THE DIFFERENTIATION OF STREPTOCOCCI BY MEANS OF FERMENTATIVE TESTS.[1]

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The question of the identity of streptococci from various sources has given the bacteriologist material for discussion and investigation for many years. Fehleisen[2] and Rosenbach[3] wished to separate the streptococci found in erysipelas from those present in the ordinary local infections, basing their classification upon differences exhibited in the ordinary fluid and solid media. Von Lingelsheim[4] made the morphological distinctions paramount and thus proposed the groups S. longus and S. brevis. More recently Schottmueller[5] suggested a new classification into S. erysipelatos, S. viridans or mitis, and S. mucosus, varieties which were to be recognized by certain distinctive appearances on blood media. Whilst the last method was gaining ground amongst the German workers, English bacteriologists were investigating the fermentative activities of streptococci with a view to ascertaining whether essential differences such as would be of value in classifying could be discovered. Working upon the assumption that the fermentative powers are biological characters of fundamental importance, Gordon,[6] and Andrewes and Horder[7] employed certain fermentable substances in culture media, and grouped the streptococci partly according to their action upon these media and partly according to their sources.

Gordon's nine tests, selected for routine employment were: (1) The clotting of milk in 3 days at 37° C.; (2) the reduction of neutral red broth during an-

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[2] Fehleisen, Ueber Erysipel, *Deut. Zeit. f. Chir.*, 1882, xvi, 391. Die Ætiologie des Erysipels, Berlin, 1883.

[3] Rosenbach, Mikroörganismen bei d. Wundinfections Krankheiten des Menschen, Wiesbaden, 1891.

[4] v. Lingelsheim, Therapie und Ætiologie der Streptokokken-Infectionen Berlin, 1899.

[5] Schottmueller, *Münch. med. Woch.*, 1903, 1, 849.

[6] Gordon, *Lancet*, 1905, lxxxiii, 1400.

[7] Andrewes and Horder, *Lancet*, 1906, lxxxiv, 708.

aërobic incubation for 2 days at 37° C.; the production of an acid reaction in aërobic cultures 3 days old at 37° C. when cultivated in various slightly alkaline broths containing severally 1 per cent. of the following: (3) saccharose; (4) lactose; (5) raffinose; (6) inulin; (7) salicin; (8) coniferin; (9) mannite.

Andrewes and Horder, making use of these tests and reviewing the results of other workers propose the following very elaborate classification of streptococci.

A. *Streptococcus equinus:* a saprophytic group in which most of the organisms ferment only saccharose and the glucosides.

B. *Streptococcus mitis:* saprophytic, in the human saliva and fæces fermenting saccharose and lactose and not the glucosides.

C. *Streptococcus pyogenes:* long chained, hemolytic, acidify milk, do not clot milk; ferment saccharose, lactose and salicin. Any of the three "cardinal" reactions may be suppressed. Certain other reactions may be added.

D. *Streptococcus salivarius:* characteristic; fermentation saccharose, lactose and raffinose.

E. *Streptococcus anginosus:* cannot always be separated from Class D; occurs in the throat; in chemical reactions practically indistinguishable from *S. salivarius.* Type-reactions are: clotting of milk, reduction of neutral red, acid formation in saccharose and lactose and often in raffinose.

F. *Streptococcus fæcalis.*

G. *Pneumococcus.*

Working along similar lines in 1904, I arrived at conclusions so different from those embodied in the recent paper of Andrewes and Horder that I think it desirable to present them in this paper.

TECHNIC.

Fermentation experiments were made with thirty-three strains of pathogenic streptococci obtained from the human body, and upon one non-pathogenic variety isolated from milk. The source of the organisms is given in the table.

1. Empyema.
2. Mastoiditis.
3. Streptococcæmia-blood.*
4. Cellulitis of foot.
5. Empyema.
6. Mouth.†
7. Liver abscess.
8. Cerebro-spinal fluid-meningitis.
9. Peritonitis.
10. Pelvic abscess.
11. Retroperitoneal abscess.
12. Suppurative arthritis; elbow.
13. Pyonephrosis.
14. Appendicitis.
15. Osteomyelitis; femur.
16. Osteomyelitis; metatarsal.
17. Cellulitis.
18. Streptococcæmia-blood.*
19. Diarrhœa; fæces.
20. Diarrhœa; fæces.
21. Diarrhœa; fæces.
22. Diarrhœa; fæces.
23. Diarrhœa; fæces.
24. Streptococcus enteritis; fæces.‡
25. Metritis.
26. Diarrhœa; fæces.
27. Milk.
28. Peritonitis.
29. Mouth.
30. Streptococcæmia; blood.*
31. Cellulitis.
32. Mouth.
33. Mouth.
34. Mouth.

* Isolated by blood culture in cases of "malignant endocarditis."

† The mouth streptococci were virulent for white mice.

‡ Through the kindness of Dr. Libman.

It was deemed important to determine the nature of the medium most favorable for the growth of the organisms. A sugar-free broth served as a basis and litmus (Kahlbaum's) was added to serve as an indicator. The results obtained showed in a number of instances that carbohydrates apparently unaffected in one medium could be easily split up in another. Therefore trials were made with the fluid media at various titers, with and without the addition of ascitic serum, and also in a medium containing beef serum and water. The following is the list of the media employed.

1. Sugar free broth	0.8 per cent. acid.
2. " "	0.5 per cent. acid.
3. " "	neutral.
4. " "	0.5 per cent. alkaline.
5. " "	0.5 per cent. acid plus one third volume ascitic fluid.
6. Beef serum and water.	

To these media one per cent. of the following carbohydrates was added.

Pentoses: arabinose, rhamnose.

Hexoses: glucose, levulose, galactose.

Hexahydric alcohols: mannite, dulcite.

Disaccharides: saccharose, lactose, maltose.

Polysaccharides: dextrin, inulin.

Sterilization was conducted on three successive days and was followed by a three-day test in the incubator. The possibility of hydrolytic change in some of the sugars, in the presence of the dilute acid medium, or transformation of some other nature during the process of sterilization had to be considered. Therefore several series of experiments (each repeated twice) were made. Flasks of sugar-free broth with litmus were sterilized, and to these, watery solutions of the various carbohydrates, separately sterilized in large tubes, were added. The amounts in each were so computed that a one per cent. solution of the fermentable substance would result. The contents of the flasks were then filled into tubes and the latter tested in the usual way. It was found that the results obtained in the media thus prepared did not differ from those made up in the usual way.

Three series of experiments were conducted with media 1, 2, 3, and 4; six series with 5 and 6. Daily observations of the cultures

indicated the advantages of the various media and the rapidity of acid formation. At the end of the fourth day the whole set of tubes together with the controls was regularly thrown out after the final readings had been recorded. To wait over a longer period of time for acid production to manifest itself, although preferable on theoretical grounds, seems disadvantageous in practice. If the chemical test is to be of any practical value, its results ought to be final within the specified time.

RESULTS OF THE EXPERIMENTS.

In presenting the results, those obtained upon the most favorable media alone will be given in detail, and the streptococci grouped accordingly. The addition of ascitic fluid not only enhances growth but seems to favor the fermentation of certain of the carbohydrates that were not attacked in the simple media.[8] Careful chemical tests of the serous fluid as to possible glucose or other sugar content were always made in advance. Fermentation tubes containing sugar free broth, litmus and the serum were inoculated with B. coli and examined for acid production. Controls of broth, serum and litmus without carbohydrates were included in the set of tubes inoculated with each strain. Keeping in mind the variations in the quality of ascitic fluid of different patients, it was decided to use a serum from the same source for a complete series of tests on all the organisms.

Inasmuch as arabinose was fermented by but one strain, rhamnose by but three strains, and dulcit (or dulcitol) by none of our series, it may be well to disregard these carbohydrates in an attempt at grouping the organisms. We may then tabulate them according to fermentation of dextrose, levulose, galactose, maltose, saccharose, lactose, inulin, dextrin and mannite, as follows:

Fermenting all but		Total Number.
1.	S. 18	1
2. Mannite	S. 3	1
3. Inulin	S. 1, 4, 6, 7, 11, 19, 20, 23, 31	9
4. Inulin, Mannite	S. 2, 5, 8, 9, 10, 12, 13, 14, 15, 16, 17, 25, 27, 28, 29, 30, 32, 33, 34	19
5. Inulin, Lactose	S. 21, 22	2
6. Inulin, Mannite, Saccharose.	S. 24, 26	2

[8] Libman, *Jour. of Med. Research*, 1901, vi, 84.

In accordance with the above we could distinguish six varieties, those failing to ferment inulin and mannite being in the majority, those fermenting all but inulin being second in number. There were therefore:

22	that	did	not	break up	mannite,
32	"	"	"	"	inulin,
2	"	"	"	"	lactose,
2	"	"	"	"	saccharose.

The carbohydrates which are most readily fermented are the hexoses, glucose, levulose and galactose (by all), the disaccharide maltose (by all), and the polysaccharide, dextrin. Acid production usually appears first in maltose, then in levulose, glucose and dextrin. Saccharose and lactose resist the action of a very few streptococci. The following table gives an insight as to the number of streptococci fermenting the various substances.

		Number of Streptococci Producing Acid in
Pentoses:	arabinose	1
	rhamnose	3
Hexoses:	glucose	34
	levulose	34
	galactose	34
Disaccharides:	saccharose	32
	lactose	32
	maltose	34
Hexahydric alcohols:	mannite	12
	dulcite	0
Polysaccharides:	dextrin	34
	inulin	2

Rhamnose was broken up by S. 19 and S. 23; rhamnose and arabinose by S. 18.

It is important to note that the fermentative properties described suffered no change from generation to generation. All the organisms were studied over a period of from six to eight months, an interval of at least six months having elapsed between the first and the final tests. The results on the serum media were uniform. This showed that artificial cultivation had no influence upon the fermentative activities.

The Results on Other Media.—Plain broth at 0.8 per cent. acid, 0.5 per cent. acid, neutral, or 0.5 per cent. alkaline titer were found to be inferior to the serum bouillon. At the 0.8 per cent. titer a number of organisms failed to attack carbohydrates which were

easily broken up in the presence of serum. These were mannite (not affected by 3 strains), lactose (2 strains), dextrin (2 strains), and saccharose (1 strain). The 0.5 per cent. acid plain broth was more favorable and fell behind the serum medium only in the case of lactose (1 strain), saccharose (1) and mannite (3). Neutral bouillon gave the same results as the last, but growths were more luxuriant. The alkaline 0.5 per cent. medium was rejected as unsuitable both because moderate acid production was indicated with difficulty, and because this titer was unfavorable for growth. Beef serum-water as suggested by Hanna[9] and Hiss,[10] although a good culture medium, was found unreliable. The results in it were variable, and frequently a streptococcus would fail to make acid from carbohydrates that were easily broken up in the presence of ascitic fluid.

Experiments on the Pneumococcus and Streptococcus Mucosus.—Fifteen strains of the former and eight of the latter were tested. The serum media are even more important for the pneumococcus than for streptococci. In plain broth with carbohydrates growths are frequently so sparse that acid production, if present at all, is too slight to bring about a visible change in the indicator. All the pneumococci and streptococci of the "mucosus" variety gave the same fermentative reactions thus differing from the ordinary streptococci. Acid was formed from dextrose, dextrin, maltose, lactose, galactose, inulin, levulose and saccharose. They failed to break up mannite, dulcit, arabinose and rhamnose.

A much larger series of pneumococci (65 strains) were tested for their power to ferment inulin. It was found that a number of them failed to produce acid in certain generations. All the organisms, however, fermented inulin at some time or other of their life history.

More recently in a study of pneumococci obtained from blood cultures it has been found by Dr. Ryttenberg and myself that the power of breaking up inulin may be lost. These observations on the mutability of fermentative properties will be published later.

[9] Hanna, *Jour. of Path. and Bact.*, 1898, v, 267.
[10] Hiss, *Jour. of Exper. Med.*, 1904. vi, 317.

SUMMARY.

If we glance again at the classification proposed by Andrewes and Horder we are struck at once by the fact that no hard and fast differences in fermentative properties characterize the various groups, and that the authors prefer to have recourse to the most frequent habitat of the organisms and to chemical tests, rather than to confine themselves to the latter method alone. From what has been said earlier in this paper, it becomes clear at once that the results gained by these authors are not convincing, for in their work they have failed to use the most favorable medium for growth of the organisms. And thus it can hardly be doubted that different reactions might have been obtained, under the conditions adopted by us. How otherwise could the fact be explained that of thirty-four of their pneumococci only eight fermented inulin. We had a similar experience while using the plain broth which led us to reject it as an unfavorable medium. Further, all our pneumococci fermented lactose with great rapidity. Andrewes and Horder report that eight of their series left lactose unchanged.

Taking our own tests into consideration we find that working with but a small number of carbohydrates we were able to find six different varieties of streptococci among only 33 pathogenic strains. Which of the various substances is to decide us in the grouping of the organisms? Would we not find even greater variations from the most common type (see No. 4 in table) if we were to extend our tests over a larger series of chemical agents? These questions are difficult to answer and only extended experimentation with a great many streptococci and many media will clear up the doubtful points.

In concluding it may be stated:

1. Streptococci vary considerably in their ability to produce acid from various carbohydrates.

2. Chemical tests of this kind should be made only in the media which are most favorable for the growth of the organisms.

3. Our results gave us six groups of streptococci, when tested upon dextrose, levulose, galactose, maltose, saccharose, lactose, inulin, dextrin and mannite, viz.: Those fermenting (1) all; (2) all but mannite; (3) all but inulin; (4) all but inulin and mannite;

(5) all but inulin and lactose; and (6) all but inulin, mannite and saccharose.

4. In view of the comparatively small number of streptococci employed we are hardly warranted in making a definite classification. Perhaps a larger series of tests upon the media employed will enable us to divide streptococci into distinct classes characterized by certain fixed fermentative properties.