Bacteriology. — Prof. M. W. BEIJERINCK presents a paper: "*On Oligonitrophilous Bacteria*".

By "Oligonitrophili" I understand those microbes, which develop in media to which are not purposely added nitrogen compounds, but without precautions having been taken to exclude the least traces of these compounds.

They give rise to two different series of „accumulation experiments", the development being caused: *First*, in the light, without any other source of carbon in the food but the carbonic acid of the atmosphere, when chromophyll-containing oligonitrophili are to be looked for. *Second*, in presence of a source of organic carbon in the medium, when colourless oligonitrophili may be expected. In both directions I have made many experiments, of which those in the light have a very slow course and are still in process; here follow some results concerning "accumulation experiments" with colourless oligonitrophili.

1. *Aërobiosis and Anaërobiosis in Oligonitrophili.*

The "elective culture" of oligonitrophili in nutrient liquids with organic carbon compounds, has first been practised by WINOGRADSKY, under circumstances which secured anaërobiosis [1]). He used 2 to 4 pCt. glucose solutions with the required mineral nutrients and 4 pCt. $CaCO^3$, but without purposely added nitrogen compounds. For the infection was used garden-soil, and he constantly obtained a culture of a microbe belonging to the butyric-acid ferments. The experiments were performed in ordinary glass jars under cotton-wool plugging, when first a rich culture of aërobics develops, which renders possible the life of the anaërobic oligonitrophilous butyric-acid ferment, called by WINOGRADSKY *Clostridium pasteurianum*. He also worked with pure cultures of this species at exclusion of air. When repeating his experiments I found that traces of nitrogen compounds are indispensable for success, and the same is the case for the aërobic oligonitrophili found by myself, so that in culture liquids, prepared with all the precautions that exclude the presence of compounds of nitrogen, as well with aërobiosis as with anaërobiosis in a nitrogen atmosphere, the growth of oligonitrophili is extremely feeble and soon ceases.

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[1]) Recherches sur l'Assimilation de l'Azote libre de l'Atmosphère par les Microbes. Archives des sciences biologiques. St. Pétersbourg. T. 3 N. 4. 1895. „Elective culture" is the name given by W. to the accumulation experiments.

My own experiments differ from those of WINOGRADSKY by my having rendered possible either aerobiosis only, or by sufficiently promoting the access of oxygen at least partly to counteract the butyric-acid fermentation. So doing I came to the discovery of a not yet described genus of oligonitrophilous bacteria, belonging to the aerobics [1]). This genus, which is easily recognisable by the large dimensions of the bacteria, I will call *Azotobacter*. Hitherto I found two well distinguished species, one, *A. chroococcum*, is extremely common in garden-soil, the other, *A. agilis*, is as common in the canal-water of Delft.

Sufficient access of oxygen is easily to be obtained in my experiments by cultivating in thin liquid layers on the bottom of spacious ERLEMEIER-jars. As the butyric-acid ferment, however, can by no means do quite without oxygen, but, being „microaërophilous", does want oxygen, albeit of low tension for vigorous development (which has been overlooked by WINOGRADSKY), the regulation of the access of oxygen is not sufficient completely to keep this ferment out of the aerobic cultures. I have therefore tried to prevent its growth by selecting carbon sources which are well assimilated by *Azotobacter*, but cannot, or can only with difficulty give rise to butyric-acid fermentation. As particularly fit for this end I found to be: mannite in 2 to 10 pCt. solutions, and calcium propionate in $^1/_2$ pCt. solutions, of which the former is hardly, the latter not at all attacked by the butyric-acid ferment [2]). Less adapted for the experiments are cane-sugar and glucose, these sugars, especially the latter, easily getting into butyric-acid fermentation. But I must remark that a feeble butyric fermentation, at least when calcium carbonate is present, is by no means prejudicial to my experiment, as calcium butyrate, too, is a source of carbon easily assimilated by *Azotobacter*.

2. *Accumulation of Azotobacter chroococcum from Garden-soil.*

This species is obtained as follows.

In an ERLEMEIER-jar is introduced a thin layer of a not sterilised culture liquid of the following composition:

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[1]) More exactly of which one species is "macroaërophilous", the other "mesoaërophilous".

[2]) It is also possible to prevent butyric-acid fermentation by introducing a piece of pure red copper into the cultures, by which *Azotobacter* is not prejudiced. This artifice occurred to me by observing the flame reaction of copper, when burning in a BUNSEN-flame, common *Azotobacter*-films, grown without addition of copper, from a crude culture, in tap-water with 2 pCt. mannite and 0,02 pCt. $K^2H\,PO^4$, and infected with garden-soil.

100 Gr. Tap-water [1]),
2 „ Mannite,
0.02 „ K^2HPO^4.

and for the infection is used a not too slight quantity, say 0.1 Gr. or more, of fresh garden-soil [2]).

Accordingly, other nitrogen compounds but the small quantities which occur in the tap-water and the infection material, are wanting. But by numerous experiments, made under very different circumstances, many of which with nutrient liquids prepared from pure destilled water, whose composition was thus perfectly known, I have, as said, come to the conclusion, that this slight quantity of compounds of nitrogen is absolutely necessary for the success of the experiments with *Azotobacter*, and that the same is true for WINOGRADSKY's *Clostridium pasteurianum*.

In presence of nitrogen compounds in a rather considerable quantity, e. g. 10 milligrams or more of potassium nitrate or ammonium phosphate per liter of culture fluid, *Azotobacter* is no more proof against the competition with the common nitrophilous microbes and does not develop. But this is by no means the case with *Clostridium pasteurianum*, which excellently develops even at much higher rates of nitrogen compounds, though only then when the nitrophilous microbes have nearly quite consumed those compounds, so that diphenylamin shows no more nitrates, NESSLER's reactive no more ammonia.

If the culture jars, prepared in the said way, are kept at 28° to 30° C. then, after 2 or 3 days, a floating film develops at the surface of the fluid, externally resembling *Mycoderma*, but consisting of *Azotobacter chroococcum*, and wherein, it is true, some other species of small bacteria are present but not in sufficient quantity to determine the character of the culture. These small bacteria have greater want of nitrogen than *Azotobacter*, but less than the common saprophytic "polynitrophilous" species; they may accordingly be called "mesonitrophilous". The best known instance of mesonitrophiii is *Bacillus radicicola* of the tubercles on the roots of the Papilionaceae, but I have not succeeded to find this species in the crude *Azotobacter* accumulations. The mesonitrophili relate to *Azotobacter* as the vinegar bacteria to *Mycoderma* in the films which are found on flat beer, and their volume, when compared with that of *Azotobacter*

[1]) The tap-water of Delft comes from the downs at Loosduinen.

[2]) From pasteurised soil aërobic oligonitrophili do not develop.

itself, is so insignificant that at a chemical analysis of the culture, they would hardly be perceived. By carrying on the experiment with $^1/_2$ pCt. calciumpropionate as carbon source, instead of 2 pCt. mannite, and with garden-soil as infection material, after 3 or 4 days *Azotobacter*-films are obtained, in which microscopically no other bacteria at all are to be found but *A. chroococcum* only, and of which culture on solid media is necessary, in order to detect the not absolutely failing strange species.

Besides these small bacteria are sooner or later found in the *Azotobacter*-films a great number of Amoebae and Monads, sometimes also Infusoria. [1])

The common soprophytic bacteria, such as the fluorescents, the various species of *Aërobacter*, *Proteobacter*, *Saccharobacter*, and the hay bacteria, are quite wanting in the *Azotobacter*-films, although their germs abound in the infection material.

Moulds and yeast species, too, are in the beginning totally absent, so that the rough culture of *A. chroococcum* can be regarded as one instance more of a "perfect accumulation experiment", of which I recently described another case. [2])

The number of carbon compounds which can be assimilated by *A. chroococcum* is considerable. Thus mannite can be replaced by 2 to 10 pCt. cane-sugar, whereby, however, a more slimy film is formed, which sooner or later sinks down. For glucose, in quantities of 2 to 6 pCt., the same may be observed. But these two sugars, especially the latter, give most easily rise to butyric-acid fermentation, which, by the free acid, acts injuriously on the growth of *Azotobacter*. At simultaneous addition of calcicum carbonate a butyric-acid fermentation may first occur, which is succeeded, in the same culture, by the growth of an *Azotobacter*-film at the expense of the butyrate, and producing crystals of calcium carbonate. With galactose, levulose and maltose, I likewise obtained magnificent *Azotobacter* cultures; galactose gives with difficulty, levulose, on the other hand, gives easily rise to butyric-acid fermentation.

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[1]) Amoebae feed with great avidity on *Azotobacter* itself, and, multiplying very rapidly, can bring about much destruction in the cultures. They belong to different species, which also easily propagate on the solid medium, fit for the pure culture of *Azotobacter*. Thereon they produce the pure "veils of amoebae", free from bacteria, described by me at another occasion (Centralblatt fur Bacteriologie Bd. 19, pag. 257, 1896 and Bd. 21, pag. 101, 1897) and hence, may be obtained in pure culture by the here described experiment, together with *Azotobacter*, and be cultivated with other microbes at will for nutriment. Brief, also for the study of Amoebae the *Azotobacter*-experiment forms the best starting point.

[2]) Centralblatt f. Bacteriologie, 2e Abt. Bd. 7, pag 35, 1901.

With glycerin the experiments have a slower course; moreover only with solutions of 2 to 5 pCt. I could obtain closed *Azotobacter*-films, whilst 10 pCt. proved to be too concentrated. Milk-sugar is not assimilated by *Azotobacter*, but quite well by the butyric-acid ferment. Furthermore, the following substances are assimilated with variable intensity, the first best, the latter with more difficulty: prōpionates, butyrates, lactates, malates, succinates, acetates and citrates. Formiates and tartrates are not attacked at all.

As from this list we may safely conclude, that *Azotobacter* is able to assimilate still various other sources of carbon beside the here mentioned, the oxidising faculty of this bactery is evidently developed in a great many directions, and may perhaps be best compared to that of the fluorescents, which, however differ from *Azotobacter* by their much greater want of nitrogen, by which they belong to the polynitrophili.

The crude *Azotobacter*-film obtained in the way described, consists at first of extremely large short-rods of ca. 4 μ thick and 5 μ to 7 μ long, with rounded ends, and which often have the shape of diplococci. [1]) Mostly all are in rest but some specimens swim stately round. Remarkable is the presence of a lateral vacuole in some individuals.

The cell-wall is slimy and easily visible, or rendered visible by introducing some small bactery into the microscopic preparation, whereby the slimy coat, which in water alone is not to be seen, becomes distinct, as the small bacteria do not penetrate into it. At nutrition with mannite most individuals are filled with exceedingly small regularly placed drops of fat.

When the cultures grow older the floating film changes color and first becomes brown, later on sometimes even black. But this does not always occur and depends on known and unknown circumstances, Thus the color changes slowly or not at all at the direct nutrition with sugars, but the change can with certainty be expected when butyrates or propionates are used as carbon food, or, with sugars, in presence of calcium carbonate, and after previous butyric-acid fermentation.

The coloring matter is not soluble in the usual solvents as water, alcohol, chloroform, ether and CS^2, and is quite different from chromo-

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[1]) On propionates and acetates as sources of carbon, and with garden-soil for infection material, I have in these accumulation experiments sometimes obtained a much smaller form, which I consider as a variety of *A. chroococcum* and not as a separate species. A second variety of *A. chroococcum* I obtained from canal-water.

phyll. It induced me to choose the word *chroococcum* for the name of the species.

With the change of color the microscopical appearance of the bacteria themselves changes also considerably. The dimensions grow smaller and the shape becomes more globulous, so that we should think to have common, even small micrococci before us, but at the partition these older cells remain united in sarcine lumps. The shapes of the involution forms of *Azotobacter* are very singular. They can attain gigantic dimensions, e. g. 10—15 μ, and remind of amoebes and yeast cells. They are especially met with in the *Azotobacter*-films of the crude cultures.

3. *Pure culture of Azotobacter chroococcum.*

The pure culture of this species from the crude floating film is easily effected by streaking it off on a culture plate of the following composition:

100	Gr.	Destilled water.
2	„	Agar.
2	„	Mannite.
0.02	„	$K^2 H PO^4$.

The 2 pCt. agar contain the other necessary mineral nutrients in sufficient quantity. Grown at 30° C. *Azotobacter* becomes after one day already visible as white, starch-like colonies, among the, for the greater part watery, transparent nitrophili. Though in the crude cultures the latter had slackened their growth, on the plates they again acquire a considerable development, evidently in consequence of the presence of nitrogen compounds in the agar. The number of the *Azotobacter*-colonies is always much smaller than might be expected from the number of germs brought on the plate, so that some attention is necessary to find them out when still young; but later they become quite distinct. On the said medium, if containing sufficient mannite, e. g. 5 to 10 pCt., the *Azotobacter*-colonies can grow a very long time, and thereby attain much greater dimensions than those of the nitrophili.

Contrary to what we have seen in the crude cultures, *Azotobacter* can develop in pure condition on the most different media. On broth gelatin it grows however slowly and little characteristically; it hardly or not liquefies the gelatin.

Grown in liquids the presence of small quantities of nitrogen compounds furthers considerably the growth of the pure cultures. Espe-

cially nitrates are easily assimilated and may even be added to an amount of 0,1 pCt. Thus I sometimes, but not always, saw an enormous growth in

100	Gr.	Tap-water
2 to 10	„	Mannite
0.02	„	$K^2H\,PO^4$
0.1	„	$K\,NO^3$

With ammonia salts the growth of the pure cultures is slower than with nitrates, and the amounts which act not deleteriously, are slight. Still I saw a considerable devolopment in

100	Gr.	Tap-water.
2 to 4	„	Glucose.
0.02	„	$K^2H\,PO^4$
0.02	„	$(NH^4)^2\,HPO^4$

Asparagin acts about as ammonia salts. Peptone is assimilated with great difficulty.

After being kept for some weeks the pure cultures, in particular with glucose as carbon food, grow dark brown, quite like the crude films mentioned above, and in other respects too, they seem somewhat to alter their character. I could at least in no way produce on nutrient liquids, with the pure cultures, the magnificent films which are obtained by the crude infections; the newly formed cells remaining constantly immersed. But I should call to mind that this is partly explained by the use of non-sterilised materials in the crude cultures, which of course cannot be used in the experiments with pure cultures.

The motility of this species is always restricted to a very small number of individuals. By this reason, as also in consequence of the slimy constitution of the cell-wall, the experiments to color the cilia had given no result in my laboratory. But Professor ZETTNOW at Berlin, whose advice I have asked, procured me very beautiful preparations, from which it is certain, that at least the great majority of the moving individuals, possess one polar cilium of nearly the same length as the body of the microbe itself.

4. *Azotobacter agilis.*

This species is obtained by the "accumulation experiment" described for *A. chroococcum*, with this difference, that the tap-water is

replaced by canal-water [1]), and that the infection with soil is omitted, as the very question is to develop the oligonitrophili present in the canal-water itself. Good *agilis*-films are produced, when

100	Gr.	Canal-water.
2	„	Mannite
0.02	„	$K^2H\,PO^4$

in a thin layer is allowed to stand for some days at 30° C.

It is true that glucose is much better assimilated by *A. agilis* than mannite, but it causes more easily butyric-acid fermentation, which should here be avoided. Nevertheless I have in some cases obtained good results with glucose, and with cane-sugar also. Likewise when using $^1/_2$ pCt. calcium lactate, or $^1/_2$ pCt. calcium acetate. Even 2 pCt. alcohol is a very good source of carbon, but, like the last mentioned organic salts, produces an *agilis*-film much later than the different sugars. With propionates I obtained less good results, as therewith very numerous monads and amoebae originate, which feed on *agilis*.

The canal-water of Delft being rich in organic matter, the addition of a little $K^2\,HPO^4$ only is mostly also sufficient to form a beautiful film of *Azotobacter agilis*, which however, as a matter of course, remains poor in bacteria material.

The pure cultures are obtained in the same way as described for *A. chroococcum*. The best medium is

100	Gr.	Destilled or tap-water
2	„	Agar
2	„	Glucose
0.02	„	$K^2H\,PO^4$

In the streaks, inoculated on this medium the colonies of *agilis*, always intermixed with those of many other kinds of bacteria, among which *Azotobacter chroococcum* commonly occurs, are easily recognised after 24 hours already.

If in this latter solid medium the glucose is replaced by $^1/_2$ pCt. calcium propionate and if streaks are made of the crude culture, then also a considerable growth follows, and around the colonies of *A. agilis* a greenish diffusion zone arises, reminding of the coloring matter of the fluorescents.

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[1]) From the water of the North sea I could not obtain oligonitrophili.

In the pure cultures of *A. agilis* on broth agar, on broth gelatin, or in broth without gelatin, the growth is not very vigorous, but the motility is great.

The microscopic appearance of this bactery, in particular of the pure cultures on glucose-agar, is extraordinary. The large, transparent, extremely motile cells, show a wall, a small cell-nucleus, a protoplast with some granules hardly discernible from the nucleus, and often a very distinct vacuole. They measure ca. 5 μ or less, sometimes however more, and are very like small monads, or, when they don't move, like small yeast-cells. At the cell-partition in the living preparation a distinct nucleus-spindle is visible in many cells.

Spores are wanting.

The cilia-coloration is difficult and did not give satisfactory results in my laboratory, I therefore addressed myself, as in the case of *A. chroococcum*, to Professor ZETTNOW in Berlin, to whom I sent *A. agilis*, with a request for his opinion. He had the kindness to supply me with magnificent preparations, which prove most convincingly that the cilia are placed in bundles at the poles. He thereabout writes as follows: „..... In Spirillen-Bouillon [1]) war kein Individuum, das sich nicht auf das lebhafteste bewegt hätte... Nach der Art der ruhigen, wogenden, wenn auch kräftigen Bewegung, welche mich sehr an derjenigen kleiner Monaden erinnerte, hatte ich 1, resp. mehrere Polgeisseln vermuthet, und diese Ansicht haben auch die Preparate aus Spirillen-Bouillon, in welcher die Kultur in vollstem Leben durch Formalin abgetötet wurde bestätigt. Es hat mir jedoch Schwierigkeit gemacht zu diesem Resultat zu kommen. Die 6 bis 10 am Pol, resp. beiden Polen befindlichen Geisseln, legen sich nämlich meistens an der mit vielem stark klebendem Ectoplasma versehenen Oberfläche so an, dass sie scheinbar von der Seite zu entspringen scheinen." I also was at first in doubt and believed to see lateral cilia, but after a minute examination of the preparations I consider Prof. ZETTNOW's description as quite correct.

The relation to nitrogen of *A. agilis* is about the same as in *Azotobacter chroococcum*; to oxygen, on the other hand, it is different, as is proved by the following experiment.

If "respiration figures" [2]) of *agilis* are formed in ordinary microscopic preparations, between object-slide and cover-glass, the most

[1]) Broth with the addition of 0.1 pCt. KNO^3 and 0.1 pCt. $(NH^4)^2SO^4$.

[2]) For this term to compare: Centralblatt fur Bacteriologie Bd. 14, pag. 827, 1893.

strongly motile cells prove, like spirils, to be "mesoaërophilous", but they accumulate somewhat nearer to the meniskus than spirils would do, so that they approach the „macroaërophilous type". When continuing to grow in the glass-room, many cells stick to the glass and then display their mesoaërophilism with great distinctness. *A. chroococcum*, on the other hand, is macroaërophilous.

If the canal-water cultures, with mannite or other sugars as carbon food, are allowed to stand for some weeks at about 18° C., many, but not all, are crowded with an exceedingly rich flora and fauna, so that sugar solutions of 2 pCt. may literally become thick with microbic life, of which, besides *A. agilis* itself, spirils and other bacteria form the main portion, but where amoebae and other protozoa too, are present in great number.

It is a remarkable fact that oligonitrophilism can be the foundation of such a profuseness of life, if only care be taken for sufficient access of air.

Chemistry. — Professor BAKHUIS ROOZEBOOM presents a paper of Dr. C. H. WIND: "*On the irregularities of the cadmium standard cell.*"

1. Some cadmium standard cells constructed in accordance with the directions of the Physikalisch-Technische Reichsanstalt exhibit abnormal phenomena as shown by the observations made in that institution [1]), and also by the researches of COHEN [2]) and others.

COHEN investigated a cell made up as follows:

$$Cd \mid \text{dilute solution of } CdSO_4 \mid \text{Cd-amalgam of } 14.3\ \%,$$

and found in the case of two cells I and II which had been constructed in accordance with this type a difference in EMF. In the cell I it amounted to 56 mV at 0° and to 50 mV at 25°, with an almost linear slope; in cell II it amounted to 51 mV at 0° and to 50 mV at 25°, with a maximum of 52 mV at an intermediate temperature.

COHEN assumes provisionally [3]) that we are dealing here with different modifications or states of equilibrium of the 14.3 per cent amalgam.

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[1]) W. JÄGER u. R. WACHSMUTH — Wied. Ann. 59, p. 575, 1896; W. JÄGER — Wied. Ann. 65, p. 106, 1898; Dr. Ann. 4, p. 123, 1901.

[2]) E. COHEN — Versl. K. A. v. W. Amst. 9, p. 125, 1900.

[3]) id. — L. c. p. 137.