THE VIRULENCE OF GROUP C HEMOLYTIC STREPTOCOCCI OF ANIMAL ORIGIN

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In the accompanying paper (1), it was shown that hemolytic streptococci obtained from chronic cervical lymphadenitis in the guinea pig, as well as the variants derived from such streptococci, failed to give rise to demonstrable protective antibodies. Furthermore, it was not possible to relate the precipitins produced by injecting rabbits with heated or formalinized vaccines to any material which was peculiar to invasive strains. That such a material existed was suggested by the presence of stainable capsules in young cultures of invasive strains, a property roughly related to the mucoidness of these strains.

The investigations to be presented were stimulated by the important observation of Kendall, Heidelberger, and Dawson (2) concerning a non-antigenic polysaccharide isolated from mucoid group A hemolytic streptococci. This polysaccharide is apparently identical with one obtained by Meyer and Palmer (3) from bovine vitreous humor and from Wharton's jelly. Synovial cavity fluid (4) has since been shown to contain the same substance. It was obtained in large amounts from mucoid group A strains; non-mucoid strains produced considerably less.

At the time these results appeared we were studying a peculiar property of the group C mucoid strains. If an 18 hour 10 per cent serum broth culture of such a mucoid strain was centrifuged and the clear supernatant acidified to about pH 4.0, a heavy flocculent precipitate developed. Similar culture supernatants of non-mucoid strains remained perfectly clear when brought to pH 4. The precipitate failed to appear in serum-free cultures. The possibility that this precipitation might be due to the presence of the mucoid carbohydrate described by Kendall, Heidelberger, and Dawson (2) suggested itself. In Meyer and Palmer's description of the vitreous humor carbohydrate (3) it was noted that this material formed a precipitate with serum globulins in the presence of acid.

The precipitation of serum protein was therefore investigated as a possible

basis for the quantitative estimation of these carbohydrates. It was found that under certain conditions, described in detail below, from 0.02 to 0.2 mg. of polysaccharide could be determined by turbidity, with an accuracy of about 5 per cent. The vitreous humor carbohydrate was prepared by a slight modification of Meyer and Palmer's method (3) and used as a standard for the streptococcus polysaccharide estimations. The reaction is sensitive to variations in salt concentration and pH, disappearing almost entirely above pH 6.0 and below 3.0. It is inhibited in the presence of an excess of either serum protein or polysaccharide, resembling an immune precipitate in this respect.

From individual strains of varying degrees of virulence, a mucoid polysaccharide has been isolated both by the method of Kendall, Heidelberger, and Dawson (2) and by another method which seems to yield purer preparations. In addition, the various strains have been examined for the presence of this substance at different culture ages and a correlation with capsule size (5–7) established. This correlation of capsules with the nonantigenic mucoid polysaccharide is in agreement with Dawson, Hobby, and Olmstead's findings with group A streptococci (7). By means of the quantitative turbidity method to be described below, it has been possible to study certain aspects of capsule behavior more accurately than would be possible on a purely morphologic basis.

Strains of Streptococci Employed.—The same group C streptococci described in detail in the previous paper (1) have been investigated. Their properties are summarized in Table I. In addition, three new strains, 9, 10, and 11, have been included. Strain 9 (group C) was isolated from a chronic cervical abscess in a horse. This strain is mucoid, that is, it possesses capsules in young cultures and has a viscid type of colony. Strain 10 (α hemolytic) was isolated from bovine mastitis by Little (8), and since it falls into the Lancefield group C it has been examined. Strain 11 is another α hemolytic strain kindly furnished by Dr. P. F. Clark. It is not classifiable in Lancefield groups A, B, or C.

With some of the unstable strains it is difficult to obtain broth cultures in which the large majority of the organisms are either mucoid or non-mucoid. However, by avoiding more than two generations in 10 per cent serum broth, and by using 10 per cent blood agar plates as the source of inoculum, the amount of dissociation is usually negligible (1).

Preparation of Vitreous Humor Polysaccharide.—The method of Sevag (9), as applied by Meyer and Palmer (3), has been used, the main difference being the avoidance of acid in purification. 750 cc. of vitreous humor (from about 60 beef eyes) were squeezed through fine gauze 3 times and precipitated with 10 volumes of cold acetone. The precipitate was washed with acetone and ether, and redissolved by grinding in 100 cc. of water gradually added. This was reprecipitated with 5 volumes of alcohol plus a few drops of 20 per cent NaCl solution. The precipitate was thrown down in the centrifuge, and redissolved by grinding with 150 cc. of water gradually added. 1.5 gm. of NaCl were dissolved in this solution, which was shaken violently for ½ hour in the presence

of 30 cc. of chloroform and 3 cc. of butyl alcohol. The chloroform-protein jell was thrown down in the centrifuge and the supernatant decanted and saved. The jell was extracted with 50 cc. of water, the resulting extract precipitated with 5 volumes of alcohol and a few drops of 20 per cent NaCl solution, and this precipitate dissolved in the supernatant from the first chloroform treatment. Shaking with fresh portions of chloroform and butyl alcohol was continued until only a faint precipitate showed at the chloroform water interface after centrifugation. The colorless syrupy supernatant was negative to trichloroacetic acid and contained no phosphorus. It was brought to neutrality with HCl, and reprecipitated twice with 5 volumes of alcohol with the addition of a few drops

TABLE I

Summary of Strains

Strain No.	Source	Colony	Virulence	Capsules in young cultures	Growth in 10 per cent horse serum, 1 per cent dextrose digest broth
1	Appeared in a chronically infected colony of guinea pigs	Stable mucoid	Very great	+++	Diffuse
2	Strain 1	Stable smooth	0	0	Granular
3	Causative agent in chronic guinea pig lymphadenitis	Unstable mucoid	Moderate	++	Diffuse
4	Appeared in a colony chronically infected with strain 3	" "	Very great	+++	Slimy rope
5	Strain 4	" "	Slight	++	Diffuse
6	Strain 4	Unstable rough	0	0	Fibrous
7	Strain 3	Unstable smooth	0	0	Granular
8	Strain 6	Stable smooth	0	0	"
9	Cervical abscess in horse	Mucoid	0	+	Diffuse
10	Bovine mastitis (α hemolysis)	Smooth	?	0	"
11	Human bacterial endocarditis (α hemolysis)	"	?	0	"

of 20 per cent NaCl solution to increase flocculation. The resulting yield was 255 mg. Analytical data appear in Table II.

Quantitative Estimation of Mucoid Polysaccharides.—Horse, bovine, human, swine, chicken, guinea pig, and rabbit serum proteins are precipitated by the vitreous humor and the mucoid streptococcus carbohydrates at pH 4.2. Horse serum was used as routine as the most convenient source of serum. Since no two samples of serum give just the same turbidity in the presence of the carbohydrate, due probably to variations in their protein content, it was necessary to make up a large amount of stock diluted serum as follows: 50 cc. of clear horse serum were mixed with 450 cc. of 0.5 M acetate buffer at pH 4.2. The acidity was brought to pH 4.2 by the addition of 2 M HCl. As a preservative, 5 cc. of merthiolate[1] 1:1000 were added, giving a final concentration of 1:100,000. This clear solution may be stored in the refrigerator and used for several months. It should stand for 24 hours before use. To 5 cc. of 0.5 M pH 4.2 acetate

[1] Eli Lilly and Company.

buffer, was added 0.4 cc. of 0.1 M pH 7.0 phosphate buffer containing the material to be tested. After mixing, 1 cc. of the dilute serum solution was added and mixed immediately. After standing at room temperature for ½ hour,[2] the turbidity was determined in a Klett photoelectric colorimeter (Duboscq type), using various thicknesses of plate glass for comparison standards. The use of serum more dilute than 1:10 increased the sensitivity of the method, but at the same time inhibition of turbidity was brought about by smaller amounts of carbohydrate.

Turbidity curves were prepared with the purified vitreous humor carbohydrate as a standard, since none of the carbohydrates isolated from the mucoid streptococci gave a greater turbidity milligram for milligram than the vitreous humor material. Indeed, the purest preparation of mucoid streptococcus polysaccharide made from washed young organisms gave precisely the same turbidity on the basis of dry weight as the vitreous humor material. Therefore, although the estimations based on this method refer to milligrams of vitreous humor polysaccharide, they may also be referred to milligrams of purified mucoid streptococcus polysaccharide. It was necessary to prepare new standard curves for each new batch of serum.

Isolation of Mucoid Polysaccharide from Streptococci

For the isolation of the mucoid carbohydrate from group C streptococci two methods were employed. One was the method of Sevag (9) given by Kendall, Heidelberger, and Dawson (2) using broth supernatants of 24 hour cultures. The other, instead of using broth into which the organisms had liberated their carbohydrate, depended upon the extraction of the polysaccharide from young washed organisms, followed by purification procedures similar to the first method. The products obtained in these two ways were compared, the results appearing in Table II.

Preparation of Mucoid Polysaccharide from Broth.—100 cc. of 10 per cent horse serum, 1 per cent dextrose digest[3] broth were inoculated with 8 to 10 colonies directly from a blood plate. After 12 hours growth, this was added to 10 liters of 1 per cent dextrose digest broth which was incubated for 24 hours with occasional stirring. 50 cc. of concentrated phenol were added and after standing 15 minutes the organisms were removed in a Sharples centrifuge. The broth was concentrated to 0.1 volume *in vacuo*, the temperature not rising above 35°C. Octyl alcohol was added from time to time to prevent foaming. The concentrated broth was precipitated with 2 volumes of alcohol, after the addition of 100 gm. of sodium acetate and 25 cc. glacial acetic acid. The resulting precipitate was dissolved in 200 cc. of 5 per cent sodium acetate solution containing 2.5

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[2] After ½ hour the precipitate begins to become flocculent, especially in the higher carbohydrate concentrations.

[3] Digest broth is prepared as follows: To 400 gm. each of ground beef heart and pig stomach add 3 liters of distilled water at 55°C. and 35 cc. of concentrated HCl. After digesting for 18 to 20 hours at 50 to 55°C., 10 cc. N/1 NaOH per liter is added, the mixture brought to a boil, and cooled. It is then filtered through gauze, adjusted to pH 7.2, filtered through paper, and autoclaved.

per cent glacial acetic acid. 50 cc. of chloroform and 5 cc. butyl alcohol were added and the solution was shaken mechanically for ½ hour. After centrifuging and discarding the chloroform layer, fresh chloroform and butyl alcohol were added to the supernatant and the shaking and centrifugation repeated until only a faint ring of precipitate appeared at the chloroform-water interface. This usually required from 5 to 10 repetitions. The polysaccharide was then reprecipitated from 5 per cent sodium acetate, 2.5 per cent glacial acetic acid solution (100 cc.), with 1.25 volumes of alcohol until it was phosphorus-free. It was redissolved in 50 cc. of water and precipitated with 5 volumes of alcohol plus a few drops of half-saturated sodium acetate solution. The precipitate was washed with alcohol and dried *in vacuo* over $CaCl_2$.

Preparation of Mucoid Polysaccharide from Washed Organisms.—8 to 10 colonies from a blood plate were suspended in 200 cc. of 10 per cent horse serum, 1 per cent dextrose digest broth, and incubated for 12 hours. The entire culture was transferred to 2 liters of 1 per cent dextrose digest broth and incubated for 3 hours with occasional stirring. After cooling in an ice bath, the organisms were centrifuged and washed twice with 100 cc. of 0.85 per cent cold salt solution and resuspended in 100 cc. of the same. 1 cc. of chloroform was added with stirring, and the suspension incubated for 24 hours at 37°C. The organisms were removed in the centrifuge, further incubated for 6 hours with 50 cc. of fresh salt solution, and again removed. To the combined supernatants (150 cc.) was added 1.5 gm. of NaCl, 25 cc. of chloroform, and 2.5 cc. of butyl alcohol. The mixture was shaken mechanically for ½ hour, centrifuged, and the supernatant withdrawn. Fresh portions of chloroform and butyl alcohol were added to the supernatant until the chloroform-water interface was practically clear. After the final shaking, the carbohydrate was precipitated with 1.25 volumes of cold alcohol. The precipitate was dissolved in 10 cc. of water. If phosphorus was present it was reprecipitated with 1.25 volumes of alcohol and redissolved in 10 cc. of water. It was finally precipitated in a weighed centrifuge tube with 3 volumes of alcohol and 0.2 cc. of 20 per cent NaCl solution. After standing for 1 hour in an ice bath, the precipitate was thrown down in the centrifuge and the supernatant discarded. The sediment was dried *in vacuo* and weighed.

Some of the properties of the products obtained from 24 hour broth supernatants are compared in Table II with the products obtained from young washed organisms. In the first place, a close correlation of yield and virulence is to be noted with the carbohydrate from washed streptococci. It would also appear that the polysaccharide from washed organisms more nearly resembles the purified bovine vitreous humor material. It has a greater capacity to precipitate proteins at pH 4.2, although products matching the vitreous humor carbohydrate in this respect were obtained only from the highly virulent strains 1 and 4. The amount of reducing substances after hydrolysis (Hagedorn-Jensen) was greater in the broth preparations; in the washed organism material it was of the same order of magnitude as the vitreous humor carbohydrate. Glucose by the orcinol method was low both in the washed organisms and the vitreous humor material. This method does not detect glucosamine; however, no signif-

TABLE II

Comparison of Mucoid Polysaccharides from 24 Hour Broth Supernatants and from 3 Hour Washed Organisms

Strain No.	Method	Yield per liter	Mucoid carbohydrate*	Glucose H.J.†	Glucose orcinol‡	N§	Relative viscosity of 0.1 per cent solution‖	Glucosamine**	Titer in group C serum††
		gm.	*per cent*	*per cent*	*per cent*	*per cent*		*per cent*	
1	24 hr. broth	0.106	45	70	36	4.1	1.43	—	10^{-5}
	3 hr. organisms	0.0067‡‡	100	42	10	2.2	2.70	23	10^{-3}
2	24 hr. broth	0.023	1	70	42	3.9	1.03	—	10^{-5}
	3 hr. organisms	None							
3	24 hr. broth	0.069	20	80	42	3.8	1.17	—	10^{-5}
	3 hr. organisms	0.007	70	52	21	2.6	2.60	23	10^{-3}
4	24 hr. broth	0.107	30	62	36	3.5	1.33	33	10^{-5}
	3 hr. organisms	0.012	100	58	11	2.1	3.80	31	10^{-3}
5	24 hr. broth	0.040	20	73	57	2.7	1.20	29	—
	3 hr. organisms	0.005	50	40	14	2.5	2.10	28	—
6	24 hr. broth	0.081	0.8	60	40	4.2	1.00	33	10^{-5}
	3 hr. organisms	None							
7	24 hr. broth	0.056	5	75	42	3.8	1.03	—	10^{-5}
	3 hr. organisms	0.0018	50	30	16	—	1.70	17	—
8	24 hr. broth	0.038	1.5	72	44	4.2	1.01	—	10^{-5}
	3 hr. organisms	None							
Vitreous humor polysaccharide.....			100	45	13	2.7	1.9	27	Negative

* Relative capacity to precipitate proteins at pH 4.2. Thus a 1 per cent preparation requires 10 mg. to produce the same turbidity as 0.1 mg. of vitreous humor carbohydrate.

† Reduction method of Hagedorn and Jensen (10) after hydrolysis in 2 M HCl for 8 hours.

‡ Orcinol method of Sørensen (11) as modified by Herriott (12).

§ Micro Kjeldahl.

‖ Measured in Ostwald viscometer in 0.85 per cent NaCl solution at 21°C. Compared to same salt solution without carbohydrate.

** Glucosamine method of Elson and Morgan (13) as used by Kunitz (14). Standard and sample both hydrolyzed with 2 M HCl in sealed pyrex tubes for 8 hours.

†† Highest dilution giving a ring when layered over undiluted serum. 10^{-3} = 1 mg. per cc.

‡‡ This yield is low since it was not realized at this time that strain 1 does not develop its maximum polysaccharide until it is 6 hours old.

icant difference in the glucosamine content was observed in any of the materials regardless of their method of preparation. Nitrogen values were, in general, lower for the washed organism material, and the viscosity of these preparations was higher than in the polysaccharides prepared from broth supernatants.

Recovery of Carbohydrate from Serum Protein Precipitates

The possibility of recovering these carbohydrates from serum protein pH 4.2 precipitates was investigated, using purified vitreous humor material and two carbohydrates obtained from broth supernatants. One was from strain 4, containing 30 per cent mucoid polysaccharide, and the other from strain 7, containing only 5 per cent. These percentages were obtained by the turbidity method.

4 mg. of vitreous humor polysaccharide, 12 mg. of strain 4 carbohydrate from broth supernatant, and 80 mg. of strain 7 carbohydrate from broth supernatant were dissolved each in 60 cc. of 0.5 M acetate buffer, pH 4.2, and 14 cc. of dilute acid serum solution added. The mixtures were stirred and iced overnight. The resulting precipitates were centrifuged and redissolved in 1.5 cc. of 0.1 M pH 7.0 phosphate buffer with the addition of 1 drop of 1 M NaOH. The solutions were immersed in boiling water, and 6 cc. of boiling 5 per cent trichloroacetic acid quickly added to each and stirred, after which they were immediately iced and the precipitates removed in the centrifuge. The supernatants were then neutralized, precipitated with 30 cc. of cold alcohol, and the flocculent precipitates centrifuged out and dried. The precipitates from these three carbohydrates were found to resemble each other in amount, in glucose by the Hagedorn-Jensen method (30 per cent), by the orcinol method (10 per cent), and in the capacity to precipitate serum protein at pH 4.2. On testing in the group C precipitating serum, no reactions were obtained.

From these results it appears that materials closely resembling the vitreous humor polysaccharide can be recovered from serum protein precipitates of impure streptococcus carbohydrate preparations. Moreover, the turbidity test applied to impure carbohydrates seems rather accurately to indicate their content of vitreous humor-like material.

The Antigenicity of the Mucoid and Vitreous Humor Carbohydrates

Perhaps the clearest indication of the greater purity of the substances obtained from washed organisms was their reaction in a strong group C precipitating serum (strain 3). Solutions containing 1 mg. per cc. gave only weak reactions when layered over the undiluted serum. Further dilution abolished the reaction. On the other hand, the carbohydrates obtained from broth supernatants reacted, frequently strongly, in a dilution of 0.01 mg. per cc. The fact that a preparation containing more of

the mucoid polysaccharide reacts much less strongly with an antiserum is evidence that the mucoid polysaccharide is not concerned with the immune reaction. The group C sera used for these reactions also precipitated the A substance from commercial peptone (15) in a dilution of 1:400,000. It therefore is likely that precipitates formed by broth supernatant polysaccharides are due to A substance rather than to the group specific (C) streptococcus carbohydrate. Hirst (16), who suggested this possibility to us, has obtained similar results with group A streptococci. Further evidence that this material fails to function as a haptene is furnished by the following observations.

Rabbits injected with highly virulent mucoid strains only rarely produce precipitins. Strain 3 is a fairly regular exception to this. When such precipitins are formed they react with Lancefield extracts (17) from any of the strains, regardless of their virulence, as well as with the substances isolated from any of the strains by the method of Kendall, Heidelberger, and Dawson (2). Furthermore, the complete absorption of such precipitins (17) may be readily accomplished with avirulent strains containing almost none of the mucoid carbohydrate.

Guinea pigs carrying chronic abscesses due to strain 3 were injected intravenously with 0.1, 1, and 10 mg. of polysaccharide from washed organisms of strains 3 and 4. No symptoms of anaphylactic shock were observed.

6 rabbits were injected intraperitoneally with 3 doses of whole fresh bovine vitreous humor at 4 day intervals. The first dose was 2 cc., the second 4 cc., and the third 6 cc. 10 days after the last injection the sera were tested with the same vitreous humor by the ring method. 2 of the 6 gave only very weak rings with undiluted vitreous humor; a 1:10 dilution was negative. No reaction whatever took place in any dilution when these 2 sera were tested with the purified polysaccharide prepared from the same vitreous humor. A similar failure to obtain antibodies for the synovial fluid polysaccharide has been reported by Meyer, Smyth, and Dawson (18).

These results, in connection with the failure to demonstrate active or passive immunity to virulent strains (1), indicate that the mucoid polysaccharide from group C streptococci does not function as an antigen even when it is on the organism. This is in agreement with the findings of Kendall, Heidelberger, and Dawson (2) with the similar, perhaps identical, material from group A streptococci.

Purified carbohydrates from washed organisms of strains 1 and 4 had no influence on the invasiveness of virulent or avirulent strains in guinea pigs. The virulent organisms suspended in 0.1 per cent solutions of the polysaccharides and injected intradermally did not kill more rapidly than did control injections. Strain 3 in the presence of 0.1 per cent solutions of these substances produced the usual chronic infection. The virulence of strains 2 and 6 was not enhanced. A solution containing 10 mg. of the

virulent strain 4 carbohydrate was mixed with 10 cc. of melted nutrient agar and a plate made. Strain 4 plated out on this medium dissociated to the R form as rapidly as on plain agar. This was also true in broth containing the mucoid carbohydrate.

The purified carbohydrate from strain 4 (0.2 mg. per cc.) had no more spreading effect (19) in skin than did salt solution. This was tested by the intradermal injection of rabbits with 1 per cent trypan blue (vital) solutions containing the material. Incidentally, the findings of Pradhan (20) using this method were not confirmed since filtrates of 2 hour and 24 hour cultures of both invasive strain 4 and non-invasive strain 6 produced equal amounts of spreading on intradermal injection in rabbits. Pradhan does not mention the spreading effect of avirulent strains. He attributes this action to a capsular material although, according to his findings, 2 hour culture filtrates in which the amount of capsular substance would be minimal show the greatest activity.

Estimation of Mucoid Polysaccharide at Different Culture Ages

To secure more accurate data on the occurrence of the mucoid polysaccharide the quantitative turbidity method was applied to cultures at different stages in their growth cycles. In applying this method to the direct determination of mucoid carbohydrate from washed organisms, it was necessary to use different techniques for the mucoid and the non-mucoid strains.

Mucoid Strains.—Cultures were inoculated as usual by resuspending 12 hour organisms grown in 10 per cent horse serum, 1 per cent dextrose broth, in 10 volumes of fresh serum dextrose broth. They were placed in a 37.5°C. constant temperature bath. From these, 5 to 20 cc., depending upon the culture age, were withdrawn, iced, and centrifuged. For most of the mucoid strains it was necessary to use a very fast angle centrifuge with flat 5 cc. tubes to obtain sedimentation. The broth supernatant was removed, and the sediment taken up in 0.1 M pH 7.0 iced phosphate buffer, using the same volume as that of the original broth. This was again centrifuged, the supernatant discarded, and the sediment suspended in 5 cc. of 0.1 M pH 7.0 phosphate buffer in a graduated 15 cc. centrifuge tube. This was immersed in boiling water for 20 minutes to free the polysaccharide from the organisms. After cooling, the organisms were sedimented and used for N determinations by micro Kjeldahl. 0.4 cc. of the clear supernatant was used for the turbidity estimation as described above.

In the broth supernatants of the mucoid strains it was possible to determine directly the amount of polysaccharide. Standard turbidity curves using broth carbohydrate mixtures were made for each batch of serum broth used. 0.4 cc. was sufficient to give readable turbidities in older cultures. The estimation of small amounts in broth is inaccurate, since the dextrose fermentation alters the pH very noticeably. Broth supernatants from the avirulent strains did not contain enough polysaccharide to give a detectable turbidity.

50 per cent horse serum broth was substituted for 10 per cent. It was found that both the nitrogen and carbohydrate values were about twice as high in this medium, but relatively the same differences between strains appeared. Therefore 10 per cent serum broth was used as routine for reasons of economy.

Non-Mucoid Strains.—For those strains which produce very small amounts of this material it was necessary to use the entire extract in the turbidity estimation. 5 to 20 cc. (generally the latter) of the culture were washed in 0.1 M pH 7.0 phosphate buffer as before, and the sedimented organisms suspended in 2.5 cc. of distilled water. 0.4 cc. of 0.1 M pH 7.0 phosphate buffer was added, the tube immersed in boiling water for 20 minutes, cooled, centrifuged, and the entire supernatant added to 2.5 cc. of 1 M pH 4.2 acetate buffer. 1 cc. of the serum solution was added and the turbidity, if any, was read as usual in ½ hour.

The results of these studies are given in Charts 1, 2, and 3. Not only is the amount of carbohydrate on the organisms of significance, but also the length of time that it persists. This is indicated by the breadth of the peak, as well as by the curve showing the amount of material liberated into the broth. In the highly virulent strains 1 and 4, the organism is encapsulated over a longer period of time than in the moderately virulent strains 3 and 5. The photographs are of Wright's stained films made at the indicated times. That there is some correlation between the amount of polysaccharide and the size of the capsule at any given time is clear, yet it is extraordinarily difficult on a purely morphologic basis to determine precise degrees of capsulation. The intermediate position occupied by the avirulent mucoid horse strain 9 is shown. It is interesting that although this strain is encapsulated at 3 hours, and devoid of its capsule at 6 hours, yet the carbohydrate curve fails to show a corresponding drop. It is very probable that this method determines not only capsular but somatic polysaccharide as well, and that the capsule in strain 9 represents only a small percentage of the total. The α hemolytic strain 10 developed about half as much of this carbohydrate as strain 9. In strain 11, also α hemolytic, none could be detected. Strain 4 with 20 mg. per cent sulfanilamide added to the serum broth gave the same carbohydrate and nitrogen values as are shown for that strain in Chart 1. The results presented in the charts are representative. At least three determinations with each strain have given good agreement.

Rate of Decapsulation

To determine under uniform conditions the speed with which the various mucoid strains lose their capsular polysaccharide the same turbidity method was employed.

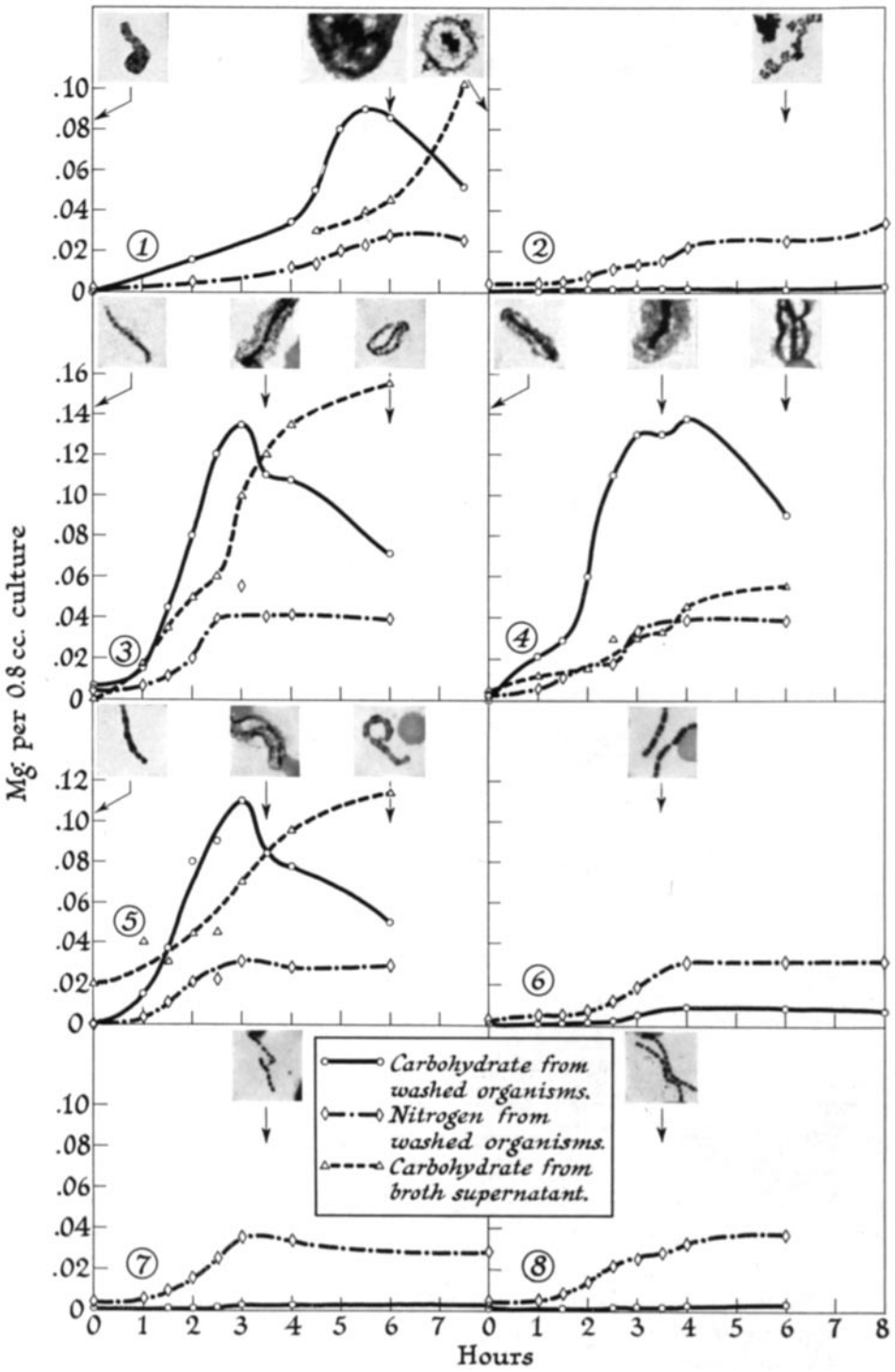

CHART 1. *1*. Very virulent mucoid. *2*. Avirulent smooth from *1*. *3*. Moderately virulent mucoid. *4*. Very virulent mucoid. *5*. Moderately virulent mucoid from *4*. *6*. Avirulent rough from *4*. *7*. Avirulent smooth from *3*. *8*. Avirulent smooth from *6*. Photographs of Wright's stained films. × 935.

The organisms were grown as usual. At the height of their capsulated phase, as determined from Chart 1, they were iced, sedimented, washed once, and resuspended in 0.1 M pH 7.0 phosphate buffer. The final volume was half that of the original culture. A sample was placed in boiling water for 20 minutes and the total mucoid carbohydrate estimated on 0.4 cc. of the resulting supernatant. The remaining suspension at 37.5°C. was sampled at various times and the carbohydrate in 0.4 cc. determined after removing the organisms in the centrifuge. On each suspension studied in this way, nitrogen estimations gave essentially the same values. The results are expressed as the percentages of total mucoid polysaccharide extracted at 100°C.

Chart 4 shows the relative rates with which the different mucoid strains liberate their polysaccharide. The virulent strains 1 and 4 liberate it more slowly than the less virulent strains 3 and 5.

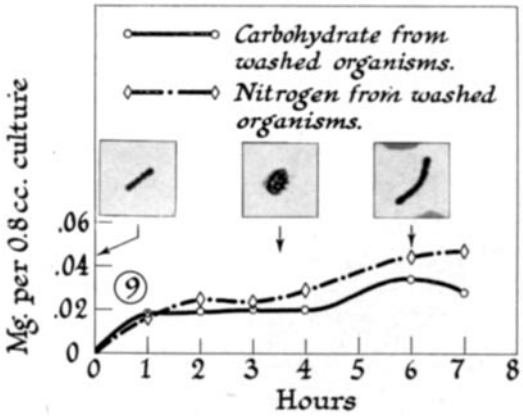

CHART 2. *9.* Avirulent mucoid from horse. Photographs of Wright's stained films. × 935.

Various factors affecting the rate of decapsulation of strain 4 were examined. Suspensions were prepared as above and adjusted to hydrogen ion concentrations ranging from pH 4.5 to 9.0. Chart 5 indicates that the minimum rate is found around pH 6.0; as more acid or more alkaline reactions are reached, the rate increases. This was confirmed by capsule stains. The presence of 0.05 per cent sodium taurocholate, as well as 3 per cent chloroform, increases the rate of decapsulation, as shown in Chart 6. Higher concentrations of sodium taurocholate are even more effective. Bile diluted 1:10, as in the pneumococcus solubility test, results in complete disappearance of the capsule in less than 15 minutes. This latter effect was necessarily determined by capsule stain, since it was found that concentrations of sodium taurocholate above 0.1 per cent act as protein precipitants at pH 4.2. Somewhat unexpectedly, it was also found that continu-

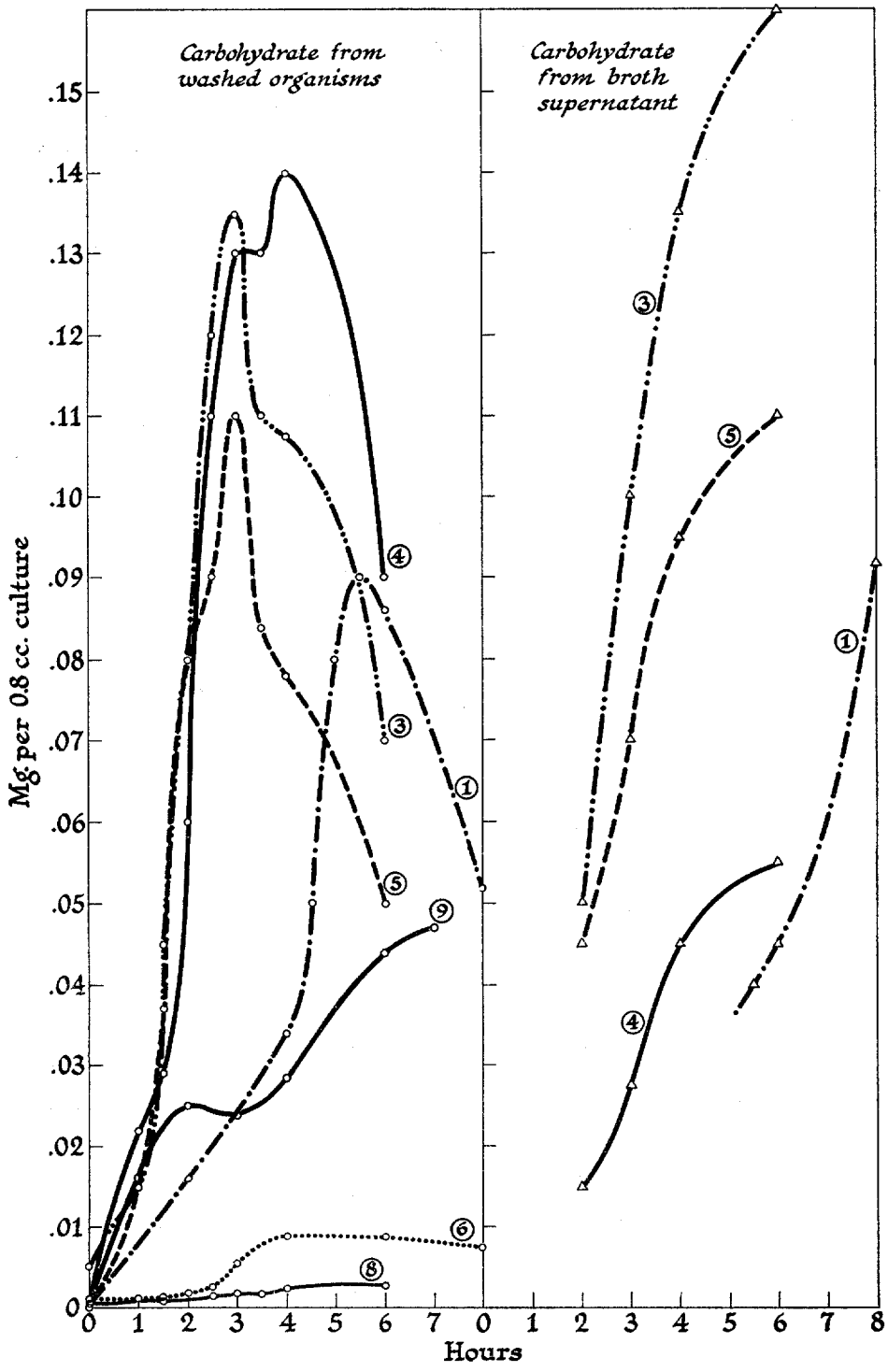

CHART 3. Carbohydrate curves from Charts 1 and 2, collected for comparison.

ous rapid stirring, or bubbling of oxygen or nitrogen through the suspension of washed streptococci, failed to affect the rate of decapsulation. The retarding effect of cold is indicated in Chart 6.

These observations show that the loss of the capsule from group C hemolytic streptococci, with the concomitant appearance of a soluble mu-

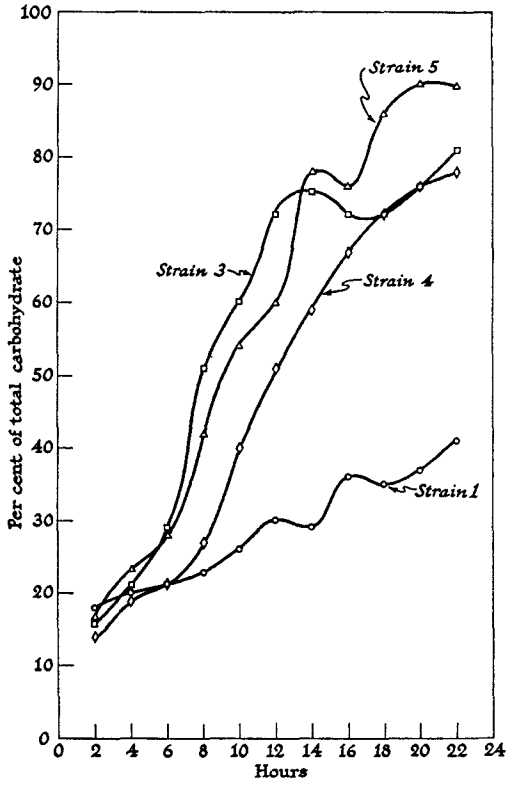

CHART 4. Rate of decapsulation of washed young organisms in 0.1 M pH 7.0 phosphate buffer at 37.5°C. Values are expressed as percentages of the total mucoid polysaccharide extracted at 100°C. *1.* Very virulent mucoid. *3.* Moderately virulent mucoid. *4.* Very virulent mucoid. *5.* Moderately virulent mucoid.

coid carbohydrate, is an autolytic process rather than simple solution. In some respects it resembles the autolysis of the pneumococcus, being accelerated by bile or bile salts. Unlike the pneumococcus autolysis, it is arrested not at all by heat, and to only a limited extent by formalin. Attempts at "flash heating" of capsulated streptococci have failed to prevent their immediate decapsulation.

DISCUSSION

It is apparent that the invasiveness of the group C hemolytic streptococcus is in some way associated with the presence of a non-antigenic polysaccharide similar to, if not identical with, that obtained from group A by Kendall, Heidelberger, and Dawson (2). Those strains which possess

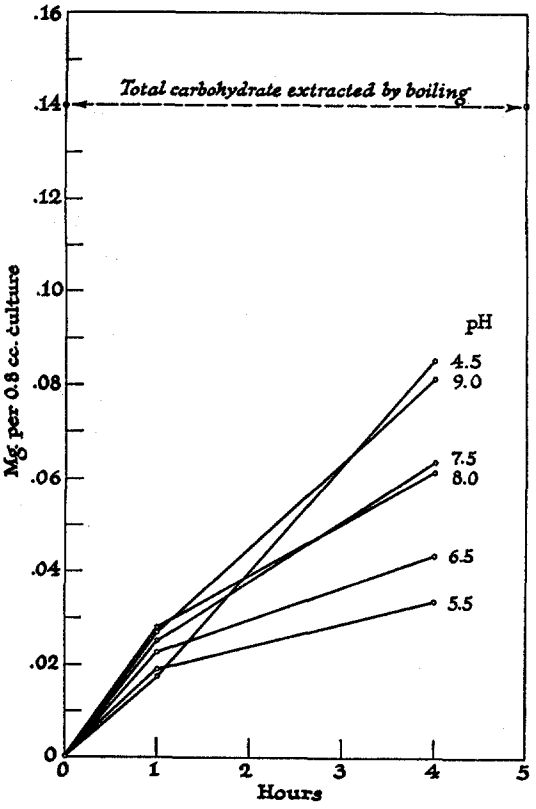

CHART 5

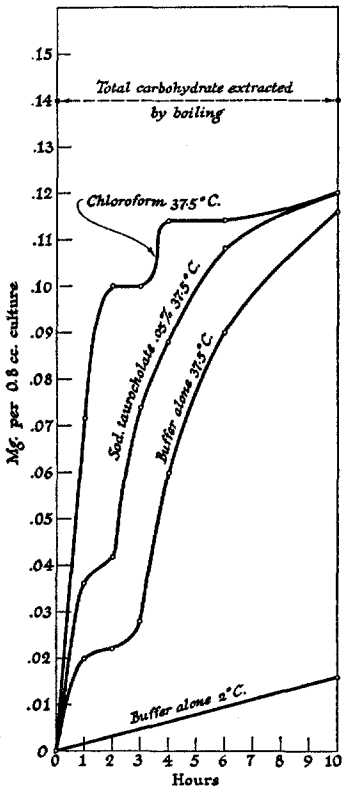

CHART 6

CHART 5. Effect of pH on decapsulation of 3 hour old, strain 4 organisms.

CHART 6. Effect of sodium taurocholate, chloroform, and cold on decapsulation of 3 hour old, strain 4 organisms.

appreciable amounts are mucoid. The correlation of the non-antigenic mucoid polysaccharide with capsules which develop in young cultures further implicates this substance as a virulence factor. These relationships have already been suggested (7) in connection with the group A hemolytic streptococci. Moreover, most of the strains isolated from serious infec-

tions in man are mucoid (21, 22). However, non-invasive mucoid capsulated strains have been encountered from time to time. The quantitative studies presented here show that there are very different degrees of mucoidness, and that the mucoid carbohydrate stays with some strains longer than with others. Similar quantitative data in connection with capsule persistence and invasiveness in the pneumococcus have been reported by Shaffer and Enders (23, 24).

These variations in the quantity and persistence of the mucoid polysaccharide may be sufficient to explain differences in invasiveness. Comparing the highly invasive strains with the entirely non-pathogenic ones, the differences in the amounts of mucoid carbohydrate produced are very significant. Perhaps less significant are the differences encountered between highly virulent strains and those which tend to localize. That no other factors influence invasiveness is unlikely. At the same time, the failure to demonstrate protective antibodies in group C (1) increases the likelihood that the mucoid polysaccharide is of considerable importance in disease production.

Protection experiments with group C streptococci differ from those reported for group A. It is possible, though sometimes difficult, to secure active and passive protection as well as to demonstrate the presence of opsonins for group A organisms. It is very probable that anti-M antibodies (25) are responsible for these effects. The examination of other group C strains might show a similar possibility. However, since the non-antigenic polysaccharide is present in group A strains, it is not out of the question that this substance also contributes to their virulence. The method which has been described for the quantitative estimation of the polysaccharide may prove useful in determining the extent to which it affects virulence in group A streptococci.

Loewenthal (26) has recently reported that vaccines made from mucoid group A strains by heating at 56°C. for 12 minutes stimulate type specific protective antibodies against group A mucoid strains with great regularity. He found that polysaccharide preparations made from broth supernatants by the method of Kendall, Heidelberger, and Dawson (2) precipitated strongly in antistreptococcal sera. The reaction was not type specific; a similarly prepared carbohydrate from a pneumococcus also precipitated. Absorption of sera with these materials failed to remove their protective antibodies. He concluded that the mucoid polysaccharide is serologically active, and that it is unrelated to invasiveness.

In the light of our experience with group C strains and that of Hirst (16) with group A, it is probable that the precipitin reactions obtained by

Loewenthal are to be attributed to the presence of A substance from peptone (15), or possibly group specific C substances in the relatively impure carbohydrate preparations he employed. Moreover, the assumption on the basis of a cross precipitin reaction that the Type III pneumococcus contains the same mucoid polysaccharide seems unjustified without further chemical characterization of this substance.

Since this study was submitted for publication, Henle and Henle (27) have presented findings which substantiate the idea that the mucoid carbohydrate is non-antigenic. They also report the presence of blood group A substance in those mucoid carbohydrates prepared from broth supernatants. Our observation that streptococcus antisera contain A substance precipitins differs from the experience of Henle, and suggests that these precipitins may constitute an important source of error. The discrepancy may be due to the fact that Henle used neopeptone broth, while our studies were carried out with a digest medium.

SUMMARY

A non-antigenic mucoid polysaccharide similar to that described by Kendall, Heidelberger, and Dawson was isolated from group C hemolytic streptococci. A simple method for its quantitative estimation is described.

By means of this quantitative method, as well as by the direct isolation of the carbohydrates, the size and persistence of capsules in young cultures of various strains have been related to the non-antigenic mucoid polysaccharide.

Similarly, invasiveness in different strains has been related to the mucoid polysaccharide. Data indicating the non-haptene nature of this material are presented.

An autolytic process, accelerated by bile, is involved in the loss of capsular material from young streptococci.

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