase activity). Well D contains a sonic extract of strain F515 grown in basal salt medium with lactose as the sole carbon source (good β-galactosidase activity).*

Thus, this immunocytological procedure yields accurate and specific localization data and demonstrates that β-galactosidase is indeed present throughout the cytoplasm of these cells.

We have shown (17) that the anti-alkaline phosphatase monomer antiserum used in this study did not cross-react with the anti-alkaline phosphatase dimer antiserum, and we had also shown that both sera were free of extraneous antibodies directed against other cell envelope constituents. We, therefore, prepared thin sections of cells of *E. coli* O8 strain F515, which had been derepressed for alkaline phosphatase, fixed, and frozen, and we reacted these sections

TABLE 1. *Distribution of ferritin-antibody conjugates on frozen thin sections*

Sample	Cytoplasm (ferritin molecules/ 100 nm²)	Cell periphery (ferritin molecules/ 100 nm²)	Formvar background (ferritin molecule/ 100 nm²)
Cells derepressed for alkaline phosphatase and exposed to an anti-alkaline phosphatase dimer conjugate.	4.0[a]	31.0	1.0
Cells repressed for alkaline phosphatase synthesis and exposed to an anti-alkaline phosphatase dimer conjugate.	0.8	0.9	0.9
Cells derepressed for alkaline phosphatase and exposed to an anti-alkaline phosphatase monomer conjugate.	0.8	3.0	0.9

[a] Determined by counting the number of ferritin molecules present in a 100-nm² area for several micrographs and reported as a mean value.

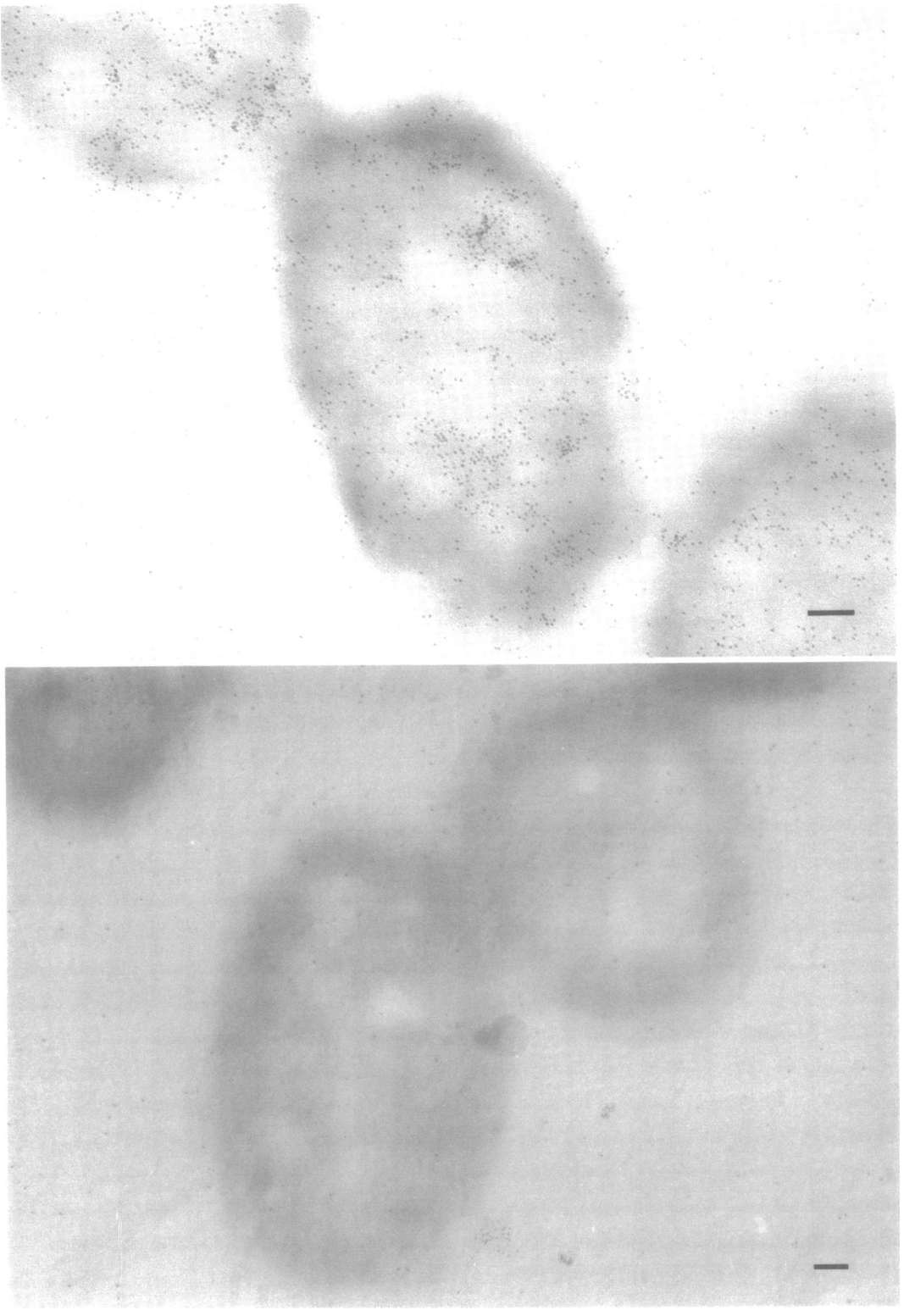

FIG. 4. *Frozen thin section of E. coli O8 strain F515 induced for β-galactosidase synthesis by growth on basal salts medium with lactose as the sole carbon source and exposed to an anti-β-galactosidase-ferritin conjugate. Note extensive labeling of the cytoplasm with the conjugate. The bar in this and subsequent electron micrographs indicates 100 nm.*

FIG. 5. *Frozen thin section of cells repressed for β-galactosidase, and derepressed for alkaline phosphatase, and exposed to an anti-β-galactosidase conjugate as a control preparation.*

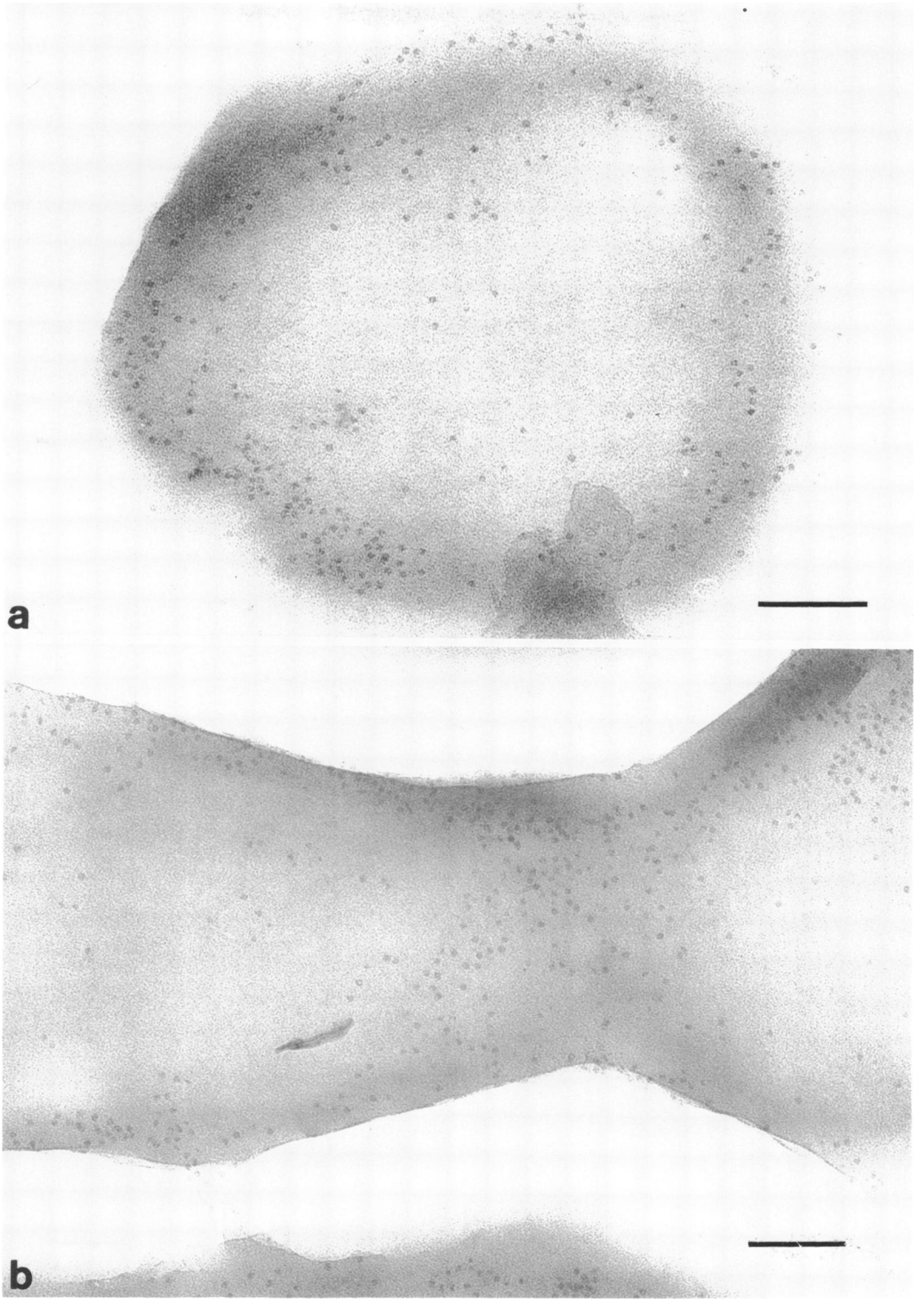

FIG. 6 a and b. *Frozen thin sections of E. coli O8 strain F515 derepressed for alkaline phosphatase synthesis by growth on phosphate-limiting media and exposed to anti-alkaline phosphatase dimer conjugate. Note the extensive labeling of the cell periphery, whereas labeling of the cytoplasm and of the formvar background is very light.*

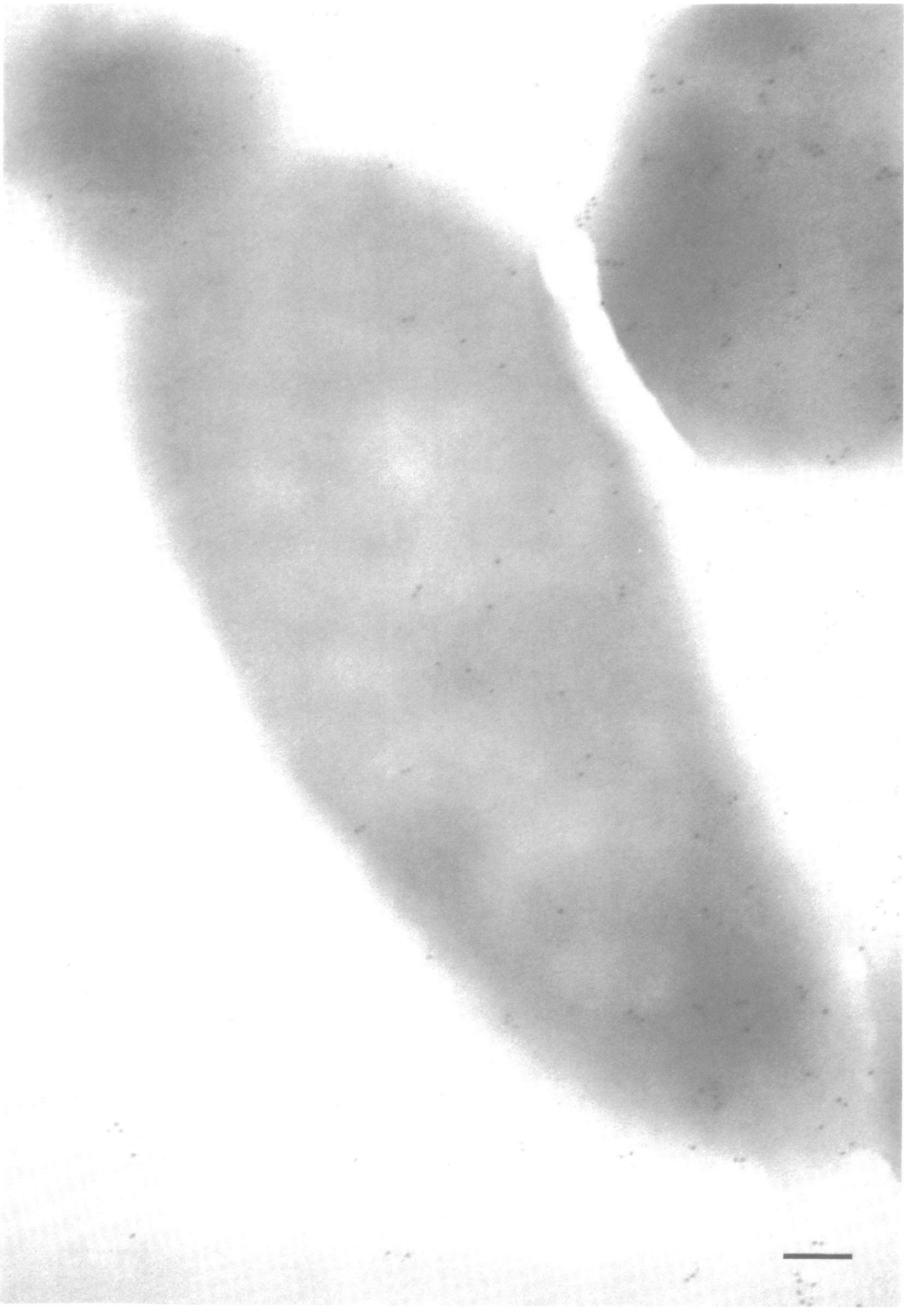

FIG. 7. *Frozen thin sections of cells repressed for the synthesis of alkaline phosphatase and exposed to an anti-alkaline phosphatase dimer conjugate as a control preparation.*

with the specific ferritin-anti-alkaline phosphatase dimer conjugate. The ferritin marker was found to be located in close association with the relatively electron-dense periphery of the cells (Fig. 6), and very low levels of background were seen in the cytoplasmic area and on the formvar. A detailed analysis (Table 1) of the number of ferritin molecules associated with peripheral area (in several micrographs) in comparison to both the cytoplasm and the background showed that this concentration of ferritin conjugate was significant. A control preparation of cells that had not been derepressed with respect to alkaline phosphatase showed an association of the ferritin-anti-alkaline phosphatase dimer conjugate that did not exceed the background level on the formvar substrate (Fig. 7). When sections of cells derepressed for alkaline phosphatase were reacted with the specific ferritin-anti-alkaline phosphatase monomer conjugate, a much less definite association of the ferritin marker with the cell periphery was noted (Fig. 8). The peripheral zone of these cells showed a degree of labeling with the conjugate which, although being much less well-defined than that obtained with the ferritin-anti-alkaline phosphatase dimer conjugate, was greater than that of the formvar support. A numerical expression of this data is presented in Table 1. This ferritin-anti-alkaline phosphatase monomer conjugate was not significantly concentrated over the cytoplasmic area of these cells (Table 1).

DISCUSSION

The location of protein synthesis in the bacterial cells is of considerable importance, but workers in this area have had to rely on two incomplete and potentially contradictory models based on morphological and physiological data, respectively.

One model was based on the observation (19, 33) that the ribosomal-messenger ribonucleic acid protein synthesis complex was directly associated with linear deoxyribonucleic acid strands in disrupted cells, and it was assumed that protein is synthesized at this locus and released into the cytoplasm. In this model, proteins that have functional locations outside the cytoplasmic membrane would accumulate in the cytoplasm (32) and be transported across the cytoplasmic membrane by an unknown mechanism (28).

The other model is based on a theoretical extrapolation (4) from protein synthesis in the eucaryotic cell (22–24) and from physiological studies of the procaryotic cell (2, 6, 9, 10, 16, 25, 29, 34). In this model, proteins that have functional locations outside the cytoplasmic membrane are synthesized by the ribosomal-messenger ribonucleic acid complex in close association with the inner aspect of the cytoplasmic membrane, and the nascent protein first associates with the hydrophobic phase of the membrane and then traverses this zone to emerge into the aqueous periplasmic zone where it assumes its folded tertiary structure and its quaternary functional associations (4).

The model of vectoral transport of nascent polypeptides is supported by specific association of ribosomes with the inner aspect of the cytoplasmic membrane of bacteria (6, 25) and the bounding membrane of eucaryotic organelles (15). Alkaline phosphatase synthesis in *E. coli* appears to occur mainly on membrane-associated polysomes (2). Cancedda and Schlesinger have suggested that the energy required for the vectoral transport of the unfolded nascent polypeptide is provided by continued protein synthesis (2). The activation energy of 55 kcal/mol for the secretion of alkaline phosphatase is consistent with the passage of a largely hydrophilic protein molecule through a hydrophobic barrier (10). Additional support is found in the differing proteolytic susceptibility of the emerging unfolded nascent polypeptides of α-amylase and protease, the fully folded native enzymes (26), and Lampen's observations that phosphatidyl serine associates with the N-terminal of the nascent protein and facilitates its passage through the hydrophobic zone of the membrane (34). It has been determined that the N-terminal of the nascent protein is lost after emergence and folding (2, 34) and that the active enzyme may then go on to form specific associations with structural elements of the cell envelope (12).

These two models are not necessarily mutually exclusive in that protein synthesis could occur at both loci, as suggested by Glew and Heath (10), with proteins destined for locations outside the cytoplasmic membrane being synthesized in a vectoral association with this membrane and extruded through it in an extended configuration, whereas proteins destined for a cytoplasmic location are synthesized at either locus.

This second model for the location of protein synthesis in bacterial cells is in good agreement with the better understood pattern in the eucaryotic cell in which "exportable" proteins are synthesized by polysomes associated with the endoplasmic reticulum (1, 7), passed into the lumen of this membrane system as linear polypeptides (22), and discharged at the cell surface from vesicles derived from this organelle (23, 24). Protein synthesis may also be associated

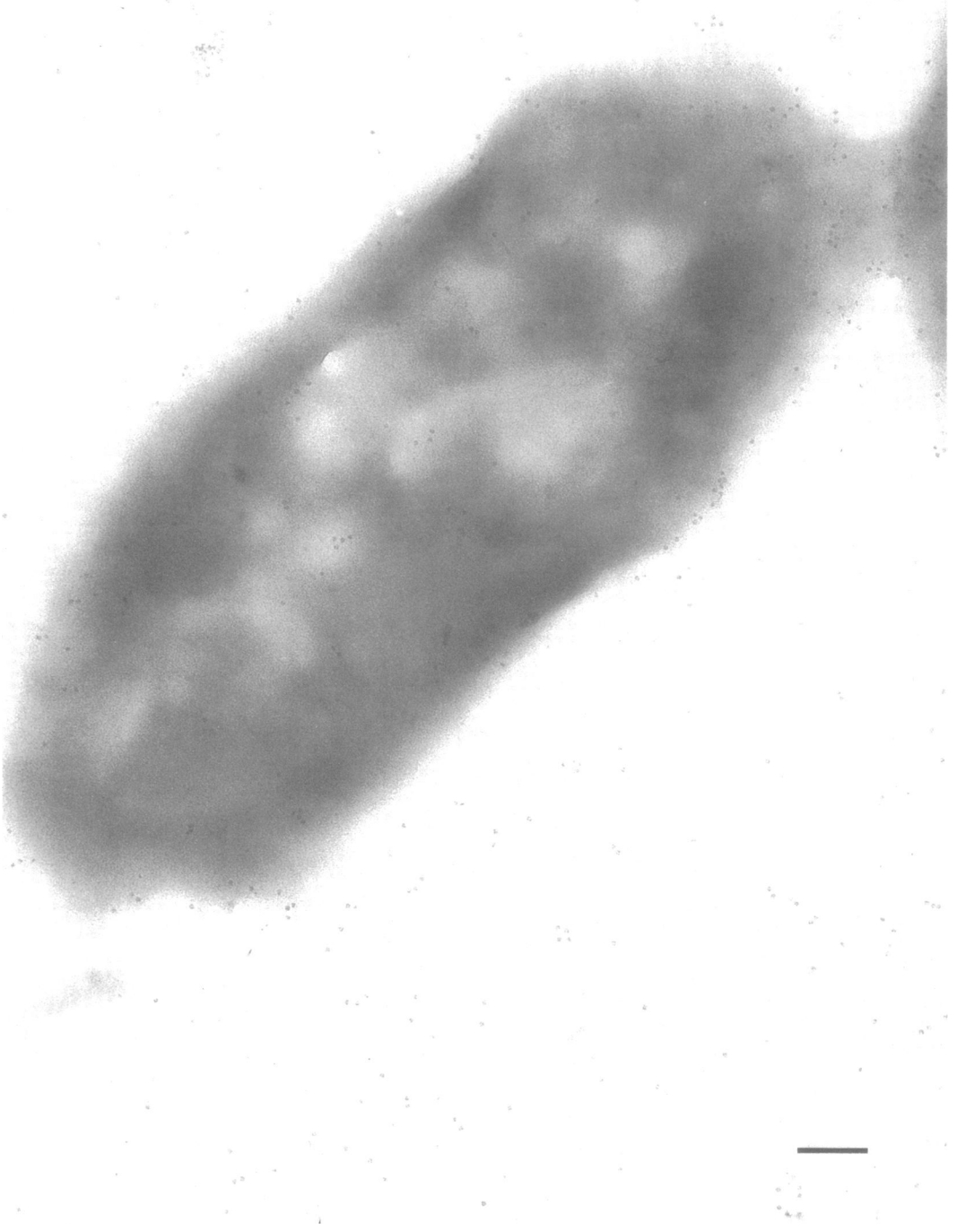

FIG. 8. *Frozen thin sections of cells derepressed for the synthesis of alkaline phosphatase and exposed to an anti-alkaline phosphatase monomer conjugate. Some reactivity was noted at the cell periphery. The reactivity of the cytoplasm is below that of the surrounding background (see Table 1).*

with the plasma membrane in eucaryotic cells (11), and polysomes are often seen in the cytoplasm without any obvious membrane associations. Thus, protein synthesis in eucaryotic cells does not appear to be rigorously limited to any one locus, but exportable proteins appear to use vectoral synthesis to traverse the bounding membranes of the cell.

More recent work (27) has shown that the initial 20 amino acids in the nascent polypeptide of an "exportable" protein of a eucaryotic cell (immunoglobulin-light chain) are hydrophobic (mostly leucine); this suggests that hydrophobic attraction may be the factor which commits the nascent polypeptides of exportable proteins to the hydrophobic interior zone of the cytoplasmic membrane. The observation that the N-terminal amino acid sequences of the nascent polypeptide of this exportable protein (20, 27), of a procaryotic periplasmic enzyme (alkaline phosphatase [2]), and a procaryotic exoenzyme (penicillinase) (34) differ from those of the fully-folded active proteins suggests that these hydrophobic initial sequences of amino acids may mediate the commitment of the proteins to the interior of the membranes and their association with phospholipid carriers (34) and then be removed when the proteins emerge on the outside of the membrane. In this way, the genetic code for a protein would determine not only its structure, function, and molecular associations but also its eventual location vis-a-vis the bounding membrane of the cell.

Immunocytology was used to provide direct morphological confirmation of the developing concensus regarding vectoral synthesis of extracellular proteins in the procaryotic cell. We reasoned that both deoxyribonucleic acid-associated and cytoplasmic membrane-associated synthesis would yield an even distribution of the resultant protein throughout the interior of the cell if it were released into the cytoplasm after synthesis. Therefore, we used a ferritin conjugate of a specific antibody to show the general cytoplasmic distribution of β-galactosidase as a control of our immunocytological methods. We then reasoned that the vectoral synthesis of a periplasmic enzyme (alkaline phosphatase) would produce a peripheral enzyme "rim" consisting of a few monomers and a large number of enzymatically active dimers. On the other hand, deoxyribonucleic acid-associated and cytoplasmic membrane-associated synthesis of this enzyme in which the protein was released into the cytoplasm would produce a cytoplasmic "pool" of monomers (32) which would be secreted to form a rim of active dimers in the periplasm. Therefore, we used ferritin conjugates of antibodies against either the monomers or the dimers of alkaline phosphatase, which had been exhaustively checked for specificity by Ouchterlony tests and in the immunocytological localization of the enzyme at the cell surface (17), and found that large amounts of the dimer and smaller amounts of the monomer were present at the cellular periphery and that there was no significant cytoplasmic concentration of the monomer. Thus, this study provides morphological support for the developing concensus that periplasmic and other extracellular proteins are synthesized at the inner aspect of the cytoplasmic membrane and traverse this membrane by vectoral synthesis.

A more precise localization of antigenic proteins in frozen sections is difficult, because the staining procedures, which would allow the definition of some cellular structures, also remove some of the conjugate and because the ferritin marker molecule is not immediately adjacent to the active site of the antibody within the conjugate.

ACKNOWLEDGMENTS

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