Lactoperoxidase-¹²⁵I Localization of Salt-Extractable Alkaline Phosphatase on the Cytoplasmic Membrane of *Bacillus licheniformis*

DONALD B. SPENCER AND F. MARION HULETT*

Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680

Previous histochemical and biochemical localizations of alkaline phosphatase in *Bacillus licheniformis* MC14 have shown that the membrane-associated form of the enzyme is located on the inner surface of the cytoplasmic membrane, and soluble forms are located in the periplasmic space and in the growth medium. The distribution of salt-extractable alkaline phosphatase on the surfaces of the cytoplasmic membrane of *B. licheniformis* MC14 was determined by using lactoperoxidase-¹²⁵I labeling techniques. Cells harvested during rapid alkaline phosphatase production were converted to protoplasts or lysed protoplasts and labeled. Analysis of the data obtained indicated that 30% of the salt-extractable, membrane-associated alkaline phosphatase was located on the outer surface of the cytoplasmic membrane, whereas 70% of the membrane-associated enzyme was localized on the inner surface. Controls for protoplast integrity (release of tritiated thymidine or examination of cytoplasmic proteins for label content) indicated excellent protoplast stability. Controls indicated that chemical labeling was not a factor in the apparent distribution of alkaline phosphatase on the membrane. These results support the previously reported histochemical localization of alkaline phosphatase on the membrane inner surface. The presence of alkaline phosphatase on the membrane outer surface is reasonable, considering the soluble forms of the enzyme found in the periplasmic region and in the culture medium.

Histochemical localizations of alkaline phosphatase (APase; orthophosphoric monoester phosphohydrolase; EC 3.1.3.1.) in *Bacillus licheniformis* MC14 have shown that the enzyme is located on the inner surface of the cytoplasmic membrane when the organism is grown in 2% Neopeptone (Difco) (27). More recent histochemical localizations of APase showed lead phosphate deposits both on the membrane inner surface and in the periplasmic region when *B. licheniformis* was grown in 1% Neopeptone (Difco) (M. Kuna and F. M. Hulett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K239, p. 226). A biochemical localization of APase as a function of culture age by Glynn et al. (11) confirmed the presence of a membrane-bound enzyme as well as a soluble enzyme between the membrane and the cell wall. These localization studies suggested that the membrane-bound APase was a precursor of the soluble enzyme (11). Magnesium-extractable membrane-bound APase has been solubilized by a heat-salt technique (14), purified to homogeneity, and characterized as a basic protein with a molecular weight of 121,000 (15) and a subunit molecular weight of 60,000 (16). The membrane-associated APase and the soluble APase (from the periplasmic region or

secreted into the medium) show immunological identity in studies using antibody prepared against the membrane-associated APase (11).

We report here the localization of salt-extractable APase on the inner and outer surfaces of *B. licheniformis* cytoplasmic membrane by using lactoperoxidase-¹²⁵I labeling techniques (30).

(This paper was presented in part at the 79th Annual Meeting of the American Society for Microbiology, Los Angeles, Calif., May, 1979.)

MATERIALS AND METHODS

Organism and growth conditions. The isolate used in this study was a facultative thermophile, *B. licheniformis* MC14 (14). Stocks were maintained on defined minimal medium agar plates at 4°C (38). Inoculation and culture growth in a defined minimal salts (DMCo²⁺) medium have been described previously (38).

Biochemical assay for APase. APase activity was assayed during cell growth as described in the accompanying paper (38). Active APase was located on nondenaturing polyacrylamide gels by using a naphthol A.S. phosphoric acid (NASP) assay (21, 28).

Lactoperoxidase-¹²⁵I labeling procedure. A 400-ml culture was grown in DMCo²⁺ medium (38) until the absorbance at 540 nm reached 1.7 and then was harvested by centrifugation at 10,000 × g for 30

min; the pellet fraction (whole cells) was suspended in 15 ml of 0.01 M Tris-acetate buffer (pH 7.3) containing 0.1 mM CoCl_2 (TAC buffer). Three equal volumes were centrifuged at $40,000 \times g$ for 30 min, and each pellet (whole cells) was suspended in 2 ml of 0.01 M TAC containing 20% glycerol (TACG) and 0.03 M MgSO_4 . Protoplast formation was carried out as described previously (38).

One of the protoplast samples was iodinated immediately. The second protoplast sample was lysed as described by Glynn et al. (11) before it was iodinated. Either lysed protoplasts were immediately iodinated, or the membranes were collected by centrifugation at $40,000 \times g$ for 30 min, suspended in 2 ml of TAC buffer, and labeled with ^{125}I by the following procedures. Protoplast labeling procedure: K^{125}I (0.5 mCi; ICN) and 6.0 mg of lactoperoxidase were added to the intact protoplast sample at room temperature. Hydrogen peroxide (0.4 μM) in TACG containing 0.05 M MgSO_4 was added at 15-s intervals (20 μl each) during a 15-min reaction period. The reaction was stopped by adding 5.0 ml of cold (0°C) TACG buffer, containing 0.05 M MgSO_4 , and centrifuging at $5,000 \times g$ for 30 min. The pellet fraction (protoplasts) was suspended in 5.0 ml of TACG containing 0.05 M MgSO_4 and centrifuged again at $40,000 \times g$ for 30 min. The pellet fraction was suspended in 2.0 ml of TAC buffer for lysis. The lysed suspension was centrifuged at $40,000 \times g$ for 30 min. The suspension in TAC buffer and centrifugation were repeated.

The second sample was subjected to the intact protoplast labeling procedure described above. Lactoperoxidase (6.0 mg) was omitted from the labeling mixture in order to monitor the occurrence of chemical labeling.

Lysed membrane labeling procedure. The lysed sample was iodinated as described above for protoplast labeling except that the $5,000 \times g$ centrifugation directly after labeling was omitted and all washes were carried out using TAC buffer.

Membrane-bound APase salt extraction. Magnesium or sodium extractions of membrane-bound APase were made by suspending labeled membrane samples in 1.0 M MgSO_4 in TAC buffer or in 0.85 M NaCl in TAC buffer, respectively, and stirring at 4°C for 30 min. Solubilized enzyme was collected in the supernatant fraction by centrifugation at $40,000 \times g$ for 30 min.

Purification of labeled APase: immunological techniques. Antiserum against heat-salt-solubilized APase was collected and prepared by the methods of Hulett and Campbell (14). ^{125}I -labeled APase was purified from 0.85 M NaCl-extracted material of lysed-labeled or intact-labeled protoplasts by incubating samples with titrated antisera (in 0.5 M NaCl) for 24 h at 4°C.

The immunoprecipitate was collected by centrifugation at $1,500 \times g$ for 30 min and washed twice in 2 ml of TAC buffer containing 0.5 M NaCl and twice again in TAC buffer. All washes were carried out at 4°C. The precipitates were subjected to electrophoresis on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (22). Reducing agents were omitted from the sample preparation (22). The gels were stained as described by Fairbanks et al. (5).

Purification of labeled APase: HPCI gel electrophoresis. Samples of the membrane proteins extracted by MgSO_4 were dialyzed to precipitate the APase (33). The APase was collected in the pellet fraction by centrifugation at $40,000 \times g$ for 30 min. A 100- μl volume of 10% hexadecylpyridinium chloride (HPCI) and 30 μl of glycerol were added to the pellet fraction, and the suspension was layered onto two identical 7.5% polyacrylamide gels (4) which had been subjected to pre-electrophoresis for 4 h at 3 mA per gel. These gels were run at 3 mA per gel for 2.0 to 3.5 h. One gel was stained for protein by the method of Fairbanks et al. (5); the other was used to locate active APase by using the NASP assay (28).

Analysis of labeled APase isolated from polyacrylamide (SDS or HPCI) gels. Gels stained for protein with Coomassie blue were scanned for protein concentration at 660 nm, sliced into 1-mm slices, and assayed for the presence of ^{125}I . Identical scales for absorbance at 600 nm or counts per minute were used in analyzing protein concentration or associated label, respectively, from lysed or intact protoplasts within each experiment. Specific activity (^{125}I per amount of protein) for each gel was determined by dividing the area weight under the APase peak from the radioactivity graph (counts per minute per slice) by the area weight under the APase peak from the protein scan.

Slab gel electrophoresis. Slab gels (7.5%) (43) were run for 1.5 h at 10 mA per slab and then for 3 h at 25 mA per slab, stained with Coomassie blue G (Sigma), and destained by the Fairbanks method (5).

Autoradiography techniques. The Coomassie blue-stained slab gel was soaked in a 70% methanol-3% glycerol solution for 2 h and dried under vacuum with mild heat on Whatman no. 1 filter paper. XR-1 X-ray film (Kodak) was exposed to the dried gel at -70°C for 48 h. The film was processed using Kodak liquid developer and fixer.

RESULTS

Analysis of label distribution in APase isolated by immunoprecipitation. Figure 1 shows the analysis of an SDS gel containing immunoprecipitated APase which had been extracted from intact ^{125}I -labeled protoplasts. At the top of Fig. 1 is an SDS gel showing a protein band of immunoglobulin G (molecular weight, 150,000) and a band of APase subunits (molecular weight, 60,000). Directly below is a scan (660 nm) of the Coomassie blue stained gel. The bottom panel is a graph of the radioactivity present in each 1-mm slice of the gel above, which shows ^{125}I to be associated only with the APase subunits. The specific activity for the APase isolated in the polyacrylamide gel was determined as described in Materials and Methods. Figure 2 shows an SDS gel, protein scan, and radioactivity graph of immunoprecipitated APase extracted from protoplasts which were lysed previous to the labeling procedure. APase protein from the intact labeled protoplasts had a specific activity which was 30% of the APase

FIG. 1. SDS gel, protein scan, and ^{125}I radioactivity graph of APase from intact labeled protoplasts. ^{125}I -labeled APase from intact labeled protoplasts was immunoprecipitated and isolated on a 7.5% SDS-polyacrylamide gel (top). The gel was stained for protein and scanned at 600 nm to determine the protein concentration of the APase subunit band (60,000 molecular weight). The gel was then sliced into 1-mm slices, which were monitored for their radioactive content in counts per minute (CPM) (bottom).

FIG. 2. SDS gel, protein scan, and radioactivity graph of APase extracted from lysed labeled protoplasts. Immunoprecipitated APase from lysed labeled protoplasts was isolated on 7.5% SDS-polyacrylamide gels (top). The concentration of APase subunits was determined by scanning the gel, stained for protein, at 660 nm (middle). The gel was cut into 1-mm slices, and each slice was monitored for gamma emissions (counts per minute; CPM) (bottom).

specific activity obtained from lysed labeled membrane.

Analysis of label distribution in APase isolated on HPCl gel. Similar labeling experiments were performed in which membrane-associated proteins, labeled in intact or lysed protoplasts, were extracted with 1.0 M MgSO_4 and isolated by HPCl-polyacrylamide gel electrophoresis. Duplicate HPCl gels containing the extracted protein were prepared, and one gel of each was used to determine the location of active APase dimers with an NASP stain. The other gel was stained for protein. Figure 3 shows representative gels indicating the location of active

FIG. 3. Localization of APase protein on HPCl gels. The gel containing the single band (left) was stained with NASP to indicate the position of active APase. The gel on the right was stained with Coomassie blue G to indicate protein concentration. Electrophoresis was carried out for 3.5 h.

APase dimers on HPCl gels stained for protein with Coomassie blue G.

Figure 4 shows the Coomassie blue-stained HPCl gel of proteins extracted with 1.0 M MgSO_4 from membranes of intact ^{125}I -labeled protoplasts. The figure also includes a 660-nm scan of the stained gel and a graph of the ^{125}I distribution in the gel. The NASP stained gel indicated that the protein band located in fractions 30 to 35 was active APase. Comparison of the radioactivity graph and the protein scan for the proteins extracted with 1.0 M MgSO_4 from membranes of enzymatically labeled intact protoplasts indicated that not all of these proteins were exposed to the labeling reaction. Note that fractions 0 to 10 and 60 to 70 did not contain counts above background readings, whereas the 660-nm protein scan indicated the presence of protein in these fractions. In contrast, the APase peak (fractions 30 to 35) was definitely labeled on intact protoplasts.

Table 1 summarizes the data collected in four separate experiments. In each case, extracted APase from protoplasts, labeled while intact, did contain radioactive label. However, APase extracted from protoplasts which were lysed before labeling contained more label per amount of protein, indicating that lysis of the protoplast exposed more APase to the labeling components than was exposed on the outer surface of intact protoplasts. The specific activities indicate that approximately 28 to 33% of the salt-extractable APase was associated with the outer surface of the cytoplasmic membrane.

Controls for protoplast integrity and chemical labeling. To insure that our labeling procedure did not cause chemical labeling (30), a second intact protoplast sample was prepared each time the labeling experiments were carried out.

Figure 5 shows the distribution of protein and radioactivity on an HPCl polyacrylamide gel of a 1.0 M MgSO_4 extract from membranes of protoplasts which were exposed to the labeling mixture minus lactoperoxidase. The radioactivity graph gives no indication of significant labeling over the entire gel and specifically not in the area of the APase dimer protein band (fractions 14 to 19, as identified by the NASP staining procedure).

Figure 6 shows an SDS slab gel and an autoradiograph of duplicate samples of cytoplasmic membrane and cytosol from protoplasts which had been treated as follows: labeled as intact protoplasts, labeled after lysis of the protoplasts, or exposed to possible chemical labeling as intact protoplasts (as described above). When lysed protoplasts were iodinated, both cytosol (Fig. 6, lanes 2 and 3) and membrane protein (lanes 4

FIG. 4. HPCL gel, protein scan, and ¹²⁵I radioactivity graph of APase from intact-labeled protoplasts. Top: HPCL gel of MgSO₄-extracted protein (electrophoresis for 3.5 h). Middle: 660-nm scan of Coomassie blue-stained gel. Bottom: Graph of counts per minute (CPM) per 1-mm slice of gel shown above. Electrophoresis was carried out for 3.5 h.

TABLE 1. *Specific activities of iodinated lysed and intact protoplasts^a*

Expt	Iodinated APase collected by:	Area wt (g) under gamma trace (A)	Area wt (g) under protein scan (B)	Sp act (A/B)	% APase labeled
I	Mg ²⁺ extraction, HPCI gel; isolation of APase from lysed ¹²⁵ I-labeled protoplasts	0.0420	0.0343	1.225	100
	Mg ²⁺ extraction, HPCI gel; isolation of APase from intact ¹²⁵ I-labeled protoplasts	0.0829	0.2091	0.396	32
II	Mg ²⁺ extraction, HPCI gel; isolation of APase from lysed ¹²⁵ I-labeled protoplasts	0.2235	0.0764	2.925	100
	Mg ²⁺ extraction, HPCI gel; isolation of APase from intact ¹²⁵ I-labeled protoplasts	0.1112	0.1375	0.809	28
III	NaCl extraction and immunoprecipitation from lysed ¹²⁵ I-labeled protoplasts	0.4406	0.1555	2.834	100
	NaCl extraction and immunoprecipitation from intact ¹²⁵ I-labeled protoplasts	0.1002	0.1059	0.946	33
IV	NaCl extraction and immunoprecipitation from lysed ¹²⁵ I-labeled protoplasts	0.3838	0.1513	2.537	100
	NaCl extraction and immunoprecipitation from intact ¹²⁵ I-labeled protoplasts	0.0801	0.1075	0.745	30

^a APase bound to membrane inner surface: 67 to 72%. APase bound to membrane outer surface: 28 to 33%.

and 5) contained label. It is clear that when the intact protoplasts were labeled, no cytoplasmic proteins were labeled (Fig. 6, lanes 6 and 7), whereas certain membrane proteins were labeled (lanes 8 and 9). No label was associated with protein in the membrane samples (Fig. 6, lanes 10 and 11) or the cytosol samples (lanes 12 and 13) when intact protoplasts were subjected to chemical labeling control conditions. The sample concentrations were not identical, and the film was not preflashed. This control system was intended to determine the presence or absence of labeling in each sample.

Protoplast integrity during the iodination reaction was also analyzed by determining the amount of [*methyl*-³H]thymidine released. Cells grown in the presence of 250 μ Ci of [*methyl*-³H]thymidine were subjected to all steps described for the iodination procedure. Analysis of samples of all supernatant fractions obtained from the centrifugation steps (protoplast-released material, cytosol, and several washes that followed) indicated that the protoplasts subjected to the ¹²⁵I reaction maintained greater than 99.0% integrity. Table 2 shows counts per minute and percent radioactivity released during protoplast formation and iodination. Only 0.03%

of the total radioactive thymidine contained within the protoplasts was released into the ¹²⁵I reaction mixture. This procedure was carried out with and without DNase in the reaction mixture to insure that released DNA was not sedimented with the intact protoplasts.

DISCUSSION

The asymmetric arrangement of protein on membrane inner and outer surfaces has been considered an established rule. Analysis of membrane structure in enveloped animal viruses (24), mitochondria (35), endoplasmic reticulum (20), platelet sarcoplasmic reticulum (29, 41), intestinal brush border (25), and erythrocyte membrane and bacterial membrane (44) have supported the premise that an absolute asymmetric orientation of proteins exists for these membranes. Postulated invertible orientation of membrane proteins has been ruled out because of the thermodynamic requirements of polar and charged group interactions with the hydrophobic bilayer (37). Modification of membrane-bound protein by proteolytic digestion (40), enzymatically catalyzed iodination (13, 30, 32), or nonpenetrating reagents (1) have allowed identification of polypeptides exposed to the outer

FIG. 5. HPCL gel, protein scan, and ^{125}I radioactivity graph of APase from protoplasts subjected to chemical labeling control conditions. Top: HPCL gel of MgSO_4 -extracted protein (subjected to electrophoresis for 2.0 h). Middle: Gel scan (660 nm) of Coomassie-stained gel. Bottom: Graph of counts per minute (CPM) per 1-mm slice of gel shown above.

surface of a membrane in intact cells, right-side-out vesicles, or resealed erythrocytes. It appeared that the only true modifications of membrane-bound proteins from both sides of the

membrane were reported for intrinsic proteins which spanned the bilayer with peptide portions being exposed on both surfaces (2, 19, 39).

Our procaryotic system has unique character-

FIG. 6. SDS slab gel (top) and autoradiogram (bottom) of membrane and cytosol from protoplasts labeled with ^{125}I as intact or lysed protoplasts, or exposed to chemical labeling control techniques. Lanes 1 and 14, Mixture of labeled proteins. Lanes 2 and 3, Cytoplasmic samples from iodinated, lysed protoplasts. Lanes 4 and 5, Membrane samples from iodinated lysed protoplasts. Lanes 6 and 7, Cytosol sample from iodinated, intact protoplasts. Lanes 8 and 9, Membrane samples from iodinated, intact protoplasts. Lanes 10 and 11, Membrane samples from chemical labeling control intact protoplasts. Lanes 12 and 13, Cytosol samples from chemical labeling control intact protoplasts.

istics with respect to enzyme distribution. It is unusual to localize active, immunologically identical enzyme on the inner surface of the cyto-

plasmic membrane (27), in the periplasmic space (11), and in the culture medium (38).

Membrane-bound intermediates of secreted

TABLE 2. *Protoplast integrity analyzed by [³H]thymidine release*

Sample type	Total ³ H cpm collected	% ³ H released
Pre-iodination wash of whole cells (supernatant fraction)	5.92×10^5	
Iodination of intact protoplasts (supernatant fraction)	2.77×10^5	<1
Supernatant fraction of protoplasts, lysed and washed after iodination	9.14×10^8	99

proteins are commonly reported in the literature (23, 31). However, the active membrane form has always been proven or assumed to be only on the membrane external surface (34, 42). These observations made a critical analysis of the distribution of APase on the membrane surface of *B. licheniformis* essential.

The lactoperoxidase-¹²⁵I labeling technique was chosen because of the innate controls in the system (30) which reduced the possibility of ambiguous interpretation. It is clear from the data presented in Table 1 that the concentrations of labeled APase extracted from membrane fractions of lysed-labeled or intact-labeled protoplasts were not influenced by the type or concentration of salt used in the extraction procedure (0.85 M NaCl or 1.0 M MgSO₄), nor by the method of isolation of the enzyme (immunoprecipitation or identification by enzymatic activity). Using these techniques, we repeatedly obtained data which indicated that 30 and 70% of the salt-extractable membrane APase was located on the outer and inner surfaces of the cytoplasmic membrane, respectively. Two assumptions were made in these calculations: (i) that all salt-extractable membrane-bound APase (inner or outer surfaces) is associated with the membrane in such a way that when exposed to the labeling components (lactoperoxidase, H₂O₂, K¹²⁵I) the susceptibility to labeling of each APase molecule is identical; and (ii) that the labeled APase from lysed-labeled protoplasts represents 100% of the labeling potential for membrane-associated APase.

These data support the observation of McNicholas and Hulett (27) that the major portion of the membrane-bound APase is located on the inner surface. When one compares the levels of enzyme production by the culture used in each of the two studies (histochemical, 0.1 U/ml; ¹²⁵I labeling, 6 U/ml), it is obvious that the amount of APase on the outer surface of membranes in the initial histochemical studies might have been either undetectable (7) or nonexistent.

Subsequent histochemical localizations (38) indicated the presence of lead phosphate deposits in the periplasmic region as well as on the membrane inner surface. This technique is not sensitive enough to determine whether these deposits resulted from active soluble APase known to exist in the periplasmic region of *B. licheniformis* cultured in 1% Neopeptone (11) or from active membrane-associated APase that might have been exposed on the membrane outer surface.

Labeling of APase on both surfaces of the cytoplasmic membrane cannot be interpreted as evidence of being a transmembrane protein since all solubilization techniques (alkaline pH, EDTA or salt extraction; 11, 14, 16) used for the membrane form of this enzyme indicate that it is peripherally bound (36, 37).

The APase (30%) assigned to the outer surface of the membrane is not the result of ruptured protoplasts. Both controls for protoplast stability ([³H]thymidine release and autoradiographic analysis of cytosol from labeled protoplasts) indicated excellent protoplast integrity. Controls also ruled out chemical labeling as a factor in the apparent APase distribution.

The specific location of APase dimers may be the direct result of the metabolic function (8, 12, 45) which required APase production due to specific culture conditions.

A number of references indicate that the APase of gram-positive organisms is located on the inner surface of the cytoplasmic membrane (27, 45), secreted through the membrane (3, 9-11, 38, 46), or both, whereas the APase of gram-negative organisms is located in the periplasmic region (17, 18, 26). In a complex medium *B. licheniformis* produces low levels of APase which is predominantly on the membrane inner surface (27). In lower concentrations of the same medium, the enzyme is associated with the inner surface of the membrane but is also secreted into the periplasmic region where it could scavenge phosphate from teichoic acids located in the cell wall (11, 12). When *B. licheniformis* is cultured in an extremely limited growth medium (DMCo²⁺), the amount of total APase is significantly increased, the APase location is extended beyond the cell wall confinement (38), and its association with the cytoplasmic membrane does not conform to an absolute asymmetric orientation, as has been reported for other systems (20, 24, 25, 35, 41).

Ghosh et al. have shown that penicillinase moieties exist in the cytosol, on the inner and outer surfaces of the membrane, and in the periplasmic region of *B. licheniformis* 749/C (6). The presence of penicillinase and alkaline phos-

phatase on both inner and outer surfaces, respectively, of different strains of *B. licheniformis* may suggest a unique situation with respect to asymmetric protein association in this species.

ACKNOWLEDGMENTS

This investigation was supported by grants from Research Corp. and from the Cottrell Research Grants Program, and by Public Health Service grant GM-21909 from the National Institute of General Medical Sciences.

LITERATURE CITED

- Berg, H. C. 1969. Sulfanilic acid diazonium salt: a label for the outside of the human erythrocyte membrane. *Biochim. Biophys. Acta* **183**:65-78.
- Carraway, K. L. 1975. Covalent labeling of membranes. *Biochim. Biophys. Acta* **415**:379-410.
- Cashel, M., and E. Freese. 1964. Excretion of alkaline phosphatase by *Bacillus subtilis*. *Biochem. Biophys. Res. Commun.* **16**:541-544.
- Davis, B. J. 1964. Disc electrophoresis. II. Method of application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404-427.
- Fairbanks, G., T. L. Steck, and D. F. Wallach. 1976. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**:2606-2617.
- Ghosh, A., and B. K. Ghosh. 1979. Immunoelectron microscopic localization of penicillinase in *Bacillus licheniformis*. *J. Bacteriol.* **137**:1374-1385.
- Ghosh, B. K., J. T. M. Wouters, and J. O. Lampen. 1971. Distribution of the sites of alkaline phosphatase(s) activity in vegetative cells of *Bacillus subtilis*. *J. Bacteriol.* **108**:928-937.
- Ghosh, R., A. Ghosh, and B. K. Ghosh. 1977. Properties of the membrane-bound alkaline phosphatase from glucose- and lactose-grown cells of *Bacillus subtilis* SB15. *J. Biol. Chem.* **252**:6813-6822.
- Glew, R. H., and E. C. Heath. 1971. Studies on the extracellular alkaline phosphatase of *Micrococcus sodonensis*: isolation and characterization. *J. Biol. Chem.* **246**:1556-1565.
- Glew, R. H., and E. C. Heath. 1971. Studies on the extracellular alkaline phosphatase of *Micrococcus sodonensis*: factors affecting secretion. *J. Biol. Chem.* **246**:1566-1574.
- Glynn, J. A., S. D. Schaffel, J. M. McNicholas, and F. M. Hulett. 1977. Biochemical localization of alkaline phosphatase of *Bacillus licheniformis* as a function of culture age. *J. Bacteriol.* **129**:1010-1019.
- Grant, W. D. 1979. Cell wall teichoic acid as reserve phosphate source in *Bacillus subtilis*. *J. Bacteriol.* **137**:35-43.
- Hubbard, A. L., and Z. A. Cohn. 1972. The enzymatic iodination of the red cell membrane. *J. Cell Biol.* **55**:390-405.
- Hulett, F. M., and L. L. Campbell. 1971. Purification and properties of an alkaline phosphatase of *Bacillus licheniformis*. *Biochemistry* **10**:1364-1370.
- Hulett, F. M., and L. L. Campbell. 1971. Molecular weight and subunits of the alkaline phosphatase of *Bacillus licheniformis*. *Biochemistry* **10**:1371-1376.
- Hulett, F. M., S. D. Schaffel, and L. L. Campbell. 1976. Subunits of alkaline phosphatase of *Bacillus licheniformis*: chemical, physicochemical, and dissociation studies. *J. Bacteriol.* **128**:651-657.
- Ihlenfeldt, M. J. A., and J. Gibson. 1975. Phosphate utilization and alkaline phosphatase activity in *Anacystis nidulans* (*Synechococcus*). *Arch. Microbiol.* **102**:23-28.
- Kier, L. D., R. Weppelman, and B. N. Ames. 1977. Resolution and purification of three periplasmic phosphatases of *Salmonella typhimurium*. *J. Bacteriol.* **130**:399-410.
- Klotz, F. N., J. E. Rothman, V. R. Lingappa, and G. Blobel. 1977. Membrane assembly *in vitro*: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. *Proc. Natl. Acad. Sci. U.S.A.* **74**:3278-3282.
- Kreibich, G., A. L. Hubbard, and D. D. Sabatini. 1974. On the spatial arrangement of proteins in microsomal membranes from rat liver. *J. Cell Biol.* **60**:616-627.
- Kreuzer, K., C. Pratt, and A. Torriano. 1975. Genetic analysis of regulatory mutants of alkaline phosphatase of *E. coli*. *Genetics* **81**:459-468.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. *Nature (London)* **227**:680-685.
- Lampen, J. O. 1973. Movement of extracellular enzymes across membranes. *Symp. Soc. Exp. Biol.* **28**:351-374.
- Lenard, J., and R. W. Compans. 1974. The membrane structure of lipid containing viruses. *Biochim. Biophys. Acta* **344**:51-94.
- Louvard, D., S. Maroux, C. Vannier, and P. Desnuelle. 1975. Topological studies on the hydrolases bound to the intestinal brush border membrane. *Biochim. Biophys. Acta* **375**:236-243.
- MacAlister, T. J., R. T. Irvin, and J. W. Costerton. 1977. Envelope protection of alkaline phosphatase against acid denaturation in *Escherichia coli*. *J. Bacteriol.* **130**:339-346.
- McNicholas, J. M., and F. M. Hulett. 1977. Electron microscope histochemical localization of alkaline phosphatase in *Bacillus licheniformis*. *J. Bacteriol.* **129**:501-515.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Nachman, R. L., A. Hubbard, and B. Ferris. 1973. Iodination of the human platelet membrane. *J. Biol. Chem.* **248**:2928-2936.
- Phillips, D. R., and M. Morrison. 1971. Exposed protein on the intact human erythrocyte. *Biochemistry* **10**:1766-1771.
- Priest, F. G. 1977. Extra-cellular enzyme synthesis. *Bacteriol. Rev.* **41**:711-753.
- Reichstein, E., and R. Blostein. 1973. Asymmetric iodination of the human erythrocyte membrane. *Biochem. Biophys. Res. Commun.* **54**:494-500.
- Schaffel, S., and F. M. Hulett. 1978. Alkaline phosphatase from *Bacillus licheniformis*. Solubility dependent on magnesium, purification and characterization. *Biochim. Biophys. Acta* **526**:457-467.
- Schlesinger, M. J., and K. Barrett. 1965. The reversible dissociation of the alkaline phosphatase of *Escherichia coli*. *J. Biol. Chem.* **240**:4284-4292.
- Schneider, D. L., Y. Kagawa, and E. Racker. 1972. Chemical modification of the inner mitochondrial membrane. *J. Biol. Chem.* **247**:4074-4079.
- Singer, S. J. 1974. The molecular organization of membranes. *Annu. Rev. Biochem.* **43**:805-833.
- Singer, S. J., and G. L. Nicholson. 1972. Fluid mosaic model. *Science* **175**:720-731.
- Spencer, D. B., C.-P. Chen, and F. M. Hulett. 1981. Effect of cobalt on synthesis and activation of *Bacillus licheniformis* alkaline phosphatase. *J. Bacteriol.* **145**:928-933.
- Staros, J. V., F. M. Richards, and B. E. Haley. 1975. Photochemical labeling of the cytoplasmic surface of the membranes in intact human erythrocytes. *J. Biol. Chem.* **250**:8174-8178.
- Steck, T. L. 1972. The organization of proteins in human erythrocyte membranes, p. 71-93. *In* C. F. Fox (ed.), *Membrane research*. Academic Press, Inc., New York.
- Thorley-Lawson, D. A., and N. M. Green. 1973. Studies

- on the location and orientation of proteins in the sarcoplasmic reticulum. *Eur. J. Biochem.* **40**:403-415.
42. **Traficante, L. J., and J. O. Lampen.** 1977. Vesicle penicillinase of *B. licheniformis*: existence of periplasmic-releasing factor(s). *J. Bacteriol.* **129**:184-190.
43. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406-4412.
44. **Wickner, W.** 1975. Asymmetric orientation of a phage coat protein in cytoplasmic membrane of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **72**:4749-4753.
45. **Wilkins, A. S.** 1972. Physiological factors in the regulation of alkaline phosphatase synthesis in *Escherichia coli*. *J. Bacteriol.* **110**:616-623.
46. **Wood, D., and H. Tristram.** 1970. Localization in the cell and extraction of alkaline phosphatase from *Bacillus subtilis*. *J. Bacteriol.* **104**:1045-1051.