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BIOLOGICAL STUDIES

OF THE

Fungi of the Human Mouth

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BIOLOGICAL STUDIES ON THE FUNGI OF THE HUMAN MOUTH.*

BY PROF. DR. W. D. MILLER, BERLIN.

In order to be able to determine upon the proper course to be taken in the attempt to remove or check the progress of any disease, it is necessary that our ideas of the cause and course of that affection be established upon the most certain, exact and scientific data which we are capable of attaining. Unfortunately for the dental profession, the attempt to furnish a scientific solution of the problems of dental caries has, until recently, been confined to a very few, and even now a majority of the investigators in dental pathology are content to restrict their observations to the clinical aspect of the question, a course which could never produce a satisfactory solution; while others even openly advocate a speculative course, and do not hesitate to ascribe to every new factor discovered in nature, a role in the production of caries of the teeth.

Consequently we have had presented to us, in turn, worms, acids, inflammation, electricity, infusoria, bacteria, putrefaction, toxic agents, etc., etc., as causes or conditions of *caries dentium*, some of

*German mycologists use the term 'Pilz' indiscriminately to designate either Schizomycetes, Blastomycetes, Hyphomycetes or Myxomycetes. When it is desirable to refer to any one of these groups in particular, they use the prefixes Spalt, Spross, Schimmel or Faden, and Schleim, giving Spaltpilz, Sprosspilz, Schimmel- or Fadenpilz and Schleimpilz. Following their example, I have in previous papers used the term fungus for all of the four groups of mycetes mentioned above, and shall also use the term in this paper, in which only Schizomycetes are treated of.

these theories containing some truth and some a surprising amount of absurdity. In the last two or three years, however, a great advance has been made in the methods of study, and a number of important points have been firmly established.

1. The observation of Leber and Rottenstein that micro-organisms are constantly present in decaying dentine, has been confirmed. (Weil, Milles, Underwood, Miller.)

2. The softening of dentine in caries has been shown to be chemically identical with that produced by certain weak organic acids. (Miller, Jeserich, Bennefeld.)

3. It has been established that various organisms found in the human mouth, produce the decalcifying acid by first converting non-fermentable sugars into fermentable varieties, and secondly, by splitting fermentable sugars into lactic acid. (Miller, Hueppe.)

4. The same organisms have been found capable of dissolving decalcified dentine, while they have no apparent effect, even after two or three years, on sound dentine. (Miller.)

5. Caries of dentine, chemically and morphologically identical with natural caries, has been produced outside of the mouth. (Miller.)

6. It has been furthermore shown that certain of the organisms of the human mouth are capable of developing under exclusion of air, thus making it possible for them to propagate within the substance of the dentine. (Miller, Hueppe.)

I propose to describe, in this and the following article, a series of experiments made for the purpose of obtaining more definite information respecting the number and morphology of the fungi of the human mouth, and their physiology, as far as is necessary to an understanding of the part which they may perform in the production of caries of the human teeth.

At the meeting of the American Dental Association, at Saratoga, a number of tubes containing pure cultures of fungi were passed around; with regard to these a reporter remarked that "they were evidently beyond the information of the majority." It is not very flattering to American dentistry if its representative Association allows a question of so great importance to remain beyond its comprehension, nor is there any excuse for such a condition of things now, so widespread have the methods of pure culture become. I

rather incline to the opinion that the reporter misinterpreted the apathy of the members of the Society. I shall, at any rate, here describe in a few words the methods now universally employed in isolating any given fungus, and then, more in detail, give the means which I have used to ascertain the physiological characteristics of the different fungi when obtained in pure culture.

We will start with a solution densely impregnated with micro-organisms, and a number of tubes of culture gelatine, perfectly sterilized. The gelatine being melted, we add to the first tube one bead (on a loop of sterilized platinum wire) of the solution. This is called the *first dilution.* From this tube we add two or three beads to a second tube (*second dilution*), and from the second five or six beads to a third tube (*third dilution.*) The gelatine is then poured upon horizontally placed, sterilized, cold glass plates. It congeals in a few seconds, and the three plates are placed in a pile (on glass benches) in a moist cell. The plates are examined after twenty-four to thirty-six hours, under a magnification of one hundred diameters.

By this means the fungi are so separated that on the third plate there will generally not be more than two to ten; (on the second there may be one hundred or two hundred, while on the first, of course, there are very many more.) As each micro-organism develops, being fixed in the gelatine, we will have at that point a pure culture of that particular kind. At another point we obtain a colony of a second kind, and so on. In general, colonies of different fungi may be distinguished with the greatest ease, by their microscopic appearance. With a sterilized platinum wire, bent at right angles at the end, we now pick up a number of the colonies of each kind, under the microscope (one hundred diameters), and transfer them directly to tubes of culture gelatine, only one colony to each tube. We have then (except in case of a possible accidental air-infection) pure cultures. Some experience is necessary to enable one to pick up the colonies under the microscope. Beginners should not attempt it with plates where more than one colony is in the field at once.

The method described in this journal (page 340, 1884), may also sometimes be used to great advantage. For fungi which do not grow on gelatine, Agar-Agar, or congealed blood serum, should be used. The former, one to one and a half per cent, has a higher

melting point than gelatine, ten per cent, and remains solid at the temperature of the human blood. When it is used for plate-cultures, it must be melted in hot water, and the infection made at a temperature of 40° to 42° C. Below this temperature it becomes solid, and cannot be poured; above it the germs would be liable to suffer. In other respects the Agar-Agar media are treated as the gelatine. Congealed blood serum cannot, of course, be poured upon plates. It is prepared in test tubes so inclined as to give the greatest possible surface, and a minimum quantity of the substance containing the fungus or fungi spread over the surface. Having obtained a pure culture of any fungus, the points to be determined regarding it are the following:

1. Its morphology; (bacillus, spirillum, micrococcus.)
2. Is it moveable? does it produce spores?
3. What are its growth-characteristics on various media, microscopically and to the naked eye?
4. What are its relations to oxygen?
5. Does it produce fermentation? If so, what fermentation, under what conditions, and with or without development of gas?
6. Does it cause putrefaction?
7. Does it have a diastatic, inverting, or peptonizing action?
8. Has it a pathogenic character?
9. Does it produce coloring matter?
10. What is its susceptibility to the action of the various antiseptics?

The first and second of these questions are, of course, determined by the microscope alone; the third, by the microscope and the naked eye combined; the fourth by the methods described in this journal, page 62, 1884, or by placing a thin strip of mica upon one half of the culture-plate before the gelatine solidifies; the mica then adapts itself closely to the surface of the gelatine, excluding the air, and if the fungus requires oxygen for its development the colonies beneath the mica either will not develop at all, or they will be very small compared with those on the other half of the plate, their growth ceasing as soon as the oxygen in the gelatine has been consumed. (Koch.) The fifth point is answered by

infecting fermentable solutions with the fungus in question, placing it under various conditions of temperature, etc., and determining the products of fermentation (if any); the sixth by analogous methods; the seventh question is determined by the action of the fungi upon starch, cane sugar, and albumen, (boiled white of egg); the eighth by experiments on animals; the ninth by the appearance or non-appearance of color in the vegetation itself, or in the surrounding medium; the tenth by experiments that will readily suggest themselves.

Other points to be investigated will be mentioned further on. Boiled potato is a medium of great value in the determination of Schizomycetes. No medium, however, requires greater care in preparation and after treatment than this, in order to obtain satisfactory results. Any sound potato *which does not become mealy or crack open on boiling*, will do for the purpose; it is first thoroughly washed and brushed, and all defective spots and deep eyes being removed, it is placed for one hour in a corrosive sublimate solution, five to one thousand, then in the steam sterilizer for one-half to one hour. In the mean time the moist cell is sterilized, and the bottom lined with filter paper wet with sublimate solution, five to one thousand. The potatoes are, while hot, removed from the sterilizer with sterilized forceps, cut into halves with a cold sterilized knife, and placed directly upon the sublimate paper (the cut surface up), and the cell closed. Potato sections prepared in this way should remain unchanged indefinitely. When the potato has become cool, the cover of the cell is carefully removed, and the fungus which is to be cultivated is spread upon a space about as large as a dime, in the centre of the section. Fungi which, morphologically as well as in their reaction upon gelatine, Agar-Agar and blood serum, show no appreciable differences, may sometimes be easily distinguished by aid of the potato culture. The potato can seldom be used to separate fungi, (*i. e.* to prepare pure culture). It is chiefly used as a reagent in distinguishing between fungi already in pure culture. For example, all comma bacilli yet discovered grow on potato, except the one found by Dencke in old cheese, which does not develop at all on potato, and is thereby at once distinguished as an entirely different fungus.

Eggs may often be used to great advantage. They are prepared as follows. The *fresh* egg is placed in sublimate, five to

one thousand, for ten minutes, then in the steam sterilizer for one hour. The cell for eggs is prepared as for potatoes, except that a sterilized glass plate, resting on a glass bench, is placed in the bottom to support the egg sections. As the eggs must be handled with the fingers, the hands must be thoroughly washed, then soaked in sublimate, five to one thousand, and then washed again in *alcohol absolutus*, to remove the sublimate. The eggs are shelled while still hot, and cut into two, three, or four sections. They are vaccinated in points upon the white; the yellow is not so well adapted to culture experiments, since it cannot be cut with a smooth surface.

I always keep on hand sections of potato and egg, also tubes of gelatine, Agar-Agar, and blood serum, and when in my practice particularly good material, or anything uncommon presents itself, a portion of it is at once transferred to these different culture media, so that it is pretty sure to develop in one of them, at least. For example, I have several times met with a fungus in the human mouth which produces a yellowish coloring matter, and which absolutely refuses to grow on anything which I have tried, except potato.

By use of the methods described I have isolated twenty-two different fungi from the secretions or deposits of the human mouth, and have endeavored to determine, as far as possible, their separate peculiarities of growth, physiological action, etc. It will, however, at once suggest itself to every one, that a thorough study of twenty-two different fungi involves an enormous amount of labor, and might constitute almost a life task for one experimenter. The task is, moreover, rendered still more difficult by reason of the fact that many of these fungi show differences of action when cultivated in different media, rendering the number of experiments necessary to come to a definite conclusion doubly great. I shall, therefore, not attempt to present an exhaustive treatment of the subject, but rather an introduction, hoping, at the same time, to establish some points which may be of use in bringing about a clearer understanding of the factors involved in the production of dental caries.

Regarding the first point to be considered—the morphology of the fungi—it is not at all necessary to enter into a minute description of all the different forms here presented. The figures will give a sufficiently clear idea of their diversity, and the appearance

of their colonies under a low power. For the rest, suffice it to say that ten of them are micro- or diplococci, five are bacteria and six bacilli. Some show more than one form of development. It would, however, lead us too far from our subject to discuss this fact here.

In liquid media, three grow out into long leptothrix, forming bundles or meshes of intertwining uni- or multicelluar threads, while one develops into spirilli; eight are motile, fourteen are non-motile, while three only have been seen to form spores. The others multiply by division alone.

With reference to the latter point, however, I have not made examinations sufficiently careful or extensive to be able to speak decidedly. Eight liquify nutritive gelatine, one converts it into a paste, thirteen leave it unchanged. On agar-agar, the differences of growth are not sufficiently pronounced to deserve particular mention. In gelatine, the microscopic appearance of the colonies of a sufficient number of these fungi, is shown in the figures (b). It will be seen that the appearance of the colonies forms a much safer means of differentiation than the morphological characteristics of the fungi, it being very seldom that, in growing, two fungi present exactly the same appearance. An exception is, however, presented by 6 and 7, which to the naked eye and under the microscope grow on gelatine exactly alike; moreover, on potato, white of egg, blood-serum, agar-agar and milk, their effect is identical. One, however, produces a yellow coloring matter, the other not, and thereby they are easily distinguished. The others may all be readily distinguished by their growth on potato.

Fig. 1

In relation to oxygen, they show great differences. Ten are strictly aerobian; i. e. they grow only where the air has free access. Four are not strictly aerobian; i. e. they propagate also when the atmospheric air is excluded, though not so rapidly. Eight grow equally well with or without access of air. Sixteen produce an acid reaction in a solution of beef extract, peptone and sugar. Four produce an alkaline reaction without the appearance of bad smelling products, and appear to leave the solution neutral. With regard to the six, however, the results were not satisfactory, sometimes the reaction being acid, at other times neutral or alkaline, depending somewhat upon the material used for the cultures.

Fig. 2

Some which produce an acid reaction in fermentable solutions, give rise to an alkaline reaction in non-fermentable solutions. The acid produced is probably, in all, or in nearly all these cases, lactic acid. This fact I established for No. 1 by chemical analysis, for No. 2 by forming the zinc salt and crystallizing, for No. 5 by the color test.* In the other cases the acid was not determined. Thirteen were repeatedly cultivated on potato. Of these, five grew rapidly, one in particular covering the whole surface of the section in forty-eight hours, and completely liquifying it to a depth of one to two mm., the liquified mass flowing off at the sides; the others develop very slowly, and attain only a limited growth. I am not able to say whether any of them possesses a diastatic action. It is, however, highly probable. Fifteen were cultivated on boiled white of egg. Four grew very rapidly, No. 19 (see fig. 13) in particular, in from two to four days, converting the egg into a semi-transparent, pasty mass, which gradually disappeared. In the first two days large quantities of sulphuretted hydrogen are developed; later, ammonia. Seven grew slowly on the white of egg, and four scarcely at all. The nourishment of the fungi naturally takes place at the expense of the albumen of the egg, which is converted into a soluble variety by the peptonizing action of the fungus. In two cases the presence of peptone could be detected in the dissolved mass after separation from the albumen, by the biuret reaction, the organisms producing more peptone than they needed for their own consumption.

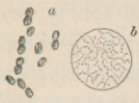

Fig. 3

Some of them produce in fermentable solutions considerable quantities of gas. If a glass bulb, with a fine stem drawn out to a point, be filled with milk inoculated with No. 3, (see fig. 2), otherwise sterile, and kept at blood temperature, in twenty-four hours so much gas will be generated that on breaking off the point the whole contents of the bulb will be ejected with considerable force. The same effect

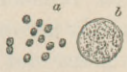

Fig. 4

*Two drops carbolic acid, one drop chloride of iron, twenty ccm. water, produce a violet color which becomes yellow on the addition of lactic acid, even in very dilute form. I am not prepared to say that this is an absolutely sure test for lactic acid. It is the test used by Prof. Ewald and others, for detecting lactic acid in the stomach, and is considered by them to be decisive. Of course the culture material itself must not give this reaction. Beef extract, for example, cannot be used, as it already contains lactic acid. A few other substances also give this reaction, but none, I believe, which are likely to be produced in these cultures.

may sometimes be produced, though not so markedly, when non-fermentable solutions are used. We may expect a similar action to take place when we seal up a dead pulp in a tooth, not only the gas itself escaping through the apical foramen, but, if its exit is hindered, ultimately forcing particles of the decomposing pulp through with it. The question suggests itself, whether certain configurations seen in carious dentine may not owe their origin in part to the pressure of gas.

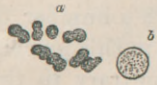

Fig. 5

Four produce coloring matter, Nos. 5 and 7 (figures 4 and 5), in gelatine cultures some days' old, forming brick-yellow masses, such as may be seen occasionally on the buccal surface of teeth which are not kept well cleaned.

On potato they appear bright yellow. Nos. 10 and 13 give the gelatine for a space one cm. in diameter around the colony, a grass-green tinge. I doubt very much whether either of these organisms has anything to do with the production of green stain, all my attempts to isolate a chromogenic fungus directly from green stain having thus far failed. Cultures of some of these fungi were made on dentine and enamel. Sections of dentine, when decalcified neutralized and soaked in saliva and sugar, formed, when kept in a perfect damp cell, a medium on which a considerable development took place, microtome sections of the dentine after two weeks showing a destruction of substance at the point of inoculation.

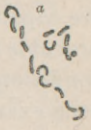

Fig. 6

On sections of normal dentine, the fungi in some cases appeared to maintain an existence until the organic matter exposed upon the surface of the section was consumed, after which the development ceased, while normal enamel, as might have been expected, formed about as good a culture substratum as glass or porcelain.

A description of the cultures in milk, blood-serum, etc., is not necessary for our present purpose. Also, experiments on animals have been made in too limited a number to lead to accurate results. It is very plain, however, that a study of the pathogenic character of twenty-two fungi is out of the question. No. 19, which possesses peculiar interest on account of its similarity to the cholera-bacillus, was tested on mice, guinea-pigs and rabbits. A small quantity from a pure culture injected into

Fig. 7

the abdominal cavity of mice, almost invariably caused death in a few hours. Guinea-pigs and rabbits have thus far shown themselves proof against it, even when large quantities were injected into the duodenum (the *ductus choledochus* not being ligated.) Experiments were made with a number of antiseptics, in adition to those reported in this journal (Vol. V. page 283.) Arsenious acid, contrary to the repeated statements of one of our journals, possesses an antiseptic power at least half as great as that of carbolic acid, and about twenty-five times greater than absolute alcohol. Chlorate of potassium, on the other hand, possesses scarcely any available power whatever. Peroxide of hydrogen proved to be particularly active. These experiments are not yet completed, and will therefore be given in a separate paper.

Fig. 8

The following practical conclusions appear to follow from the experiments above recorded:

1. A great majority of the fungi found in the human mouth are capable of producing acid from cane or grape sugar, and it is probable that, *with very few exceptions,* all can, when the proper conditions are presented to them. In nearly all cases which have been examined with special reference to this question, the acid has appeared to be lactic. The acetic acid fermentation, which cannot go on at temperatures above 35° C. (Fluegge), is out of the question in the human mouth, nor is there, as yet, any proof of the presence of more than minute traces of butyric acid.

2. In non-fermentable substances, the reaction will be found either neutral or alkaline, in some cases considerable quantities of ammonia and sulphuretted hydrogen being produced. If, therefore, a decomposing pulp is sealed up in a tooth, its reaction cannot be acid, and caries cannot take place either in the pulp chamber or root canals.

3. Of considerable interest is the fact that the same fungus may produce an acid reaction in one substratum, and an alkaline in another. If, for example, No. 19 (Fig. 13) be cultivated in certain neutral, non-fermentable substances, an alkaline reaction will appear. If, then, sugar be added, the reaction will in a few hours change to acid. In such a case we undoubtedly have two distinct processes going on; first, the nutrition of the organ-

Fig. 9

ism accompanied by the appearance of alkaline products, and secondly, its fermentive action accompanied by acid products. Ordinarily, the latter so outweigh the former that the resultant reaction will be acid. This is, however, by no means necessarily the case. On the other hand, conditions may readily be produced under which the resultant reaction will be neutral or alkaline, especially in the human mouth, where so many different fungi and so various conditions are present. In such a case, the result would be to put a temporary check upon the advance of the decalcifying process; in other words, upon the caries itself. In the case of particularly foul-mouthed persons, the foulness itself may become a preventive of caries.

Fig. 10

4. The possession of a peptonizing action by a large number of these fungi, readily accounts for the solution of the decalcified dentine.*

5. Any one of these fungi which can produce acid by fermentation of carbohydrates, or can dissolve the decalcified dentine, may aid in the production of caries, while any one which combines both these properties, as many of them do, may alone bring about the phenomenon of dental carries. A solution of the dentine or enamel, without previous decalcification, cannot take place. The fact which I have so often affirmed, and which was denied by Milles and Underwood, that one continually meets with large tracts of softened, non-infected dentine, has been completely confirmed by Arkovy and Matrai. They say, "the invasion extends, however, only to a certain depth, and only isolated tubules show a deeper in-

Fig. 11

*Not a little confusion has been introduced by attempted artificial definitions of putrefication and fermentation. The idea that every change in nitrogenous organic substances must be of the nature of putrefaction, is particularly misleading. A ferment of the nature of pepsine, which dissolves coagulated albumen, is widely distributed among the fungi of fermentation, as well as putrefaction, and the schizomycetes in general require nitrogenous substances in some shape, for their nutrition. The dissolution of the organic portion of dentine is by no means dependent upon the presence of putrefactive organism, but may be accomplished equally well by fermentation. As stated in previous papers, I never found a putrefactive organism in the deeper portions of carious dentine. Moreover, the acid reaction of carious dentine is highly unfavorable to the development of such organisms. I intend to repeat and extend my experiments on this point. The presence of putrefactive organisms, while it would accelerate the second stage of caries, could only retard the first

vasion, sometimes to twice the depth, and reach the border of the normal dentine," the whole territory between the isolated tubules being free from invasion.

Fig. 12

6. The comparative or complete independence of many of these organisms of the free access of air, renders their propagation within the dentine, or under fillings where softened, non-sterilized dentine has been left, an easy matter.

7. The fact that dentine and enamel form so exceedingly poor culture substrata for schizomycetes, is an additional proof of the position that their attack upon the teeth is only secondary; i. e., they owe their rapid development to the secretions, deposits, etc., of the oral cavity, and not until the tissue of the tooth has undergone a certain change—first decalcification, second peptonization—can they adapt it to their nourishment. The decalcification is produced chiefly by acid, resulting from the action of the organisms upon certain carbohydrates in the human mouth, while the peptonization is produced, either by the direct action of the protoplasm of the organisms upon the decalcified dentine, or by the action of a ferment which they produce.

A knowledge of the properties of the fungi of the human mouth, as given above, combined with a microscopic and chemical examination of carious tissue, and comparative studies of caries of living and dead teeth, appear to me to furnish a fair solution of the phenomena of dental caries. That other agents than those of a parasitic nature are also often concerned, there can be no doubt. To say nothing of predisposing causes, an acid reaction of the oral secretions, acid medicines, acid foods, etc., may give rise to caries at points which otherwise probably would have escaped.

Fig. 13