On photosynthesis of the Thiorhodaceae

ON PHOTOSYNTHESIS OF THE THIORHODACEAE

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PROEFSCHRIFT

TER VERKRIJGING VAN DEN GRAAD VAN DOCTOR IN DE WIS- EN NATUURKUNDE AAN DE RIJKS-UNIVERSITEIT TE UTRECHT, OP GEZAG VAN DEN RECTOR-MAGNIFICUS Dr. H. BOLKESTEIN, HOOGLEERAAR IN DE FACULTEIT DER LETTEREN EN WIJSBEGEERTE, VOLGENS BESLUIT VAN DEN SENAAT DER UNIVERSITEIT TEGEN DE BEDENKINGEN VAN DE FACULTEIT DER WIS- EN NATUURKUNDE TE VERDEDIGEN OP

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PIETER ANTON ROELOFSEN

GEBOREN TE BANDOENG (JAVA)

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Aan mijn aanstaande vrouw

VOORWOORD.

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CONTENTS.

INTRODUCTION.

The extensive and careful investigations of VAN NIEL (1931) on the coloured sulphur bacteria have thrown a new light on the morphology, physiology and ecology of this group of organisms. The chief result of his study is undoubtedly the elucidation of the main principles underlying the metabolism of these organisms. This is the more important, since previous investigators held very divergent and partly conflicting opinions regarding this subject. VAN NIEL succeeded in bringing experimental proof for his theory that in purple and green sulphur bacteria we are dealing with a group of organisms which for their proliferation are dependent on a very special type of photochemical carbon dioxide assimilation. Whilst in photosynthesis of the ordinary green plants the carbon dioxide reacts with water, in the case of the purple sulphur bacteria the water is replaced by oxidizable sulphur compounds or organic substances.

VAN NIEL and MULLER (1931) have rightly emphasized the great importance of a closer study of the photochemical carbon dioxide assimilation in the *Thiorhodaceae*[1]) for our conception of photosynthesis in general.

The theoretical considerations of these authors made me decide to study the process in question with the aid of the methods which had been so successfully applied by WARBURG and NEGELEIN (1923) to the study of the photosynthesis of unicellular chlorophyll-containing organisms (green algae) (C. f. chapter IV).

Such a study seemed the more interesting, since the anaerobic nature of the purple sulphur bacteria implies the ab-

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[1]) As *Thiorhodaceae* or purple sulphur bacteria, or in the abbreviated form p. s. b., I will designate those *Rhodobacteriales*, which are obligate anaerobes and which are characterized by a photosynthetic autotrophic mode of life. *Athiorhodaceae*, or purple bacteria (p.b.), are those *Rhodobacteriales*, which are facultative-anaerobes, obligate heterotrophonts and which are obligately photosynthetic in an oxygen-free medium. (VAN NIEL and MULLER 1931).

sence of a true respiration process, which in the study of the green algae complicates the situation. Yet it appeared probable that in the *Thiorhodaceae* too some energy yielding process of still unknown nature would proceed during periods of darkness and therefore also a study of this point might be deemed of interest (C. f. Chapter III).

Finally it seemed attractive to study the energy relations in the photochemical carbon dioxide assimilation of the *Thiorhodaceae*. The fact that on the one hand this process is closely related to ordinary photosynthesis, but that on the other hand other photochemically active pigments and different reaction components are involved, seems to offer favourable conditions for a deeper penetration into the mechanism of the energy transference in the photochemical processes of both green plants and purple bacteria. As a first step in this direction a determination of the number of light quanta required for the reduction of one molecule of carbon dioxide appeared to be of much importance, especially since theoretically it must be considered possible to determine this number for different wave lengths and whilst employing different hydrogen donators (C.f. Chapter V).

It may be remarked in advance that I had no opportunity to exhaust completely the programme as outlined above.

It seems superfluous to give here a survey of the older literature on the metabolism of the coloured sulphur bacteria, since a very thorough and critical discussion of it has recently been given by VAN NIEL in his publication mentioned above.

It will suffice to give here a brief review of the publications which have appeared since VAN NIEL's paper was published. For the rest I will restrict myself to a short survey of the present state of our knowledge regarding the points at issue in the introductory remarks to each of the various chapters.

In the first place mention may be made of the book published by ELLIS in 1932 on the sulphur bacteria. However, new points of view regarding the metabolism of the coloured sulphur bacteria have not been opened in this monograph. Neither is this the case in the publications by GINSBURG-KARAGITSCHEWA (1932) on methods of cultivating *Thiorho-*

daceae and by VON DEINES (1933) on the nature of the sulphur stored in these organisms.

The important question of the behaviour of the *Thiorhodaceae* in organic media, which was only more or less incidentally struck by VAN NIEL, has been more fully investigated by MULLER (1933[1]). The principal result of this study is undoubtedly that although these bacteria can thrive on various of the most commonly accepted carbon sources, they obviously are unable to utilize these compounds directly in the synthesis of their cell substances. The *Thiorhodaceae* — and probably also the *Athiorhodaceae,* when growing anaerobically in the light in organic media — have the remarkable property of utilizing the organic nutritional compounds only indirectly, viz. as hydrogen donators in the photochemical reduction of carbon dioxide.

GAFFRON (1933, 1935) has published several investigations on the metabolism of the *Athiorhodaceae.* This author too has realized the great advantages of the application of the manometric method for the study of this subject. Though one should interpret the results of GAFFRON with great reserve, since he did not use pure cultures, but worked with impure enrichment cultures, yet on the whole the results obtained give support to the idea, that the metabolism of the *Athiorhodaceae* and that of the *Thiorhodaceae* in an organic medium under anaerobic conditions in the light are fundamentally the same. It must be emphasized, however, that in another communication (1934) in which the metabolism of the *Thiorhodaceae* is studied, GAFFRON himself rejects this idea. In this paper a quite new conception of the metabolism of these bacteria has been given. Although my own investigation was already in full swing at the time when this paper was published, I felt it necessary to give due attention to this question. I have therefore repeated his experiments and subjected them to a critical examination (c.f. pag. 49).

Finally important work has been published of late on the chemical nature of the pigments of the purple bacteria. VAN NIEL (1933) has published some preliminary results of a study of the chemical composition of bacterio-erythrine, the red pigment of the *Rhodobacteriales.* VAN NIEL's results

leave no doubt that this pigment belongs to the carotinoids. NOACK and SCHNEIDER (1934), SCHNEIDER (1934) and FISCHER and HASENKAMP (1935) have made extensive studies of the chemistry of the green pigment of the purple bacteria. As was already assumed by other investigators, this pigment appears to be closely related to chlorophyll and in all probability the same will hold for bacterioviridine, the pigment of the green sulphur bacteria.

In a preliminary communication (1934) some of the results of my own work have already been published.

CHAPTER I.

THE ISOLATION AND CULTIVATION OF THE STRAINS USED.

§ 1. *Introductory remarks.*

The majority of old and recent investigators of the physiology of autotrophic organisms, with which I mean bacteria as well as green plants, has probably underrated the dangers, caused by the use of material, in which the organism to be investigated is not in a pure condition.

Now I will not pretend that the results of the study of the physiology of the green plants have been greatly impaired by this. Nevertheless I think that a simple reference to the publications of VAN NIEL (1931) about the photosynthetic autotrophic *Thiorhodaceae* and to that of KINGMA BOLTJES (1934) about the chemosynthetic autotrophic nitrifying bacteria will suffice to demonstrate the necessity of the purity of the material employed in a physiological study of autotrophic organisms also.

While an apparently wholly inorganic medium may be still an excellent substrate for heterotrophic specialists like *Bac. oligocarbophilus* and *Hyphomicrobium vulgare,* such a medium allows a good development of even very common heterotrophic bacteria, as soon as autotrophic organisms are thriving or have thrived in it.

Many investigators in cultivating algae have been greatly encumbered by the presence of bacteria in these cultures. So one can easily imagine that in those cases where no pure, but so-called "sound algae" were used for metabolic experiments, these bacteria have contributed markedly to the respiration observed. Yet definite statements regarding this point are usually lacking.

A recent example of the confusion, which may arise from neglecting the requirement of purity of the cell material em-

ployed, is to be found in the investigation of GAFFRON (1934) previously mentioned. I shall revert to this later on.

§ 2. *Origin of the strains used.*

At the outset of my investigations the strains *1, 4, 7, 9, 12, 19, a, b,* and *c* employed by F. M. MULLER were still available. However, these strains had all been contaminated since, but by applying the method described by VAN NIEL (1931), it was an easy task to purify them once more. The contamination was so slight, that I could make use of shake cultures in peptone agar (peptone "Poulenc" 1%, NaCl 2%, 2% agar in tapwater) thus profiting by the more rapid growth in organic media, as already mentioned by VAN NIEL (1931, p. 102).

The greater majority of my experiments was carried out, however, with a strain (*d*), which was isolated by me from an enrichment culture sent by H. GAFFRON to the Microbiological Laboratory, Delft. This bacterium, it is true, was present in the enrichment culture as the prevailing organism, but for its isolation it still proved to be necessary to employ the inorganic medium, as described by VAN NIEL (1931, p. 22). A short description of this strain follows here.

Being obligately anaerobic and obligately photosynthetic, it belongs to the group of the *Thiorhodaceae*. Under the conditions of cultivation as employed by me, in casu in an inorganic or organic medium with a pH of about 8,0 and a low Na_2S concentration, mainly highly motile diplococcus forms may be observed, though micrococcus forms are not rare.

Neither conglomerates of three or more cells nor the formation of capsules or the production of mucus were ever observed. The cells have an average diameter of 1,5 μ. In some cases, specially in the organic media, they may be larger, up to 4 μ. Under certain conditions the cells may contain one or more highly refractive sulphur globules. The bacterium reminds one of the organisms described by VAN NIEL as the *Thiocystis*-type.

It proved impossible to bring this strain into a known genus with the aid of one of the proposed systems of the *Thiorhodaceae*. Considering the exceedingly unsatisfactory state of

the taxonomy of this group this can hardly be wondered.

§ 3. *Cultivation and control of the pure cultures.*

The pure cultures obtained were kept as stab cultures in BURRI-tubes with organic agar media. The tubes were provided with a second plug of absorbent cotton-wool, with potassium pyrogallate, and were rubber-stoppered. It appeared, that the modification of the BURRI-method as indicated by RITTER and DORNER (1932), viz. a substitution of the caustic soda by sodium carbonate, offered no advantage in this case. Thus placed in artificial light the cultures are easily kept alive for one or two months.

The strains *a*, *b*, *c*, *9* and *19* were kept in the mentioned peptone agar medium. The other ones, which did not grow so well in this medium, were kept in a Na-malate medium. This was prepared by adding 0,1% Na-malate, 0,1% $Na_2S_2O_3$ and the usual 2% agar to the standard salt-solution, i.e. NaCl 2%, $(NH_4)_2SO_4$ 0,1%, K_2HPO_4 0,05%, $MgSO_4$ 0,02%, in tap-water.

Before the inoculation 0,005% Na_2S was added and the pH was adjusted to 8,0 for strain *d*, to 8,5 for the strains *4* and *12* and to 8,7 for the strains *1* and 7. This was carried out by adding sterile 10% solutions of H_3PO_4 or of Na_2CO_3, using the indicators cresol red and brom thymol blue. Of course these media may also be used without agar; however, in using the liquid medium in culture tubes, a weekly transfer is necessary. I have abandoned the use of glass-stoppered bottles for keeping the pure cultures, because of the danger of contaminations entering between neck and stopper. The mentioned substrates are to be preferred to inorganic media in that they are more easily prepared, since $NaHCO_3$, which requires a separate sterilization by filtration, may be left out. Hence the risk of contamination is smaller, whereas the growth is more abundant. As a carbon source Na-malate was chosen by me, since in consequence of its high "oxidation value" 1,2 mol. carbon dioxide is produced per mol. Na-malate taken up by the bacteria (MULLER 1933[1], p. 150). As a result the pH of the medium remains constant during a much longer time, which

favours the growth of the bacteria and their viability.

Experience has taught me, that the addition of thiosulphate has a favourable influence. Probably hereby the carbon dioxide, originating from the Na-malate, is reduced again under simultaneous formation of sulphuric acid and hence the malic acid can be more fully converted into cell material.

The purity of the strains was controlled with each transfer by inoculating peptone agar plates and by incubating these during several days aerobically and anaerobically at a temperature of 30° C. For the anaerobic cultures I used the convenient "anaerobic jars" as devised by Mc INTOSH and FILDES. These jars are in general use for the cultivation of anaerobes in the Delft laboratory.

§ 4. *Cultivation of the bacteria used in the experiments.*

It was not only of paramount importance to find a strain specially suitable for my experiments, but also to offer that strain such favourable culture conditions, that I could daily have a proper quantity of young and vigorous bacteria at my disposal. The requirements for the material to be used were fairly high. In the first place all cells should be in optimal conditions, specially for the experiments mentioned in Chapter V. Cultures, in which a part of the bacteria would have sunk to the bottom already or would have produced mucus and formed cell conglomerates, were to be excluded a priori and it was essential to select a strain which would not, or only in a very slight degree, show these properties in the culture medium employed.

In the second place the bacteria should have to thrive well in a culture medium of a pH preferably below 8,0 (group 2 of VAN NIEL 1931, p. 46). The reason for this is, that the pH of the suspension liquid, which is to be used in the experiments, may not differ much from that of the medium in which the bacteria have been grown.

If one wants to measure changes in the carbon dioxide pressure manometrically, the primary requirement is to use a suspension liquid, which is at the most slightly alkaline. At a pH $>$ 8,0 one is too much encumbered by the so-called

"carbon dioxide retention" (see p. 33). So the strains *1*, *4*, *7* and *12*, which were adapted to a pH of 8,5—8,7, had to be rejected for the majority of my experiments.

It appeared that of the remaining strains, strain *d* best complied with the demands, at least when only the behaviour in inorganic culture media was considered. Although the majority of the experiments were carried out with this strain *d*, I have gained so much experience with the strains *a*, *b*, *c*, *9* and *19*, that it seems justified to conclude that these strains behave fundamentally in a completely similar way. As a matter of fact none but quantitative differences have appeared.

In order to obtain the material needed for the experiments, bottles of 600 cm^3 were inoculated from pure cultures. Like the pure culture tubes the glass-stoppered bottles were incubated in a light cabinet especially made for this purpose (VAN NIEL 1931). The temperature measured in the culture bottles could be varied between 30° and 36° C. They were placed inside at a distance of 25 cm. from a 60—75 Watt electric bulb. In some preliminary growth experiments, carried out with strain *a* in peptone broth I could easily ascertain a distinct temperature optimum at 31°C. I made these experiments with a "temperature organ" fundamentally similar to the apparatus described by RUINEN (1933). It was placed before a window facing the east.

In the light cabinet, however, my strains thrived best at 35° C. This is probably due to the greater quantity of light, which is available there. Under these conditions the cultures in suitable organic media attained their maximal development within a week, whereas those in inorganic media reached it in the second week.

The majority of the experiments was carried out with material originating from an inorganic medium. In case other media have been used, this has always been mentioned. The inorganic medium, which finally proved to be the best for my purpose, was prepared by adding to the mentioned standard salt solution 0,1—0,15% $Na_2S_2O_3$ and by just boiling up, cooling and filtering it, before sterilization. In this way the formation of a precipitate in sterilizing was prevented. Before using this medium it was freed from oxygen by boiling

it up, followed by a rapid cooling and by adding 0,005% Na_2S. After adding 0,7—1% $NaHCO_3$ and adjusting the pH to 7,8 in the manner previously mentioned, the culture bottles were filled almost to their necks and inoculated from culture tubes with a liquid Na-malate medium, which served as a link between the stab cultures and the cultures in the glass-stoppered bottles. In order to exclude the air as much as possible, the bottles were as yet provided with a layer of pure sterilized (120°C.) paraffine oil. In dry sterilization (200°C.) of bottles with paraffine rests, substances may be formed, which appear to be harmful to the bacteria; hence the used bottles should be very thoroughly cleaned.

The $NaHCO_3$ was added by means of the apparatus as shown in fig. 1, by adding a suitable quantity of a 7% solution of $NaHCO_3$ to the standard solution. The 7% $NaHCO_3$

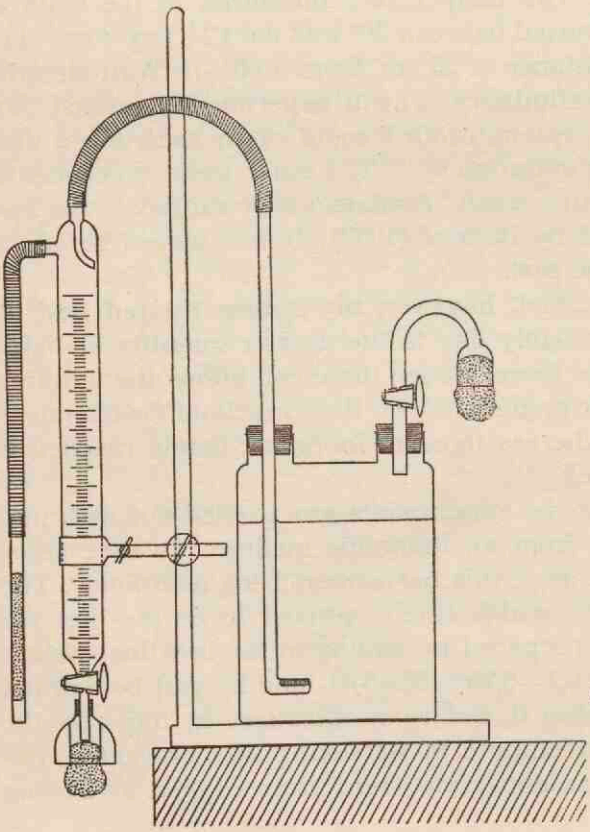

fig. 1.

Apparatus for the preservation and the sterile sampling of Na-bicarbonate solutions.

solution was sterilized with the aid of a Seitz-filter, which previously had been thoroughly coated with tin. Preceding this, carbon dioxide, sterilized by passing a plug of cotton-wool, was led through the solution in the apparatus until the pH was about 7,8. This was done because in the long run a $NaHCO_3$ solution exposed to the air passes into a Na_2CO_3 solution. As moreover a $NaHCO_3$ solution with a pH of 7,8 contains much carbonic acid, the bacteria have more CO_2 at their disposal.

The concentrations of the carbon dioxide available, of the $Na_2S_2O_3$ and of the Na_2S were chosen in such a way that the bacteria only temporarily contained sulphur. Just previous to their maximal development, the cultures lost in most cases the calcareous appearance and turned a dark red colour. On microscopic examination it then appeared that nearly all cells had lost the characteristic highly refractive sulphur globules. This production and subsequent consumption of sulphur is advantageous for three reasons.

In the first place it is conducive to an efficient absorption by the bacterial pigments of the light used (see pag. 29), which is of special importance for the experiments to be mentioned in Chapter V.

Secondly under these conditions it is excluded, that the bacteria use their reserve sulphur for the carbon dioxide assimilation which is important in those cases, where the assimilation with the aid of hydrogen donators added on purpose, had to be studied.

Finally the pH in the cultures is kept constant on account of the formation of sulphuric acid in a quantity equivalent to that of the alkali liberated from the $NaHCO_3$. The advantages thereof are that the crop per bottle is larger and that the bacteria are accustomed to a pH which is suitable for the use in the manometric method (see pag. 33). A bottle of 600 cm^3 produced about 450 mm^3 centrifuged bacteria with a dry weight of about 120 mgr.

By using 0,2% Na_2SO_3 instead of 0,1% $Na_2S_2O_3$, I tried to prevent the formation of reserve sulphur. The growth in this medium, however, was highly unsatisfactory.

For the experiments to be described later on, it was neces-

sary to know the absorption coëfficients of nitrogen and carbon dioxide in the suspension liquid. Since these coëfficients are unknown for a 2% salt solution, I have tried to adapt strain *d* to a concentration of 0,5% NaCl. The growth herein, however, was never as satisfactory as in a medium with 2% NaCl and consequently I have made very little use of this medium.

Furthermore it may be stated that an addition of f.i. Na-malate to a quantity of 0,01% exerts a distinctly accelerating influence on the growth of the bacteria and I availed myself of this expedient, either when I wanted material within a short time, or when I had to make transfers from old cultures.

The use of bacteria cultivated in wholly organic media, involved a number of peculiar difficulties (see page 54). Considering that by the sterilizing and by the repeated boiling of the media with $(NH_4)_2SO_4$ as a nitrogen source, a varying and therefore unknown quantity of NH_3 got lost, I investigated whether Na-nitrate or urea were suitable to replace the $(NH_4)_2SO_4$. For this purpose I made with these substances a so-called N-auxanogram according to BEIJERINCK (1889) using as a substrate the previously mentioned malate-thiosulphate-medium without $(NH_4)_2SO_4$ in leached agar. The PETRI dishes were provided with potassium pyrogallate (see f. i. KOCH 1934) and after having been sealed, incubated in the light cabinet. Strain *d* showed a pronounced preference to $(NH_4)_2SO_4$, even when the agar was inoculated with bacteria from a culture in which they had urea as the only nitrogen and carbon source.

CHAPTER II.

METHODS EMPLOYED.

§ 1. *Manometers.*

In those experiments, where I have restricted myself to the study of the metabolism of the bacteria in the dark, I have mostly used the single constant-volume type of manometer, generally known as the WARBURG-type. All vessels were provided with one or two side-bulbs. Useful indications regarding details in the technique, the calibration of the apparatus, the calculation of the constants, the shaking apparatus, etc. are given by DIXON (1934) and by KREBS (1929).

All experiments, where besides the metabolism in the dark also that in the light was studied, were carried out with two differential manometers placed side by side. They were quite similar to those which WARBURG (1923) employed for his experiments on the CO_2-assimilation of *Chlorella*. For further technical data on these manometers and for indications concerning their calibration I refer to the original description of WARBURG and NEGELEIN (1923), to WARBURG (1926) and to GAFFRON (1929).

The special requirements of the p.s.b. with respect to their environment made it necessary to deviate in certain respects from the technical procedures as applied by WARBURG. Thus I worked at different temperatures, with other suspension liquids and gases, etc. For some experiments with the differential manometers I used vessels which differed from the normal ones, in so far that they were provided with a side-bulb. The two differential manometers which stood at my disposal enabled me to make control or duplicate experiments. A double kathetometer such as WARBURG employed for the reading of his manometers, did not seem to me to be essential; I did the reading with the naked eye. The menisci were illuminated by "Everreadies", which were fed by a bell trans-

former. For the experiments described in Chapter V, I used silvered WARBURG-vessels. The silver coating was deposited on the outside and in order to protect it against the harmful effect of the water in the bath, it was not coppered as WARBURG recommends, but simply coated with an aluminium varnish.

§ 2. *Light sources.*

With those experiments, where the use of monochromatic light was not required, the two WARBURG-vessels containing the bacterial suspensions and the two compensation vessels filled with suspension liquid were illuminated by a half silvered 100 Watt PHILIPS incandescent lamp, length of the filament 25 cm; distance from the vessels 5 cm. This lamp, made watertight in the simple way shown in fig. 2, was placed in the water bath in such a manner as to allow the two vessels with bacteria to be illuminated with practically equal intensity. This was controlled by a series of assimilation measurements.

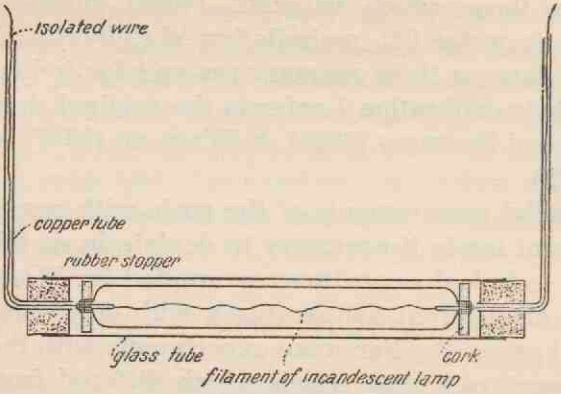

fig. 2.

Arrangement for the immersion of the incandescent lamp.

As the light of this lamp, when directly connected with 220 Volt ∿, led to solarisation phenomena (URSPRUNG 1917), I used a 75 Watt electric bulb as a resistance. The intensity of the visible light of the 100 Watt lamp is then very low and a rough comparison of it with the intensities of yellow, green

and blue light, which would lead to as high an assimilation velocity (see Ch. IV) as caused by this lamp, makes it probable that the assimilation observed was mainly due to infra-red light. As a matter of fact a $CuSO_4$ filter (6% aqueous solution of 1 cm thickness) diminished the assimilation velocity to 10% of its original value.

The intensity of this light source was never consciously varied, nor controlled or levelled. Fluctuations in the voltage indeed caused perceptible, though for these experiments no troublesome, changes in the assimilation velocity.

Small changes in pressure occurred in the manometers immediately after switching on and off the light. This is caused by the fact that the suspension with bacteria absorbs more light and hence becomes warmer (0,03—0,06°C.) than the suspension liquid in the control vessel. These small fluctuations were of no importance for my experiments, since they did not last longer than a few minutes.

For the experiments with monochromatic light, described in Chapter V, I used a quartz mercury lamp burning on 110 Volt = or a 50 Watt Philora sodium lamp from PHILIPS, which burnt on 220 Volt ~ with the use of a suitable transformer and a choke-coil. The light intensity of the mercury lamp was not constant, but since I only used it in experiments in which I did not make high demands upon the absolute value of the results, I made no special arrangements for the control of this intensity.

The sodium lamp, however, burnt very constantly without any special arrangement, at least after the first hour had passed by. Fluctuations of 2%, inherent to the sodium lamp under these conditions, were within the experimental error. I isolated the yellow (578 $m\mu$), the green (546 $m\mu$) and the blue (436 $m\mu$) emission lines from the mercury light by means of a $CuSO_4$ filter and coloured glasses from SCHOTT & GEN., Jena, placed in a cuvette filled with water. Hereby the indications of NUERNBERGHK (1933) were followed. There further data can be found regarding the amount of the optical impurities still present in the filtered light. The sodium light was applied without purification. According to an information, kindly given by Prof. G. HOLST of the Physical Labo-

ratory of the PHILIPS-works, the impurities present in the sodium light of this type of lamp amount to about 5% of the total light intensity.

The assimilation effected by the impurities in the light of the mercury lamp and of the sodium lamp was determined with the aid of special dye solutions or coloured glasses which absorbed the wave length selected for the experiments, but not the impurities in question. Hereby I paid special attention to select such control filters through which the infra-red light also passed.

In order to control the green light (546 mμ) I used a 1 cm thick layer of 0,02% erythrosine in water. This absorbs the green light almost wholly, but allows the red and infra-red light to pass. The influence of impurities of the blue light I could determine by means of the coloured glass OG_2 of SCHOTT which wholly absorbs the blue. Finally for the control of the yellow light (578 mμ and 589 mμ) I employed the coloured glass BG 11 of SCHOTT, which almost wholly absorbs the yellow light and allows all the other wave lengths to pass. These control filters were placed into the water bath in order to prevent the loss of light by reflection. In this manner I could determine the correction term which had to be introduced for assimilation on false light.

With certain experiments it was necessary to diminish the light intensity by a known amount. This was carried out with blackened wire cloth screens with varying mesh and thickness of wire. The brass wire cloth obtained from the „Metaaldraadweverij Dinxperloo, Holland" was coppered and blackened, strictly keeping to the prescription in SCHÜRER-WALDHEIM (1921, p. 286). By means of the spectral-pyrometer, still to be described, the extinction coëfficients of these screens were determined. As is known the absorption of the light by these filters is independent of the wave length of the light.

As is shown in fig. 3, a homogenic beam of light was cast on the bottom of a vessel with bacteria by means of the lenses L_1 and L_2. For this purpose the water bath was provided with a window of plate glass, while a mirror (S_2) placed under the vessel with bacteria threw the light upwards. In order to protect this mirror against deterioration, I had cemented

a glass plate against the silvered side with so-called "de Khotinsky cement"[1]).

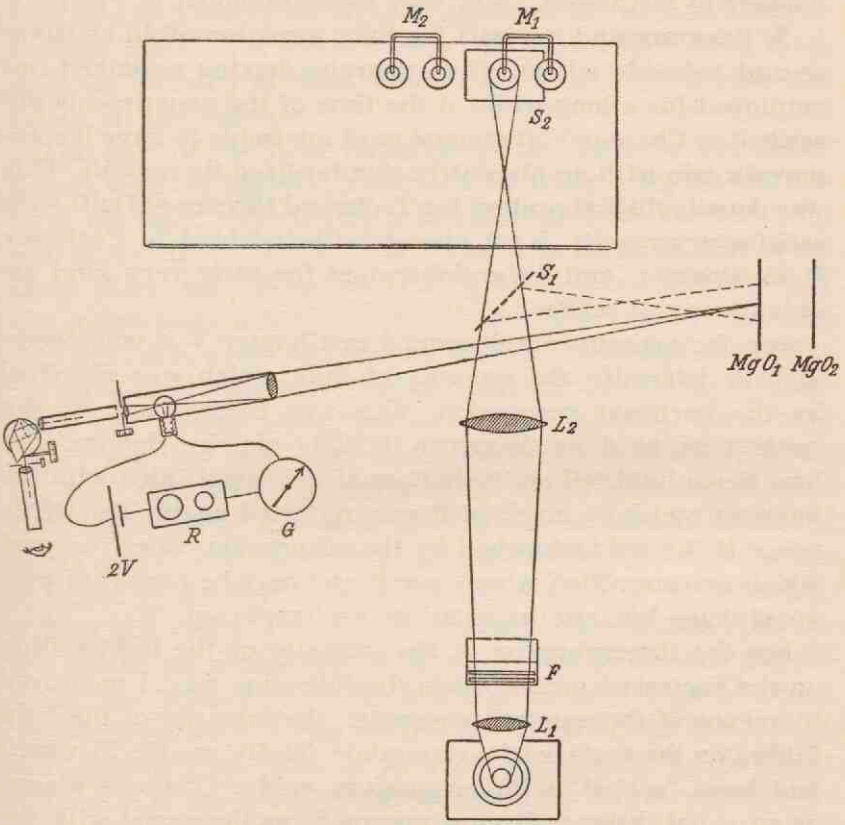

fig. 3.
Explanation in text.

§ 3. *Determination of light intensities.*

All determinations of the intensity of the monochromatic light were carried out in absolute units (erg/cm²/sec.) with the aid of a spectral-pyrometer, a somewhat simplified form of the apparatus described by EYMERS, ORNSTEIN and VERMEULEN (1932). I refer to this publication for the construction,

[1]) A cheaper substitute may be obtained by melting 3 parts of brown shellac with 1—2 parts of Venetian turpentine.

manipulation and standardization of this apparatus. It was constructed and standardized in the Physical Institute of the University of Utrecht. I am very much indebted to Professor L. S. ORNSTEIN and his staff for their kind, manifold assistance and valuable advice. The apparatus having remained unemployed for a long while at the time of the experiments described in Chapter V, it seemed most advisable to have it compared again with an absolutely standardized thermopile. This was done in the Laboratory for Technical Physics at Delft with satisfactory results. I am also greatly indebted to Professor H. B. DORGELO and his collaborators for their very kind assistance in this matter.

For the experiments described in Chapter V it was necessary to determine the quantity of light which was absorbed by the bacterial suspension. This was carried out by the method as used by WARBURG (1923), viz. by determining how much light fell on the bottom of the vessel filled with the bacteria and how much of it was reflected again. The difference is the light absorbed by the suspension, since the part which is transmitted is very small and may be neglected provided dense bacterial suspensions are employed.

For the determination of the intensity of the light falling on the bacteria I proceeded in the following way. I measured by means of the spectral-pyrometer the intensity of the light falling on the surface of a glass plate (MgO^1, see fig. 3) which had been "sooted" with magnesium oxide. This was placed at an equal distance from a mirror S^1 as the vessel with the bacteria. The intensity of the light on a second magnesium oxide surface (MgO^2) had previously been compared with that of the light falling on a third magnesia surface located in the air above the spot where normally the vessel with the bacteria was placed. Thereby another calculated correction was made for the extra reflection on the water-air boundary introduced. In this manner once for all the correction terms were determined which had to be introduced for the determination of the light intensity and from this moment on they were all carried out on the plate MgO^1. It may be stated by the way, that the water in the thermostat was kept clear by refreshing it daily. It is evident that the mirror S^1 was inter-

posed only when the intensity of the light was determined. This was carried out before and after each illumination period and as I found the light intensity of the sodium lamp to be very constant, it seemed allowable to calculate from these data the quantity of light, which during the experiments had fallen on the bacteria.

The part of the light reflected by the bacteria was determined with the spectral-pyrometer by comparing the intensity of the light, reflected by a magnesium oxide surface, with that of the light reflected by a vessel with bacteria kept in the same place (see WARBURG l.c.). It is assumed thereby that the magnesium oxide reflects all the light and furthermore that this ideal white surface and the bacterial suspension have an analogical type of reflection distribution. These assumptions will strictly taken not have been realized, but I may safely say that they have been closely approximated. When the light falls perpendicularly on the vessel with bacteria, the light reflected by the suspension of course must be observed at an angle with the perpendicular. In doing so one is not troubled by the light reflected by the surface of the glass bottom of the vessel. The size of this angle had, within reasonable limits, no perceptible influence. Thus after each experiment, the reflection of the bacteria in the WARBURG-vessel was determined and also for this a correction was introduced. An aqueous suspension of 15 mm^3 sulphur-free bacteria per cm^3 reflects about 3% of the sodium light thrown on it, whereas bacteria containing sulphur, under similar conditions, can show a diffuse reflection of even 20% of the light.

The whole of the optical equipment and the water bath were placed in a dark room improvised by means of heavy curtains.

By the way I wish to draw the attention to the great possibilities of application of the spectral-pyrometer in biology. Particularly in studying the reflection and dispersion of the light thrown on leaves (SEYBOLD 1933) and other objects it undoubtedly is a most useful instrument. The great simplicity as well as the relatively great reliability of the once standardized instrument make it, even in the hands of non-physicists, an extremely convenient apparatus.

§ 4. *Water bath.*

The thermostat consisted of a zinc cistern of 30×30×72 cm filled with water and provided with a piece of plate glass, inserted in one wall. The water was properly stirred and could be kept at any temperature between 25—35°C. at 0,01°C. constant. The heating was done by means of ten heating tubes connected in parallel, an arrangement which has been in use for years in the Delft laboratory. The tubes which were U-shaped were each of them 1.40 M. long (of which 1 M. submerged) and had a resistance of 100 Ω. They consisted of a copper tube, through which an asbestos-isolated commercial resistance wire was drawn. The heating tubes were connected with 50 Volt alternate current in order to exclude danger in case of short-circuit. The thermoregulator was constructed according to WÜST (1930). The electric current for this regulator was obtained with a PHILIPS rectifier 1016/1017.

The advantages of this uniform heating of the whole thermostat and the accurate regulation of it by the mentioned thermoregulator specially were of great profit in the experiments with the single manometers. The simple way in which the thermoregulator could be adjusted to special temperatures was very convenient.

§ 5. *Gas mixtures.*

The gases or gas mixtures used in the WARBURG-vessels greatly varied in different experiments. Except for the special cases in which oxygen or hydrogen mixtures were employed, it was necessary to deprive them most rigorously of all traces of hydrogen or oxygen. The *Thiorhodaceae* namely are anaerobic and as will appear further on they also involve hydrogen in their metabolism. For the said purpose all gases always were led through red hot copper wool or wire cloth (partly converted into copper oxide), put in a Pyrex glass tube (compare fig. 4). A good device for the electrical heating of the copper can be found in a publication of KENDALL (1931). The purified gas passed through a $CaCl_2$-tube and then was led either to a washing-bottle with a suspension liquid which

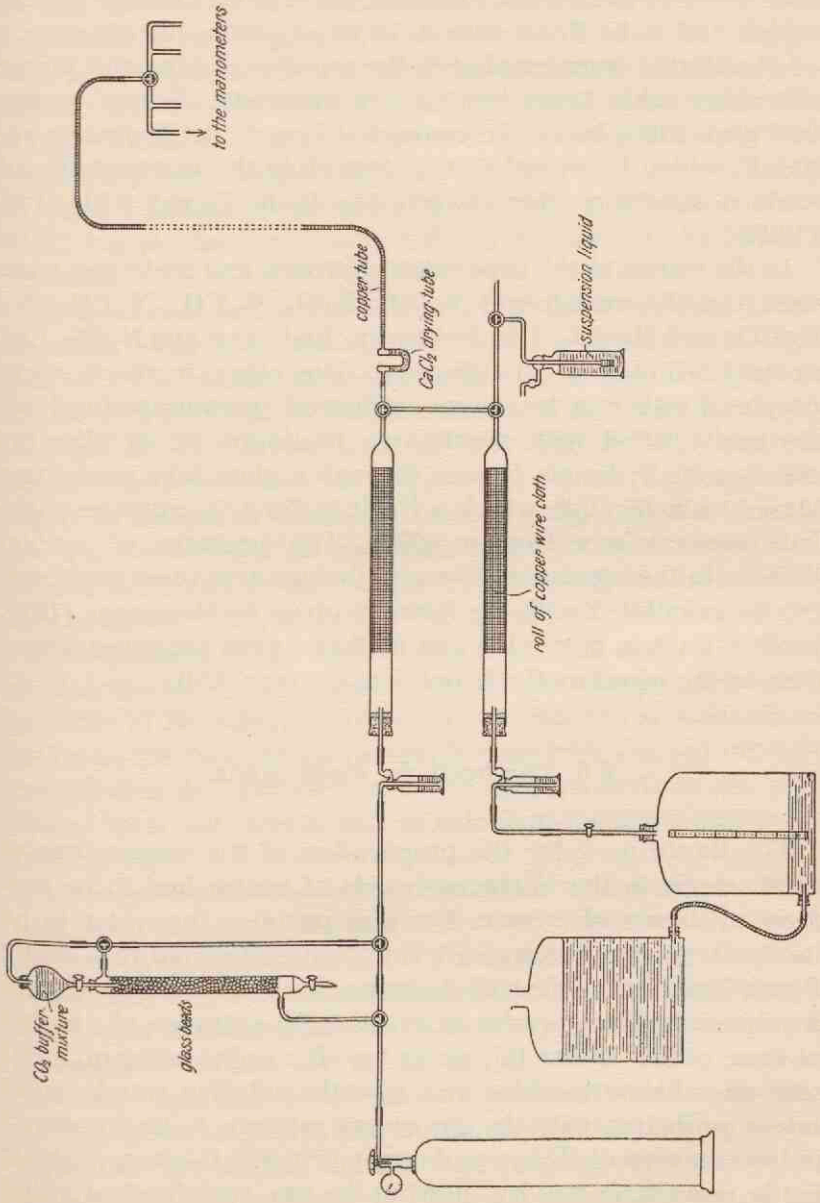

fig. 4.

Equipment for the preparation of oxygen-free gas mixtures and for the filling of the manometer vessels with these gases.

was to be saturated with the gas, or to the WARBURG-vessels, which had to be filled with it. In these arrangements copper or glass tubes were employed, the number of taps and pieces of rubber tubes being limited to a minimum. Where copper and glass tubes had to be connected I used "de Khotinsky cement", which is gas-tight. Data regarding the permeability of various substances for oxygen are to be found with HILL (1928).

In the course of the experiment nitrogen and hydrogen were used and also mixtures of N_2/CO_2, N_2/H_2, N_2/CO, N_2/CH_4, $N_2/H_2/CO_2$ and H_2/CO_2. I had nitrogen, hydrogen and $N_2/5\%$ CO_2 in steel cylinders at my disposal. The other mixtures were prepared either in two large calibrated communicating bottles partly filled with paraffinum liquidum, or by allowing nitrogen or hydrogen to pass through a glass tube containing glass beads, through which a $NaHCO_3/Na_2CO_3$ mixture trickled (see VAN DEN HONERT 1930). The pressure of carbon dioxide in the atmosphere in equilibrium with these mixtures, can be calculated with the formula given by WARBURG (1919, p. 230). Carbon monoxide and methane were prepared according to the usual methods (c.f. TREADWELL 1930).

§ 6. *The bacterial suspensions.*

The liquid used for the preparation of the suspensions of the bacteria in the WARBURG-vessels of course had to be free from all traces of oxygen. For that purpose, the water to be used, after the addition of 2% NaCl, was boiled out in a strong Pyrex flask and while still steaming the flask was closed with a rubber stopper in order to prevent the entrance of air and at once cooled under the water-tap. If needed inorganic and organic salts were added and now the solution was brought into equilibrium with the gas or gas mixture to be employed in the experiment. This was done by bringing it into a washing bottle (see fig. 4) and by allowing the gas, very finely divided by means of a piece of rattan, to bubble through it during 30—60 min.

In those cases where I actually wanted to put carbon dioxide

at the disposal of the bacteria, but wished to observe their behaviour with respect to another gas, I used CO_2 buffer mixtures, which are solutions of Na-carbonate and Na-bicarbonate in a certain ratio. The corresponding buffered carbon dioxide pressure can be calculated as indicated above. Both calculation and experience show that the buffering capacity of these solutions is considerable. The pH, it is true, is fairly high, but the bacteria employed did not seem to be harmed thereby.

In the cases where I wanted to test the behaviour of the bacteria towards carbon dioxide itself I generally took nitrogen with 5% carbon dioxide as a gas phase and a suspension liquid with about 0,5% Na-bicarbonate, which was previously brought in equilibrium with the gas mixture. The addition of Na-bicarbonate had to adjust the pH of the suspension liquid to the pH to which the bacteria were adapted. The latter was determined colorimetrically before each experiment on the clear culture medium after removing the bacteria by centrifuging. The quantity of Na-bicarbonate, to be added to the suspension liquid in order to reach the same pH, could be calculated. I had namely previously determined which quantity of Na-bicarbonate had to be added to an aqueous solution of 2% NaCl being in equilibrium with a given nitrogen carbon dioxide mixture, in order to obtain a definite pH. With two of these data one is able to calculate the third (see DIXON 1934, p. 78).

These precautions as to the adjustment of the pH are essential, since the p.s.b. are especially very sensitive to a lower pH. Bacteria, originating from a medium with a pH 8,0 show a distinctly reduced assimilation when the pH of the suspension liquid is lower than 7,6. In most experiments I employed a suspension liquid with a pH of 7,8.

In the beginning I have indeed doubted whether in using such alkaline solutions, one would not be troubled by the retention of the carbon dioxide and perhaps with reason because even in a solution of 0,5% Na-bicarbonate in equilibrium with 5% carbon dioxide, Na-carbonate will still be present. A calculation of this retention according to the principles given by WARBURG (1926, p. 110) shows, however, that it is to

be neglected in case of such high carbon dioxide tensions.

The pH remained fairly well constant during the experiment as only slight acid production took place and because a bicarbonate-carbonic acid mixture is a pH buffer owing to the property of compensating acid or alkali production by driving out or taking up carbon dioxide.

The slight acid production by the bacteria in the dark, as mentioned on page 41, consequently led to a carbon dioxide formation not entirely to be neglected. When more than relative value is to be attached to the observed changes in the carbon dioxide pressure a production or consumption of acid has to be taken into account. As is known (see f.i. DIXON 1934) one cannot buffer the pH with other buffer mixtures without increasing the carbon dioxide retention considerably, at least at this pH.

Finally I still have to mention that the constants of the different vessels have been calculated with the well-known absorption coëfficients of gases in water. However, by the addition of 2% NaCl the solubility of the gases will undoubtedly decrease. The absorption coëfficients for this solution were not available. Those of carbon dioxide in 15% NaCl and in RINGER-solution at 38° C. are known. If one approximately calculates the absorption coëfficient of carbon dioxide in 2% NaCl from these data, then it appears that in consequence of it the constants of the vessels are somewhat reduced. With the differential manometers this reduction is about 2%, in the other cases it is to be neglected.

After having described how the gas mixtures were prepared and purified and how the suspension liquid was handled, I will now deal with the treatment the bacteria had to undergo before the experiment was started. In many cases it was advisable for reasons to be mentioned later on, to put the culture bottles with bacteria into an incubator of 35°C. in the dark the day before. Then the content of the bottles was centrifuged. In the beginning I was afraid of poisoning the bacteria by the oxygen from the air and I therefore excluded the air from the liquid in the centrifuge tubes by means of paraffine oil. As appeared afterwards this was, however, superfluous. The centrifuged bacteria were then suspended in

the liquid mentioned before and centrifuged again. Not until the bacteria were washed out in this manner, were they suspended again and put into the WARBURG-vessels.

The gas or gas mixture to be used, was blown through the manometer vessels before and after the bacterial suspension was put into them. The manometers were then placed into the thermostat and they were shaken for some five minutes. The taps were just opened from time to time in order to allow the gas (which had expanded with the rise of the temperature) to escape. Then all joints were worked in and made to bind fairly firmly, and the shaking apparatus was set going anew. After some ten minutes equilibrium was obtained and now the experiments could be started.

It may be remarked, that in consequence of all these operations with non sterile glassware the bacterial suspensions were no doubt contaminated. However, the short duration of the experiments excludes any possibility of a perceptible influence of this contamination.

The quantity of bacteria in the vessels varied considerably. A quantity of 34 cm^3 suspension solution with 50—450 mm^3 bacteria came into the vessels of the differential manometers, which had a capacity of about 55 cm^3. For the vessels of the single manometers with a capacity of 7—20 cm^3, 1—4 cm^3 bacterial suspension were used. The quantity of the bacteria present was determined after the end of the experiment, by centrifuging an aliquote part of the suspension in calibrated so-called TROMSDORFF-tubes, during 10 minutes with 3000 revolutions per min. It appeared that working in this way 100 mm^3 of bacteria had a dry-weight of about 27 mgr.

CHAPTER III.

THE METABOLISM OF THE THIORHODACEAE IN THE DARK.

§ 1. *Introductory remarks.*

A question, arising immediately while studying the physiology of the photosynthetic autotrophic organisms, is how they keep alive during the nocturnal periods of darkness. MULLER (1933[1], p. 163) says in reference to the *Thiorhodaceae:* "..... and they certainly do not die or pass into a resting-stage overnight. Neither can we expect that they will be able to survive a period of several hours without some process taking place inside their cells, which maintains the potential differences in their physico-chemical structure."

This question, which at that time could not yet be solved on the basis of the available experimental data, is closely related to another question, viz. whether the *Thiorhodaceae* are able to grow in the dark and, if so, under which conditions.

VAN NIEL (1931, p. 105) in a careful survey of the literature concerning this question, mentions two instances where coloured sulphur bacteria were reported to have grown in the dark. NADSON should have been successful in cultivating his green sulphur bacteria and MIYOSHI his *Thiorhodaceae* in the absence of light.

However, none of the later investigators of coloured sulphur bacteria have succeeded in corroborating these observations. Now it is possible to explain negative results in all those cases, where one did not use pure cultures, by accepting an overgrowing of the sulphur bacteria by more rapidly growing heterotrophic organisms. This seems the more plausible, since for growth experiments in the dark of course only organic media can be applied.

However, this explanation does not hold in those cases where

pure cultures have been used. VAN NIEL (1931, p. 106) and MULLER (1933[1], p. 140) have inoculated a number of pure cultures of *Thiorhodaceae* in yeast water, peptone water and synthetic glucose, fructose, lactate and pyruvate media. Even after a prolonged incubation at 25°C in the dark they could not observe any growth, either under anaerobic, or under aerobic conditions. However, they always found growth when these culture bottles were exposed to the light.

This behaviour is in contrast with that of the *Athiorhodaceae*, pure cultures of which grow abundantly in such media in the dark, as MOLISCH (1907) and VAN NIEL (VAN NIEL and MULLER 1931, p. 259) observed. The latter investigator made the important observation, that contrary to what holds for the growth in the light, the growth in the dark only can take place in the presence of oxygen. The strictly anaerobic character of the *Thiorhodaceae* should therefore be the reason, why these organisms are fully unable to grow in the dark.

How to conceive — in the light of the foregoing considerations — the process or the processes, which maintain the life of the *Thiorhodaceae* and *Athiorhodaceae* in periods of darkness? As obviously neither the *Thiorhodaceae*, nor the *Athiorhodaceae* are able to grow in the dark in media, which are very suitable for the cultivation of bacteria in general, VAN NIEL (1931, p. 106) rejected for both groups the idea of a fermentation process being the base of their metabolism in periods of darkness. Since these organisms in nature usually are found in a more or less complete anaerobic environment, the idea of an oxidative dissimilation has to be discarded as well.

As a matter of fact VAN NIEL finishes his discussion with the words: ".....their metabolism under these conditions still remains an open question". On the other hand MULLER (1933[1], p. 164) states in this connection: "However I think that it is quite possible, that an organism can effect some special fermentation process, without being able to synthesize its cell material from the fermentation substrate, without the aid of an external source of energy. Such a special fermentation process might be the conversion of sugars into lactic acid. It

is quite conceivable that the p. s. b. are able to "maintain" themselves on a lactic acid fermentation in the dark, but are able to develop only in the light, because they need radiant energy for some of the dehydrogenations, involved in the synthesis of cell material".

In my opinion this conception is fully justified, since one can find analogous cases in another group of photosynthetic autotrophic organisms, namely among the green plants. Although a great number of algae can grow in the dark on a medium containing for instance glucose, there are also simply organized algae, which in pure culture do not tolerate organic matter at all, e.g. the blue algae. (BEIJERINCK 1898, 1901). Now nobody will venture to conclude only from this, that these blue algae are unable to maintain themselves for some time on a dissimilatory conversion of their reserve food compounds. As a matter of fact it will be shown that the suppositions of MULLER very nearly approximated the truth. He apparently did not intend to say, that, if the *Thiorhodaceae* have a sufficient quantity of a suitable substrate at their disposal, for instance a carbohydrate, they should be able to maintain themselves in the dark for an unlimited time. One should not forget that also essential cell compounds will be consumed (maybe by autolysis) and that a defective rebuilding of these substances in the dark ultimately can cause the death of the bacteria. This idea is supported by the experience that bacteria in an organic medium cannot withstand longer periods of darkness than a culture of the same organisms in an inorganic medium. The contrary is even more likely to be true.

Summarizing we may say that on ground of negative or positive results of some growth experiments some ideas regarding the metabolism of the *Thiorhodaceae* in the dark had been given. However, a direct experimental proof of the correctness of these ideas was still lacking. Therefore it seemed worth while to investigate this question.

§ 2. *Experiments with bacteria cultivated in an inorganic medium.*

a. Autofermentation. When bacteria cultivated in the com-

mon inorganic medium, mentioned on p. 19, were centrifuged, washed, and suspended in an oxygen-free solution of sodium chloride, either provided or not with sodium bicarbonate, and then brought into the WARBURG-vessels in an atmosphere of nitrogen or in a nitrogen carbon dioxide mixture, in the dark an increase of pressure was always observed. Moreover this increase in pressure proved to be constant for many hours. All strains investigated by me showed the same behaviour in this respect.

Evidently this increased pressure was the result of a production of carbon dioxide, since it failed to turn up, when alkali was put in a side-bulb of the manometer vessel, or when the bacteria were suspended in one of the carbon dioxide buffer mixtures mentioned previously.

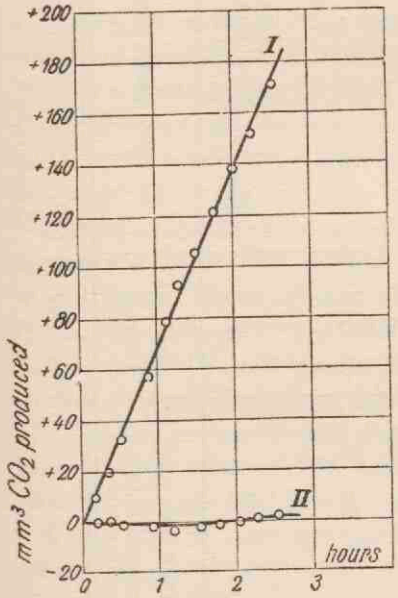

fig. 5.

Carbon dioxide production in the dark.

Curve I. Carbon dioxide production by bacteria of strain d, cultivated in the inorganic medium, suspended in tap-water with 2% NaCl and 0,5% $NaHCO_3$, in equilibrium with N_2/7% CO_2 at a temperature of 30°C.

Curve II. The same arrangement except the presence of alkali in a side-bulb of the manometer vessel.

Fig. 5 gives a graph of the course of such an experiment, while in table 1 the data relating to a few experiments are presented. The figures indicate the production of carbon dioxide in mm^3 gas of 0° and 760 mm pressure per hour by a certain quantity of bacteria. These are the averages of measurements, which usually were continued for some hours.

TABLE 1.

Carbon dioxide production in the dark.

Experiment No.	Description of experimental conditions	Manometer[2])	CO_2 production in mm^3 per hour	Change in pressure in mm. Brodie[1]) in control, alkali in side-bulb
1.	Bacteria of strain d, cultivated in the inorganic medium, suspended in distilled water with 2% NaCl, in equilibrium with N_2, temperature 33°C.	S. M. 4 6 1	 39 — —	 — —1 0
2.	Conformable to experiment No. 1.	S. M. 1 4 3	 36 — —	 — —1 0
3.	170 mm^3 bacteria of strain d, cultivated in the organic medium, "starved" during 20 hours at 36°C., suspended in: a. tap-water, 2% NaCl, with a 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ and 1 part Na_2CO_3. (manometer III, filled with N_2). b. tap-water, 2% NaCl, 0,4% $NaHCO_3$, in equilibrium with N_2/5% CO_2. (manometer I) temperature 30°C.	D. M. I III	 46 —	 — 0
4.	Conformable to experiment no. 3, except temperature of starvation period, which was 30°C., manometer 1 with CO_2 buffer mixture. Manometer 3 with $NaHCO_3$-solution.	D. M. 3 1	 115 —	 — 0

[1]) Brodie solution is the manometric fluid used and has the following composition: 23 gr. NaCl, 5 gr. Na-choleinate, in 500 cm^3 water, with a trace of thymol. 10.000 mm Brodie is equivalent to 760 mm Hg.

[2]) Differential manometer is abbreviated in all tables as D.M.
Single manometer (constant volume type) is abbreviated as S.M.

At the end of all experiments the bacteria were examined under the microscope in order to verify whether they still were in a good condition. As a rule the result of this examination was satisfactory, but sometimes the bacteria had obviously come into contact with the caustic soda in the side-bulb of the manometer vessel. Of course such experiments were discarded.

Because the production of carbon dioxide, after having been constant for some hours, shows a slight decrease rather than an increase, it may be concluded that this production of carbon dioxide is a normal physiological process ("autofermentation"). Under these conditions it is tempting to identify this process with the already mentioned fermentation process, suggested by MULLER (1933 [1]).

Further I have considered the question, whether the carbon dioxide evolved, was indeed produced by the bacteria, or whether it was not, at least partly, due to the Na-bicarbonate in the solution, from which it could have been expelled by acid, formed in the autofermentation process. In the first place acid production should have been reflected in a higher increase in pressure due to carbon dioxide production by bacteria suspended in a solution containing bicarbonate, as compared with the carbon dioxide liberation by an equal quantity of the same bacteria suspended in a solution free from bicarbonate or carbonate. Furthermore one can also detect a possible acid production by adding an excess of acid to one suspension before and to a second (similar) suspension after the experiment and determining the quantity of the carbon dioxide liberated in both cases. The results of the experiments concerning this question are to be found in table 2.

From this it may be inferred, that as a matter of fact, a development of acid takes place corresponding to about 30% of the totally liberated carbon dioxide.

As a rule this acid production has not been taken into account, since in most experiments only relative values mattered. The assimilation velocity was always determined by measuring the difference between the changes in pressure in the dark and in the light and therefore the error caused by evolution of carbon dioxide as a result of acid production,

TABLE 2.

Acid production in the dark.

Exp. No.	Description of experimental conditions	Manometer	CO_2 present in the suspension, determined by addition of excess acid.		Total CO_2 production in mm^3	% of total CO_2, driven out by acid produced
			At the beginning	At the end of the experiment		
1.	Equal quantities of bacteria of strain d, cultivated in the inorganic medium, suspended in tap-water 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at a temperature of 32°C., put in different manometers.	S. M.				
		1	85	—	—	—
		2	86	—	—	—
		4	—	69	70	23
		6	—	70	68	23
2.	Conformable to experiment no. 1.	S. M.				
		4	108	—	—	—
		6	108	—	—	—
		1	—	91	91	20
		2	—	86	68	25
3.	Conformable to experiment no. 1 except the suspension solution, i.e.	D. M.			CO_2 production in mm^3 per hour	
	a. tap-water 2% NaCl, in equilibrium with N_2. Manometer no. 1.	1	—	—	113	—
	b. tap-water 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2. Manometer no. 3.	3	—	—	175	35
	Temperature 30°C.					

was eliminated.

As will be expounded on page 93 this assumption is not quite correct, because it appears, that these acids are assimilated again in the light, at least to some extent. Such an assimilation of acids will of course lead to a carbon dioxide uptake in surplus to that due to the carbon dioxide consumption of the cells.

TABLE 3.

Autofermentation in the dark of bacteria, which had been exposed to various starvation periods.

Exp. No.	Description of experimental conditions	Differential manometer	Starvation period	CO_2 production in mm^3 per 1 cm^3 bacteria per hour.[1])
1.	200 mm^3 bacteria of strain d, cultivated in the inorganic medium, suspended in 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/7% CO_2, temperature 30°C.	1	no st. per.	1300
		3	no st. per.	1100
2.	Conformable to experiment no. 1. 260 mm^3 bacteria p. manom. vessel	1	no st. per.	1050
		3	no st. per.	950
3.	Conformable to experiment no. 1. 170 mm^3 bacteria p. manom. vessel	1	no st. per.	1120
		3	no st. per.	1120
4.	Conformable to experiment no. 1. 160 mm^3 bacteria p. manom. vessel	1	18 h. at 30°C	825
5.	Conformable to experiment no. 1. 180 mm^3 bacteria p. manom. vessel	1	18 h. at 30°C	710
		3		
6.	Conformable to experiment no. 1. 290 mm^3 bacteria p. manom. vessel	1	20 h. at 35°C	475
7.	Conformable to experiment no. 1. 220 mm^3 bacteria p. manom. vessel	1	24 h. at 35°C	410
8.	Conformable to experiment no. 1. 220 mm^3 bacteria p. manom. vessel	1	24 h. at 35°C	390
9.	Conformable to experiment no. 1. 200 mm^3 bacteria p. manom. vessel	1	24 h. at 36°C	360
		3	24 h. at 36°C	360

[1]) In this and all following tables the data regarding carbon dioxide production indicate the sum of the carbon dioxide produced by the bacteria and the carbon dioxide driven out of the suspension liquid by acid formation.

However, a determination of the quantity of acid formed or assimilated in the experiments with the differential manometers presented insurmountable difficulties, since the quantity of bicarbonate in the medium was too large to permit its measurement in the manometers. On the other hand it was impossible to lower the quantity of bicarbonate because the pH and the concentration of carbon dioxide in the medium were dependent on the bicarbonate concentration.

b. The influence of a *preceding period of darkness* on the rate of the production of carbon dioxide was in agreement with the conception, that the phenomena described are due to an autofermentation. In table 3 a number of experiments is reported concerning the velocity of the autofermentation of bacteria from cultures, which had passed different periods of darkness. In the table the figures relating to the carbon dioxide production indicate the sum of the carbon dioxide driven out of the solution by acid and of the carbon dioxide produced directly by the bacteria.

From these data it can be concluded that in consequence of a preceding darkness of 24 hours at 36°C., the autofermentation has fallen to 30% of its original value. The vitality of the bacteria, judged by their motility had not decreased thereby and I found in addition that also the assimilatory capacities were only slightly diminished.

c. The *influence of the temperature* on the velocity of the autofermentation was very marked. As may be seen from table 4, the value of the Q_{10} was high. In this connection it must be pointed out, that the temperatures used were not higher than the temperature of the light cabinet in which the bacteria were cultivated. Hence a harmful influence of these temperatures is out of question.

d. I was unable to make direct experiments about the *influence of light* on the autofermentation, since the assimilation cannot be excluded.

Similar difficulties have been encountered by the investigators, who have studied the influence of light upon respiration of green plants. In this connection I restrict myself to drawing the attention to the reliable experiments of VAN DER PAAUW (1932, p. 522). This investigator observed an increase of the

TABLE 4.

Influence of temperature on the autofermentation.

Exp. No.	Description of experimental conditions	D.M.	CO_2 produced at various temperatures in mm^3 per hour.		Q_{10}
1.	Bacteria of strain d, cultivated in the inorganic medium, starved during 20 h. at 35°C. suspended in tap-water 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at various temperatures.	1	114 at 25°C.	180 at 30°C.	3,2
2.	Conformable to exp. no. 1.	1	100 at 26°C.	178 at30,5°	3,9
3.	Conformable to exp. no. 1.	1	119 at 28°C.	255 at 33°C.	4,3

respiration of algae, under the influence of exposure to the light from an ordinary electric bulb. He could prevent a strong assimilation in this light, by absorbing all carbon dioxide by alkali. The respiration was measured by the absorption of oxygen.

Strictly speaking it remains doubtful whether the respiration under conditions favouring a strong assimilation, i.e. in the simultaneous presence of light and sufficient carbon dioxide, is influenced in the same sense and to the same extent by the exposure to light. Moreover it is not at all excluded that the occurrence of the assimilation process in itself exerts an influence on the respiration velocity (MULLER 1933 [1]) [1]).

For these reasons one certainly has to consider the possibility of a change in the autofermentation of the *Thiorhodaceae* as a consequence of an exposure of these organisms to light. Since we are dealing here with fermentation which cannot be measured independently from the assimilation — as in the

[1]) In this connection it may be mentioned, that the *Athiorhodaceae* are the only photosynthetic organisms where the influence of light and of the carbon dioxide assimilation on the respiration can be studied without meeting complications caused by the liberation of oxygen in the assimilation process.

case of respiration by oxygen uptake —, no direct experiments to decide this question could be made. Yet in none of my experiments concerning the assimilation of carbon dioxide, I could observe any after-effect of exposure to light upon the rate of autofermentation. The velocity of the carbon dioxide production attained its original value almost immediately after the end of the light periods. This is in contrast with the experiences of VAN DER PAAUW (1932) with algae.

Hence it seems allowable to conclude that under the conditions prevailing in my experiments the light did not exert any influence upon the autofermentation of the bacteria and that thus this process proceeded with unchanged velocity during the assimilation. At best the autofermentation will increase, be it very slightly, in consequence of the small increase in temperature caused by the illumination (vide p. 25).

e. The question arises which compound can be the *substrate of the autofermentation.* The only observations which seem appropriate to give a clue to the solution of this problem are those of H. G. DERX, quoted by VAN NIEL (1931, p. 106), who found indications for the formation in the light of a reserve product, which could be coloured violet by iodine. However, in spite of repeated attempts, I was unable to corroborate these observations.

Experiments made in order to investigate the possibility of influencing the rate of the fermentation by adding organic substances of known constitution, e.g. glucose, Na-lactate, Na-butyrate and Na-malate yielded quite negative results (see tables 5 and 6). Hence I could not get any indication regarding the nature of the reserve substance by this indirect way either. It is, however, not excluded that one will be able to detect an influence of certain added substances upon the fermentation, by using bacteria, which were previously exposed to a starvation period. It remains possible that the bacteria used in my experiments did not react on these additions because they had an excess of stored substrates for the autofermentation process at their disposal.

In the meantime it is in my opinion not justified to conclude, either from a positive or from a negative result of the addition of glucose upon the autofermentation, whether the

TABLE 5.

Experiments on the influence of organic substances and sulphates on the autofermentation.

Experiment No.	Description of experimental conditions	Manometer	CO_2 production in mm^3 per hour.		
			Without any addition	Added substance	After addition of the mentioned substance
1.	Bacteria of strain d, cultivated in the inorganic medium, suspended in distilled water with 2% NaCl p.a. and 0,5% $NaHCO_3$ p.a., in equilibrium with N_2/5% CO_2, temperature 30°C.	D. M. 3	400	—	—
		I	—	0,3% glucose	400
2.	Bacteria of strain d, cultivated in the standard solution with 0,1% Na-butyrate, 0,1% $Na_2S_2O_3$ and 0,7% $NaHCO_3$, suspended in the same solution as used in experiment No. 1.	D. M. III	270	—	—
		IV	—	0,2% Na-butyrate	270
3.	Conformable to experiment No. 1.	D. M. IV	150	—	—
		III	—	0,1% Na-lactate	155
4.	Conformable to experiment No. 1.	S. M. 4	128	—	—
		1	—	0,3% Na-malate	125
		6	—	0,3% Na-malate + 0,2% Na_2SO_4	130
5.	Conformable to experiment No. 1.	D. M. 1	285	0,2% Na-malate	270
		3	—	0,2% Na-malate	245
		3	—	0,2% Na-malate + 0,5% Na_2SO_4	245
6.	Conformable to experiment No. 1.	D. M. 1	—	0,2% glucose	270
		1	—	0,2% glucose + 0,5% Na_2SO_4	260

TABLE 6.

Experiments on the influence of organic substances and sulphates on the auto-fermentation of bacteria from the peptone medium.

Exp. No.	Description of experimental conditions	Manometer	Change in pressure in mm. Brodie per hour calculated for equal volumina, caused by hydrogen and carbon dioxide production.		
			Without any addition	Added substance	With the addition of the mentioned substance
1.	Bacteria of strain 9, cultivated in the peptone medium suspended in distilled water with 2% NaCl p.a., 0,5% $NaHCO_3$ p.a., in equilibrium with N_2/5% CO_2, temperature 30°C.	D. M. 3	+ 28	—	—
		1	—	0.6% Na_2SO_4 + 0,2% Na-malate	+ 30
2.	Conformable to experiment No. 1	S. M. II	+ 41	—	
		V	—	0,3% Na-malate	+ 40
		VI	—	0,3% Na-malate + 0,5% Na_2SO_4	+ 39
3.	Conformable to experiment No. 1	S. M. IV	+ 31	—	—
		V	—	0,3% Na-butyrate	+ 31
		VI	—	0,5% Na_2SO_4 + 0,3% Na-butyrate	+ 32

reserve product which is fermented, is a carbohydrate or not. For not rarely it happens, that one observes acceleration of the respiration or of the fermentation of an organism, by adding substances, which certainly cannot be substrates of these processes. I cite in this connection only the examples of ethylene (MACK and LIVINGSTONE 1933), cyanide in certain cases, methylene blue, etc. Even if one observes a fermentation of the added substance, it would mean only, that the *Thiorhodaceae* are able to ferment that substance, but not that their reserve substances are identical with it. On the other hand it is possible not to find any influence of a certain substance, which yet very probably is the substrate of the autofermentation. So WATANABE (1933) observed no influence of the addition of 1% glucose upon the respiration of *Ulva*, although with other algae he could confirm the positive results found by other investigators. In all probability the *Ulva* thallus used has been rich in reserve products, but such considerations have evidently not been taken into account by WATANABE. *f.* In connection with the phenomenon of the autofermentation I must also draw attention to the publication of GAFFRON (1934). According to him the *Thiorhodaceae* should be able *to oxidize in the dark several organic substances with sulphate as a hydrogen acceptor*, which is then reduced to sulphide. At least this is the explanation he offers for the increase in carbon dioxide production, observed by him after addition of glucose, Na-malate etc. together with sodium sulphate to the medium. GAFFRON even goes so far as to conclude that the presence of sulphates is indispensable for the use of organic substances.

These conclusions seemed of so far-reaching importance that I decided to repeat these experiments. I used in this case both strain *d* — the same organism as used by GAFFRON — cultivated in the ordinary inorganic culture medium, and strain *9*, cultivated in the peptone medium. As the graphs in fig. 6 and 7, and the experiments in tables 5 and 6, show unmistakably, the result of these experiments was fully negative. Hence I have to reject the explanation GAFFRON gave for the observations made by him.

As I know that GAFFRON used for his experiments the en-

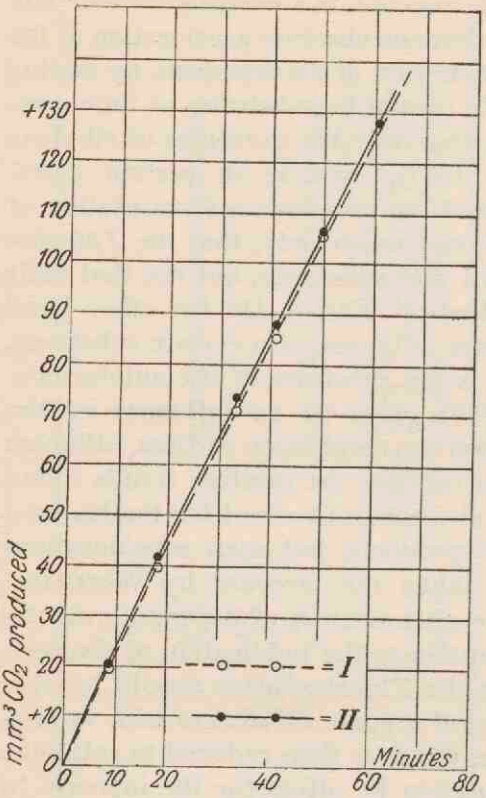

fig. 6

Carbon dioxide production in connection with a possible sulphate reduction.

Curve I. Carbon dioxide production by bacteria of strain **a**, cultivated in the inorganic medium, suspended in distilled water with 2% NaCl pro analyse and 0,5% $NaHCO_3$ pro analyse, in equilibrium with N_2/7% CO_2 at a temperature of 30°C. No SO_4-ions present.

Curve II. The same arrangement except the presence of 0,2% Na_2SO_4 and 0,3% Na-malate in the suspension liquid.

richment culture, from which I isolated strain *d* and as moreover the method of cultivation was pretty well the same, it seems impossible to explain the difference in results by a difference in the properties of the bacteria used. A more plausible explanation I see in the fact, that GAFFRON did not use pure cultures. As mentioned before, heterotrophic organisms are very well able to grow in an inorganic medium, provided that autotrophic organisms are present. In this case one is tempted to suppose, that the enrichment culture of GAFFRON has been infected with the sulphate reducing anaerobic *Vibrio desulfuricans* and with *Bacterium coli*, which as a rule obstinately accompanies the former organism (vide BAARS, 1930). The only guarantee GAFFRON gives that in this case actually the physiology of the *Thiorhodaceae* was studied, is to be found in the following passage (GAFFRON l. c. p. 449): „Da

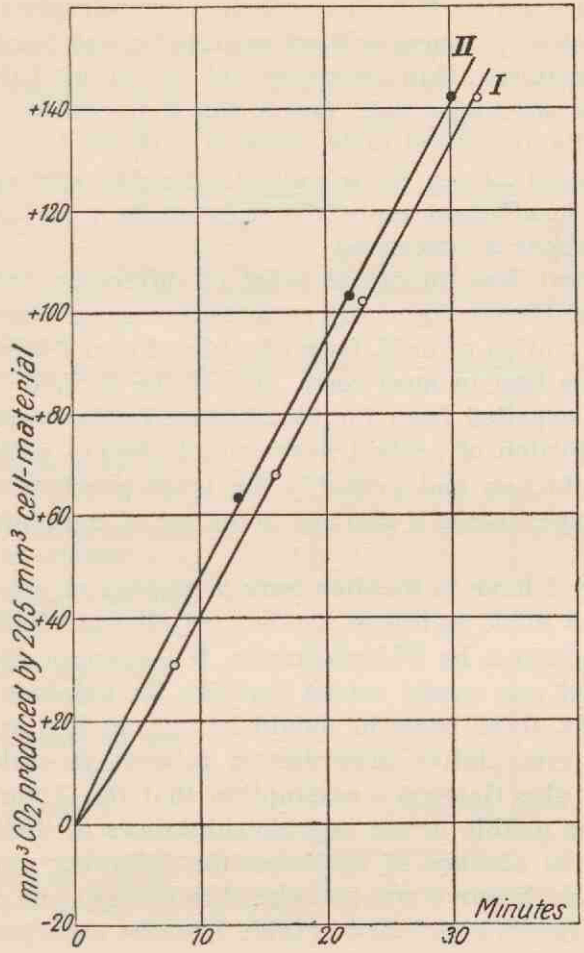

fig. 7.

Carbon dioxide production in connection with a possible sulphate reduction.

Curve I. Carbon dioxide production by bacteria of strain **d**, cultivated in the inorganic medium, suspended in distilled water with 2% NaCl pro analyse, 0,5% $NaHCO_3$ pro analyse and 0,2% glucose pro analyse, in equilibrium with N_2/7% CO_2 at a temperature of 30°C. No SO_4-ions present.

Curve II. The same bacteria as in I, after the addition of 0,5% Na_2SO_4.

in der genannten Nährlösung keine heterotrophen Organismen wachsen können, gelangt man mit etwas Sorgfalt rasch zu Reinkulturen. Man überzeugt sich davon mit Hilfe des Mikroskops, am besten unter Zusatz von Methylenblau, das nicht nur die Bakterien gut färbt, sondern auch die lebhafte Bewegung sistiert". Even for a trained microbiologist such a criterion is insufficient, especially as far as the very small *Vibrio desulfuricans* is concerned.

A second, less important point of difference between the results of GAFFRON and my own concerns the very considerable formation of acid, found by him. From GAFFRON's data it appears that in most cases 70% of the formed carbon dioxide is expelled from the bicarbonate in the suspension by the production of acids. I never could observe such a strong acid production and probably the large production in GAFFRON's experiments is also due to the use of contaminated cultures.

Finally I have to mention here a number of growth experiments I made to test in another way the possibility of sulphate reduction by *Thiorhodaceae*. If this supposition would be correct, one would expect that like the sulphate reducing organisms, these bacteria should be able to live in the dark on this fermentative dissimilation process. In order to test this and also GAFFRON's assumption that the *Thiorhodaceae* should be unable to use organic substances in their metabolism in the absence of sulphates the following experiments were made. Strain *d* was inoculated in bottles of 60 cm^3, filled with the media mentioned in table 7, whilst these bottles were incubated partly in the dark and partly in the light. In the standard solution I had substituted in this case the sulphates by chlorides. All organic and inorganic salts were pure and had especially been tested on the absence of sulphates. In the cultures, where no thiosulphate or sulphate had been added, the only source of sulphur the bacteria had at their disposal, was a little Na_2S. This may have been partly oxidized to sulphate.

The results of these experiments leave, however, no doubt that the *Thiorhodaceae* are unable to grow with a sulphate reduction as a base, either in the light, or in the dark. (Cf.

TABLE 7

Dependence of growth of strain d on the presence of sulphate and of organic substances with different "oxidation value" in the dark and in the light.

Strain d inoculated in 60 cm^3 glass-stoppered bottles, completely filled up with a sulphate-free standard salt solution with 0,005% Na_2S, and with addition of the following substances.	Incubated in the dark at a temperature of 30°C.	Incubated in the light cabinet at a temperature of 30°C.
1. 0,1% Na-butyrate	—	—
2. 0,1% Na-butyrate and 0,1% Na-thiosulphate	—	—
3. 0,1% Na-butyrate and 0,1% Na-sulphate	—	—
4. 0,1% Na-butyrate and 0,3% Na-bicarbonate	—	+
5. 0,1% Na-butyrate and 0,1% Na-malate	—	+
6. 0,1% Na-butyrate and 0,05% Na-formate	—	+
7. 0,1% Na-malate	—	+

\+ indicates growth.
— indicates absence of growth.

experiments 1, 2 and 3, table 7.). Moreover these experiments prove that they are able to grow in the light with organic compounds in media free from sulphates or bicarbonate, provided that the "oxidation-value" of the compounds in question is high enough (C.f. table 7 no. 5, 6 and 7 and further MULLER 1933[1], p. 161.)

In my opinion the foregoing experimental results warrant the conclusion that the above mentioned statements of GAFFRON regarding the metabolic activities of the *Thiorhodaceae* are untenable.

§ 3. *Experiments with bacteria, cultivated in an organic medium.*

Since I had found that the rate of growth and the yield of bacteria in organic media were markedly higher than in inorganic media I decided to test the suitability of the bacteria grown in the former media for my experiments.

Bacteria of one of the strains previously mentioned, were cultivated in the peptone culture medium (see pag. 16), centrifuged, washed and suspended in the ordinary inorganic aqueous salt solution. If these suspensions were put in the WARBURG-manometers with a nitrogen atmosphere I observed an increase in pressure, which calculated per cm^3 cell material, only amounted to about 10—20% of the increase in pressure caused by bacteria of the same strain, when cultivated in the inorganic medium.

It appeared further that these "peptone bacteria" on microscopic examination always showed big and quaint forms. Besides, they showed a much reduced motility as compared with bacteria of the same strain from inorganic media. Moreover this motility very soon decreased and disappeared completely after a relatively short stay in the manometer vessels. Hence it must be concluded that these bacteria were abnormal in a morphological as well as in a physiological sense.

The reduced autofermentation — and as will be shown later on also the low assimilatory activities — is obviously caused by a reduced vitality in general, apparently due to the cultivation of the bacteria in the organic medium.

Although the peptone medium differs considerably from the medium preferred by the *Thiorhodaceae* in nature, such a reduced vitality was not expected, since the growth in the peptone medium is as a rule much more abundant than in the inorganic culture media.

How remarkable this may be, still more astonishing was the observation that the increase in pressure in the dark as a consequence of the autofermentation, was not quantitatively due to carbon dioxide production. It appeared namely that in the presence of alkali in a side-bulb of the manometer vessel, there still remained a measurable increase in pressure.

(fig. 8 and tables 8, 9 and 10). This can by no means be attributed to H_2S, since of course this gas would have also been absorbed by the alkali. The idea of the unknown gas being oxygen had also to be rejected, as it was not absorbed by potassium pyrogallate.

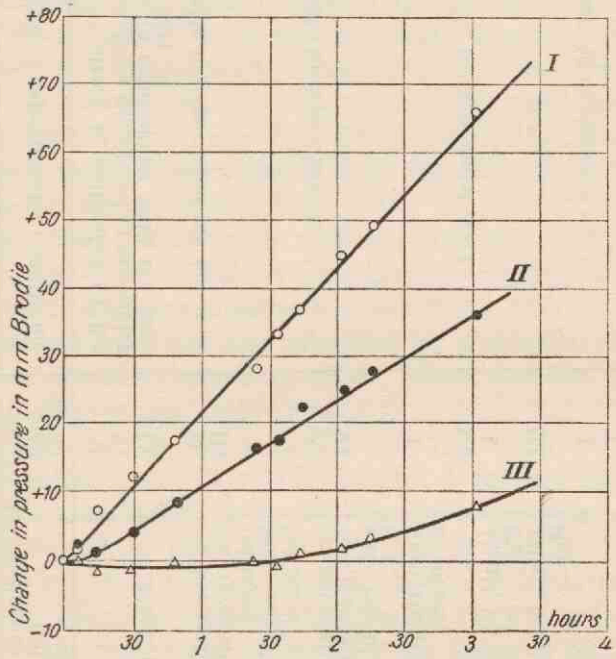

fig. 8.

Carbon dioxide and hydrogen production by bacteria from a peptone culture medium.

Curve I. Changes in pressure effected by bacteria of strain a, cultivated in the peptone medium, suspended in distilled water with 2% NaCl, in equilibrium with N_2 at a temperature of 30°C. CO_2 production amounting to 20 mm³ per hour when the H_2 production is assumed to be 15 mm³/hour.

Curve II. The same arrangement as in I, except the presence of Na-pyrogallate with excess alkali in a side-bulb of the manometer vessel. Hydrogen production amounting to 15 mm³ per hour.

Curve III. The same arrangement as in II, except the presence of Pd-black in a second side-bulb of the manometer vessel. After two hours the Pd is apparently poisoned by some metabolic product of the bacteria.

TABLE 8.

Experiments demonstrating the nature of the gas produced besides carbon dioxide by bacteria from the peptone medium.

Exp. No.	Description of experimental conditions	Single manometer no.	Substances present in side-bulbs of the manometer vessel	Change in pressure in mm. Brodie per hour in equal volumina.
1.	Bacteria of strain a, cultivated in the peptone medium, suspended in tap-water 2% NaCl, in equilibrium with N_2, temperature 30°C.	V	Alkali.	+15
		VI	Alkali.	+13
		IV	Alkali and Pd black.	0
2.	Conformable to experiment no. 1.	IV	Na-pyrogallate, excess alkali.	+13
		III	Na-pyrogallate, excess alkali, Pd black.	— 1
		I	Na-pyrogallate, excess alkali, Pd black.	— 1
3.	Conformable to experiment no. 1.	IV	Na-pyrogallate, excess alkali.	+16
		VI	Na-pyrogallate, excess alkali and Pd black poisoned by H_2S.	+15
		I	Na-pyrogallate, excess alkali, Pd black.	0
4.	Conformable to experiment no. 1.	VI	Alkali.	+14
		I	Alkali, Pd black, poisoned by H_2S.	+13
		III	Alkali, Pd black, poisoned by H_2S.	+13
5.	Conformable to experiment no. 1.	I	Alkali.	+17
		V	Alkali.	+17
		IV	Alkali, cupro oxide in ammonia.	+18
		VI	Alkali, cupro oxide in ammonia.	+16

However, when besides alkali, palladium black was put in another side-bulb of the manometer vessel, no change of pressure was detectable as can be seen from fig. 8 and in table 8. Apparently the unknown gas was adsorbed by the palladium. It must be remarked that in spite of the presence of Pd in experiments like those represented by curve III in fig. 8, there always could be detected a slight increase in pressure, when the experiments were continued too long. Probably in these cases the Pd was poisoned by volatile products of the bacterial metabolism. By adding a little H_2S, the palladium was poisoned immediately and did not adsorb the unknown gas anymore. (table 8 no. 3 and 4). Now it is a well-known fact that palladium is a strong adsorbent for gaseous hydrogen, but it also adsorbs considerable quantities of carbon monoxide and unsaturated hydrocarbons. However, these last-mentioned gases are also absorbed by a solution of cupro oxide in ammonia (TREADWELL 1930), which was not the case with the unknown gas. (table 8 no. 5.)

On the base of all this it seems safe to conclude that the unknown gas in question was hydrogen. A further confirmation of this conclusion is to be found in experiments like those mentioned on page 71 and in fig. 10 and 11. There it is demonstrated that the gas in question was assimilated again in the light, which as is shown in Chapter IV, § 5, is in accordance with the behaviour of the bacteria towards hydrogen.

Furthermore it was remarkable, that the ratio of hydrogen and carbon dioxide produced varied a good deal, when different strains were tested. When using different cultures of the same strain this ratio varied also, although to a far smaller degree. The hydrogen production amounted to 8—30% of the total gas production (table 9.). I got the impression, that the larger and the stranger the bacteria were and the sooner their motility in the WARBURG-vessels fell off, the more hydrogen they produced. The minute bacteria of strain *9* ordinarily produced H_2, amounting to 8 vol. % of the total gas production; the large bacteria of strain *a*, however, gave off 30 vol. % H_2.

The quite different behaviour of bacteria of a strain cultivated in the peptone culture medium, as compared with bacteria of the same strain from the inorganic medium, as well

TABLE 9.

Ratio of carbon dioxide and hydrogen produced by bacteria from the peptone medium.

Experiment No.	Description of experimental conditions	Manometer	CO_2 production in mm^3 hour.	H_2 production in mm^3 per hour.	H_2 production in % of total gas produced.
1.	Bacteria of strain **d**, cultivated in the peptone medium, suspended in tap-water 2% NaCl, in equilibrium with N_2, temperature 30°C. Alkali present in side-bulb of manometer no. 1 and 3.	S. M.			
		4	14	—	—
		1	—	5	25
		3	—	5	
2.	Conformable to experiment no. 1. Alkali present in side-bulb of manometer no. IV and VI. Bacteria of strain **9**.	S. M.			
		III	30	—	—
		IV	—	2	7
		VI	—	2,5	
3.	Bacteria of strain **c**, cultivated in the peptone medium, suspended in own culture medium, in equilibrium with N_2 at a temperature of 30°C. Alkali present in manometer no V.	S. M.			
		VI	22	—	—
		V	—	3	12
4.	Bacteria of strain **a**, cultivated in the peptone medium, suspended in:	D. M.			
	a. tap-water 2% NaCl, 0,3% $NaHCO_3$ in equilibrium with N_2/5% CO_2. Manometer no. 3.	3	127	—	—
	b. tap-water 2% NaCl, 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ and 1 part Na_2CO_3. Manometer no.1. Temperature 30°C.	1	—	48	27
5.	Conformable to experiment no. 4.	D. M.			
		3	87	—	—
		1	—	37	30

as the differences in the ratio CO_2/H_2 in the total gas production of the different, in other aspects similar strains, also speak for the idea that the "peptone bacteria" must be considered as abnormal. In my opinion, the hydrogen production must be considered from the same point of view; probably it is connected with autolytic phenomena.

That the bacteria from the peptone culture medium must be more liable to autolysis than bacteria from the inorganic media, is clear, when their abnormal morphological habit and their reduced vitality is taken into consideration. The cause of the autolysis is not to be looked for in the transference of the peptone bacteria into the synthetic solution, since they also produced gaseous hydrogen after being suspended in their own culture medium.

In my opinion the explanation must be sought either in the vigorous shaking of the WARBURG-manometers, or in the denseness of the bacterial suspension. For I never could detect any hydrogen production in culture bottles, even after their exposure to darkness during several days at a temperature of 35°C. In agreement with the above ideas I always could detect ammonia in the suspension liquid after experiments with bacteria from peptone cultures, this being in contrast to my experience with bacteria cultivated in an inorganic medium.

However, not all organic culture media exerted such an influence on my bacteria. Bacteria of the strains *a*, *b* or *9*, cultivated in a Na-malate-Na-thiosulphate medium for instance, did not produce hydrogen in my experiments. Their microscopic aspect was quite normal too. The influence of long periods of darkness on the autofermentation of bacteria from peptone culture media, was much greater in comparison with the effect of this treatment on the behaviour of bacteria from inorganic media. After an exposure to a period of darkness of twelve hours at a temperature of 35°C., most of the bacteria did not move anymore and the total gas production, as well as the assimilatory activity of the bacteria was as a rule reduced very considerably.

Special experiments showed that the gas production of the bacteria from the peptone medium could not be influenced

by the addition of organic compounds. (table 6.). In this regard they behave like those from inorganic media.

Nor could I detect any increase of the gas production on addition of Na-butyrate to suspensions of bacteria cultivated in a Na-butyrate-Na-bicarbonate culture medium, as can be concluded from the data in experiment No. 2 of table 5.

Anticipating the results mentioned in § 4 of Chapter IV, I wish to state that in all probability the gaseous hydrogen, produced by the bacteria, is partly assimilated again. In this assimilation process, the hydrogen serves as a hydrogen donator for the reduction of carbon dioxide. The change in pressure detected in the manometers evidently results from the difference between the rate of hydrogen production and the rate of hydrogen assimilation. However, the uptake of hydrogen will be very small in consequence of the very low pressure of this gas in the experiments described above. This may be concluded from the experiments mentioned on page 82, where it is shown, that even the presence of 1% hydrogen in the gas phase of the manometer vessels only had a relatively small depressing effect on the total increase in pressure per minute.

Finally I wish to draw the attention to the remark of GAFFRON in his publication on the metabolism of the *Athiorhodaceae* (1933, p. 7) „Irgend welche Stoffwechselprodukte werden von den Purpurbakterien, wenigstens nach meinen bisherigen Befunden, nicht ausgeschieden."[1]) This statement was not amended in a further publication of GAFFRON on this subject (1935). In the light of the results of my experiments on the *Thiorhodaceae*, it seems rather improbable and it would be advisable to reinvestigate the *Athiorhodaceae* in this respect.[2])

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[1]) His remark, that MULLER (1933 [1]) observed the same thing with the *Thiorhodaceae* does not hold, since in this statement the production of carbon dioxide in MULLER's experiments is neglected.

[2]) In a private letter Mr. C. S. FRENCH of the California Institute of Technology informed me that he observed a CO_2-production in the dark with the *Athiorhodaceae: Spirillum rubrum.*

§ 4. *Behaviour of the* THIORHODACEAE *towards oxygen.*

Although I did not intend to study the problem of possible relations between the *Thiorhodaceae* and oxygen, I could not avoid making some observations on this question. Dr. C. B. VAN NIEL had informed me in a private letter that he had observed an oxygen consumption by suspensions of *Thiorhodaceae* and this observation was always confirmed in my experiments. In consequence in fermentation and assimilation experiments the suspension liquid and the atmosphere in the manometer vessels must always be thoroughly freed from traces of oxygen. Especially the oxygen uptake of bacteria, which had been cultivated in inorganic media, was very vigorous.

I did not investigate the phenomenon of the oxygen consumption in details supposing that this question was studied by VAN NIEL. So I do not know, whether the uptake either is constant, decreases after some time, or even stops at all. However, I have to draw the attention to the fact, that the *Thiorhodaceae* are catalase-positive (VAN NIEL and MULLER 1931) and that I got the impression, that these bacteria are not so strictly anaerobic as generally is supposed. As a matter of fact I found, that strain *a*, inoculated in culture tubes, nearly completely filled up with previously boiled out and rapidly cooled peptone broth with 0,005% Na_2S, always showed abundant development, although the air had free access to the media.

In addition one can use shake cultures in peptone agar for the isolation of these bacteria, even without shutting them off from the air by means of a rubber stopper, paraffine etc. Only the upper part of the agar column did not contain any colonies under these conditions.

Furthermore peptone agar plates with colonies of *Thiorhodaceae* which had developed during an anaerobic incubation in the light, could be exposed to the air without any harmful influence on the bacteria, at least in so far as a microscopic examination allows one to judge. Even after having been in contact with the air during several days, all bacteria in such colonies still showed the normal motility, provided the plates

had been kept in the light.

I must mention, however, that since streak cultures on aerobic agar plates always yielded negative results in the light as well as in the dark, it is obvious that the *Thiorhodaceae* are not able to grow with an oxidative dissimilation as a base. They even seem to die when exposed to the full oxygen tension of the air. The facts mentioned above can be understood on the assumption that the reducing capacity of a large number of bacteria already present is able to stand the harmful effect of the air, in contrast with the reducing capacity of one isolated bacterium.

In view of this, I cannot recommend for the present to interpret this oxygen consumption by suspensions of *Thiorhodaceae* as a consequence of a normal respiration process. Still it is not impossible that a certain restricted supply of oxygen stimulates the metabolism and the development of these bacteria. Even if this is the case, one should distinguish between a favourable influence, caused by a direct participation of the oxygen in the metabolism and a possibly favourable influence caused by a limited oxidation of the medium surrounding the cells, which medium will be reduced under the influence of the bacterial metabolism. Only when the first alternative holds true, one should speak of respiration.

§ 5. *Summary.*

1. The *Thiorhodaceae* in the dark as well as in the light, maintain themselves with a fermentative dissimilation of reserve food material, which process is accompanied by a production of carbon dioxide and acids.
2. The rate of this autofermentation highly depends on the temperature and on a previous exposure to a period of darkness.
3. Under the conditions of my experiments in all probability the illumination does not exert any influence on the autofermentation.
4. Neither the addition of any of the organic substances mentioned nor that of the inorganic substances tried, affect the autofermentation.

5. The *Thiorhodaceae* produce hydrogen in the dark under certain conditions, provided they are cultivated in a peptone medium.
6. Oxygen is rapidly consumed by suspensions of these bacteria, but it remains open whether this process should be termed respiration or not.

CHAPTER IV.

THE CARBON DIOXIDE ASSIMILATION OF THE THIORHODACEAE.

§ 1. *Introductory remarks.*

A few years ago only deficient and rather confused opinions existed on the question, how *Thiorhodaceae* assimilate carbon dioxide in a medium where they only have carbon dioxide and oxidizable sulphur compounds at their disposal besides the other necessary inorganic salts. In fact there was not yet given a satisfactory explanation, why in such a medium the presence of carbon dioxide and oxidizable sulphur compounds as well as an adequate supply of light energy were essential for the development of these organisms.

It was generally accepted that the p. s. b. were able to assimilate carbon dioxide photosynthetically in the same way as is done by the green plants. The negative or at best very doubtful results of all attempts to detect oxygen formation by the p. s. b. were, however, a serious obstacle for this conception. On the other hand it was suggested that the carbon dioxide could also be assimilated chemosynthetically at the cost of the energy liberated in the oxidation of hydrogen sulphide to sulphuric acid. According to BUDER (1919) the latter process would occur with the aid of the oxygen produced in the photochemical process. However, in this line of thought the problem remained unsolved, why the *Thiorhodaceae* require both the presence of H_2S and light simultaneously, i. o.w., why they cannot reduce carbon dioxide either with the energy of the H_2S oxidation (colourless sulphur bacteria!) or with light energy (green plants!) alone.

It was VAN NIEL (1931), who, applying the general considerations of KLUYVER and DONKER (1926, 1930) regarding the unity in the biochemical activities of all living cells on the

problem in question, offered a fully satisfactory explanation. His experimental results can be best interpreted on the base of his theory, that the water used by the green plants as a hydrogen donator in the hydrogenation of the carbon dioxide cannot be used as such by the *Thiorhodaceae*, but that these organisms in inorganic media require oxidizable sulphur compounds for this purpose[1]).

VAN NIEL (1931) summarizes the results of his study as follows:

"It is shown that the metabolism of the purple and green sulphur bacteria is a truly photosynthetic process, which can best be considered as one of a number of possible photosynthetic reactions of the general type: $CO_2 + 2\,H_2A = CH_2O + H_2O + 2\,A$."

Hence the *Thiorhodaceae* are able to build up the carbon compounds necessary for their cell material, using one or more of the following photosynthetic reactions as a base:

$$CO_2 + 2\,H_2S = CH_2O + H_2O + 2\,S$$
$$CO_2 + 2\,S(H_2O) = CH_2O + H_2O + 2\,SO$$
$$CO_2 + 2\,SO(H_2O) = CH_2O + H_2O + 2\,SO_2$$
$$CO_2 + 2\,SO_2(H_2O) = CH_2O + H_2O + 2\,SO_3$$

Although as early as 1907 MOLISCH concluded: „dass die Ernährungsversuche mit Purpurbakterien uns mit einer neuen Art von Photosynthese bekannt gemacht haben, bei der organische Substanz im Lichte assimiliert wird.....", it was VAN NIEL (1931, p. 100), who suggested that such an assimilation of organic substance has to be considered as an assimilation (reduction) of carbon dioxide, the organic compounds acting solely as hydrogen donators.

MULLER (1933[1]) investigated the metabolism of the *Thiorhodaceae* in a number of organic media. The results of his experiments were fully in favour of the conception given and we may therefore conclude that in the equation: $CO_2 +$

[1]) Of course one can also stick to the idea, that the assimilation process itself is still accompanied by an oxygen production, but that the presence of the oxidizable sulphur compounds in the culture medium is indispensable for the removal of the oxygen, which would exert a harmful influence on the bacteria. In my opinion there are no arguments which speak for this less simple theory.

$2\ H_2A = CH_2O + H_2O + 2\ A$. H_2A and A may also represent organic substances, both for the carbon dioxide assimilation of the *Thiorhodaceae* and in all probability for that of the *Athiorhodaceae*.

It seemed to be of great importance to investigate in how far these ideas about the carbon dioxide assimilation of the *Thiorhodaceae*, which were mainly based on the results of analyses of outgrown cultures, could be confirmed and extended with the aid of a quite different technique. In the first place it seemed desirable to study the velocity of the carbon dioxide assimilation under various conditions.

As mentioned already in Chapter II, I used for all experiments on assimilation the two differential manometers. Except for the experiments in Chapter V always an incandescent lamp was used as a light source. For further technical details compare Chapter II.

§ 2. *Auto-assimilation of carbon dioxide.*

a. Experiments with bacteria from an inorganic culture medium.

When suspending *Thiorhodaceae*, cultivated in an inorganic medium, in an isotonic solution of sodium chloride and 0,5% Na-bicarbonate in previously boiled and rapidly cooled water, and on bringing this suspension in manometer vessels with an atmosphere of for instance pure nitrogen with 5% carbon dioxide, I always could observe a decrease of pressure on illuminating the vessels. This is of course exactly the contrary of what happens in the dark (compare Chapter III.)

This phenomenon can only be explained by assuming that either nitrogen or carbon dioxide, or both, are assimilated in the light. When I did not suspend the bacteria in a mixture of bicarbonate and carbonic acid, but in a carbon dioxide buffer mixture of Na-bicarbonate and Na-carbonate, I could not observe any change in pressure either in the light, or in the dark, although the bacteria remained quite healthy in this solution, at least as far as may be judged from a microscopic examination. Hence the conclusion seems justified that the bacteria are able to assimilate carbon dioxide in the light,

although no hydrogen donators had been added to the medium. When asking which substance served as a hydrogen donator under the conditions of the experiment, we must conclude that it cannot be water, since then an equivalent amount of oxygen would have been produced. But in this case no decrease of pressure would have been observed. Under these circumstances the bacteria in the suspensions had no other hydrogen atoms at their disposal than either those from organic reserve food compounds possibly present in the cells (1) or those from oxidizable inorganic reserve food compounds (2) or those from oxidizable metabolic products (3).

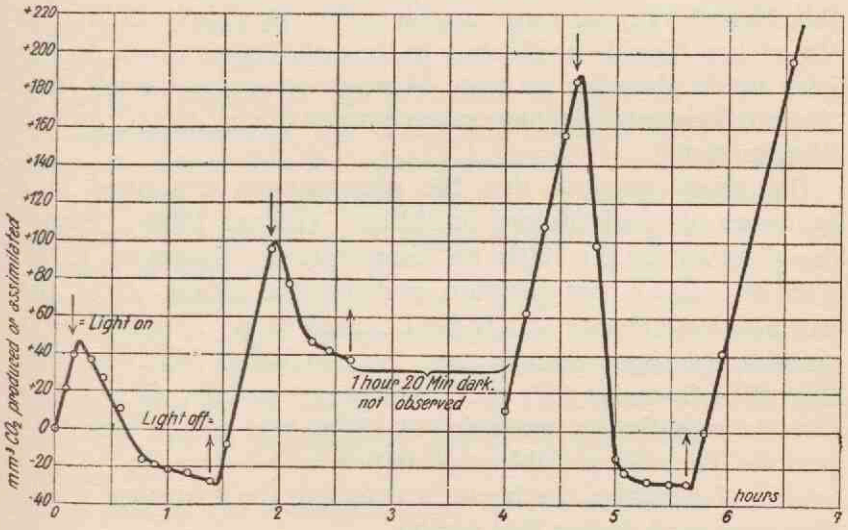

fig. 9.

Carbon dioxide uptake by a suspension of 260 mm³ bacteria of strain a, cultivated in the inorganic medium, suspended in distilled water with 2% NaCl pro analyse, 0,5% $NaHCO_3$ pro analyse, free from SO_4-ions, in equilibrium with N_2/7% CO_2 at a temperature of 30°C. For explanation see text.

From experiments like those represented graphically by fig. 9 one must conclude that under the conditions mentioned, the bacteria mainly used as hydrogen donators the organic products of their metabolism. During these experiments the same vessel with bacteria was exposed to intermittent pe-

riods of light and darkness. The arrows in the figure indicate the moments when the light was switched on and off. A closer examination of the graph learns that at the beginning the uptake of carbon dioxide in the light is rapid and slower later on. Evidently this phenomenon returns after each period of darkness and apparently the assimilation velocity remains high during a longer period, if the previous period of darkness is longer. This behaviour is of course incompatible with the idea that the reserve food compounds themselves are responsible for the assimilation observed (at zero time in fig. 9 the experiment was already some time going on.). The slower uptake of carbon dioxide (in fig. 9 at 0 h. 45, 2 h. 15 and 5 h.) was still considerable, viz. nearly as high as the carbon dioxide production in the autofermentation. As has been made plausible on page 44 we must accept namely that the autofermentation goes on unchanged during the periods of illumination[1]).

One might suggest, that the phenomenon described is a symptom of "assimilatory inhibition" (EWART 1898), "Ermüdung" (PANTANELLI 1904) or "Solarisation" (URSPRUNG 1917). That this was out of question, and that the falling off of the assimilation velocity was indeed caused solely by a lack of a suitable hydrogen donator, may be concluded from the fact, that if H_2S or Na_2SO_3 were added previously, the carbon dioxide assimilation proceeded at a high rate during very long periods. The only possible explanation is therefore that after a period of darkness the bacteria consume the hydrogen donators, produced during that period.

In agreement with this conception was the fact, that the velocity of the uptake of carbon dioxide in the absence of added hydrogen donators — which I will call auto-assimilation — decreased considerably when the bacteria had been exposed to a period of darkness of some twenty hours at 35°C., before the experiment started[2]). Still the capacity of

[1]) Numerous experiments like the one described above were performed, always with similar results. However, I will refrain from publishing more of them, since they do not lend themselves for a simple tabular representation.

[2]) The fermentation products formed during that period were of course removed by the preceding centrifuging of the cultures.

assimilating carbon dioxide after addition of hydrogen donators was but little influenced by this treatment. Since it seemed probable that such "starved" bacteria would offer special advantages for the study of the suitability of different hydrogen donators, such bacteria have been used in most of the following experiments.

The slow auto-assimilation which on continued illumination follows the rapid one, will in all probability proceed at least partly with the same hydrogen donators as those which are responsible for the rapid assimilation. However, in the light these donators do not accumulate as they do in the dark. It seemed possible, that part of this rest-assimilation must be ascribed to sulphur stored in the cells. As a matter of fact I was much surprised to find that the part played by this stored sulphur apparently was very small. I never could observe any striking difference in the behaviour of bacteria of the same strain containing or not containing sulphur. One must conclude therefore, that the sulphur present in the *Thiorhodaceae* as a reserve food compound can only slowly be utilized.

From the experiment to be mentioned on page 77 it will appear in addition that the oxidation of hydrogen sulphide proceeds rapidly up to the level of sulphur, but that this stage once being reached the assimilation velocity falls down considerably. Evidently the hydratation and subsequent dehydrogenation of the sulphur hydrate is a slow process, limiting the rate of assimilation under the conditions of the experiments.

In raising the question which is the nature of the compounds which accumulate during the periods of darkness and which compounds can serve as hydrogen donators for the auto-assimilation, it seems plausible to think of oxidizable products of a carbohydrate fermentation.

Now on page 41 it was shown that during the autofermentation acids were produced. The experiments mentioned in table 14 however, showed that bacteria cultivated in the ordinary inorganic medium could but use Na-malate, Na-lactate and Na-butyrate as a hydrogen donator to a slight extent. Hence it is improbable that the hydrogen donators, produced during the autofermentation, would be organic acids, since the

auto-assimilation can proceed very rapidly. Only a small part of the auto-assimilation can possibly be ascribed to these substances.

It must be possible in principle, to demonstrate a consumption of acids as a result of the assimilation, since this will be reflected during the assimilation in an increase of the carbon dioxide present in the suspension as bicarbonate or as carbonic acid. It would be feasible to detect this in a duplicate experiment by adding an excess of acid in one vessel before and in the other after the assimilation, in the same way as was done in the experiments mentioned sub 1 and 2 in table 2.

However, in the dehydrogenation of the acids other acids will be formed first. Only if the successive dehydrogenations lead to the production of pyruvic acid, it is probable, that this acid will be involved in the assimilatory metabolism of the cells and hence will disappear from the medium (MULLER 1933[1], p. 153).

Considering all this, it seems allowable to assume that the assimilation of the acids produced by the bacteria, whenever it would take place, will only be very slight. Hence it was accepted that the uptake of carbon dioxide as calculated from the difference between the changes of pressure per unit of time during periods of light and of darkness, was quantitatively due to a carbon dioxide assimilation and not partly to a conversion of carbonate into bicarbonate. This is an essential point in the interpretation of the results of those experiments in which an absolute value was attached to the amount of carbon dioxide taken up and where no correction for auto-assimilation could be applied.

Since the autofermentation of the *Thiorhodaceae* might be a combined lactic acid and alcoholic fermentation, I have also tried whether ethyl alcohol could be used as a hydrogen donator. Under the conditions of my experiments this compound is unsuitable as such, as can be judged from the figures in table 14 no. 4. Acetaldehyde, however, can be used very well for that purpose (table 14, no. 3); it may be expected that under these conditions it will be oxidized to acetic acid. If acetaldehyde or some other aldehyde would be responsible for the

greater part of the auto-assimilation, in particular for the rapid part of this process, this would imply that during the assimilation acid will be produced and hence carbon dioxide will be expelled from the suspension. In consequence of this the real assimilation would be larger than the apparent one. On the other hand it seems extremely unlikely that substances as reactive as aldehydes would be the final products of the autofermentation.

If the conclusion of GAFFRON (1934) that the *Thiorhodaceae* are able to reduce sulphates to sulphides in the dark, could have been confirmed, the auto-assimilation could have been explained as an assimilation with the aid of those sulphides. As set forth earlier for this assumption no support whatever has been found. Moreover, it appeared that the typical symptoms of auto-assimilation could be observed as well, when there were no sulphate ions present in the suspension, as is shown for instance in the experiment represented in fig. 9. Still it remains possible that the auto-assimilation proceeds at the cost of organic or inorganic SH-compounds produced indirectly, e.g. in the autolytic breakdown of proteins.

It must be admitted that the unknown chemical nature of the products of the metabolism in the dark and in particular the unknown nature of the hydrogen donators active in the auto-assimilation, involves a factor of uncertainty in the interpretation of the experiments, especially of those described in Chapter V.

b. Experiments with bacteria from an organic culture medium.

In using bacteria from a peptone culture medium, which as shown before, produce hydrogen in the dark, the auto-assimilation is still more complicated, because there also will be a carbon dioxide assimilation with gaseous hydrogen as a donator. As will be shown in § 4 and 5 of this Chapter, the *Thiorhodaceae* are capable of performing this, in the dark as well as in the light. In the latter case, however, the process is much accelerated.

When the bacteria are not illuminated and when they have only the traces of hydrogen produced by themselves at their

disposal, the hydrogen uptake will be scarcely perceptible and hence the production of hydrogen will predominate by far.

In the light, however, this is no more the case. In agreement with the considerations given there could be observed an increase of the pressure in the manometer vessels in the dark and a decrease in the light, even when these bacteria were suspended in a carbon dioxide buffer mixture, this observation being in contrast to what is observed when bacteria cultivated in an inorganic medium are used. To give an idea of these phe-

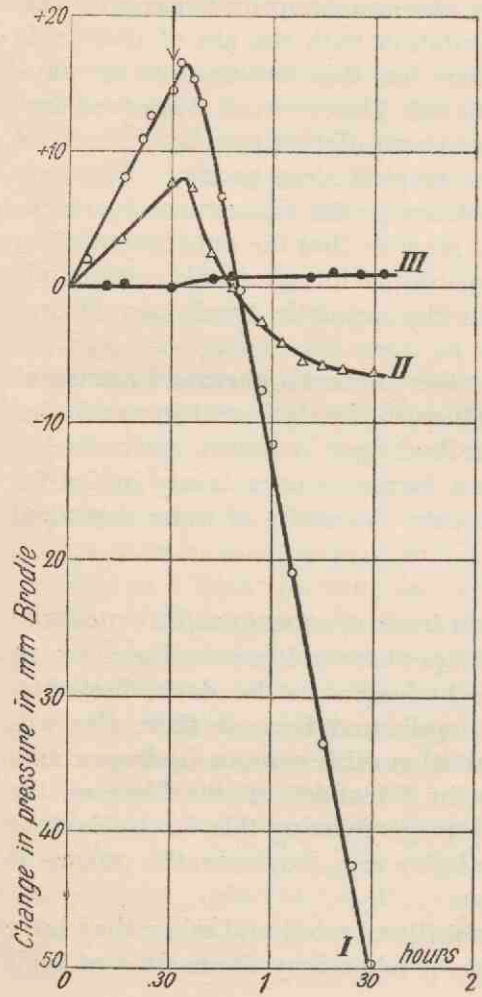

fig. 10

Curve I. Changes in pressure effected by bacteria of strain **a**, cultivated in the peptone medium, suspended in tap-water with 1,8% NaCl, 0,3% $NaHCO_3$ and 0,01% Na_2S, in equilibrium with N_2/3% CO_2 at 30°C. Differential manometer No. 3.

Curve II. Changes in pressure effected by an equal quantity of the same bacteria as in I, suspended in 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ to 1 part Na_2CO_3, in equilibrium with a N_2/CO_2 atmosphere at 30°C. Differential manometer No. 3.

Curve III. Changes in pressure effected by another sample of bacteria of strain **d**, cultivated in the inorganic culture medium, and suspended in the same solution as in II, at the same temperature. Differential manometer No. 3.

nomena some type experiments are represented graphically in fig. 10. The difference in behaviour between bacteria from organic and those from inorganic media, when suspended in a carbon dioxide buffer mixture, is strikingly demonstrated by the curves II and III. That the former are able to assimilate carbon dioxide with H_2S as a hydrogen donator as well, appears from a comparison of the curves I and II in fig. 10.

In the foregoing paragraph it was shown that when using bacteria cultivated in the inorganic medium, the auto-assimilation was the higher the longer the previous period of darkness had lasted. Therefore it was expected that this would

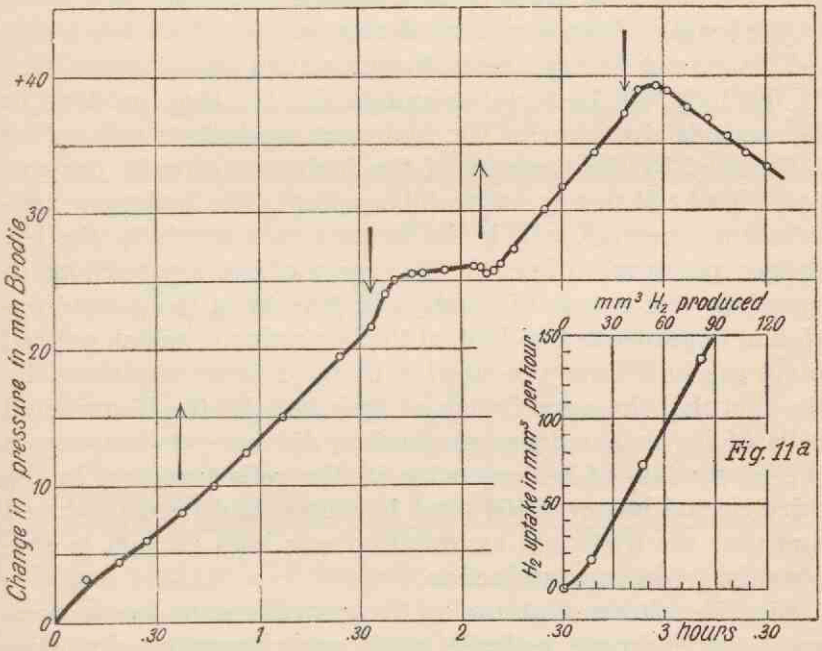

fig. 11 and fig. 11a.

Changes in pressure effected by bacteria of strain a, cultivated in the peptone medium, suspended in a 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ to 1 part Na_2CO_3 in distilled water with 2% NaCl, in equilibrium with a N_2/CO_2 atmosphere at 30°C. Differential manometer No. 3.

fig. 11a. Velocity of the H_2 uptake in the first, second and third period of illumination of the experiment in fig. 11, in dependence on the quantity of H_2, present in the vessel at the times in question.

hold as well for the assimilation of the gaseous hydrogen which accumulates in the dark periods when bacteria from peptone culture media are used. Such an experiment is represented graphically in fig. 11.

The bacteria used were cultivated in peptone water, suspended in a carbon dioxide buffer mixture[1]) and as soon as possible the experiment was started. Hence I could assume that there were but traces of hydrogen in the manometer vessel at zero time, that is at the moment when equilibrium was attained in the vessels. The bacteria were exposed alternatively to light and darkness and apparently the assimilation of a gas, different from carbon dioxide, increased according to the length of the period of darkness.

This cannot be explained otherwise than by assuming, that in the light the bacteria assimilate the gas they produce in the periods of darkness. The hydrogen production will not be influenced by the pressure of the hydrogen already present, or at least not to any appreciable extent. The hydrogen assimilation, however, will be influenced very much by the hydrogen pressure, at least in the region of the low tensions in question. The course of changes in tension in the second period of exposure to the light, at the beginning of which only 60 mm^3 gas had been produced, will have to be explained by the fact that the assimilation of hydrogen by the illuminated cells of the rather dense suspension, did not yet compensate the production of hydrogen by all the cells together. In the third period however 100 mm^3 hydrogen had been produced and now the hydrogen assimilation was high enough to surpass the hydrogen production.

Fig. 11*a* shows that under the conditions of the experiment the hydrogen assimilation is approximately proportional to the hydrogen tension, as may be expected in view of the principle of the limiting factors (BLACKMAN, 1905).

In order to prevent confusion it must be remarked that at the beginning of the experiment of fig. 10 curves I and II a considerable amount of hydrogen already had been produ-

[1]) This means of course that in the experiment to be described, only changes in tension of gases different from carbon dioxide could be observed.

ced, because this time I had not started the observations immediately after equilibrium had been attained.

Finally I wish to mention an experiment in which it was shown that the assimilation of the hydrogen produced by the bacteria themselves was more rapid, depending on the concentration of the carbon dioxide they had at their disposal. The uptake of hydrogen could therefore be limited either by the hydrogen tension or by that of the carbon dioxide. This is shown by the experiment represented in table 10.

TABLE 10.

Assimilation of hydrogen produced in the dark when illuminated and its dependence on the carbon dioxide concentration.

Exp. No.	Description of experimental conditions	D.M.	H_2 production in the dark in mm^3 per hour	H_2 uptake in the light in mm^3 per hour
1.	Bacteria of strain a, cultivated in the peptone medium, suspended in:			
	a. tap-water 2% NaCl, in equilibrium with N_2. Manometer no. I. Alkali present in side-bulb.	I	31	37
	b. tap-water 2% NaCl, with 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ and 1 part Na_2CO_3. Manometer no. II.	II	32,5	66
	Temperature 30°C. Light source incandescent lamp.			

Equal quantities of bacteria of the same culture from the peptone medium were suspended in tap-water with 2% NaCl and in a carbon dioxide buffer mixture. In the first mentioned case alkali was present in a side-bulb of the manometer vessel, in order to absorb the carbon dioxide produced by the bacteria. Hence in both cases only changes in tension of gases other than carbon dioxide could be measured. It appears from the data in table 10, that the bacteria which had an excess of carbon dioxide at their disposal, assimilated more hydrogen per unit of time than the bacteria which obtained a small quantity of carbon dioxide. This notwithstanding the

fact that the quantities of hydrogen available in both cases must have been equal, since the same amounts of hydrogen will have been produced per unit of time. That also in the second case an absorption of hydrogen could still be observed must probably be ascribed to the fact, that the alkali in the side-bulb was unable to absorb immediately all carbon dioxide formed in the autofermentation process.

All this may be considered as a proof of the view that the tension of the carbon dioxide limits the assimilation of the gas produced by the bacteria. Therefore this gas must be a hydrogen donator and hence this experiment can also be considered in support of the view, expressed in Chapter III, that the unknown gas produced by the "peptone bacteria" during the autofermentation, is hydrogen indeed.

I have called the phenomenon described in this paragraph: "auto-assimilation of carbon dioxide". This term is in so far misleading, as it may give the impression that we are dealing here with a quite new phenomenon. However, since the green plants can assimilate the carbon dioxide and water formed in their respiration process, we meet here exactly the same situation. The only difference is that the *Thiorhodaceae* use other hydrogen donators instead of water.

The foregoing also throws new light on the following statements of MULLER (1933 [1], p. 165): "In these cultures practically no metabolic products other than relatively small amounts of CO_2 have been detected" and "it has been shown that in all probability the substrate is completely converted into cell material and CO_2, i.o.w. that the assimilation predominates in the metabolism." From the facts reported above it follows that this predomination of the assimilation process should not be seen as a suppression of the other metabolic processes, but rather that the substances produced in these processes are for the greater part reassimilated again.

The latest experiences of GAFFRON (1935) show that the *Athiorhodaceae* too are characterized by a rapid auto-assimilation, followed by a slow one and also in this respect there appears to be a close resemblance between *Athiorhodaceae* and *Thiorhodaceae*.

§ 3. *The carbon dioxide assimilation with oxidizable sulphur compounds.*

For the study of the assimilation of carbon dioxide with hydrogen donators specially added, I always used "starved" bacteria, because under these conditions one meets with fewer difficulties since the auto-assimilation is so much decreased. Yet it remained necessary to determine the extent of the auto-assimilation under the same conditions in the control manometer and to introduce a correction for the auto-assimilation in the proper experiment.

Because the assimilatory capacity of bacteria from an organic medium is always considerably less than that of bacteria from an inorganic medium, for the greater majority of these experiments only the latter bacteria were used. Other than quantitative differences, however, could not be observed. The bacteria nearly always were suspended in tap-water with 2% NaCl, in most cases with the addition of 0,5% sodium-bicarbonate, in equilibrium with nitrogen containing 5% carbon dioxide.

a. Experiments with H_2S. As may be derived from fig. 12, the assimilation of carbon dioxide with hydrogen sulphide as a donator proceeded at a high speed. It was constant for a long time and apparently remained so until all H_2S had been consumed (at 1.30 in fig. 12), whereafter it fell off to the level of the auto-assimilation, which was of the same order as that in the control manometer without H_2S. Only in very low concentrations H_2S was the limiting factor for the assimilation of carbon dioxide. By determining the amount of H_2S added[1]), it was possible to calculate how many molecules of carbon dioxide were assimilated per molecule of H_2S.

The result of such a calculation is given in table 11.

In this connection it has to be remarked that for the calculation the changes in pressure during the periods of light were supposed to be quantitatively due to the assimilation of carbon dioxide. Hence it was assumed that a possible assimila-

[1]) This amount was determined by volumetric measurement of a saturated aqueous solution of known temperature.

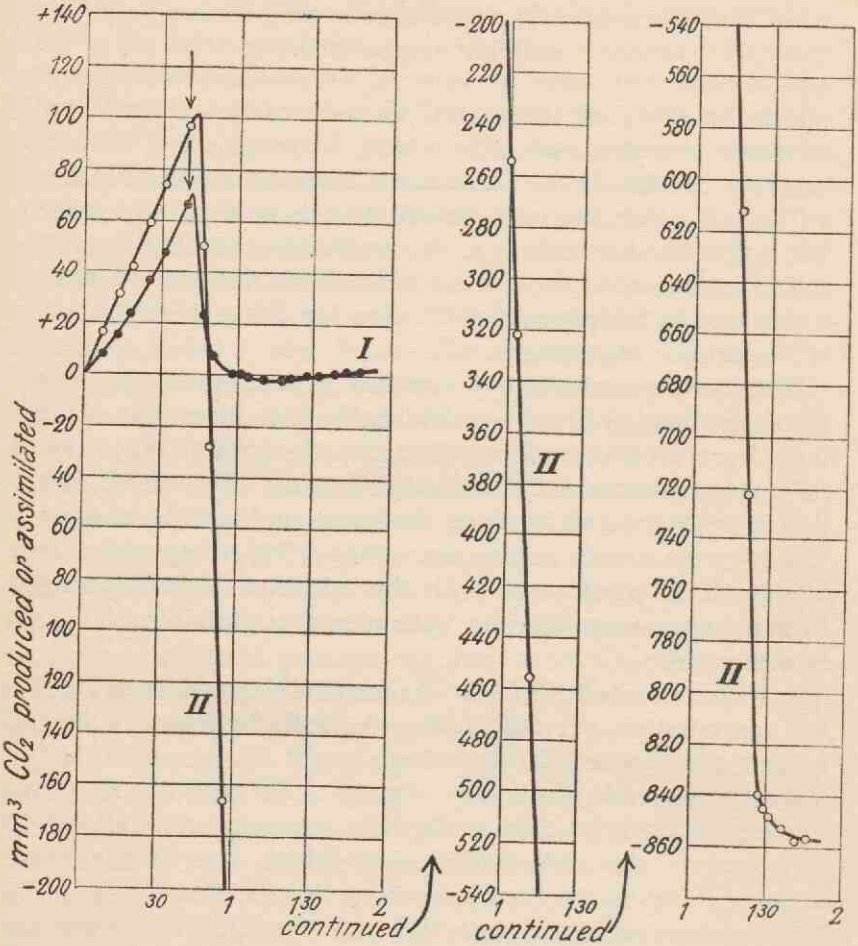

fig. 12.

Carbon dioxide assimilation with H_2S as a hydrogen donator.

Curve I. CO_2 production and auto-assimilation of ± 300 mm³ bacteria of strain **d**, cultivated in the inorganic medium, exposed to a period of darkness of 20 hours at 36°C,, and suspended in tap-water with 2% NaCl and 0,5% $NaHCO_3$, in equilibrium with N_2/7% CO_2 at 30°C. Differential manometer No. 3.

Curve II. Same arrangement, except the addition of 0,6 cm³ saturated solution of H_2S in water of 14°C.

TABLE 11.

Carbon dioxide assimilation with hydrogen sulphide.

Exp. No.	Description of experimental conditions	D.M.	mm³ H_2S added	mm³ CO_2 assimilated[1])	Ratio H_2S CO_2
1.	Bacteria of strain d, cultivated in the inorganic medium, starved during 18 h. at 35°C., suspended in tap-water 2% NaCl and 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at 30°C.	1	1540	910	1,7
2.	Conformable to exper. no. 1.	1	1470	840	1,8
3.	Conformable to exper. no. 1.	1	1030	610	1,7
4.	Conformable to exper. no. 1.	1	290	160	1,8

tion of acids from the autofermentation could be neglected. For a documentation of this assumption the reader may be referred to p. 69.

The decrease in pressure caused by absorption of H_2S could also be neglected, as may be concluded from the following. By calculating with the aid of the dissociation constant of H_2S (for the first $H^{\cdot}$) and with the absorption coëfficient of H_2S in distilled water the H_2S pressure in equilibrium with water of pH 7,8, it appears that this pressure can have been at most $3{,}5 \times 10^{-4}$ atm. This involves that only 0,2% of the changes in pressure observed have been due to an absorption of H_2S.

Considering, that for all these reasons the found average quotient H_2S/CO_2 of 1,75 will still be on the low side, one may conclude, that two H_2S molecules are used for the reduction of one CO_2 molecule. As the equations on p. 65 show, one has to assume therefore, that in the first instance the H_2S was only oxidized to the oxidation level of sulphur. Anyhow the assimilation with sulphur as a donator was too slow to permit its study by the manometric method. This is in agreement

[1]) These data are corrected for the carbon dioxide consumed by auto-assimilation under the same conditions, measured in a control manometer.

with the facts, mentioned on p. 69, concerning the auto-assimilation of *Thiorhodaceae* either or not containing sulphur. Up to the present the remarkable difference in oxidation velocity of H_2S and sulphur had escaped notice, since the earlier investigations were restricted chiefly to the chemical analysis of out-grown cultures.

Another example of the desirability to complete the results

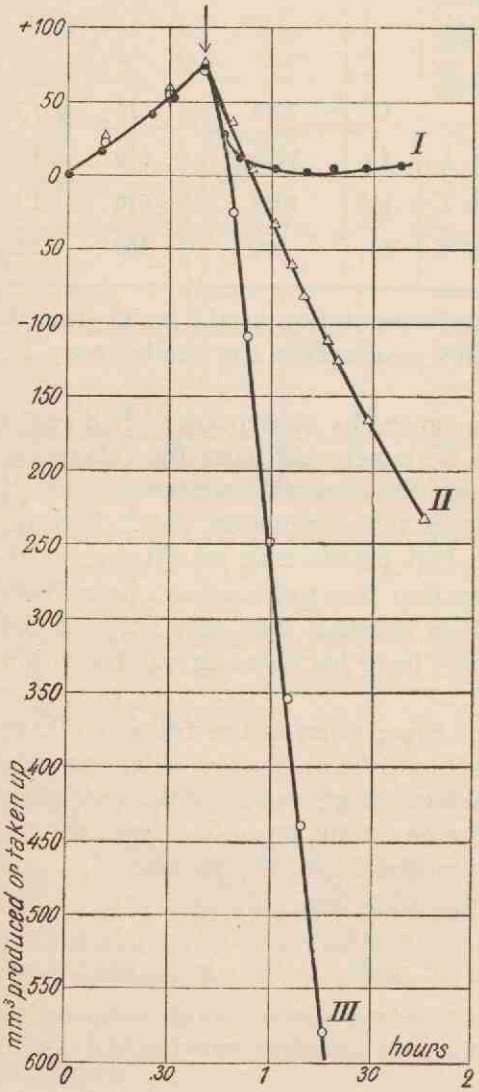

fig. 13.

Carbon dioxide assimilation Na_2SO_3 as a hydrogen donator, in comparison with the auto-assimilation and with the carbon dioxide assimilation with H_2S as a hydrogen donator.

Curve I. CO_2 production and CO_2 uptake of ± 230 mm³ bacteria of strain **a**, cultivated in the inorganic medium, exposed to a period of darkness of 20 hours at 36°C. and suspended in tap-water with 2% NaCl and 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at 30°C. Velocity of CO_2 assimilation 95 mm³/hour. Differential manometer No. 1.

Curve II. Same arrangement as in I, except the addition of 0,2% Na_2SO_3 7 H_2O. Assimilation velocity 370 mm³/hour. Differential manometer No. 1.

Curve III. Same arrangement as in I, except the addition of an aqueous solution of 2500 mm³ H_2S. Assimilation velocity 1230 mm³/hour. Differential manometer No. 1.

of growth experiments with the outcome of different methods of investigation is to be found in the observation of GAFFRON (1933) regarding the assimilation of carbon dioxide with H_2S as a donator by an *Athiorhodaceae*. Still as far as known these organisms are unable to grow in an inorganic medium.

b. Experiments with sulphite and thiosulphate. The assimilation of carbon dioxide with an excess of Na_2SO_3 as a hydrogen donator proceeded at a well measurable rate, though much slower than with H_2S under the same conditions. Vide fig. 13.

In contrast to what had been found with H_2S, I could observe a distinct influence of the Na_2SO_3 concentration upon the assimilation velocity. I first determined the assimilation velocity with 0,05% Na_2SO_3 7 H_2O and after addition of more Na_2SO_3 the assimilation velocity was determined again, all other conditions remaining unchanged. Thus by comparing bacteria under similar circumstances, it became evident that below 0,2% Na_2SO_3 the assimilation velocity increased with higher concentrations, but that above 0,3%, the sodium sulphite exerted a harmful influence upon the bacteria which may be due to the fact that they were not accustomed to such high concentrations of sulphite.

I will not venture to decide, whether a Na_2SO_3 concentration above 0,2% is indeed no longer a limiting factor in the assimilation process, as possibly the harmful influence of sulphite becomes already manifest at concentrations below 0,3%.

Since for low concentrations of sulphite the assimilation velocity is very small, I refrained from determining the quotient Na_2SO_3/CO_2 as has been done for H_2S.

The carbon dioxide assimilation with sodium thiosulphate proceeds nearly as quick as that with sodium sulphite under similar conditions. Detailed experiments were not carried out. GAFFRON (1934) stated, that the sulphur from the sodium thiosulphate is not oxidized further. The average of the quotient Na_2SO_3/CO_2, as determined by him, was 2,2. This author gives as his opinion that polythionic acids will be formed out of the $Na_2S_2O_3$. More probable it seems to me, that in the first instance the thiosulphate will be converted into Na_2SO_4 and sulphur.

§ 4. *The carbon dioxide assimilation with gaseous hydrogen in the dark.*

The experiments with the *Thiorhodaceae* cultivated in peptone water as reported in Chapter III § 3 and in § 2*b* of this Chapter induced me to study the behaviour of these bacteria

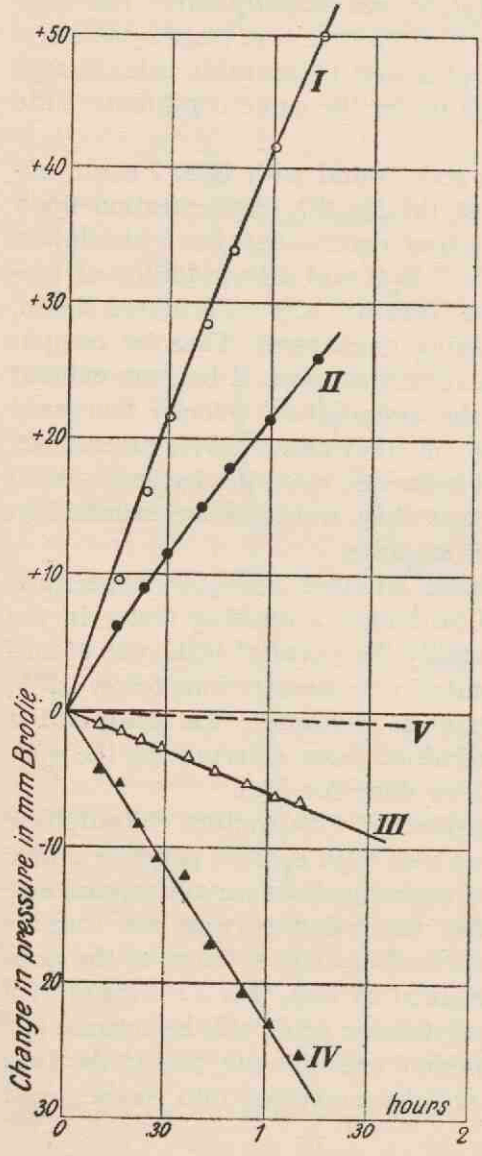

fig. 14.

Carbon dioxide and hydrogen assimilation in the dark.

Curve I. Change in pressure effected by the CO_2 and H_2 production of ± 800 mm^3 bacteria of strain **a**, cultivated in the peptone medium, suspended in tap-water with 2% NaCl and 0,3% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at a temperature of 30°C. Differential manometer No. 3.

Curve II. Change in pressure effected by the H_2 production of an equal quantity of the same bacteria as in I, suspended in a 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ to 1 part Na_2CO_3 in tap-water with 2% NaCl, in equilibrium with a N_2/CO_2 atmosphere at 30°C. Differential manometer No. 1.

Curve III. Continuation of the experiment represented by curve I, after the introduction of 65% N_2, 30% H_2 and 5% CO_2 in the manometer vessel.

Curve IV. Continuation of the experiment represented by curve II, after the introduction of 70% N_2 and 30% H_2 in the manometer vessel.

Curve V. See text.

towards hydrogen.

Restricting myself for the moment to what happens in the dark, I wish to examine more closely the experiment represented in fig. 14. The course of this experiment was as follows. Bacteria of strain *a*, cultivated in peptone water, were centrifuged in such a way that finally two equal quantities of bacteria were obtained. One half was suspended in a solution of sodium bicarbonate in a manometer vessel with nitrogen containing 5% carbon dioxide. The other half was suspended in a carbon dioxide buffer mixture. The changes in pressure in the two manometers may be seen from the lines I and II of fig. 14; they do not show anything particular, as these results are in agreement with the experiences mentioned in Chapter III § 3. This experiment only served to determine, how much hydrogen and how much carbon dioxide was given off per unit of time. These quantities were 41 mm^3 and 92 mm^3 per hour respectively.

Thereupon the manometers were taken out of the water bath and now a mixture of 65% nitrogen, 30% hydrogen and 5% carbon dioxide was introduced in the manometer vessel containing the bicarbonate solution. The other manometer was filled with 70% nitrogen, 30% hydrogen. After having been replaced into the water bath, they showed, when equilibrium had been established, the changes in pressure, which are represented in the lines III and IV. These results leave no doubt that hydrogen was absorbed in the dark. The difference in the changes in pressure per unit of time, indicated by the curves III and IV, enabled me to calculate how much hydrogen was absorbed per unit of time. In the corresponding manometer namely, changes in pressure resulting from changes in carbon dioxide pressure were excluded. The H_2-uptake was 82 mm^3 per hour.

If one substracts from the increase in pressure of curve I the value, corresponding with the absorption of hydrogen of 82 mm^3 per hour, then the dotted line V is obtained. Now, on the understanding that the absorption of hydrogen in the two manometers has been the same[1]) the difference between the

[1]) The only difference in the two experiments was a different pressure of carbon dioxide and a different pH. A lowering of the H_2-uptake by a limiting CO_2 supply in the case of line III still seems to be possible.

changes in pressure of curves V and III can only be explained by an absorption of carbon dioxide. This calculated CO_2-uptake attained the value of 35 mm^3 per hour. The quotient of the hydrogen and the carbon dioxide taken up is then 2,4.

It seems premature to conclude definitely from these few experiments, which certainly are liable to objections, that per molecule of carbon dioxide two molecules hydrogen are absorbed.

However, the experiments prove that a carbon dioxide and hydrogen absorption by the *Thiorhodaceae* occurs in the dark. Furthermore there are distinct indications, that at least two hydrogen molecules are wanted for the reduction of one carbon dioxide molecule. This is in accordance with the general formula for the carbon dioxide assimilation.

For further investigations in this direction it is, of course, recommendable to use the non-hydrogen forming bacteria from inorganic media. These moreover take up the hydrogen much more rapidly and only by the use of very dilute suspensions of bacteria and a high hydrogen pressure, it will be possible to measure the velocity of hydrogen and carbon dioxide uptake, without being encumbered by the slow supply of hydrogen as a consequence of the small solubility of this gas in water.

Casually I studied with bacteria cultivated in peptone water how quick the absorption of hydrogen was, if the pressure of this gas was decreased. With 1% hydrogen in the atmosphere in the manometer vessels the hydrogen absorption was only 20% of the hydrogen production. With 2% hydrogen it was still only 40% and not until the hydrogen concentration reached 4% it was high enough to surpass the hydrogen production. Practically the gaseous hydrogen produced by the bacteria themselves is not taken up again to any degree. However, this is no longer the case when the suspension is illuminated (Compare § 2*b* of this Chapter.)

§ 5. *Carbon dioxide assimilation with gaseous hydrogen in the light.*

When I illuminated manometer vessels with bacteria which

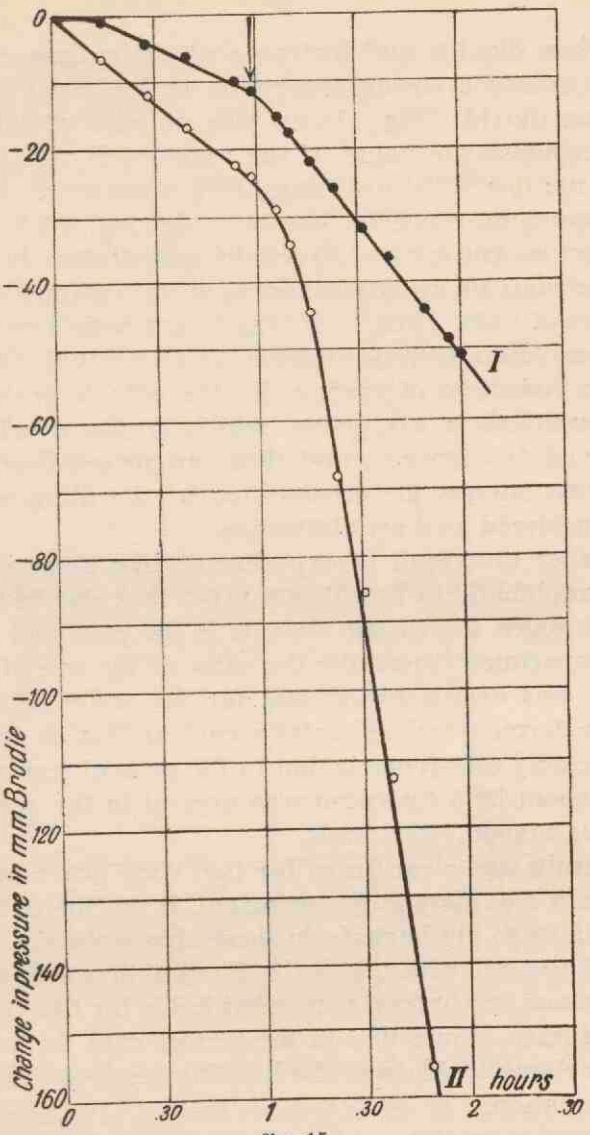

fig. 15.

CO_2-and H_2-assimilation in the dark and in the light.

Curve I. Change in pressure effected by the H_2 uptake of bacteria of strain d, cultivated in the inorganic medium, suspended in 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ to 1 part Na_2CO_3 in tap-water with 2% NaCl, in equilibrium with a N_2/CO_2 atmosphere at 30°C. Differential manometer No. 3.

Curve II. Change in pressure effected by CO_2 and H_2 uptake of an equal quantity of the same bacteria, suspended in tap-water with 2% NaCl and 0,5% $NaHCO_3$, in equilibrium with N_2/7% CO_2 at 30°C. Differential manometer no. 1.

had carbon dioxide and hydrogen at their disposal, I could always observe a strong absorption of hydrogen as well as of carbon dioxide. Fig. 15 can give an idea of this. I have not determined the value of the quotient H_2/CO_2, since too great a number of complicating factors were involved.

This quotient, however, has been determined by GAFFRON (1935) by measuring the hydrogen assimilation in the light of suspensions of an *Athiorhodaceae* on addition of known quantities of soda. Thus he obtained very satisfactory results, amounting to an average of 2 for the quotient H_2/CO_2.

As the reduction of carbon dioxide with hydrogen under these conditions is a reaction, which is characterized by a decrease of free energy, and thus can proceed without the aid of light energy, the acceleration by the illumination has to be considered as a sensibilisation.

I consider the result of experiments like those which are shown graphically in fig. 16 as a proof that also in this uptake of hydrogen the carbon dioxide is the principal acceptor.

This experiment was quite the same as the one of table 10, where it was demonstrated that for the assimilation of the hydrogen formed by the bacteria carbon dioxide was necessary. The only difference is that in the present series of experiments about 30% hydrogen was present in the gas mixture within the manometer vessels.

The results are clear, in so far that they prove once more that carbon dioxide was the principal, if not the only, acceptor available to the bacteria in these circumstances. The fact that in the vessel with alkali the illumination does not exert any influence, in contrast with what holds for the vessel with carbon dioxide, shows that in the former case the concentration of carbon dioxide was the limiting factor for the hydrogen assimilation.

§ 6. *Assimilation of hydrogen with other hydrogen acceptors.*

In a few experiments of a preliminary character I investigated whether special reducible substances could replace the carbon dioxide as a hydrogen acceptor for gaseous hydrogen. For this purpose I compared the velocity of absorption of

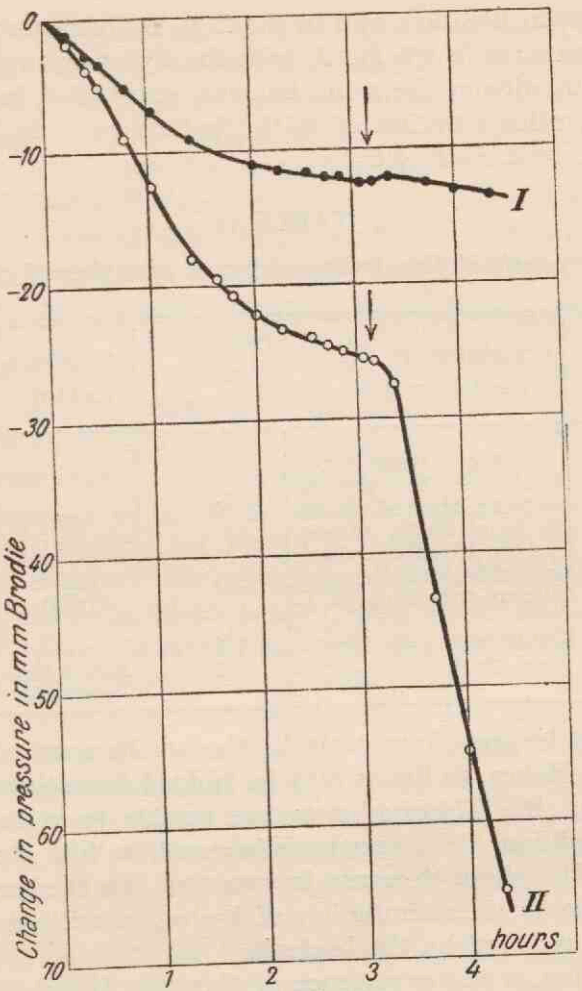

fig. 16.

H_2 uptake dependent on the available quantity of CO_2.

Curve I. Change in pressure effected by the H_2 uptake of bacteria of strain a, cultivated in the peptone medium, suspended in tap-water with 2% NaCl, in equilibrium with N_2/30% H_2 at 30°C. Alkali present in a side-bulb of the manometer vessel. Differential manometer No. 3.

Curve II. Change in pressure effected by the H_2 uptake of an equal quantity of the same bacteria, suspended in a 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ and 1 part Na_2CO_3 in tap-water with 2% NaCl, in equilibrium with a N_2/CO_2 atmosphere at 30°C. Differential manometer No. 2.

hydrogen in the dark and in the light by bacteria suspended in tap-water with 2% NaCl, with the hydrogen uptake of an equal quantity of the same bacteria suspended in the same solution after addition of 0,1% Na-fumarate, or 0,05% Ca-formate, or 0,1% $NaNO_3$.

TABLE 12.

Hydrogen assimilation in the presence of different compounds

Experim. No.	Description of experimental conditions	Differential manometer	Substance added	H_2 uptake in mm^3 per hour
1.	Bacteria of strain d, cultivated in the peptone medium, starved during 24 h. at 36°C., suspended in distilled water with 2% NaCl, in equilibrium with H_2 at 30°C.	3	—	40
		1	0,05% $NaNO_3$	39
2.	Conformable to experiment no. 1.	1	—	17
		3	0,05% Ca-formate	17,5

As may be seen from table 12, the results were completely negative. Hence, as far as may be judged from these few observations, the *Thiorhodaceae* are unable to reduce under these conditions the compounds in question with gaseous hydrogen. The slight decrease in pressure observed was apparently due to an assimilation of hydrogen with the carbon dioxide produced by the bacteria.

According to the experience of GAFFRON (1935) the behaviour of an *Athiorhodaceae* cultivated in yeast extract was quite different.

§ 7. *Cultivation of* Thiorhodaceae *in a hydrogen carbon dioxide atmosphere.*

It seemed interesting to ascertain whether the *Thiorhodaceae* are able to grow in an inorganic medium devoid of any oxidizable sulphur compound, provided they have hydrogen and carbon dioxide at their disposal. For this purpose tubes

containing standard solution to which 0,5% $NaHCO_3$ and 0,005% Na_2S had been added, were inoculated and placed in an "anaerobic jar". The jar was then filled with an atmosphere of hydrogen with 5% carbon dioxide, free from oxygen, and was placed before an electric bulb at 30°C.

Already after a few days definite signs of development of the bacteria were present. As I was able to cultivate several generations in this way, it is certainly out of question, that the growth had taken place exclusively at the cost of the thiosulphate introduced with the first inoculation.

The 0,005% Na_2S was added to bring about a sufficiently reduced state of the medium in the tubes and was necessary to ensure a good start of the bacteria. If one calculates, how much organic matter could be synthesized with the amount of Na_2S present per culture tube, then — assuming a complete oxidation of the Na_2S tot Na_2SO_4 — this appears to amount to 0,5 mgr. at most. This organic matter corresponds with a quantity of bacteria, which is not perceptible in that dilution, of which I convinced myself by preparing such a suspension. It may be added that I always could observe a definite decrease in pressure of the atmosphere in the anaerobic jar after the bacteria had been growing in it for some time.

Further experiments will have to decide how much hydrogen and how much carbon dioxide is consumed in such growth experiments, and whether also under these conditions carbon dioxide really is the only acceptor which can be used by the *Thiorhodaceae*.

In connection with the hydrogen and carbon dioxide assimilation performed by the *Thiorhodaceae* without the aid of radiant energy, it seemed to be of interest to ascertain whether it would also be possible to cultivate the bacteria under such conditions. Organisms capable of growth at the expense of a hydrogen and carbon dioxide mixture have already been found before (see for instance SÖHNGEN 1906, STEPHENSON and STICKLAND 1933). To investigate this the anaerobic jars with tubes containing young cultures of bacteria in a H_2/CO_2 atmosphere, were put in an ordinary (dark) incubator at a temperature of 30°C. Without exception the bacteria ceased to develop. Even cultures in a Na-malate-Na-thiosulphate me-

dium brought in the hydrogen carbon dioxide atmosphere, refused to grow in the dark.

I did not investigate whether *Athiorhodaceae* too are able to grow in a hydrogen carbon dioxide atmosphere in the light. If so, this would be the first case in which these organisms can be cultivated in an inorganic medium. Even when they would require the presence of an organic substance under such conditions in the dark, this would be a remarkable fact, as hitherto *Athiorhodaceae* have never been cultivated in complete darkness under anaerobic conditions. The only instance I could find in the literature about the cultivation of *Athiorhodaceae* in a hydrogen atmosphere, was the following statement of MIGULA (1900): „Nach meinen Versuchen gedeiht *Spirillum rubrum* auf schrägem Agar auch in Wasserstoffatmosphäre vorzüglich, bildet aber ebenfalls keinen Farbstoff". Whether in this case the cultures were illuminated remains an open question.

I have also made a casual observation regarding a possible assimilation of hydrogen by green plants. Though BOUSSINGAULT (1868) and especially EWART (1896) have found that hydrogen and hydrogen carbon dioxide mixtures depressed the rate of carbon dioxide assimilation of *Chara* and *Elodea*, and even could inhibit this process, this might have been only due to a lack of oxygen. Therefore I have tried whether the green alga *Stichococcus bacillaris* could assimilate hydrogen in an atmosphere of air with 20% hydrogen, either in the dark or in the light. For this purpose a pure culture of this alga was cultivated in daylight in the solution used by EILERS (1926). The algae were suspended in a 0,05 molar carbon dioxide buffer mixture of 8 $KHCO_3$ against 2 K_2CO_3.

The result was negative; green plants do not seem to be able to derive from hydrogen gas the H-atoms necessary for the reduction of carbon dioxide.

In connection herewith I will remark, that it would be interesting to ascertain whether the green plants like the *Rhodobacteriales* are able to involve organic acids into the photochemical reduction of carbon dioxide.

The only fact established in this connection is that in gro-

wing cultures of green algae, organic compounds can disappear from the nutritive medium in the course of time. This may obviously be attributed to an oxidation of these compounds with oxygen (respiration). The possibility that the compounds in question will partly have acted as donators for the photochemical carbon dioxide reduction, is however not to be excluded. An indication for this could possibly be obtained by determining in how far in such experiments the assimilation quotient deviates from 1, provided the final product of the assimilation remains a carbohydrate.

§ 8. *Experiments with carbon monoxide and methane.*

For the sake of completeness I will mention some experiments carried out in order to establish whether carbon monoxide and methane are gases, which either in the dark or in the light, could be utilized by the purple sulphur bacteria. For this purpose the bacteria were suspended in the bicarbonate solution and in a carbon dioxide buffer mixture, while the gas phase in the manometer consisted of a mixture of nitrogen, carbon dioxide and 30% of the gas to be studied (table 13).

Neither in the dark, nor in the light, could I observe anything particular, whilst also the microscopic appearance of the bacteria at the end of the experiment was normal. Apparently carbon monoxide and methane are completely inert gases for the *Thiorhodaceae*, at least as far as can be judged from these few observations.

§ 9. *Assimilation of carbon dioxide with organic substances.*

Since the researches of MOLISCH (1907) on the *Athiorhodaceae* and of VAN NIEL (1931) on the *Thiorhodaceae*, it is known that both groups of organisms can assimilate the organic substances offered to them. MULLER (1933 [1]) has given experimental proof that the *Thiorhodaceae* also under these conditions use carbon dioxide as an acceptor. The organic compounds should undergo a series of dehydrogenations until they are converted into pyruvic acid, acetaldehyde or carbon dioxide. With a view to the great similarity in behaviour of *Thiorhodaceae* and *Athiorhodaceae* MULLER concluded that

TABLE 13.

The unsuitability of carbon monoxide or methane as assimilation substrates.

Experiment No.	Description of experimental conditions	Differential manometer no.	Gas phase in manometer vessel	Change of pressure in mm. Brodie per hour. in the dark	in the light
1.	Bacteria of strain **a**, cultivated in the peptone medium, suspended in 0,05 molar CO_2 buffer mixture of 9 parts $NaHCO_3$ and 1 part Na_2CO_3 in tap-water 2% NaCl, at 30°C. Pd black present in side-bulb.	I	70% N_2, 30% CH_4 and ± 0,4% CO_2	+ 2	— 1
2.	Bacteria of strain **d**, cultivated in the inorganic medium, starved during 20 h. at 36°C., suspended in 0,05 molar CO_2 buffer mixture of 8 $NaHCO_3$ and 2 Na_2CO_3 in tap-water with 2% NaCl at 30°C.	1	50% N_2, 50% CH_4 and ± 0,15% CO_2	0	— 1
3.	Bacteria of strain **d**, cultivated and treated as the bacteria in experiment no. 2, suspended in:				
	a. 0,1 molar CO_3 buffer mixture of 9 $NaHCO_3$ and Na_2CO_3 in tap-water 2%NaCl. Manometer no. 1.	1	80% N_2, 20% CO 0,4% CO_2	0	0
	b. tap-water 2% NaCl, with 0,5% $NaHCO_3$. Manometer no. 3.	3	75% N_2, 20% CO 5% CO_2	+ 25	— 23
	Temperature 30°C.				

in all probability the metabolism of both groups of organisms in a suitable organic medium under anaerobic conditions in the light will be the same.

The correctness of this assumption was then confirmed by experiments of GAFFRON (1933). In a later investigation (1934), however, this author made observations, which led him to the following conclusion: (l.c., p. 447) „Der Unterschied des Stoffwechsels von roten Schwefelbakterien *(Thiocystis)* und Purpurbakterien *(Rhodovibrio)* ist sehr deutlich. Gibt man zu einer Suspension von *Rhodovibrio* etwas buttersaures Natrium und belichtet, so werden für jedes Molekül Buttersäure 0,4 Moleküle Kohlensäure assimiliert, ausserdem wird die Carboxylgruppe reduziert. Macht man denselben Versuch mit *Thiocystis*, so geschieht gar nichts. Trotzdem wächst *Thiocystis* sehr gut in Medien, die statt Sulfid organische Verbindungen enthalten. Die Erklärung hierfür liegt in einer Reaktion, die von der Kohlensäure-assimilation unabhängig ist und im Dunkeln (anaerob) abläuft. Während *Rhodovibrio* Butyrat im Dunkeln unverändert lässt, benützt *Thiocystis* die organische Substanz zur Reduktion von Sulfaten. Hierbei entstehen Sulfide, Kohlensäure und organische Säuren. Die gebildeten Sulfide ermöglichen eine Assimilation von Kohlensäure wenn die Bakterien belichtet werden."

The experiments mentioned on p. 49 and p. 52 carried out by me in order to test this point, justify the conclusion that the explanation given by GAFFRON of the difference in behaviour of *Rhodovibrio* and *Thiocystis* in his experiments, is incorrect.

I had already made the observation, that the bacteria cultivated in peptone water indeed could use Na-malate as donator (table 14 No. 5 and 8), whereas the facts reported in § 2 of this Chapter show that the *Thiorhodaceae* very probably are also able to use as hydrogen donators the organic products formed in their dark metabolism. Hence I was very sceptical of GAFFRON's statement regarding the unsuitability of malate, glucose, a.o., to serve as hydrogen donators for the *Thiorhodaceae*.

When I repeated his experiments and used strain *d*, cultivated in an inorganic medium, it appeared that the assimila-

TABLE 14.

Carbon dioxide assimilation with organic substances.

Exp. No.	Description of experimental conditions.	D.M.	Compound added, either at the beginning, or in the second part of the experiment	CO_2 assimilation in mm^3 per hour. Control auto-assimilation	CO_2 assimilation in mm^3 per hour. Total assimilation	CO_2 assimilation on added comp., expressed in % of total CO_2 assimil.
1.	Bacteria of strain d, cultivated in the inorganic medium, suspended in tap-water 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/7% CO_2 at 35° C.	1	—	280	—	—
		3	0,3% Na-malate	—	310	10
2.	Conformable to experiment no. 1.	4	—	305	—	—
		3	0,3% Na-lactate	—	450	30
3.	Conformable to experiment no. 1.	3	0,1% acetaldehyde	—	440	70
		4	0,1% acetaldehyde later on	135	450	
4.	Conformable to experiment no. 1.	3	0,1% ethyl alcohol later on	250	255	0
5.	Conformable to experiment no. 1, except the use of bacteria cultivated in the peptone medium.	3	—	160	—	—
		1	0,2% Na-malate	—	330	50
6.	Conformable to experiment no. 1, except the use of bacteria cultivated in a medium with, 0,2% Na-malate and 0,7% $NaHCO_3$.	3	0,1% Na-malate later on	270	475	45
7.	Conformable to experiment no. 1, except the use of bacteria cultivated in a medium with 0,2% Na-butyrate and 0,7% $NaHCO_3$.	4	0,1% Na-butyrate later on	250	390	35
8.	Conformable to experiment no. 1, except the use of bacteria of strain 9, cultivated in the peptone medium.	1	0,2% Na-malate later on	160	530	70
		2	0,2% Na-malate later on	155	520	
9.	Conformable to experiment no. 1. (Representing the ordinary assimilation velocity with H_2S as a donator.)	3				
		1	H_2S solution	300	1300	77

tion with the organic donators mentioned was indeed very weak as compared with the assimilation with H_2S as donator[1]). Experiment no. 4 on table 14 shows that there is no question of a carbon dioxide assimilation with ethyl alcohol, since the assimilation was exactly equal to that in the control manometer. Sodium malate and sodium lactate, however, were assimilated unmistakebly, although very slowly (vide table 14 No. 1 and 2). Acetaldehyde caused a much stronger assimilation (table 14 No. 3.)

However, far stronger assimilation of sodium malate could be observed, when I used bacteria from the same strain *d*, which were cultivated in peptone water (table 14 No. 5) or in a Na-malate-bicarbonate medium (table 14 No. 6). It proved to be the same case with the assimilation of sodium butyrate by bacteria, cultivated in a sodium butyrate-bicarbonate medium. (table 14 no. 7.).

In these experiments concerning the influence of the "history" of the bacteria on the ability to use special organic substances as donators, it appeared that this ability was to a high degree dependent on the concentration in which these donators were added. In other words, the supply of these organic compounds to those parts of the cells in which the photochemical reaction occurred, was a limiting factor in the carbon dioxide assimilation, at least in those cells which were under the most favourable conditions of illumination. One therefore gets the impression, that the observed differences are caused by changes in the permeability as a result of the culture medium used.

The differences stated by GAFFRON (1934) between *Athiorhodaceae* and *Thiorhodaceae* thus appear to bear only a quantitative character. Particularly if one realizes that this investigator has compared *Athiorhodaceae,* cultivated in yeast water, with *Thiorhodaceae* cultivated in an inorganic medium, these differences lose their significance.

If one wishes to investigate the behaviour of *Thiorhodaceae* towards certain compounds, one should use organisms which

[1]) In the beginning, viz. in my paper in 1934, I even believed that under these conditions, the bacteria were unable to use organic hydrogen donators.

are adapted to grow in a pure culture in media containing the same compounds. Only then one can draw reliable conclusions from a negative result of such an experiment.

I am fully aware that still many experiments must be performed before a clear insight in these problems can be obtained. The experiments carried out only had an orientating character and aimed chiefly at testing the correctness of the assumption of GAFFRON (1934) regarding the essential difference between the *Athiorhodaceae* and the *Thiorhodaceae*.

§ 10. *The oxidation-reduction potential in suspensions of* THIORHODACEAE.

It is a well-known fact, that the nature of the metabolism of organisms is reflected in the oxidation-reduction potential occurring in the surrounding medium. For an extensive survey of the literature on this subject up to 1932 I refer to ELEMA (1932). This investigator was the first, who successfully tried to link up the nature of the predominating metabolic process

TABLE 15.

E_h in suspensions of strain a, *cultivated in a peptone medium.*

Exp. No.	Description of experimental conditions	E_h in the dark on three electrodes in m. Volt.	E_h in the light on the same electrodes in m. Volt.
1.	Bacteria of strain a, cultivated in the peptone medium, suspended in tap-water with 2% NaCl, 0,3% $NaHCO_3$ and 0,2% Na_2SO_3, in equilibrium with N_2/5% CO_2, temperature 30°C. pH 7,5. Light source was an electric bulb of 40 Watt burning on a distance of 10 cm from the suspension.	— 230 — 235 — 240	— 125 — 130 — 130
2.	Conformable to experiment No. 1.	— 225 — 230 — 235	— 140 — 135 — 135

with the value of the potential. For further publications I refer to: ELEMA, KLUYVER and VAN DALFSEN (1934); KLUYVER and HOOGERHEIDE (1934); KINGMA BOLTJES (1934); LIPMANN

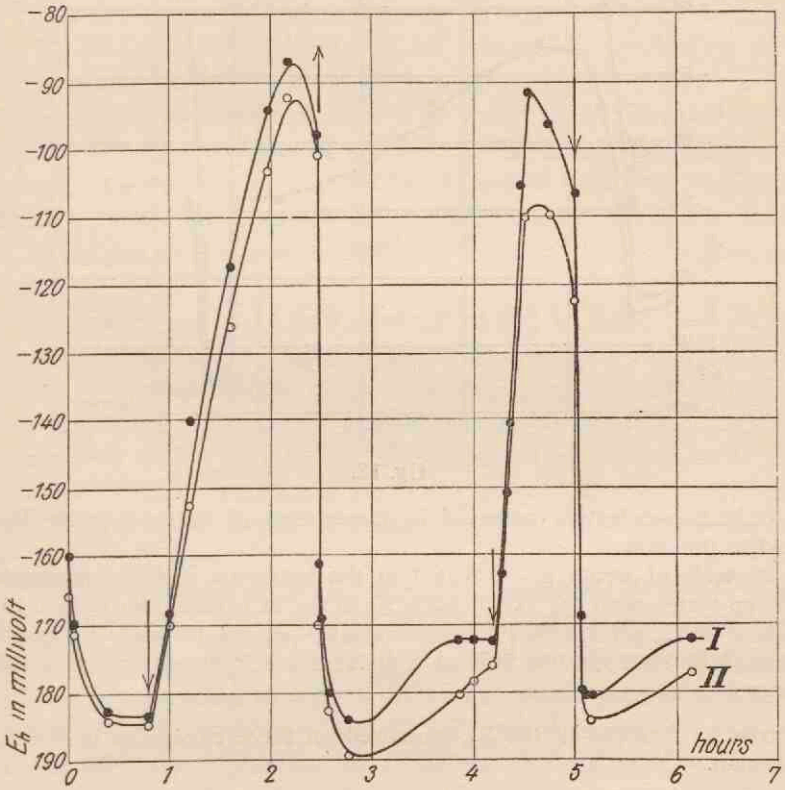

fig. 17.

Oxidation-reduction potential in suspensions of bacteria in the light and in the dark.

Bacteria of strain d, cultivated in the inorganic medium, suspended in tap-water with 2% NaCl, 0,7% $NaHCO_3$ and 0,2% Na_2SO_3, in equilibrium with N_2/5% CO_2 at 30°C, pH 8.1. N_2/5% CO_2 constantly passing through the suspension. A 40 Watt electric bulb at a distance of 10 cm serving as a light source.

Curve I. E_h measured on a platinum electrode.

Curve II. E_h measured on a gold electrode.

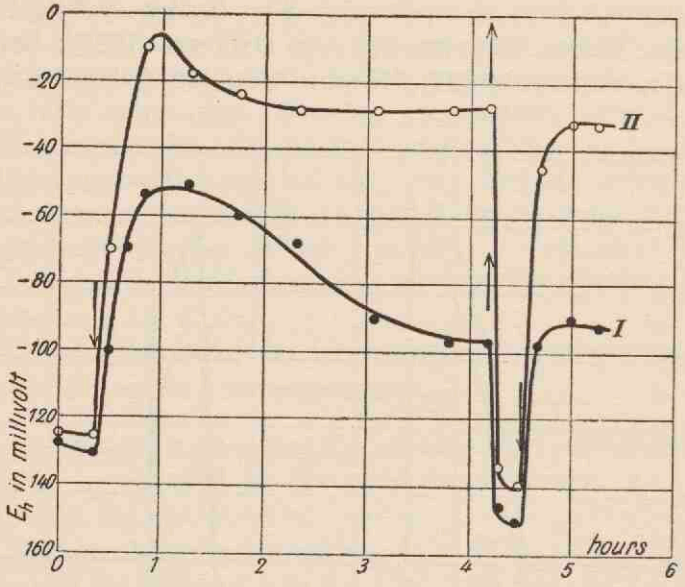

fig. 18.

Oxidation-reduction potential in suspensions of bacteria in the light and in the dark.

Bacteria of strain d, cultivated in the inorganic medium, suspended in tap-water with 2% NaCl, 0,3% $NaHCO_3$, in equilibrium with N_2/5% CO_2 at 30°C., pH 7,5. N_2/5% CO_2 constantly passing through the suspension. A 40 Watt electric bulb at a distance of 10 cm serving as a light source.

Curve I. Average of the E_h, measured on three electrodes in the suspension.

Curve II. Same arrangement as in I, except the addition of 0,2% Na_2SO_3 to the medium.

(1933, 1934).

I decided therefore to investigate, to which degree and in which sense the redoxpotential in suspensions of *Thiorhodaceae* is altered by the carbon dioxide assimilation, i.o.w. indirectly by exposure of these suspensions to light. The results of the experiments in question are represented in fig. 17 and 18 and in table 15. After being centrifuged and washed, the bacteria were suspended in the ordinary solution of ± 0,5% $NaHCO_3$ in tap-water with 2% sodium chloride, previously

brought in equilibrium with nitrogen containing 5% carbon dioxide. As a hydrogen donator 0,2% Na_2SO_3 or 0,2% $Na_2S_2O_3$ was added, as these compounds, in contrast to H_2S, do not directly influence the potential of the electrodes.

With the apparatus described by ELEMA (1932) I measured the E_h in these suspensions under anaerobic conditions at a temperature of 30°C. in the dark and in the light. During the whole experiment an oxygen-free mixture of nitrogen containing 5% carbon dioxide passed through the suspension. At the end the pH was measured by means of the glass electrode (ELEMA 1932). As a light source an ordinary electric bulb of 40 Watt, put at a distance of 10 cm from the bacterial suspension, was used.

The curves in fig. 17 and curve II of fig. 18 relate to suspensions to which a hydrogen donator had been added, curve I of fig. 18 represents the change of the E_h in the same suspension without a hydrogen donator. From the results it may be concluded, that under the conditions of these experiments the auto-assimilation and the carbon dioxide assimilation with the added hydrogen donators induced an increase of the E_h of $\pm$ 60 m Volt. and 120 m Volt. respectively.

The rise brought about by the auto-assimilation pretty soon changed into a considerable decrease during the exposure. These facts correspond with the course of the auto-assimilation as represented in fig. 9. After the short period of darkness in fig. 18 from 4,15 until 4,32 the difference between the E_h of the suspension of bacteria without and with hydrogen donator is still more striking. In consequence of the shortness of the dark period the bacteria did not have at their disposal products of autofermentation which could act as a hydrogen donator.

Table 15 represents the results of experiments with bacteria cultivated in a peptone medium. As might be inferred from the property of these bacteria to produce hydrogen the potential of these suspensions was much lower than in the experiments with bacteria from an inorganic medium. It may be remarked, that the measured E_h was still much higher than the E_h of the hydrogen electrode for atmospheric pressure and for the pH of the medium. This may be due to the

fact that the hydrogen tensions, which occurred in the bacterial suspensions, will have been extremely low, owing to the nitrogen 5% carbon dioxide mixture, which was continuously passing through the media. At these low tensions the E_h of the hydrogen electrode is accordingly higher; under the conditions of the experiment an E_h of 250 mV will correspond to the potential of a hydrogen electrode at a hydrogen tension of 10^{-6600} atmosphere. Moreover it must be emphasized that the E_h in the suspension cannot be anything else but the resultant of the different E_h characteristic of the various oxido-reduction processes, which together constitute the autofermentation, the assimilation and the hydrogen production.

In such a heterogeneous system like the cell-protoplasm, it will be possible that these reactions proceed independent of each other, and hence the E_h, occurring in the surrounding medium, is nothing else but a rough indication of the total metabolism. In those cases, where one special metabolic process, for instance alcoholic fermentation or denitrification, is strongly predominating, more absolute significance can be attached to the values of the E_h observed.

In view of the changes in the E_h in aqueous solutions of inorganic salts, observed by PINCUSSEN (1934) as a result of the exposure of these solutions to the light, it seemed advisable to try whether this phenomenon could have interfered in my experiments. However, in the absence of bacteria I could not observe any change in the E_h in the suspension liquid on exposure to the light.

Finally the possibility that changes of the temperature of the suspensions with bacteria, caused by the exposure to the light, might have induced the changes in the E_h observed, may practically be discarded. Firstly the changes of the E_h on exposure were too rapid and secondly the observed difference in the E_h of the suspensions with and without added hydrogen donators cannot be accounted for.

In a way suspensions of *Thiorhodaceae* behave therefore as a photo-half-cell, provided substrates for the assimilation are present.

§ 11. *Summary.*

1. The *Thiorhodaceae* are able to assimilate carbon dioxide with special, still unknown products of their own dark metabolism.
2. The results of my manometric experiments are in agreement with the theory of VAN NIEL (1931) concerning the assimilation of carbon dioxide with oxidizable sulpur compounds.
3. The manometric method is unsuitable to prove the hydrogen donator character of the sulphur stored in the cells of the *Thiorhodaceae* or produced during the oxidation of H_2S or $Na_2S_2O_3$; the assimilation with sulphur evidently proceeds too slowly.
4. The *Thiorhodaceae* assimilate both hydrogen and carbon dioxide in the light as well as in the dark; however, in the former case with much higher velocity.
5. The *Thiorhodaceae* are able to grow in the light in a wholly inorganic medium, devoid of an appreciable quantity of oxidizable sulphur compounds, provided they have gaseous hydrogen at their disposal.
6. The ability of the *Rhodobacteriales* to use organic substances as a hydrogen donator for the reduction of carbon dioxide is to a high degree dependent on the nature of the medium, in which the organisms were cultivated.
7. The *Thiorhodaceae* are unable to assimilate carbon monoxide or methane.
8. The oxidation-reduction potential in suspensions of *Thiorhodaceae* increases considerably on exposure of these suspensions to the light.

CHAPTER V.

ON ENERGY RELATIONS OF THE CARBON DIOXIDE ASSIMILATION OF THE THIORHODACEAE.

§ 1. *Introductory remarks.*

In this chapter I want to discuss the importance of a determination of the quantity of light required for the carbon dioxide assimilation by the *Thiorhodaceae*. Moreover I shall report about a series of experiments which I have undertaken for these determinations. I want to emphasize in advance, however, that these experiments have not led to ultimate results, they rather have a merely preliminary character. However, private circumstances forced me to bring my investigations to an end. Still it seems to me that it is justified to publish the results obtained until now, since they may be helpful to other investigators, who would feel inclined to aim at a more definite solution of the problem in question.

§ 2. *Outline of the problem.*

On discussing the energy relations of the carbon dioxide assimilation of the *Thiorhodaceae* it seems appropriate to give first attention to what is known about these relations in the corresponding process in green plants.

The thermodynamic side of this process has been very elaborately dealt with by STERN (1933), to whose survey may be referred here. The view held by STERN, BRIGGS (1929), WURMSER (1929) and which is also found in the monographs of SPOEHR (1926) and of STILES (1925) is as follows. The actual photochemical process can be represented by the general equation $CO_2 + H_2O \rightarrow CH_2O + O_2$. The increase in thermodynamic potential of this reaction is, when the actual concentrations of primary and final products in the cell are taken into consideration, about 114.600 cal. [1]) (C.f. STERN l.c.)

This corresponds to 2,7 quanta of the wave length 660 mμ, 2,3 quanta of 578 mμ and 1,8 quanta of 436 mμ (STERN l.c. p. 370). With a view to this the authors mentioned are of opinion, that one would expect to find three quanta of red or yellow light and two quanta of blue light sufficient for the reduction of one molecule of carbon dioxide by the green plants.

Now our knowledge of the actual energy efficiency of this reaction is very scant .The results of the older investigators endeavouring to arrive at a determination of this efficiency (I refer for this to SPOEHR 1926), have lost the greater part of their interest. The reasons for this lack of reliable data partly lie in the inaccuracy of the methods applied and are for the rest due to a neglect of the necessity to control all the factors which determine the rate of photosynthesis.

WARBURG and NEGELEIN (1923), however, approached the said problem in a new and refined manner. Their investigation on the carbon dioxide assimilation in thick suspensions of the alga *Chlorella* led to the conclusion, that 4,4 quanta of red and yellow and 5,1 quanta of blue light are required to attain the assimilation of one molecule of carbon dioxide.

Since the appearance of the fundamental publications of WARBURG and NEGELEIN, other attempts to arrive at an experimental solution of the problem in question have been made by WURMSER (1923, 1924, 1926, 1929), BRIGGS (1929), SCHMUCKER (1930) and BURNS (1933). Whilst SCHMUCKER comes to results, which are quite identical with those of WARBURG and NEGELEIN, the other authors found considerably higher values. However, even the numbers of WARBURG and of SCHMUCKER turn out higher (at least 50% for red and yellow light and 150% for blue light) than might be expected solely on the ground of the above mentioned energetic considerations. This low efficiency, so they argue, may be accounted for by assuming that not all quanta absorbed need have been photochemically active. Some quanta would have been

[1]) On the ground of measurements of the rH in assimilating cells, Wurmser (1926) comes, it is true, for the carbon dioxide reduction proper to only 43.800 cal. However, the starting point of his calculation is too much hypothetical to attach much value to this conclusion.

lost as fluorescence energy and other quanta would have been converted into heat, f. i. if the activated chlorophyll or chlorophyll-carbonic acid complex loses its energy in collisions with other than the chemically reactive molecules. Moreover other coloured and colourless photochemically inactive substances in the cell, as f.i. the carotines and protoplasm, may have been responsible for the absorption of a portion of the light.

Though thermodynamics can only tell us that a definite photochemical reaction *may* take place under uptake of such and so many quanta, the foregoing authors apparently take it for granted, that the only condition to be fulfilled is to supply the carbon dioxide molecule or the carbonic acid-chlorophyll-complex with the amount of energy thermodynamically required.

It is characteristic of these considerations that therein EINSTEIN's law of photochemical equivalence, according to which an equal number of quanta of various wave lengths produces the same photochemical effect, is neglected.

WARBURG and NEGELEIN (l.c.) have concluded from the results of their investigations that EINSTEIN's law holds also in the case of the photochemical carbon dioxide reduction. However, they did not find fully equal quantum numbers with different wave lengths, but they ascribe the differences observed to experimental complications and hence consider them as being unimportant and only apparent. Whilst these authors thus emphasize the probability of finding an equal number of quanta with different wave lengths, they are on the other hand relatively indifferent with respect to the absolute value of this number. They only strive to attain as high an energy efficiency of the carbon dioxide assimilation as possible.

This is due to the fact, that they do not try to visualize the internal mechanism of the undoubtedly complex photosynthetic process. We may conclude directly to this complexity from the extreme improbability that the photochemically active substance would be capable of gathering a number of quanta, before the reaction $CO_2 + H_2O \rightarrow CH_2O + O_2$ proceeds. From a physical point of view namely, the degree of probability for a molecule once activated by the uptake of

one quantum, to find the opportunity to absorb one or even two other quanta in addition, is exceedingly small, considering the short lifetimes of activated molecules in general (see f.i. BONHOEFFER and HARTECK, 1933)[1]).

From this it seems quite indispensable to study the internal mechanism of the carbon dioxide reduction. However, it is superfluous to enter into a detailed discussion of the theories existing thereabout. I refer to the monographs of HOLLUTA (1926) and of SPOEHR (1926) and to the publication of MULLER (1933[2]). For the question in consideration it will suffice to state that various of the theories proposed are characterized by the opinion that in reducing one carbon dioxide molecule several separate endothermic reactions have to proceed successively. This implies that in this line of thought several photochemical processes have to follow one another before a carbon dioxide molecule has been brought to the reduction level of a carbohydrate.

This conception underlies the theories of VAN NIEL and MULLER (1931) and of SHIBATA (1933). These investigators assume that in the carbon dioxide reduction process more or less stable intermediate stages are formed. VAN NIEL and MULLER have postulated formic acid as an intermediate product, whereas SHIBATA apparently assumes, that four more or less stable stages are passed through in the carbon dioxide reduction[2]). Which intermediate stages are involved, which pigments are responsible for the photochemical reactions, whether hydrogen atoms or carbon dioxide are activated thereby etc., is as yet of minor importance. The only essential point for the moment is that the mentioned investigators accept that four so-called photochemical primary processes[3]) are

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[1]) This view is corroborated by the experimental result, that at least with low light intensities, the velocity of the carbon dioxide assimilation is directly proportional to the intensity (I) of the absorbed light. If a simultaneous absorption of n quanta would occur, it would have been proportional to I^n (JAMES 1934).

[2]) This assumption is to a certain extent supported by the recent views regarding the rôle of free radicals in biochemical processes. C.f. WILLSTÄTTER and HABER (1931).

[3]) With photochemical primary process is meant the initial process wherein absorbed radiant energy is converted into chemical energy.

involved in the reduction of CO_2 to CH_2O.

The fact, that WARBURG and NEGELEIN (1923) and all other authors previously mentioned have never found a smaller number of quanta than just four to be sufficient for the carbon dioxide reduction, may be considered a material support for the above conceptions regarding the internal mechanism of the carbon dioxide reduction process. For as is known, the EINSTEIN law of photochemical equivalence requires light absorption only to take place in quanta.

So if we assume the presence of four photochemical primary processes in the carbon dioxide reduction, then this necessarily means that just four quanta are absorbed in this process, since, as was already mentioned, the idea of a simultaneous absorption of two or more quanta by the reacting molecule is to be rejected.

On summarizing the foregoing considerations we may conclude, that if we only take into account the requirements of thermodynamics, we must assume, that for the process of the carbon dioxide assimilation by the green plants three, or for special wave lengths two quanta, per molecule carbon dioxide will suffice. On the contrary the investigators, who assume that four photochemical primary processes are involved, have to accept that this quantum number has to amount to four. This means that an experimental determination of this number offers a way to test experimentally the correctness of these conceptions.

The trouble, however, is that the margin between the numbers of quanta postulated in both conceptions — three against four quanta — is rather small, whilst an experimental determination of this number, as will appear from the following paragraphs, is linked up with great difficulties and inaccuracies. Hence it is doubtful whether it is possible to come to a definite answer when using green plants for the experiments.

For this reason it seemed highly important to study this problem with the *Thiorhodaceae*. As has been set forth already in the Introduction, VAN NIEL has shown that it is characteristic of the photochemical carbon dioxide reduction with this group of bacteria, that not water, but oxidizable sulphur compounds or organic compounds act as hydrogen donators

in this process. This involves that the increase of the free energy in this case is much smaller than in the case of the green plants, because in the latter the carbon dioxide reduction includes the splitting of water under liberation of oxygen. In the case where hydrogen sulphide acts as a hydrogen donator, STERN calculates the increase of the free energy of the reaction $CO_2 + 2\ H_2S \rightarrow CH_2O + H_2O + 2\ S$ to only a good 17.000 cal. under standard conditions. This implies that in this case one quantum of visible or neighbouring ultrared light will already be amply sufficient to fulfil the thermodynamical requirements of this reaction. On the other hand VAN NIEL's experimental proof that in *Thiorhodaceae* four hydrogen atoms are involved in the photochemical assimilation of one carbon dioxide molecule, makes it extremely probable that, here as well as in the carbon dioxide assimilation of the green plants, four photochemical primary processes will occur. It then necessarily follows, that in both processes four quanta are required per molecule of carbon dioxide assimilated.

When the experiments would show, that indeed in spite of the very low energy requirements of the process, under no conditions a lower quantum number than four can be found, this might be considered as a strong indication, that in the reduction of each carbon dioxide molecule four photochemical primary processes are involved. This would mean, that prior to the introduction of each hydrogen atom in the carbon dioxide molecule a photochemical primary process is required. Of course such a result would strongly plead for an analogous situation in the carbon dioxide assimilation of green plants[1]).

If on the other hand we should find, that less than four quanta — f.i. one — would suffice, then this would mean in

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1) Whether the hydrogen atoms of the donators, or the carbon dioxide and its subsequent reduction stages are activated, is another question, which can be left out of consideration here. I refer in this connection to the different views of NOACK (1926), of VAN NIEL and MULLER (1931), of MULLER (1933[2]), of STOLL (1932), of SHIBATA (1933) and of DHAR (1934).

all probability that the carbon dioxide molecule would be the substrate of the photochemical primary process. The reduction of this molecule would then proceed in secondary consecutive reactions of non photochemical nature.

Furthermore I can point here to a second advantage of using in particular the *Thiorhodaceae* for the determination of the number of quanta required. As has been previously remarked, the determination in question is attended with great experimental difficulties, principally due to the fact that it is hardly to realize that all the light falling on the bacteria acts photochemically. If in the case where hydrogen sulphide is the donator we should find that no quantum number lower than four can be reached, we may still stick to the opinion that only one quantum has been photochemically active and that the others have been lost by absorption, by conversion into fluorescence energy etc. The advantage of operating with purple bacteria is, however, the fact that these organisms can use such greatly different hydrogen donators. Among these there will be such as to require, from an energetic point of view, the co-operation of more than one quantum[1]). If now in using these donators the number of quanta determined should likewise prove to be four — or if at least this number would not change with the nature of the different donators used — this would certainly speak in favour of the assumption that in all cases four photochemical primary processes are involved.

Finally it seemed that the determination of the number of quanta might be of importance to decide which of the two pigments of the *Thiorhodaceae* would be the photochemically active one. It is generally assumed that it would be the green pigment, named bacteriochlorine by MOLISCH (1907) and bacteriochlorophyll by NOACK and SCHNEIDER (1933). This assumption is supported by the results of SCHNEIDER (1934) and of FISCHER and HASENKAMP (1935), who have shown that this pigment is closely related to chlorophyll. VAN NIEL and MULLER (1931) are inclined to the view that the red

[1]) The carbon dioxide reduction, whereby $S(H_2O)$ is converted in three stages into H_2SO_4, means an increase of free energy of not less than 100.000 cal. One of these stages will therefore certainly require more than one quantum of ultra-red light.

pigment too acts as a photocatalyst, namely in the activation of the hydrogen atoms in the hydrogen donators. Apparently these authors accept that this red pigment is activated directly by the light absorbed; MULLER (1933[2]), later suggests that it may be activated indirectly by light absorbed by the bacteriochlorophyll.

The absorption spectra of both pigments being known (MOLISCH 1907, BUDER 1919), a comparison of the quantum number for different wave lengths probably will permit a decision in these problems.

The considerations given above will suffice to show the importance of a determination of the number of quanta involved in the carbon dioxide assimilation of the *Thiorhodaceae*, if possible with different hydrogen donators and for different wave lengths.

§ 3. *Discussion of methods.*

Having outlined in the preceding paragraph the problem to be investigated, I wish to discuss the experimental methods to be employed.

In the first place I will consider the methods formerly used in studying this problem in the carbon dioxide assimilation of the green plants and which of these methods would be most suitable for my purpose. It is clear that one must determine simultaneously, under conditions optimal for the utilization of the light supplied, the number of molecules of carbon dioxide absorbed by the bacteria and the number of quanta of a certain wave length absorbed by the photochemically active agent.

Which conditions can now be considered to be "optimal for the utilization of the light supplied"?

As has already been remarked, the complete process of the carbon dioxide assimilation must be seen as a chain of at least three different groups of processes. Firstly the diffusion of the carbon dioxide and of the hydrogen donators into the cell and the adsorption of these by the photochemically active surfaces, secondly one or more photochemical reac-

tions and finally a number of reactions in which the ultimate products of the photochemical process undergo further conversions. Now the velocity of a chain process is mainly determined by the slowest reaction participating in it. After BLACKMAN (1905) has applied this theory to the study of the physiology of plants it is known as the "principle of limiting factors". In particular the investigations on the carbon dioxide assimilation of the green plants by VAN DEN HONERT (1930), VAN DER PAAUW (1932), JAMES (1934) and others, have demonstrated that the photochemical part of the carbon dioxide assimilation process can be studied by investigating this process as a whole, provided light is the "limiting factor". This means that the velocity of the photochemical process should not be limited by a low rate of a preceding or of a subsequent physical or chemical process. These conditions are realized, if small changes of one of the external factors (except the light) do not influence the rate of assimilation at all. Changes in the intensity of light, however, ought to cause a proportional change in the assimilation velocity. For instance slight variations of the carbon dioxide concentration, of the pH, of the temperature, of the concentration of the hydrogen donators etc. should not exert any influence. Moreover the conditions of cultivation and the age of the organisms used must be optimal since also "internal conditions" can exert an influence upon the velocity of the carbon dioxide assimilation (VAN DER PAAUW 1932). So WARBURG and NEGELEIN (1923) point out that it is advantageous to use organisms that have been grown in feeble light.

For the determination of the number of carbon dioxide molecules assimilated I followed the method of WARBURG and NEGELEIN (1923) using the differential manometers previously mentioned. Other methods for the determination of the carbon dioxide assimilation mentioned in literature appeared to be either too inaccurate, or not applicable in the case of cell suspensions. It is evident that methods based on a determination of the quantity of carbohydrates, c.q. oxygen produced in the assimilation process, could not be applied either.

It seems necessary to give here due attention to objections raised against the method of WARBURG and NEGELEIN, as ap-

plied by me.

VAN DEN HONERT (1930) objected to the application of cell suspensions because of the unequal illumination of the cells, which is inevitable even when dilute suspensions are used. Sound as this argument may be under certain conditions, this unequal illumination does not interfere with the study of the photochemical part of the process, since the applied intensities of light have to be so small that even in the best illuminated cells the light intensity is the limiting factor in the carbon dioxide assimilation process. That it is possible to fulfil this requirement follows directly from the observation that under suitable conditions the assimilation is indeed proportional to the light intensity.

I also will mention two of the objections raised by BRIGGS (1929) to the procedure of WARBURG and NEGELEIN. In the first place BRIGGS is of opinion that the carbon dioxide assimilation measured was too small in comparison with the respiration, which predominated strongly. BRIGGS points out, that whenever the respiration would be influenced by light (see VAN DER PAAUW 1932) this would lead to a relatively great error in the calculation of carbon dioxide assimilation from the difference between the decrease of pressure in the light and in the dark. It seems difficult to overcome this objection, since the only way to increase the assimilation would be the use of higher light intensities, but in all probability this would accordingly have had a stronger influence on the respiration too and no improvement would result. Moreover this remedy would interfere with the necessity to use light which still is in minimum. Since all points to the probability that the autofermentation of the *Thiorhodaceae* is but very little or not at all influenced by the light intensities used (see page 44), I do not think this objection of any importance as far as my own experiments are concerned.

Another of the objections made by BRIGGS bears upon the intermittent illumination (10 min. light, 10 min. darkness) as applied by WARBURG and NEGELEIN. The establishment of the gas equilibrium between the suspension liquid and the gas phase would be so slow that, notwithstanding the vigorous shaking of the vessels, it would have levelled the observed differences

between the changes of pressure in the periods of light and of darkness. As I felt this objection more or less justified I have deviated on this point from the procedure of WARBURG and NEGELEIN. I always prolonged the periods of light and darkness to such an extent that the changes of pressure per unit of time in such a period became constant.

I now come to the question how to determine the number of quanta absorbed by the photochemically active agent. From a perusal of the literature I concluded that here also the method of WARBURG and NEGELEIN (1923) was the most suitable one. The methods applied by the greater majority of the other investigators for the measurement of the light absorbed, could not be used when cell suspensions instead of leaves or pieces of thallus were to be employed.

In the method of WARBURG and NEGELEIN (1923) practically all the light falling on the cell suspension is absorbed because very dense suspensions are used. If the part of the light that is absorbed by the suspension liquid and by the photochemically inactive parts of the cells, may be neglected, the "number of the quanta absorbed by the photochemicallly active agent" may be assumed to be equal to the number of quanta falling on the bacterial suspension. The amount of radiant energy which is actually lost cannot be determined, either by by experiment or by calculation; compare BRIGGS (1929) and SEYBOLD (1933). The latter estimated the fraction absorbed by the colourless parts of green leaves at 10% of the whole amount absorbed. This fraction will be considerably smaller in the case of a suspension of green algae as this approximates a suspension of chloroplasts. A further postulate is, that the fraction of the light that gets lost for the photochemical process by its conversion into heat, or into fluorescence energy, even after its absorption by the photochemically active agent, may be neglected. In consequence of all this one will always find a larger number of quanta per carbon dioxide molecule than is indeed used for its reduction. From the results of WARBURG and NEGELEIN it appears that this surplus has not necessarily to be large; these investigators have obtained results which are 50% higher than thermodynamically required and but 10% higher than is expected by some investigators

from a photochemical point of view (see p. 106). Even when taking into account a loss of 50% or 100%, it remains possible that for the carbon dioxide assimilation of the *Thiorhodaceae* one might obtain numbers less than four. For the reasons set forth in the foregoing paragraph this would already mean a satisfactory result.

As has been mentioned previously I used bacteria which did not contain sulphur, in order to make the error caused by the dispersion of the light by the cells as small as possible. Nevertheless the quantity of dispersed light appeared to be rather considerable. Consequently this amount has been measured in the way described and taken into account in the calculations made.

§ 4. *Experimental.*

In the preceding paragraph already a survey has been given of the factors to be taken into account if one wishes to study the carbon dioxide assimilation of certain organisms under conditions optimal for the most efficient utilization of radiant energy. I will now deal with the consequence of the application of these principles on the *Thiorhodaceae.*

In the first place it is desirable to use very young and vigorous bacteria, whilst furthermore the carbon dioxide assimilation has to be studied in conditions as much "physiological" as possible, i.o.w. in a medium which is more or less identical with the culture medium. Thus the following precautions were always taken. The pH of the suspension liquid was adjusted to that of the medium in the culture bottle from which the bacteria were collected; the concentrations of NaCl and $NaHCO_3$ used were made practically equal to those in the culture media, whilst the temperature in the experiment was the same as that in the light cabinet in which the bacteria had been grown.

As I had already observed, that a prolonged stay in the WARBURG-vessels had an unfavourable influence upon the assimilatory ability of the bacteria, the duration of the experiments was kept as short as possible.

With a view to the low viability of bacteria from organic

culture media (c.f. pag. 54) it is self-evident that for the experiments in this Chapter I only used bacteria cultivated in the inorganic culture medium.

Whilst for the experiments mentioned in Chapter IV I could use bacteria, which had been subjected to a "starvation period", this was no longer allowed here, since the assimilatory capacity as a rule was appreciably diminished by this treatment. The consequence of the use of fresh bacteria, however, was, that the auto-assimilation began to play a much more prominent part. This was still promoted by the fact that I had to use dense suspensions of bacteria ($\pm$ 13 mm^3 bacteria per cm^3). In consequence not only the auto-assimilation came to the front, but also the carbon dioxide production became extremely high. This was the more troublesome, since the assimilation velocity had to be kept low. It was namely imperative to apply low light intensities only (compare the considerations on page 110). Preliminary experiments showed that only with very low light intensities a region was found in which there was a direct proportion between the light intensity and the rate of assimilation. It may be remarked, that in these circumstances photosynthesis does not manifest itself by a decrease of pressure but by a slower rate of increase.

After having ascertained, that the use of lower temperatures, as e.g. 25°C., did not alter the relation between the value of the autofermentation and that of the assimilation in a favourable sense, I performed all further experiments at 35°C. This also was the temperature at which the bacteria were cultivated.

With some experiments I first proved that lowering the carbon dioxide concentration in the gaseous phase from 5% to 3% did not have the least influence upon the rate of assimilation, provided the pH was kept constant by a suitable lowering of the bicarbonate concentration. From this result it may be concluded that in my experiments neither the concentration of the carbon dioxide nor that of the carbonate or bicarbonate ions have been limiting factors.

The accumulation of unfavourable factors mentioned above, forced me to give up the study of a number of questions mentioned in § 2 of this Chapter. For instance it proved to

be impossible, to make the bacteria use exclusively the hydrogen donators added, since assimilation only on the donators produced in the autofermentation proceeded with the same rate as after addition of hydrogen donators to the medium. Even an addition of H_2S did not cause the least acceleration of the assimilation velocity. Yet as has been previously shown, H_2S was the donator which caused a more rapid assimilation than whatever other oxidizable sulphur compound or organic substance. Of course it remains possible, that the bacteria will have used H_2S besides the self-produced hydrogen donators because this compound was present in excess. However, a definite answer as to the relative quantities of H_2S and of fermentation products used as donators cannot be given.

In these circumstances I was forced to use the unknown self-produced substances as hydrogen donators and to determine the number of quanta required for this reaction with an unknown reactant. Yet I did not feel this a reason to give up its determination. For a first approximation it seems of secondary importance from which donators the hydrogen atoms necessary for the reduction of the carbon dioxide are obtained. With a view to the efficient utilization of the radiant energy during the auto-assimilation, which was apparent from the impossibility to accelerate the carbon dioxide assimilation by adding H_2S, one may conclude that for the reduction of a carbon dioxide molecule certainly no more quanta will be necessary than if H_2S had acted as a hydrogen donator. This implies, that also with the auto-assimilation one quantum of the visible or neighbouring infra-red light already will be amply sufficient to meet the thermodynamic demands of this reaction. In view of a further answering of the questions posed, it is to be regretted that for the reason mentioned above, I was incapable to test different hydrogen donators.

I wish to give a more detailed description of the experiments than has hitherto been done with a view to the far-reaching conclusions, which might be drawn from them.

Young cultures in the inorganic medium were centrifuged and the bacteria washed and suspended in the ordinary oxygen-free 2% salt solution with about 0,5% Na-bicarbonate, in

equilibrium with nitrogen containing 5% carbon dioxide. The suspension came into a manometer vessel (vide p. 24) coated with silver on the outside (bottom excepted) and containing the same gas mixture. The filled manometer was placed into the water bath. The velocity of the shaking of the manometer was such that a permanent equilibrium between the suspension and the gas phase was maintained, which could easily be controlled by varying the speed of rotation. The number of turns in my experiments was usually 500—600 per minute. As soon as the increase of pressure per unit of time, due to the production of carbon dioxide by autofermentation, had become constant, I calculated how many mm^3 carbon dioxide were produced per hour. Then the suspension was exposed to monochromatic light of known intensity and when the change in pressure per unit of time had grown constant, (usually after 20 min.) the increase in pressure per unit of time was determined again. Usually the carbon dioxide production in the dark was determined once more and thereupon the suspension was illuminated again, but now with a lower light intensity, in order to control whether the light was indeed the limiting factor.

The quantity of carbon dioxide, which was assimilated per unit of time and which is mentioned in table 16, thus was calculated in the assumption, that the difference of the increase of pressure per unit of time in the dark and in the light quantitatively was due to the assimilation of carbon dioxide. A possible formation or disappearance of acids was thus neglected (see p. 69). Moreover a slight error has been introduced in the calculation of the number of mm^3 carbon dioxide taken up, since vessel constants were used which had been calculated in the assumption that the solubility of carbon dioxide in the suspension liquid did not differ from that in distilled water.

Actually this is not quite correct, since the solubility of CO_2 in water is reduced a little by the addition of 2% NaCl. As is mentioned on p. 34, the error due to this cause can be estimated to be 2%, i.o.w. the figures given for carbon dioxide production or uptake are all 2% too high.

Finally a part of the carbon dioxide assimilation had to be ascribed to "false light", not measured with the spectral

pyrometer. As explained on p. 26, this additional assimilation was determined by means of special dye solutions or coloured glasses. The sodium light used contained admixtures apparently causing an assimilation, which amounted to ± 8% of the total assimilation.

The necessary corrections being introduced, I knew, how many molecules carbon dioxide were assimilated by the bacterial suspensions under conditions, which were optimal for a most efficient utilization of the radiant energy.

It only remained to determine how many light quanta were absorbed by the suspension per unit of time. I determined the intensity of the light, which fell upon the suspension, by means of the spectral pyrometer as described on p. 27. This enabled me to measure the intensity of the light in ergs/cm²/sec. That part of the light, which was reflected by the suspension, was determined after each experiment, using the method, described on pag. 29. The light transmitted by the bacteria could be safely neglected, of which I convinced myself by means of the spectral pyrometer. Moreover in the experiment itself the greater part of this light was reflected again in the suspension by the silver coating.

As already mentioned before (p. 25) the determination of the light intensity before and after the experiment, always showed that the sodium lamp burned with a very constant intensity. This, however, was not the case with the mercury vapour lamp; variations of 5 to 10% in light intensity were not rare. In connection with the very low photochemical efficiency of the monochromatic yellow, green and blue light furnished by the mercury lamp, it seemed superfluous to take special precautions to prevent these variations. Consequently the data in table 16 No. 4—7 are less accurate than those which refer to the intensity of the sodium light.

Assuming that the light had been constant during the entire period of illumination and after the introduction of the necessary corrections, I could calculate how many ergs had been absorbed by the suspension per unit of time. This value was divided by the number of ergs of one quant of the wave length used, and thus the number of quanta absorbed per

TABLE 16.

Number of light quanta of different wave lengths required for the assimilation of one carbon dioxide molecule.

Experiment No.	Description of experimental conditions.	Wave length in mμ	Radiant energy absorbed in erg/cm²/sec.	CO_2 assimilation in mm³ p. hour.	Number of quanta absorbed per molec. CO_2
1.	Bacteria of strain d, cultivated in the inorganic medium, at 35°C., suspended in tap-water 2% NaCl, 0,5% $NaHCO_3$, in equilibrium with N_2/5% CO_2, temperature 35°C.	589	4050	320	7,3
2.	Conformable to experiment No. 1.	589	4050 2250 1550	345 190 140	6,9 6,9 6,6
3.	Conformable to experiment No. 1.	589	2200 1500	175 120	7,4 7,4
4.	Conformable to experiment No. 1.	578	± 5000 ± 2500	140 70	± 20 ± 20
5.	Conformable to experiment No. 1.	578	± 6500	140	± 25
6.	Conformable to experiment No. 1.	546	± 6000	60	± 55
7.	Conformable to experiment No. 1.	546 436	± 6000 ± 3000	50 20	± 60 ± 60

unit of time was obtained[1]). This was divided by the number of molecules of carbon dioxide assimilated per unit of time; the quotient gave the value of the number of quanta absorbed by the suspension per molecule carbon dioxide assimilated.

§ 5. *Discussion of the results obtained.*

A consideration of the results given in table 16 shows in the first place that the number of quanta found to be necessary for the reduction of one carbon dioxide molecule varies a good deal. It is impossible, that this should be caused by a different absorption of the light by the suspension liquid, or by the "colourless" parts of the protoplasm. In that case also WARBURG and NEGELEIN (1923) ought to have found with the same method similar deviations in the assimilation of carbon dioxide by *Chlorella*. Obviously the cause of these differences lies in the light absorption by the bacterio-erythrine, the red pigment present in the bacteria used.

A comparison of the absorption spectra of the green and the red pigment of the *Thiorhodaceae* as given by MOLISCH (1907) and BUDER (1919) shows that the wave lengths with low assimilatory efficiency for the greater part will have been absorbed by the bacterio-erythrine.

Regarding the slight difference in wave length of the sodium light (589 mμ) and the yellow band of the mercury lamp (578 mμ) the difference in quantum numbers in these cases is surprisingly large. It seems probable that the explanation of this must be found in the following. In the region in question the bacteriochlorophyll shows a very distinct absorption band, which, however, ends abruptly between 589 and 578 mμ. This implies that at 578 mμ a much larger portion of the light will be absorbed by the red pigment, the more so, because this pigment has a strong absorption band in this region.

These facts justify the conclusion that the light absorbed

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1) $h\nu = 3{,}33 \times 10^{-12}$ ergs for 589 mμ
„ $= 3{,}40 \times 10^{-12}$ „ „ 578 „
„ $= 3{,}62 \times 10^{-12}$ „ „ 546 „
„ $= 4{,}53 \times 10^{-12}$ „ „ 436 „

by the bacterio-erythrine is lost for the carbon dioxide assimilation, at least with the hydrogen donators at issue (e.g. unknown metabolic products, H_2S, Na_2SO_3).

The number of quanta of the wave length 589 mμ needed for the assimilation of one molecule of carbon dioxide by the suspension, is many times higher than might be expected on account of exclusively thermodynamical or photochemical considerations. However, it seems probable that also from this wave length a considerable part has been absorbed by the red pigment. In order to make an estimation of this, I extracted in the simple way as indicated by MOLISCH (1907) the green pigment of a given quantity of bacteria, viz. with absolute ethyl alcohol. Thereupon I dissolved the red pigment from the same bacteria in an equal quantity of chloroform. The extinction coefficients of the green and of the red solution for the wave length 589 mμ which I determined with the aid of the spectral pyrometer, were in the ratio 1,2 : 1. As undoubtedly the separation of the two pigments has not been complete, in reality this ratio will be higher. Furthermore the absorption bands in solutions of alcohol and chloroform may possibly be shifted a little as compared with the bands in the living bacteria.

On the assumption that the light absorbed by the red pigment is lost for the assimilation and accepting furthermore that the ratio 1,2 : 1 can be applied for the conditions in the cell also, the number of quanta absorbed by the green pigment is 3,6—4,4. Considering the incomplete separation of the pigments by the method of extraction used, the number of quanta absorbed by the green pigments in the cell, will be somewhat higher.

It seems to me, that these results point to the necessity of four quanta per molecule carbon dioxide and certainly one may conclude from it, that the number of one quantum per molecule carbon dioxide, required thermodynamically, is entirely insufficient.

Further experiments, however, will be necessary before definite and reliable conclusions may be drawn. Then it will be advisable to determine more accurately the absorption spectra of both pigments of the purple bacteria. If possible

the assimilation experiments should be made with those wave lengths, which only are absorbed by the bacteriochlorophyll. As the absorption band of this pigment, near the D-line, is the only one in the visible spectrum and since the red pigment apparently has a not negligible share in the absorption in this region, it is probable, that one will have to have recourse to monochromatic infra-red light.[1])

Furthermore it does not seem impossible that by using other bacteria, e.g. *Athiorhodaceae* or other strains of *Thiorhodaceae*, one will not be hindered by the auto-assimilation and the autofermentation to such an extent as in my experiments. If so, different hydrogen donators could be applied.

Finally, judging from our — it is true, limited — knowledge of the metabolism of the *green* sulphur bacteria, it must have great advantages, to study the problem under discussion on these organisms. In this case one gets entirely rid of the disturbing influence of the absorption of light by the bacterio-erythrine. Furthermore in all probability there will be no question of auto-assimilation in this case, since up to the present hydrogen sulphide has appeared to be the only suitable hydrogen donator for the carbon dioxide assimilation of these organisms. It seems, however, difficult to cultivate them.

Addendum.

Growth experiments in sodium light.

In a preliminary communication (1934), I came to the conclusion that, in view of the good growth of *Thiorhodaceae* in sodium light with sodium thiosulphate as a hydrogen donator, the light absorbed by the bacterio-erythrine is lost for the carbon dioxide assimilation of the *Thiorhodaceae*. This was contradictory to the hypothesis of VAN NIEL and MULLER (1931) according to which the activation of the bacterio-erythrine by light absorption would be essential for the activation of the hydrogen atoms in the donators.

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1) Perhaps it is possible to use a potassium or caesium lamp for this purpose.

My conclusion was based on the assumption, that sodium light would practically only be absorbed by the bacteriochlorophyll. This conclusion seemed warranted by the absorption spectra of these pigments, as given by MOLISCH (1907) and by BUDER (1919). Since, however, we have seen that the absorption of sodium light by the bacterio-erythrine cannot be neglected at all, the mentioned growth experiments fail to uphold the conclusion in question.

On p. 119, however, it is set forth that there are other reasons why it seems extremely improbable that bacterio-erythrine is active as a photocatalyst in the carbon dioxide assimilation of the *Thiorhodaceae*.

Of course it remains possible that — as was suggested by MULLER (1933[2]) — an indirect activation of the bacterio-erythrine takes place by radiant energy absorbed by the bacteriochlorophyll.

§ 6. *Summary.*

In this Chapter some theoretical considerations have been given about the importance of the determination of the number of quanta in the carbon dioxide assimilation in general and of this number in the carbon dioxide assimilation of the *Thiorhodaceae* in particular. The experimental results obtained give strong indications that the light absorbed by the bacterio-erythrine under the conditions of my experiments is lost for the carbon dioxide assimilation. Furthermore it seems probable that also for the carbon dioxide assimilation with the most suitable hydrogen donators not less than four quanta are required for the reduction of one molecule of carbon dioxide.

GENERAL SUMMARY.

A study was made of the metabolism of the carbon dioxide assimilation of the *Thiorhodaceae* or purple sulphur bacteria, with the aid of the manometric method.

It appeared that these anaerobic bacteria can only maintain themselves in periods of darkness on a fermentation of an — as yet unknown — reserve food substance. The production of carbon dioxide and acids (under special conditions also of hydrogen) could be demonstrated.

The conceptions of VAN NIEL (1931) and MULLER (1933[1]) regarding the metabolism of purple sulphur bacteria in certain inorganic and organic media were fully corroborated by the results of the manometric method. In addition it was shown that as yet unknown products of the autofermentation and also gaseous hydrogen can also act as hydrogen donators for the carbon dioxide assimilation. Whereas carbon dioxide assimilation with hydrogen as a donator occurred in the dark as well as in the light, growth occurred only in the latter case.

The carbon dioxide assimilation, caused by the illumination of bacterial suspensions, was reflected in the oxidation-reduction potential occurring in the medium.

Experiments with monochromatic light of known intensity showed that the light, absorbed by the red pigment of the purple sulphur bacteria, is of no use for the carbon dioxide assimilation.

It has been found that more than one quantum — probably four quanta — have to be absorbed by the green pigment for the assimilation of each carbon dioxide molecule. The importance of a further extension of these experiments for our insight into the mechanism of the photochemical carbon dioxide reduction in general was stressed.

For a more extensive survey of the results obtained the reader is referred to p. 62 and p. 101.

The investigations were carried out in the *Microbiological Laboratory, Technical University, Delft.* I am very much indebted to *Prof. Dr. Ir. A. J. Kluyver* for his thorough and indispensable help and criticism.

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STELLINGEN.

I.

In de purperen zwavel bacteriën speelt zich bij voortduring een zelfgisting af; de eindproducten hiervan worden in het licht weer geassimileerd.

II.

De meening van GAFFRON, dat de *Thiorhodaceae* in staat zijn in het donker sulfaten te reduceeren, is onjuist.

H. Gaffron, Biochem. Zschr. *269*, 447, 1934.

III.

De assimilatie van een koolzuurmolecuul door de *Thiorhodaceae* vereischt naar alle waarschijnlijkheid de absorptie van vier licht-quanten door het bacteriochlorophyll.

IV.

De rangschikking van de micellen in de celwanden van *Phycomyces Blakesleeanus* geschiedt onder den invloed van de protoplasmastrooming.

V.

De onderzoekingen van WILLSTÄTTER en ROHDEWALD over de amylasen der leucocyten brengen een sterk element van onzekerheid in de uitkomsten van een groot deel der tot dusver gepubliceerde enzymologische onderzoekingen.

R. Willstätter und *M. Rohdewald*, Zeitschr. f. physiol. Chem. *221*, 13, 1933.

VI.

Bij de bepaling der enzymatische activiteit dient men ook rekening te houden met de oxydatie-reductiepotentiaal van het medium.

VII.

De micro-pedologische bestudeering van in den bodem voorkomende ziekteverwekkende organismen is van grooter belang voor de phytopathologie dan een onderzoek van de physiologie dier organismen in reincultuur.

VIII.

De samenstelling van de recente flora van Zuid-Amerika, Afrika, Australië en Nieuw-Zeeland is tot nu toe slechts te verklaren volgens het beginsel van de theorie van *Wegener* over het ontstaan der continenten.

IX.

Het gehalte aan kiemen van *Azotobacter chroococcum* is geen maatstaf voor de vruchtbaarheid van den bodem.

X.

Naar alle waarschijnlijkheid wordt de cacao-fermentatie ingeleid door *Bacterium aerogenes*.

XI.

Het met meer succes volgen van het hooger onderwijs in de natuurwetenschappen door abituriënten van het gymnasium in vergelijking met die der Hoogere Burgerscholen wettigt nog niet de conclusie, dat het gymnasium de meest geschikte voor-opleiding voor de natuurwetenschappelijke studie aan de universiteiten biedt.