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# Comparative Electrophoretic and Amino Acid Analyses of Isolated Membranes from *Streptococcus pyogenes* and Stabilized L-Form[1]

C. PANOS,[2] G. FAGAN, AND C. G. ZARKADAS

*Department of Biochemistry, Albert Einstein Medical Center, Northern Division, Philadelphia, Pennsylvania 19141*

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Major quantitative, but not qualitative, differences in the various species of proteins in purified membranes from *Streptococcus pyogenes* and its stabilized L-form have been demonstrated by acidic and alkaline disc gel electrophoresis with and without urea. The fact that no significant differences in the amino acid content or composition between these two membranes could be demonstrated emphasizes that these results are probably due to changes in the relative amounts of the various species of proteins in this subcellular component. The possibility of these protein changes in the L-form membrane being related to its inability to synthesize a rigid cell wall is discussed. Finally, phage-associated lysin, routinely used for removal of the group A streptococcal cell wall, does not appear to affect the protein profile or amino acid composition of the membrane either metabolically or nonmetabolically.

Recent publications have appeared on the utilization of disc gel electrophoresis for identification and differentiation of microorganisms. With regard to bacterial L-forms, Theodore et al. (14) employed crude membrane preparations to demonstrate gel electrophoretic differences between L-forms of *Proteus*, *Streptobacillus*, *Staphylococcus*, and *Streptococcus*. Although L-form strain specificity could not be established, it was suggested that such differentiation might be achieved if purified rather than crude membrane material were examined. Also, others have stated that the genetic identity of microorganisms seems to be reflected in the electrophoretic patterns of their cell proteins (11). By using whole cells, Razin and Shafer (12) were able to confirm the parentage of certain L-forms by comparing their patterns with those of bacteria from which they supposedly had been derived. Their survey also included the L-form and parental *Streptococcus pyogenes* used in the present study.

This stabilized L-form from *S. pyogenes* is devoid of a rigid cell wall (7) and is known to form excess membrane (Fig. 12 in reference 2). Also, the lipid composition of this organism is known to differ from that of the parent bacterium (8). Recently, it has been suggested that the almost complete absence of a cell wall polysaccharide (polymeric rhamnose) in the L-form might be due to alterations in the protein content of the membrane matrix (1). This conclusion was prompted by our unpublished results, from polyacrylamide gel electrophoretic studies, showing a marked reduction in certain protein bands in purified membranes from the L-form in comparison with its parental coccus. This possibility was enhanced, in part, by the inability to remove labeled polymeric rhamnose from streptococcal membrane fragments by lipid solvent extraction (1). The results of these electrophoretic studies now form the basis for this report and represent the first such comparison of purified membranes of a bacterium and its L-form. The validity of these electrophoretic results being due to changes in relative amounts of various protein species, and not amino acid differences, is indicated by a comparison of the amino acid and amino sugar composition of these two membrane preparations.

## MATERIALS AND METHODS

**Organisms and growth conditions.** *S. pyogenes* (12) and its stabilized L-form were the same as used

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[2] Present address: Department of Microbiology, Jefferson Medical College, Philadelphia, Pa. 19107.

previously (1). Cultures of each were harvested at mid-exponential growth phase. Completely synthetic (16) and complex (9) media were used for growth of both organisms. All media were preextracted to remove lipid before use; otherwise the growth conditions and washing procedures for these whole cells from either medium were as before (9). Streptococcal protoplasts were obtained with a group C streptococcal-phage lysate (1), and streptococcal and L-form membranes were prepared essentially as has been detailed previously (10). Also, such membrane preparations, when examined by electron microscopy, were found to be free of cell wall fragments and ribosomes.

**Polyacrylamide disc gel electrophoresis.** Electrophoretic analyses were made on large-size (0.6 by 7.0 cm) acidic and alkaline gels prepared in destaining tubes (Canalco, Inc., Rockville, Md.). For acetic acid gels, the method of Rottem and Razin (13) was followed, including the solubilization of membranes with phenol-acetic acid-water. The method of Ornstein (6), as modified by Weinbaum and Markman (17) by the addition of 8 M urea, was used for alkaline (*p*H 8.6) polyacrylamide disc gel electrophoresis. For these alkaline gels, membrane suspensions in water (0.03 ml) were solubilized by the addition of 0.01 ml of sodium dodecyl sulfate (2%), 0.01 ml of mercaptoethanol (1%), and water (0.05 ml) after mixing and heating (37 C) for 0.5 hr. No stacking gels were used with either gel system. Each gel was loaded with 0.3 to 0.4 mg of coccal membrane protein, whereas slightly greater amounts (up to 0.5 mg) of L-form membrane protein were used to obtain comparable intensities of the major bands. Acidic gels were run for 2.5 hr, whereas alkaline gels required an additional 20 min after loss (runoff) of the dye marker (bromophenol blue, total time approximately 2 hr). Staining was with Amido black, and destaining was performed nonelectrically with acetic acid (7%), as has been described (13). All gels were photographed by using a green filter and printed on high-contrast paper to accentuate stained-band intensity differences. On such photographs (Fig. 1 and 3), all lightly-stained bands appear to be absent. Densitometric tracings of all gels were obtained with a model 1050 linear drive (Vicon Industries, Huntington Station, N.Y.) attached to a Zeiss PMQ II spectrophotometer.

**Chemical methods.** Samples of membranes (1.0 to 1.1 mg) were hydrolyzed in evacuated, deaerated, and sealed tubes with 6 N HCl (1 ml) for various periods of time (1, 2, 3, 6, 12, 24, 48, and 72 hr). After removal of residual HCl in vacuo, each sample was dissolved in *p*H 2.2 citrate buffer (3 ml), and a portion was used for the determination of the amino acid and amino sugar content with a Beckman automatic ion exchange, amino acid analyzer. Identification of each peak was accomplished by comparing its position with similar elution patterns previously confirmed by paper chromatography. Protein determinations were done according to Lowry et al. (in 3) with bovine serum albumin (Armour and Co., Kankakee, Ill.) as the standard. The protein content of these streptococcal membranes was 75.5%. L-form membranes, treated with or without lysin (see below), had a similar, but lower (65.5%), protein content.

## RESULTS

**Comparative electrophoretic and densitometric studies.** Polyacrylamide disc gel electrophoresis was carried out in three different systems: acetic acid plus urea, alkaline conditions (*p*H 8.6), and alkaline conditions (*p*H 8.6) plus urea. Also, these comparative studies were performed with coccal and L-form extracts used simultaneously on gels prepared at the same time and from the same reagents. This permitted the direct comparison of such gels by negating the slight variations noted from day to day, upon repetitive analyses in the migration rates of their bands, because of gel consistency differences. Finally, mixtures of L-form and coccal extracts run on the same gel always demonstrated combined protein and densitometric patterns, indicating that comparable bands in both preparations have similar protein species.

Figure 1 illustrates protein patterns of purified membranes from *S. pyogenes* (gel A) and its stabilized L-form (gel B) obtained in the acetic acid plus urea system. The major areas of interest are indicated (arrows 1 and 2). Although it appears as though a major band (arrow 1) in the L-form membrane is missing (Fig. 1, gel B), it should be emphasized that all

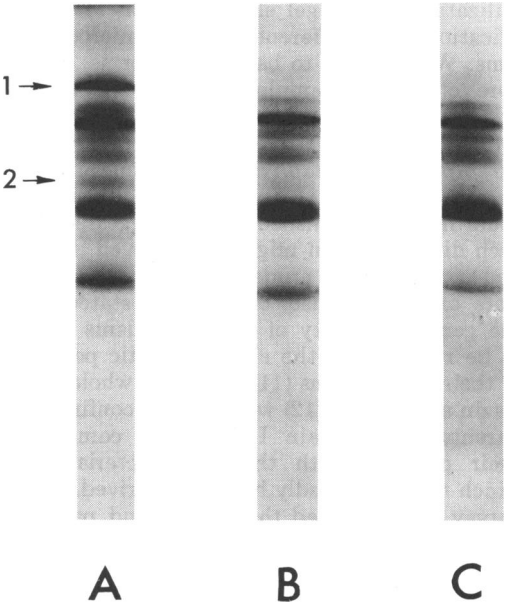


FIG. 1. *Polyacrylamide-gel electrophoretic patterns of proteins from purified streptococcal and L-form membranes in acetic acid plus urea. A, S. pyogenes (protoplast) membrane; B, L-form membrane without lysin treatment; and C, L-form membrane after lysin treatment; see text. Arrows indicate major areas of change.*

bands were present in both of these gels (see also Materials and Methods). This is confirmed by comparing the densitometric tracings of such gels, typical examples of which appear in Fig. 2 (corresponding arrows 1 and 2). Therefore, only marked quantitative differences appear to exist between the membrane proteins from these two organisms. It should also be pointed out that there were no differences noted in the electrophoretic patterns of membranes from this group A streptococcus or its L-form when harvested from crude or synthetic media. Likewise, although electrophoretically imperceptible quantities of lysin were used for streptococcal protoplast formation prior to membrane isolation, the migration rate of purified lysin was determined (by acetic acid gel electrophoresis) and found to be faster than that of the fastest-moving band in either membrane preparation. Controls included the treatment of intact L-form cells with lysin to simulate parental streptococcal membrane isolation. No quantitative (band intensities) or qualitative protein differences were noted in membranes from L-form cells treated with or without lysin (Fig. 1, gels B and C). Similarly, no change in the protein profile of the membrane was apparent upon prior exposure of the intact L-form to inactivated lysin (60 C, 1 hr) for 1 hr at 4 or 37 C. Finally, Coomassie blue is purported to be more sensitive than Amido black in the staining of acrylamide gels for the detection of protein. However, in these studies use of both stains gave identical results.

Figure 3 illustrates gels of streptococcal and L-form membrane proteins obtained at an alkaline *p*H. Gel A shows a single, sharply defined, and fast-moving band. However, in the presence of urea (gels B and C) this single component was disaggregated, as is evident by the lack of any of this fast-moving component of gel A remaining. Again, typical examples of densitometric tracings of the B and C gels (Fig. 4) illustrate a quantitative (arrows 1 and

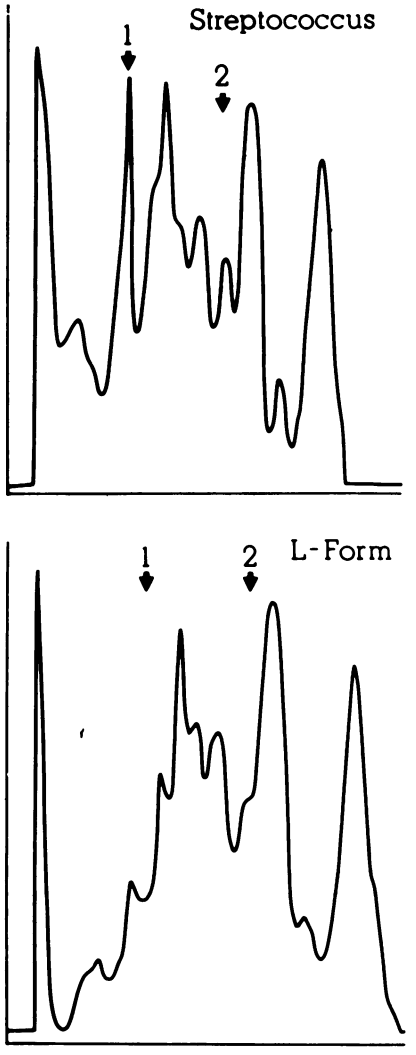


FIG. 2. *Typical densitometric tracings of gels such as in Fig. 1. Corresponding areas of change are indicated by arrows.*

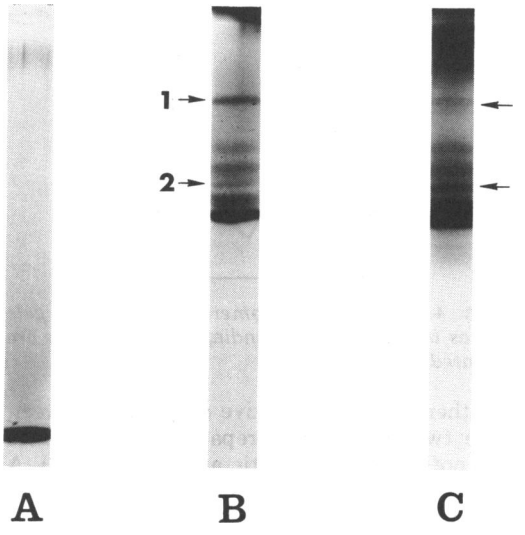


FIG. 3. *Polyacrylamide-gel electrophoretic patterns of proteins from purified streptococcal and L-form membranes at pH 8.6 with and without addition of urea. A, S. pyogenes (protoplast) and L-form membrane without urea; B, protoplast membrane with urea; and C, L-form membrane with urea. B and C, Membranes isolated by or treated with lysin, respectively. Arrows indicate major areas of change.*

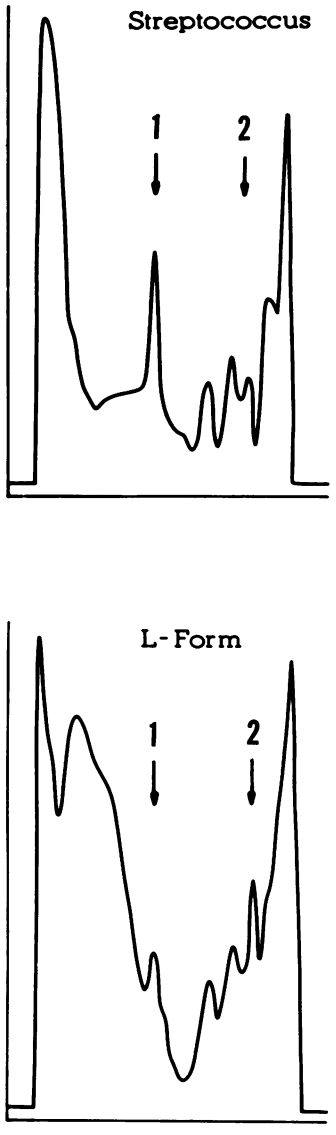


FIG. 4. *Typical densitometric tracings of gels such as in Fig. 3. Corresponding areas of change are indicated by arrows.*

2) rather than a qualitative difference between these two membrane preparations, similar to that noted with the acetic acid gels (Fig. 1). A characteristic of these alkaline gels is their inability to be destained completely.

**Amino acid and amino sugar comparisons.** Table 1 shows the amino acid and amino sugar composition of the membranes from *S. pyogenes* and its L-form treated with and without lysin. The recovery of material applied to such columns ranged from 92.8 to 97.4% for all three membrane preparations, as determined by Kjeldahl nitrogen. Only serine and threonine in the L-form and serine in coccal membrane preparations were found to be unstable with increasing time of hydrolysis. Any explanation at this time for this difference in threonine stability between these two membrane preparations would be only speculative. The final concentration of serine and threonine in each preparation was determined by extrapolating to zero time the values obtained after the various times of hydrolysis (15). Similarly, hexosamines are known to decompose markedly upon acid hydrolysis (4). Plotting of the results obtained for glucosamine indicated maximal release after 4 hr, followed by rapid destruction with increasing time of hydrolysis for membranes from this coccus and its L-form.

As is apparent, streptococcal and L-form membrane preparations were devoid of all amino acids with sulfhydryl groups (no cysteine or cysteic acid), and the hexosamine, muramic acid, was not detected. Also, no major differences were apparent in the amino acid content or composition between the L-

TABLE 1. *Amino acid and amino sugar composition of membranes from Streptococcus pyogenes and stabilized L-form*[a]

| Amino acids | Coccus | L-form (no lysin)[b] | L-form (with lysin)[c] |
|---|---|---|---|
| Lysine | 5.29 ± 0.21 | 5.18 ± 0.22 | 4.95 |
| Histidine | 1.37 ± 0.15 | 1.30 ± 0.18 | 1.33 |
| Ammonia | 1.02 ± 0.18 | 1.00 ± 0.11 | 0.89 |
| Arginine | 3.49 ± 0.13 | 3.35 ± 0.17 | 3.75 |
| Aspartic acid | 6.31 ± 0.06 | 5.99 ± 0.11 | 6.36 |
| Threonine | 3.52 ± 0.11 | 3.49[d] | 3.29 |
| Serine | 3.25[d] | 2.91[d] | 2.63 |
| Glutamic acid | 7.24 ± 0.22 | 6.65 ± 0.12 | 7.71 |
| Proline | 2.25 ± 0.11 | 2.07 ± 0.10 | 2.16 |
| Glycine | 3.10 ± 0.16 | 2.85 ± 0.09 | 2.95 |
| Alanine | 4.46 ± 0.18 | 4.25 ± 0.13 | 4.35 |
| Valine | 4.95 ± 0.12 | 4.45 ± 0.29 | 4.54 |
| Methionine | 2.01 ± 0.06 | 1.66 ± 0.07 | 1.74 |
| Isoleucine | 5.21 ± 0.30 | 4.60 ± 0.28 | 4.61 |
| Leucine | 6.73 ± 0.29 | 5.99 ± 0.25 | 6.02 |
| Tyrosine | 2.78 ± 0.13 | 2.46 ± 0.07 | 2.69 |
| Phenylalanine | 3.72 ± 0.13 | 3.58 ± 0.23 | 3.54 |
| Glucosamine | 0.17[d] | 1.20[d] | 0.26[d] |

[a] Average of at least six determinations. Values given are the mean ± standard deviation of the mean and are expressed as grams per 100 g of membrane.

[b] Intact L-form not treated with lysin prior to membrane isolation.

[c] Intact L-form treated with lysin prior to membrane isolation; 24 hr hydrolysate only.

[d] Corrected values; see text.

form and coccal membranes or the L-form membrane treated with or without lysin. Worthy of note, however, was the finding of seven times more glucosamine in the membrane of the L-form as compared with that of parental *S. pyogenes*.

## DISCUSSION

These results demonstrate for the first time significant quantitative differences in the protein content of membranes from *S. pyogenes* and its stabilized L-form. The fact that there are no pronounced differences in the amino acid content or composition of the membranes from each of these organisms only emphasizes that a change in the relative amounts of the various species of proteins has occurred. These results, together with the newly described coiled morphology of the L-form membrane (Fig. 12 in ref. 2) and changes in L-form membrane lipid content and composition (8, 10) and polysaccharide synthesis (1, 9), now adequately demonstrate some examples of structural, morphological, and enzymatic alterations that may occur after conversion of a bacterium to a stabilized L-form. The use of the group C, streptococcal, phage-associated lysin for the removal of the rigid group A streptococcal cell wall is well known. The membrane electrophoretic patterns and amino acid results obtained with the intact L-form, after treatment with and without lysin, illustrates that use of lysin for cell wall removal does not result in continued compositional or structural alterations of the membrane. The use of heat-inactivated lysin, on the other hand, also shows its inability to contribute to such electrophoretic and chemical analyses in a nonenzymatic manner by mere adsorption onto membrane.

Earlier, we had speculated that the L-form inability for continued cell wall synthesis might be related to this observed electrophoretic difference between relative amounts of the various protein species of the membrane of *S. pyogenes* and its L-form (1). This would be probable if glucosamine and muramic acid of the group A coccal cell wall were found as components of certain of these protein species in the coccal membrane. This possibility would be further enhanced if, in turn, the involvement of these amino sugars during synthesis, or in the attachment of a cell wall polymer to these protein species, could be demonstrated. The finding of a significantly greater amount of glucosamine in the L-form rather than the streptococcal membrane (Table 1), together with the ability of only membrane particles from the parent streptococcus to synthesize a cell wall polysaccharide containing carbohydrates common to at least two different "wall-type" polymers in vitro (1), seems to add to this speculation. Studies in this direction are currently in progress.

### ACKNOWLEDGMENTS

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