DEGENERATION AND VARIATION OF GONOCOCCI

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I. THE CULTURAL BEHAVIOUR OF DEGENERATING GONOCOCCI

In a previous paper (Casper, 1937a) we reported our investigations concerning the serological typing of 109 strains from acute cases, and some strains from chronic cases, of gonorrhea. Using two completely type-specific sera, we were able to classify a large number of the strains by means of comparative agglutination.

Several difficulties arose in the classification of the remaining strains. Some strains which we could not classify by comparative agglutination were also unclassifiable by the agglutinin-absorption test. At times, the agglutinin-absorption test gave inconsistent results even with our test strains used for immunization.

We also called attention to the fact that the cultivation of suitable test strains of gonococci is exceedingly difficult. In view of the lack of suitable culture media and of our incomplete knowledge of the optimum conditions for the cultivation of this organism, the danger of degeneration must always be considered. Experience has shown that degenerative changes in the cocci, which are not necessarily accompanied by a change in morphology, often lead to changes in their antigenic properties. Thus, the test strains with which diagnostic sera are prepared and which serve as controls in the specific absorption of strains under examination, offer numerous sources of error.

It is well known that the type-specific carbohydrate of the

pneumococcus, which is always present in organisms freshly isolated from human cases and virulent for mice, may be lost rapidly upon cultivation on artificial media. It is also known that the loss of type-specific carbohydrate is accompanied not only by a loss of virulence for mice, but also by a loss of specificity in all immune reactions. Conversely, pneumococci which by mouse-passage have become very virulent for these animals, give strongly type-specific immune reactions. Thus, we have at our disposal a means of maintaining the type-specificity of these organisms. Griffith (1928) points out that virulent pneumococci are entirely insensitive to the antibodies of the avirulent cells (R-pneumococci) and, consequently, are unable to exhaust the agglutinins from an R-serum. He also showed that the R-variant was unable to remove the type-specific agglutinins of the mother-strain. The same author (1920) was able to split off a serological variant of a type-specific meningococcus. This variant, without showing any cultural signs of degeneration, could not remove the agglutinins from a type-specific serum. He directed attention to the fact that such cultures could give rise to diagnostic errors.

Schiemann (1929) reported on the occurrence of "pseudotypes" in pneumococci and meningococci. In agar cultures of old laboratory strains of meningococci, he found a characteristic variation in colony morphology. The colonies were very small and represented a new-serological type. These strains were not agglutinated by type I or type II serum, but only by the serum of a degenerated type I strain. This "pseudotype" he never found in strains freshly isolated by lumbar puncture. Sometimes, however, it was found in strains obtained from pharyngeal cultures of meningococcus carriers. The sera of such "pseudotypes" were, naturally, unfit for the diagnosis of cases which actually occurred in practice.

Atkin (1925) attempted a classification of the gonococcus on the basis of colony morphology. He found, in fresh cultures of acute cases of gonorrhea, large papilla-bearing colonies which he designated type I. After prolonged incubation of these, a small papilla-free variant was split off. This variant he called type II.

He considered it especially noteworthy that he was able to isolate strains identical with his type II variant from cases of chronic gonorrhea. These strains were found most frequently in the cervix. According to Atkin's observations, the small papilla-free colonies are overgrown soon after isolation by the large papilla-bearing colonies but again gain predominance in aging cultures. He claimed that the papilla-free organisms were an individual pathogenic type, and assumed that classification based on colony morphology was at least as valuable as classification by serological methods. He prepared a "type I" serum with a strain freshly isolated from a male patient and his "type II" serum with an old laboratory strain showing only papilla-free colonies and originally isolated from a cervix. With these sera he was able to distinguish: (1) early forms which gave no serological reactions, but which, on further development, reacted with his "type I" serum and after the splitting off of "type II" reacted with his "type II" serum, (2) "type I" strains, (3) "type II" strains, (4) strains reacting with both "type I" and "type II" sera. A few strains were not classifiable according to his serological methods.

These investigations appear particularly interesting if one considers our observations that, in chronic gonorrhea, we found numerous strains which reacted as strongly with our type I serum as with our type II serum.

Experimental

Atkin has stated that the degenerated forms can be observed only on a semi-solid pea-broth or horse-serum agar at a pH of 7.8. While optimum conditions for growth are provided on horse serum or ascitic agar at a pH of 7.5, the gonococci tend to autolyze because of rapid growth and, as a consequence, die before the formation of papillae has begun. A pH of 7.8, on the other hand, delays growth, impedes autolysis and thus creates the conditions conducive to degeneration.

In our experiments we closely observed several strains of gonococci which had been cultivated for a long time. After testing a large group of media, we found that the best results

were obtained with our blood-water agar (Casper, 1929). On this medium, which has a pH of 7.5 to 7.6, large single papilla-bearing colonies were formed, so that it appears as if the semi-solid state plays a more important rôle than the pH. After three to five days on this medium, transplants could still be made from every portion of the colony. According to Atkin, degeneration proceeds in the following manner: At the time of cultivation on alkaline medium, a group of papillae are formed on the colony. Transplantation after five days reveals that only the papillae-bearing portions of the colony are living. Lenz and Schaefer (1936) assume that the papillae are centers of regeneration of the colony while all the other parts of the colony are dead.

We did not find dissociation of papilla-free from papilla-bearing colonies so simple. In our transplants, firstly, the papilla-free portion was not dead and, secondly, papilla-bearing colonies like those with which we started were obtained from both the papilla-free portions and from the individual papillae. In the first cultures, some completely smooth colonies were found after transplantation. These, however, proved to be non-viable, and we were not able to obtain pure papilla-free cultures in this way.

In order to obtain papilla-free colonies, we turned to culturing the papilla-free central part of the colony which, according to Atkin, is supposed to consist of confluent papillae. By this means, we obtained some colonies which produced only a few papillae after numerous transplants. As a rule, transfers were made after 4 to 5 days growth in the incubator at 37°C. By transferring only the smooth part of the colony, we eventually obtained pure papilla-free cultures. The smooth part of the colony was triturated in saline and one drop of a 1:100,000 dilution placed on a blood-water agar plate and spread with a platinum spatula, so that as many and as large colonies as possible might be obtained.

The original plate of the test strain, transplanted simultaneously, exhibited the same characteristics as on the first day. In the case of our type I gonococcus, the first transplants showed some colonies with both mesial and marginal papillae (fig. 1).

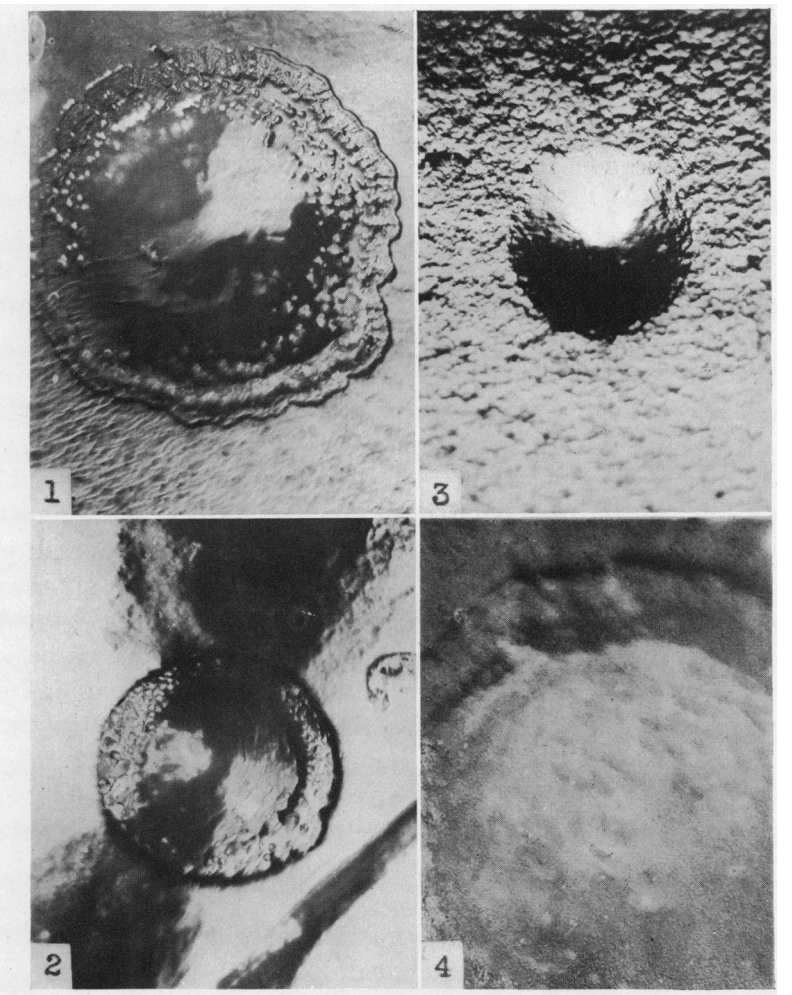

FIG. 1. SPECIFIC COLONY WITH PAPILLAE, AND FLAT, CONVOLUTED BORDERS
Papillae both marginal and central

FIG. 2. PAPILLA-BEARING COLONY AFTER CULTIVATION AND IN THE PROCESS OF TRANSFORMATION TO THE SMOOTH VARIANT

Papillae only marginal, center almost completely smooth, border no longer convoluted but almost round.

FIG. 3. TYPICAL SMOOTH FORM

Colony has no border, conic, completely smooth, no papillae. The apparent irregularities are merely the reflexion of the rough surface of the agar.

FIG. 4. VARIANT OF THE SMOOTH FORM

Colony concave (center has sunk). Border quite round. No papillae.

These papillae became very small and marginal only, then finally disappeared (fig. 2). In the subcultures, which showed only papilla-free colonies, there occurred a split into colonies of two different sizes. One, a small hemispherical (fig. 3) and the other, a more concave colony which was larger than the first (fig. 4).

Papilla-free colonies were picked out of mixed cultures for the initial injections in the production of antisera. Later the pure papilla-free cultures could be used. The procedure for preparation of the antisera has already been described (Casper, 1937a).

The transformation of our type II gonococcus culture followed a similar course. In this case, as in type I, most of the colonies also had numerous papillae and there were a few papilla-free colonies to begin with. These, however, proved to be non-viable and transplants were made from the papilla-free portion of a papilla-bearing colony. Type II also underwent several reversions to the papilla-bearing form. Strangely, there appeared large, papilla-free, staphylococcus-like whitish colonies, which, however, proved to consist of gram-negative bean-shaped diplococci. As in case of type I, we first had to use the papilla-free colonies of suitable cultures for the immunization of rabbits. Later, we obtained serviceable papilla-free cultures.

The papilla-free colonies appeared sooner than those of type I and two modifications arose, large and small smooth colonies. By subculturing, we obtained both of them in pure form.

We found no differences in the gram-staining properties of the gonococci in the original culture, in the concave papilla-free colonies, or in the large and small papilla-free colonies. We never observed those gram-positive gonococci reported by some authors (Raven 1934, etc.).

In similar fashion, a number of other gonococcus cultures which had been cultivated over a period of several months to over a year, produced both types of colonies, with and without papillae, on our blood-water agar. In every case we were able to obtain both forms in pure culture. This, however, required about three months after the original transplants had been made. We examined four each of our type I and type II and some of

our overlapping strains. The papilla-free and papilla-bearing forms of the overlapping strains were not tested serologically.

Cross-serological reactions in reciprocal sera were now undertaken with the type I strain (98) and the type II strain (116). The results of the tests of these strains, with and without papillae, and the sera obtained from them are given in table 1.

From this table it is seen that we were unable to establish those sharp serological differences between the serological types and their variants which Atkin described. We must consider, however, that Atkin produced the "type II" serum used in his diagnostic experiments with laboratory strains which had been cultivated for two years, and that he was not completely successful in verifying, by serological examination, the derivation of his "type II" from a "type I" strain. Similar to our findings, he could only describe an increase in the related reactions, which in our experiments, were most pronounced with the smooth variant.

To us, however, it seems most important that this table clearly shows that type II, as far as regards its ability to form papillae as well as its serological specificity, is to be considered as an individual type and not a derivative of type I transformed by degeneration.

Since immunization of our rabbits was begun at a time when our strains had not yet completely lost their ability to form papillae, the experiment had to be enlarged so that more exact knowledge might be gained as to how great the serological differences due to degeneration may become. Nevertheless, our results show that degenerated strains are more sensitive to overlapping antibodies.

According to the prevailing conception, we must assume that, as in the case of pneumococci, the type-specific carbohydrate masks the remaining antigenic valencies of the gonococcus.

In this experiment it would have been possible to classify our papilla-bearing type I strain on the basis of its reactions with the antisera of the degenerated variants of both types. Classification of the type II strain, however, could not have been reached on this same basis since its reactions with the sera of the degen-

TABLE 1

Cross agglutination tests of the typical (papillae-bearing) strains of types I and II and their smooth variants with the corresponding antisera

STRAINS TESTED	NORMAL RABBIT SERUM			ANTISERUM OF THE PAPILLAE BEARING STRAIN 98						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 98						ANTISERUM OF THE PAPILLAE BEARING STRAIN 116						ANTISERUM OF THE SMOOTH VARIANT OF STRAIN 116					
	25	50	100	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800	25	50	100	200	400	800
Strain 98 type I with papillae	0	0	0	++	++	++	++	++	+	++	++	+	±	±	−	±	−	−	−	−	−	±	−	−	−	−	−
Smooth variant of strain 98	0	0	0	+	++	+	+	±	−	++	++	++	++	+	+	+	+	+	±	±	−	+	±	±	−	−	−
Strain 116 type II with papillae	0	0	0	±	−	−	−	−	−	++	+	±	−	−	−	++	++	++	++	+	+	++	++	±	−	−	−
Smooth variant of strain 116	±	±	±	+	+	±	±	−	−	+	±	±	±	−	−	++	++	++	++	++	+	+	++	++	±	±	−

erated types were appreciably different. Apparently the smooth variants differed in the degree of their degeneration. This is seen from the fact that the smooth variant of type II gave a ± reaction in 1:100 normal serum while the smooth variant of type I gave a negative reaction.

We have not investigated the question as to whether there is a specific early form without papillae, which, as Atkin assumes, is responsible for the relatively frequent lack of reactivity between freshly isolated type I gonococcus strains and their homologous sera and which, after a short period of cultivation gives a type-specific reaction. On the basis of our serological experiments, however, there is no indication for the assumption of such a form.

The use of unclassified papilla-bearing strains for the preparation of antiserum seems to assure no absolute protection against the appearance of co-agglutinins. Despite this, it should be valuable to carry out a control of the cultural behaviour, on suitable media, of the strains which are to be used for immunization and for testing the serum. Of course, the demonstration of type-specific precipitating antibodies in diagnostic sera with the aid of the specific carbohydrates (Casper, 1937b) offers far greater certainty.

Summary

Strains from fresh cases of acute gonorrhea in the male and classified as two distinct serological types were transformable from the papilla-bearing to the papilla-free form.

The formation of papilla-free colonies is a sign of degeneration probably due to growth on artificial media. Diagnostic sera prepared with such degenerated cultures are very likely to give rise to errors in classification.

Classification based only on colony morphology does not serve to exclude the possibility that we may be dealing, not with different types, but with degenerated forms of the same type which have only lost their type-specific carbohydrate.

II. THE SEROLOGICAL BEHAVIOUR OF DEGENERATED GONOCOCCUS STRAINS

The difficulties arising in the classification of freshly-isolated gonococci (Casper, 1937a) caused by non-specific reactions with type-specific immune sera have been previously indicated. In order to clarify the question as to whether the test-strains with which our immune sera were prepared had degenerated, or whether the immune sera themselves had changed, we resorted to the agglutinin-absorption test.

By means of absorption we were able to classify some strains which had given overlapping reaction with our type-specific immune sera. No definite conclusions, however, could be reached by this method with strains from chronic gonorrhea since all these strains absorbed the agglutinins of both the type I and type II sera.

It is well known that pathogenic bacteria in artificial cultures degenerate easily, and, as has been shown in the case of pneumococci, lose their ability to produce the type-specific factor. In this way they become agglutinable by the antisera of a type which does not influence the undegenerated strain. For, every pneumococcus possesses, besides the type-specific carbohydrate, factors common to all types. These common factors cannot be demonstrated because, as the cell is intact, the type-specific factor masks the reaction. In fact, due to the precipitation of these overlapping parts of the antigen, autolyzed type-specific cultures react with the antisera of heterologous types. In the same way, a partially degenerated culture might show overlapping reactions. In such cases, therefore, the types could be differentiated from each other only by absorption test. But, in an absorption test, the question always arises as to whether the overlapping reaction was caused, providing that one works with type-specific test strains and test sera, by the common protein antigen or by the presence of an individual type-specific carbohydrate related to two different type-specific factors.

We observed the serological behaviour of gonococcus test strains, previously type-specific, but which, as a consequence of

long cultivation, had undergone degeneration. A few strains had been cultivated over a period of several years. Thus, strain 1 (type I) and strain 25 (type II) were observed for six and five years respectively. From time to time, these strains were tested by agglutination in homologous and heterologous sera and also by agglutinin-absorption. Agglutination tests with one typical strain after degeneration are described in table 2.

These strains retained their specificity for a long time. Later, however, simultaneous with the drop in the titer of the homologous serum from 1:1600 to 1:400, there appeared strong agglutination in heterologous serum and also spontaneous agglutination.

TABLE 2

Changing serological behaviour of a type I gonococcus after long cultivation. Reversion to type specific agglutination by cultivation in citrated blood after apparent complete degeneration

STRAIN 1 (TYPE I)	TYPE I SERUM	TYPE II SERUM	NORMAL SERUM	SALINE
At the time of isolation, November, 1927..	1:1600+	0	0	0
After cultivation, October 1930.....	1:400+	1:400+	1:400+	±
After transplantation to citrated guinea pig blood.....	1:400+	0	0	0

We were able to eliminate the spontaneous agglutination in the following manner: The culture was transplanted on 14 successive days in bouillon, to which increasing quantities (10, 25, 50 per cent) of citrated guinea pig blood had been added. This strain now gave blood water agar cultures which did not agglutinate in either saline or normal serum. At this time we found practically no co-agglutination in heterologous serum.

In table 3 are seen the results of absorption tests with strains 1a and 25a, i.e., strains 1 and 25 after degeneration. These strains were tested with antisera prepared from them while they were in the degenerated state. At the same time, they were tested with the antisera of type-specific strains.

Table 3 reveals the following facts: A degenerated strain is capable of absorbing the agglutinins from a type-specific serum

(tests 1, 2, 4, 5). Thus, it remains possible to classify type-specific strains even though degenerated strains be used for absorption.

The agglutinins of the serum prepared with a degenerated strain are not absorbed by a fresh heterologous strain (test 8). This shows that a serum prepared with a degenerated strain, which has lost most of its specific carbohydrates, may still retain some of its type-specific qualities. Although these de-

TABLE 3

Absorption tests with freshly isolated and old laboratory strains and their corresponding antisera

TEST NUMBER	TYPE SPECIFIC IMMUNE SERUM	STRAIN USED FOR ABSORPTION	EXAMINATION OF THE NON-ABSORBED SERUM WITH					EXAMINATION OF THE ABSORBED SERUM WITH				
			Type I strains		Type II strains			Type I strains		Type II strains		
			1a	76	25a	57	72	1a	76	25a	57	72
1	41 type I	1a	1,600	800	50		50	50	25	50		50
2	57 type II	1a	100			1,600		25			1,600	
3	57 type II	25a			1,600					800		
4	72 type II	25a			1,600		1,600			400		1,600
5	72 type II	25a					1,600					400
6	72 type II	72					1,600					100
7	72 type II	1a					1,600					1,600
8	1a type I	72	800					800				
9	1a type I	1a	800					200				
10	1a type I	25a	800					200				
11	1a type I	25a	400					200				
12	25a type II	25a			400					50		
13	25a type II	1a			400					50		
14	25a type II	72			400					50		

generated strains can evoke a type-specific immunological response *in vivo*, they themselves are no longer capable of reacting type-specifically. This can be seen from the overlapping reactions between the degenerated strains and their reciprocal antisera (tests 10, 11, 13).

The degree of degeneration of any particular strain may vary from day to day. In a very degenerated state, it cannot absorb the agglutinins from a type-specific serum (test 3). At such time it would be unsuitable for use as a test strain. In a more

specific state, however, the agglutinins are very strongly absorbed (test 1, 2, 4, 5). This demonstrates that a degenerated strain under certain unknown conditions may regain its type-specific factor.

Discussion

The gonococcus, like the streptococcus and pneumococcus, has been classified into several serological types. Upon cultivation on artificial media, these types undergo changes which induce the loss of their type-specific characteristics. Thus, it is seen that the behaviour of the gonococcus is quite analogous to that of the pneumococcus and streptococcus.

Investigations on the classification of streptococci (Griffith, 1934) have revealed the occurrence of certain types which are capable of absorbing the specific agglutinins from heterologous sera. Cultivation of a type-specific strain may so alter its agglutinability that it can be agglutinated, not only by its homologous antiserum, but also by a heterologous antiserum. This may occur even though there are no apparent changes in morphological or cultural characteristics. Similar observations have been made on the pneumococcus. Griffith (1928) and Schiemann (1929), have demonstrated the existence of cultural variants which have lost their type-specificity. Thus, S-organisms are not agglutinated by a serum prepared with an R-strain. It is well known that those changes which induce the loss of type-specificity of pneumococci are due to a loss of specific carbohydrate and are accompanied by a loss of virulence.

The peculiar behaviour of gonococci observed in agglutination and agglutinin-absorption tests might also be attributed to certain changes which affect their type-specificity, that is to say, the characteristic or function upon which their serological identity depends.

The loss of type-specificity which is responsible for such pronounced variations in serological behaviour is a phenomenon observed in the serology of many bacterial species which differ widely in morphology, antigenic composition and pathogenic action. In the light of these considerations it is easily under-

standable that difficulties must arise in the classification of long-cultured strains if one uses diagnostic immune sera which have not been tested on many freshly isolated strains. The fact that the so-called "pseudotype" described by Schiemann reverted into a known type after seeming to be a new one for some time, shows that freshly isolated strains must be tested in the establishment of the pathogenicity of any serologically "peculiar" type.

The loss of type-specific carbohydrate and the concurrent loss of type-specific immune reactivity is not a sudden one, but as we showed for the gonococcus (Casper 1937 b), proceeds slowly and gradually. It is, therefore, probable that at certain times a degenerating strain may possess sufficient carbohydrate for the recognition of its original type, while at other times it will give quite non-specific reactions. Along the same lines, we have seen that many reversions took place during our attempts to transform papilla-bearing to papilla-free strains, and that this transformation could only be accomplished after a very considerable number of transplants. These papilla-free forms are not capable of eliciting a type-specific immune response and the antisera prepared with them cannot be used in the classification of type-specific strains. Thus, one may say, that the loss of type-specific carbohydrate increases the ability to elicite type-specific immune response decreases. However, the 5-and 6-year old laboratory strains must have retained some of their original qualities so that at times they were able to give type-specific reactions. Whether all three changes; loss of type-specific carbohydrate, transformation to papilla-free forms and overlapping reactivity occur at the same time, we have not determined. Probability speaks for it.

If we recognize the fact that the type-specific antigenic complex is apt to be broken up before reaching the antibody-forming cells so that it becomes exceedingly difficult to prepare type-specific immune serum with them, the possibility must be considered of a change of the same degree of rapidity, but of reverse order. In other words, in the transference of gonococci, even from acute gonorrhea, to artificial media, such rapid degenera-

tion may occur that they may not only give overlapping reactions but may also be agglutinated by normal serum or saline. That these are signs of degeneration can be seen from table 2. This may account for the relatively high frequency of non-type-specific, overlapping strains, isolated from acute cases of gonorrhea.

We must now consider still another possible source of degeneration. It is known that throat cultures of meningococcus-carriers (Griffith, 1920) have yielded strains serologically different from those obtained by lumbar puncture. These strains, for the most part, are not of any definite type, but correspond to an intermediate stage, i.e., their overlapping valencies are more pronounced than those of strains from acute infections. The strains which we isolated from chronic gonorrhea never were a definite type, but always showed overlapping valencies and agglutination in saline and normal serum. Thus, we may assume that the gonococcus, by adaptation to human tissue, undergoes the same degenerative process which we see after adaptation to artificial media. This degenerative process, including the decrease of type-specific carbohydrate, carries with it a decrease of virulence. We have seen that under certain conditions type-specificity may be regained and thus may explain the exacerbation of symptom-free infections.

Summary and conclusions

1. Associated with the loss of carbohydrate in old gonococcus cultures, there is a developing relationship between formerly heterologous strains.

2. Since, at the time of isolation of gonococci from acute cases of gonorrhea, one cannot estimate the degree of their degeneration, this fact may explain the relatively high frequency of overlapping strains.

3. A theory has been advanced that in chronic gonorrhea the gonococcus, by adaptation to the human tissue, may undergo the same degenerative processes that occur after prolonged cultivation on artificial media.

III. THE SLIDE AGGLUTINATION OF TEST STRAINS OF GONOCOCCI

In view of the relatively frequent occurrence of gonococcus strains which were equally strongly agglutinated by each of two type-specific sera (Casper, 1937 a) we decided to investigate the question as to whether it was also possible to split off colonies from type-specific strains which would react with the serum of the heterologous type.

It is well known that in the cultivation of the specific phases of paratyphoid bacilli, subcultures with mixed phases are found so that unless special precautions are taken (adequate serological controls and cultivation of pure phases) most of the sera are mixed phase (Andrewes, 1922). Despite the presence of the non-specific phase in freshly isolated strains these mixed-phase sera may be sufficient for differential diagnosis, since, in these sera, the non-specific phase is "masked" and, therefore, does not exert any influence on the agglutinins of the other phase.

Bearing in mind the behaviour of the paratyphoid bacilli, we examined the antisera of our type I and type II gonococci with the assumption that, in the cultivation of these organisms, a change of phase may eradicate the serological differences between the types.

Technique of Slide agglutination: The strains tested were definitely classified by the comparative agglutination method (with the exception of the degenerated strain 25a). After diluting them with saline to 1:100,000 so that large single colonies might be obtained, the cultures were streaked on blood-water agar plates. Following the procedure used with paratyphoid bacilli and Griffith's technique with scarlatinal streptococci (Griffith, 1926 and 1927) drops of each of the concentrated sera being examined were placed next to each other on a slide and a part of a single colony emulsified in each of them. The results were read immediately as to (1) rapidity and (2) intensity of floculation. Some were read with a magnifying glass, but the majority with the naked eye. The following grades were distinguished: ++, flaky (a few large flocculi); +, flaky (several, but smaller flocculi); (+), coarsely granular (numerous fine flocculi); ±, finely granular.

TABLE 4

STRAIN	TYPE	TOTAL NUMBER OF COLONIES TESTED	SPONTANEOUSLY AGGLUTINATED	IMMUNE SERA PREPARED WITH LIVING GONOCOCCI								IMMUNE SERA PREPARED WITH KILLED GONOCOCCI															
				Type I serum 83				Type II serum 84				Type I sera								Type II sera							
												75				76				57				25a			
				++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0	++	+	±	0
76	I	10	0	10	0	0	0	0	0	2	8	0	2	1	7	0	2	1	7	0	2	1	7				
76a		10	5	5	0	0	0					0	(5)	0	0	0	(5)	0	0	0	2	3	0				
Degenerated mixed culture																											
76b		13	0	13	0	0	0									0	(5)	3	5	0	0	2	11				
Specific subculture																											
57	II	12	0	0	8	0	4	0	8	0	4									12	0	0	0	0	6	0	6
Old strain																											
25a	II	5	0	5	0	0	0	0	5	0	0									0	5	0	0	0	5	0	0

The first two occurred rapidly, showing a specific phase reaction. The last two were not so striking. In a few cases the so-called specific colonies were transferred to new plates and examined again after 24 hours incubation at 37 degrees C. Table 4 is an excerpt of numerous experiments with many different strains and their corresponding antisera.

An examination of the table reveals that strain 76 reacts specifically with serum 83 (type I). Two colonies, moreover, react quite strongly with serum 57 (type II) while one colony gives a weaker reaction. Subcultures of the former colonies yielded strain 76a. Testing of this subculture demonstrated the continued presence of mixed-phasic properties, although it could still be identified as a type I. Five of the ten colonies agglutinated spontaneously. Strain 76b was derived from that colony which gave a ± reaction with serum 57. Agglutinations done with this strain proved to be type-specific.

The presence of mixed phases is easily seen with strains 57 and 25a. Despite this fact, strain 57 can be recognized as a type II. Strain 25a, on the other hand, due to its long cultivation, has reached such a state of degeneration that it is no longer possible to classify it.

Discussion

By means of the slide-agglutination test, we were able to reveal relationships between heterologous strains of gonococci which could not be seen by a titration of agglutinins. It must be concluded from this that there may be factors which are active in concentrated sera, but which cannot be detected in diluted sera. This phenomenon appears comprehensible in view of the important rôle which the colloidal state of the medium plays in the agglutination reaction.

Let us point once more to the fact that Griffith (1926, 1937) in his investigations on the classification of scarlatinal streptococci believed that he was justified in assuming the existence of different phases of the specific types. Furthermore, let us refer to earlier experiments of Griffith (1920) in which he examined a very large number of meningococcus-carriers and discovered the

striking facts that (in contrast to their results with organisms from lumbar-puncture), in the organisms growing on the mucous membrane, type II was more frequent than type I; and that stages intermediate between both types, which seldom occur in cultures from lumbar puncture, were exceedingly frequent. At that time they assumed that they were dealing with a labile antigen which was able to develop in both directions. This theory approaches the conception of the change of phase.

Overlapping reactions in comparative agglutination have been seen, for the most part, in strains which have undergone degeneration. According to the relationships revealed by slide-agglutination, we must now consider the possibility of these relationships being due either to the loss of type-specific carbohydrate and consequent preëminence of a common protein antigen or to the existence of a carbohydrate related to each of two heterologous types (C-substance of Boor and Miller (1931)).

Atkin classified gonococci by cultural behaviour into two types. Type II, isolated from chronic gonorrhea, was designated as a papilla-free variant of his type I; the latter, isolated from acute gonorrhea, was characterized by papilla-bearing colonies. In view of the findings of Griffith and Scott with meningococcus-carriers and our experiments with gonococci, we think it advisable to consider Atkin's experience with chronic gonorrhea in a different light.

By means of comparative agglutination, we were able to classify a large number of gonococcus strains freshly isolated from acute gonorrhea into two heterologous types. We also demonstrated the existence of a completely type-specific carbohydrate for each of these (Casper, 1937b). Slide-agglutination tests performed with representative strains, on the other hand, detected the presence of mixed-phasic colonies. By picking individual colonies, we could obtain both mixed and specific phases of the same strain. Moreover, we showed that continued cultivation of a type-specific strain led to degeneration and overlapping serological reactivity due to the loss of its carbohydrate. In addition, each of the papilla-bearing type-specific strains could be transformed to papilla-free variants by prolonged cul-

tivation. Similar to the condition in carriers of the meningococcus, it is probable that chronic gonorrhea is not caused by an individual pathogenic type of gonococcus, but rather that degenerative processes like those occurring after prolonged cultivation on artificial media, may take place due to the adaptation of the organism to its environment.

Inasmuch as Atkin used old laboratory strains for the preparation of his diagnostic type II antiserum, it must be emphasized that, as the above mentioned evidence for degeneration proves, errors in classification were bound to arise.

A number of the practically observed difficulties in classification by serological methods can be accounted for by 1) preparation of diagnostic sera with organisms which were not sufficiently specific and 2) rapid degeneration of the strains immediately after isolation or accidental transplantation of a degenerated colony in purification of the culture. Whether such errors in classification can be avoided by slide-agglutination or any other method cannot yet be answered from our experiments.

Further study of variability is necessary for the solution of the difficulties of serological analysis. Many phenomena are still unexplained.

SUMMARY

1. After cultivation on artificial media, strains of gonococci which were type-specific in the comparative agglutination test were shown by slide-agglutination to have mixed-phase colonies.

2. We have discussed the possible errors in serological classification which may be caused by the use of mixed-phasic cultures.

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