rapid isolation and identification of other enteroviruses.

1. Paul, J. R., Melnick, J. L., in *Diagnostic Procedures for Virus and Rickettsial Diseases* 2nd Edit. Am. Public Health Assn., New York, 1956.

2. Melnick, J. L., *Am. J. Public Health,* 1958, v48, 1170.

3. Baron, S., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 760.

Received January 4, 1961. P.S.E.B.M., 1961, v106.

Extracellular Proteins of Staphylococci.* (26472)

ALAN W. BERNHEIMER AND LOIS L. SCHWARTZ
Department of Microbiology, New York University School of Medicine, New York

Staphylococci are known to release into the environment in which they grow a number of enzymes and toxins. Knowledge of the origin and function of these extracellular proteins may clarify the manner and degree in which they influence virulence and perhaps communicability. To this end strains of differing pathogenicity were selected with a view to examining the number and distribution of proteins that could be revealed by starch gel electrophoresis, a method capable of giving remarkable resolution as has been demonstrated in studies of proteins of serum (1,2) and other materials(3,4).

Materials and methods. Strains. Strains Mendita and 3895 are examples of classically avirulent coagulase-negative staphylococci of the albus type. Strain Mam-der is regarded as occupying a position intermediate between nonpathogenic and pathogenic staphylococci (5). Strains 4423 and 26925 are examples of *Staphylococcus aureus* phage type 80/81 isolated from the human nasopharynx. Strain 4423 was freely communicable among premature infants in a nursery but was accompanied by an abnormally low lesion rate; strain 26925 was obtained from a healthy person who had no history of recent suppurative lesions. Strains Smith-diffuse and Smith-compact are variants of *Staphylococcus aureus* originally isolated from a case of osteomyelitis. The former is virulent for mice intraperitoneally and is lysed by phage 44A; the latter is avirulent for mice intraperitoneally and apparently is not phage-typeable(6).

Production and Concentration of Extracellular Proteins. The medium used was similar in composition to that of Gladstone and van Heyningen(7) but was modified to exclude substances of high molecular weight that might be present in small amounts in some of the constituents. Two liters of doubly concentrated medium, pH 7.1 - 7.2, were placed in a 6 × 18 in pyrex cylinder fitted with a plexiglass cover containing a large central hole and 2 smaller holes eccentrically located. A large cellophane sac (uninflated width 2¾ in) containing 2000 ml distilled water was suspended from the plexiglass cover by bringing the open end of the sac through the central hole and attaching it to a short length of large-diameter glass tubing. The latter was closed with a rubber stopper carrying 0.7 mm inlet and outlet tubes so that the solution inside the sac could be mixed during incubation by bubbling with gas. The 2 smaller holes carried additional inlet and outlet tubes for mixing of solution outside the sac. The assembly was autoclaved for 30 minutes at 15 lb per square inch, allowed to cool, and placed in a 36° incubator for temperature equilibration.

The inoculum, consisting of saline-washed cocci derived from 2 ml of a 16-hour broth culture, was aseptically introduced into the cellophane sac. Incubation was allowed to proceed for 24 hours during which time the

* This investigation was supported in part by research grants from Life Insurance Medical Research Fund and Nat. Inst. of Allergy and Infect. Dis., U. S. Public Health Service. The authors are indebted to Dr. David E. Rogers and Dr. Frederick H. Wentworth for cultures as well as for information regarding their history.

culture inside the sac, and the medium outside as well, were continuously bubbled with a mixture of 20% CO_2 and 80% O_2, cotton-filtered, at a rate of approximately 120 bubbles per minute. Heavy growth occurred inside the sac while none occurred, as a rule, in the external solution. After centrifugation the clear supernate was concentrated 5- to 8-fold by pervaporation, dialyzed against running tap water for 12 to 24 hours, and repervaporated to 100 to 200 ml. The solution was dialyzed in the cold against 4 volumes of saturated ammonium sulfate for 2 to 3 days, with intermittent mixing, so that it equilibrated at 80% saturation. The precipitate was recovered by centrifugation, dissolved in 10 ml distilled water, and dialyzed in the cold against 5 l distilled water for 2 to 3 days. The solution was centrifuged, the insoluble sediment discarded, and the supernate lyophilized.

Starch gel electrophoresis. The method used was essentially that described by Smithies(1). As a routine, 10 mg staphylococcal protein preparation were subjected to electrophoresis (borate, pH 8.6) following which the gel was sliced in a horizontal plane and stained with amido black(1).

Distribution of Toxic and Enzymatic Activities. The product obtained from strain 4423 was employed. *Hemolytic activity*, apparently alpha hemolysin, was localized (a) by slicing the gel in the broad plane, as for staining, and bringing the cut surface into contact with rabbit blood agar, and also (b) by titrating against rabbit red cells eluates of slices that had been transversely cut at appropriate intervals along the length of the gel. The amounts of staphylococcal product applied to the gel were 1 and 10 mg respectively. Elution was carried out by slow-freezing at −16° each transverse slice together with 1 ml phosphate-buffered saline containing 0.1% bovine serum albumin, followed by thawing and mechanical compression of the gel sections to express fluid. *Lipase* was detected by placing strips of gel on tributyrin agar(8) and incubating for one hour at 37° followed by removal of the gel strips and examination for clearing. Addition of eluates to egg yolk broth (McGaughey and Chu(9) resulted in the development of opalescence. The most intense effect occurred in zones corresponding to those of maximal lipase activity suggesting that the opalescence is related to lipolytic activity on triglycerides as proposed by Gillespie and Alder(10). *Desoxyribonuclease* was detected by allowing gel strips to remain in contact with one per cent agar containing 0.2% sodium desoxyribonucleate, 0.03 M borate, pH 8.6, and 0.01 M $MgSO_4$. After 1 hour at 37°C, the gel strips were removed, the agar flooded with 95% ethanol and examined for differences in opacity. *Hyaluronidase* was estimated in eluates using the method of Tolksdorf *et al.*(11). *Staphylokinase* was detected by placing small volumes of eluates on fibrin plates(12) containing plasminogen, and subsequently observing for areas of fibrin digestion. Control plates from which plasminogen was omitted were used to differentiate direct proteolytic action, if present, from that brought about by staphylokinase. Significant amounts of *proteolytic activity* were absent as judged from failure of fibrin to be digested in plates lacking plasminogen. As no *coagulase* could be detected in 0.1% solutions of unfractionated staphylococcal proteins, it appears that little or no soluble coagulase is formed under the conditions used for cultivation.

Results. Yield and nature of extracellular material. Five cultures of the Smith-diffuse strain were grown and processed as described. Bacterial growth, expressed as OD_{650} varied from 0.37 to 0.68, and total weight of non-dialyzable extracellular product obtained from the supernates varied from 156 to 316 mg. Under the conditions of cultivation used, the Smith-diffuse strain formed non-dialyzable substances averaging 45% of its own weight.

Comparable data for all the strains studied are given in the first 4 columns of Table I. Strain 4423 formed as little as 10% of its weight in non-dialyzable substances while the compact variant of the Smith strain produced 68%. The results suggest that strains of staphylococci vary widely in quantity of extracellular material of high molecular weight that they elaborate during growth.

The non-dialyzable products obtained from

TABLE I. Growth, Yield and Properties of Extracellular Products of Diverse Strains.

Strain	Bacterial growth, OD_{650}	Yield of extracellular non-dialyzable product, mg	$\frac{\text{Wt of product (mg)} \times 100}{\text{Wt of growth (mg)}}$ %	OD_{280}* per 0.5 mg	$\frac{OD_{280}}{OD_{260}}$	Folin-Ciocalteau color as tyrosine, μg/mg	Orcinol color as ribose, μg/mg
Mendita	.615	99	16	1.36	1.38	47	18.5
3895	.452	190	43	.904	1.26	57	12.4
Mam-der	.462	175	39	1.25	1.55	69	23.6
4423	.660	65	10	1.05	.95	94	7.5
26925	.390	84	22	.83	1.02	69	6.7
Smith-compact	.290	195	68	.85	1.12	61	7.5
Smith-diffuse	.445	186	43	.925	1.07	66	9.3

* 0.03 M borate buffer, pH 8.6.

the culture supernates were assayed for protein using the Folin-Ciocalteau phenol reagent(13) and the results (Table I) indicate that the products consist very largely of protein. However, the values of the ultra-violet absorption ratio, $OD_{260} : OD_{280}$, suggest that substantial amounts of nucleic acid are also present. The products were therefore analyzed for ribose, using the orcinol reagent (14), and from the color developed (Table I) it can be estimated that ribonucleic acid is present to the extent of 3 to 12% of the weight of the products. It is notable that the products of the 2 coagulase-negative strains and the "intermediate" strain contain distinctly larger amounts of ribonucleic acid (9.3 to 11.8%) than those of the 4 coagulase-positive strains (3.8 to 4.7%).

Description and analysis of electrophoretic patterns. The 4 examples of potentially pathogenic staphylococci (strains 4423, 26925, Smith-diffuse and Smith-compact) each show 12 to 14 bands and the patterns of these 4 strains are remarkably similar although not identical. All 4 strains show 3 bands of protein (lettered A, B and C in Fig. 1) which have separated to the left of the sample application slit. (Band B could not be detected in the starch slice of Smith-compact chosen for portrayal in the figure but was present as a faint band in a pattern of another preparation of the same strain). Band B may be of particular interest because it is the heaviest staining band of the 12 formed by the penicillin-resistant phage type 80/81 strain 4423, and because it, as well as bands A and C, is absent from Mendita, 3895 and Mam-der. Labelling of bands of different strains by the same letter does not imply that these bands necessarily represent the same substance but is intended to indicate similarity to the observer's eye, of staining intensity, width, or position in relation to other bands.

The bands labelled H, I, K and L of strain 4423 appear to recur in Smith-diffuse and

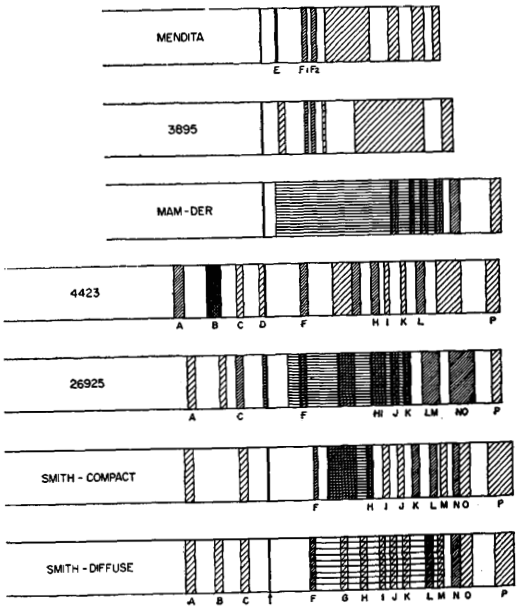

FIG. 1. Representation of starch gel electrophoretic patterns for 7 strains of staphylococci. Arrow shows location at which samples were applied. Bands E, F, G, etc. are in the direction of the positive electrode. Diagonal shading indicates degree of intensity of protein staining; horizontal shading indicates a moderately dark background of diffusely staining material. Because the representation is diagrammatic the sharpness and clarity of the patterns tend to be somewhat exaggerated.

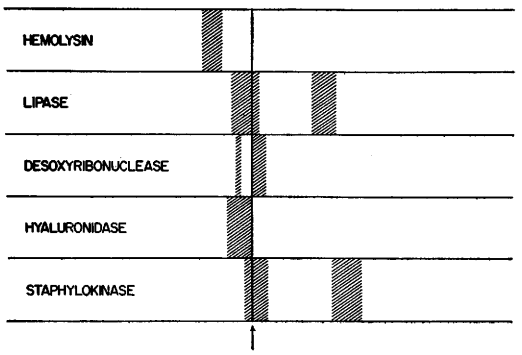

FIG. 2. Localization of hemolytic and enzymatic activities after electrophoresis in starch gel. Strain 4423. Arrow indicates origin. Migration toward the right is in the direction of positive electrode.

Smith-compact. It is less certain that some of the bands of Mam-der are homologous with H, I, K and L of 4423 and the Smith strains, and it is likewise difficult to relate to H, I, J and K the bands in the corresponding region of the 26925 pattern. It is notable that a fast-moving band, P, is present in all patterns except those of Mendita and 3895.

The patterns of the 2 nonpathogenic strains, Mendita and 3895, differ strikingly from those of the potentially pathogenic staphylococci. Only 7 and 6 bands, respectively, are evident and about half of them are relatively faint. The pattern of the "intermediate" strain, Mam-der, shows no very strong resemblance to either those of the pathogenic staphylococci or to those of Mendita and 3895.

To make sure the observed bands actually represent products of staphylococcal growth, medium was assembled, incubated and concentrated in the usual way but instead of inoculating it, 15 mg crystalline ovalbumin were added as a carrier to the medium to be processed. Electrophoresis of the resulting concentrate yielded a pattern indistinguishable from that given by a solution of crystalline ovalbumin, no additional bands being discernible.

The information provided by assays for toxic and enzyme activities is summarized in Fig. 2 in which shaded areas represent zones of maximal activity. In some instances the width of the zones is arbitrary inasmuch as increasing the sensitivity of the test, as can be done by lengthening the time of development, tends to broaden the zone of detectable activity. Although lipase and staphylokinase each show 2 zones of maximal activity, it need not be inferred that staphylococci synthesize or secrete 2 kinds of lipase and 2 kinds of staphylokinase. It may be that some of the enzyme molecules are free to move in the gel while the remainder may be aggregated or complexed with a second substance that tends to retain the enzyme at or near the sample slit.

Discussion. The results show that growth of both potentially pathogenic (coagulase-positive) and nonpathogenic (coagulase-negative) staphylococci is accompanied by the accumulation of a large amount of extracellular substance, chiefly protein. Quantities of protein found are comparable with those reported by Rogers(15). The presence of appreciable amounts of ribonucleic acid in the culture supernates here studied indicates that cell lysis occurred to a significant degree, and at first sight (Table I) it appears that lysis occurred to a greater extent among nonpathogenic than among potentially pathogenic strains. Organisms of the latter type, however, are known(16,17) to produce a thermostable nuclease which degrades both DNA and synthetic polyribonucleotides. Ribonuclease activity was readily detectable in the extracellular products of potentially pathogenic strains and it is possible that this enzyme accounts for the difference in orcinol-reactive material found. Even though extensive autolysis occurred in cultures of nonpathogenic staphylococci the electrophoretic patterns yielded by them are distinctly less complex than those of the potentially pathogenic strains.

The existence of a multiplicity of extracellular staphylococcal proteins is deducible not only from the diversity of biological and biochemical effects exhibited by cultures and culture filtrates(18) but also from the results of studies based on an immunological double diffusion technic(19) which revealed as many as 10 antigens to be associated with the Wood 46 strain. Using commercial antitoxin prepared against this strain, staphylococci isolated from lesions produced, on the average, between 6 and 7 lines of precipitate while co-

agulase-negative strains formed none. Further work in this direction(20) showed the number of lines of precipitate to be directly related to mouse virulence.

The technics used in this investigation permit detection of as many as 15 diffusible products (14 stainable bands and one white band) in the case of coagulase-positive staphylococci, and the majority of these, judging from the patterns of activity shown in Fig. 2, do not appear identifiable with previously described staphylococcal enzymes and toxins. It is notable also that many of these proteins are absent from cultures of saprophytic strains. The number of strains that have been examined is small but the results suggest that investigation of the new proteins, and possibly those of additional strains, may yield information that will help elucidate the problem of staphylococcal pathogenicity.

Summary. Strains representative of potentially pathogenic and nonpathogenic staphylococci were studied with respect to the extracellular proteins that accumulate in cultures. Starch gel electrophoresis of the non-dialyzable substances isolated from culture supernates revealed that 12 to 14 species of proteins are elaborated by potentially pathogenic strains while about half as many were found to be associated with nonpathogenic staphylococci. Additional findings permitted the loci of hemolytic and several enzymatic activities to be mapped and compared with those of protein. Most of the proteins do not appear to be identifiable with previously described products of staphylococcal growth, and therefore, represent new extracellular substances. Several of these occur consistently and perhaps exclusively in association with potentially pathogenic strains.

1. Smithies, O., *Biochem. J.,* 1955, v61, 629.
2. ———, *ibid.,* 1959, v71, 585.
3. Poulick, M. D., *J. Immunol.,* 1959, v82, 502.
4. Markert, C. L., Moller, F., *Proc. Nat. Acad. Sci.,* 1959, v45, 753.
5. Smith, J. M., Dubos, R. J., *J. Exp. Med.,* 1956, v103, 87.
6. Hunt, G. A., Moses, A. J., *Science,* 1958, v128, 1574.
7. Gladstone, G. P., van Heyningen, W. E., *Brit. J. Exp. Path.,* 1957, v38, 123.
8. Marks, J., *J. Path. and Bact.,* 1952, v64, 175.
9. McGaughey, C. A., Chu, H. P., *J. Gen. Microb.,* 1948, v2, 334.
10. Gillespie, W. A., Alder, V. G., *J. Path. and Bact.,* 1952, v64, 187.
11. Tolksdorf, S., McCready, M. H., McCullagh, D. R., Schwenk, E., *J. Lab. Clin. Med.,* 1949, v34, 74.
12. Astrup, T., Müllertz, S., *Arch. Biochem. and Biophysics,* 1952, v40, 346.
13. Heidelberger, M., MacPherson, C. F. C., *Science,* 1943, v97, 405 and v98, 63.
14. Mejbaum, W., *Z. Physiol. Chem.,* 1939, v258, 117.
15. Rogers, H. J., *J. Gen. Microb.,* 1954, v10, 209.
16. Cunningham, L., Catlin, B. W., de Garilhe, M. P., *J. Am. Chem. Soc.,* 1956, v78, 4642.
17. Cunningham, L., *Ann. N. Y. Acad. Sci.,* 1959, v81, 788.
18. Elek, S. D., *Staphylococcus pyogenes and its relation to disease,* Edinburgh, 1959.
19. Elek, S. D., Levy, E., *Brit. J. Exp. Path.,* 1950, v31, 358.
20. Howard, J. G., *J. Path. and Bact.,* 1954, v68, 177.

Received January 6, 1961. P.S.E.B.M., 1961, v106.