# On the metabolism of the purple sulphur bacteria in organic media

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PROEFSCHRIFT

TER VERKRIJGING VAN DEN GRAAD VAN DOCTOR IN DE WIS- EN NATUURKUNDE AAN DE RIJKSUNIVERSITEIT TE UTRECHT OP GEZAG VAN DEN RECTOR MAGNIFICUS, DR. C. G. N. DE VOOYS, 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 MAANDAG 30 JANUARI 1933 DES NAMIDDAGS TE 4 UUR

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AAN MIJN OUDERS
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## Introduction.

Though it has long been known that the *purple sulphur bacteria* are able to develop autotrophically in a mineral medium containing hydrogen sulphide, many points concerning their metabolism remained difficult to explain.

*Winogradsky* (43) concluded from his experiments that $H_2S$ was indispensable for the development and was oxidized to $H_2SO_4$, but also that these bacteria had to be considered as strongly anaerobic. The investigations of *Engelmann* (10), *Buder* (7) and others showed that radiant energy was very stimulating, if not necessary for their development. Finally *Bavendamm* (3) stated that both $H_2S$ and light were indispensable, but failed to give an explanation of this phenomenon.

It was not before *van Niel*'s important contribution to the subject (28) appeared, that we got a clear insight into the metabolism of these bacteria as it occurs in a mineral medium containing $H_2S$ and $CO_2$[1]. From his experiments *van Niel* concluded that, under uptake of radiant energy, the $CO_2$ is reduced by the $H_2S$ according to the equation:

$$CO_2 + 2\,H_2S \rightarrow CH_2O + 2\,S + H_2O.$$

The sulphur appears as an intermediate product inside or outside the cells and in its turn reduces $CO_2$, thereby being oxidized to $H_2SO_4$, so that we get the final equation:

$$2\,CO_2 + H_2S + 2\,H_2O \rightarrow 2\,CH_2O\,^2 + H_2SO_4.$$

*Van Niel* further pointed out that, in agreement with the theory of *Kluyver* and *Donker* (19) about metabolic processes in general, we may consider this process as a dehydrogenation of $H_2S$ in subsequent steps[3] to $H_2SO_4$ with $CO_2$ as the hydrogen acceptor. Comparing the equations given above with that of the photosynthesis of the green plants:

$$CO_2 + 2\,H_2O \rightarrow CH_2O + O_2 + H_2O$$

he arrived at the conclusion that the metabolism of the *purple sulphur bacteria* in a mineral medium containing $H_2S$ and $CO_2$ has to be considered as a photosynthetic process, representing a special case of the general equation of photosynthesis:

$$CO_2 + 2\,H_2A \rightarrow C_2HO + 2\,A + H_2O.$$

*Van Niel* also showed that not only $H_2S$ is dehydrogenated to $H_2SO_4$ (with sulphur as an intermediate product), but that, in the absence of $H_2S$, sulphur, sulphite and thiosulphate can serve as hydrogen donators in the photosynthetic process, thereby likewise being converted into $H_2SO_4$.

Moreover *van Niel* carried out some experiments with media containing organic substances, which settled an old controversy, as will appear from the following survey of the literature.

*Winogradsky* (43) added to his culture medium 0.005—0.01 % Ca-butyrate, Ca-formiate or Na-acetate and obtained no better results with very dilute peptone or meat extract. On account of the fact that the bacteria in his cultures did not develop without $H_2S$, he considered them as "An-

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[1] My own work is a direct continuation of that of *van Niel*. In these pages I have given a brief review of his most important results and conclusions, but for a good understanding of my own work the reader is recommended first to make himself familiar with *van Niel*'s paper.

[2] In these equations $CH_2O$ denotes the primary product of photosynthesis from which all organic cell constituents are derived.

[3] $H_2S$ to S, $S.H_2O$ to SO, $H_2SO_2$ to $SO_2$, $H_2SO_3$ to $SO_3$.

orgoxydanten", i. e. as organisms which derive their energy from the oxidation of inorganic compounds (in casu $H_2S$) and emphasized that they needed very little organic matter. Since at that time he had not yet got the idea of the carbon autotrophy of the *purple sulphur bacteria*, he naturally considered these small amounts of organic matter as necessary for their development.

*Molisch* (24) held the opinion that organic matter in relatively large quantities was absolutely indispensable, but based his conclusion chiefly upon experiments with *Athiorhodaceae*, which can not oxidize $H_2S$ and therefore can not live autotrophically. He mentioned however that a typical, sulphur storing *Chromatium* developed very well in a peptone-dextrine medium without $H_2S$.

In connection with the favorable influence of light on the development *Molisch* expressed the opinion that (l. c. p. 91) „zwischen der Assimilation der organischen Substanz, dem Lichte und den Farbstoffen irgendein Zusammenhang besteht", and (p. 92) „daß die Ernährungsversuche mit *Purpurbakterien* uns mit einer neuen Art von Photosynthese bekannt gemacht haben, bei der organische Substanz im Lichte assimiliert wird"

*Buder* (7) considered the *purple sulphur bacteria* as obligately autotrophic organisms, which do not need any organic matter for their development, but build up their cell material exclusively from $CO_2$. He refuted the idea that the typical *purple sulphur bacteria* will be able to grow in media containing organic substances in the absence of $H_2S$. He mentioned however the possibility that intermediate forms between *Thiorhodaceae* and *Athiorhodaceae* may exist which are able to develop both autotrophically and heterotrophically. *Bavendamm* (3) shared *Buder*'s opinions.

*Van Niel* however made the remarkable observation that his strains, most of which undoubtedly belong to the typical *purple sulphur bacteria*, are able to grow in a medium *devoid of oxidizable sulphur compounds*, but containing leucomethylene blue or reduced indigo carmine. In the course of the metabolism these leuco-dyes were dehydrogenated to the corresponding dyes which further remained intact. Moreover *van Niel* found that a good development of all his purple strains took place in more normal organic media, in the absence of $H_2S$. Good results were obtained with media containing peptone, yeast extract, Na-lactate or Na-pyruvate, whereas glucose permitted only a scanty development. As was the case in mineral media containing oxidizable sulphur compounds, growth only occurred under anaerobic conditions in the light, whereas in the dark or in contact with the air no development was observed.

Lactic and pyruvic acids — in contrast with peptone, yeast extract and leuco-dyes — are simple organic substances. Therefore it seemed possible to carry out a quantitative chemical investigation of the metabolism of the *purple sulphur bacteria* in media free from $H_2S$, but containing these or other simple organic compounds.

The remarkable point is again that growth in these media only occurs in the light. It is evident that this necessity of radiant energy for organisms thriving on organic substances constitutes a most interesting problem. Moreover one might expect that a more profound

knowledge of this remarkable metabolism would contribute to our insight into the metabolism of the *Athiorhodaceae*. When connected with *van Niel*'s data regarding the metabolism of the *purple sulphur bacteria* in mineral media, such knowledge might even deepen our insight into photosynthesis in general [cf. *van Niel* and *Muller* (29)].

## Chapter I.

## Material and technique.

### *§ 1. Material and methods of culture employed.*

All my experiments were carried out with pure cultures, which were obtained by the method described by *van Niel* (28, p. 22—24). I used the following strains of *van Niel*: nrs. 1, 4, 7, 9, 12 and 19 (28, p. 27) and three strains, named *a*, *b* and *c*, which I had brought from Holland. Strain *a* was obtained by Dr. *J. B. van der Lek* from an enrichment culture started with mud from the Zuiderzee near the island of Wieringen; strains *b* and *c* were obtained by myself as infections from the air in bottles with a $NaHCO_3$—$Na_2S$ mineral medium in the laboratory for microbiology of the Technical University at Delft.

Strains *a*, *b* and *c* are very similar to each other and to strains 9 and 19; they all belong to *van Niel*'s *Chromatium* type. Strains 4 and 12 represent his *Thiocystis* type and strains 1 and 7 his *Pseudomonas* type. For the greater majority of my experiments I have taken one representative of each of the three types.

The pure cultures were kept in glass-stoppered bottles of 30 ccm with a layer of paraffine oil on top of the medium, which filled up the remaining part of the bottle completely. Contaminations which might fall on the bottle can not enter through the paraffine oil between neck and stopper and are killed before opening the bottle by heating the neck in a burner flame.

A mineral $Na_2S$—$NaHCO_3$ medium as well as yeast extract and Na-pyruvate media were used. The mineral medium had the following composition:

| | |
|---|---|
| $NaCl$ | 2 % |
| $(NH_4)_2SO_4$ | 0.1 % |
| $K_2HPO_4$ | 0.05 % |
| $MgSO_4$ | 0.02 % |
| $NaHCO_3$ | 0.5 % |

with the addition of 0.1 % $Na_2S$ . 9 aq. for strains *a*, *b*, *c*, 1 and 7 and 0.05 % $Na_2S$ . 9 aq. for strains 9, 19, 4 and 12.

$NaHCO_3$ and $Na_2S$ have to be added after sterilisation, because they lose part of their $CO_2$ and $H_2S$ when heated in solution, on account of hydrolysis. The $NaHCO_3$ was dissolved and sterilised separately by filtration

through a *Seitz* filter; the $Na_2S$ was added from a stock solution of 10% $Na_2S$ . 9 aq. which can be sterilised in an autoclave without loss of $H_2S$.

The $p_H$ was adjusted to 8.0 for strains *a*, *b*, *c*, 9 and 19; to 8.5 for 4 and 12 and to 8.7 for 1 and 7, with drops of sterile 10% solutions of $H_3PO_4$ and $Na_2CO_3$.

The yeast extract medium contained 2% NaCl and had by itself a $p_H$ of about 7.2, which was suitable for strains *a*, *b*, *c*, 9 and 19; for the other strains adjustments to $p_H$'s 8.5 and 8.7 were made.

The pyruvate medium contained 0,25% Na-pyruvate and the same inorganic salts as the mineral medium, with the omission of $NaHCO_3$ and was adjusted to the same $p_H$'s of 8.0, 8.5 and 8.7 for the different strains.

In order to exclude the oxygen as much as possible, the media were either used immediately after opening the autoclave or after boiling, followed by a rapid cooling; then the $NaHCO_3$ was added and in the case of the organic media one drop of a sterile solution of 10% $Na_2S$ . 9 aq. for each 100 ccm of culture medium, in order to remove the last trace of oxygen. This quantity of $Na_2S$ by itself is too small to cause an appreciable development of the bacteria.

For the analytical experiments $^1/_2$ l- and 125 ccm-bottles were used, which were completely filled with the medium, without adding paraffine oil. Neck and stopper were covered with paraffine after inoculation. The media employed contained the same base of inorganic salts as the pyruvate pure cultures, to which 0.25—1% of the organic substance which was to be investigated and, if necessary, 0.25 or 0.5% $NaHCO_3$ were added. A little $Na_2S$ was added and the $p_H$ was adjusted in the same way as with the pure cultures.

Together with each culture a blank was prepared.

The bottles were kept under continuous illumination in a light cabinet as described by *van Niel* (28, p. 12).

As the *p. s. b.*[1] grow rather slowly, cultures and blanks often had to be kept in the light cabinet for three weeks or longer. It sometimes happened that during this long stay in the light *p. s. b.* developed in the blanks.

In all probability this infection will have originated from the air. When pouring large quantities of medium from a flask into the culture bottles, much air is drawn into the flask and in a room where much work is done with *p. s. b.*, these organisms will be present also in the air[2]. The development of *p. s. b.* in the blanks however could be prevented easily by adding a little mercuric iodide.

[1] The term "*purple sulphur bacteria*" will be abbreviated from now on as *p. s. b.*

[2] Apparently these strongly anaerobic bacteria can occur in the air in a form which is resistant to oxygen.

### § 2. *Analytical methods.*

The following substrates were selected for analytical experiments: lactic, pyruvic, succinic, malic, acetic and butyric acids, all used in the form of the sodium salts.

The procedure of the analysis of the $^1/_2$ l-cultures was as follows: Immediately after opening the bottle two portions of 25 ccm were taken out for the determination of $CO_2$, which was carried out in the same way as described by *van Niel* (28, p. 86). Following this the $p_H$ was estimated with *Clark*'s and *La Motte*'s indicators.

The culture liquid was then filtered in order to get rid of the larger agglomerations of bacteria and brought to a $p_H$ of about 9.5. Two thirds of the original volume were destilled off, the destillate was acidified and $^2/_3$ of its volume were destilled off. This was repeated, till the volume of the destillate was less than 100 ccm. The destillate from the alcaline culture liquid contained the neutral volatile products eventually formed, and was tested for ethyl alcohol in the following manner: 10 ccm were mixed with the same volume of concentrated sulphuric acid and a few drops of a 0.2 n solution of potassium dichromate were added. When ethyl alcohol is present, the characteristic smell of acetaldehyde is easily noticed. Control experiments proved this test to be very sensitive. Of the destillates from the lactate and pyruvate cultures the refractive index was determined by means of a *Bausch* and *Lomb* refractometer. The destillates from the succinate, malate, acetate and butyrate cultures were also tested for acetone with a 1 % solution of 2, 4-dinitrophenylhydrazine in dilute HCl [cf. *van der Lek* (23)].

The residue of the alcaline destillation was filtered from a precipitate originating from the bacteria and acidified with $H_2SO_4$ to a $p_H$ of about 2.5. Nine tenths of the original volume were destilled off and the destillate was neutralised with 0.1 n baryta. In the case of the acetate and butyrate cultures twice or thrice $^9/_{10}$ of the original volume were destilled off, in order to collect all the remaining substrate from the medium. The combined neutralised destillates were concentrated and acidified with an amount of $H_2SO_4$ equivalent to the amount of baryta used for the neutralisation. Following this a few drops of a saturated $Ag_2SO_4$-solution were added in order to precipitate the HCl originating from the NaCl in the culture medium. Especially when the volume of the liquid in the destillation flask becomes small, some HCl will go over together with the fatty acids.

The liquid was then brought to a volume of 100 ccm and three times 90 ccm were destilled off. With butyric as well as with acetic

acid three destillations of $^9/_{10}$ of the original volume are sufficient to collect all the acid present. In these three destillates the acid could be titrated with sufficient accuracy.

In order to check whether the volatile acid remaining in the medium was pure acetic or butyric acid, the titrated destillates were concentrated and after addition of the calculated amount of $H_2SO_4$, a *Duclaux*-destillation was carried out in the manner described by *van der Lek* (23): From a volume of 110 ccm 5 times 20 ccm are destilled off and collected in volumetric flasks of 20 ccm. The figures so obtained were compared with those from destillations of the pure acids.

During the acid destillation another precipitate of bacterial origin was formed. The residue was neutralised and treated with magnesia mixture to remove the phosphate[1]. After filtration and evaporation to dryness, 2 ccm of 50 % $H_2SO_4$ were added and enough anhydrous sodium sulphate to get a dry mass, which was extracted with ether in a *Soxhlet* apparatus during 8 hours. The ether extract, which contained the non-volatile acids, was dried over night with anhydrous $Na_2SO_4$, filtered and the ether was destilled off. After dissolving the residue in water and filtering, the aqueous solution was neutralised with 0.1 n baryta. The barium salts of succinic and malic acids were then precipitated with the 14-fold volume of 95 % ethyl alcohol, the precipitate being allowed to settle over night. The next day it was collected on a Jena glass filter nr. 4 and thoroughly washed with hot alcohol of the same concentration as that in which the precipitate had been formed. After drying and weighing the filter the Ba-salt was dissolved in HCl and washed out with hot water. The filter was then dried and weighed again in order to correct for small quantities of $BaSO_4$, which may originate from a little $H_2SO_4$ in spite of the treatment of the ether extract with anhydrous $Na_2SO_4$. The amount of unattacked succinate or malate in the succinate and malate cultures can be determined in this way with sufficient accuracy, if only in the case of malate the neutralisation of the acid is carried out at boiling temperature, in order to decompose the malic anhydride.

The nature of the alcohol-insoluble Ba-salt (succinate or malate) can be established by determining its barium content. This was done by adding 1 ccm of 50 % $H_2SO_4$ to the HCl-filtrate and collecting the precipitate of $BaSO_4$ on a Jena glass filter nr. 4, which was washed,

[1] Phosphoric acid is soluble in ether to some extent and, when the extract is neutralised with $Ba(OH)_2$, it is precipitated as $BaHPO_4$. Since this salt is soluble in HCl, it interferes with the determination of the non-volatile acids as described below and therefore it is necessary to remove the phosphate before the extraction.

dried and weighed. In some cases the non-volatile acid in the filtrate from the $BaSO_4$-precipitate was further identified by the vacuum sublimation method of *Klein* and *Werner* (16). In the sublimate succinic acid was identified microchemically as Pb-succinate, malic acid as Ag-malate.

The alcoholic filtrate was tested for lactic acid after removal of the alcohol, in the manner indicated below and in some cases by the microchemical reaction with yttrium nitrate.

When lactate was used as a substrate the amount remaining in the medium was determined directly in the culture liquid by the method of *Ulzer* and *Seidel*, following the prescription of *Smit* (35). The lactic acid is oxidized to oxalic acid with permanganate in an alcaline medium and the excess permanganate removed with $Na_2SO_3$. After filtration from the precipitate of $MnO_2$ the liquid is acidified with $H_2SO_4$ and boiled to drive out the $SO_2$. The oxalic acid is then titrated with standard $KMnO_4$. It is advisable to avoid a large excess of $Na_2SO_3$, since this substance may react with $MnO_2$ to form non-volatile sulphur compounds (e. g. dithionic acid) which also reduce permanganate in an acid medium. The lactic acid determinations were carried out in duplo or in triplo.

The pyruvic acid remaining in the pyruvate cultures was determined by the method of *Kayser*, as described by *van Niel* (27). This method is based on the reaction of CO-groups with hydroxylamine. When $NH_2OH.HCl$ is used, HCl is set free which can be titrated with standard alcali. Unfortunately it appeared that in my case this method yielded results which were not quite satisfactory (cf. Chapter III, § 1, b).

No indications of the presence of non-volatile neutral products (e. g. glycerol) were found, except traces of acetylmethylcarbinol in the pyruvate cultures which substance was determined by the well-known method of *Lemoigne* in the manner described by *van Niel* (26).

The 125 ccm-cultures were analysed only for $CO_2$ and for the remaining substrate in the manners indicated above. Furthermore the amount of carbon present in the bacteria was determined by the method developed by *Heslinga* (12) for the elementary analysis of organic substances. After the 2 samples of 25 ccm for the $CO_2$-determination had been taken out of the bottle, the remaining culture liquid was centrifuged in order to collect the bacteria. The supernating liquid was still turbid with breakdown products of the bacteria, but after adding a few drops of 50 % sulfuric acid (up to a $p_H$ of about 2.5), a precipitate was formed which could be centrifuged, leaving a clear

supernatant liquid which was then used for the determination of the remaining substrate.

The liquid used for the $CO_2$-determinations was also centrifuged, so that the bacteria from the whole bottle were collected. The combined precipitates were washed twice on the centrifuge with destilled water. The bacteria were then rinsed from the centrifuge tubes into a porcelain boat, dried at 45° C and burned in a quartz tube in a stream of oxygen, using a mixture of equal amounts of manganese dioxide and lead peroxide as a catalyst. The amount of $CO_2$ resulting from the combustion was determined in the usual way.

By running analyses of pure succinic acid I convinced myself that I had this method well in hand.

## Chapter II.

### Experiments on the development of the purple sulphur bacteria in organic media.

As has been mentioned in the introduction, *van Niel* had already obtained a good growth of the *p. s. b.* in yeast extract, peptone, Na-lactate and Na-pyruvate media, in the absence of $H_2S$, which results I could fully confirm for all strains. In order to get a notion which other simple organic compounds were suitable substrates for the *p. s. b.*, I prepared cultures in 30 ccm-bottles (in the same way as described in Chapter I, § 1 for the pure cultures), containing the following substances in quantities of 0.25, 0.5 or 1 % with the addition of 0.25 or 0.5 % bicarbonate:

*Nitrogen-free organic acids* (as Na-salts): formic, acetic, propionic, butyric, succinic, fumaric, glycolic, malic and tartaric acids.

*Alcohols*: ethyl alcohol, glycerol.

*Sugars*: glucose.

I want to emphasize that, with the exception of a special series of control experiments (see below), all experiments were carried out under anaerobic conditions in the light.

A good growth of all three types of *p. s. b.* occurred with acetate, propionate, succinate, fumarate and malate. With butyrate only the *Chromatium* and *Pseudomonas* types showed a good development, whereas glucose was a suitable substrate for the *Thiocystis* and *Pseudomonas* types only. The higher $p_H$ to which the media for the last mentioned types are adjusted (8.5 and 8.7 respectively) as compared with that of the media for the *Chromatium* type (8.0), may be favorable to an attack of the glucose molecule, as sugars are known to undergo purely chemical changes even in a slightly alcaline medium. Further investigation will be necessary to test this assumption.

That the cultures I employed were really pure was shown by inoculating yeast extract and peptone agar plates from them, which remained sterile after prolonged incubation at 30° C[1].

As already stated in the introduction, *van Niel* (28, p. 106) found that also in organic media no development occurred in the dark. I have had the same experience with yeast extract, peptone, pyruvate and glucose cultures (other substrates were not tested). The cultures with the two last mentioned substrates were brought into the light after a stay of a week or more in the dark at 25° C, whereupon they developed readily into normal cultures.

That development with an organic substrate can occur in the *complete* absence of $H_2S$, is shown by the following experiment: a pyruvate medium was cooled immediately after sterilisation in a stream of oxygen-free nitrogen. Cultures with this medium and with a pyruvate medium prepared in the ordinary way with a small amount of $Na_2S$, developed equally well, whereas in cultures in a medium prepared without either of these precautions to remove the last trace of oxygen, development was either very much retarded or did not occur at all.

*Van Niel* (l. c. p. 103) also tackled the question whether prolonged cultivation in organic media might render the *p. s. b.* incapable of growing in a mineral medium with oxidizable sulphur compounds and reported that four months of cultivation in yeast extract had no effect in this direction. When I transferred my strains to a mineral $Na_2S$-medium after cultivation in yeast extract for nine months, they all developed well, showing the characteristic sulphur droplets inside or outside the cells. Even after cultivation in yeast extract for a year and a half, strain *a*, when transferred to a mineral $Na_2S$-medium, showed a distinct growth and all the cells stuffed with sulphur the next day! The results of these experiments make it highly improbable that the *p. s. b.* will ever loose their ability to grow autotrophically after cultivation in organic media.

## Chapter III.

## Investigation of the metabolism of the purple sulphur bacteria in organic media.

### *§ 1. The nature and the quantities of the metabolic products formed.*

In order to establish the nature of the metabolic products formed from the different substrates, analyses of cultures in $^1/_2$ l-bottles

[1] Of course this only shows that aerobic contaminations were absent; ordinary anaerobic contaminations however would have been easily noticed, because they would have developed much faster than the slow growing *p. s. b.*

were carried out. I used the following substrates for these experiments: lactate, pyruvate, acetate, succinate, malate and butyrate. I did not obtain a good growth with other substrates until shortly before the close of my experimental work. An analysis of e. g. glucose and propionate cultures certainly would have yielded interesting results, but the experiments with the above mentioned substrates were so time-consuming that I could no more analyse cultures with these two substrates.

a) Lactate.

11 cultures were analysed, all with the same result, viz. that, *whereas a luxurious development of the bacteria took place, no products other than a small quantity of carbon dioxide and a very small quantity of volatile acid could be detected.* The alcaline destillate was practically pure destilled water and the ether extract of the residue of the acid destillation contained no non-volatile acids other than lactic acid. Only about $^1/_5$ of the substrate (1 % Na-lactate) had been used up. The $p_H$ of the medium had increased markedly, especially in the cultures without $NaHCO_3$, as a consequence of the formation of NaOH from the lactate which had been consumed. Details of the analyses of five cultures are given in Table I.

*Table I.*

Cultures on 1 % Na-lactate in $^1/_2$ l-bottles.

| Strain | Age in days | Change of $p_H$ | Lactate used up, millimols per 100 ccm. | $CO_2$ formed, millimols per 100 ccm. | Vol. acid, milliequivalents per 100 ccm. | |
|---|---|---|---|---|---|---|
| 9 | 81 | 7.8— 9.3 | 1.17 | 0.45 | 0.04 | no $NaHCO_3$ |
| 7 | 73 | 9.0—10.5 | 2.63 | 0.10 | 0.05 | id. |
| *b* | 88 | 7.8— 9.5 | 1.62 | 0.60 | 0.04 | id. |
| 9 | 69 | 8.0— 8.8 | 2.40 | 0.79 | 0.05 | 0.75% $NaHCO_3$ |
| 7 | 69 | 8.6—10.2 | 2.82 | 0.64 | — | id. |

b) Pyruvate.

The results I obtained with this substrate are similar to those of the lactate cultures in so far that I did not find any neutral volatile products or non-volatile acids other than pyruvic acid and that only relatively small amounts of volatile acid were formed. The quantity of $CO_2$ however was much larger and I could detect traces of acetylmethylcarbinol[1]. The culture liquid had the characteristic smell of

[1] The residue of the carbinol determination was tested for 2, 3-butyleneglycol by boiling with bromine [*van Niel* (26)], but I found no indication of the presence of this substance.

diacetyl, which substance was probably formed in very small traces from the carbinol by oxidation by the air after the opening of the bottle. I never noticed this smell in cultures with other substrates.

The $p_H$ as a rule decreased. Table II shows the figures for the six cultures I analysed.

*Table II.*

Cultures on 1% Na-pyruvate in $^1/_2$ l-bottles.

| Strain | Age in days | Change in $p_H$ | Pyruvate used up, millimols per 100 ccm. | $CO_2$ formed, millimols per 100 ccm. | Vol. acid, milliequivalents per 100 ccm. |
|---|---|---|---|---|---|
| *b* | 57 | 7.8—8.0 | 3.37 | 3.77 | 0.17 |
| 9 | 53 | 7.8—8.3 | 3.22 | 2.03 | 0.28 |
| 7 | 51 | 8.7—8.3 | 3.36 | 2.50 | 0.27 |
| *b* | 50 | 7.5—7.4 | 4.51 | 4.11 | 0.27 |
| 9 | 59 | 7.5—6.8 | 4.62 | 3.99 | 0.46 |
| 7 | 58 | 8.4—6.9 | 3.12 | 2.81 | 0.24 |

I used an *Eastman Kodak* preparation of pyruvic acid, which was a clear liquid with a slight yellow colour. The Na-salt was prepared by adding $Na_2CO_3$. The determination of pyruvic acid in the blanks always gave much lower figures than expected on the base of the quantity of pyruvic acid added to the medium, which was calculated from direct titration with 0.1 n KOH of a certain volume of the stock preparation. Therefore not much significance can be attached to the values for "pyruvate used up" in Table II. The explanation of this discrepancy probably lies in the great reactivity of the carbonyl group in the pyruvic acid molecule, which causes polymerisation, through which process the number of free CO-groups is diminished and therefore lower values in the determination by the *Kayser* method are obtained. I tried to purify the acid by fractionate destillation in vacuo, followed by a preparation of the Na-salt, which was precipitated with alcohol in the form of purely white needles which were free from water. However when I titrated the free CO-groups in a fresh solution of this salt by the *Kayser* method, I got a value which was 22% lower than the calculated one.

Even when a really pure preparation of pyruvic acid is used, there still remains the probability that, owing to the long duration of the experiments (the cultures have to stay some days in the light at 25° C), the pyruvate in the slightly alcaline culture liquid will undergo changes which will render the determinations unreliable [cf. *de Jong* (14)].

Since it would take considerable time to work out this problem in such a way that reliable results could be obtained, I have not used pyruvate as a substrate for quantitative experiments as described in § 2.

c) Acetate.

The metabolism in acetate closely resembles that in lactate. I analysed three cultures of strain 7[1] after 1, 2 and 3 weeks; the quantity of $CO_2$ formed was likewise small and the $p_H$ in the oldest culture had increased considerably, as is shown in Table III.

*Table III.*

Cultures on 0.25 % Na-acetate with 0.5 % $NaHCO_3$ in $^1/_2$ l-bottles.

| Strain | Age in days | Change of $p_H$ | Acetate used up, millimols per 100 ccm. | $CO_2$ formed, millimols per 100 ccm. |
|---|---|---|---|---|
| 7 | 7 | 8.7—9.0 | 1.50 | 0.24 |
| 7 | 14 | 8.7—9.5 | 1.54 | 0.35 |
| 7 | 21 | 8.7—> 9.5 | 2.63 | 0.41 |

No neutral volatile products or non-volatile acids were formed. *Duclaux*-destillations showed that the remaining volatile acid was practically pure acetic acid.

d) Succinate and Malate.

The amounts of $CO_2$ formed from these substrates are larger than those formed from lactate and acetate; more $CO_2$ is formed from malate

*Table IV.*

Cultures on 0.25 % Na-succinate with 0.5 % $NaHCO_3$ and on 0.25 % Na-malate in $^1/_2$ l-bottles.

| Strain | Age in days | Change of $p_H$ | Substrate used up, millimols per 100 ccm. | $CO_2$ formed, millimols per 100 ccm. | Vol. acid, milliequivalents per 100 ccm. |
|---|---|---|---|---|---|
| | | Succinate cultures. | | | |
| *b* | 7 | 8.0—9.0 | 0.91 | 0.81 | 0.02 |
| 1 | 21 | 8.7—9.2 | 0.92 | 0.77 | 0.06 |
| 12 | 25 | 8.5—8.8 | 0.63 | 0.45 | 0.04 |
| | | Malate cultures. | | | |
| *a* | 20 | 8.0—8.8 | 1.63 | 2.09 | 0.05 |
| 1 | 24 | 8.7—9.0 | 0.70 | 0.95 | 0.04 |
| 12 | 32 | 8.5—8.9 | 0.75 | 0.78 | 0.07 |

[1] My first attempts to grow the other strains in acetate were unsuccessful and it was not but shortly before the close of the experimental work that I obtained well developed acetate cultures of them. For this reason I have not carried out any analyses of acetate cultures of strains other than nr. 7.

than from succinate. Therefore the $p_H$ does not become as high as in the lactate and acetate cultures. The malate medium did not contain any $NaHCO_3$; sufficient poising action was obtained from the $CO_2$ produced. No neutral volatile products and only very small quantities of volatile acid are formed; besides the remaining substrates no non-volatile acids could be detected. The Ba-content of the alcohol-insoluble Ba-salts prepared from the substrates remaining in the medium agreed fairly well with that calculated for succinate, resp. malate; in the succinate and malate cultures of strain 12 the acids were moreover identified as succinic, resp. malic acid by sublimation in vacuo. The figures for these analyses are collected in Table IV.

### e) Butyrate[1].

With this substrate a very remarkable phenomenon was observed: $CO_2$, instead of being formed, *was taken up from the medium*. Again no volatile neutral products or non-volatile acids were formed. The figures for the *Duclaux*-destillations of the volatile acid remaining in the medium agreed closely with those obtained from pure butyric acid. The results obtained with butyrate are given in Table V.

*Table V.*

Cultures on 0.25% Na-butyrate with 0.5% $NaHCO_3$ in $^1/_2$ l-bottles.

| Strain | Age in days | Change of $p_H$ | Butyrate used up, millimols per 100 ccm. | $CO_2$ taken up, millimols per 100 ccm. |
|---|---|---|---|---|
| *b* | 21 | 8.0—> 9.5 | 1.38 | 1.71 |
| 1 | 33 | 8.7—9.5 | 1.09 | 0.75 |

### f) Conclusions.

From the results of the analyses of the $^1/_2$ l-cultures it appears that the metabolism of the *p. s. b.* in organic media is not a fermentation process, such as occurs with other anaerobic microorganisms (e. g. alcoholic and butyric acid fermentations and sulphate reduction), which convert their substrates for 90% or more into metabolites, under liberation of considerable amounts of energy and only for 10% or less into cell material. With the exception of $CO_2$ in relatively small quantities, no waste products of the metabolism have been found[2]. It seems

[1] Since development of strains 4 and 12 (*Thiocystis* type) in a butyrate medium was very irregular and insufficient for an accurate analysis, no data from cultures of these strains are available.

[2] The very small quantities of volatile acid found in the cultures with non-volatile acids as substrates are probably products of autolysis.

therefore that the substrate is almost completely converted into cell material and $CO_2$, i. o. w. that the assimilation predominates in the metabolism. In order to establish this more firmly, I have carried out analyses of cultures in 125 ccm-bottles in which, besides the amount of substrate consumed and of $CO_2$ formed (or taken up), the amount of carbon present in the bacteria was determined, so that it was possible to draw up a carbon balance of the cultures. The results of these analyses are given in the next paragraph.

### § 2. *Experiments on the significance of the assimilation in the metabolism.*

Before giving the detailed results obtained with the 125 ccm-cultures, I want to point out that as a rule a perfect carbon balance (in so far that 100 % of the carbon of the consumed substrate can be accounted for as carbon in bacteria and in $CO_2$) was not obtained. This may be due to the fact that a certain portion of the bacteria had undergone autolysis, which means that the cell constituents had been broken down to either soluble products or to fragments so small that they were not carried down by centrifuging, not even after acidification, which always gave a slight precipitate. It is true that in very young cultures but little autolysis had occurred, but then only small amounts of substrate had been used up. This impaired the accuracy of the analyses, especially of the $CO_2$-determination, since the quantity of $CO_2$ formed was often not larger than a few milligrams per 25 ccm.

As was already mentioned, no 125 ccm-cultures with pyruvate were analysed. The results obtained with the other five substrates follow below. In the tables the amounts of substrate used up, of $CO_2$ formed and of carbon in bacteria are given in milligramatoms of carbon per 100 ccm of medium; the figures in the last column represent the percentage of the carbon of the substrate accounted for as bacteria and $CO_2$: $\frac{\text{carbon in bact.} + \text{carbon in } CO_2}{\text{carbon in substrate}} \times 100$. In the analyses of the butyrate cultures the carbon of the $CO_2$, which has been taken up, is counted negative, so that a figure is obtained which is directly comparable with the figures for the other substrates:

$$\frac{\text{carbon in bact.} - \text{carbon in } CO_2}{\text{carbon in substrate}} \times 100.$$

#### a) Lactate and Malate.

Table VI shows the results obtained with these substrates.

*Table VI.*

Cultures on 0.35 % Na-lactate and 0.25 % Na-malate in 125 ccm-bottles.

| Strain | Age in days | Change of $p_H$ | Substrate used up | $CO_2$ formed | Carbon in bacteria | Percentage recovered |
|---|---|---|---|---|---|---|
| | | | Lactate cultures. | | | |
| *b* | 30 | 8.0–9.5 | 3.29 | 0.41 | 2.95 | 102 |
| 12 | 30 | 8.5—9.2 | 1.04 | 0.12 | 0.82 | 90 |
| 1 | 30 | 8.7—10.0 | 3.70 | 0.25 | 3.15 | 92 |
| | | | Malate cultures. | | | |
| 19 | 25 | 8.0—8.8 | 4.48 | 1.25 | 2.72 | 89 |
| 7 | 28 | 8.7— — | 5.41 | 1.77 | 2.94 | 87 |
| 12 | 30 | 8.5—9.2 | 2.92 | 0.86 | 1.93 | 96 |

We may consider these results as highly satisfactory, when we bear in mind that the cultures were quite old at the time they were analysed and that the $p_H$ had increased markedly in all cases both factors favoring autolysis. The high yield in the first experiment is probably due to a somewhat too high value for the lactate remaining in the medium. With a view to the limitations of the analytical methods, a percentage of recovery of about 90 % may be considered as a strong indication that indeed the substrate is converted quantitatively into cell material and $CO_2$.

b) Acetate, Succinate and Butyrate.

With these substrates serious difficulties were encountered. Autolysis, which was rather limited in lactate and malate cultures, appeared to be quite strong even in young cultures with acetate, succinate and butyrate, as I could tell already from the peculiar smell of the culture liquid. Another striking difference with lactate and malate cultures was the nature of the bacteria: when grown in these two substrates they were rather slimy and stuck together at the bottom of the centrifuge tubes, so that it was easy to get them into the porcelain boat for the combustion. When grown in acetate, succinate and butyrate however, they formed a very finely divided precipitate which adhered strongly to the glass and therefore was only difficultly removed from the tubes.

Owing to the strong autolysis the first analyses with these substrates showed percentages of recovery of only 60—80 %, though the cultures mostly were younger than 20 and sometimes not older than 7 days.

Now we must bear in mind that in acetate, succinate and butyrate cultures the $p_H$ will rise strongly from the start of the development: with the first two compounds one equivalent of alcali is set free for every two gramatoms of carbon that are assimilated, which is only

counterbalanced by the formation of small amounts of $CO_2$ and o a little $H_2SO_4$, originating from $(NH_4)_2SO_4$ after the assimilation of $NH_3$. With butyrate one equivalent of alcali is produced for every four gramatoms of carbon assimilated, but here $CO_2$ is taken up instead of being formed. With lactate however one equivalent of alcali is set free for every three gramatoms of carbon assimilated and with malate much more $CO_2$ is produced.

Moreover I observed that in many cases development with these three substrates was much more rapid than with lactate or malate; in one acetate culture 0.25% Na-acetate had disappeared completely after a week! It is evident that such a rapid development will be accompanied by a very rapid rise in $p_H$, so that the bacteria will be exposed to a high $p_H$ during a longer period than in a slow-growing culture. I therefore started some more cultures, which I analysed after only a few days, i. e. as soon as I thought that they would have developed enough to warrant a sufficiently accurate analysis. Indeed I

*Table VII.*

Cultures with 0.25% Na-acetate, 0.25% Na-succinate and 0.25% Na-butyrate, all with 0.5% $NaHCO_3$, in 125 ccm-bottles.

| Strain | Age in days | Change of $p_H$ | Substrate used up | $CO_2$ formed | Carbon in bacteria | Percentage recovered |
|---|---|---|---|---|---|---|
| | | | Acetate cultures. | | | |
| 7 | 10 | 8.7—9.5 | 3.72 | 0.28 | 2.30 | 69 |
| 7 | 20 | 8.7—9.1 | 3.61 | 0.16 | 2.36 | 70 |
| 7 | 17 | 8.7— > 9.5 | 4.28 | 0.43 | 2.26 | 63 |
| 7 | 7 | 8.7— > 9.5 | 5.84 | 0.38 | 3.94 | 74 |
| 7 | 3 | 8.7— > 9.5 | 3.70 | 0.30[1] | 2.72 | 82 |
| 7 | 7 | 8.7— > 9.5 | 4.72 | 0.49 | 2.69 | 67 |
| | | | Succinate cultures. | | | |
| *a* | 13 | 8.0—9.3 | 4.73 | 0.73 | 2.51 | 69 |
| 12 | 17 | 8.5—9.3 | 4.01 | 0.63 | 2.10 | 68 |
| 1 | 12 | 8.7—9.3 | 5.06 | 1.10 | 2.83 | 78 |
| *a* | 7 | 8.0—8.9 | 4.30 | 0.63 | 2.21 | 66 |
| *a* | 4 | 8.0—8.9 | 3.27 | 0.45 | 1.70 | 66 |
| 1 | 5 | 8.7—9.4 | 3.60 | 0.55 | 2.20 | 76 |
| | | | Butyrate cultures. | | | |
| *a* | 28 | 8.0—8.7 | 2.74 | — 0.35 | 2.38 | 74 |
| 1 | 24 | 8.5—8.7 | 1.38 | — 0.28 | 1.35 | 78 |
| *a* | 15 | 8.0—9.5 | 5.40 | — 0.84 | 4.53 | 66 |
| 1 | 8 | 8.7— > 9.5 | 3.33 | — 0.45 | 3.53 | 92 |
| *a* | 7 | 8.0—9.5 | 6.45 | — 0.79 | 5.90 | 79 |
| *a* | 3 | 8.0—9.0 | 1.52 | — 0.39 | 1.97 | 104[2] |

[1] The $CO_2$-determinations in this analysis were lost; the figure in the table is the mean calculated on the basis of the other analyses (cf. p. 150).

[2] As only little butyrate has been consumed in this culture, the errors in the analytical procedures have been relatively large, which may account for the high percentage of recovery.

obtained better results with butyrate in this way; with succinate and acetate results were still disappointing, but then it is difficult to judge from the appearance of a culture when the right mean between a development too small for accurate analysis and too large on account of autolysis, has been reached.

In Table VII I have collected the results obtained with these three substrates; only those cultures which have been analysed after a short time and in which not too much of the substrate has been used up, show a percentage of recovery of more than 80 %.

Certainly the carbon balances of the acetate, succinate and butyrate cultures are not as satisfactory as those of the lactate and malate cultures. Nevertheless the percentages of recovery actually obtained with the three former substrates, together with the fact that in the $^1/_2$ l-cultures no metabolic products other than $CO_2$ could be found, point towards a total conversion also of these substrates into cell material and $CO_2$.

## Chapter IV.

### Preliminary survey of the experimental results obtained.

The experiments described in the two foregoing chapters enable us to give here an outline of the metabolism of the *p. s. b.* in organic media.

In the first place the experiments reported in Chapter II show convincingly that a perfectly normal development of the *p. s. b.* is possible in media containing, besides the necessary inorganic salts, only one simple, nitrogen-free organic compound. The most important point in this connection however is, that these media are free from oxidizable sulphur compounds. The significance of this fact can not be easily overrated, since an authority on the subject like *Winogradsky*, even as recently as 1931, still expressed the opinion that the development of the *p. s. b.* was absolutely confined to mineral media containing hydrogen sulphide (44).

We must remember in this connection that *van Niel* (28) already made some preliminary observations, which contradicted *Winogradsky*'s statement. These observations however were restricted to a small number of organic media, mostly of a complex nature. My own experiments fully corroborated those of *van Niel* and moreover they showed the suitability of a larger number of simple organic compounds as substrates for the *p. s. b.* Herewith the problem of the metabolism of the *p. s. b.* in organic media had become accessible to a quantitative chemical treatment.

In the meantime it has to be kept in mind that the experiments referred to above yielded another fact of primary importance, viz. that also in this heterotrophic metabolism the cooperation of radiant energy is an essential factor. We must conclude from this fact that photochemical processes play a part in the conversion of the organic substrates, which is a most remarkable phenomenon. Besides the *p. s. b.* and the *Athiorhodaceae*, no heterotrophic organisms are known in whose metabolism radiant energy plays such an important part. Moreover, with the *Athiorhodaceae* radiant energy is an essential factor for development only under anaerobic conditions; they are able to develop in the dark when oxygen is available [cf. *van Niel* and *Muller* (29)]. The *p. s. b.* however, being strictly anaerobic, do not develop in the presence of oxygen. Among the *autotrophic* organisms the green plants and the *coloured sulphur bacteria* are known to need radiant energy for their development; this external source of energy renders possible the reduction of $CO_2$, the only source of carbon for organisms living autotrophically. Now the *p. s. b.* are able to develop autotrophically (in the presence of oxidizable sulphur compounds) as well as heterotrophically, in both cases only when radiant energy is available. This suggests the possibility that there is some connection between the metabolism under autotrophic and under heterotrophic conditions. In the next chapter I shall return to this point.

The fact that the *p. s. b.* convert their substrates practically completely into cell material and relatively small quantities of $CO_2$, is certainly not less remarkable than the necessity of radiant energy. Whilst aerobic organisms may convert up to 50 % of their substrate into cell material [cf. the figures given for moulds by *Waksman* (40, p. 417)], anaerobic organisms as a rule convert their substrates only for 10 % or less into cell material and for 90 % or more into metabolic products. In order to grasp the full meaning of these facts, we must realize what the function of metabolism is. We can consider this function to be twofold; firstly to provide the building stones for the cell material (assimilation) and secondly to provide the organism with the necessary energy (dissimilation). Since aerobic dissimilation processes (i. e. dehydrogenations with oxygen as a hydrogen acceptor) yield more energy than anaerobic ones (i. e. dehydrogenations with organic substances or sulphate as hydrogen acceptors) per unit of substrate consumed, it is easy to understand that aerobic organisms dissimilate a smaller fraction of their substrate than anaerobic ones.

The *p. s. b.* apparently do not derive the energy they need from a chemical conversion of their substrates. This question will be dealt with later on.

Finally the experiments have revealed another phenomenon to which we must pay attention, viz. that from the various substrates different amounts of $CO_2$ are formed. This is brought forward clearly when we consider the amounts of $CO_2$ produced in relation to the amounts of substrate consumed. The figures collected in Table VIII denote the number of millimols of $CO_2$ produced per millimol of substrate consumed for the various substrates, both of $^1/_2$ l- and of 125 ccm-cultures. The figures for the butyrate cultures are negative because in this medium $CO_2$ is taken up instead of being formed. At the bottom of the table the mean values for each substrate are given. Since the amounts of substrate consumed in the pyruvate cultures are not accurately known, no figures for these cultures have been included.

Apart from the very low second value, the figures for lactate do not differ too much. The acetate, malate and succinate figures are also fairly uniform, but between the highest and the lowest figure for butyrate there is a considerable difference. When we compare the mean figures among each other, we see that those for lactate and acetate do not differ very much, but that the mean figure for succinate is distinctly higher than that for lactate or acetate and that the malate mean is again distinctly higher than the succinate mean, whereas the mean figure for butyrate stands out by its negative value.

*Table VIII.*

Number of millimols of $CO_2$ produced per millimol of substrate consumed for the cultures treated in Chapter III.

| Lactate | Acetate | Succinate | Malate | Butyrate |
|---|---|---|---|---|
| 0.38 | 0.16 | 0.89 | 1.28 | — 1.24 |
| 0.05 | 0.23 | 0.84 | 1.36 | — 0.69 |
| 0.37 | 0.16 | 0.71 | 1.04 | — 0.51 |
| 0.33 | 0.15 | 0.62 | 1.12 | — 0.81 |
| 0.23 | 0.09 | 0.63 | 1.31 | — 0.62 |
| 0.38 | 0.20 | 0.87 | 1.18 | — 0.54 |
| 0.35 | 0.13 | 0.59 | | — 0.49 |
| 0.20 | 0.21 | 0.55 | | — 1.03 |
| | | 0.61 | | |
| mean 0.29 | mean 0.17 | mean 0.70 | mean 1.22 | mean — 0.74 |

The significance of these characteristic differences among the mean figures for the various substrates becomes clear when we consider the various oxidation levels of the substrates. In this connection it seems

desirable to introduce a measure for the oxidation level of a compound, i. e. for the degree in which the average carbon atom in the compound is oxidized. It is obvious that this state of oxidation must be compared with the state of oxidation of the average carbon atom in a standard compound. As such I should like to take the average carbon atom in a carbohydrate. Since every organic compound can be supposed to be derived from a carbohydrate with the same number of carbon atoms, either by addition or by substraction of hydrogen atoms (eventually under uptake or loss of water), it seems indicated to accept as the unit of oxidation the removal of 2 H-atoms and to define the "oxidation value" of a given compound as half the number of hydrogen atoms involved in the said transformation, divided by the number of carbon atoms present in the compound. When hydrogen has to be withdrawn from the compound, the oxidation value will be taken as negative, in the opposite case as positive.

We can now calculate the oxidation values of the various substrates. Lactic and acetic acids have the empirical formulae of carbohydrates: $C_3H_6O_3$, resp. $C_2H_4O_2$; therefore their oxidation value will be 0. Succinic acid ($C_4H_6O_4$) possesses 2 H-atoms less than a $C_4$-carbohydrate ($C_4H_8O_4$), so its oxidation value will be $+ {}^1/_4 = + 0.25$. The empirical formula of malic acid is $C_4H_6O_5$: by loss of 1 $H_2O$ this becomes $C_4H_4O_4$ or 4 H-atoms less than a $C_4$-carbohydrate, so that the oxidation value of malic acid will be $+ {}^2/_4 = + 0.50$. Finally we get for butyric acid: $C_4H_8O_2 + 2\ H_2O = C_4H_{12}O_4 = C_4H_8O_4 + 4\ H$. Oxidation value: $- {}^2/_4 = - 0.50$. If we arrange the various substrates according to their oxidation values, we obtain the following series:

$$\text{butyric} < \text{lactic and acetic} < \text{succinic} < \text{malic acid}.$$

We see immediately that the amounts of $CO_2$ produced per unit of substrate consumed vary in the same way. Now we must bear in mind that, when $CO_2$ is split off from a substance, the oxidation value of the product of this reaction will be lower than that of the substance itself:

$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2.$$

In this reaction the oxidation value is lowered from + 0.33 (pyruvic acid) to − 0.50 (acetaldehyde).

Realizing that the substrate is practically completely converted into cell material, we arrive at the conclusion that, when $CO_2$ is produced, the oxidation value of the cell material taken as a whole will be lower than that of the substrate. When however the synthesis of cell material from the organic substrate includes an uptake of $CO_2$, the oxidation value of the cell material will be higher than that of the organic substrate.

Now we can calculate the mean oxidation value of the cell material in cultures with the various substrates from the figures in Table VIII. I shall give the calculations for lactate and acetate as an example. The mean value for the number of millimols of $CO_2$ produced per millimol of lactate consumed is 0.29. We therefore substract 0.29 ($CO_2$) from $C_3H_6O_3$:

| | | |
|---|---|---|
| C 3.00 | H 6.00 | O 3.00 |
| C 0.29 | | O 0.58 − |
| C 2.71 | H 6.00 | O 2.42 |

Now we add so much $H_2O$ that we obtain equal numbers for oxygen and carbon:

| | | |
|---|---|---|
| C 2.71 | H 6.00 | O 2.42 |
| | H 0.58 | O 0.29 + |
| C 2.71 | H 6.58 | O 2.71 |

and calculate the excess of hydrogen as compared with an imaginary carbohydrate of the same carbon content: $6.58 - 2 \times 2.71 = 1.16$. This figure divided by 2 gives the number of oxidation units that the cell material is more reduced than this imaginary carbohydrate: 0.58. This figure, divided by the number of carbon atoms in a "molecule" of the imaginary carbohydrate and provided with a minus sign, gives the mean oxidation value of the cell material in lactate cultures: $-0.58/2.71 = -0.21$.

For the mean oxidation value of the cell material in acetate cultures we get the following calculation:

| | | |
|---|---|---|
| C 2.00 | H 4.00 | O 2.00 |
| C 0.17 | | O 0.34 − |
| C 1.83 | H 4.00 | O 1.66 |
| | H 0.34 | O 0.17 + |
| C 1.83 | H 4.34 | O 1.83 |

$4.34 - 2 \times 1.83 = 0.68$. Oxidation value of the cell material:

$$-\frac{0.68}{2 \times 1.83} = 0.19.$$

In the same way we obtain the following mean oxidation values for the cell material in the other substrates:

| | |
|---|---|
| succinate | − 0.12 |
| malate | − 0.16 |
| butyrate | − 0.11 |

Though the oxidation values of the cell material in the various substrates are not identical (moreover Table VIII shows that, especially

for butyrate, there are differences among the individual cultures with the same substrate), they indicate clearly that the cell material taken as a whole is always a little more reduced than carbohydrate. Now the cell material of bacteria in general will consist chiefly of proteins[1] [cf. the figures given by *Hopkins*, *Peterson* and *Fred* (13) for *Clostridium acetobutylicum* and *Lactobacillus Leichmanni*]. For the composition of the average protein we may take the following figures: C 52.6%, H 6.9%, N 16.3%, O 22.5%, S 1.4% [cf. *Oppenheimer* (30, p. 121)], from which we obtain after division by the atomic weights: C 4.38, H 6.85, N 1.16, O 1.41, S 0.04. We can eliminate N and S as $NH_3$ and $H_2S$, substituting for one molecule of $NH_3$ or $H_2S$ one molecule of $H_2O$, which gives: C 4.38, H 5.79, O 2.61. The oxidation value calculated from these figures is $-0.07$.

Besides proteins bacteria will contain small amounts of substances which have a much lower oxidation value than proteins, e. g. fats, lipoids and, in the case of the *p. s. b.*, the red (carotinoid) pigments.

These facts make us understand why the oxidation values found for the cell material of the *p. s. b.* are slightly negative.

In conclusion we may say that the differences in the amounts of $CO_2$ produced or taken up in cultures with the various substrates are in agreement with the observation that these substrates are practically completely converted into cell material, the oxidation value of which is approximately the same with all substrates.

In the next chapter I shall try to give a more detailed analysis of the metabolism of the *p. s. b.* in organic media.

## Chapter V.

### Analysis of the metabolism of the purple sulphur bacteria in organic media.

Some 70 years ago *Pasteur* discovered that microorganisms exist, which are able to develop in a medium containing only one simple, nitrogen-free organic compound. Still the important consequence of this discovery, that these heterotrophic organisms must build up the wide variety of organic substances which are present in their cell material, exclusively from this one compound, has seldom been realized. When we bear in mind that the composition of microorganisms in general is about as complex as that of the higher plants [cf. the data

[1] Bacteria producing large amounts of cell wall material of a carbohydrate nature will form an exception.

collected by *Buchanan* and *Fulmer* (6)], we realize how remarkable the synthesizing power of such a small single-celled organism is. We are still more impressed in the case of the *p. s. b.*, which, in synthesizing their cell material, use their organic substrate in such an economical way, that only small quantities of $CO_2$ appear as waste products. But the synthesizing power of the *p. s. b.* appears in its most impressive form in a mineral medium containing oxidizable sulphur compounds, where the cell material is built up exclusively from carbon dioxide. The same holds for media in which these sulphur compounds are replaced by leuco-dyes like leucomethylene blue, because these leuco-dyes are only dehydrogenated to the corresponding dyes, which further remain intact. In these cases the synthesizing power of the *p. s. b.* equals that of the green plants.

These introductory remarks may suffice to elucidate the remarkable character of the phenomenon which is usually designated with the simple term of assimilation. It seems well worth while to enter into a more detailed consideration of the chemical processes underlying the synthesis of cell material.

Nowadays there can be scarcely any doubt that acetaldehyde (fat formation) and α-ketoacids, especially pyruvic acid (synthesis of aminoacids[1]) are the building stones of the complex organic substances which constitute the cell material [cf. *Kluyver* (17) and *Quastel* (32)]. To these we may add simple sugars, especially glucose, from which a. o. the polysaccharides of the cell wall must be formed.

When we consider this viewpoint to be generally applicable for an understanding of the synthesis of cell material, it is clear that in every special case we have to account for the formation of these "building stones" from the substrate. It will be useful to illustrate this with a few examples.

As such I choose the metabolism of different organisms with lactic acid as the organic substrate. This substance will be dissimilated in some way under liberation of energy. An aerobic organism will dehydrogenate lactic acid with oxygen as a hydrogen acceptor. For

[1] The carbon chain of pyruvic acid itself is found in alanine and serine and also in tyrosine and tryptophane. However, also the aminoacids which are substitution products of valeric acid (arginine and histidine), isocaproic acid (leucine) and glutaric acid (glutaminic acid) are most likely derived from pyruvic acid, because the reactivity of this substance makes it extremely suitable for coupling reactions with other carbonyl compounds, leading to the formation of α-ketoacids with more complex carbon chains, such as occur in the above mentioned aminoacids.

the removal of lactic acid in muscle tissue during the aerobic phase *Kluyver* (17) has suggested the following scheme:

$$CH_3CHOHCOOH + O \longrightarrow CH_3COCOOH + H_2O,$$

$$CH_3COCOOH \longrightarrow CH_3CHO + CO_2,$$

$$CH_3CHO + H_2O \rightleftharpoons CH_3CH(OH)_2,$$

$$\begin{array}{l} \quad H \quad H \\ CH.COH + O \longrightarrow CH_2CHOH + H_2O, \\ \quad H \quad OH \qquad\quad O \end{array}$$

$$\begin{array}{l} CH_2CHOH \rightleftharpoons CH_2OHCHO. \\ \quad O \end{array}$$

The same chain of reactions may occur with any aerobic organism thriving on lactic acid. Of course it is quite possible that part of the acetaldehyde is dehydrogenated to acetic acid (which can be further dehydrogenated or be excreted). Also the glycolaldehyde may be dehydrogenated. However, the main feature of the scheme is that the organism in question can obtain the "building stones" pyruvic acid, acetaldehyde and carbohydrate, from the substrate by a series of dehydrogenations with oxygen as an acceptor, in combination with a decarboxylation and a hydratation. It appears from the scheme that acetaldehyde and carbohydrate are obtained from the substrate via pyruvic acid, so that we may call this compound the "mother substance of the cell material".

It is evident that other suitable hydrogen acceptors can take the place of oxygen, e. g. nitrate in the case of the *denitrifying bacteria* and sulphate in the case of the *sulphate reducing bacteria*. The last mentioned microorganisms are highly anaerobic and as far as we know sulphate and other reducible sulphur compounds are the only suitable acceptors for them [cf. *Baars* (2)].

We see then that in the above mentioned cases the "building stones" occur as intermediate products in the dissimilation process. We may expect that in the case of the *p. s. b.* the "building stones" are likewise obtained from lactic acid by dehydrogenation, the difference with the above mentioned organisms being only, that these "building stones" are converted exclusively into cell material, instead of being converted to a large extent into waste products in a dissimilation process. The nature of the acceptor used by the *p. s. b.* will be discussed later on.

The scheme for the assimilation of lactic acid aids to the understanding of the assimilation of pyruvic acid as well. Two features of

the metabolism of the *p. s. b.* in pyruvate cultures may be explained here, viz. the formation of traces of acetylmethylcarbinol and the amount of volatile acid, which is not as negligible as in the lactate, succinate and malate cultures (compare Table II with Tables I and IV). It appears from the scheme that in a pyruvate medium the first step — apart from an assimilation of the substrate as such — is a decarboxylation of pyruvic acid to acetaldehyde, a reaction which is easily brought about by a great variety of living cells. Therefore the suggestion lies at hand that this reaction will proceed with a higher speed than the assimilation of the acetaldehyde or its dehydrogenation (in the hydrated form) to glycolaldehyde. This will lead to an accumulation of acetaldehyde which will favor other conversions of this highly reactive substance. It is a well-known fact that for instance yeast will convert an excess of acetaldehyde into acetylmethylcarbinol [cf. *Kluyver*, *Donker* and *Visser 't Hooft* (20)]. Another possible consequence of an accumulation of acetaldehyde is its dehydrogenation to acetic acid instead of to glycolaldehyde. Though acetic acid is also assimilated by the *p. s. b.*, it is quite acceptable that, as long as pyruvic acid is present, it will be left untouched, since it is much less reactive than the latter compound. Assuming that the volatile acid formed in the pyruvate cultures was indeed acetic acid, its appearance in these cultures becomes quite comprehensible.

Before giving schemes for the assimilation of the other organic substances which I have used as substrates in my experiments, I must say a few words about the assimilation of the *p. s. b.* in mineral media containing oxidizable sulphur compounds. Here, as with autotrophic organisms in general, the organic cell constituents are formed exclusively from $CO_2$. This involves a reduction of $CO_2$, which we may assume to yield $CH_2O$ as the primary product. Apart from the experimental indications of the occurrence of formaldehyde as an intermediate product in the $CO_2$-assimilation of the green plants and of certain autotrophic bacteria, a theoretical chemical consideration of $CO_2$-assimilation in general naturally leads to the assumption that $CH_2O$ is the primary product of this process. Whether we are dealing with the conversion of $CO_2$ into hexoses (in the case of the green plants), or into pyruvic acid (in those cases where no hexoses or their derivatives appear as storage products, but apparently a direct conversion of $CO_2$ into cell material takes place), we are facing the problem of the formation of compounds with more than one C-atom per molecule from a $C_1$-compound of a much higher oxidation value. The obvious course for such a process to take is a reduction of the $CO_2$ to a $C_1$-compound having approximately the same oxidation value as the $C_3$- or $C_6$-compounds that are ultimately formed, i. e. a reduction to $CH_2O$. This compound

needs not be identical with the stable formaldehyde as it is kept in the laboratory, but may well be a reactive form, which, on account of its reactivity, is immediately polymerised to sugars or involved in other reactions. The formation of pyruvic acid from $CH_2O$ may proceed as follows:

$3\,CH_2O \rightarrow CH_2OHCHOHCHO$ (glyceric aldehyde)
$CH_2OHCHOHCHO \rightarrow CH_3COCH(OH)_2$ (methylglyoxal hydrate)
$CH_3COCH(OH)_2 + \text{acc.} \rightarrow CH_3COCOOH + H_2\text{-acc.}$

This conversion may take place with the *p. s. b.* in mineral media; acetaldehyde can be formed from pyruvic acid by decarboxylation, whereas sugars may be obtained directly from $CH_2O$ by polymerisation.

Now that I have drawn up a scheme for the formation of the "building stones" from lactic acid, it is an easy task to do the same for the other substrates in a similar way. Also acetic, succinic, malic and butyric acids will have to be dehydrogenated in order to obtain pyruvic acid from them; with this compound we have reached the scheme given above for lactic acid.

For the conversion of acetic acid into pyruvic acid the following scheme has often been suggested [cf. for instance *Wieland* (42, p. 264)]:

$$2\,\begin{matrix}H\\ \diagdown\\ HCCOOH\\ \diagup\\ H\end{matrix} + \text{acc.} \longrightarrow HOOCCH_2CH_2COOH + H_2\text{-acc.},$$

$$HOOCC\overset{H}{\overset{|}{-}}\overset{H}{\overset{|}{C}}COOH + \text{acc.} \longrightarrow HOOCC{=}CCOOH + H_2\text{-acc.},$$

$$HOOCC{=}CCOOH + H_2O \rightleftharpoons HOOCCH_2CHOHCOOH,$$

$$HOOCCH_2CHOHCOOH + \text{acc.} \longrightarrow HOOCCH_2COCOOH + H_2\text{-acc.},$$

$$HOOCCH_2COCOOH \longrightarrow CH_3COCOOH + CO_2.$$

The dehydrogenation of acetic acid to succinic acid has been postulated for a wide variety of aerobic and facultatively anaerobic organisms: for muscle tissue by *Thunberg* (38) and *Ahlgren* (1) and for bacteria by *Quastel* (33); for moulds by various investigators [cf. *Bernhauer* (4)]. Also *Wieland* (41) has expressed himself in favor of this mode of biochemical oxidation of acetic acid. A purely chemical conversion of acetic acid into succinic acid was effected by *Moritz* and *Wolffenstein* (25), using persulphate as an oxidizing agent, which result was confirmed by *Bernhauer* and *Stein* (5). Very few obligately anaerobic organisms can thrive on acetic acid as the only source of carbon: apart from the *p. s. b.* (and probably the *Athiorhodaceae*) only the *methane*

*producing bacteria* [*Söhngen* (36)] and the sulphate reducing *Vibrio Rübentschickii* [*Baars* (2)]. Whereas the former ferments acetic acid to methane and carbon dioxide, the latter dehydrogenates acetic acid to $CO_2$ with sulphate as an acceptor; also here the first step may well be a dehydrogenation to succinic acid.

The dehydrogenation of succinic acid to fumaric acid, followed by a hydratation of the latter compound to malic acid, by "resting bacteria", was proved by *Quastel* and *Dampier Whetham* (34). The same processes probably occur with moulds [cf. *Bernhauer* (4)].

The dehydrogenation of malic acid to oxaloacetic acid and the subsequent decarboxylation of the latter to pyruvic acid was first postulated by *Quastel* (31) for *Bacterium coli* and *Pseudomonas pyocyanea* and seems indeed the most probable way of conversion.

The assimilation of succinic and malic acids is also explained by the acetic acid scheme.

With butyrate matters are more complex. It is possible to conceive of an application of *Knoop's* well-known theory of $\beta$-oxidation of fatty acids [(21); cf. also *Coppock, Subramaniam* and *Walker* (9); *Kay* and *Raper* (15)], in terms of dehydrogenation and hydratation, as follows:

$$CH_3\overset{H}{\underset{H}{C}}-\overset{H}{\underset{H}{C}}COOH + \text{acc.} \longrightarrow CH_3\overset{H}{C}=\overset{H}{C}COOH + H_2\text{-acc.},$$

$$CH_3\overset{H}{C}=\overset{H}{C}COOH + H_2O \rightleftharpoons CH_3CHOHCH_2COOH,$$

$$CH_3CHOHCH_2COOH + \text{acc.} \longrightarrow CH_3COCH_2COOH + H_2\text{-acc.},$$

$$CH_3COCH_2COOH + H_2O \longrightarrow CH_3COOH + CH_3COOH.$$

We would thus have obtained acetic acid from butyric acid by withdrawal of 4 H-atoms and by addition of 2 molecules of $H_2O$.

In connection with the possibility of $\beta$-oxidation of butyric acid I tried to grow the three types of *p. s. b.* with Na-$\alpha$- and $\beta$-hydroxybutyrate (two 30 ccm-cultures of each type with each compound) as substrates, with the result that only the *Chromatium* and *Pseudomonas* types developed in $\beta$-hydroxybutyrate and that none of the types developed in $\alpha$-hydroxybutyrate. Now these two types grow well in butyrate, whereas the *Thiocystis* type does not, so that this result is in agreement with the assumption of $\beta$-oxidation and seems to exclude the possibility of $\alpha$-oxidation. However I only disposed of small quantities of $\alpha$- and $\beta$-hydroxybutyric acids, which were by no means pure and from which I prepared the Na-salts via the Ba- and Ca-salts. For this reason I do not want to attach much value to these experiments.

Now there is an other way possible for the assimilation of butyric acid, viz. a conversion of the methyl group into a carboxyl group, yielding succinic acid. *Verkade* and his collaborators (39), who found that undecylic acid is oxidized to undecane-diacid in the human body, introduced for this mode of oxidation of fatty acids the term "$\omega$-oxidation". In terms of dehydrogenation and hydratation this conversion of butyric acid might proceed as follows:

$$\begin{array}{l} \overset{H\ H}{\diagdown\ \diagup} \\ CH-\overset{\cdot}{C}CH_2COOH + \text{acc.} \longrightarrow CH_2{=}CHCH_2COOH + H_2\text{-acc.}, \\ \underset{H\ H}{\diagup\ \diagdown} \end{array}$$

$$CH_2{=}CHCH_2COOH + H_2O \rightleftharpoons CH_2OHCH_2CH_2COOH,$$

$$\begin{array}{l} H \\ HO\overset{\cdot}{C}CH_2CH_2COOH + \text{acc.} \longrightarrow OHCCH_2CH_2COOH + H_2\text{-acc.}, \\ H \end{array}$$

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\quad H \\ OHCCH_2CH_2COOH + H_2O \rightleftharpoons HO\overset{\cdot}{C}CH_2CH_2COOH, \\ \qquad\qquad\qquad\qquad\qquad\quad HO \end{array}$$

$$\begin{array}{l} H \\ HO\overset{\cdot}{C}CH_2CH_2COOH + \text{acc.} \longrightarrow HOOCCH_2CH_2COOH + H_2\text{-acc.} \\ HO \end{array}$$

In this way succinic acid is obtained from butyric acid by withdrawal of 6 H-atoms and by addition of 2 molecules of $H_2O$.

A chemical conversion of butyric acid into succinic acid was effected by *Cahen* and *Hurtley* (8), who oxidized butyric acid with hydrogen peroxide. *Stant, Subramaniam* and *Walker* (37) isolated succinic acid from a culture of *Aspergillus niger* on Ca-butyrate and mentioned the possibility that it had originated directly from butyric acid by oxidation of the methyl group. I have sought very carefully for traces of succinic acid in $^1/_2$ l-cultures with butyrate as well as with acetate, which were started especially for this purpose, but no indication of the presence of this acid could be found. This may mean however that succinic acid and the other possible intermediate products are more easily attacked than butyric and acetic acids themselves, so that they do not appear in the medium, but are further converted inside the cells as soon as they are formed.

The two ways of assimilation of butyric acid outlined above seem the most acceptable ones. As yet it is impossible to say which of them is to be preferred.

In the schemes given in this chapter I have discussed how the various substrates can be converted into the "building stones" of the cell material by dehydrogenation, decarboxylation and hydratation.

Besides the "building stones" and $CO_2$, no other compounds are formed from the substrates by the postulated reactions.

Now dehydrogenation of a substance will lead to the formation of compounds with a higher oxidation value than that of the substrate itself. In Chapter IV I have shown that the cell material taken as a whole has a lower oxidation value than all the substrates used in my experiments, with the exception of butyric acid. Therefore the rise of oxidation value caused by the dehydrogenation of the substrate must be compensated in some way or other in the course of the assimilation process. Such a compensation is obtained by the decarboxylations:

oxaloacetic acid (ox. val. + 0.75) → pyruvic acid (ox. val. + 0.33) → acetaldehyde (ox. val. — 0.50).

However we can not account for the formation of highly reduced substances containing long hydrocarbon chains (e. g. the higher fatty acids and aminoacids like leucine) by means of a decarboxylation of less reduced compounds. The most acceptable scheme for the formation of the higher fatty acids is the following one [cf. *Haehn* and *Kinttof* (11)]: a coupling of two molecules of acetaldehyde, yielding acetaldol, which, by splitting off water, passes into crotonaldehyde. This unsaturated aldehyde is hydrogenated to butyric aldehyde, which in its turn combines with acetaldehyde, etc. Thus we see that in this process unsaturated compounds act as hydrogen acceptors.

A hydrogenation is moreover an essential link in the process of the formation of aminoacids from ketoacids [cf. *Knoop* and *Oesterlin* (22)]:

$$R . COCOOH + NH_3 \rightleftharpoons R . C{=}NH . COOH + H_2O,$$
$$R . C{=}NH . COOH + 2\,H \longrightarrow R . CHNH_2 . COOH.$$

Now that we have come to the conclusion that the assimilation o the organic substrates involves a series of dehydrogenations as well as a series of hydrogenations, it is logical to combine these processes to an oxidoreduction process in which the hydrogen of the substrate is transferred to organic acceptors, which are the precursors of the more reduced cell constituents. These acceptors are derived from dehydrogenation products of the substrate, viz. from the "building stones"; for instance the unsaturated aldehydes from acetaldehyde and the iminoacids via ketoacids from pyruvic acid. However, the above mentioned acceptors can not be the only ones which come into play in the dehydrogenation of the substrate. We have seen in Chapter III that in butyrate cultures $CO_2$ is taken up from the medium. This means that $CO_2$ has been hydrogenated, i. o. w. that *$CO_2$ has acted as a hydrogen acceptor*. The only possible hydrogen donators for the hydrogenation of the $CO_2$ are butyric acid itself or compounds derived from it; so

we arrive at the conclusion that here *$CO_2$ is reduced by an organic substance*. Since the oxidation value of butyric acid is lower than that of the cell material, this substrate has to be dehydrogenated to a certain extent by a "foreign" acceptor, i. e. by an acceptor which is not a derivative of the substrate's own dehydrogenation products. We must therefore conclude that the presence of $CO_2$ in butyrate cultures will be indispensable for the development of the *p. s. b.* The following experimental results confirm the correctness of this conclusion.

Butyrate cultures without special addition of $NaHCO_3$, notwithstanding the fact that these cultures were adjusted to the usual initial $p_H$, scarcely showed any development. When $NaHCO_3$ was added afterwards, the cultures immediately developed in a normal way. I performed this experiment with nine cultures, always with the same result. The very slight development observed in cultures without the addition of $NaHCO_3$ must be attributed to small amounts of $CO_2$ introduced with the inoculum and, in the form of $Na_2CO_3$, when adjusting the $p_H$. But this development did not yield more than 10 milligrams of dry bacteria per 100 ccm of medium. When 0.02 % $NaHCO_3$ was added, the yield of dry bacteria became 4—5 times as large. This effect of $NaHCO_3$ was not due to its poising action, because the $p_H$ in the cultures without $NaHCO_3$ had not changed from its initial value.

The hydrogenation of $CO_2$ will yield primarily $CH_2O$, which also will be used for the synthesis of cell material; therefore in the butyrate cultures there are two sources of bacterial substance, viz. butyric acid and $CO_2$.

When $CO_2$ can act as an acceptor for the dehydrogenation of butyric acid, the suggestion lies at hand that $CO_2$ can do the same for the dehydrogenation of the other substrates[1]. However, in cultures with the other substrates $CO_2$ is formed instead of being taken up, so that we have no direct proof that also in these cases $CO_2$ acts as a hydrogen acceptor. Nevertheless there is an indication that, at least in acetate and succinate cultures, $CO_2$ must play a part as an acceptor in the assimilation process.

On p. 154. I have pointed out that the synthesis of the greater majority of organic cell constituents probably passes the pyruvic acid stage. When we look at the scheme given on p. 157 for the conversion of acetic and succinic acids into pyruvic acid, we see that in the case

[1] I want to point out that *van Niel* (28, p. 102), when dealing with the growth of the *p. s. b.* in organic media in the absence of sulphur compounds, already suggested that lactic acid might function as a hydrogen donator for $CO_2$.

of acetic acid 0.5 mols of $CO_2$ are produced per mol of substrate converted into pyruvic acid and that in the case of succinic acid 1 mol of $CO_2$ is produced per mol of substrate converted. When we bear in mind that part of the pyruvic acid has to be decarboxylated in order to obtain the other "building stones", we see that the number of mols of $CO_2$ produced per mol of substrate *assimilated to cell material*, must be even larger than 0.5 resp. 1. Actually I found that the average number is 0.17 for acetic acid and 0.70 for succinic acid (see Table VIII). The only way to explain this discrepancy is to assume that a hydrogenation of $CO_2$ proceeds simultaneously with the assimilation of these substrates, i. o. w. that also here $CO_2$ acts as an acceptor for the dehydrogenation of the substrate.

The formation of pyruvic acid from lactic acid does not involve a decarboxylation; in the conversion of malic acid into pyruvic acid 1 mol of $CO_2$ is split off per mol of substrate converted into pyruvic acid, but in the assimilation of malic acid to cell material 1.22 mols of $CO_2$ are split off per mol of substrate assimilated. Therefore there is no indication that $CO_2$ will act as an acceptor in the dehydrogenation of lactic and malic acids, such as there is in the case of acetic and succinic acids. But it is quite possible that the amount of precursors of highly reduced substances is insufficient to effect the dehydrogenation of the whole of the consumed substrate, so that also in the assimilation of lactic and malic acids the acceptor $CO_2$ must come into play.

The conceptions given above may well apply to the metabolism of the *Athiorhodaceae* also. These organisms need organic substances for their development; they can thrive aerobically both in the dark and in the light, but anaerobically only in the light. *Van Niel* and *I* (29), when discussing this remarkable situation, arrived at the following conclusions (l. c. p. 262): "In case the metabolism of *purple bacteria* in organic media under anaerobic conditions and with the supply of radiant energy is chemically similar to the metabolism under aerobic conditions in the dark, we are dealing with processes in which the dehydrogenations involved in the synthetic processes are carried out with oxygen as the final acceptor in the dark, whereas in the light the oxygen is replaced by some other compound which requires activation by radiant energy".

With a view to the results obtained in this paper, the suggestion lies at hand that this "other compound" will be carbon dioxide. The *Athiorhodaceae* possess the same system of pigments as the *p. s. b.*, so that we may expect them to be also capable of using $CO_2$, under uptake of radiant energy, as an acceptor for the hydrogen of organic compounds.

Now that we have duly considered the chemical side of the assimilation, we must devote our attention to the energy side of this process. *Kluyver* (17) has made it acceptable that the chemical processes involved in the synthesis of cell material are spontaneous reactions, i. e. reactions accompanied by a decrease of free energy. Now the assimilation of the *p. s. b.*, in mineral media containing oxidizable sulphur compounds as well as in organic media, involves the use of $CO_2$ as a hydrogen acceptor. We may expect that a dehydrogenation of these sulphur compounds and of organic substances with $CO_2$ as an acceptor will be accompanied by an *increase* of free energy; if so, the addition of radiant energy will be a thermodynamical necessity in order to make these reactions proceed. As yet it is impossible to say whether radiant energy will have the same function in one or more dehydrogenations of the organic substrates with organic acceptors like those mentioned above. However this does not seem very probable, since most of these oxidoreductions will also occur in the synthesis of cell material of ordinary heterotrophic anaerobic organisms, which do not require the cooperation of light energy.

In connection with the energy requirements of the assimilation process, a few remarks about the possible other energy requirements of the *p. s. b.* may be made. Besides for the performance of internal and external mechanical work, energy will be necessary for the "maintenance" of the organism. The various physico-chemical structures inside the cell, which are indispensable for its existence as a living unit, need a constant supply of energy to protect them against a breakdown. *Kluyver* (18) has pointed out that this "maintenance" may be effected by an oxidoreduction process which establishes potential differences at the interfaces which are the essential elements in these physico-chemical structures. The various dissimilation processes which we encounter in living cells are finally nothing but oxidoreductions, but the same also holds for the assimilation processes. Therefore it does not seem excluded that the photosynthetic process taking place in the cells of the *coloured sulphur bacteria* not only provides the building stones for the cell material, but also maintains the structures inside these cells which are essential for their existence. This means that as long as assimilation proceeds, these bacteria will not "need" a dissimilation process.

But what will happen in the dark? In their natural environment the *p. s. b.* are submitted to diurnal periods of darkness 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. Similar views are expressed by *van Niel* (28, p. 104—107). He considered the possibility that some fermentation process might occur in the dark, but he refuted this idea because the *p. s. b.* are not able to develop in the dark, not even in media containing yeast extract, pyruvate or glucose. 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 connection with this possibility an observation made by *Derx* [cf. *van Niel* (l. c. p. 106)] is of importance: *p. s. b.* kept in the daylight stained violet with iodine at the end of the afternoon, whereas early in the morning no such staining could be observed. These facts point towards the production of a reserve carbohydrate in the light, which is metabolised in the dark. Supposing that the bacteria indeed "maintain" themselves in the dark by a lactic acid fermentation of a reserve carbohydrate, one might expect that the lactic acid formed will be assimilated during daytime. Such a situation can be compared with the discharge and charge of an accumulator; when the bacteria are kept in the dark for a long time, the accumulator will be totally discharged and the bacteria must either die or pass into a resting stage.

However, further investigation will be required before anything more definite regarding the dissimilation of the *p. s. b.* can be said.

In summarizing the considerations given in this chapter on the metabolism of the *p. s. b.*, we can say that all evidence is in favor of the assumption that with these organisms also organic substances can act as donators for the hydrogenation of $CO_2$, and that also in organic media, one or more photosynthetic processes are involved in the metabolism of the *p. s. b.*

This metabolism is thus linked up with that of the *p. s. b.* in mineral media, where oxidizable sulphur compounds act as donators for the hydrogenation of $CO_2$ and with photosynthesis in general. In the equation:

$$CO_2 + 2H_2A \rightarrow CH_2O + 2A + H_2O$$

we may now replace $H_2A$ by organic substances as well as by $H_2S$ or $H_2O$.

**Summary.**

1. The *purple sulphur bacteria* are able to develop in media containing only one simple, nitrogen-free organic compound, in the absence of oxidizable sulphur compounds.

2. Radiant energy is indispensable for development in these media.

3. A quantitative chemical investigation has been carried out of the metabolism in cultures containing lactate, pyruvate, acetate, succinate, malate or butyrate as the organic substrate.

4. In these cultures practically no metabolic products other than relatively small amounts of $CO_2$ have been detected; in the butyrate cultures $CO_2$ is taken up instead of being formed.

5. By determining the carbon content of the bacterial substance synthesized in the cultures, 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.

6. The differences in the amount of $CO_2$ formed (or taken up) per unit of substrate consumed in cultures with different substrates are caused by the different oxidation values of the various substrates, the average oxidation value of the cell material of the bacteria being approximately the same with all substrates.

7. Since a consideration of assimilation in general leads to the insight that the greater majority of organic cell constituents is formed from the substrate via pyruvic acid, the ways in which this acid can be formed from the various substrates used in the experiments have been discussed.

8. The conversion of the substrates into pyruvic acid involves one or more dehydrogenations; a consideration of the hydrogen acceptors which may effect this dehydrogenation shows that $CO_2$ must play a prominent part as an acceptor in this process.

9. In connection with point 2 this leads to the conclusion that photosynthetic processes are involved in the metabolism of the *purple sulphur bacteria* in organic media.

10. In the equation for photosynthesis in general:

$$CO_2 + 2H_2A \rightarrow CH_2O + 2A + H_2O.$$

$H_2A$ may now be replaced by organic substances as well as by $H_2S$ or $H_2O$.

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# STELLINGEN

1.

De roode Zwavelbacteriën zijn mixotrooph.

2.

De roode Zwavelbacteriën zetten hun organische substraten quantitatief om in bacteriemateriaal en koolzuur, welks hoeveelheid afhangt van het oxydatieniveau van het substraat.

3.

Ook bij de — onder invloed van het licht verloopende — ontwikkeling der roode Zwavelbacteriën in organische media wordt koolzuur gereduceerd.

4.

De Californische Coniferae — voorzoover zij een beperkt areaal hebben — zijn te beschouwen als relictendemen.

5.

De door *Henze* gevonden reactie van methylglyoxaal met acetylazijnzuur geeft een ongedwongen verklaring van het verband, dat bestaat tusschen vetafbraak en koolhydraatstofwisseling in het dierlijk lichaam.

*M. Henze*, Zeitschr. f. physiol. Chem. **189**, 121, 1930; **195**, 248, 1931.

*R. Stöhr* u. *M. Henze*, ebenda **206**, 1, 1932; **212**, 111, 1932.

6.

Het homologiebegrip van *Jacobshagen* verschilt in wezen niet van het historische homologiebegrip, zoodat de definitie van *Gegenbaur* boven die van *Jacobshagen* te verkiezen is.

*E. Jacobshagen*, Allgemeine vergleichende Formenlehre der Tiere. Leipzig 1925, S. 73—132.

7.

De infectie van Hazelnoten met *Nematospora Coryli* Peglion geschiedt door stekende insecten.

8.

De voorstelling van *Bernhauer* over het ontstaan der polyenen in de plant verdient de voorkeur boven die van *Emde*.

*K. Bernhauer*, Biochem. Zeitschr. **249**, 199, 1932.

*H. Emde*, Helv. chim. Acta **14**, 888, 1921.

9.

Voor het electrocapillaire verschijnsel van *Becquerel* hebben *Freundlich* en *Söllner*, en *Bikerman* een bevredigende verklaring gegeven.

*H. Freundlich* u. *K. Söllner*, Zeitschr. f. physik. Chem., Abt. A, **138**, 349, 1928; **152**, 313, 1931.

*J. J. Bikerman*, ebenda **153**, 451, 1931.

10.

De door *Clark* gegeven verklaring van het voorkomen van Diatomeeën in sedimenten, die buiten de poolstreken ontstaan zijn, is te verwerpen.

*Bruce L. Clark*, J. of Geol. **19**, 583, 1921.

11.

Het verdient geen aanbeveling, in het internationale wetenschappelijke verkeer, naast Engelsch, Duitsch en Fransch, een vierde taal te gebruiken.