VARIANTS OF HEMOLYTIC STREPTOCOCCI; THEIR RELATION TO TYPE-SPECIFIC SUBSTANCE, VIRULENCE, AND TOXIN.

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In previous communications one of us (1) described three substances which can be extracted from hemolytic streptococci: (1) *The nucleoprotein P* is common to all strains of hemolytic streptococci and is serologically related to the nucleoproteins of pneumococci and of green streptococci. (2) *The non-protein substance C*, which appears to be a carbohydrate, is found in all strains of hemolytic streptococci but is species-specific and serologically distinct from the carbohydrate fractions of pneumococci and green streptococci. (3) *The type-specific fraction M*, which is probably protein in nature, has not been isolated from other species of microorganisms; it occurs in serologically distinguishable forms which serve to differentiate hemolytic streptococci into types.

One of us (2) has previously described two forms of hemolytic streptococci distinguishable by the morphology of their colonies. The general appearances of these colonies, when grown on a special medium and viewed by reflected light, are the same as those which distinguish the rough and smooth varieties of other bacteria but the terms "R" and "S" have not been used and the colonies have been designated "matt" and "glossy" to avoid confusion which would otherwise certainly arise from the circumstance that the rough, or matt, colonies are the virulent type while the smooth, or glossy, forms are relatively avirulent. It is the purpose of this paper to show that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies.

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It will also be shown that filtrates from both matt and glossy cultures of hemolytic streptococci contain skin-reactive toxin.

Some Characteristics of the Type-Specific Substance M.

The type-specific substance M is prepared by a modification of Porges' (3) method. The bacterial bodies are extracted with N/20 HCl in salt solution at the temperature of boiling water; and after neutralization and clarification by centrifuging the resulting clear slightly yellow fluid is used as an antigen for precipitin reactions. Bacterial extracts prepared in this manner contain the type-specific substance M and in addition they also contain small quantities of the non-type-specific fractions, P and C, which may cause some precipitation with sera prepared against any strain of hemolytic streptococcus. To avoid the appearance of these confusing precipitates the serum may be absorbed with any heterologous strain of hemolytic streptococcus and by this means a specific anti-M serum is obtained which will only precipitate in the presence of the homologous M substance.

In the original work (1) which led to the recognition of the type-specific substance M thirteen strains were used which Dochez, Avery and Lancefield (4) had classified, some years earlier, into types by agglutination and protection tests. Ten of these strains yielded type-specific precipitating substances which differentiated them into types corresponding to those originally determined. The remaining three strains failed to yield any type-specific substance although they had been classified by agglutination and protection in the earlier work. It was suggested that prolonged cultivation in the laboratory had caused these three strains to lose their type-specific characteristics. In the present communication it will be shown that matt cultures containing the type-specific substance can, by various means, be reduced to the glossy form in which the type-specific substance is no longer present.

The Preparation of Anti-M Serum.

As the type-specific substance M, after separation from the bacterial cell, does not produce any demonstrable antibody, when injected into animals, the only antigen available for preparing anti-M serum

is a suspension of bacteria in the matt form. Matt cocci contain the three substances P, C and M and sera prepared with these organisms, therefore, contain antibodies to each of the three substances. On the other hand, cocci in the glossy form contain the two substances P and C but are devoid of the type-specific substance M; consequently, antibacterial sera prepared with glossy strains contain antibodies to P and C but do not contain any type-specific antibody.

Anti-M sera were prepared by inoculating rabbits intravenously with 16 hour cultures of matt cocci grown in tryptic broth. Immunization was commenced with four injections on consecutive days of 1 cc. of heat-killed culture, followed a week later by four doses of 2 cc. of the same vaccine. During the 3rd week the rabbits received four doses of 0.5 cc. of living culture and in the 4th week this dose was doubled. A final series of doses of 2 cc. of living culture was given during the 5th week and the animals were bled 10 days after the last injection. The sera of animals immunized in this way usually contained a satisfactory quantity of antibody to the type-specific substance M but with some strains it was necessary to continue immunization with 5 cc. and even 10 cc. of living culture before useful sera could be secured. A few strains have been encountered which, although they were in the matt form and moderately virulent for mice (0.001 cc. or 0.0001 cc.), produced only traces of type-specific antibody in rabbits even after intensive immunization. Twelve rats were immunized with a strain (New York V E14) which had previously failed to produce more than traces of type-specific antibody in the sera of twelve immunized rabbits. The rats, which remained perfectly well during immunization, received the following intraperitoneal doses: 1st week 3 doses of 0.25 cc. of heat-killed culture; 2nd week 4 doses of 0.5 cc. of heat-killed culture; 3rd week 4 doses of 1.0 cc. of heat-killed culture; 4th week 3 doses of 1.0 cc. of living culture; 5th week 4 doses of 2.0 cc. of living culture; 6th week 4 doses of 2.0 cc. of living culture. Seven of the immunized rats yielded moderately good anti-M sera; the remaining five sera contained traces of type-specific antibody. As rats appear to be able to tolerate relatively larger doses of culture than rabbits it is possible that they may be more suitable for the preparation of anti-M serum; but the small yield of serum makes this method impracticable for routine purposes.

The Absence of the Type-Specific Substance M from Organisms Which Form Glossy Colonies.

It has already been stated that the type-specific substance M is found in HCl extracts of matt hemolytic streptococci and that it is not found in similar extracts prepared from the glossy variants.

This is demonstrated by the following experiment:

Four type-specific anti-M sera were prepared by immunizing rabbits with four matt strains of hemolytic streptococci belonging to different serological types and by subsequently removing the non-type-specific antibodies from the sera by absorption with heterologous strains.

Table I gives the precipitin reactions of the four sera with HCl extracts prepared from cultures of the homologous cocci (1) in the matt form and (2) in the glossy variant form.

TABLE I.

Precipitin Reactions of Type-Specific Anti-M Sera with Extracts of the Homologous Strains (1) in the Matt Form, (2) in the Glossy Form.

Volumes of extracts*	Strain S43		Strain S23		Strain C203		Strain London	
	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form	1 Extract from matt form	2 Extract from glossy form
cc.								
0.4	+++	−	++++	−	++	±	++	−
0.1	++	−	++	−	++±	−	++±	−
0.025	+	−	+	−	++	−	+±	−

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

It will be seen from Table I that the extract prepared from the matt form of each strain gave a good precipitin reaction with the homologous antiserum; on the other hand, the extract prepared from the glossy form of each strain gave a negative precipitin reaction with the single exception of Strain C203 which gave a faintly positive reaction. Although this experiment seems to show that each of the four strains lost its type-specific substance in the process of degradation to the glossy variant form, yet it will be seen from the results of experiments with highly concentrated extracts that, in reality, only one strain had completely lost its type-specific substance. Highly concentrated extracts were prepared from each of the four glossy variants referred to in Table I in the following manner: The centrifuged deposit from 9 liters of broth culture was extracted with HCl; and, after concentration by alcoholic precipitation, the precipitate was redissolved in

5 cc. of salt solution. Precipitin tests, with the concentrated extract prepared from Strain S23 were negative showing that this strain was completely devoid of type-specific substance. Precipitin tests, with the other three concentrated extracts and their homologous specific anti-M sera, were weakly positive showing that three of the glossy cultures retained traces of type-specific substance. The minute amounts of specific substance remaining in these cultures can be judged from the following figures—9 liters of broth culture were used in preparing extracts from the glossy forms—50 cc. of broth culture were used in preparing extracts from the matt forms—180 times more culture was, therefore, used in the preparation of the glossy extracts than in the preparation of the matt extracts and, in spite of these disproportionate quantities, the latter extracts contained the larger quantity of the type-specific substance M. It appears from these experiments that hemolytic streptococci are rarely degraded to the point at which type-specific substance completely disappears.

Some Characteristics of Matt Cultures of Hemolytic Streptococci.

Twenty-eight strains of hemolytic streptococci were examined immediately after isolation from pathological conditions in the human body. The sources of these cultures included cases of puerperal septicemia, pleural effusion, scarlet fever, pneumonia and sinusitis; strains were also isolated from the depths of enucleated tonsils and from throat swabs. In twenty-one cases the cultures when freshly isolated, were entirely composed of matt colonies; in five cases both matt and glossy colonies were seen on the plates; and in two cases the cultures were entirely glossy, but as both the glossy strains were obtained from throat swabs and were accompanied by other bacteria there was no evidence that the hemolytic streptococci were playing a pathogenic rôle. Table II gives the source and character of the cultures.

There is, therefore, some evidence that cultures freshly isolated from human sources are usually of the matt variety and this statement particularly applies to diseases such as septicemia in which the streptococci are the undoubted causal agent.

It is frequently found that matt strains of hemolytic streptococci, isolated from human lesions and undoubtedly pathogenic for man, are

TABLE II.

The Morphological Appearance of Colonies of Freshly Isolated Strains of Hemolytic Streptococci and the Source of the Cultures.

No.	Disease	Source of culture	Pure culture or mixed flora	Morphology of colonies
1	Puerperal septicemia	Blood culture	Pure culture	Matt
2	" "	" "	" "	"
3	" "	" "	" "	"
4	Nephritis and septicemia	" "	" "	"
5	Endocarditis	Postmortem culture from spleen	" "	"
6	Pneumonia and pleural effusion	Chest fluid	" "	"
7	Sinusitis	Nasal mucus	" "	"
8	Scarlet fever	Throat swab	Almost pure culture	"
9	" "	" "	" " "	"
10	Tonsillitis	" "	" " "	"
11	Enlarged tonsils	From depths of enucleated tonsils	" " "	"
12	" "	" "	" " "	"
13	" "	" "	" " "	"
14	" "	" "	" " "	"
15	Pharyngitis	Swab from pharynx	" " "	"
16	Sinusitis	" " nose	" " "	"
17	Tonsillitis	" " tonsils	" " "	"
18	"	" " "	" " "	"
19	Pneumonia	Sputum	Chiefly *S. hæmolyticus; B. influenzæ*	"
20	" (Type IV pneumococcus)	"	Mixed flora	"
21	" " " "	"	Equal numbers hemolytic streptococci and pneumococci	"

22	Pneumonia (Type IV pneumococcus)	Sputum	Mixed flora	Matt and glossy
23	Enlarged tonsils	From depths of enucleated tonsils	Pure culture	" " "
24	" " "	" "	Almost pure culture	" " "
25	" " "	" "	" " "	" " "
26	" " "	" "	" " "	" " "
27	Tonsillitis	Swab from tonsils	Mixed flora, about 5 per cent hemolytic streptococci	Glossy
28	Pneumonia (Type IV pneumococcus)	" " "	Mixed flora	"

avirulent for mice (M.L.D. 0.5 cc. or 1.0 cc.). Such a culture entirely composed of matt colonies will be referred to in this paper as the matt attenuated form because it is avirulent for mice yet possesses the colony characteristics and the specific substance of the virulent form. Attempts to increase the virulence of matt attenuated cultures, by mouse passage, have always been successful although some of the strains tested have required very many passages before the maximal virulence of 0.000001 cc. has been attained and in some cases the virulence has never risen above 0.0001 cc. even after 80 or 90 consecutive passages through mice.

Virulence is the only quality which distinguishes the matt virulent form from the matt attenuated variety as these colonies are identical in appearance, and serological examination of HCl extracts does not show any significant difference in the quantity of type-specific substance which can be extracted from equal volumes of the two cultures. From these experiments it appears that the matt form, which is always potentially virulent, may occur in, at least, two separate varieties characterized by quantitative differences in virulence for mice; and it is probable that, by suitable passage experiments, additional forms can be obtained distinguishable by different degrees of virulence for other species of animals.

Methods of Converting the Matt Form to the Glossy Variant.

The degree of ease with which glossy variants can be obtained from different strains varies enormously. In some cases great difficulty is experienced in maintaining laboratory stock cultures of the matt, or potentially virulent, form as they spontaneously change to the glossy variant even when stored in blood broth in the ice box. In these cases it is necessary to resort to frequent mouse passages to prevent the total loss of the matt form. On the other hand, matt strains have been encountered which do not show any tendency to change to the glossy variant after repeated subcultivations on agar. Intermediate between these extremes are strains which develop a small proportion of glossy colonies after repeated subcultivations on agar. When one of the glossy colonies derived from these strains is subcultured in broth and replated on agar a mixture of matt and glossy colonies usually appears but occasionally a pure glossy culture

may be obtained by this method. By repeated selection of glossy colonies and subcultivation in broth a culture composed entirely of glossy colonies can often be obtained and in some instances a pure culture of the glossy variant can be secured by the simple process of repeated subcultivation on agar slants.

Griffith (5) and others (6–8) have shown that smooth pneumococci can be converted to the rough form by cultivation in the homologous anti-S serum. We have applied this technique to hemolytic streptococci and have found that the cultivation of matt strains in undiluted homologous anti-M serum of high titer is the quickest and most reliable method of obtaining glossy variants, and with some highly stable matt strains this is the only method by which we have been able to secure the glossy form. Here again there are wide differences between individual strains; in some cases, a few transfers in serum suffice to convert a virulent matt culture, containing abundant M substance, into the glossy avirulent variant devoid of any specific substance; in other cases, after as many as 90 transfers in high titer serum traces of the type-specific substance M can still be detected in concentrated bacterial extracts, although the colonies appear to be glossy and the organisms have lost their virulence for mice. Attempts have been made to rid these cultures of the remaining traces of type-specific substance by alternately cultivating the cocci in immune serum, plating out, selecting the most glossy colonies and again subculturing in immune serum; one strain was subjected to this treatment thirty times after 90 previous consecutive transfers in immune serum but at the end of the experiment it still retained traces of the specific substance.

Some Characteristics of Glossy Cultures of Hemolytic Streptococci.

The glossy variant is avirulent for mice in comparison with the matt virulent culture from which it is derived and attempts to raise the virulence of the variants by mouse passage have usually been unsuccessful. It is, however, possible to obtain glossy cultures which are partially virulent for mice. A strain which, in the matt virulent form, killed mice regularly in doses of 0.000001 cc. or 0.0000001 cc. was cultivated in the homologous anti-M serum and, after 55 transfers in 50 per cent serum, a pure culture of the glossy variant was ob-

tained which did not contain any type-specific substance, yet this glossy culture was sufficiently virulent to kill mice regularly in doses of 0.01 cc. and occasionally in doses of 0.001 cc. More prolonged cultivation in anti-M serum did not cause any further decrease in virulence. The partially virulent glossy culture was passed through twenty-five mice intraperitoneally but the virulence remained unchanged and there was no reappearance of type-specific substance. This appears to be an exceptional strain as the M.L.D. of the majority of glossy strains is 0.5 cc. or 1.0 cc.

During the process of conversion from matt to glossy various types of colonies appear, which may possibly represent intermediate forms or may be due to individual colonies containing a mixture of matt and glossy cocci. We have observed that different strains sometimes show peculiarities in the morphology of their colonies which are so striking that the strain can be recognized either in the matt or glossy form. In addition to these strain peculiarities other varieties of colonies appear during the gradual change from matt to glossy. Griffith (9) has noted that in spite of the apparently diverse appearances of streptococcal colonies three forms can generally be distinguished. Two of his forms appear to correspond to our matt and glossy colonies and the third is characterized by a soft consistency, a whitish opaque raised center and a thin translucent margin. In a previous communication one of us (10) described a similar form of colony, which differed in the important respect of being tough instead of watery; but further observation has shown that colonies characterized by a flat marginal zone surrounding a central eminence may occur in two forms, corresponding to the matt or to the glossy state. The matt form of this colony is opaque and of tough consistency with a central eminence surrounded by a flat marginal zone; the glossy form has a similar contour but is soft and watery. The irregular shape of these colonies causes difficulty in observing the light-reflecting character of their surfaces but the matt and glossy forms can generally be distinguished by other characteristics. These observations seem to indicate that this third type of colony is not a distinct entity separate from the matt and glossy forms; but we have failed to determine the significance of these very characteristic colonies.

The classification of colonies is further complicated by the occasional appearance of pseudoglossy forms. When a matt culture is spread on a plate the colonies in close proximity to each other may present the typical matt appearance but widely separated colonies in the same culture may be glossy in appearance. If one of the latter colonies is selected and spread on a fresh plate a pure culture of typical matt colonies may result. Pseudoglossy colonies are generally larger than true matt or true glossy colonies but they so nearly resemble the true glossy form that they are liable to cause confusion.

Owing to these variations in the appearance of colonies we have been unable to rely entirely on the colony form as a guide to the character of cultures. The criteria we have used to determine when a culture is completely degraded from the matt state are: (1) that concentrated HCl extracts of glossy cultures should not cause any precipitation when mixed with pure homologous anti-M serum (absorbed with a heterologous strain to remove the antibodies to P and C); (2) that the result of the above test should remain unchanged after the culture has been passed through a mouse.

It will be seen in the detailed description of experiments that we have only been able to secure one strain in this completely degraded state.

Reversion of Glossy Cultures to the Matt Form.

Dawson and Avery (11) have shown that many strains of R pneumococci can be reverted to the S form by repeated mouse passages or by cultivation *in vitro* in anti-R serum. Griffith (12) has shown that R pneumococci frequently revert when they are mixed with large doses of heat-killed S pneumococci and inoculated subcutaneously into mice.

We have attempted to revert glossy hemolytic streptococci to the matt form by each of these three methods.

1. Mouse Passage.—Passage experiments have been done with glossy cultures derived from five different strains (S3, S23, Henson, S43, C203) but no definite evidence has been obtained that reversion can be achieved by this method. In two cases, S3 (ten passages) and Henson (twenty-five passages), the glossy character of the cultures was judged entirely by the appearance of the colonies as no anti-M serum was available for these strains.

The virulence (0.1 cc.) and colony form of Strain S3 remained unchanged after passage through ten mice.

Passage of Strain Henson through twenty-five mice caused the virulence of the culture to rise from 0.5 cc. to 0.01 cc. and this change was accompanied by a slight alteration in colony form, many of the colonies in the passage culture having flat tops instead of the typical dome-shaped appearance of glossy colonies. In this instance the partial restoration of virulence and the accompanying change in colony structure may possibly indicate that reversion had commenced but the evidence is inconclusive in the absence of serological proof that the culture had been completely degraded before the mouse passages were commenced.

The glossy culture of Strain S23 appeared to be completely degraded as no trace of precipitate was formed when highly concentrated HCl extracts were mixed with the homologous type-specific antibody. This culture was passed through twenty-seven mice and at the end of the experiment the virulence for mice (0.01 cc.) and the colony form remained unchanged and there was no reappearance of type-specific substance.

The glossy culture of Strain S43 was not completely degraded as, although unconcentrated extracts of the variant culture did not precipitate the homologous anti-M serum, yet traces of type-specific substance could be demonstrated in concentrated HCl extracts. This culture was passed through a series of mice and examination of unconcentrated extracts, after each passage, showed the gradual reappearance of type-specific substance so that after eight passages the culture was equal to the original matt form in its yield of type-specific substance. The virulence (0.1 cc.) and colony form were unaltered by eight passages but this experiment seems to indicate that a culture which has lost the major part of its type-specific substance and yet retained a fraction of its original specificity can be reverted to the original form with comparative ease.

The variant culture of Strain C203 formed typical glossy colonies but concentrated HCl extracts of the cocci contained traces of type-specific substance. After passage through ten mice the virulence (0.1 cc.) and the colony form of the culture remained unchanged and there was no increase in the quantity of type-specific substance which could be extracted from the cocci.

2. Cultivation in Immune Serum.—A glossy culture of Strain New York V was obtained which failed to kill mice in a dose of 0.5 cc. Rabbits were immunized with this culture and a serum was obtained which agglutinated the glossy culture up to a dilution of 1 in 2,560. The glossy culture was grown in various dilutions of this serum (5 per cent, 10 per cent, 50 per cent, 100 per cent) for a number of transfers but in no case was there any evidence of reversion—50 per cent serum was selected as the concentration in which the cocci appeared to multiply most freely and the culture was carried 118 transfers in this medium. At the end of the experiment the form of the colonies and the virulence of the culture remained unchanged.

Immune serum prepared against glossy cocci contains antibodies to the two non-type-specific fractions, P and C. In the following experiment the influence

of pure anti-P serum on glossy cultures of four strains was tested. High titer anti-P serum was prepared by immunizing rabbits with purified nucleoprotein extracted from hemolytic streptococci. The glossy variant forms of four strains (S43, S23, C203, London) were cultivated for twelve transfers in a 10 per cent dilution of this serum. This treatment did not alter the colony forms of the cultures although the virulence of two strains (S23 and London) was definitely increased. The quantities of type-specific substance, however, which could be extracted from the cocci of all four strains remained unchanged.

3. Subcutaneous Inoculation of Mice with Glossy Cultures in Combination with the Homologous Matt Cocci Killed by Heat.—A few experiments have been done with one of our glossy strains (Henson) in an attempt to revert this culture to the matt form by the technique devised by Griffith for the reversion of R pneumococci to the S form. A heavy suspension of the matt culture was prepared by heating, at 60°C. for 30 minutes, the deposit from 50 cc. of culture, concentrated to 2 cc. 0.5 cc. of the heat-killed suspension was mixed with 0.05 cc. of living glossy culture and injected subcutaneously into a mouse. Cultures from the lesion in the mouse contained both matt and glossy colonies although controls indicated that the matt organisms of the heated suspension were dead. Unfortunately, this technique, so successful with pneumococci, is not altogether satisfactory for hemolytic streptococci as it has been found impossible to avoid ulceration when large numbers of heat-killed matt organisms are combined with glossy cultures and even small doses of glossy culture alone frequently cause ulceration. Matt colonies isolated from these open ulcers must be viewed with suspicion since they may arise from contaminating cocci, but it seems probable that further work with this technique may yield convincing evidence of the reversion of the glossy cocci to the matt form.

So far as any conclusions can be drawn from the limited number of observations recorded it seems that the glossy variant is a highly stable form but that reversion may occur under certain conditions.

A Comparison of the Toxigenicity of Matt and Glossy Cultures of the Same Strain.

The method used for comparing the toxigenicity, the virulence for mice and the colony appearance of matt and glossy cultures of the same strain was as follows:

Young broth cultures of the different forms of each strain were sown in 50 cc. of tryptic digest broth. After 16 hours incubation the virulence and colony appearance of a sample taken from each culture were determined; and the flasks were then returned to the incubator. The cultures were filtered after 4 days of incubation and the filtrates were tested by injecting 0.1 cc. of diluted filtrate into

TABLE III.

A Comparison of the Toxigenicity and Virulence of Matt and Glossy Cultures of the Same Strain.

	Identification number of patient	Filtrate from matt virulent culture	Filtrate from matt attenuated culture	Filtrate from glossy culture
1. Bronchopneumonia strains				
Strain S43. Active filtrate diluted 1 in 500	1	15 mm. (10^{-7})	15 mm. (10^{-1})	13 mm. (10^{-1})
" " Boiled " " 1 " 500		11 "	10 "	8 "
" " Active " " 1 " 50	1	27 "	23 "	26 "
" " Boiled " " 1 " 50		18 "	9 "	14 "
" S23. Active " " 1 " 100	1	22 " (10^{-7})	—	20 " (10^{-2})
" " Boiled " " 1 " 100		6 "	—	11 "
2. Scarlet fever strains				
Strain C203. Active filtrate diluted 1 in 1,000	1	28 " (10^{-7})	—	20 " (less than 10^{-1})
" " Boiled " " 1 " 1,000		2 "	—	7 "
" N. Y. V. Active " " 1 " 1,000	2	18 " (10^{-6})	—	19 " (10^{-1})
" " Boiled " " 1 " 1,000		0		0
" " Active " " 1 " 1,000	3	26 mm. (10^{-5})	—	32 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0
" " Active " " 1 " 1,000	4	10 mm. (10^{-5})	—	10 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0
" " Active " " 1 " 1,000	5	11 mm. (10^{-5})	—	18 mm. (10^{-1})
" " Boiled " " 1 " 1,000		0	—	0

The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.
0 indicates no reaction; — indicates not tested.

the skin of one, or more, known positive reactors. Table III gives the dimensions of the reactions which followed the injection of active filtrate and of the same filtrate after heating in boiling water for 2 hours. Measurements, which are given in mm. representing the average diameter of the skin reactions, were taken 24 hours after injection. The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.

The two strains isolated from cases of bronchopneumonia produced weak toxic filtrates in comparison with the scarlet fever strains and were therefore used in greater concentration.

It will be seen from Table III that the filtrates from the different forms of each strain caused approximately equal reactions and that no correlation could be established between virulence and toxigenicity.

DISCUSSION.

It may be stated as a broad generalization that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies. This is analogous to the invariable presence of the soluble specific substance S in virulent cultures of pneumococci and its absence from avirulent R cultures, but here the analogy breaks down as far as virulence for mice is concerned, since it is possible to prepare matt cultures of hemolytic streptococci which contain large quantities of the type-specific substance M and yet are avirulent for mice.

One of the most striking characteristics of hemolytic streptococci is the difficulty which has always been experienced in securing highly virulent cultures of a large number of strains. This is undoubtedly due in part to the fact that the glossy variant is a highly stable avirulent form but even when we exclude this variant form and confine our attention to the matt or potentially virulent varieties we are still unable to secure highly virulent cultures with any degree of ease. The behavior of matt attenuated cultures undergoing mouse passage is frequently capricious; virulence generally rises to a moderate level after the initial ten, or twenty passages and it may then increase suddenly, or it may gradually increase after many more passages, or it may remain for an indefinite period in a state of mediocrity. This is in contrast to pneumococci which appear to be either rough and avirulent for mice or smooth and of maximal virulence for this

species. The virulence of pneumococci appears to be intimately associated with the presence or absence of the soluble specific substance S; in the case of hemolytic streptococci, however, virulence is not entirely dependent on the presence or absence of the type-specific substance M; some additional unknown factor is operative. Glossy variants, when fully degraded, contain no type-specific substance and are avirulent for mice; matt organisms occur in two forms equally rich in type-specific substance—one of these forms is no more virulent for mice than the glossy variant, the other is highly virulent.

It is possible that this contrast between pneumococci and hemolytic streptococci may be partly due to differences in bacterial structure. In the case of pneumococci the soluble specific substance S is disposed in a capsular layer over the surface of the organism; but microscopic examination of hemolytic streptococci gives no information as to the situation of the type-specific substance M in the bacterial bodies; and it is possible that the distribution of this substance throughout the organisms may render it less accessible and therefore less susceptible to external influences.

Certain strains of hemolytic streptococci exhibit an unexpected stability in all forms. Cultures which are partially degraded so that they contain only small quantities of type-specific substance may be passed through a number of mice without any accumulation of type-specific substance and without any alteration of virulence. Conversely, when the virulence of a matt strain has become established it is difficult to reduce the culture to the matt attenuated state and at the same time to avoid conversion to the glossy variant form.

No relationship could be established between toxigenicity and virulence; in some instances highly virulent matt cultures produced weak toxic filtrates and the glossy variant avirulent forms were equally toxigenic; in other instances relatively avirulent matt strains produced highly toxic filtrates.

It appears therefore that virulence is not determined by toxigenicity and is not entirely dependent on the presence or absence of type-specific substance although cultures which have lost their type specificity are invariably avirulent.

An unknown factor determines whether hemolytic streptococci,

which contain their full quota of type-specific substance, are virulent or attenuated.

SUMMARY.

Hemolytic streptococci, when freshly isolated from pathogenic lesions, form characteristic matt colonies and contain the type-specific substance M.

Two varieties of matt cultures, equally rich in type-specific substance, can be distinguished by the virulence of the organisms for mice: (1) the matt virulent variety, (2) the matt attenuated variety.

The matt forms of hemolytic streptococci can be degraded to a third variety which forms glossy colonies and is always relatively avirulent. This is accomplished by prolonged cultivation on artificial media, by selection of colonies or by cultivation in homologous anti-M serum. In the process of degradation the cocci lose the major part of their type-specific substance but complete disappearance of type-specific substance rarely occurs.

The glossy variant form, when fully degraded, is highly stable; but glossy cultures which have retained some type-specific substance can occasionally be reverted to the original matt form.

Toxic filtrates from matt and glossy cultures are approximately equal in skin reactivity.

No relationship appears to exist between virulence and toxigenicity.

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