THE EFFECT OF A POLYSACCHARIDE-SPLITTING ENZYME ON STREPTOCOCCAL INFECTION

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PLATE 26

(Received for publication, December 31, 1940)

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When Kendall, Heidelberger, and Dawson (1) isolated a polysaccharide from group A hemolytic streptococcus cultures, they gave renewed interest to the subject of streptococcus capsules. Somewhat later, Seastone (2) showed that the capsules of group C streptococci were chemically similar to those of group A. In both groups, this capsular polysaccharide is non-type-specific; with heat-killed vaccines prepared from both group A and group C strains, it has been impossible to stimulate in rabbits the production of any antibodies against this capsular substance. A further interesting fact is that a chemically similar substance occurs widely throughout the mammalian organism, and has been isolated by Meyer and his collaborators from vitreous humor, Wharton's jelly, synovial fluid, and the fluid from a mesothelial tumor (3–5). Moreover, enzymes capable of splitting this polysaccharide occur in certain bacteria (6, 7) and also in some mammalian tissues (8, 7).

All of the above facts raise interesting questions concerning the rôle of the capsule in infection with streptococci. How may a capsule, made up of a substance normally present in the host, influence the pathogenesis and virulence of an invading streptococcus? It is with this question that the present paper is principally concerned.

Preparation of the Capsular Polysaccharide

Kendall, Heidelberger, and Dawson obtained the polysaccharide from the supernatant fluids of broth cultures of group A streptococci following centrifugation. Upon repeating their work, we found that all of our preparations contained about 50 per cent of blood group A substance. This contaminating material has been shown to be present in large quantities in peptone (9). In order to eliminate this and other sources of error, all the media, before they were used for culture, were prepared as follows:—

The medium was essentially that of Todd and Hewitt (10) except that beef heart or vegex was substituted for the horse meat, and the alcohol-insoluble fraction was

removed before use. The beef heart infusion for 60 liters of medium was first concentrated *in vacuo* to 4 liters; when vegex was used 600 gm. of the paste was dissolved in 4 liters of water. In this concentrate 1,500 gm. of Pfanstiehl peptone was dissolved, then 2.5 volumes of ethyl alcohol was added. The precipitate which settled out overnight was discarded; and the alcohol in the supernatant fluid was removed by distillation *in vacuo*. This material was then dissolved in 60 liters of tap water; the pH was adjusted to 7.4; and the salt mixture was added, bringing the final pH to 7.8. It was then filtered through Chamberland filters No. L 5 and inoculated immediately by adding 100 cc. of young actively growing culture to each 4 liter flask. The growth was always heavy. After overnight incubation, the organisms were killed by adding either formalin or acetic acid to a final concentration of 2 per cent. The microorganisms were removed by centrifugation in a Sharples supercentrifuge; and the clear supernatant fluid was concentrated *in vacuo* to one-tenth the original volume. To this concentrate were added 0.1 volume of glacial acetic acid, 500 gm. of sodium acetate, and 1.25 volumes of ethyl alcohol. The precipitate which formed was redissolved in 2 liters of water containing acetic acid and sodium acetate as before, and again precipitated with 1.25

TABLE I

Analyses of Carbohydrate Capsular Preparations from Group A Hemolytic Streptococci

Lot No.	Strain	Type	Specific rotation	Total N	Total C	Acetyl	Ash
				per cent	*per cent*	*per cent*	*per cent*
90	S23	14	−60.2	3.30	42.8	11.50	1.80
91	S43	6	−61.7	3.55	42.7	12.12	1.78
92	S23	14	−66.1	3.16	44.7	10.70	1.58

volumes of ethyl alcohol. The precipitate, already fairly free of protein, was again dissolved in water and shaken with chloroform and butyl alcohol (11) until a precipitate no longer formed at the interface. After a third precipitation with alcohol, the polysaccharide was dissolved in water and dialyzed 1 or 2 days against distilled water. The fourth and final precipitation was in 10 volumes of cold acetone, after adding a drop of HCl to the carbohydrate solution. This precipitate was washed in acetone and dried *in vacuo*. The final yield, at best about 120 mg. per liter of original culture, varied considerably among the different microorganisms used.

In Table I are shown certain chemical data on preparations made in the above manner with type 6 and type 14 group A hemolytic streptococci.[1] From this table it can readily be seen that the several products were fairly uniform. Furthermore, these data correspond fairly well with some of those obtained by Meyer and his collaborators in analyzing similar carbohydrates from mammalian sources (3–5). These two types of group A

[1] The type 6 strain used was S43 and the type 14 strain was S23. Both were obtained originally from the 1918 streptococcus epidemic in Texas and both had been rendered virulent for mice by repeated mouse passage.

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streptococci also yielded essentially similar products. In none of the preparations was there more than a trace of blood group A substance. We were unable to remove the ash present in our preparations, either by repeated precipitation with alcohol and acetone or by prolonged dialysis against distilled water.

All of our immunological studies on the above preparations have confirmed the work of Kendall, Heidelberger, and Dawson (1), and of Seastone (2), who were unable to find any circulating antibodies in rabbits after the injection of group A and group C streptococcal vaccines. We have tried many varieties of group A streptococcal vaccines, and have employed many different strains. The vaccines were killed by heat, acetic acid, and formalin, respectively; and both young and old cultures were used. None of these vaccines, nor the prolonged injection of living cultures intravenously, ever gave rise to sera in which even a trace of precipitins for the capsular polysaccharide occurred. It seems therefore that Loewenthal (12), who has reported obtaining capsular antibodies in rabbits, was probably dealing with some impurity in his carbohydrate preparations, possibly blood group A substance.

Effect of Specific Enzymes on the Capsular Polysaccharide

In order to carry out certain *in vivo* tests on capsules, it was necessary to have a potent enzyme non-toxic for experimental animals, but still capable of splitting the capsular carbohydrate. From pneumococci, streptococci, *Bacillus welchii* (4, 7), and from soil bacilli (13), carbohydrate-splitting enzymes have been isolated. Recently, Chain and Duthie (8) described the presence of a similar enzyme in bovine testicular extract and they believe that this enzyme may be the same as the spreading factor of Duran-Reynals. Since a watery extract of leech heads has been described as the most potent source of this spreading factor (14), we decided to test the potency of such an extract for carbohydrate-splitting enzyme.[2] Leech extract proved to be very potent in this respect; and furthermore, this particular enzymatic activity was confined entirely to extracts of those portions of the leech containing the salivary glands.

Although such preparations contain a potent enzyme, it was necessary to eliminate the hirudin which occurs in abundance in leech extract. If the hirudin were not removed, animals treated with the extract bled easily following injection and often succumbed to a fatal hemorrhage from a simple hypodermic needle puncture. Since there exist no definite data on the

[2] Recently both Claude (15) and Meyer, Hobby, Chaffee, and Dawson (16) have reported the presence of a mucolytic enzyme in leech extract.

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chemical nature of hirudin, we assumed that it might be similar chemically to heparin, *i.e.*, a high molecular weight polysulfuric acid ester of a polysaccharide, and hence we used as a means of getting rid of the leech anticoagulant two substances which are known to precipitate heparin. With the first of these precipitants, clupein (17), it was possible to remove about 85 per cent of the hirudin present in the leech extract; while with the second, toluidine blue (18), all but traces of the remaining 15 per cent were precipitated and removed. Both substances were used in preparing the leech extract described below because clupein removes other non-enzymatic materials from the extract besides hirudin, while toluidine blue removes the hirudin more completely.

The method of preparing leech extract began essentially as described by Claude (14): The heads of 48 leeches were cut off just behind the salivary glands, minced with scissors and ground with sand. Following this, they were extracted several times with a total of 50 cc. of water. To this solution was added about 50 mg. of clupein; and the heavy precipitate thus formed was removed and discarded. At this point the addition of more clupein did not cause further precipitation, although only 85 per cent of the hirudin had been removed. 50 mg. of toluidine blue was then added and the resulting small precipitate was removed by centrifugation. The excess of toluidine blue was removed by dialysis against distilled water. During this dialysis another precipitate formed which was also discarded. The final water-clear preparation was only slightly less potent in enzyme than the original extract. It was non-toxic when given intraperitoneally to mice, and also contained the spreading factor. There was usually about 0.5 mg. of solid material per cc. of solution.

When this leech extract was incubated with the capsular polysaccharide under suitable conditions, reducing groups were released. The enzyme was active against the similar polysaccharide of the umbilical cord and the carbohydrate obtained from a pleural tumor (3, 5).[3] It was inactive against other polysaccharides tried such as chondroitin sulfuric acid and blood group A substance. The optimum activity was obtained between pH 5.0 and 6.0, although it was active from pH 4.5 to 9.0. It was completely inactivated by heating at 50°C. for one hour.

One peculiarity of this enzyme is that when added to its substrate, even in considerable excess, the final yield of reducing sugar (calculated as glucose) was only 45 to 52 per cent, whereas the theoretical yield should have been 90 per cent, assuming that the capsular polysaccharide is made up of equivalent amounts of N-acetyl glucosamine and glucuronic acid, and that the polysaccharide was completely split to these derivatives. Most of the previously described enzymes have split the substrate to the

[3] These preparations were kindly given to us by Dr. M. H. Dawson.

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point of yielding reducing sugar values close to the theoretical 90 per cent level. This may mean that the enzyme in leech extract is able to split only one of the carbohydrate linkages and the end product is then a disaccharide, while other enzymes or enzyme combinations split the polysaccharide down to monosaccharide derivatives. Table II shows typical activity figures for the enzyme used in various concentrations against the same amount of streptococcus capsular polysaccharide. It is clear that even when the enzyme was present in excess, the end point remained around 50 per cent.

TABLE II

Per Cent of Reducing Groups Released from Streptococcus Capsular Carbohydrate by Leech Extract

Time	Concentration of leech extract					
	0.06 per cent	0.03 per cent	0.015 per cent	0.007 per cent	0.003 per cent	0.0015 per cent
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	32.2	23.5	12.7	7.5	2.5	1.0
3	42.3	43.0	36.5	29.2	18.0	13.0
7	46.5	47.2	40.0	32.0	31.5	23.3
24	50.5	52.5	47.7	48.7	47.7	39.5

The per cent concentration of leech extract is expressed in terms of dry weight of the material in the extract used. Hanes' modification of the Hagedorn-Jensen method was used for determination of reducing groups which are expressed in terms of glucose. Corrections have been made for the proper blanks in each case. The initial carbohydrate concentration in each tube was 0.1 per cent.

Effect of Leech Extract Enzyme on Streptococcus Capsules

Many authors have demonstrated the presence of capsules on group A streptococci (19, 20). One of the most satisfactory methods has been that of Seastone, who grew streptococci in high concentrations of serum. One can also demonstrate capsules surrounding microorganisms grown on a moist sealed blood agar plate. Although they are usually very difficult to detect on cocci grown in ordinary fluid media, it was discovered in the course of another investigation (21) that group A streptococci often have excellent capsules when grown in Todd-Hewitt filtered broth. In such media, the capsules are most readily made visible by the use of moist India ink preparations, in which they stand out sharply against a dark granular background. In Todd-Hewitt broth cultures of group A streptococci, the capsules appear only when the microorganisms are growing vigorously, and they are largest when multiplication of the microorganisms is most rapid. With the slowing of growth, the capsules decrease in size and shortly disappear, probably by diffusion into the medium. Likewise streptococci

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virulent for mice are well encapsulated, while growing in the peritoneal cavity of the mouse. On the other hand, many strains of group A streptococci, especially those cultured long in the laboratory, are very poorly encapsulated either *in vivo* or *in vitro*. However, glossy strains as well as matt strains may be equally well encapsulated.

Similar, but much more striking and very much larger, capsules may be found on mouse virulent and guinea pig virulent group C streptococci, grown either *in vitro* or *in vivo*. The group C strains which cause nonlethal endemic infection in guinea pigs, usually have small capsules, comparable in size to those of virulent group A strains, while group C strains from human beings frequently have no demonstrable capsules at all. The capsules of the mouse virulent group C streptococci[4] seem much more stable and last much longer in fluid culture than those of the group A strains.

If, to a drop of culture of encapsulated streptococci of either group A or group C, one adds a drop of leech extract just before making an India ink preparation, the capsules disappear almost instantly. The demonstration works equally well with encapsulated microorganisms from mouse peritoneal washings, and is most striking with the virulent group C strain (D181) since the capsules are so large. The lytic effect on the capsule was also demonstrable *in vivo* with streptococci of either group.

Two mice were each inoculated intraperitoneally with 0.5 cc. of culture. One-half hour later, one mouse was injected intraperitoneally with 0.1 cc. of leech extract. At 30 minute intervals exudate was removed from each animal and mixed with India ink. In the untreated mouse, the microorganisms remained fully capsulated, while in the one treated with enzyme, they were devoid of capsules for 2 to 4 hours, after which time they suddenly became encapsulated (see Figs. 1, 2, and 3). Another intraperitoneal injection of leech extract again removed them.

Effect of Leech Extract Enzyme on Streptococcal Infections

Having determined that the enzyme in leech extract was capable of acting on the capsules *in vivo*, the next experiment was to test the effect of the enzyme on the course of an infection. In Tables III and IV are recorded the results with two group A strains, D58[5] and S23. Both strains were virulent for mice and both had good capsules. The mice, divided into several groups, were inoculated intraperitoneally with the indicated dilution of culture; and then one-half of each group received 0.1 cc. of purified

[4] The mouse virulent group C strain referred to here and used throughout this paper was obtained from Dr. Seastone and is referred to in his paper (2) as strain No. 4. Our designation for this strain is D181.

[5] D58 is a mouse virulent strain of a type 3 group A hemolytic streptococcus, obtained by Colebrook from puerperal sepsis, and is called by him strain "Richards."

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leech extract diluted to 0.5 cc. in saline. The extract was given intraperitoneally a few minutes following the inoculation, then every 8 hours

TABLE III

Protective Action of Leech Extract in Mice Infected with Group A Streptococcus, Type 14 (Strain S23)

Mouse No.	Treated with leech extract — Dilution of culture			
	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-8} cc.
1	D1	D1	D2	S
2	D1	D1	D3	S
3	D1	D1	D3	S
4	D1	D1	S	S
5	D2	D2	S	S
6	D2	D3	S	S
7	D3	D10	S	S
8	D3	D10	S	S
9	D4	S	S	S
10	D5	S	S	S

Mouse No.	Virulence controls — Dilution of culture			
	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-8} cc.
1	D1	D1	D1	D1
2	D1	D1	D1	D10
3	D1	D1	D1	S
4	D1	D1	S	S
5	D3	D1	S	S
6	D3	D2	S	S
7	D3	D2	S	S
8	D10	D2	S	S
9	S	D3	S	S
10	S	S	S	S

All mice were inoculated first with respective dilutions of culture. Within one-half hour after infection, all treated mice received 0.1 cc. of leech extract intraperitoneally. The treated mice were then given 0.1 cc. of leech extract at 8 hour intervals for 48 hours, and at 12 hour intervals during the 3rd and 4th days. The experiments shown in Tables IV and V were done in a similar way.

D, with a number, indicates death within that number of days. S means survival throughout time of experiment, 10 days.

for 2 days, and every 12 hours on the 3rd and 4th days. Under the conditions of these experiments, there was only suggestive evidence that the enzyme had a slight influence on the lethal action of strain S23, in that the groups of mice inoculated with 10^{-6} and 10^{-7} cc. of culture survived

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longer when treated with the leech extract, even though there was no significant difference in the number of survivors. This tendency to delayed death was distinctly more marked in mice inoculated with strain D58.

TABLE IV

Protective Action of Leech Extract in Mice Infected with Group A Streptococcus, Type 3 (D58, Strain "Richards")

Mouse No.	Treated with leech extract			
	Dilution of culture			
	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-8} cc.
1	D1	D1	D2	D1
2	D2	D1	D2	D1
3	D2	D1	D4	D2
4	D2	D1	D4	D4
5	D3	D2	S	D7
6	D3	D3	S	S
7	D4	D3	S	S
8	D4	D3	S	S
9	S	D3	S	S
10	S	S	S	S

Mouse No.	Virulence controls			
	Dilution of culture			
	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.	10^{-8} cc.
1	D1	D1	D1	D1
2	D1	D1	D1	D1
3	D1	D1	D1	D1
4	D1	D1	S	D1
5	D1	D1	S	S
6	D1	D1	S	S
7	D1	D1	S	S
8	D1	S	S	S
9	D1	S	S	S
10	S	S	S	S

See note under Table III.

When, on the other hand, a similar experiment (Table V) was made with the virulent group C streptococcus, strain D181, a marked protective influence of the leech extract was demonstrated. The enzyme protected the mice completely up to and including 10,000 M.L.D.'s, which was the maximum employed. When still another group of controls was treated with the same leech extract, which had been inactivated by heating at 50°C. for 40 minutes, it no longer offered protection, showing that the protective

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factor has about the same heat lability as the carbohydrate-splitting enzyme. If the animals receiving the active enzyme were not treated intensively following infection, the amount of protection was much less; and even those treated effectively for 4 days occasionally suffered recurrences and died 4 to 8 days after the treatment was discontinued.

TABLE V

Protective Action of Leech Extract in Mice Infected with Group C Streptococcus Strain (D181)

Treated with leech extract					
Dilution of culture					
10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.
S	S	S	S	S	S
S	S	S	S	S	S
S	S	S	S	S	S
S	S	S	S	S	S
Treated with leech extract heated (56° 40 minutes)					
Dilution of culture					
10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.
D1	D1	D2	D2	D2	S
D1	D1	D2	D2	D2	S
D1	D2	D2	D2	D2	S
D2	D2	D2	D4	S	S
Virulence controls					
Dilution of culture					
10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.	10^{-7} cc.
D1	D1	D2	D2	D2	S
D1	D1	D2	D2	D2	S
D1	D2	D2	D2	D2	S
D2	D2	D2	D3	D2	S

In this experiment the mice were treated for 4 days and the experiment was terminated on the 6th day. See note under Table III.

A similar experiment was carried out in guinea pigs, since they are the natural hosts of this infectious agent. In this experiment, summarized in Table VI, the guinea pigs were infected intraperitoneally with graded doses of the same group C strain used in mice. The controls died in 1 to 6 days, all having peritonitis and blood stream invasion. The treated guinea pigs were given 0.3 cc. of leech extract intraperitoneally every 8 hours for 3 days and every 12 hours for 3 additional days, beginning a few minutes after

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inoculation. All the treated guinea pigs were sacrificed at the end of a week; and cultures of the blood, abdominal abscesses, and peritoneal surface were made. In no case was there any peritonitis or peritoneal abscess. The four guinea pigs receiving the highest doses of inoculum had subcutaneous abscesses and varying degrees of bacteremia, while in the remaining two, no evidence of infection was found. Since the control animals all died of peritonitis, while the treated ones had none, it seems plain that the extract was able to control this phase of the infection. Those guinea

TABLE VI

Protective Action of Leech Extract in Guinea Pigs Infected with Group C Streptococcus, Strain D181

	Infecting dose					
	10^{-1} cc.	10^{-2} cc.	10^{-3} cc.	10^{-4} cc.	10^{-5} cc.	10^{-6} cc.
Treated						
	S	S	S	S	S	S
Blood culture	+	+++	++++	++	–	–
Peritoneal culture	–	–	–	–	–	–
Skin abscess culture	++++	++++	++++	++++	No abscess	No abscess
Controls						
	D1	D6	D2	D2	D5	D2
Blood culture	++++	++++	++++	++++	++++	++++
Peritoneal culture	++++	++++	++++	++++	++++	++++

Treatment was started within 30 minutes after the infecting dose had been given. Treated guinea pigs received 0.3 cc. of leech extract, intraperitoneally, every 8 hours for 72 hours, and every 12 hours thereafter, until the experiment was terminated at one week. See Table III for further explanation.

pigs that had the abscesses and blood stream infection, probably would have eventually succumbed even had the treatment been continued. Doubtless, these animals received a small subcutaneous infection along the tract of the needle used for intraperitoneal inoculation; and it seems likely that the enzyme preparation had little or no effect on the infection in this site, and from this abscess the organisms were able to multiply and invade the blood stream.

DISCUSSION

The capsule of group A and group C streptococci does not seem to be an organized structure in the same sense that a cell wall is a structure. Rather it seems to be merely an accretion around the microorganism of a slowly

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diffusible substance, which during the active phase of bacterial growth is produced faster than it diffuses away. The capacity to produce this capsular substance varies enormously among the different strains, but is always well developed in mouse virulent strains of both groups of streptococci; and in some mouse virulent group C strains its production is very marked.

Seastone (2) has presented evidence indicating that the large capsules of virulent group C strains are responsible in part for their increased virulence. In his infected colony of guinea pigs, the endemic microorganism was a group C streptococcus which had the power of infecting the animals without killing them; and the infection remained localized in lymph nodes. When, however, a virulent group C strain appeared in the colony, probably by variation of the endemic strain, it killed large numbers of the guinea pig population with an acute disease. This new strain was always much more heavily capsulated than the endemic strain. Seastone tried in vain to protect animals against this virulent infection, both by active and passive immunization; but only in those animals having chronic infection with the endemic strain was any protection observed, and even here was very slight.

In our experiments with group C streptococcal infections of both mice and guinea pigs, we obtained good protection by the use of an enzyme which would effectively remove these large capsules, and it seems at least possible that the saccharolytic and protective agent are the same. This offers further substantial evidence that the capsule of these group C streptococci is an important factor in making these strains virulent for mice and guinea pigs. This *in vivo* demonstration of the effect of the enzyme on the capsule was limited in these experiments to peritoneal infections, where the infectious process was so circumscribed that the microorganisms were readily accessible to the therapeutic agent. The presence of the spreading factor in these leech extracts made it impossible to show a similar effect on subcutaneous infections, since the spreading factor, if applied there, would probably have extended the local area of infection. The fact that repeated intraperitoneal administration of the enzyme is necessary for protection, and that a demonstrable decapsulating effect lasts only a few hours is probably due to inactivation of the active principle, possibly by exhaustion on a similar substrate in the host.

With group A streptococci, the story is somewhat different. In this group we have never encountered a strain which has capsules comparable in size to the virulent group C strain. While there is some parallelism between the appearance of mucoid colonies and virulence of many strains of group A, nevertheless, the importance of another factor associated with virulence, the M substance, has been amply demonstrated (22). The M

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substance is always present in mouse virulent strains; and sera containing sufficient anti-M antibody has a marked protective effect against group A streptococcus infection of mice. From the results of the present experiments, it would seem that the capsule plays a minor rôle in the virulence and invasiveness of these particular microorganisms. Here again it seems unwise to generalize from the very special type of infection where bacteria are put into the peritoneal cavity of the mouse and the decapsulating enzyme is applied at the same site, to instances where the infection is in other tissues in which it is impossible to bring the enzyme so closely into contact with the microorganism.

SUMMARY

1. Confirming the observations of other experimenters, it has been found that group A hemolytic streptococci produce a capsule containing a polysaccharide which is similar to, if not identical with, certain high molecular weight sugars found in the mammalian body.

2. Leech extract possesses a powerful enzyme capable of splitting one of the linkages in this polysaccharide and of decapsulating group A and group C hemolytic streptococci *in vitro* and *in vivo*.

3. Mice and guinea pigs can be protected from intraperitoneal infection with a virulent group C streptococcus by the intraperitoneal administration of leech extract. In contrast there is little protective action of leech extract in mice infected with group A hemolytic streptococci.

4. The protective effect of leech extract against streptococcal group C infection is probably due to the removal of the capsule *in vivo*.

5. The capsule of mouse virulent group C streptococci plays a major rôle in the virulence of that microorganism, while the capsule of certain mouse virulent group A streptococci plays little, if any, rôle in virulence, at least when the infection is intraperitoneal in the mouse.

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EXPLANATION OF PLATE 26

Fig. 1. Capsule on group A hemolytic streptococci (strain S23), grown in mouse peritoneum. India ink preparation. × 1,000.

Fig. 2. Capsule on group C hemolytic streptococci (strain D181), grown in mouse peritoneum. India ink preparation. × 1,000.

Fig. 3. Decapsulated group C hemolytic streptococci (strain D181), grown in mouse peritoneum, and withdrawn 30 minutes after intraperitoneal injection of leech extract. India ink preparation. × 1,000.

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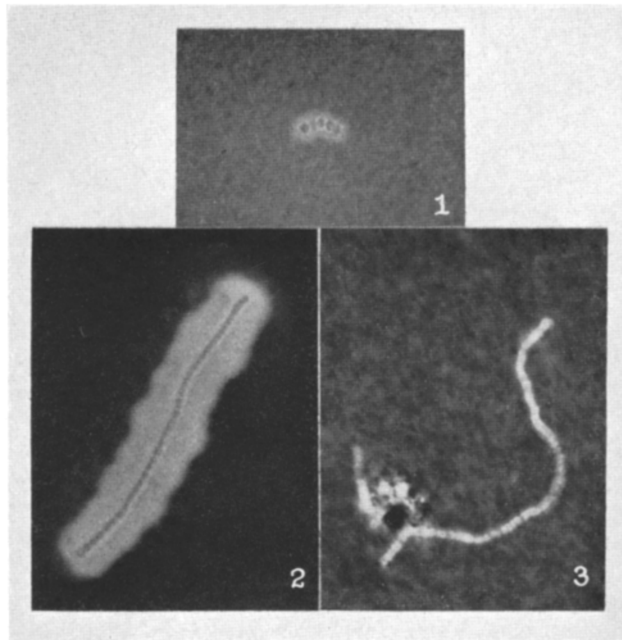

(Hirst: Streptococcal capsular splitting enzyme)

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