6. Later stage of division ; the chromatin-spheres are massed on the thread joining the blepharoplasts.
7. The chromatin-masses have passed to either pole of the dividing monad ; the line joining the blepharoplasts still persists.
8. Division is almost complete, and the line between the blepharoplasts has disappeared.
9. One of the products of a recent division ; the nucleus has not yet been reconstructed.
10. Early stage of conjugation.
11. Active zygote-stage showing the approximation of the blepharoplasts and large vacuoles.
12. Early stage of encystation of the zygote ; the wall at this stage is quite soft and stains with eosin.
13. Unripe cyst, showing reserve-body. Iron-hæmatoxylin preparation.
14. Unripe cyst, showing possibly reduction-division on the part of the nuclei.
15. Almost ripe zygote cyst, with elongated synkaryon. Hæmalum-preparation ; does not show reserve-body.
16. Ripe cyst, with round synkaryon. Hæmalum-preparation ; does not show reserve-body.

Further Observations on the Variability of Streptococci in Relation to Certain Fermentation Tests, together with some Considerations Bearing on its Possible Meaning.

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The fermentation tests devised by Mr. H. Gordon (1905, 1) were employed by him and by a number of subsequent observers as a means of differentiating streptococci into definite varieties.

This application of the tests necessarily presupposes the stability of the reactions yielded by any given strain of streptococci in the test media. But after a prolonged examination of a number of different strains of streptococci I was unable to confirm this primary requirement. Consequently, I ventured to call in question the supposed constancy of the tests (1910, 2; 1911, 3). A considerable mass of evidence of the variability of streptococci under cultivation in respect of their reactions in "Gordon's media" was submitted. The observations recorded made it clear that the

reactions in these media of a series of selected strains of streptococci varied independently from time to time under the conditions of ordinary laboratory cultivation, and could in certain cases be made to vary very greatly by growing the organisms for longer or shorter periods in media containing particular "sugars."

The conclusions which were drawn from these and similar observations have been strongly controverted by Gordon (1911, 4), who issued an article containing a series of criticisms of my results, and reasserted his claim to have differentiated distinct types of streptococci by means of his tests. The grounds on which he based this contention, unfortunately, appear to involve some slight misapprehension of the question under discussion. This was whether the "types" thus differentiated are fixed and independent, or merely temporary and liable to variation and interchange of characters.

On the other hand, the later work of Beattie and Yates (1911, 5) completely confirmed the views which I had put forward, since they found it impossible to differentiate between the various strains of streptococci which they used, and state that in their hands "Gordon's tests have proved quite unreliable for this purpose."

For the purpose of throwing further light upon the subject, I have examined the behaviour of a single streptococcus freshly isolated from the human subject in regard to Gordon's tests, both after growth in certain ordinary culture media, and after a series of successive passages through the mouse. I have also incidentally re-examined the reactions of some of the streptococci employed in my previous experiments after the lapse of a further period of 18 months. The results obtained are in entire agreement with my former conclusions.

Methods.

The methods and precautions used were the same as those employed in my previous work (1911, 3). But, in view of certain doubts expressed by Gordon, it may be well to state that throughout the whole of my investigation of the fermentation tests the "sugars" used in the preparation of the media have been pure substances obtained from Merck. Further, in every case where the reaction appeared to give a negative result, the streptococci in the tube in question were proved by successful sub-culture to be actually living before the result was accepted as negative.

The streptococcus A was isolated *post mortem* from the blood of a case of ulcerative endocarditis, and appeared to be present in pure culture. It was three times plated out, and propagated on each occasion from a single isolated colony. Its reactions were then tested in Gordon's media.

A stock culture was established in stab agar, and propagated in sub-culture

every week. Cultures were also established in bouillon, gelatin, and milk. These were all incubated at 37° C., and were sub-cultivated every three days—the bouillon culture into fresh bouillon, the gelatin culture into gelatin, and the milk culture to milk.

After the lapse of a month, and again after two months, the streptococci growing in bouillon, gelatin, and milk respectively were examined as regards their reactions in the test media. In the meantime the organism from the original stock was being passed through a succession of mice. It was recovered in each case *post mortem* from the blood of the mouse, plated for purity, tested in Gordon's media, and again inoculated into a fresh mouse. In this manner five successive passages were carried out, and the reactions of the streptococcus recorded after each passage.

Results.

In Table I are shown the reactions of the streptococcus A as originally isolated, and after its continued cultivation in bouillon, gelatin, and milk at 37° C. for periods of one month and two months respectively. It is seen that in every case the original reactions underwent important changes. Fifteen changes of individual reactions occurred in total, and four new sets of reactions made their appearance in the course of the experiment in question. The power of clotting milk was lost in every case, and only the neutral red, saccharose, and lactose reactions remained entirely unchanged. Thus, so far as the fermentation tests afford information, streptococcus A and its derivatives in bouillon, gelatin, and milk present five distinct varieties or strains of streptococcus.

Table I.—Showing the Reactions of Streptococcus A when first Isolated and after Cultivation in different Media.

Streptococcus A.	Milk.		Neutral red.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.	Changes after one month.	Changes after the second month.
	Acid.	Clot.									
When originally isolated.....	+	+	0	+	+	+	+	0	0		
After 1 month in bouillon	+	0	0	+	+	+	+	+	0	2	
„ 2 months „	+	0	0	+	+	0	0	+	+		3
After 1 month in gelatine	+	0	0	+	+	+	0	+	0	2	
„ 2 months „	+	0	0	+	+	+	+	+	0		1
After 1 month in milk.....	0	0	0	+	+	+	+	0	+	3	
„ 2 months „	+	0	0	+	+	0	0	+	+		4

In all the tables the sign + indicates the production of an acid reaction in the sugar medium, or the reduction of neutral red, or the production of a clot in milk where the property of clotting milk is under examination. The sign 0 indicates that the change in question did not occur within the limits of time prescribed for Gordon's tests.

Table II contains the results obtained by testing the streptococcus A in its original form, and after each successive passage through the mouse. There are eight changes of individual reactions. All six series of reactions are different, and each of them is different from the reactions shown in Table I from the cultures grown in bouillon, gelatin, and milk.

Table II.—Showing the Reactions of Streptococcus A when first Isolated and after each of Five Passages through the Mouse.

Streptococcus A.	Milk.		Neutral red.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.	Number of changes.
	Acid.	Clot.								
When originally isolated	+	+	0	+	+	+	+	0	0	
After one passage	+	+	0	+	+	+	+	+	0	1
„ two passages	+	0	0	+	+	0	+	0	0	3
„ three „	+	+	0	+	+	0	+	+	0	2
„ four „	+	+	0	+	+	+	+	+	0	1
„ five „	+	+	0	+	+	+	0	+	0	1

Thus it appears that, starting from a single freshly isolated streptococcus of undoubted purity, no less than ten distinct varieties, or variants, were obtained. The only instances of identical reactions which occurred were those exhibited by the variants obtained after two months' growth in bouillon and two months in milk, and the variants got by one month's growth in bouillon and two months' growth in gelatin.

In Table III are given the reactions of the streptococci L, P, G, M, V, E, in January, 1912, along with the results which they had yielded previously from stock agar cultures in 1908 and 1910, as shown in my previous papers. G is the only streptococcus which, in 1912, exhibits the same series of reactions as in 1910. Numerous changes are seen in the reactions of the streptococci. Streptococci S and H of my previous list could not be re-examined as they had unfortunately been allowed to die out. Streptococcus E was one originally isolated from horse-dung, all the others were of human derivation.

Table III.—Showing the Reactions of certain Streptococci at Different Dates after continued Cultivation in Stab Agar.

Streptococcus.	Date of testing.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.	Number of changes.
L	June, 1908	+	+	0	0	0	0	0	
	„ 1910	0	0	+	0	0	+	0	4
	January, 1912	+	0	+	+	0	+	0	2
P	June, 1908	+	0	+	+	0	+	0	
	„ 1910	0	0	+	0	0	+	0	2
	January, 1912	+	0	+	+	0	+	0	2
G	June, 1908	+	+	+	0	+	+	+	
	„ 1910	+	+	+	+	0	+	0	3
	January, 1912	+	+	+	+	0	+	0	0
M	June, 1908	+	+	+	+	0	+	0	
	„ 1910	0	+	+	0	0	+	0	2
	January, 1912	+	0	+	+	0	+	0	3
V	June, 1908	+	+	0	0	+	+	0	
	„ 1910	0	0	+	0	0	+	0	4
	January, 1912	+	+	+	+	0	+	0	3
E	August, 1910	0	+	+	0	+	+	+	
	November, 1910	0	+	+	+	0	+	+	2
	January, 1912	+	+	+	+	+	+	+	2

Stability of the Reactions.

The evidence for and against the stability of Gordon's test reactions may now briefly be summarised.

In bringing out the tests originally Gordon stated (1905, 1) that he had "found on repeating the tests three times over that each of the ten streptococci responded on each subsequent occasion to each test in the same fashion as it had done in the first instance." He also wrote as follows (1905, 6): "On several occasions I have re-examined a streptococcus in the differential tests after it had been in culture *for periods up to a fortnight*,* and up to the present time I have always found that such streptococci respond to the test in exactly the same manner as when first isolated." He also re-examined 11 different streptococci after one passage through the mouse, and found variation in two cases only, affecting one reaction in each case. These results he regarded as "reassuring" in relation

* The italics are mine.—E. W. A. W.

to the question of the stability of the reactions. Thus it is seen that the original evidence in favour of the stability of the reactions was somewhat slight.

Andrewes and Horder (1906, 7), recognising that constancy of the tests was "clearly a cardinal point," investigated the question with considerable care. They eventually concluded that the reactions were sufficiently stable for the purpose which they had in view, though they frankly stated the existence of some degree of variation within their experience. Indeed, the evidence which they adduce of constancy of the reactions after prolonged sub-culture, after passage, and under varying external conditions, is in each case associated with an admission of the occurrence of instances in which the reactions exhibited variation.

Cumpston (1907, 8), who examined streptococci from the throat and tissues in scarlatina, 50 per cent. or more of which gave identical reactions, stated that he re-examined many of the cultures after periods ranging up to as much as three months, and was able to corroborate the assertion that each strain of streptococcus preserves its characters unchanged.

On the other hand, Beattie and Yates (1911, 5), who examined the same question, found one change of reaction in each of three strains of streptococci after prolonged sub-culture, and one or more changes of reaction in four strains of streptococci out of five after a single passage through the rabbit, one strain remaining unchanged.

In my own earlier experiments (1911, 3) streptococcus L exhibited seven different series of reactions in the course of the investigation, P six different series, S nine, G seven, M four, V three, and H two, while in the present observations streptococcus A has been found to offer no less than 10 distinct and independent series of reactions. The January, 1912, examination of the old streptococci adds a new series of reactions in the case of L, P, M, and V.

Thus it is clear that it may justifiably be stated (as I stated in my former paper) that streptococci, which are at one time different in respect of the tests, may at another time give identical reactions, while those which are apparently identical at a given date may later on exhibit totally different reactions. That is to say, the test reactions are by no means stable.

Gordon maintains emphatically (1911, 4) that variation on sub-culture does not discount the value of "approved types" of streptococci as defined by him. This attitude is extremely difficult to comprehend, since the question of stability of the reactions can only be approached at all by the examination of successive sub-cultures.

It will, of course, be admitted as a self-evident proposition that, if a given micro-organism varies in such a manner as to become entirely indistinguishable

from another given micro-organism, the two (so long as they remain indistinguishable) can no longer be regarded as distinct varieties. Until some differentiating character has been discovered, they must be looked upon as identical. It has been shown in my experiments that these considerations apply to streptococci in respect of their reactions in Gordon's media. And it has also been shown that, *on sub-culture through the animal body*, as well as on sub-culture in artificial media, the test reactions of particular streptococci tend to exhibit striking variations.

The conclusion seems to follow unavoidably that Gordon's tests do not afford an adequate basis for a classification of streptococci. This conclusion, however, does not necessarily imply that the tests are valueless. Nor does it suggest that fixed and definite distinct varieties do not exist among the streptococci. But it maintains that the existence of such varieties has not been proved by the application of the tests, nor even rendered in the least degree probable.

Even had the tests been found to be reasonably constant under ordinary conditions, the results obtained by their use do not on close examination appear to supply so clear and decisive a differentiation of streptococci as might be supposed from writings on this subject. Thus, among six variants of the *S. equinus* described by Andrewes and Horder (1906, 7)—a type of streptococcus "which seems entitled to rank as a species" in their opinion—no less than three are identical with variants of their *S. pyogenes*, so far as Gordon's tests themselves afford any indication. The distinction which they actually draw between the organisms is made to rest on characters which form no part of Gordon's tests, such as pathogenicity, the presence or absence of growth in gelatin, and so forth.

Again, if one looks into the reactions actually laid down by Gordon himself in his latest communication (1911, 4) as typical of *S. salivarius*, *S. pyogenes*, and *S. fæcalis*, one finds a range of admitted possible variation such as brings *S. pyogenes* very near *S. salivarius* on the one hand, and equally near *S. fæcalis* on the other. For it is to be noted that, in the form in which the reactions are now given, we have two alternatives for *S. salivarius*, and no less than eight alternatives for *S. pyogenes* (see Table IV). Further, the only absolutely fixed distinction, as regards the test reactions between what may pass as a *S. pyogenes* and what is to be classed as a raffinose-negative *S. salivarius*, is the difference in the reaction to neutral red. Similarly, the only distinction in the test reaction between another form of *S. pyogenes* and the *S. fæcalis* is again the reaction in the neutral red test—a test which Andrewes and Horder have noted (along with salicin) as "more liable to vary than most of the others."

It appears, therefore, that even in these selected cases the types differentiated by the tests lack something in definition and distinction.

Table IV.—Showing the Reactions indicated by Gordon for certain "Approved Types" of Streptococci expressed in a Table.

Streptococcal type.	Milk.		Neutral red.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.	Number of possible forms allowed by this series of reactions.
	Acid.	Clot.								
Salivarius	+	+	+	+	+	+ or 0	0	0	0	2
Pyogenes.....	+	0 or +	0	+	+	0	0	+ or 0	0 or +	8
Fæcalis	+	+	+	+	+	0	0	+	+	1

Possible Meaning of the Reactions.

Hitherto, attention has been directed to the lack of constancy of the tests, and with that object examples have chiefly been used in illustration which showed a noteworthy degree of variability. But it is not to be supposed on that account that every streptococcus will at any moment necessarily exhibit variation. Within the limits of my own experiments, it has not always been possible to produce variation in particular streptococci even after several months of manipulation, though at another period, or under different conditions, the same streptococci frequently exhibited considerable variability. Again, streptococci which have given a series of variations over a considerable period may later on preserve an appearance of constancy even after the lapse of a long interval of time, as in the case of streptococcus G in agar culture in June, 1910, and January, 1912. But no streptococcus yet examined in these experiments has preserved unchanged the whole of its reactions throughout the whole period of observation.

We do not at present sufficiently understand the conditions governing streptococcal variation or the conditions of temporary constancy, but much of the evidence seems to point in the direction of a distinct environmental influence.

The question is not an easy one to investigate, owing to the difficulty of supplying absolutely constant conditions of environment even in the laboratory cultivation of streptococci. But it is at least possible that particular nutritive and other conditions in the environment favour the

development of particular series of reactions. When a given streptococcus has reached the particular (or one of the particular) series of reactions favoured by particular surroundings, it is quite possible that under continued cultivation in the same surroundings it may exhibit no changes of reaction during long periods of time. And it might even be somewhat slow to change when introduced into a new environment.

It appears to me that this hypothesis of adaptation to the particular environment affords a more probable explanation of the differences observed among streptococci in the test reactions than the theory of fixed types. For example, the differences observed between the common streptococci of the saliva and those of the fæces may find their explanation along these lines, since it must be presumed that the fæcal streptococci originally reach the intestine by descending from the mouth. The change apparently is a very gradual one, for Andrewes and Horder state (1906, 7), with regard to the reactions of these streptococcal types, that "*S. salivarius* passes by insensible gradations into *S. fæcalis*."

Further, it may be regarded as at least possible that the streptococci present in saliva actually acquire the *S. salivarius* type (or types) during and owing to their residence in the mouth. If this is not the case, how does so comparatively great a degree of uniformity of reactions as is described come to occur among the streptococci found in an open cavity like the mouth? Or again, how comes it that the common streptococcus of the air does not appear among the types stated to be most common in the saliva?

The fact that, although the total number of types described in the saliva and in fæces is very considerable (in saliva 48 and in fæces 40), yet the great majority of all the streptococci found have been shown to fall into a few main groups, itself suggests the possibility that a persistent adaptation goes on in these situations, as a result of which the streptococci entering the mouth or reaching the intestine constantly tend to assume particular series of reactions.

The findings in a number of my own earlier experiments led me to suggest the possibility that in particular surroundings streptococci tend to assume particular types in relation to Gordon's tests—in fact, that the reactions differentiate particular environments rather than particular varieties of streptococci. The evidence, which is somewhat striking and undoubtedly suggestive, is shown in Tables V, VI, VII, and VIII.

Table V.—Showing the Reactions of certain Streptococci in 1910 after Continuous Cultivation in Stab Agar for Two Years.

Six of the Streptococci agree in every reaction but one.

Streptococcus.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
L	0	0	+	0	0	+	0
P	0	0	+	0	0	+	0
V	0	0	+	0	0	+	0
S	0	+	+	0	0	+	0
M	0	+	+	0	0	+	0
H	0	+	+	0	0	+	0
G	+	+	+	+	0	+	0

Table VI.—Showing the Reactions of some of the Streptococci from Table V after Eight Weeks' (in the case of P Nine Weeks') Cultivation in Inulin Medium.

Three are identical, and one other differs only in respect of one reaction.

Streptococcus.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
L	+	+	+	+	+	+	+
S	+	+	+	+	+	+	+
G	+	+	+	+	+	+	+
M	0	+	+	+	+	+	+
P	+	0	+	0	+	+	0

Table VII.—Showing the Reactions of the Streptococci of Table VI after their return for Three Months to Cultivation in Stab Agar.

Three are identical, and show the same reactions as L, P, V, *from agar in* 1910, *and* G *only differs as regards the saccharose reaction.*

Streptococcus.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
L	0	0	+	0	0	+	0
P	0	0	+	0	0	+	0
S	0	0	+	0	0	+	0
G	0	+	+	0	0	+	0
M	0	+	+	+	+	+	+

Table VIII.—Showing the Reactions of some of the Streptococci from Table V after Four Months' Cultivation in Mannite Medium.

Three are identical, and V *differs only in regard to the clotting of milk.*

Streptococcus.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
L	0	0	+	0	0	+	0
P	0	0	+	0	0	+	0
M	0	0	+	0	0	+	0
V	+	0	+	0	0	+	0
S	0	+	0	0	0	0	0

The 1912 reactions of the streptococci L, P, G, M, V (all of human derivation) tested after 18 months' continuous cultivation in stab agar further support the hypothesis, since with the exception of two differences in the saccharose reaction they are seen in Table IX to present an absolute identity.

Table IX.—Showing the Reactions of some of the Streptococci from Table V after 18 Months' further Cultivation in Stab Agar.

Three are identical, and the other two differ only in regard to the saccharose reaction. The reason why the reactions differ from those obtained with agar cultures in 1910 is unknown. It is not known, for example, whether the agar has undergone any change. The leading reactions are the same as those of P from agar in 1908.

Streptococcus.	Milk clot.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Mannite.
L	+	0	+	+	0	+	0
P	+	0	+	+	0	+	0
M	+	0	+	+	0	+	0
G	+	+	+	+	0	+	0
V	+	+	+	+	0	+	0

Accordingly I conclude that the tests may assist in the determination of the source and origin of particular strains of streptococci met with. It is, however, necessary to speak with a good deal of reserve upon this point because at present we do not possess enough exact evidence as to the length

of time that streptococci can retain particular characters in respect of their test reactions, nor as to the length of time and other conditions necessary for the impression of new characters upon them. Meanwhile it is probably true that in certain cases the source of particular streptococci may safely be deduced from their reactions in the test media, and that, as Andrewes and Horder clearly pointed out, "a streptococcus from the mouth fermenting only saccharose, salicin, and coniferin, non-pathogenic, and incapable of growth on gelatin at 20° C. may with some confidence be referred to inhaled horse-dung." The question is clearly worth pursuing further.

On the other hand, in at any rate a considerable proportion of cases, the results obtained by applying Gordon's tests must at present remain inconclusive owing to the lack of sharpness and definition in the demarcation of the different streptococcal types to which attention has already been called. Thus, as Houston (1905, 9) pointed out in discussing the possibility of recognising fæcal contamination by means of streptococcal tests, "the fact of there being so many groups" of fæcal streptococci "unfortunately suggests the likelihood of streptococci isolated from various and perhaps unobjectional sources falling almost necessarily under one or other of the 40 groups." And again, "unfortunately, with so many groups to select from, a particular streptococcus . . . . derived from a non-fæcal source could hardly fail to fall under one or another of the 40 groups of fæcal streptococci."

This is perhaps to state the case more strongly than necessary, since the 40 fæcal groups present after all only a fraction of the 512 different series of reactions theoretically possible with Gordon's nine tests. Nevertheless, it is to be remembered on the other side that the *à priori* probability of the appearance of the different series is by no means equal, since all the sugars are not equally easily fermented.

It is of interest to note in this connection that if the varieties of streptococci already described by different observers who have used the tests were to be accepted as fixed and definite independent organisms more than 100 different kinds of streptococci have probably already been identified.

Conclusions.

1. There is at present no proof of the existence of more than one kind of streptococcus pathogenetic for man.

2. The reactions obtained with streptococci in Gordon's media are largely dependent on the character of the environment in which the organisms have previously been growing.

REFERENCES.

1. Gordon. "Report on some Characters by which various Streptococci and Staphylococci may be Differentiated and Identified," 'Reports Med. Officer Local Gov. Board,' 1905, p. 388.
2. Ainley Walker. "On the Value of 'Sugar' Tests for Streptococci," 'Journal of Pathology and Bacteriology,' 1910, vol. 15, p. 124; 'Proceedings of the Pathological Society of Great Britain and Ireland.'
3. Ainley Walker. "On Variation and Adaptation in Bacteria, Illustrated by Observations upon Streptococci, with special Reference to the Value of Fermentation Tests as Applied to these Organisms," 'Roy. Soc. Proc.,' 1911, B, vol. 83, p. 541.
4. Gordon. "The Differentiation of Streptococci," 'Journal of Pathology and Bacteriology,' 1911, vol. 15, p. 323.
5. Beattie and Yates. "Sugar Tests and Pathogenicity in the Differentiation of Streptococci," 'Journal of Pathology and Bacteriology,' 1911, vol. 15, p. 247.
6. Gordon. "A Ready Method of Differentiating Streptococci, and some Results already Obtained by its Application," 'Lancet,' 1905, vol. 2, p. 1400.
7. Andrewes and Horder. "A Study of the Streptococci Pathogenic for Man," 'Lancet,' 1906, vol. 2, pp. 708, 775, 852.
8. Cumpston. "The Relative Frequency of Various Types of Streptococci in Scarlatina," 'Journal of Hygiene,' 1907, vol. 7, p. 599.
9. Houston. "Report on the Bacteriological Examination of (1) The Normal Stools of Healthy Persons; (2) The Intestinal Contents of Sea-fowl and Fish; and (3) Certain of our Public Water Supplies," 'Reports Med. Officer Local Gov. Board,' 1905, p. 472.