The Initial Stage in Peptidoglycan Synthesis. IV. Solubilization of Phospho-*N*-acetylmuramyl-pentapeptide Translocase*

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ABSTRACT: The initial reaction in the biosynthesis of peptidoglycan is catalyzed by phospho-*N*-acetylmuramyl-pentapeptide translocase (UDP-MurNAc-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala:C_{55}-isoprenoid alcohol phosphate phospho-MurNAc-pentapeptide transferase). In addition to the transfer reaction, the enzyme catalyzes the exchange of [^{3}H]uridine monophosphate with the uridine monophosphate moiety of uridine diphosphate-*N*-acetylmuramyl-pentapeptide. The solubilization of the enzyme from membrane fragments of *Staphylococcus aureus* Copenhagen has been achieved with the following diverse reagents: (1) sodium lauroyl sarcosinate (3 μmoles/mg of protein) at 4°; (2) 10 M urea (pH 7.8) at 20°; (3) 0.08 M KOH at 4°. In the case of sodium lauroyl sarcosinate, two distinct activity peaks for the exchange reaction were detected by sucrose density gradient centrifugation and gel filtration on Bio-Gel A-50m. The major peak of activity has an exclusion volume corresponding to a weight-average molecular weight of 2×10^6, whereas the second peak of exchange activity corresponds to a molecular weight in the range of 100,000–200,000. The activity of the translocase from urea-solubilized membrane fragments was recovered after dialysis against buffers containing KCl. The major peak of activity corresponds to a molecular weight of 2×10^6. Additional peaks of transfer and exchange activity were observed. In the case of the KOH-solubilized preparations, two major peaks of exchange activity were detected. The exclusion volumes of these peaks on Bio-Gel A-50m were identical with those observed with membrane fragments solubilized with sodium lauroyl sarcosinate. From these results, we suggest that the translocase exists in more than one form. The low molecular weight form provides a source for the purification and study of the enzyme.

The biosynthesis of peptidoglycan, the major structural polymer of bacterial cell walls, is catalyzed by a series of enzymes associated with the bacterial membrane (Anderson *et al.*, 1966, 1967; Araki *et al.*, 1966; Chatterjee and Park, 1964; Meadow *et al.*, 1964; Matz and Strominger, 1968). The initial reaction in this series is catalyzed by phospho-MurNAc[1]-pentapeptide translocase (UMP) (Anderson *et al.*, 1965; Struve and Neuhaus, 1965; Struve *et al.*, 1966) according to reaction 1, where the lipid acceptor is C_{55}-isoprenoid alcohol (Higashi *et al.*, 1967). On the basis of kinetic studies, effects of surfactants, and uncoupling by dodecylamine, we proposed that an enzymephospho-MurNAc-pentapeptide intermediate participates in the reaction catalyzed by the translocase (Heydanek *et al.*, 1969). In order to identify and study the postulated intermediate, solubilization of the translocase was attempted with three different reagents. These are (1) sodium lauroyl sarcosinate, (2) urea, and (3) KOH. It is the purpose of this paper to compare the solubilized preparations by sucrose density gradient centrifugation, filtration on Bio-Gel A-50m, and disc gel electrophoresis. These comparisons indicate that phospho-MurNAc-pentapeptide translocase exists in at least two forms. The preparations described in this paper provide a basis for the further purification of these forms.

UDP-MurNAc-pentapeptide + lipid acceptor-phosphate $\xrightleftharpoons{Mg^{2+}}$ UMP + lipid acceptor-diphosphate-MurNAc-pentapeptide (1)

Experimental Section

Materials. The preparation of membrane fragments from *Staphylococcus aureus* Copenhagen, nucleotides, surfactants, bacteria, and other chemicals have been previously described (Struve *et al.*, 1966; Stickgold and Neuhaus, 1967; Heydanek *et al.*, 1969). Bio-Gel A-50m was purchased from Bio-Rad Laboratories. Sodium lauroyl sarcosinate and sodium oleoyl sarcosinate were kindly given by Geigy Chemical Corp. Cutscum was purchased from Fisher Scientific Co.

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[1] Abbreviation used: MurNAc, *N*-acetylmuramyl.

Assays. Transfer assay A, B, and the exchange assay have been previously described in detail (Heydanek *et al.*, 1969). In summary, transfer assay A measures the synthesis of lipid diphosphate-MurNAc-[^{14}C]-pentapeptide from UDP-MurNAc-[^{14}C]pentapeptide according to reaction 1. Since alkaline phosphatase is not incorporated into the reaction mixture, this assay is a measure of the equilibrium position of reaction 1 and, thus, depends primarily upon the lipid acceptor concentration. In contrast, transfer assay B contains bacterial alkaline phosphatase so that only the forward direction of reaction 1 is observed. This assay measures the release of [^{3}H]UMP from [^{3}H]UDP-MurNAc-pentapeptide followed by the hydrolysis of [^{3}H]UMP to [^{3}H]uridine and is a function of the enzyme and lipid acceptor concentrations. The exchange assay involves the exchange of UMP with the [^{3}H]UMP moiety of [^{3}H]UDP-MurNAc-pentapeptide according to reaction 2. Heydanek *et al.* (1969) proposed reactions

$$\text{UMP} + [^{3}\text{H}]\text{UDP-MurNAc-pentapeptide} \overset{Mg^{2+}}{\rightleftharpoons} \text{UDP-NAc-muramyl-pentapeptide} + [^{3}\text{H}]\text{UMP} \quad (2)$$

3–8, where EUMPPMp is the Michaelis complex of

$$\text{E} + \text{UMPPMp} \rightleftharpoons \text{E} \cdots \text{UMPPMp} \quad (3)$$
$$\text{E} \cdots \text{UMPPMp} \rightleftharpoons \text{UMP} \cdots \text{E-PMp} \quad (4)$$
$$\text{UMP} \cdots \text{E-PMp} \rightleftharpoons \text{E-PMp} + \text{UMP} \quad (5)$$
$$\text{E-PMp} + \text{acceptor-P} \rightleftharpoons \text{acceptor-P} \cdots \text{E-PMp} \quad (6)$$
$$\text{acceptor-P} \cdots \text{E-PMp} \rightleftharpoons \text{E} \cdots \text{acceptor-PPMp} \quad (7)$$
$$\text{E} \cdots \text{acceptor-PPMp} \rightleftharpoons \text{E} + \text{acceptor-PPMp} \quad (8)$$

enzyme (E) and UDP-MurNAc-pentapeptide, E-PMp is the covalent intermediate between phospho-MurNAc-pentapeptide and enzyme, and UMP $\cdots$ E-PMp is the Michaelis complex of the covalent intermediate and UMP. The exchange assay involves reactions 3–5, whereas the transfer assay involves reactions 3–8. The addition of K^+ stimulates the transfer and exchange assays (Heydanek *et al.*, 1969). Thus, the assays used for the work described in this paper contain 0.2 M KCl. The rate of exchange, R, is presented in moles per liter per minute.

The amount of available lipid acceptor was measured in a modification of transfer assay A. Bacterial alkaline phosphatase (0.5 unit) was included in the reaction mixture. The mixture was incubated at 25° for 4 hr and the reaction was terminated by the addition of 0.5 ml of cold 0.3 M $HClO_4$. The amount of lipid product was determined by the Millipore procedure described for transfer assay A (Heydanek *et al.*, 1969).

Acid phosphatase was measured by the release of [^{3}H]uridine from [^{3}H]UMP. The reaction mixture contained 0.17 M magnesium acetate (pH 5.5), 3.2×10^{-5} M [^{3}H]UMP (14.3 cpm/pmole), and membrane fragments in a total volume of 0.06 ml. The mixture was incubated for 30 min at 25° and the reaction was terminated by the addition of 0.2 ml of cold 0.2 M acetic acid. The mixture was quantitatively applied to a Dowex 1 (acetate) X-8 (200–400 mesh) column (5×20 mm). Two portions (0.2 ml) of 0.2 M acetic acid which were used to wash the reaction tube were applied to the column. The effluents (0.66 ml) were collected in a polyethylene vial and assayed for radioactivity. A unit of acid phosphatase is the amount of enzyme required to catalyze the formation of 1 μmole of [^{3}H]uridine/hr at 25°.

Analytical Methods. Measurements of radioactivity were made as previously described (Heydanek *et al.*, 1969). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. For protein analyses in the fractions from the sucrose density gradients, the modification of Schuell and Schuell (1967) was used. Disc electrophoresis was performed with the acid–urea gel system described by Neville (1967).

Sucrose Gradient Separation. Samples were layered onto a linear sucrose density gradient (4.5 ml, 5–40%) containing 0.02 M Tris-HCl (pH 7.8) and centrifuged in a type 65 SW rotor for 8 hr at 65,000 rpm at 4° in a Beckman L2-65 ultracentrifuge. The contents of the tubes were removed from the bottom of the tube with an autogradient elution device (Chromatography Corp. of America).

Gel Filtration. Columns of Bio-Gel A-50m (50×1.6 cm) were equilibrated with 0.02 M Tris-HCl (pH 7.8). The columns were developed at 4° with the above buffer at a flow rate of 0.6 ml/min. Calibration of the columns was performed with blue dextran (weight-average molecular weight 2×10^{6}) and bovine serum albumin.

Extraction Procedures. All preparations were stored at −196° until used for assay.

Sodium Lauroyl Sarcosinate (*NL*-97). Membrane fragments (67 mg of protein) were centrifuged at 100,000*g* for 30 min at 4°. The pellet was suspended with a glass-Teflon homogenizer in 6.8 ml of 0.7% (w/v) sodium lauroyl sarcosinate (3.0 μmoles/mg of protein) in 0.02 M Tris-HCl (pH 7.8) at 4°. The suspension was maintained at this temperature for 10 min and then centrifuged at 100,000*g* for 30 min at 4°. The supernatant solution was dialyzed for 2 hr against 1 l. of 0.02 M Tris-HCl (pH 7.8). The resulting solution (8.2 ml, 4.6 mg/ml of protein) was used as the "NL-97 soluble" enzyme.

Urea. Membrane fragments (15 mg of protein) were centrifuged at 100,000*g* for 30 min at 4°. The pellet was homogenized at 20° in a glass-Teflon homogenizer in 2.0 ml of 10 M urea in 0.02 M Tris-HCl (pH 7.8). The suspension was centrifuged at 100,000*g* for 30 min at 15°. The supernatant fraction was removed and used as a source of the "urea-soluble" enzyme. Since no activity could be detected in the presence of urea, dialysis of the solution against 0.005 M Tris-HCl (pH 7.8) containing 1.0 M KCl was routinely used to recover the activity.

Potassium Hydroxide. Membrane fragments (10 mg of protein) were centrifuged at 100,000*g* for 30 min at 4°. The pellet was suspended with a glass-Teflon homogenizer in 0.5 ml of 0.08 M KOH at 4° and allowed to stand for 1.0 hr at 4°. The solution was centrifuged at 100,000*g* for 30 min at 4°, and the supernatant fraction was removed. The pellet was resuspended in 0.5 ml of 0.02 M Tris-HCl (pH 7.8) and the pH was adjusted to 7.8 if necessary. The supernatant fraction was adjusted to pH 8.0 and used as a source of the KOH-soluble enzyme.

TABLE I: Detergent Extraction of Membrane Fragments.[a]

Detergent	μmoles/mg	Protein Extracted (%)	Exchange (pmoles/min)	Transfer (pmoles/min)	E/T^c
None			2780[b]	118	24
Buffer extraction		11	75	3.6	21
Sodium lauroyl sarcosinate	0.5	25	162	9.5	17
	3.0	73	1200	16.3	74
Sodium oleoyl sarcosinate	0.5	51	616	16.7	37
	3.0	90	69	35.5	2
Cutscum	0.5	36	364	8.0	45
	3.0	46	241	13.0	18
Triton X-100	0.5	33	210	8.7	24
	3.0	33	401	7.1	56
Deoxycholate	0.5	19	170	7.3	23
	3.0	41	24	5.8	4
Sodium dodecyl sulfate	0.5	40	333	6.53	51
	3.0	55	245	12.1	20

[a] The membrane fragments (6.7 mg) were extracted with 2 ml of detergent containing 0.02 M Tris-HCl (pH 7.8) according to the procedure described in the Experimental Section. The supernatant fractions were dialyzed for 12 hr against 0.02 M Tris-HCl (pH 7.8). The exchange assay and transfer assay B were used with samples of the dialyzed supernatant fractions. [b] Specific exchange activity (R/mg) = 0.69×10^{-5} mole/l. per min per mg of protein. [c] E/T is the ratio of exchange activity to transfer activity.

Results

The results of kinetic studies, differential effects of surfactants, and uncoupling by dodecylamine provided evidence for an enzyme-phospho-MurNAc-pentapeptide intermediate in the reaction catalyzed by the translocase (Heydanek *et al.*, 1969). Owing to the heterogeneous nature of the membrane fragments (Figure 1), classical protein methods could not be readily used for the purification and detection of this intermediate. Thus, solubilization of the translocase from the membranes of *S. aureus* Copenhagen was undertaken. A component was considered soluble if it did not sediment at 100,000*g* for 30 min. We chose three different reagents on the basis of their diverse properties. Disaggregation of the membrane fragments by different reagents provides comparisons that enable one to assess the presence of reagent-induced artifacts, *e.g.*, protein–detergent or protein–lipid–detergent complexes.

Phospho-MurNAc-pentapeptide translocase and available lipid acceptor were measured during aerobic growth of *S. aureus* Copenhagen. As illustrated in Figure 2, the specific activity of the enzyme increases fourfold by late-logarithmic phase. As the culture enters stationary phase, the specific activity decreases to 25% of that observed in the late-logarithmic phase. In addition, the amount of available lipid acceptor decreases and parallels the amount of translocase measured by the exchange reaction. In addition to the translocase, a second enzyme associated with the membrane (Mitchell, 1963), acid phosphatase, was chosen as a marker to evaluate the solubilized enzyme preparations (Figure 2).

Detergent Extraction of Membrane Fragments from S. aureus Copenhagen. Many standard surfactants, *e.g.*, sodium dodecyl sulfate and Triton X-100, were not satisfactory because high concentrations irreversibly inhibited the translocase. Another major problem with detergent extraction concerned the different effects on the exchange and transfer assays (Heydanek *et al.*, 1969). Table I shows the results of extracting membranes from *S. aureus* Copenhagen with six different

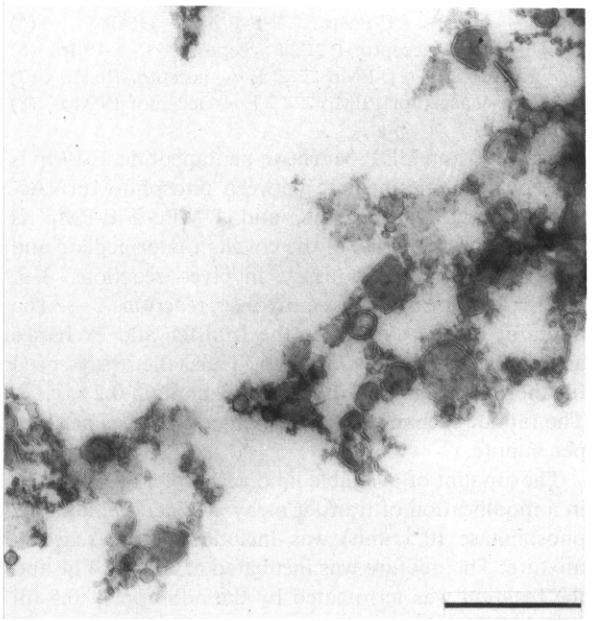

FIGURE 1: Electron photomicrograph of membrane fragments from *S. aureus* Copenhagen. The membrane preparation was dialyzed 8 hr against 0.005 M Tris-HCl (pH 7.8) containing 0.01 M $MgCl_2$ and was stained with 0.05% uranyl acetate (pH 4.2). Scale marker represents 0.5 μ, × 52,000.

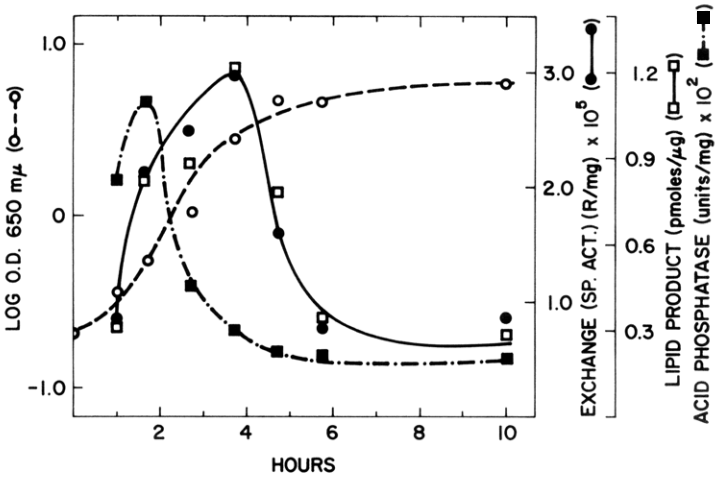

FIGURE 2: Translocase, available acceptor, and acid phosphatase as a function of growth. *S. aureus* Copenhagen was grown with aeration and stirring in a New Brunswick 14-l. fermentor that was maintained at 37°. The growth medium (12 l.) contained: 1% glucose, 0.5% K_2HPO_4, 1% yeast extract, and 1% peptone T. The medium was inoculated with 500 ml of an overnight culture and samples were withdrawn at the indicated time. Membrane fragments were prepared from the bacteria as previously described (Struve *et al.*, 1966). The exchange and acid phosphatase assays were used. The amount of available lipid acceptor was measured as described in the Experimental Section.

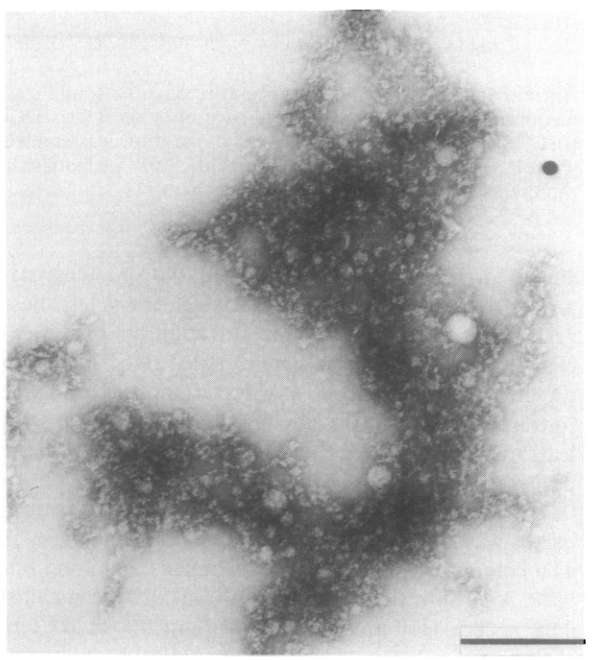

FIGURE 3: Electron photomicrograph of membrane fragments solubilized with sodium lauroyl sarcosinate. The membrane preparation was dialyzed 8 hr against 0.02 M Tris-HCl (pH 7.8) and stained with 0.05% uranyl acetate (pH 4.2). Scale marker represents 0.5 μ, × 45,000.

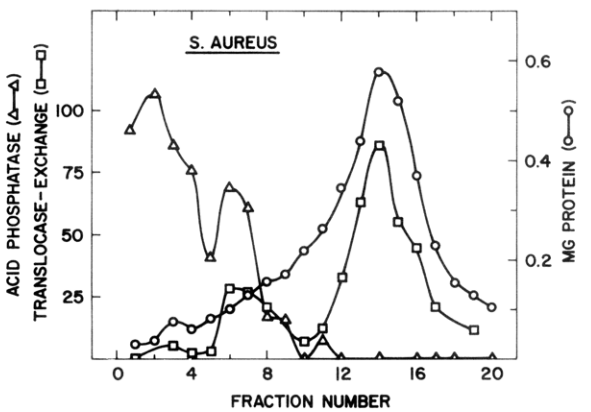

FIGURE 4: Sucrose gradient profile of membranes from *S. aureus* Copenhagen solubilized with sodium lauroyl sarcosinate. The samples were layered on a 5–40% sucrose gradient and centrifuged as described in the Experimental Section. Samples of the fractions (0.24 ml) were assayed for acid phosphatase (μmoles/min per fraction × 10^6) (△—△), translocase exchange (pmoles/min per fraction) (□—□), and protein (mg/fraction) (○—○).

surfactants. Of the detergents tested, sodium lauroyl sarcosinate was the most effective agent for extracting the enzyme from membrane fragments isolated from *S. aureus* Copenhagen. This conclusion is based on the total number of exchange units. In contrast to the other preparations, membrane fragments solubilized with sodium lauroyl sarcosinate have a lower level of transfer activity (E/T = 74). Although sodium oleoyl sarcosinate (3 μmoles/mg) solubilized 90% of the protein, only 2.5% of the activity could be recovered after dialysis. Other surfactants such as cutscum, Triton X-100, deoxycholate, and sodium dodecyl sulfate were not as effective as sodium lauroyl sarcosinate in solubilizing the translocase. It is difficult to establish whether all of the detergent is removed by dialysis. Terry *et al.* (1967) observed that sodium dodecyl sulfate is easily removed by dialysis from solubilized membranes prepared from *Mycoplasma laidlawii*.

In Figure 3 an electron photomicrograph of membrane fragments solubilized with sodium lauroyl sarcosinate and stained with uranyl acetate is shown. The size distribution of the fragments has been greatly reduced by treatment with detergent. A sucrose gradient profile of this preparation is shown in Figure 4. The membrane-associated acid phosphatase described by Mitchell (1963) was clearly resolved from the translocase. Two distinct activity peaks were observed for the translocase. There was, however, very little transfer activity associated with the fractions from the sucrose gradient.

The detergent-solubilized enzyme preparation from *S. aureus* was filtered on Bio-Gel A-50m (Figure 5). The main peak of activity has an exclusion volume

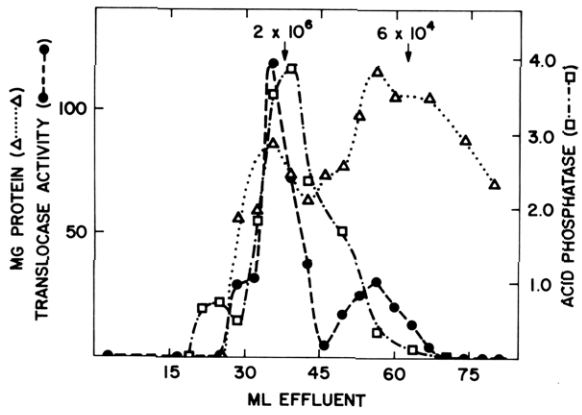

FIGURE 5: Gel filtration on Bio-Gel A-50m of membrane fragments solubilized with sodium lauroyl sarcosinate. The solubilized membrane preparation (14.7 mg) was filtered at 4° on a column (50 × 1.6 cm) with a flow rate of 0.7 ml/min. Samples of the fractions (3.5 ml) were assayed for acid phosphatase (pmoles/hr per fraction × 10^4) (□---□), translocase exchange (pmoles/min per fraction) (○--○), and protein (mg × 10^2) (△···△). The elution volumes of blue dextran (2 × 10^6) and bovine serum albumin (66,500) are shown at the top of the profile. No significant transfer activity was observed.

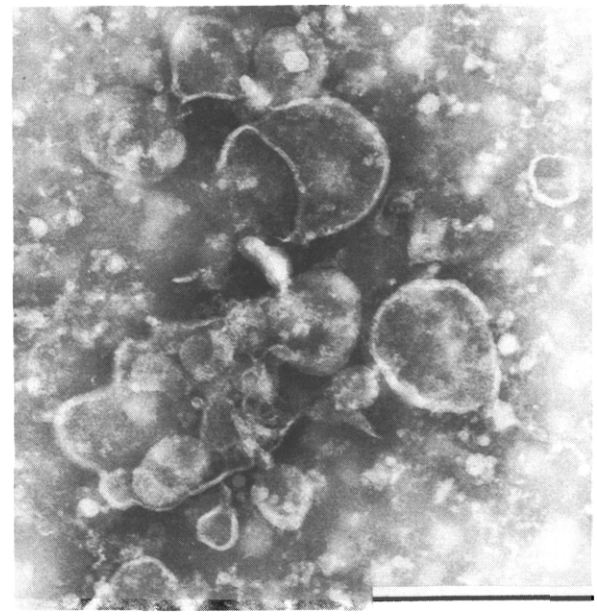

FIGURE 6: Electron photomicrograph of urea-solubilized membranes aggregated in the presence of 0.005 M Tris-HCl (pH 7.8) containing 1 M KCl. The preparation was stained with 1% sodium phosphotungstate (pH 7.0). Scale marker represents 0.5 μ, × 45,000.

corresponding to blue dextran that has a weight-average molecular weight of 2 × 10^6. As in the case of the sucrose density gradient profile, two peaks of activity are observed. In this gel filtration, however, the acid phosphatase is not resolved from the major peak of translocase. The second peak of translocase corresponds to an approximate molecular weight range of 100,000–200,000. It should be noted that the major peak in both methods of characterization is not symmetrical. Thus, on the basis of these analyses it would appear that at least two and perhaps three forms of the translocase are present in the detergent-solubilized membrane fragments.

Urea Extraction of Membrane Fragments from S. aureus. Extraction of the membrane fragments from *S. aureus* Copenhagen in 10 M urea (pH 7.8) at 20° solubilized more than 90% of the fragments. Although the enzyme was not active in the presence of urea, dialysis of the urea extract resulted in 85% recovery of the activity if 1.0 M KCl was present in the dialysis buffer. In contrast, dialysis against 1.0 M NaCl or 1.0 M $MgCl_2$ gave 29 and 21% recovery of the enzyme units, respectively. However, after dialysis, the material aggregated and readily sedimented at 100,000*g* (30 min). An electron photomicrograph of the aggregated material from *S. aureus* Copenhagen is shown in Figure 6. A large fraction of the material reaggregated into membranelike structures. Many unsuccessful attempts were made to dialyze the urea-soluble preparation against a buffer in which the protein would remain soluble. In contrast to the translocase solubilized with sodium lauroyl sarcosinate, the dialyzed urea-soluble enzyme preparation showed no reduction in transfer activity (E/T = 13).

The sucrose gradient profile of the urea-soluble enzyme preparations from *S. aureus* Copenhagen is shown in Figure 7. In contrast to the gradients described for the detergent-solubilized membranes, these gradients contained 8 M urea. In addition to the exchange activity, transfer activity was associated with these fractions. However, a wide variation in exchange to transfer ratio was observed in the different peaks. At least four peaks of transfer activity were observed in the preparation from *S. aureus* Copenhagen.

The urea-solubilized membrane preparation from *S. aureus* was also analyzed on Bio-Gel A-50m (Figure 8). The column was developed with buffer containing 8 M urea. After filtration, each fraction was dialyzed against 0.02 M Tris-HCl (pH 7.8) containing 0.3 M KCl to recover the activity. As in the case of the detergent-solubilized preparation, the major peak of translocase corresponds to a molecular weight of 2 × 10^6. Again a wide variation in the exchange to transfer ratio is observed for the multiple peaks. In contrast to the sucrose gradient profile (Figure 6), the acid phosphatase is well resolved from the major peak of translocase. An additional attempt to analyze the urea-soluble membrane preparation from *S. aureus* was made by electrofocusing in a pH gradient (3–8) containing 8 M urea. Although the protein was separated into three major fractions, the translocase and acid phosphatase were eluted from the column at a pI = 4.9.

Potassium Hydroxide Extraction of Membrane Fragments. A third method of solubilizing the membrane fragments involves extraction with KOH at 4° for 1–2.5 hr. This method is based on the stability studies described by Struve (1966). In Figure 9, the effect of KOH concentration on the translocase remaining in the pellet is shown. The yield of enzyme in the pellet is 50% of that found in the intact membranes. The amount of

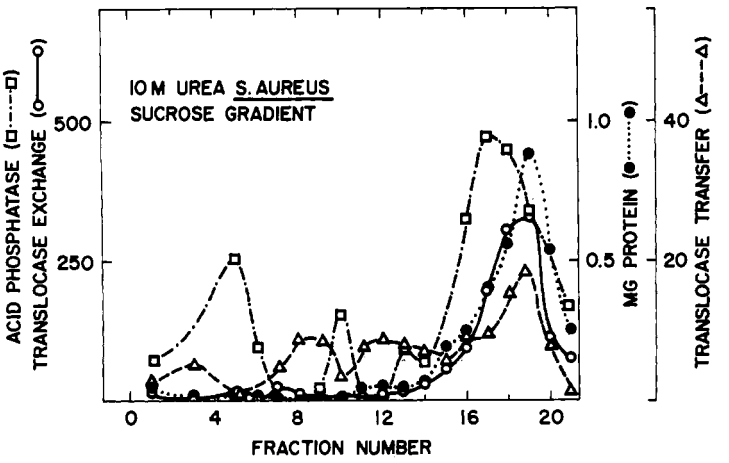

FIGURE 7: Sucrose gradient profile of urea-solubilized membrane fragments. The samples were applied to a 5–40% sucrose gradient containing 8 M urea, centrifuged, and eluted as described in the Experimental Section. The fractions were dialyzed for 2 hr at 4° against 0.005 M Tris-HCl (pH 7.8) containing 1.0 M KCl. Aliquots of the fractions were assayed for protein (mg/fraction) (●···●), translocase exchange (pmoles/min per fraction) (○—○), acid phosphatase (pmoles/hr per fraction) (□···□), and translocase transfer (pmoles/min per tube) (△--△).

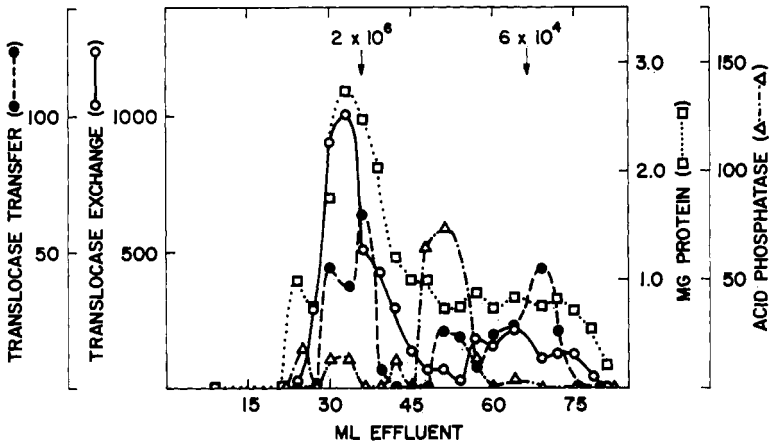

FIGURE 8: Gel filtration of urea-solubilized membrane fragments on Bio-Gel A-50m. The urea-solubilized membrane preparation (21 mg) was applied to a column (51 × 1.6 cm) of Bio-Gel A-50m that had been equilibrated with 0.02 M Tris-HCl (pH 7.8) containing 8 M urea. The column was developed at 4° with the above buffer at a flow rate of 0.13 ml/min. Each fraction (3.0 ml) was dialyzed for 3.5 hr at 4° against 0.02 M Tris-HCl (pH 7.8) containing 0.3 M KCl. Samples of the fractions were analyzed for protein (mg/fraction) (□···□), acid phosphatase (pmoles/hr per fraction) (△···△), translocase exchange (pmoles/min per fraction) (○—○), and translocase transfer (pmoles/min per fraction) (●--●).

pellet protein approaches 15% of the total membrane protein. The specific activity of the translocase in the pellet increases fourfold.

In a separate series of experiments the KOH supernatant fraction was examined for translocase. When this fraction was adjusted to pH 8.0, the remaining activity was demonstrated in a soluble form. This preparation showed little tendency to aggregate at the lower pH and retained complete activity for at least 2 months when stored at −196°. In Table II the distribution of acid phosphatase and translocase from membrane fragments of *S. aureus* after extraction with 0.08 M KOH at 4° for 1 hr is shown. In contrast to the translocase, the acid phosphatase was found only in the soluble fraction. Extraction of intact cells of *S. aureus* Copenhagen with 0.3 M KOH did not result in the release of significant amounts of translocase.

The KOH supernatant fraction was filtered on Bio-Gel A-50m (Figure 10). Two peaks of exchange activity were resolved with exclusion volumes similar to those found with the preparations solubilized with sodium lauroyl sarcosinate. No effect on the elution profile was observed if mercaptoethanol was included in the buffer. The acid phosphatase was not resolved from the major peak of translocase.

Analysis of Solubilized Fractions by Disc Gel Electrophoresis. Salton *et al.* (1967) successfully analyzed the detergent- (Nonidet P. 40) solubilized membranes from *M. lysodeikticus* by the disc gel procedure of Neville (1967). The gel patterns of the three soluble preparations described in this paper are shown in Figure 11. The supernatant fractions from each method, *i.e.*, sodium lauroyl sarcosinate, urea, and KOH, give essentially similar patterns. There are, however, significant differences from the intact membrane fragments. The disc gel procedure described by Neville (1967) introduces β-mercaptoethanol into the sample before it enters the stacking gel. Removal of this component from the sample results in an inability of the soluble preparation to enter the gel.

Discussion

An analysis of the transfer mechanism catalyzed by phospho-MurNAc-pentapeptide translocase would be greatly facilitated if the enzyme preparation were soluble and monodisperse. In order to solubilize this enzyme the effects of three reagents on membrane fragments from *S. aureus* Copenhagen have been examined and compared in this paper. The primary means of evaluating these preparations depended upon measuring the acid phosphatase and the activities

TABLE II: Extraction of Membrane Fragments with 0.08 M KOH at 4°.[a]

Fraction	Activity			
	Translocase (R/mg × 10^5)	Yield (%)	Acid Phosphatase (units/mg × 10^3)	Yield (%)
Membranes	1.23	(100)	1.07	(100)
Supernatant	0.98	35.5	0.77	33.4
Pellet	2.97	42.7	0.0	0.0

[a] The KOH extracts were prepared as described in the Experimental Section. The exchange and phosphatase assays were used to measure the translocase and acid phosphatase, respectively.

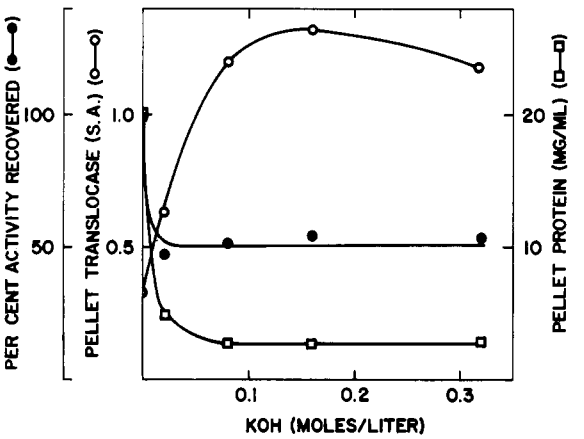

FIGURE 9: Effect of KOH concentration on the translocase associated with the pellet. The membrane fragments (10 mg) were extracted with KOH as described in the Experimental Section. The pellets were assayed for protein (□—□) and translocase. The per cent of starting activity (translocase exchange) (●—●) and the specific activity (pmoles/min per mg × 10^3) (○—○) are plotted. The exchange assay was used to measure the translocase.

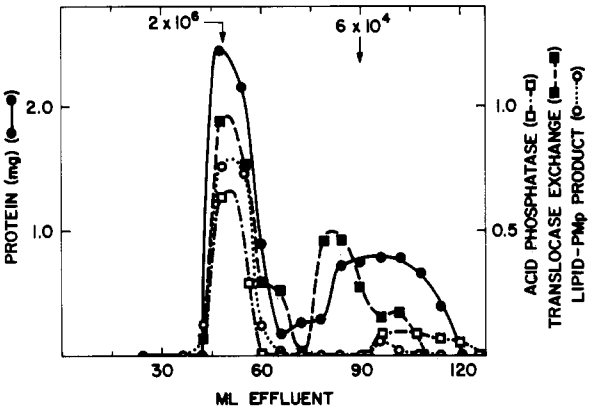

FIGURE 10: Gel filtration on Bio-Gel A-50m of the KOH-soluble fraction. For this experiment, 0.25 M KOH was used to prepare the soluble fraction. This fraction was filtered at 4° on an Bio-Gel A-50m column (69 × 1.6 cm) with a flow rate of 0.5 ml/min. Portions of the fractions (6.0 ml) were assayed for protein (mg/fraction) (●—●), acid phosphatase (μmoles/hr per fraction × 10^4) (□···□), translocase exchange (pmoles/min per fraction) (■—■), and available acceptor (pmoles lipid acceptor-diphosphate-MurNAc-pentapeptide (lipid-PMp) formed per fraction) (○···○). The amount of available acceptor was measured as described in the Experimental Section.

catalyzed by the translocase, *i.e.*, exchange and transfer.

The three methods resulted in preparations with some distinctly different properties as well as some common properties. For example, a comparison of the detergent- and KOH-solubilized systems by sucrose gradient centrifugation and filtration on Bio-Gel A-50m revealed the presence of at least two forms of the translocase. The similarities between these preparations suggest that these reagents are probably not causing artifacts during the course of solubilization. In contrast to the detergent-solubilized membrane fragments, KOH solubilized a smaller fraction (40%) of the translocase. The remaining activity was resistant to solubilization.

The alkali-solubilized fraction showed no reduction in the transfer activity when compared with the fraction solubilized with sodium lauroyl sarcosinate. The urea-soluble enzyme preparation was not active in its soluble form but regained enzyme activity when the urea was removed by dialysis. After dialysis the protein aggregated and appeared to re-form membrane-like structures. In addition, the exchange to transfer ratio of this preparation is similar to that of intact membrane fragments. In contrast, the detergent- and KOH-solubilized protein showed no tendency to aggregate after dialysis.

In spite of several distinctly different properties, the three soluble preparations gave essentially similar patterns on disc gel electrophoresis.

Since the velocity of transfer in transfer assay B is a function of both the enzyme concentration and the lipid acceptor, it is suggested that the detergent-solubilized preparation is either greatly depleted of lipid acceptor or the lipid is not as accessible to the acceptor site as it is in the case of intact membrane fragments. In the case of the urea-solubilized preparations, there appear to be multiple peaks of translocase with varying amounts of lipid acceptor. Since the exchange assay is primarily a function of the enzyme concentration, this assay is better than transfer assay B for establishing the actual amount of translocase.

A number of attempts have been made to increase the yield of the low molecular weight form of the translocase. Although treatment of membrane fragments with sodium lauroyl sarcosinate resulted in a significant shift in the soluble protein to a lower exclusion volume on Bio-Gel A-50m, when compared with solubilization by KOH, the percentage of total activity in the low

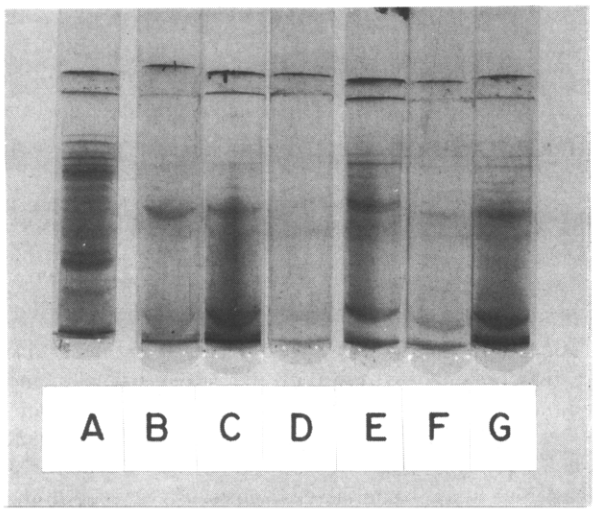

FIGURE 11: Disc electrophoresis of fractions from *S. aureus* Copenhagen. Disc electrophoresis was performed with the acid–urea–gel system described by Neville (1967). The samples (0.15 ml) contained: 10% β-mercaptoethanol, 5.6 M urea, 0.05 M K_2CO_3, 100–200 μg of protein, and a trace of methyl green. Electrophoresis was carried out toward the cathode at 1.5 mA/tube. The samples in each tube were: (A) membrane fragments, (B) NL-97 pellet, (C) NL-97 supernatant fraction, (D) KOH pellet, (E) KOH supernatant fraction, (F) urea pellet, and (G) urea supernatant fraction. Approximately equal amounts of protein were applied to each gel. The proteins in gels were stained with coomassie blue for 2 hr in 12.5% trichloroacetic acid.

molecular weight peak was similar for both preparations. Thus, it would appear that the smaller peak is not an artifact of the preparation and may represent a distinct form of the enzyme.

An examination of the membrane preparation solubilized with sodium lauroyl sarcosinate in the analytical ultracentrifuge revealed a single symmetrical peak with a $s_{20,w}$ of 4.95 S (M. G. Heydanek and F. C. Neuhaus, unpublished observation). The utility of the schlieren pattern as a criterion for the homogeneity of membrane preparations has been questioned by Engelman *et al.* (1967). The data presented in this paper suggest that the translocase is not associated with a single well-defined subunit. On the other hand, it might be argued that the reagents and conditions that have been described could lead to extensive destruction of a "membrane subunit."

It is proposed that the translocase exists in more than one form. This is clearly indicated in the detergent- and KOH-solubilized preparations. The occurrence of the translocase in both the mesosome and plasma membrane is a distinct possibility. Fitz-James (1968) found that the mesosome is the chief site of membrane lipid synthesis and may be the site of new membrane synthesis. It was suggested that a precursor–product relationship exists between mesosomes and the plasma membrane. Thus, the multiple forms of the translocase described in this paper may reflect their presence in these two types of membranes. On the basis of the experiments described in this paper, we feel that the low molecular weight form of the translocase isolated from either the sodium lauroyl sarcosinate soluble system or the KOH-soluble system provides the best preparation for the further purification and study of this enzyme.

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