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A Mechanism for Penicillinase Secretion in *Bacillus licheniformis**

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Abstract. Cell-bound isozymes of penicillinase are distinguished from extracellular enzyme by their capacity to bind deoxycholate and to elute with an apparent molecular weight of 45,000 on gel filtration in its presence. By methods that are unlikely to involve changes in primary structure, the cell-bound forms (both from the plasma membrane and from the periplasmic vesicles) can be converted to forms that are very similar if not identical to the exo-form (i.e., eluting with a molecular weight of 24,000 in the presence and absence of deoxycholate). In the case of plasma membrane penicillinase, addition of 25 per cent potassium phosphate at pH 9.0 leads to a 65 per cent conversion in 20 minutes at 30°. Vesicle fraction penicillinase can be converted by pH 9.0 treatment alone. We suggest that the conversion involves a change from a hydrophobic to a hydrophilic conformational type, and that this is the crucial step for enzyme secretion in microorganisms. A model is presented to account for existing data in which we postulate that monomers of the newly synthesized penicillinase in an extended hydrophobic conformation are inserted into the membrane at special growing points where they may change to a hydrophilic exo-form, or polymerize to the major plasma membrane type of penicillinase.

Introduction. For the study of enzyme secretion, a system is desirable in which there is a rate-limiting step between synthesis and release. Pollock was able to demonstrate that a part of the penicillinase synthesized by *Bacillus licheniformis* (strain 749) passed through a cell-bound state before release.[1] Later, we showed that only about 25 per cent of the penicillinase secreted at any one time during active synthesis was derived from a cell-bound intermediate and the remainder was released by a process coupled to synthesis.[2] Two entirely separate and different processes could be involved but it is equally possible to visualize a unified scheme of secretion which is only partly inhibited by chloramphenicol (see below).

The cell-bound penicillinase is present in the plasma membrane and in a periplasmic vesicle fraction released by protoplasting.[3] Chloramphenicol-insensitive penicillinase release occurs at the expense of the vesicle fraction.[4]

Pollock[1, 5] and later Lampen[6] examined the cell-bound penicillinase, and indicated that it may be covalently bound to membrane components; if so, enzyme secretion must involve scission of this bond.

Recently we have shown (using gel filtration) that the molecular weight of

vesicle fraction and of plasma-membrane penicillinase was 45,000 in the presence of deoxycholate, whereas that of exoenzyme was 24,000.[7] When deoxycholate is removed, plasma membrane penicillinase polymerizes to a molecular weight of 600,000; in contrast, vesicle penicillinase decreases to 24,000 but will return to 45,000 in the presence of deoxycholate. We regard this as evidence that about 50 molecules of deoxycholate were bound per molecule of vesicle fraction penicillinase.

We offer evidence that these cell-bound forms of penicillinase are conformational variants of the exo-form and that secretion could involve a change from a hydrophobic form, capable of traversing the cell membrane, to a hydrophilic form, stable in aqueous media. A detailed model of secretion is presented.

Preparation of Membrane and Vesicle Fractions. As described previously.[7]

Analytical Methods. *Penicillinase* was assayed by the method of Sargent[8] where necessary modified to overcome deoxycholate interference.[7] All assays of penicillinase derived from membranes were conducted in the presence of 10 mg per assay of sodium taurocholate.[7] *Deoxycholate* was determined as in reference 9, and *protein* as in reference 10. In experiments involving high salt concentrations (Fig. 1*A*), the amount of protein in the supernatant represents the difference between the sediment (resuspended in distilled water) and the starting material. *Exopenicillinase* was determined quantitatively as described previously.[4]

Gel filtration: Columns (47 × 2 cm) containing Biogel A5M were equilibrated in 0.05 *M* pH 9.0 pyrophosphate containing 0.1% deoxycholate (DOC-PP), or in 0.05 *M* NaCl. Molecular weights were determined as described previously.[4] The apparent molecular weight of 24,000 for exopenicillinase[4] is clearly anomalous as amino acid sequence and ultracentrifugation indicate about 29,000.[11]

Preparation of solubilizates: Plasma membrane (200 μg of protein/ml) and vesicle fraction (50 μg of protein/ml) were solubilized in either DOC-PP or 0.1% sodium taurocholate in pyrophosphate.

Results and Discussion. Conversion of plasma-membrane penicillinase to the exo-form: If high concentrations of salt (ammonium sulfate, pH 8.0 or potassium phosphate, pH 9.0) are added to deoxycholate or taurocholate-solubilizates of plasma membrane, the penicillinase is converted to a form that can penetrate Sephadex G-75 in the absence of deoxycholate, with an elution volume identical to that of exopenicillinase. The effect of phosphate concentration on deoxycholate-solubilizates is illustrated in Figure 1*A*. Deoxycholate precipitates at about 15 per cent (w/v) together with a substantial proportion of protein. Above about 20 per cent, the penicillinase is converted to the exo-form and is precipitated above 35 per cent. In this experiment samples were incubated for five minutes at room temperature (24°) after which precipitates were spun down. The conversion is both temperature and time dependent; the reaction rate being much faster at 30° than at 0° (Fig. 1*B*). After 20 minutes, 65 per cent conversion has occurred. When membranes are treated at pH 9.0 with salt alone there is a moderate conversion to the exo-form. Deoxycholate alone has no effect. The effect of deoxycholate and salt is evidently cooperative.

Previously, we have shown that the penicillinase of plasma membrane solubilized in DOC-PP, elutes with a molecular weight of 45,000 on both Sephadex G-100 and Biogel A5M equilibrated in DOC-PP.[7] After high salt and deoxycholate treatment, the molecular weight decreases to that of exopenicillinase (24,000) both in DOC-PP and in NaCl (Fig. 2*A* and *C*).

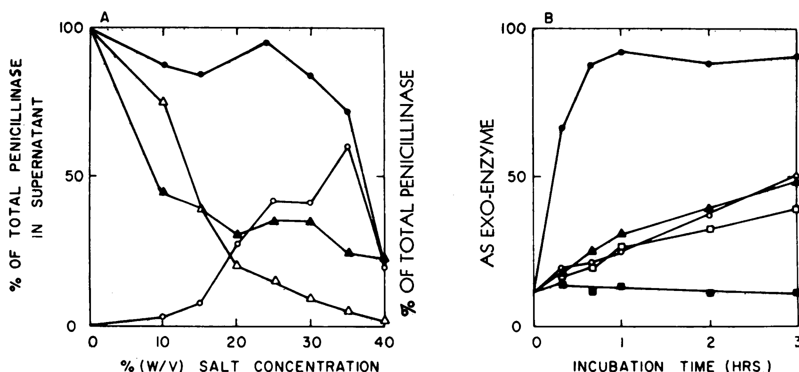


FIG. 1.—Conversion of plasma membrane penicillinase to exopenicillinase.

(*A*) Effect of salt concentration on conversion to exoenzyme of plasma membrane penicillinase. Reaction mixtures (12.5 ml final volume) contain plasma membrane (0.3 mg/ml) in DOC-PP. 50% (w/v) potassium dihydrogen phosphate adjusted to pH 9.0 with KOH was added to give the concentration of salt shown. After 5 min at 24°, precipitates were sedimented at 12,000 × *g* for 30 min. The percentage of the total present in the supernatant was determined: (●—●) penicillinase; (○—○) exopenicillinase; (▲—▲) protein; (△—△) deoxycholate.

(*B*) Factors affecting rate of conversion to exoenzyme. Reaction mixtures (2 ml) contain plasma membrane (20 mg) in pyrophosphate. Samples were incubated with the additions stated, under the following final conditions. At the times shown 100 μl samples were removed, diluted in 400 μl of distilled water and then chromatographed on Sephadex G-75 for exopenicillinase determination as described above. (●—●), 30°, 0.1% deoxycholate, and 25% w/v phosphate (see text); (○—○), 0°, 0.1% deoxycholate, 25% phosphate; (▲—▲) 30° C, 0.1% deoxycholate, 37.5% phosphate; (□—□) 30°, no deoxycholate, 25% phosphate; (■—■) 30°, 0.1% deoxycholate, no phosphate.

Conversion of vesicle-fraction penicillinase to the exo-form: The principal form of penicillinase in the vesicle fraction has a molecular weight of 24,000 and can bind deoxycholate to become 45,000 in DOC-PP[7] (Fig. 2*B*). Previously we noted that at pH 9.0 the vesicle penicillinase in intact washed cells is released as the exo-form.[4]

In order to relate the organization of the vesicle fraction with the observed *in vivo* alkaline lability of vesicle penicillinase, we have compared the isolated penicillinase molecules from the vesicle fraction before and after pH 9.0 treatment. Vesicle fraction dissolved in 0.1 per cent sodium taurocholate in pyrophosphate and incubated at 30° for 30 and 60 minutes was chromatographed on Biogel A5M in DOC-PP at 2° (Fig. 2*B*). After 30 minutes a shoulder on the leading edge is still evident but at 60 minutes this completely disappears. In sharp contrast, partially purified plasma membrane penicillinase is unaffected by pH 9.0 conditions.

Nature of the cell-bound penicillinase: The most economical interpretation of our data is that cell-bound penicillinase is composed of a monomer of molecular weight 24,000 that can exist in two different conformations which are similar in that they can bind deoxycholate and elute with a molecular weight of 45,000 in DOC-PP but which differ in size after removal of deoxycholate. Thus

plasma membrane penicillinase polymerizes to 600,000, whereas vesicle fraction penicillinase remains as the monomer.[7]

The evidence for deoxycholate binding is that the molecular weight of the monomer increases from 24,000 in the absence of deoxycholate, to 45,000 in its presence (Fig. 2*B*). The only feasible alternative is that deoxycholate in some way affects the volume of Biogel available to the penicillinase molecule. Against this point of view is the observation that five other proteins show no change in elution volume in the presence or absence of deoxycholate.[7, 12]

There is no direct evidence that plasma membrane penicillinase contains a monomer with the same amino acid sequence as the vesicle penicillinase; however, this is the simplest explanation of the observations (*a*) that both forms assume a molecular weight of 45,000 in the presence of deoxycholate and (*b*) that both can be converted by high salt or pH 9.0 treatment to a 24,000 form.

The mechanism of conversion to exopenicillinase: The conversion to exopenicillinase can involve a change either in primary structure or in conformation. Definitive evidence that exopenicillinase and the monomeric cell-bound form are identical in primary structure cannot be obtained easily, although recently the amino acid sequence of exopenicillinase has been determined.[11] Cell-bound penicillinase prepared by trypsin treatment of whole cells differs from the exo-form only by the absence of the terminal lysine residue which presumably is removed by trypsin, but a larger peptide distinguishing cell-bound penicillinase from exopenicillinase may be removed with it. Using gel filtration on Biogel A5M or Sephadex G-100, we have found no difference in molecular

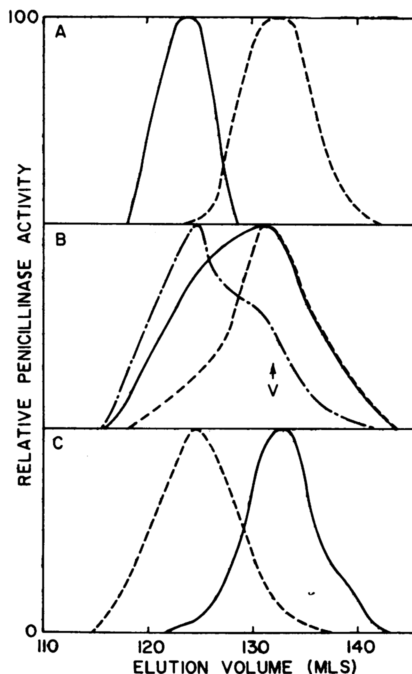


FIG. 2.—Demonstration of conversion to exo-form of cell-bound penicillinases. Columns (47 x 2 cm) of Biogel A5M equilibrated in DOC-PP were employed. Void volume = 56 ml.

(*A*) Conversion of plasma-membrane penicillinase to the exo-form: (—) plasma membrane dissolved in DOC-PP; (—) plasma membrane converted to exo-form (see text).

(*B*) Conversion of vesicle fraction penicillinase to the exo-form; (—·—) vesicle fraction dissolved in DOC-PP; (—) vesicle fraction after 30 min in 0.1% sodium taurocholate in pyrophosphate at 30°; (—) vesicle fraction after 60 min in STC-PP at 30°. Exopenicillinase elution volume shown by (V).

(*C*) Elution diagram of standards. (—) Exopenicillinase; (—) ovalbumin.

weight between the monomeric form from the vesicle fraction and exopenicillinase. A difference of 3000 would certainly have been detected.

A less direct argument suggests that any differences in primary structure between conversion products and exoenzyme are unimportant. Any change in primary structure would probably be mediated by an enzyme, so the conversion reactions may involve either enzymatic cleavage of a covalent bond or dissociation of noncovalent bonding. Evidence for disruption of a noncovalent bond is provided if conversion to exoenzyme occurs in very high yield and at a rate many times the endogenous release rate, under conditions that would not break covalent bonds or stimulate normal enzyme reactions.

In the case of plasma membrane penicillinase these requirements are clearly met (Fig. 1). Thus 65 per cent conversion occurred in 20 minutes at 30°, whereas endogenous release of exopenicillinase was negligible. The rate and extent of the reaction under these conditions is sufficient to suggest strongly that conversion is the result of a change in noncovalent bonding and not due to an endogenous hydrolytic release factor. Activation of a latent release factor masked by a permeability barrier is also unlikely as the addition of deoxycholate alone should achieve this.

Conversion of vesicle fraction penicillinase to the exo-form at pH 9.0 does not provide critical evidence in favor of either argument, as a pH dependent conversion can be either enzymatic or nonenzymatic, although there is no significant change in molecular weight.

Enzyme secretion as a conformation change: Although it has not been shown that conversion to exo-form involves no change in primary structure, there is certainly evidence that the monomeric cell-bound form differs from the exo-form in conformation. This is expressed in terms of the deoxycholate binding that characterizes all species of cell-bound penicillinase (Fig. 2). Previously, we suggested that the binding is essentially hydrophobic because taurocholate, which has a hydroxyl group in the 7 position (on the hydrophobic face), is not capable of depolymerizing the membrane bound penicillinase.[7] Furthermore, in view of the well-known ability of bile salts to form micelles, it would not be surprising if their interactions with proteins were hydrophobic.[13] If so, then the surface of the monomeric cell-bound form is relatively hydrophobic compared with that of the exoenzyme. We suggest, therefore, that the essential step in enzyme secretion involves a conformation change from a hydrophobic form, adapted to interacting with and traversing the cell membrane, to a hydrophilic one stable in aqueous solution.

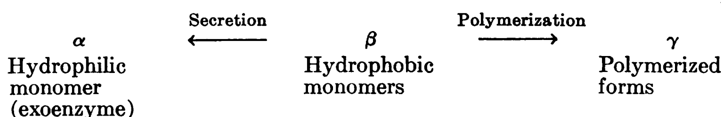
The monomers of the cell-bound forms can be viewed as minor conformational variants of a basic hydrophobic cell-bound type. Plasma membrane penicillinase monomers are apparently most hydrophobic as they polymerize readily. Presumably when salt is added to deoxycholate-solubilizates of this form and deoxycholate is precipitated, conversion to the hydrophilic form occurs rather than repolymerization because in the high polarity medium a hydrophilic form represents the lowest free energy content. Significantly, high salt alone causes some conversion to the exo-form (Fig. 1*B*).

The vesicle fraction monomer, while sufficiently hydrophobic to bind deoxy-

cholate, is less so than plasma membrane penicillinase as it does not polymerize after removal of deoxycholate, but it is sufficiently sensitive to the polarity of the medium to aggregate in the presence of highly cross-linked gel filtration media.[7]

A model for penicillinase secretion: We have incorporated these findings and previously published data into the following model of penicillinase secretion to give a coherent explanation of the secretion phenomenon. Thus, as in Lampen's earlier model,[14] we visualize a membrane growing point at which penicillinase and certain other membrane components are inserted into the membrane. In view of the absence of soluble internal penicillinase, and the sensitivity to chloramphenicol of penicillinase secretion, it appears that the growing point and the polysomes synthesizing penicillinase are unlikely to be widely separated, and the process of synthesis may extrude the polypeptide into and through the membrane in the manner suggested by Redman.[15]

We envisage the newly inserted form of penicillinase as an extended configuration with surface hydrophobic sites (at which deoxycholate binding can occur) that render the molecule capable of entering the membrane. Once in the membrane, the essential secretion step involves a conformation change that internally masks hydrophobic groups. Alternatively, if not secreted, penicillinase must aggregate to the polymerized forms discussed above. For convenience this can be expressed in terms of the following reactions and nomenclature.



We define the β forms as any penicillinase molecule of apparent molecular weight 24,000 which is capable of binding deoxycholate. In addition to the small vesicle fraction penicillinase this may include nascent polypeptide chains and any unaggregated monomers of the γ forms, yielding a small range of conformational types.

Under the growth conditions used, about 75 per cent of the penicillinase secreted at any one time is derived from a process that is tightly coupled to enzyme synthesis (chloramphenicol sensitive). The remaining 25 per cent is derived from the vesicle fraction. Tightly coupled release can be visualized in the context of this model, if the growing point is regarded as a delicate and unstructured area in the vicinity of the ribosome, at which newly synthesized penicillinase has its highest chance of undergoing the $\beta \rightarrow \alpha$ transition that is required for secretion. If one assumes that protein synthesis is the only cellular process inhibited by chloramphenicol, then the chloramphenicol susceptibility of the tightly coupled fraction indicates that release from the ribosome is the rate-limiting reaction for the release of these molecules. Penicillinase not secreted immediately after synthesis is incorporated into the membrane at the growing point. We suggest that it retains some capacity to undergo the necessary conformation change for secretion but that this step is now rate limiting so that its release is chloramphenicol insensitive. Penicillinase not secreted by either process must develop

into the polymerized penicillinase of the plasma membrane. The relationships between the reactions are illustrated in Figure 3. Because the $\beta \rightarrow \alpha$ conversion is favored by high pH, exoenzyme secretion is strongly pH dependent.[4]

We have no data that indicate the origin of the plasma-membrane penicillinase. It may be derived from vesicle fraction penicillinase, perhaps during displacement from the growing point, or the morphological features[16] may be such that enzyme formed on one side of the growing point structure polymerizes directly, whereas that on the other side becomes vesicle fraction penicillinase and is eventually released.

Inhibition of chloramphenicol-insensitive release by deoxycholate, Triton X 100, and the aromatic tertiary amines[4] (all amphipathic molecules) might be explained as follows: If deoxycholate, for example, is bound to the β-form hydrophobically, its carboxyl groups will render the molecule hydrophilic and thus stabilize the β-form. Taurocholate is a much less effective inhibitor of pH-dependent release. This probably reflects its lower hydrophobicity, its interactions with penicillinase not being sufficiently strong to "freeze" the β-configuration.

The inhibition of pH-dependent release noted after protoplasting[4] is probably caused by sucrose, as 50 per cent inhibition of release by washed cells is found in 0.5 *M* sucrose (unpublished data). Lampen[17] suggested that either a soluble release factor was lost by dilution after protoplasting, or else, protoplasting *per se* disorganized the release mechanism. However, if as the evidence indicates, penicillinase is held by noncovalent bonds, the effect of sucrose must be to strengthen the bonds between penicillinase and the particle fraction. We have noted previously that in the presence of sucrose, taurocholate cannot solubilize vesicle and membrane penicillinase;[7] also passage through highly cross-linked

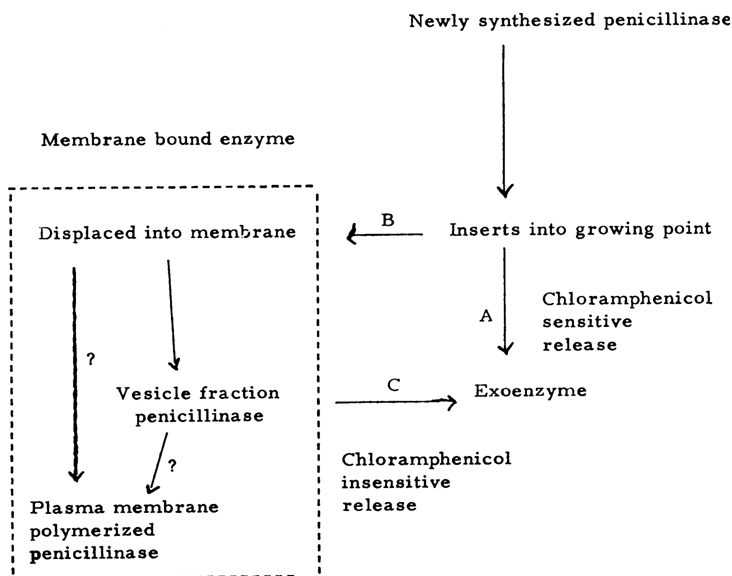


FIG. 3. Postulated flow diagram of penicillinase secretion.

dextran and agarose columns causes polymerization of the monomeric vesicle fraction penicillinase,[7] an effect that appears analogous to that of sucrose.

Relationship to other secretion systems: The flow diagram illustrating the secretion of penicillinase (Fig. 3) may serve as a general model. The relative rates of A, B, and C may differ, responding independently or in unison to environmental conditions. Secretion appears tightly coupled to enzyme synthesis in many microbial systems[18] (reaction C is absent); cell-bound enzyme is not found in some of these but others contain substantial amounts indicating that B may vary relative to A. Certain β-configurations could be inactive in order to secure a hydrophobic molecule and mask autolytic enzymes while in transit.

We propose that a conformation change from a hydrophobic molecule adapted to traverse the plasma membrane at special sites, to a hydrophilic one stable in an aqueous environment may be the basic mechanism of secretion. Support of this scheme can only be obtained by identifying a cell-bound intermediate with a conformation demonstrably different from the exo-form. If, as in *B. licheniformis,* some release continues in the absence of enzyme synthesis, the theory can be tested; but with systems showing no uncoupled release, it cannot. Uncoupled secretion might be obtained in these systems by manipulating the growth conditions. Our model suggests that there will be conformational differences between cell-bound and extracellular forms of the same enzyme even if the former is not the precursor of the latter. There is strong evidence for this in the case of *Bacillus cereus* penicillinase.[19, 20]

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[1] Pollock, M. R., *J. Gen. Microbiol.*, **26**, 267 (1961).
[2] Sargent, M. G., B. K. Ghosh, and J. O. Lampen, *J. Bacteriol.*, **97**, 820 (1969).
[3] *Ibid.*, 1329 (1968).
[4] *Ibid.*, 1231 (1968).
[5] Kushner, D. J., and M. R. Pollock, *J. Gen. Microbiol.*, **26**, 255 (1961).
[6] Lampen, J. O., *J. Gen. Microbiol.*, **48**, 249 (1967).
[7] Sargent, M. G., and J. O. Lampen, *Arch. Biochem. Biophys.*, **136**, 167 (1970).
[8] Sargent, M. G., *J. Bacteriol.*, **95**, 1493 (1968).
[9] Eriksson, S., and J. Sjorvall, *Arkiv. Kemi*, **8**, 299 (1955).
[10] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
[11] Ambler, R. P., and R. J. Meadway, *Nature*, **222**, 24 (1969).
[12] Morgan, R. G. H., J. Barrowman, and B. Borgström, *Biochim. Biophys. Acta*, **175**, 65 (1969).
[13] Small, D. M., S. A. Renkett, and D. Chapman, *Biochim. Biophys. Acta*, **176**, 178 (1969); and Kratohvil, J. P., and H. T. Dellicolli, *Can. J. Biochem.*, **46**, 945 (1968).
[14] Lampen, J. O., in *Society of General Microbiology Symposium*, ed. M. R. Pollock and M. Richmond (London: Oxford University Press, 1965), vol. 15, p. 115.
[15] Redman, C. M., *J. Biol. Chem.*, **242**, 761 (1967).
[16] Ghosh, B. K., M. G. Sargent, and J. O. Lampen, *J. Bacteriol.*, **96**, 1314 (1968).
[17] Lampen, J. O., *J. Gen. Microbiol.*, **48**, 261 (1967).
[18] Coles, N. W., and R. Gross, *J. Bacteriol.*, **98**, 659 (1969); Coleman, G., and W. H. Elliott, *Biochem. J.*, **83**, 256 (1962); *Ibid.*, **95**, 699 (1965); May, B. K., and W. H. Elliott, *Biochim. Biophys. Acta*, **157**, 607 (1968). Casas, I. A., and L. N. Zimmerman, *J. Bacteriol.*, **97**, 307 (1969).
[19] Citri, N., N. Garber, and M. Sela, *J. Biol. Chem.*, **235**, 3454 (1960).
[20] Citri, N., *Biochim. Biophys. Acta*, **27**, 277 (1958).