STREPTOCOCCUS MUCOSUS LYTICUS.

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Introduction.

Seven strains of a Streptococcus which have been isolated from patients suffering from acute infections of the respiratory tract, in some instances with severe general symptoms, have been fully investigated. The organism was isolated from the throat, sputum, and in one case from pus removed from the chest. The characteristic features of every strain were a mucous growth on certain solid media such as serum agar and egg, rapid lysis of the mucous colonies on serum agar, so that after a few days' incubation of the growth at 37° C. the lysis was complete, and formation of fresh mucous growth when the lysed colonies were transferred to serum agar even after many generations.

Literature.

The organism first described by Schottmüller (1903) produced small mucous colonies on serum agar, showed no growth on gelatin at 22° C., did not ferment inulin, and acidified milk with the formation of clot. The organism mentioned by Howard and Perkins (1901) grew well on plain agar and gelatin, and that described by Park and Williams (1905) where serum culture media were used, after two or three generations lost its characteristics. Dochez and Gillespie (1913) described a mucous Streptococcus which was insoluble in bile and failed to ferment inulin. Buerger (1906) described a group of encapsulated streptococci of mucous character whose morphological and cultural characteristics distinguished them from certain types of Pneumococcus, namely the "mucous" type. Lysis which is the most characteristic feature of our Streptococcus is not referred to by any of these authors.

Mair (1929), however, in discussing the mucous variety of the pneumococci, says: "The mucous variety shows large transparent watery colonies well raised at first above the surface and apt to form confluent streaks. On further incubation, these dry down so as to become almost invisible." And again, in discussing the organism of Schottmüller, he says: "After 2 days' incubation (on blood agar) the mucoid character disappeared and no haemolysis was observed until after several days."

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Morphology and staining reactions.

In direct smears from the tissues, oval, capsulated, Gram-positive diplococci were seen. Smears made from pure cultures 24 hours old and stained with Gram's stain show medium and long-chained forms, both with and without capsule formation depending on the medium which had been used. Apart from Dorset's egg medium, the incorporation of blood serum or whole blood in a medium is essential for demonstrating capsules. After 24 hours' growth at 37° C. autolytic forms appear in film preparations from the various solid media as swollen Gram-negative cocci and ghosts together with cocci which still retain Gram's stain. The numbers of Gram-negative cocci rapidly increase with continuous incubation at 37° C. The organisms are non-motile and stain with all the ordinary dyes.

Cultural characteristics.

Repeated reference in the text is made to serum broth, and serum agar which were prepared by the addition of 0·15 c.c. of sterile unheated human serum to every 5 c.c. of beef broth or agar. The serum was added to the melted agar at 45° C. The organisms form mucous colonies on serum agar, Dorset's egg medium and media to which whole blood has been added. The most characteristic feature of this Streptococcus is lysis, which occurs after 24 hours' growth at 37° C. on serum agar and is described in detail below. Optimum growth is at 37° C. The organism grows anaerobically, and in an atmosphere of 20 per cent. CO_2 better than aerobically. Lysis takes place in any atmosphere.

Lysis on serum agar and egg. Serum agar: "mucous" colonies appear after 24 hours at 37° C. as discrete, smooth, dome-shaped colonies, varying considerably in size from pin-point to 3 mm. in diameter, and becoming confluent where crowded. The colonies are clear and moist when examined by reflected light, and opalescent by transmitted light. The "mucous" viewed with a hand lens is granular and viscid.

Lysis first appears as a slight depression of the dome of the colony which is opaque by transmitted light and, as lysis proceeds, this opacity gradually spreads outwards and the colony becomes flatter. Subsequently the colony appears as a flat granular area with minute islets of "mucous" at the periphery, and after about 5 days at 37° C. the lysed colonies are visible as more or less circular greyish discs flush with the surrounding medium when viewed by transmitted light. Colonies of streptococci or staphylococci growing on the serum agar plates in contact with the *Strept. mucosus* were unaffected by the lysis of the "mucous" colonies.

Lysed serum-agar plates. Serum-agar plates inoculated with the *Strept. mucosus* were allowed to lyse at 37° C. When the lysed plates were reinoculated from emulsions in beef broth of any of our strains of *Strept. mucosus*, *Staph. aureus* or *B. coli*, no growth occurred; if, however, the plates were heavily

reinoculated, then *B. coli* and *Staph. aureus* grew well, but no growth of the *Strept. mucosus* occurred.

Dorset's egg medium. A good "mucous" growth was present after incubation for 24 hours at 37° C. with no change after 48 hours, and some mucous growth was still present on this medium after 7 days at 37° C., especially at the periphery and bottom of the egg slopes. A good "mucous" growth developed on egg slopes which had previously been covered with a thick deposit of heat-killed organisms. Emulsions of the organism in saline from egg slopes, when mixed with washings from lysed plates, had not undergone lysis after 4 hours' incubation at 37° C. when estimated with Brown's density tubes.

Blood agar. In 24 hours at 37° C. a good "mucous" growth was obtained on blood agar prepared by adding 0·15 c.c. of sterile oxalated human blood to every 5 c.c. of agar at 52° C. There was a tendency to pallor of the medium limited to the area occupied by the transparent colonies. In 48 hours lysis commenced as shown by "ditching" of the colonies with a definite α-type of haemolysis spreading into the area around the colonies. At the end of a week at 37° C. the growth had almost completely disappeared.

Serum broth. Uniform turbidity followed by gradual lysis.

Chocolate agar. A good granular growth with a little "mucous" developed in 24 hours which rapidly became entirely granular with decoloration of the medium.

Inspissated blood serum. In 24 hours a "mucous" growth was present which rapidly lysed and then formed a granular surface on the medium.

Agar. In 24 hours at 37° C. a very fine "mucous" pin-point growth appeared, the larger colonies of which reached 1 mm. in diameter. Lysis commenced after 48 hours at 37° C. and continued very slowly. When the agar plates were reinoculated with the same strains after lysis was complete, no growth occurred.

Litmus lactose agar. No growth occurred on this medium.

Gelatin. Incubated at 22° C. No growth after 10 days.

BIOCHEMISTRY.

In Table I is shown the fermentation reactions of six strains.

Table I.

Strain ...	1		2		3		4		5		6	
Time in days ...	1	7	1	7	1	7	1	7	1	7	1	7
Serum:												
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	+	+	−	+	+	+	+	+	−	+	+	+
Lactose	+	+	−	+	+	+	+	+	+	+	+	+
Saccharose	+	+	−	+	+	+	+	+	+	+	+	+
Salicin	−	sl. +	−	−	−	+	−	+	+	+	−	−
Mannite	−	−	−	−	−	+	−	−	−	+	−	−
Milk	+	+	−	+	+	+	+	+	+	+	+	+

+ = acid reaction. − = no acidity.

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In the preparation of these media 0·15 c.c. of sterile unheated human serum was added to each tube of approximately 5 c.c. of the "sugar" medium.

Hiss' serum water. Tubes of 5 c.c. of Hiss' medium were inoculated with each strain and incubated for 48 hours at 37° C., but no change occurred. Two tubes of the medium were inoculated in each instance, one with litmus and the other with phenol red as the indicators. When, however, 5 c.c. of Hiss' medium was added to a 24 hours' growth of these streptococci on egg slopes the medium was acidified and clotted after 24 hours at 37° C., and the clotting increased with further incubation. No change occurred in the controls made by pouring Hiss' medium on to sterile egg slopes. Strains of the Pneumococcus reacted in a similar manner to the *Strept. mucosus*.

Haemolysis. (*a*) Blood agar: all strains showed an α-type of haemolysis after incubation for 48 hours at 37° C.

(*b*) Blood peptone water: 0·1 c.c. of sterile human red cells free from plasma was added to each tube of 5 c.c. of peptone water prepared as follows:

Tube 1. 0·5 per cent. NaCl,
0·1 ,, agar,
1·0 ,, peptone,
0·1 c.c. of red cells.

Tube 2. 0·85 per cent. NaCl,
0·1 ,, agar,
1·0 ,, peptone,
0·1 c.c. of red cells.

Control tubes were used in every experiment. The media were incubated after inoculation with each strain of *Strept. mucosus* in the upright position at 37° C. for 24 hours, but no haemolysis occurred.

Bile salt solubility. Thick emulsions of each strain were made in normal saline and in distilled water from egg slopes grown for 24 hours at 37° C. To each emulsion was added an equal amount of 4 per cent. bile salt in distilled water and in saline. Control tubes without bile were also employed. The density of each emulsion was estimated immediately, and again after 20 min. at room temperature. All strains showed partial solubility in bile salts. Lysis was also demonstrated in film preparations from bile salt media.

Viability.

At 37° C. and in ice safe. Serum-agar slopes were inoculated with each strain and incubated at 37° C. for 24 hours and for 6 days. The serum-agar tubes which had been incubated at 37° C. for 24 hours were then stored in the ice safe for 6 days. Lysis occurred in all the tubes, though very slowly in the ice safe; but, at 37° C. and on ice, scattered droplets of "mucous" were still present at the periphery of the growth at the end of 6 days. Sub-cultures made on egg slopes from the lysed growths and incubated at 37° C. for 24 hours all showed a good "mucous" growth. Similar experiments made with egg

cultures confirmed that lysis was considerably less in the cultures stored on ice than at 37° C.

At 22° C. No growth occurred in 10 days on gelatin slopes which had been inoculated with each strain and incubated at 22° C., but when egg media were inoculated with scrapings from the surface of the gelatin tubes and incubated at 37° C. for 24 hours a good "mucous" growth developed.

Effect of heat. Saline emulsions of each strain were made from egg slopes which had been grown for 24 hours at 37° C. Each emulsion was divided into two parts, one of which was heated in a water-bath at 55° C. for 10 min., the other for 20 min. Sub-cultures were then made on to egg slopes which were incubated at 37° C. for 24 hours. It was found that three of the strains were killed by 10 min. exposure at 55° C. and the others succumbed in 20 min. at the same temperature.

Cultivation on immune sera.

Anti-sera obtained from rabbits which had been immunised with our strains of the mucous streptococci were added to melted agar in the proportion of 0·6 c.c. of the unheated immune serum to 15 c.c. of agar. Serum agar prepared in this way when inoculated with any of the strains of *Strept. mucosus* gave a good "mucous" growth after 24 hours at 37° C., but no difference was detected in the growth of the organism cultivated in its own immune serum. Similar results were obtained when 0·15 c.c. of any of the unheated immune sera was added to 5 c.c. of beef broth and incubated at 37° C.

PATHOGENICITY.

(*a*) *For animals.*

(1) *Intradermal inoculation.* Rabbits previously sensitised with a dose of the live Streptococcus intravenously did not react when injected intradermally 6 days later with 0·2 c.c. of the live emulsion.

Guinea-pigs previously sensitised with a dose of the live Streptococcus intraperitoneally, followed 6 days later by 0·2 c.c. of a live emulsion intradermally, also gave no reaction.

(2) *Intravenous injections* of the live streptococci into rabbits in doses amounting to 2000 million per c.c. produced loss of weight and the animals were obviously ill. When they were killed about 7 days later, numerous infective processes were found including bi-lateral sero-fibrinous pleurisy and pericarditis with effusion. The *Strept. mucosus* was recovered in pure culture from the heart's blood, pericardial and peritoneal fluids and the spleen.

(3) *Intraperitoneal inoculation.* A guinea-pig inoculated intraperitoneally with 300 millions of the live streptococci died after 20 days with general fibrinous peritonitis and sero-fibrinous pleurisy. Pure cultures of the Streptococcus were obtained from the spleen, heart's blood and peritoneal fluid.

The virulence of these organisms varied considerably as shown experimentally when inoculated into rabbits and guinea-pigs. To quote extreme

examples, one rabbit inoculated intravenously with a total of 4700 million live organisms, in ascending doses over a period of 12 days, and a guinea-pig inoculated intraperitoneally with 1000 million, showed no ill effects after a lapse of 3 months and 6 weeks respectively. On the other hand a rabbit became seriously ill 7 days after an intravenous inoculation of 2000 million and a guinea-pig died 20 days after an intraperitoneal inoculation of 330 million of these organisms.

(*b*) *For man.*

Toxicity of vaccines. A very severe local and considerable general reaction may follow a subcutaneous dose of only 10 million of these streptococci killed by formalin, and some patients appear to be unable to withstand a dose greater than 50 million. The local reaction may be severe without a general reaction or the general reaction may mask the local effect.

Immunity.

(*a*) *Precipitins. Preparation of antigens.* Each strain was inoculated from a 24 hours' growth on egg slopes into large tubes of serum broth which were grown at 37° C. for 2 days and 7 days respectively.

It was found that a 7 days' growth at 37° C. furnished the better antigen.

(1) The cultures were centrifugalised at high speed to clear the medium as far as possible by such means; the supernatant fluid which was glass clear was used as the antigen.

(2) Cultures of the different strains were mixed and passed through Seitz filters or Berkefeld V candles to obtain clear sterile filtrates.

(3) 1 c.c. of distilled water was added to a 6 days' growth of each strain on egg. A thick bacterial suspension was obtained which was poured into sterilised bottles containing fine glass beads and shaken in the electric shaker for 1 hour. It was then centrifugalised at high speed, the supernatant fluid pipetted off and an equal quantity of 1·8 per cent. sodium chloride added so as to give a 0·9 per cent. salt content.

(*b*) *Preparation of antisera.* Rabbits were inoculated intravenously in doses of 500 and 1000 million at a 15 days' interval with live organisms grown on egg slopes and suspended in saline, with formolised vaccines and with Seitz filtrates. The sera were heated at 55° C. for half an hour and then tested.

Technique of reaction.

Each tube contained 1 c.c. of the diluted antiserum and 0·5 c.c. of undiluted antigen, making 1·5 c.c. in each tube. The serum dilutions ranged from 1 : 2 to 1 : 200. The reactions were carried out at 52° C. for 24 hours, and then allowed to cool for 30 min. before the results were read with a hand lens. Serum and antigen controls were used in each experiment.

Details of results.

(*a*) *Precipitins.*

The following terms are used to express our results:

(1) "Complete" = Thick precipitate at bottom of tube: clear supernatant fluid.

(2) "Incomplete" = Precipitate at bottom of tube: granular suspension.

(3) "Marked" = No precipitate at bottom of tube: granular suspension visible to naked eye.

(4) "Trace" = Fine granular suspension visible with a hand lens.

(5) "Faint trace" = Very fine granular suspension visible with a hand lens.

All the precipitates were granular when viewed microscopically. Samples of blood from each rabbit tested before inoculation failed to give any reaction with any of the antigens referred to above.

Reactions were "marked" up to a dilution of 1 : 100 and showed a "trace" up to 1 : 200. Similar results occurred when rabbits were given four doses of mixed strains extending over a period of 12 days, each rabbit receiving a total of 4000 million organisms. Stock pneumococcal sera types I and II gave similar results with a 7 days' serum-broth antigen of the *Strept. mucosus*. None of the reactions were so marked when the egg antigens referred to in the text were employed. The serum-broth antigens after the addition of 0·1 per cent. formalin showed some decrease in potency after 1 week in the ice safe.

The immune sera gave no reaction with *B. coli* and *Staph. aureus* precipitin antigens prepared by growing these organisms in beef broth for 1 month at 37° C. and then filtering through a Seitz filter, although strong reactions were obtained with these antigens and their antisera.

A summary of our precipitin results is as follows:

(1) Our antigens were prepared by growing these streptococci in serum broth at 37° C., then centrifugalising the cultures at high speed until they were glass clear.

(2) Bacterial cultures filtered through Seitz filters or Berkefeld V candles gave no reaction with the immune sera.

(3) Immune sera were readily prepared by injecting the live organisms intravenously into rabbits.

(4) We were unable to obtain immune sera by inoculating rabbits with bacterial suspensions killed with formalin or heat, or with filtered toxins.

(5) No reaction occurred with normal rabbits' sera.

(6) Precipitin antigens of equal value were obtained with the different strains.

(*b*) *Agglutinins.*

Owing to the presence of auto-agglutinins these experiments were not proceeded with: the colonies themselves were granular and emulsions of "mucous" colonies in normal saline showed a similar granularity, but this

granularity was more in evidence when specific immune rabbits' serum was added to an emulsion of the mucous growth.

Summary.

1. Seven strains of *Strept. mucosus* are described which have the following characteristics: large "mucous" colonies on serum agar and other media referred to in the text, with a subsequent particular form of lysis; no growth occurred on gelatin at 22° C., although the organisms were still viable on this medium after 10 days at this temperature; inulin was fermented and milk acidified without any clot formation; the organisms were only partially soluble in bile.

2. All our strains were isolated from patients suffering from acute infections of the respiratory tract.

3. Inflammatory and suppurative lesions were produced in rabbits and guinea-pigs by inoculation of the live organisms.

4. Vaccines may prove highly toxic when injected subcutaneously even in small doses into patients suffering from these infections.

5. Immune sera containing precipitins were formed in rabbits inoculated with the live organisms, but not when filtered toxins or dead cultures were employed.

6. Precipitin antigens were obtained by high-speed centrifugalisation of serum-broth cultures until the supernatant fluid was glass clear, and from centrifugalised suspensions in saline from egg cultures, but after filtration of the cultures through a Seitz filter or Berkefeld V candle the filtrates were inert.

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