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Physical Separation of Streptococcal Nicotinamide Adenine Dinucleotide Glycohydrolase from Streptolysin O

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Streptococcal nicotinamide adenine dinucleotide glycohydrolase (NADase) with a molecular weight of about 55,000 and an isoelectric pH of 8.55 was isolated from crude streptolysin O (SLO) preparations. NADase differed from SLO in size, charge, and immunological behavior. Streptococcal NADase is considered to have no role in the hemolytic process because it has no hemolytic activity; conversely, partially purified SLO showed no NADase activity. The hemolytic activity of crude SLO was completely inhibited by anti-tetanolysin, whereas the NADase activity in the same reaction mixture was unaffected. Experiments involving double diffusion in agar also demonstrated immunological nonidentity of the two proteins.

Streptolysin O (SLO) belongs to a group of closely similar oxygen-labile, cholesterol-sensitive, cytolytic toxins produced by diverse gram-positive bacteria. Other examples are cereolysin, pneumolysin, and tetanolysin (1). These toxins are antigenically related and presumably have similar mechanisms of action (1, 13, 16).

Besides SLO a number of other extracellular products are released into the culture fluid during streptococcal growth, including: streptokinase (8), streptococcal proteinase (15), and nicotinamide adenine dinucleotide glycohydrolase (NADase) (EC 3.2.2.5) (7). Recently, Fehrenbach (10) reported that SLO and NADase are the same substance, and in a later paper (11) he concluded that the enzymic breakdown of cellular nicotinamide adenine dinucleotide (NAD) is the key process in SLO-induced hemolysis.

The present report describes the separation of streptococcal NADase and SLO from culture supernatant fluids and shows, in addition, that the two substances are immunologically distinguishable. The possibility that NADase has a role in the hemolytic process was also investigated.

MATERIALS AND METHODS

Organism. The C203U mutant strain of group A *Streptococcus pyogenes* was used because it produced no streptolysin S.

Cultivation of organism. An overnight broth culture of C203U was slowly frozen in 2-ml samples, stored at −20 C, and quickly thawed before use. Five 2-ml samples were routinely inoculated into 9.6 liters of yeast diffusate medium prepared as previously described by Bernheimer and Schwartz (5) for staphylococcus, but modified to contain 0.5% glucose and 1% thioglycolate (Eastman Organic Chemicals).

The organisms were grown at 37 C with slow stirring and without aeration in a 14-liter fermentor (Microferm model MF 14, New Brunswick Scientific Co., New Brunswick, N.J.). After 16 h glucose was added stepwise to a final concentration of 2%. For the next 6 to 12 h, pH was monitored and maintained at 7.0 by the addition of 5 N NaOH. Growth was measured by absorbance at 650 nm. Approximately 2 h after the pH became stabilized and growth no longer increased, the culture was chilled, centrifuged, and the cocci were discarded. The supernatant fluid was brought to 80% saturation by addition of solid ammonium sulfate. After standing overnight at 4 C, the precipitate was recovered by centrifugation, collected in approximately 100 ml of 80% saturated ammonium sulfate, and stored at 4 C.

Hemolytic activity. Prior to titration, test solutions were activated by mixing an equal volume of 0.1 M dithiothreitol (Nutritional Biochemicals Corp.) dissolved in 0.067 M sodium phosphate (pH 7.4) and allowing the mixture to stand for 15 min at room temperature. Streptolysin O was assayed with rabbit erythrocytes by the method described for the assay of cereolysin (2). A hemolytic unit (HU) is defined as the smallest amount of lysin required to produce 50% lysis of a 0.7% erythrocyte suspension after incubation at 37 C for 30 min.

NADase. The assay of NADase was performed by the cyanide method of Kaplan et al. (14), as modified by Carlson et al. (7). A unit of enzyme activity is defined as the amount of enzyme which destroys 0.01 μmol of NAD in 7.5 min at 37 C.

Gel filtration chromatography. Crude SLO dialysate was filtered through Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.02 M phosphate buffer (pH 6.8) in a water-cooled K25/100 column (Pharmacia). Fractions (7 ml) were collected in a refrigerated fraction collector and assayed for NADase and hemolytic activities.

Isoelectric focusing. The method of Vesterberg et al. (18) employing conditions described by Bernheimer et al. (3) was followed for the characterization of isoelectric point (pI). Isoelectric focusing of partially purified SLO was carried out at 4 C in a 110-ml focusing column (LKB Instruments, Rockville, Md.) by using pH 3 to 10 ampholine. Fractions (4 ml) were assayed for hemolytic activity, NADase activity, absorbance at 280 nm, and pH.

Ion-exchange chromatography. Whatman wet microgranular diethylaminoethyl (DEAE)-cellulose (DE 52) was suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (Sigma 7-9, Sigma Chemical Co.) buffer (pH 8.4), packed in a water-cooled K26/40 column (Pharmacia), and equilibrated in the same buffer. After dialysis of precipitated crude SLO against this buffer, the material was added to the column and washed with approximately 720 ml of equilibrating buffer. Gradient elution was carried out by use of an upper reservoir containing 0.05 M Tris-hydrochloride buffer (pH 8.4) and 0.6 M NaCl which fed into a constant mixing chamber containing 300 ml of 0.05 M Tris-hydrochloride buffer (pH 8.4). Fractions (9 ml) were collected and assayed for absorbance at 280 nm, NADase, and hemolytic activities.

SDS-polyacrylamide gel electrophoresis. Molecular weights were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Weber and Osborn (19) by using a 10% acrylamide system with model 12 Canalco equipment (Canal Industrial Corp.). The gels were stained with Coomassie brilliant blue (CoLab Laboratories) and destained as described by the same authors.

The mobility was calculated as the ratio of distance of test material migration to that of cytochrome *c* (Boehringer and Soehne GmbH, Mannheim). The mobilities of bovine plasma albumin (Armour Pharmaceutical Co., Chicago, Ill.), ovalbumin (Sigma Chemical Corp., St. Louis, Mo.), and cytochrome *c* were plotted against their molecular weights on a semilogarithmic scale. The molecular weights of the test material were determined from their mobilities on the standard curve (Fig. 3).

Gel diffusion. Gel diffusion on slides, using 1% Noble agar (Difco, Detroit, Mich.), was performed by the method of Campbell et al. (6). Samples were tested against horse antistreptolysin globulins containing 20,000 U/ml (batch no. 7, 19-9-47; Serum Institute, Carshalton, England) and against horse anti-tetanolysin (supplied by W. C. Latham; Massachusetts Public Health Biologic Laboratories, Boston) containing an antistreptolysin O titer of 5,000 U/ml. Gel diffusion was carried out in a humidified chamber at approximately 25 C for 24 to 48 h.

Streptolysin O-anti-tetanolysin titration. Crude SLO was dialyzed against 0.05 M phosphate buffer, pH 6.8. The crude SLO dialysate containing 40,000 HU/ml and NADase activity of 381,000 U/ml was diluted 1:100 with the dialysis buffer. Decreasing volumes of this dilution were incubated for 15 min at 37 C with increasing volumes of anti-tetanolysin diluted 1:100 with the above buffer as described in Table 1. Hemolytic and NADase activities were assayed.

RESULTS AND DISCUSSION

Precipitated crude SLO was redissolved by dialysis against 0.02 M phosphate buffer (pH 6.8) and subjected to gel filtration. This procedure removed most of the pigments and low-molecular-weight peptides. The material obtained (partially purified SLO) contained both hemolytic and NADase activities.

Isoelectric focusing of the partially purified SLO in a pH gradient (Fig. 1) revealed two major peaks of hemolytic activity at pH 6.1 and 7.55 and a separate peak of NADase activity at pH 8.55. The pI of 7.55 obtained for one of the SLO peaks is in good agreement with the pI of 7.5 reported by Halbert (12). The second hemolytic peak of pI 6.1 may be related to the pI of 5.8 obtained in the presence of 1 M glycine also described by Halbert (12). The pI of streptococcal NADase does not seem to have been measured previously, but the results of Carlson et al. (7) by using paper electrophoresis indicate that NADase has a higher pI than SLO, since it migrated further toward the cathode.

Crude SLO precipitate was dialyzed against 0.05 M Tris-hydrochloride buffer (pH 8.4) at 4 C. A 25.5-ml sample of this dialysate containing 2.6×10^6 HU and 22.4×10^6 NADase units was added to a DEAE-cellulose column. The NADase was eluted from the column by 0.05 M Tris-hydrochloride buffer (pH 8.4), whereas the

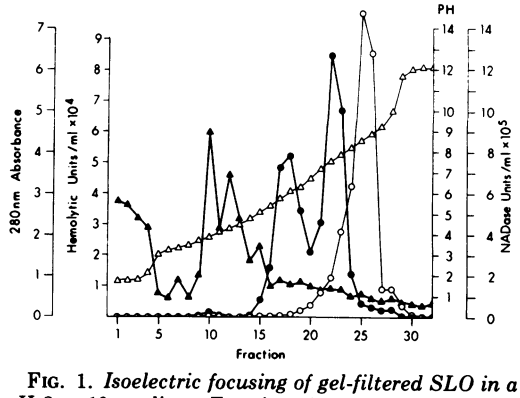

FIG. 1. *Isoelectric focusing of gel-filtered SLO in a pH 3 to 10 gradient. Fractions (4 ml) were examined for absorbance at 280 nm (▲), hemolytic activity (●), NADase activity (○), and pH (△).*

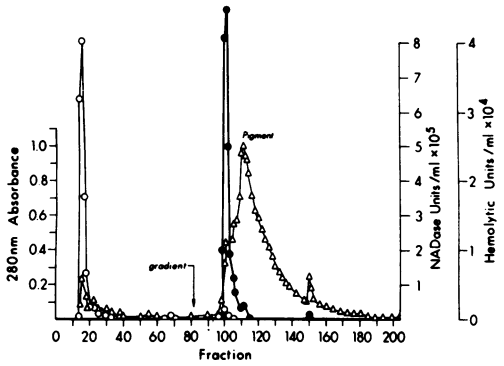

FIG. 2. *DEAE-cellulose chromatography of crude SLO dialysate. Fractions (9 ml) were assayed for hemolytic activity (●), NADase activity (○), and absorbance at 280 nm (△).*

hemolytic activity was eluted only after a 0.6 M NaCl gradient in the same buffer was applied (Fig. 2). This separation gave a sharp NADase peak with a specific activity of 4.0×10^6 NADase units per unit of absorption at 280 nm, and it contained no hemolytic activity. Although the first hemolytic peak obtained from the DEAE-cellulose column possessed some residual NADase activity, the latter was not congruent with the hemolytic peak and it could subsequently be removed by means of cation exchangers. The second hemolytic peak contained no NADase activity.

SDS gel electrophoresis of purified NADase gave a single band having a mobility corresponding to a molecular weight of 55,000. Under the same conditions, partially purified SLO gave a major band of molecular weight 67,000 to 69,000 (Fig. 3).

Gel diffusion of crude SLO dialysate, partially purified SLO, and purified NADase was carried out against antistreptolysin O (Fig. 4A). Crude SLO formed several precipitin lines; partially purified SLO formed one heavy line which was continuous with the heavy line from the crude dialysate. NADase formed a single thin precipitin line which was also continuous with one of the thin lines produced by the crude dialysate. The formation of two separate lines which crossed indicates immunological nonidentity of SLO and streptococcal NADase. Gel diffusion of the same samples against anti-tetanolysin (Fig. 4B) produced a single continuous precipitin line from the crude SLO and the partially purified SLO; no precipitin line was formed from the NADase.

Oxygen-labile hemolysins have been shown to be antigenically related, since they are neutralized by hyperimmune horse antistreptolysin and anti-tetanolysin (1, 16). In contrast, the other extracellular products produced by streptococcus are not known to be antigenically related to the extracellular products of other gram-positive organisms. This explains the formation of a single precipitin line obtained when crude SLO is diffused against anti-tetanolysin

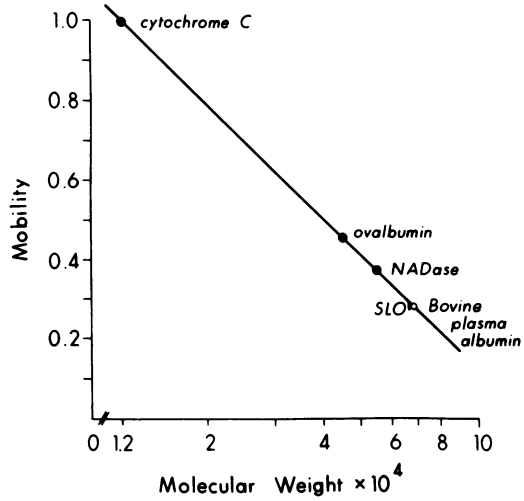

FIG. 3. *Molecular-weight determination by SDS-gel electrophoresis of NADase and SLO. The mobility is the distance of migration divided by the distance of migration of cytochrome c.*

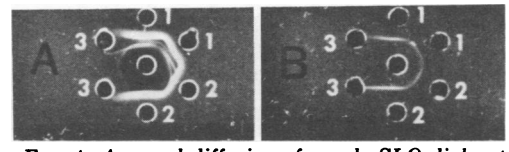

FIG. 4. *Agar gel diffusion of: crude SLO dialysate (1), partially purified SLO (DEAE) (2), streptococcal NADase (3), against (A) antistreptolysin and (B) anti-tetanolysin.*

TABLE 1. *Neutralization of streptolysin activity with persistence of NADase activity*[a]

Tube no.	Crude SLO[b] (ml)	ATL[c] (ml)	SLO:ATL (vol/vol)	Hemolytic units per ml of crude SLO	NADase units per ml of crude SLO
1	4.0	0.0	∞	40,000	381,000
2	3.5	0.5	7:1	4,000	383,000
3	3.0	1.0	3:1	0	398,000
4	2.0	2.0	1:1	0	371,000
5	1.0	3.0	1:3	0	383,700
6	0.5	3.5	1:7	0	379,600

[a] Abbreviations: SLO, streptolysin O; ATL, anti-tetanolysin.
[b] Dilution, 1:100.
[c] Dilution, 1:100.

compared with the several lines obtained when it is diffused against antistreptolysin. For the same reason, the absence of an immunological reaction between streptococcal NADase and anti-tetanolysin is in agreement with expectation.

Immunological nonidentity is further demonstrated by the results of Table 1. Titration of various amounts of crude SLO with various concentrations of anti-tetanolysin shows complete inhibition of hemolytic activity. In the same samples NADase activity remained unchanged. Earlier, it was reported by Carlson et al. (7) that cholesterol, although inhibiting SLO activity, did not affect NADase activity.

These results clearly indicate that NADase activity is not related to the hemolytic activity of SLO. NADase is not generally found as an extracellular product of bacteria (4). No NADase activity has been detected in preparations of other oxygen-labile hemolysins that have been tested, namely, pneumolysin (13) and cereolysin (unpublished experiments).

The absence of hemolytic activity in the streptococcal NADase isolated in the present study is analogous to the absence of hemolytic activity found by Johnson (13) in NADase isolated from *Neurospora crassa*.

It is evident that the molecular weight of NADase (approximately 55,000) is close to that of SLO [61,000, Van Epps and Anderson (17); 67,000 to 69,000, Fig. 3 of this paper]. Sephadex gel filtration cannot be expected to provide more than a partial separation of two globular proteins having these molecular weights. Since Sephadex gel filtration was the only purification step reported to have been used by Fehrenbach (10), the two peaks of NADase activity corresponding to molecular weights of 55,000 and 63,000 (9) may well have been mixtures of NADase and SLO. The very low specific activities for both NADase and SLO, as well as only a 2.5-fold purification (10), are also consistent with an incomplete separation.

Our results lead to the conclusion that hemolytic and NADase activities of crude SLO are two distinct activities attributable to two different proteins which differ in size, charge, and immunological properties.

It should be of interest to investigate further the existence of more than one form or variety of SLO (Fig. 1).

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LITERATURE CITED

1. Bernheimer, A. W. 1970. Cytolytic toxins of bacteria, p. 183–212. *In* S. J. Ajl, T. C. Montie, and S. Kadis (ed.), Microbial toxins, vol. 1. Academic Press Inc., New York.
2. Bernheimer, A. W., and P. Grushoff. 1967. Cereolysin: production, purification and partial characterization. J. Gen. Microbiol. **46**:143–150.
3. Bernheimer, A. W., P. Grushoff, and L. S. Avigad. 1968. Isoelectric analysis of cytolytic proteins. J. Bacteriol. **95**:2439–2441.
4. Bernheimer, A. W., P. D. Lazarides, and A. T. Wilson. 1957. Diphosphopyridine nucleotidase as an extracellular product of streptococcal growth and its possible relationship to leucotoxicity. J. Exp. Med. **106**:27–37.
5. Bernheimer, A. W., and L. L. Schwartz. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**:455–468.
6. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1970. Methods in immunology, p. 252–255, 2nd ed. W. A. Benjamin, New York.
7. Carlson, A. S., A. Kellner, A. W. Bernheimer, and E. B. Freeman. 1957. A streptococcal enzyme that acts specifically upon diphosphopyridine nucleotide: characterization of the enzyme and its separation from streptolysin O. J. Exp. Med. **106**:15–26.
8. Dillon, H. C., Jr., and L. W. Wannamaker. 1965. Physical and immunological differences among streptokinases. J. Exp. Med. **121**:351–371.
9. Fehrenbach, F. J. 1969. Gel-filtration behavior and molecular weight of NAD-glycohydrolase (EC 3.2.2.5) from streptococci in column chromatography on sephadex gels. J. Chromatogr. **41**:43–52.
10. Fehrenbach, F. J. 1971. Identity of streptolysin-O and NAD-glycohydrolase (EC 3.2.2.5). Z. Naturforsch. Ser. B **26**:1336–1338.
11. Fehrenbach, F. J. 1972. NAD-glycohydrolase (streptolysin-O), EC 3.2.2.5 and its role in cytolysis. Biochem. Biophys. Res. Commun. **48**:828–831.
12. Halbert, S. P. 1970. Streptolysin O, p. 69–98. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
13. Johnson, M. 1972. Properties of purified pneumococcal hemolysin. Infect. Immunity **6**:755–760.
14. Kaplan, N. O., S. P. Colowick, and A. Nason. 1951. Neurospora diphosphopyridine nucleotidase. J. Biol. Chem. **191**:473–483.
15. Liu, T. Y., N. P. Neumann, S. D. Elliot, S. Moore, and W. H. Stein. 1963. Chemical properties of streptococcal proteinase and its zymogen. J. Biol. Chem. **238**:251–256.
16. Todd, E. W. 1941. The oxygen-labile hemolysin or θ toxin of *Clostridium welchii*. Brit. J. Exp. Pathol. **22**:172–178.
17. Van Epps, D. E., and B. R. Anderson. 1969. Streptolysin O: sedimentation coefficient and molecular weight determinations. J. Bacteriol. **100**:526–527.
18. Vesterberg, O., T. Wadstrom, K. Vesterberg, H. Svensson, and B. Malmgren. 1967. Studies on extracellular proteins from *Staphylococcus aureus*. I. Separation and characterization of enzymes and toxins by isoelectric focusing. Biochim. Biophys. Acta **168**:228–242.
19. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.