Microbiology. — *"Chemosynthesis at denitrification with sulfur as source of energy."* By Prof. M. W. BEIJERINCK.

(Communicated at the meeting of February 28, 1920).

In photosynthesis organic matter results from the reduction of carbonic acid by light as source of energy; the same takes place in chemosynthesis by chemical energy. Organisms with photo- or chemosynthesis are called autotrophes; those which feed on other organic substances are heterotrophes. The product of chemosynthesis is the body substance of the producers, always spore-free bacteria.

I described chemosynthesis at denitrification with sulfur as source of energy, on 16 April 1903 at the 9th Dutch Congress of Natural and Medical Science [1]). I then thought that in the process a facultatively anaerobic bacterium was concerned difficult to isolate by the plate-culture method. It was further presumed, that this species produced so much organic substance by chemosynthesis that the many directly visible bacteria, denitrifying with organic food, might live thereon. This supposition has proved to be erroneous; the latter themselves are in fact the operators of the sulfur denitrification as well as of the chemosynthesis. They are easily cultivated on broth-agar or broth-gelatin, but then they lose, and this is the new view, their autotrophy together with the power of sulfur denitrification, whilst preserving this power with organic food. The loss is caused by the growth with organic food and this loss being hereditarily constant, we have a case here similar to that which I described earlier for the nitrate ferment, and which I called "physiological species formation" [2]). Just as I then distinguished the oligotrophic from the polytrophic state we may in this case speak of the *autotrophic* and the *heterotrophic* condition of the operators [3]). The heterotrophic form is thus some common denitrifying bacterium.

On account of the little acquaintance with chemosynthesis acquired until now, I will begin with describing once more the original experiment [4]).

[1]) Phénomènes de réduction produits par les microbes. Archives Néerland. Sér. 2. T. 9, Pag. 153. 1904.

[2]) These Proceedings. Vol. 23, Pag 1163, March 28 (10 April) 1914.

[3]) As the existence of chemosynthesis is proved with certainty for the sulfur denitrification, but not for the nitration, the same nomenclature could not be followed in the two cases.

[4]) An enumeration of the chief processes accompanied with chemosynthesis is to be found in my paper: Bildung und Verbrauch von Stickstoffoxydul durch Bakteriën. Centralbl. f. Bakteriologie 2te Abt. Bd. 25, Pag. 30, 1910.

Arrangemeut and course of the experiment.

If a mixture of sulfur and chalk is introduced into a saltpetre solution with addition of some garden soil or canal mud, there will soon evolve at room temperature or at 25° to 30° C., a current of gas consisting of free nitrogen and carbonic acid. Thereby the saltpetre is denitrified, the sulfur is oxidised to sulfuric acid, found back as gypsum and potassiumsulfate, after the formula

$$6\,KNO_3 + 5\,S + 2\,CaCO_3 = 3\,K_2SO_4 + 2\,CaSO_4 + 2\,CO_2 + 3N_2$$

whereby per gram of decomposed nitrate about 1 cal. is produced. When after some days the process has become intense, the mud with the gas rises to the surface, and if the experiment is carried out in a flask, the contents can flow out with the gas as a slimy mass. This is bacterial slime, which keeps the sediment together.

If using distilled water with 10 % chalk, 10 % sulfur, 2 % potassium-saltpetre, 0.02 % bipotassium phosphate, 0.02 % magnesium chloride, and infecting with a small floccule from the said denitrification, we see after some days at 25° to 30° C. the very same phenomena as when using soil, only less intense; so the presence of soil is not necessary, but it clearly acts favourably. If the soil or mud is beforehand left a few days under a dilute saltpetre solution, so that all the organic substances fit for denitrification are removed, the soil remains quite as good for the sulfur-chalk experiment, hence the organic matter cannot be the cause of the favourable action on the process. It seems to result from the presence in the soil of colloidal silicic acid and aluminium silicate, which are to be considered as catalyzers that hasten the decomposition. So, in a thiosulfate denitrification the reaction goes on much swifter in presence of chalk and bolus (aluminium silicate) than with chalk only.

The saltpetre solution can be used in the most different concentrations. Even in 10 % solutions in tapwater made to a pap with sulfur and chalk, I saw at room temperature a spontaneous, intense gas production, with slime formation. The gas was nitrogen and carbonic acid; nitrogen oxydul seemed quite absent. The slime is bacterial slime, for the greater part consisting of different varieties of *Bacterium stutzeri* and *B. denitrificans*. It is so voluminous that its formation can only be explained by admitting that the said bacteria themselves produce this slime from the carbonic acid by chemosynthesis. With distilled water the result of the experiment is the same. In a closed bottle and with distilled water the process goes on as with accession of air, which proves convincingly, that presence of organic substance is not required for the development of the rich bacterial

flora which encloses the chalk and sulfur, and where at last many infusoria and monads, that feed on the bacteria, may be observed. As said the organic matter of the bacterial bodies must here be formed from the carbonic acid, whilst the required chemical energy is produced by the oxidation of the sulfur. Consequently this is a case of chemosynthesis and no other analogous process is known which produces organic substance in a simpler and more profuse way. [1])

By decanting and renovating the saltpetre solution as soon as the evolution of gas diminishes, the activity returns. [2]) This being repeated a few times the precipitate changes into a slimy mass, so rich in slime-forming bacteria that at heating on a platinum plate in the BUNSEN burner carbon is separated. With concentrated sulfuric acid carbonisation is also easily demonstrated. As the rate of nitrogen of this slime is less than 3 %, it must chiefly consist of wall substance, which is evidently the chief product of the chemosynthesis. [3]) It results from the carbonic acid after the same formula as the starch in the chlorophyll granules by photosynthesis, thus

$$6\,CO_2 + 5\,H_2O = C_6H_{10}O_5 + 6O_2$$

so that oxygen is set free, which explains the ready course of the process in a closed bottle, when considering that all denitrifying bacteria require a little free oxygen.

Just as the organic denitrification, that with sulfur may as well take place in the dark as in the light. After pasteurisation no sulfur-denitrification or oxydation is observed.

The quantitative estimation of the carbon fixed by chemosynthesis was made as follows. The sediment was treated with hydrochloric acid and later with alkali to remove the chalk and the sulfur, whereby certainly a great portion of the organic substance is lost. In the remaining precipitate, which still contains gypsum, the organic matter was determined as carbonic acid after the method of HERZFELD-

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[1]) It is true that chemosynthesis at the oxidation of hydrogen in presence of carbonic acid and soil, described by NIKLEWSKY and LEBEDEFF, is as productive in organic substance, but the experiment is less simple.

[2]) Addition of soda instead of decantation and renovation, also acts favourably. Evidently the dissolved sulfuric acid is difficultly neutralised by the chalk of the precipitate.

[3]) See also: A. J. LEBEDEFF, Ueber die Assimilation des Kohlenstoffs durch Wasserstoff-oxydierenden Bakteriën. Berichte d. Deutschen Botan. Gesellsch. Bd 27, Pag. 598, 1909. He says that the bacterium can oxidise hydrogen in absence of CO_2; this, however, is manifestly erroneous. Nor does he take into consideration the oxygen produced at the denitrification by the hydrogen of the saltpetre, used by him as source of nitrogen. His fear that by using ammonsalts nitrification would follow, is under these conditions unfounded.

WOLFF-DEGENER,[1]) by oxydation with bichromate and sulfuric acid. After a culture of about six weeks there was in this way found about 0.05 gram of carbonic acid per gram of oxidised sulfur, which corresponds to 0.013 gr. of organic carbon.[2]) This quantity, however, must certainly be doubled, for at the extraction of the chalk and sulfur at least half the weight of the bacterial substance is lost. I therefore esteem the production of organic carbon in relation to the oxidised sulfur at 2 % in weight.

Old cultures containing much organic matter and in which the nitrate has disappeared produce H_2S, obviously in consequence of sulfate reduction, and perhaps, too, directly from the still present sulfur, whilst the hydrogen wanted for this originates from the organic material formed by chemosynthesis. Such liquids finally teem with infusoria and monads, and various other members of the so remarkable "sulfur-flora" and "-fauna".

The microscopical image during the period of chemosynthesis is that of very small, partly motile rodlets and micrococci. Spore-formers with chemosynthesis do not exist.

Plate culture.

The agents of the denitrification with sulfur were isolated on different solid media, but always with the result that the pure cultures, grown on organic media did not, or only feebly denitrify in the anorganic mixture; *only those of the silicic plates were but slightly enfeebled* in this function. The media used were: washed agar dissolved in distilled water, with salts; or tapwater-agar with $^1/_2$ % thiosulfate, 0,1 % saltpetre and 0,02 % bipotassium fosfate; or silicic plates with the same mixture with or without addition of chalk, and finally broth-agar and broth-gelatin.

If on the media containing organic matter floccules of the sulfur denitrification are streaked off and cultivated at 30° C., there appear, already within 24 hours, denitrifying colonies which, especially on the broth plates grow with a remarkable rapidity. The two or three chief species recognisable among the denitrificators may be easily distinguished. On the media containing sulfur or thiosulfate and chalk, and on the silicic plates, the colonies remain small

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[1]) F. TIEMANN und A. GÄRTNER. Die chemische, mikroskop. und bakteriol. Untersuchungen des Wassers. 3te Aufl. Pag. 247, 1889.

[2]) The quantity found by Mr. JACOBSEN at the direct oxidation of sulfur by bacteria was of the same order. (Die Oxydation des elementaren Schwefels durch Bakterien. Folia Microbiol. Jahrg. 1, Pag. 487, 1912).

and cannot be well recognised on account of the opaqueness of the medium. Yet I have further examined these colonies by making streaks of them on broth-agar plates, always finding that they more or less readily develop; colonies failing in this respect I did not find.

I have also tried to obtain anorganic denitrifications with those portions of the streaks on the sulfur- and thio-sulfate plates lying between the colonies, but as well in aërobic as in anaërobic condition always in vain. Neither microscopically nor by colouring, bacteria or microbes of other nature could be found in these parts.

Hence it follows with certainty that the agents of the anorganic denitrification grow to colonies both on the sulfur-chalk and the thio-sulfate plates and besides, as will be still further proved below, on the ordinary broth plates. The highly improbable hypothesis that they might be obligative anaërobes is disproved by these experiments, which are, however, well in accordance with the conception that by growth on organic matter their power of autotrophy gets lost.

To compare the broth with the thiosulfate medium I made the following experiment.

A platinum wire was bent so as to form at one end a loop, with which droplets of the same size could easily be taken up; the other end was curved to a circular base, which made it possible to place it on the balance and determine the weight of the droplet. Now drops of equal size were taken up with this loop from the anorganic denitrifications and transported for comparison to a thiosulfate- and to a broth-plate. The result was that the number as well as the species of the developing colonies were about the same. All the colonies grown on the thiosulfate plates, after being sown on broth-plates, developed very well, quite in accordance with what was observed already for the colonies grown on the sulfur-chalk plates.

So it is certain that the microbes causing the anorganic denitrification produce colonies on the organic plates.

This statement is of particular interest as the colonies, when again transferred to the anorganic sulfur-chalk mixture, do not, or only very feebly, denitrify, which means that they have almost or quite lost their power of chemosynthesis [1]).

This is not only true for the pure colonies separately, but likewise for the combinations that may be made of them. Even when the whole bacterial mixture on the plates is transported to the anorganic medium, only a slight or no chemosynthesis or denitrification

[1]) In "Untersuchungen über die Physiologie denitrifizirender Schwefelbakteriën, Sitzungsberichte Heidelberger Akademie. Biol. Abt. 1912", R. LIESKE has come to another result.

at all occurs. On the thiosulfate plates the germs preserve their autotrophy longer than on the broth plates, but there too, this power finally gets lost. The real cause of this loss is not yet quite explained. With certainty it can only be said to take place when the concerned germs *augment when fed with organic food.*

Especially on the broth plates at 30° C. the colonies develop rapidly. It seems that four or five species are thereby active. Three or four denitrify strongly in broth bouillon with 0.1 to 1 % potassium-nitrate, and they predominate so much that non-denitrifying species are not easily found. There is even no surer and easier method to obtain bacteria denitrifying with organic food than this anorganic denitrification, for although it is often difficult to isolate the active bacteria from the organic denitrifications, this is here by no means the case [1]).

Among the colonies obtained from the anorganic mixture there are, as said, some which do not denitrify with organic food. Probably they live in the sulfur-chalk cultures as saprophytes at the expense of the organic matter formed by the autotrophes.

On silicic-thiosulfate-nitrate-chalk plates develop, after two or three weeks, yellowish colonies of 1 to 1½ mm. in diameter and nearly 1 mm. high, evidently autotrophic. In the anorganic mixture, freed from air by boiling, they cause a vigorous denitrification after 24 hours at 28° C. already. When sown on broth-gelatin the colonies appear to consist of two soft varieties [2]) of *B. stutzeri*, which do not melt the gelatin and of which one shows the usual structure; the other, the commonest by far, lacks that structure completely, nevertheless it resembles *B. stutzeri* in the other cultural aspects. It consists of a white soft mass of extremely small rodlets. In broth nitrate both show strong denitrification, especially the soft form, so that it is one of the most intensely denitrifying bacteria I know. At re-inoculation from the organic into the anorganic food we also find here that the autotrophy and the power of anorganic denitrification are lost.

[1]) The most important denitrifying soil bacterium, the spore-forming *Bacillus nitroxus*, loses its denitrifying power quite or partly by growing on aërobic plates. Other species, such as *Bacterium pyocyaneum*, *B. stutzeri*, *B. denitrofluorescens* preserve, in aërobic plate cultures and in the collections, their denitrifying power unchanged for years.

[2]) In reality there are three varieties, but the third which shows the character of the ordinary tough, folded colonies of *B. stutzeri*, is rarer. — It must be admitted that the difference between the soft colonies and the typical *B. stutzeri* is, superficially, considerable, and I think that many other observers would bring them to distinct species.

The principal species.

The colonies from the sulfur-chalk denitrifications, which develop on the broth plates are for a part coloured yellow or reddish brown by carotin[1]), for the greater part, however, colourless. The brown species is a Micrococcus; it liquefies the gelatin and the micrococci differ much in size; the smaller ones are highly motile, but they lose their motility when transferred to broth-agar, whereby their denitrifying power, too, disappears. The yellow species is related to the brown and consists of small very motile rodlets. Here also the same variability.

The uncoloured colonies are of two types: soft, and tough or slimy.

All the soft ones liquefy the gelatin on which they grow intensely; sugars are not fermented, no fluorescence; they belong to three classes different by their size: 1. Extensive, rapidly growing, strongly denitrifying. 2. Middle sized, less rapidly growing, as strongly denitrifying. These two classes are allied by intermediate forms and may be brought to one single species, *Bacterium denitrificans*. 3. Very small and feebly growing, non-denitrifying bacteria, manifestly living at the expense of organic food produced by the other species through chemosynthesis.

With the pure cultures on an organic medium of the second form, I have succeeded in obtaining very feeble anorganic denitrifications, hence, chemosynthesis. This could, however, only be observed in the quite young cultures that had but for a short time grown on the broth medium. Cultures which have longer than two or three days been in contact with organic food and the air, can no more denitrify with sulfur and chalk, but still very well in saltpetre broth. For demonstrating the anorganic denitrification, test tubes are partly filled with mud, previously deprived of organic matter by keeping the mud under a saltpetre solution. To the mud sulfur and chalk are added and subsequently 1 % saltpetre; the dissolved oxygen and the germs are removed by boiling; sterilisation is not wanted, as spore-formers with chemosynthesis do not exist.

Entrance of air is prevented by a hollow glass sphere, well fitting in the tube and floating on the liquid, but this precaution is not necessary.

With the pure cultures of the soft colonies I could not obtain any evolution of gas in this mixture, they manifestly lose their autotrophy still sooner than those of the second group.

The more or less tough, or slimy, or cartilaginous colonies belong

[1]) This pigment is soluble in CS_2 and turns blue or violet with concentrated sulfuric acid.

all to *Bacterium stutzeri*, if taking the conception of species in a broad sense; superficially there is a great difference between the colonies of this group. The usual form, which is very remarkable and easily recognisable by the shape of the colonies, has been described in these Proceedings by Professor VAN ITERSON [1]. Even in the smallest floccules of the sulfur denitrifications some form of *B. stutzeri* is found, although the soft colonies prevail. But besides, other varieties of *B. stutzeri* occur, for example such which slightly liquefy gelatin, or such which are light brown or rose-coloured, or whose colonies lack the so characteristic structure, and again others with that structure, but wanting the denitrifying power. There are, too, intermediate forms between the tough and the soft class, and I think it possible that they originate from each other by mutation.

That *Bacterium stutzeri* in the anorganic denitrifications possesses autotrophy, follows from the above described experiment with the silicic plates. But this may also be proved for colonies of "organic" origin, if only the right moment be chosen for experimenting with them. In the organic plate cultures the autotrophy of this species gets however rapidly lost. Only with quite fresh colonies, grown on thiosulfate-agar plates, and transferred to the anorganic medium, just at the time of their becoming visible a feeble but distinct anorganic denitrification could be obtained, which continued during several days with the same degree of intensity, only much feebler than the spontaneous denitrification. So it seems proved that the autotrophy does not disappear as an indivisable factor, but may get lost in parts.

That the autotrophy is really lost in the originally active colonies, is corroborated by the fact that not only the single colonies of the organic plates, but likewise the combinations of the colonies of the different species are quite inactive. Even all the colonies of broth-agar plates together, mixed with the undeveloped germs lying between them, do not produce any denitrification in the anorganic mixture. And this must be true for all the different species which produce anorganic denitrifications and evidently possess the power of chemo-synthesis in their natural habitat.

This form of variability is obviously analogous to that of the nitrate ferment, which I formerly described [2]) and as said called physiological species-formation. In both cases a new elementary species is produced. It is remarkable that a number of species or varieties living under the same conditions are subject to this trans-

[1]) Ophoopingsproeven met denitrificeerende bakteriën. Acad. of sciences. Amsterdam, July 1902.

[2]) Ueber das Nitratferment und über physiologische Artbildung. Folia micro-

formation, and that between the principal form and the one that has completely lost its original character, some feebly denitrifying intermediate forms are found, which may be compared to subspecies.

Taking *B. denitrificans* as an example we can speak of *B. denitrificans autotrophus* and of *B. denitrificans heterotrophus*, the change being possible only in one direction, at least with our present knowledge.

This change is not a mutation in the accepted sense, as thereby the primitive stock continues to exist with the mutant under the same conditions under which the latter was formed. Here on the contrary all germs change simultaneously, so that in this case we have to do with a hereditarily constant modification, comparable to the pleomorphy of many Fungi, and to a certain extent, to alternation of generation. Comparable also to the production of somatic cells from germ cells during the ontogony of higher animals and plants, a fact certainly of general physiological signification. But modification and mutation are conceptions not sharply distinguishable and gradually related.

Another case of variability, similar to the loss of chemosynthesis by feeding with organic substances, I observed in various lower Algae respecting photosynthesis. For a long time I have been cultivating the gonidia of the lichen *Xanthorea parietina*, which are identical with the Protococcacee *Cystococcus humicola*. The first isolation was made by streaking off the said lichen, rubbed to a mash, on pure agar with salts and cultivating it in light. The thus obtained green, pure colonies, develop very readily as well in the light as in the dark on maltextract-agar and form large green masses, which, however, in course of time completely lose the power of photosynthesis, so that neither on agar with salts, nor in anorganic liquid media any growth takes place. Microscopically no difference is to be seen between the inactive chloroplasts of these cells and the active ones of normal *Cysto coccus* cells.

The very same I observed in cultures of *Pleurococcus vulgaris*, isolated from the bark of trees and long cultivated on maltextract-gelatin, on which it grows vigorously in the dark without losing the green colour. Hence it is clear that for photosynthesis the presence of chlorophyll in the living protoplasm is not sufficient, but the process requires still another factor, which may get lost through cultivation with organic food.

The greater part of the chlorella's of *Hydra viridis*, undoubtedly

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biologica, 3e Jahrg. Heft. 2, Pag. 1, 1914. Recently I found that the ferment which produces nitrous acid from ammonium salts behaves in the same manner and changes, when fed with organic food into a saprophytous non-nitrifying form

belonging to the so easily cultivable species *Chlorella vulgaris*, lose, when out of the *Hydra* body, howsoever fed, as well the power of photosynthesis as that of growth, so that it is very difficult to cultivate them. So, here is a case where change of food causes the loss as well of the function of photosynthesis as of that of growth.

CONCLUSION.

Some of the common denitrifying bacteria, such as *B, denitrificans* and *B. stutzeri* (these names taken in a broad sense), and probably some other species, may occur under two physiologically different modifications, which are hereditarily constant, when their feeding conditions remain unchanged. One form, the autotropic, is adapted to the anorganic medium (sulfur- or thiosulfate-chalk-nitrate) and shows chemosynthesis; the other, the heterotrophic form, requires organic food. They may be compared to the oligotrophic and the polytrophic condition of the nitrate ferment. Intermediate forms, feebly denitrifying in the anorganic medium, also occur, hence the autotrophy may be lost gradually.

The heterotrophic forms preserve the power of denitrification with organic food.

The nitrite ferments of the ammonium salts are also related to hereditary modifications with the character of saprophytes, living on organic food and unable to oxidise ammonium salts.

Great changes in the nature of the food may thus be the cause of hereditary modifications of certain factors, and this seems to throw some light on the causes which underlie ontogony.