The Capsule of *Streptococcus equi*

By J. B. WOOLCOCK

*Department of Veterinary Paraclinical Sciences, School of Veterinary Science,
University of Melbourne, Parkville, Victoria 3052, Australia*

(Received 6 August 1974)

INTRODUCTION

The capsular material of mucoid group A streptococci is identical with the polysaccharide isolated from various body tissues and has been identified as hyaluronic acid (Kendall, Heidelberger & Dawson, 1937). Group C streptococci also produce a similar component (Seastone, 1939) and the size and persistence of the capsules in young cultures of various group C strains can be related to the presence of this mucopolysaccharide. Among the group C streptococcal strains examined quantitatively for the production of hyaluronic acid, no representatives of *Streptococcus equi* have been examined. Most determinations of hyaluronic acid production by streptococci have utilized turbidimetry, but the results obtained by such procedures are dependent on the degree of polymerization of the hyaluronic acid (Greiling, 1963).

This communication describes the production of hyaluronic acid by *S. equi*, by quantitation of the polysaccharide separated from the culture supernatant. In addition, the effect on experimental *S. equi* infection of repeated treatment with hyaluronidase is recorded.

METHODS

Micro-organism, source and growth medium. *Streptococcus equi* was kindly supplied by Dr A. Gulasekharam, Commonwealth Serum Laboratories, Melbourne, Australia, and had been recovered from an equine submandibular lymph node abscess. It conformed to the description of this organism as given by Bazeley & Battle (1940). To obtain minimal interference in direct hyaluronic acid estimation, a simple medium was devised. This consisted of (g/l water) Bacteriological Peptone (Evans), 20.0; glucose, 10.0; sodium bicarbonate, 2.0; sodium chloride, 2.0; disodium phosphate, 0.4; adjusted to pH 7.8 and sterilized by filtration.

Growth of organism. Overnight seed cultures were centrifuged, and the deposited cells resuspended and added to fresh growth medium. These cells were obtained from a volume of seed culture equal to one-tenth of the volume to be inoculated. Cultures were kept at pH 7.0 to 7.6 during growth by the addition of sterile NaOH. A replicate culture containing 0.002 % phenol red was used as indicator.

Determination of hyaluronic acid in culture supernatant. (i) Precipitation from supernatant. The method of Scott (1960) was used in which a quaternary ammonium salt is utilized to produce insoluble mucopolysaccharide complexes. Cetyltrimethylammonium bromide (CTAB) (1 %, w/v) in 0.3 M-NaCl was used, together with a small amount of a filter aid (washed Celite 545, Johns-Manville, U.S.A.). Cationic exchange was performed with saturated alcoholic potassium thiocyanate.

(ii) Estimation of hyaluronic acid. The modified uronic acid carbazole reaction of Bitter & Muir (1962) was used, with glucuronolactone as the standard. Confirmation that the

Table 1. *Hyaluronic acid recovery from culture supernatants of S. equi*

Time (h)	Hyaluronic production (mg/100 ml culture)	
	Supernatant analysed immediately	Supernatant analysed after holding at 37 °C for 16 h
4	6.0	4.8
6	11.6	12.0
7	18.4	18.4
8	29.6	30.4
10	36.6	36.0
12	38.2	37.6
24	52.0	53.6

colour reaction was specifically due to hyaluronic acid was obtained by the addition of streptococcal hyaluronidase (kindly supplied by Dr G. Jacobs, Organon, Holland), an enzyme specific for the mucopolysaccharide (Linker, 1966).

Treatment with hyaluronidase. Groups of 18 to 20 g mice were injected intraperitoneally with 0.5 ml of appropriate dilutions of a 5 h culture. Treatment with hyaluronidase (from bovine testes, approximate activity 300 i.u./mg, Koch-Light Laboratories) began 2 h later, using intraperitoneal injections of 0.5 ml of a 375 i.u./ml solution. Injections with the enzymes were given every 2 h for the first 12 h, every 4 h for the next 36 h, and every 12 h for the last 48 h. Control groups were treated with heated hyaluronidase or saline.

RESULTS

Recovery of hyaluronic acid from growth medium

Precipitation with CTAB permitted the recovery of 80 % hyaluronic acid from the growth medium. To achieve this recovery, it was necessary to dilute the medium 1 in 2 with water before CTAB treatment. Omission of this step resulted in a 65 % recovery rate. Growth of *S. equi* in this medium (which is relatively unenriched compared with the usual culture media for this organism) was satisfactory, given a long lag phase.

Production of hyaluronic acid by S. equi

By 24 h, the organism had produced 52 mg hyaluronic acid/100 ml culture, with the most rapid production during the 4 to 8 h period (Table 1). This coincided with the time when *S. equi* was multiplying rapidly and when frequent adjustment of the pH of the culture was necessary to neutralize the copious acid production. Indian ink preparations indicated that at 8 h the bacterial capsule was at its maximum size. By 12 h the number of fully encapsulated cells had dropped considerably and by 16 h only a small proportion of the bacteria showed any capsular material at all. By 24 h no capsulated cells could be found.

Hyaluronidase production by S. equi

Evidence from Table 1 also suggests that this organism does not produce hyaluronidase. Firstly, the level of hyaluronic acid production did not fall over the 24 h period. Readings taken at 48 h gave comparable figures to the 24 h estimation. Secondly, controls were included to determine whether continued incubation of the culture supernatant resulted in a fall in hyaluronic acid recovery. Samples which were incubated (with the addition of merthiolate to prevent any bacterial growth) gave similar values to non-incubated samples.

Effect of hyaluronidase on S. equi infection

Enzyme treatment failed to alter the course of the infection. No protection was afforded even though dosing with hyaluronidase followed very soon after infection and was maintained intensively. The group receiving heated hyaluronidase likewise showed no evidence of protection.

DISCUSSION

The accuracy of determination of hyaluronic acid production by measurement of the polysaccharide in the culture supernatant is dependent on the use of media in which growth medium constituents do not interfere with the methods being used for such determinations. Thus, the colorimetric method of Greiling (1963) and the spectrophotometric method of Greiling (1958) were found unsatisfactory for the present work. The medium used in this study had to be mucopolysaccharide-free, as well as capable of supporting satisfactory growth of *S. equi*, an organism normally requiring enrichment for cultivation.

Gerlach & Köhler (1970) examined hyaluronic acid production by group A and one unspecified group C streptococci, using quaternary ammonium salt precipitation. The highest yielding organism (a group A strain) produced 24 mg/100 ml culture, and medium composition greatly influenced the production of the mucopolysaccharide. MacLennan (1956*a, b*) is one of the few workers who have determined hyaluronic acid production by group C streptococci, but his isolates did not include any strain specifically identified as *S. equi*. Three strains (one each of *S. zooepidemicus*, *S. dysgalactiae* and *Streptococcus* sp.) produced between 40 and 56 mg hyaluronic acid/100 ml culture after 24 h growth, and turbidimetric methods were used for these determinations. That such strains should produce amounts of hyaluronic acid similar to that recorded in this study for *S. equi* is not surprising. Although *S. equi* is regularly endowed with very large capsules, and has a characteristic mucoid colony, other strains of streptococci, particularly *S. zooepidemicus*, are occasionally encountered which resemble *S. equi* colonially and can only be distinguished biochemically.

While some group C streptococci produce both hyaluronic acid and hyaluronidase, production of the former component by these strains can be detected only very early in the growth period (MacLennan, 1956*b*). The absence of hyaluronidase production by the strain of *S. equi* used in this study is consistent with the results obtained with the three group C strains mentioned above, which also produced copious amounts of hyaluronic acid. The loss of a demonstrable capsule from *S. equi* over a 24 h growth period cannot therefore be explained on the basis of the action of hyaluronidase.

The protective effect of hyaluronidase on experimental group C streptococcal infection in mice observed by Hirst (1941) and Rothbard (1948) was not confirmed. McClean (1942) also failed to achieve protection with repeated hyaluronidase treatment, and suggested that this failure may have been due to rapid elimination from the circulation of the highly purified enzyme used in his study. Whatever the reason for the different effect of hyaluronidase treatment observed in group A and group C streptococcal infections (Kass & Seastone, 1944), subsequent workers have attributed to the capsule of group C streptococci the unquestioned role of a virulence factor while at the same time equivocating concerning its role on group A cells. It is probable, however, that this procedure for assessing the role of the capsule may not be meaningful, because of the ability of streptococci stripped of their hyaluronic acid capsule by treatment with hyaluronidase to regenerate new capsules in 5 or 10 min (Stollerman, 1972). Nevertheless, the possession of a capsule whether by a group A or a group C strain must endow that organism with an antiphagocytic capacity

and its relative role in determining virulence will depend on the presence or absence of other factors exhibiting a similar capacity, especially M protein. Such a component has recently been demonstrated in *S. equi* (Woolcock, 1974).

The advice and discussion provided by Professor D. Lowther, Biochemistry Department, Monash University, are gratefully acknowledged.

REFERENCES

- BAZELEY, P. L. & BATTLE, J. (1940). Studies with equine streptococci. I. A survey of beta-haemolytic streptococci in equine infections. *Australian Veterinary Journal* **16**, 140–146.
- BITTER, T. & MUIR, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry* **4**, 330–334.
- GERLACH, D. & KÖHLER, W. (1970). Die Bildung und Isolierung von Streptokokken Hyaluronsäure. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* (Abteilung I) **215**, 187–195.
- GREILING, H. (1958). Eine spektrophotometrische Methode für die Bestimmung der Bakterien-Hyaluronidase. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **309**, 239–242.
- GREILING, H. (1963). Hyaluronic acid. In *Methods of Enzymatic Analysis*, pp. 87–92. Edited by H.-U. Bergmeyer. New York: Academic Press.
- HIRST, G. K. (1941). The effect of a polysaccharide-splitting enzyme on streptococcal infection. *Journal of Experimental Medicine* **73**, 493–505.
- KASS, E. H. & SEASTONE, C. V. (1944). The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of group A haemolytic streptococci. *Journal of Experimental Medicine* **79**, 319–330.
- KENDALL, F. E., HEIDELBERGER, M. & DAWSON, M. H. (1937). A serologically inactive polysaccharide elaborated by mucoid strains of group A haemolytic streptococcus. *Journal of Biological Chemistry* **118**, 61–69.
- LINKER, A. (1966). Bacterial mucopolysaccharidases (mucopolysaccharide lyases). In *Methods in Enzymology VIII Complex Carbohydrates*, pp. 650–654. Edited by E. F. Neufeld and V. Ginsberg. New York: Academic Press.
- MACLENNAN, A. P. (1956a). The production of capsules, hyaluronic acid and hyaluronidase by group A and group C streptococci. *Journal of General Microbiology* **14**, 134–142.
- MACLENNAN, A. P. (1956b). The production of capsules, hyaluronic acid and hyaluronidase by 25 strains of group C streptococci. *Journal of General Microbiology* **15**, 485–491.
- MCCLEAN, D. (1942). The *in-vivo* decapsulation of streptococci by hyaluronidase. *Journal of Pathology and Bacteriology* **54**, 284–286.
- ROTHBARD, S. (1948). Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A streptococcus infection in mice. *Journal of Experimental Medicine* **88**, 325–341.
- SCOTT, J. E. (1960). Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. In *Methods in Biochemical Analysis*, vol. 8, pp. 145–197. Edited by D. Glick. New York: Interscience.
- SEASTONE, C. V. (1939). The virulence of group C haemolytic streptococci of animal origin. *Journal of Experimental Medicine* **70**, 361–378.
- STOLLERMAN, G. H. (1972). In discussion. In *Streptococci and Streptococcal Diseases: Recognition, Understanding and Management*, p. 405. Edited by L. W. Wannamaker and J. M. Matsen. New York: Academic Press.
- WOOLCOCK, J. B. (1974). Purification and antigenicity of an M-like protein of *Streptococcus equi*. *Infection and Immunity* **10**, 116–122.