OXIDATION OF ALCOHOLS BY NON-SULFUR PHOTOSYNTHETIC BACTERIA

JACKSON W. FOSTER[1]

Hopkins Marine Station of Stanford University, Pacific Grove, California

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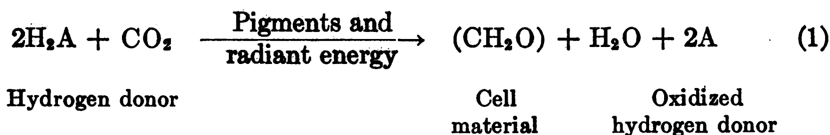
The photosynthetic purple bacteria are characterized by their ability to assimilate carbon dioxide in the light in the presence of suitable substrates and under anaerobic condtions. Bacterial photosynthesis represents special cases of the fundamental equation of photosynthesis (van Niel, 1931, 1935):



$$2H_2A + CO_2 \xrightarrow[\text{radiant energy}]{\text{Pigments and}} (CH_2O) + H_2O + 2A \qquad (1)$$

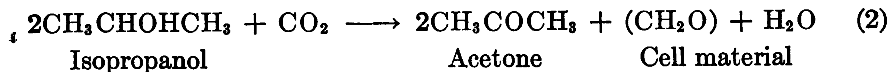
Hydrogen donor — Cell material — Oxidized hydrogen donor

Carbon dixoide is reduced anaerobically to cell substance and water by the hydrogen donor H_2A through the agency of photosynthetic pigments with the absorption of radiant energy.

Two more or less clear-cut groups can be distinguished among the purple bacteria on the basis of their physiological characteristics. One group, the sulfur purple bacteria (family *Thiorhodaceae* Molisch, 1907) can develop in strictly inorganic media containing H_2S or other reduced sulfur compounds, whereas the non-sulfur purple bacteria (family *Athiorhodaceae* Molisch) require organic substrates as hydrogen donors. The distinction is, however, not rigid because (1) the sulfur purple bacteria can all utilize organic substrates instead of sulfide (Muller, 1933; Gaffron, 1935; van Niel, 1936), and (2) various non-sulfur purple bacteria can accomplish photosynthesis with molecular hydrogen as hydrogen donor.

It is now recognized that the non-sulfur purples represent a group of bacteria possessing quite heterogeneous physiological properties (van Niel, 1941, p. 280).

Until recently, studies of the metabolism of photosynthetic purple bacteria on organic substrates have dealt exclusively with salts of simple organic acids. This paper deals with the isolation of non-sulfur purple bacteria capable of attacking various alcohols for the photosynthetic reduction of CO_2, and with some of their physiological aspects. Of especial interest was the isolation of one organism which quantitatively converts isopropanol into acetone with the concomitant reduction of CO_2 in the light (Foster, 1940).



$$2CH_3CHOHCH_3 + CO_2 \longrightarrow 2CH_3COCH_3 + (CH_2O) + H_2O \qquad (2)$$

Isopropanol — Acetone — Cell material

[1]National Research Council Fellow, 1939–40. Present address: Research Laboratory, Merck & Co., Inc., Rahway, N. J.

ENRICHMENT AND ISOLATION OF ALCOHOL-OXIDIZING PHOTOSYNTHETIC BACTERIA

A basal mineral medium consisting of 1000 ml of distilled water, 1 g of $(NH_4)_2SO_4$, 2 g of KH_2PO_4, 0.5 g of $MgCl_2$, and 4 g of $NaHCO_3$, was divided into two portions, one receiving 0.1% yeast autolysate and the other none. The final pH was between 7.0 and 8.0. Smaller portions of each of these two basal media were then supplemented separately with various alcohols to the final concentration stated: methanol, 0.2%; ethanol, 0.2%; n-propanol, 0.2%; isopropanol, 0.2% n-butanol, 0.1%; and n-amyl alcohol, 0.1%. The various media were then inoculated with a grám or so of soil or mud, transferred into small glass-stoppered bottles which were filled to the neck to exclude air bubbles and placed in a light cabinet in full exposure to incandescent lamps. The temperature ranged from 25–28°C.

The highly anaerobic character of these cultures together with the presence of organic matter and sulfates render conditions favorable for the development of non-photosynthetic sulfate-reducing bacteria (van Delden, 1904; Baars, 1930; Starkey, 1938). As a result of their activities the initial medium is soon converted into a sulfide solution ideally suited for the enrichment of sulfur purple bacteria which often develop to such an extent that isolation of non-sulfur purple bacteria becomes impossible. This complication could be avoided by eliminating sulfate from the enrichment media, substituting NH_4Cl for $(NH_4)_2SO_4$. In such cultures, the non-sulfur purple bacteria usually grow, unaccompanied by the photosynthetic sulfur bacteria. Development depends, of course, upon the introduction of traces of sulfur compounds with the inoculum. Occasionally, this may not suffice for normal growth. In one experiment, for example, where no sulfate was added and soil inoculum was used, growth after 6 days was still insignificant. Addition of 1–10 mg of $(NH_4)_2SO_4$ per 60 ml bottle caused a spectacular increase of growth over night, demonstrating that development had been limited by the absence of sulfur.

The most satisfactory procedure for the enrichment of non-sulfur purple bacteria consists of omitting sulfate from the medium for the first culture and the first transfer. In this way the sulfate-reducing bacteria are deprived of conditions necessary for their development and the non-sulfur purple bacteria soon outnumber the accompanying sulfur forms. For further subcultures in liquid media, sulfate can be added without causing the undesirable secondary flora to appear, while ensuring an abundant growth of the non-sulfur purple organisms.

An external supply of CO_2, e.g. $NaHCO_3$, should be provided when alcohols serve as hydrogen donors for photosynthesis. Alcohols are more reduced than the cell material, so that their dehydrogenation, with CO_2 as the final acceptor, results in a net uptake of CO_2. A consideration of the general equation (2) will make this clear. The situation is comparable with that of photosynthesis in the presence of the higher fatty acids, for which Gaffron (1933) has demonstrated the same process (see also Muller, 1933). In those cases where an outside supply of CO_2 is not provided, a scanty development of the organism is obtained, due to traces of CO_2 present in the medium.

Usually after the third serial transfer in liquid media, enrichment was con-

sidered adequate for the isolation of pure cultures. The solid or "shake" agar method described in detail for photosynthetic bacteria by van Niel (1931) proved excellent for these organisms.

The first series of shake tubes usually showed several different types of colonies. This was verified by microscopic examination of the colonies with the low power lens and by examination of slides. The dilution tubes showing well-isolated colonies were chosen for further purification. Cells from the different colony types were suspended in sterile tap water and shake cultures prepared as before. This process was repeated at least a third time in every case and, if necessary, thereafter continued until microscopic examination indicated the purity of the cultures.

Often there was a preponderance of one or a few types of bacteria in the enrichment cultures so that at the high dilution required to obtain well-isolated colonies, those organisms present in smaller numbers would fail to show up. Careful examination of the lower crowded dilutions always revealed the presence of other colony types. Transplants of these were, of course, heavily contaminated but they could be purified in the regular manner. As many as six to eight series of shake tubes were required before some cultures could be deemed pure.

For stock cultures, the freshly purified strains were stored in a light cabinet as stabs in the regular alcohol—$NaHCO_3$—agar medium with paraffin seals. All of these strains also grow luxuriantly without alcohol in yeast autolysate agar (Foster, 1940). A total of 70 different strains of alcohol-oxidizing photosynthetic purple bacteria were isolated.

GENERAL CHARACTERISTICS OF THE ISOLATED STRAINS

Extensive morphological and cultural studies of these organisms are being conducted by van Niel. All strains belong to the non-sulfur purple bacteria. They comprise a variety of red and brown types. Morphologically, several forms can be distinguished; these may be designated as spirilla, vibrios, cocci and short and long rods. The color of individual cultures ranges from a light tan to dark brown, and from light pink to dark purple.

All are capable of utilizing various single alcohols as sole hydrogen donors for the photosynthetic reduction of CO_2. Moreover, of a number of strains tested all can develop on most or all of the alcohols tried.

In common with previously isolated strains of the non-sulfur purple bacteria they require special, as yet undetermined, nutrients, probably growth factors. This was not apparent from the enrichment cultures where often good development was observed in media without addition of growth factors (yeast extract). However, the pure cultures invariably failed to grow in liquid media prepared without the addition of yeast extract. It is, therefore, likely that, in the crude cultures, contaminating organisms provided the growth factors necessary for the development of the purple bacteria. This is borne out by observations on the growth in shake cultures. In crowded tubes a beneficial influence of contaminating colonies upon the growth of photosynthetic bacteria could frequently be noted. For example, within a zone of approximately 3 mm around a large

purple colony, there was a marked increase in size of colonies of a brown photosynthetic organism; these latter were inconspicuous throughout the rest of the tube. Apparently, the large well-developed colony was excreting substances favorable for growth of the adjacent bacteria. In addition, the agar used for the isolation of pure cultures is itself a source of growth factors. Washed agar proved much inferior in this respect.

The individual strains also differ with respect to their tolerance to oxygen, as shown by the depth at which growth commenced in stabs.

Of the numerous isolates two strains were selected for more detailed physiological studies on alcohol oxidation.

The first was obtained from enrichment cultures containing iso-propanol. It is a spherical to short rod-shaped bacterium, often occurring in chains. It bears a certain resemblance to Ewart's *Streptococcus varians*. Under anaerobic conditions, the cultures are brown; in the presence of air the pigmentation appears red. While a number of strains of this type were available, most studies were carried out with an isolate here designated as strain C.

The second is a spiral-shaped organism isolated from an ethanol enrichment culture. A number of similar, if not identical, strains have been obtained from various other alcohols; the particular strain used for most experiments, designated as *Rhodospirillum* sp., can develop rapidly in the presence of any one of a number of alcohols. Characteristic for this organism is the light pink color of its cultures in alcohol media, and the curious consistency of colonies in agar shake cultures. The organism is closely related to, if not identical with, *Spirillum rubrum* Esmarch. In shake and stab cultures in alcohol media the growth has a very light pink color as contrasted to a dark reddish-purple in yeast extract media. The same held true for liquid cultures. The colonies in solid alcohol media were exceedingly hard and brittle, quite unlike the usual butyrous consistency of bacterial colonies. The whole colony dislodged when touched with a needle, and great difficulty was encountered in smashing the colonies to get suspensions suitable for microscopic study and for transplants. Crushed between two glass slides and examined microscopically, the picture was one of dense clusters of solidly packed nonmotile spirilla.

CULTURAL CHARACTERISTICS OF RHODOSPIRILLUM SP. REQUIREMENT FOR CALCIUM

Attempts to cultivate this "hard" spirillum in liquid, alcohol media resulted in a slow but fair growth of a very peculiar type. The cells tended to settle out as clumps so that only flocculent, agglutinated clumps of growth accumulated on the bottom of the vessels, leaving the main part of the medium perfectly clear. There was a marked tendency to adhere to the walls of the glass vessels in the form of a thin, tough film, so that the culture vessels gave the appearance of being painted on the inside, and of being filled with the clear medium. The film fell away from the glass upon shaking. It could be pulled off like dried paint from a surface. Figure 1 (right) shows the appearance of such a culture compared with a normal culture (left).

Microscopically, the flocculent sediment and film were composed of densely

packed, nonmotile spirilla. Study of the conditions resulting in this unusual type of growth showed that the critical factor was not the concentration of ethanol or the pH of the medium, growth being the same from 0.05% to 0.3% and from pH 6.5 to 8.0 respectively. Some retardation was noted at pH 6.5. Development was the same whether the bottles were sealed against air or plugged with cotton.

FIG. 1. CALCIUM DEFICIENCY IN RHODOSPIRILLUM SP.

Bottle on the left had 10 ppm added calcium; the one on the right had no added calcium. Note the marked adherence of the growth in the form of a membrane to the surface of the bottle in the calcium deficient culture. Certain areas of the membrane have fallen away, showing the perfectly clear medium. The light patch on the upper right portion of the bottle is where the membrane has separated from the glass in the front and back of the bottle. The other light areas are where the membrane has peeled from the front surface only. The overhanging membrane can be detected at the upper edge of the membrane in the front, where its doubling up photographs as a thin, irregular, dark band.

The calcium-containing culture on the left has no membrane whatsoever and the dark color is due wholly to the intense development of the bacteria as a suspension throughout the medium. The cells in this culture were very actively motile.

When the organism was inoculated into 10% yeast autolysate medium with or without 0.1% ethanol, growth started fairly slowly but rapidly became abundant. Although some settling did occur, an excellent suspended type of growth was obtained without any of the film-like growth (fig. 1, left). Microscopically the spirilla from this culture were vigorously motile; cells consisting of 2 to 20 coils could be observed. Quite different purplish hues were apparent, the new isolate being considerably brighter and lighter in alcohol media than in

yeast autolysate medium alone. Cultivation, both aerobic and anaerobic, in 0.3% Na malate medium containing 0.1% yeast autolysate, an excellent substrate for most purple bacteria, still gave only the atypical, agglutinated growth. By increasing the yeast autolysate concentration to 0.5%–1.0% in ethanol or malate medium, the typical suspended growth was obtained, indicating that the critical factor was present in the yeast autolysate.

A clue as to the cause of the peculiar growth habit of this spirillum was provided by a chance observation. In preparing anaerobic cultures of purple bacteria it was the custom to fill sterile glass-stoppered bottles about two-thirds with sterile basal medium, add aseptically required supplements such as alcohol and $NaHCO_3$, inoculate, and then fill the bottles to the neck with sterile distilled water. In one experiment, consisting of a large series of treatments, the sterile distilled water supply became exhausted just at the last flask. Sterile tap water was here used as a substitute. After 2–3 days incubation, out of all the bottles

TABLE 1

Concentration of calcium and development of Rhodospirillum sp.

Ca AS $CaCl_2$	CULTURE DEVELOPMENT AFTER 2 DAYS
ppm	
0	Good; completely agglutinated at bottom
1.8	Good; suspended
3.6	Abundant; entirely suspended; grew most rapidly
5.4	Abundant; entirely suspended
7.6	Abundant; entirely suspended
15.2	Abundant; entirely suspended
30.4	Abundant; entirely suspended
45.6	Abundant; entirely suspended
60.8	Poor; suspended and clumped; distinctly repressed
KCl	Good; completely agglutinated on bottom
Tap water control	Abundant; suspended

only the one which had received tap water showed abundant suspended growth; in all the other flasks growth was clumped and membrane-like. Fresh cultures made with tap water confirmed the conclusion that something present in tap water was capable of inducing the suspended type of growth of this organism. The effect could be obtained with the neutralized HCl solution of the residue left on ignition of the total solids of the tap water. A series of salts of metallic elements known to occur in tap water including $ZnSO_4$, $MnSO_4$, Na_2MoO_4, $CuSO_4$, $CaCl_2$ and $FeCl_3$ was tested. The $CaCl_2$ treatment was the only one which showed abundant suspended growth and complete absence of clumping. Tests with KCl showed that the effect was due to a requirement for calcium by this organism.

Table 1 shows the influence of concentration of calcium on growth of the alcohol-oxidizing spirillum in distilled-water malate medium containing 0.2% yeast autolysate.

The culture containing 3.6 ppm calcium grew most rapidly, although the others

up to 45 ppm were close behind. There were no apparent differences over this range and approximately 3 ppm was the optimum under the conditions of this experiment. The culture with 1.8 ppm showed good suspended growth but considerably less than the optimum. The controls (KCl and no $CaCl_2$) both showed good growth but in each case it was clumped and membranous, leaving a perfectly clear supernatant medium.

Levels of $CaCl_2$ higher than 45 ppm were distinctly repressive. Not only does calcium affect the type of growth but it is also a limiting factor in the amount. This was shown by measuring in hematocrit tubes the volumes of cell yield per 5 ml of culture. The cultures without added $CaCl_2$ contained 1.6 mm^3 of cells per 5 ml as compared to 2.2 mm^3 in the 3.6 ppm culture. It should be mentioned that no special precautions were taken to eliminate completely the last traces of calcium present as impurities in the basal medium. It is probable that greater responses to added calcium could be obtained by using a specially purified medium and calcium-free culture vessels. The attachment of the growth to the glass in the form of a film suggests that the glass may have been a source of calcium.

METABOLIC STUDIES ON ALCOHOL-OXIDIZING PHOTOSYNTHETIC BACTERIA

Oxidation of n-alcohols

Most of the strains isolated from the *n*-alcohols made luxuriant growth, anaerobically, in liquid media containing a single alcohol and 0.2% yeast autolysate. This indicated efficient utilization of the alcohol.

It seemed possible that among the isolates a number of cultures might be found which could oxidize normal alcohols only to the corresponding fatty acids without being able to effect a further oxidation of the latter. Such organisms could be compared physiologically with the *Acetobacter suboxydans* type among the acetic acid bacteria, or with *Methanobacterium omelianskii* among the methane-producing bacteria. The existence of this type of alcohol oxidation among the purple bacteria would also furnish additional evidence for the view that the organic substrate functions exclusively as hydrogen donor in the photosynthetic reaction.

Since an incomplete oxidation of the alcohol would naturally result in less abundant development of the causative organism, studies were carried out with ten specially selected strains of purple bacteria, originally isolated from ethanol cultures, which appeared to grow less abundantly than the others in alcohol media. Each of these was inoculated into 1-liter quantities of 0.2% ethanol medium and incubated anaerobically in the light. After five days, the cultures were acidified to pH 1.5 with H_2SO_4 and steam distilled. The negligible titration values of the distillates of all the cultures showed that acetic acid was not a normal end product. Two of these cultures grown in 0.1% *n*-butanol also failed to yield significant amounts of volatile acid.

Further experiments with strain C, characterized by its ability to cause an incomplete oxidation of the secondary alcohol isopropanol (Foster, 1940), in media containing 0.1% methyl, ethyl and *n*-propyl alcohol led to similar results. Qualitative tests on distillates obtained from the methanol cultures were negative

for formaldehyde (hydrazone test) whereas formic acid was present in traces ($HgCl_2$ test). The ethanol cultures contained no detectable acetaldehyde or volatile acid. The first several drops of distillate from the *n*-propanol culture yielded a small amount of crystalline 2,4-dinitrophenylhydrazone. The amount was too small to recrystallize. Microscopically these crystals were short, stubby spindles. Control tests on media without the alcohols were negative in each case. It is apparent from these results that strain C degrades the lower aliphatic primary alcohols quite completely although apparently traces of intermediates can be detected. It appears that *n*-alcohols are oxidized completely by the non-sulfur purple bacteria during photosynthesis, and that intermediary oxidation products, such as the corresponding fatty acids, do not accumulate to any appreciable extent.

Oxidation of secondary alcohols

In contrast to the abundant development of these purple bacteria in media containing primary alcohols is the scanty growth in cultures with secondary alcohols. Development in such media is not only slow, but it remains extremely slight. Experiments designed to study the optimal conditions as to alcohol concentration etc., showed that initial isopropanol concentrations above 0.25% exert a toxic effect, and that in the lower concentrations growth is proportional to the amount of alcohol present in the medium. Fairly abundant development could be achieved by repeated additions of secondary alcohols in 0.02% quantities at weekly intervals.

The very poor growth in media containing secondary alcohols seemed quite logical if the oxidation of the alcohol molecule were incomplete, for relatively little hydrogen would then be available for photosynthetic reduction of CO_2 (i.e. growth). The logical products to expect from the incomplete utilization of the secondary carbinols are the corresponding ketones.

This possibility was tested experimentally by analysis of cultures of 3 strains in isopropanol media. To obviate the disadvantages of the extremely slight growth and the long time required to obtain it, the following technique was adopted. The organisms were cultivated in 10% yeast autolysate medium in which they all grew luxuriantly. After centrifugation, the cells were resuspended in 0.1% $NaHCO_3$ containing 0.1% isopropanol and further incubated anaerobically in the light for three days. The cultures were then centrifuged and 10 ml portions of the supernatants slowly distilled in a microstill after acidification with H_2SO_4 and addition of a few drops of oleic acid as antifoam. The first ml of distillate was caught in 0.5 ml of 1% 2,4-dinitrophenylhydrazine in 2*n* HCl. Appearance of a yellow precipitate from all three cultures indicated hydrazone formation and the presence of a volatile ketone which was identified as acetone. Detailed quantitative analyses of both growing cultures and "resting" cells acting on isopropanol have already appeared in a previous publication (Foster, 1940).

These experiments have not only shown that isopropanol gives rise to the formation of acetone during the photosynthetic activity of non-sulfur purple bacteria, but they have provided the demonstration that such conversion is quantitative.

(See equation (2).) Thus was furnished the first incontrovertible evidence that an organic substrate functions as hydrogen donor, and in this capacity exclusively, in the photosynthetic metabolism of the purple bacteria.

The activity of strain C towards other secondary alcohols during photosynthesis has now been investigated. Bottles of 1 liter capacity were filled completely with the basal medium supplemented with 0.05 and 0.1% respectively of each of the alcohols listed below, inoculated with strain C, stoppered tightly for anaerobiosis, and incubated in the light for 1 month. The following summary indicates the growth with respect to control cultures without the respective alcohol.

TABLE 2

Identification of 2,4-dinitrophenylhydrazones from cultures of photosynthetic bacteria

ORIGINAL SECONDARY ALCOHOL	KETONE	MELTING POINTS			N CONTENT*	
		Bacterial product	Authentic	Mixed	Bacterial product	Calculated
		°C	*°C*	*°C*	*per cent*	*per cent*
Methyl-ethyl-carbinol	Methyl-ethyl-	109	108	108.5	22.00	22.21
Methyl-*n*-propyl-carbinol	Methyl-*n*-propyl-	139	139	139	20.53	21.05
Methyl-iso-propyl-carbinol	Methyl-iso-propyl-	120.5	120.5	120.5	20.71	21.05
Diethyl-carbinol	Diethyl-	142	142	142	20.71	21.05

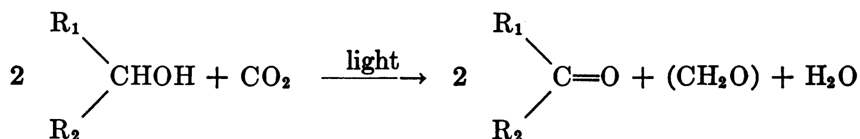
N analysis of authentic methyl *n*-propyl 2,4-dinitrophenylhydrazone = 20.98 per cent. Calculated = 21.05.

* I am indebted to Dr. A. M. Haagen Smit of the California Institute of Technology for the N-analyses.

	0.05%	*0.1%*
Methyl ethyl carbinol	Slight	Slight
Methyl *n*-propyl carbinol	Slight	None
Diethyl carbinol	Slight	Slight
Methyl isopropyl carbinol	Slight	Slight
Methyl *n*-butyl carbinol	None	None
Methyl *n*-hexyl carbinol	None	None
Trimethyl carbinol	Slight	Slight
Acetyl methyl carbinol	Slight	Slight
;:3-butylene glycol	Slight	Slight

Failure to grow in 0.1 % methyl *n*-propyl-, methyl *n*-butyl- and methyl *n*-hexyl carbinols is probably due to the high toxicity of those higher alcohols. The first four listed cultures were acidified and steam distilled into cold 2,4-dinitrophenylhydrazine in 2*n* HCl. The copious hydrazone precipitates were filtered, washed, and recrystallized from ethanol-chloroform mixture. Authentic ketone 2,4-dinitrophenylhydrazones were prepared from the corresponding carbinols by oxidation with $K_2Cr_2O_7$-H_2SO_4 mixture and steam distillation as above. Melting points and nitrogen analyses of the bacterial products proved their identity with the ketones obtained chemically from the corresponding alcohols (table 2). Thus, the secondary alcohols listed in table 2 are photo-

synthetically converted to the corresponding ketones. Although quantitative balance sheets were not made, it is, in view of the decisive results obtained with isopropanol (Foster, 1940), almost certain that these carbinols are quantitatively converted to the ketones with the concomitant reduction of stoichiometric amounts of CO_2 and synthesis of cell material. The photosynthetic conversion of secondary alcohols may then be represented by the following general equation:



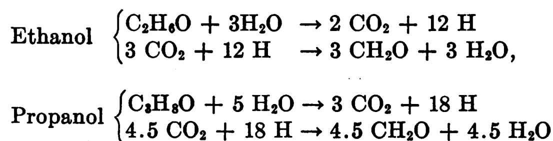
$$2\ \begin{matrix} R_1 \\ \diagdown \\ \end{matrix}\!\!CHOH\!\!\begin{matrix}\\ \diagup \\ R_2\end{matrix} + CO_2 \xrightarrow{\text{light}} 2\ (R_1)(R_2)C{=}O + (CH_2O) + H_2O$$

The products of the conversion of the trimethyl carbinol, acetyl methyl carbinol and 2,3-butylene glycol cultures were not identified. From the limited but distinctly positive growth made in these media it seems likely that, in these cases also, the oxidation of the alcohols had not gone to completion.

MANOMETRIC EXPERIMENTS ON ALCOHOL OXIDATION

The manometric experiments have all been carried out with suspensions of *Rhodospirillum* sp. The methods were those used and described earlier (Foster, 1940). In these experiments, it is important to add the substrate alcohols as soon as temperature equilibrium has been reached else the alcohol gradually distills from the side arm, dissolves in the bacterial suspension and is continuously attacked by the cell suspension, thus obscuring the true zero time reading.

Figure 2 shows the CO_2 uptake by *Rhodospirillum* sp. suspensions in the presence of 0.005 mM of methanol, ethanol, etc. The cells used in this experiment had been grown in a medium containing 0.1% *n*-butanol. It will be seen that the rate of CO_2 assimilation is practically the same for ethanol and *n*-propanol, but that the total assimilation is greater in the case of the latter. If the two alcohols were completely dehydrogenated with a subsequent transfer of hydrogen to CO_2, the following equations would roughly represent the two reactions:



$$\text{Ethanol}\ \begin{cases} C_2H_6O + 3H_2O \rightarrow 2\ CO_2 + 12\ H \\ 3\ CO_2 + 12\ H \rightarrow 3\ CH_2O + 3\ H_2O, \end{cases}$$

$$\text{Propanol}\ \begin{cases} C_3H_8O + 5\ H_2O \rightarrow 3\ CO_2 + 18\ H \\ 4.5\ CO_2 + 18\ H \rightarrow 4.5\ CH_2O + 4.5\ H_2O \end{cases}$$

From these, it appears that the CO_2 assimilation with ethanol should result in a net uptake of 1 mole per mole of substrate, and with *n*-propanol of 1.5 mole. Such high values were, however, never observed. This must be ascribed to the fact that the over-all composition of the product of photosynthesis is more reduced than that of carbohydrate (See van Niel, 1936, 1941). The results of several experiments show that the best values for CO_2 assimilation per 0.01 mM of ethanol and *n*-propanol respectively are approximately 150 and 240 mm. This is in good agreement with the data reported previously for fatty acids which show that for every 4 H atoms transferred less than 1 mole of CO_2 is consumed. (Gaffron, 1933; van Niel, 1936, 1941).

n-Butanol is obviously attacked more slowly; iso-propanol and methanol are apparently not utilized at all. An accurate value for CO_2 assimilation with *n*-butanol has been hard to obtain, due to the gradual falling off of the assimilation rate with consequent difficulties in determining just where the break representing the complete utilization of the substrate occurred. The best estimates range around 270 mm^3 per 0.01 mM of butanol.

The slow rate of assimilation with *n*-butanol is not an inherent characteristic of suspensions of *Rhodospirillum* sp. In other experiments (see, e.g., figs. 4 and 5) results were obtained which indicate that the rates in the presence of ethanol and butanol may be essentially the same. It seems likely that the results represented in figure 2 must be ascribed to the use of a cell suspension which was not in the best of conditions, and that butanol has a more toxic effect than the lower alcohols.

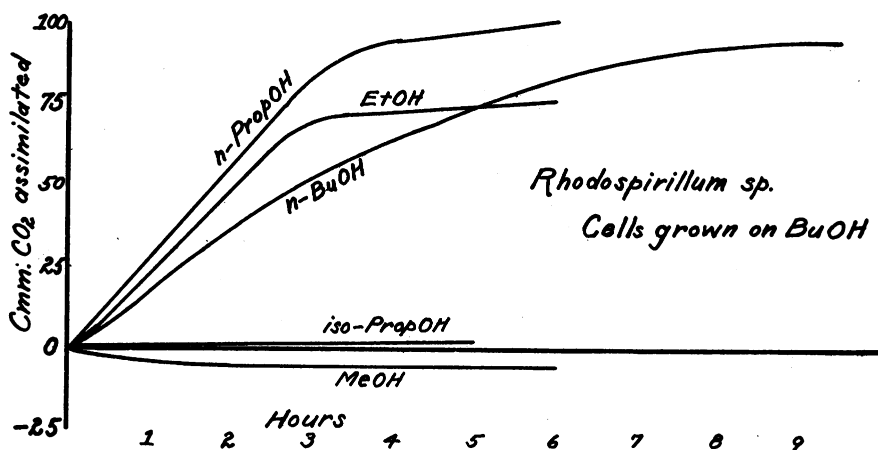


FIG. 2. PHOTOSYNTHETIC REDUCTION OF CO_2 BY RHODOSPIRILLUM SP. WITH LOWER ALCOHOLS AS SUBSTRATES

Results of experiments with higher alcohols are shown in figure 3. It is clear that the branched-chain alcohols are attacked much more slowly than the corresponding straight-chain compounds. This is in accord with biological properties generally ascribed to these compounds, and with similar observations of Gaffron (1935) on the rates of decomposition of straight-chain and branched fatty acids by non-sulfur purple bacteria. Of especial interest is the high rate of assimilation with the 6-carbon alcohol hexanol. The quantitative relations between the amount of CO_2 assimilation with different alcohols indicate an additional uptake of about 50 mm^3 of CO_2 per 0.005 mM CH_2, again in substantial agreement with previous findings for the behavior of non-sulfur purple bacteria in the presence of the homologous fatty acids (Gaffron, 1933; van Niel, 1935).

Determinations of initial and final bicarbonate in suspensions of *Rhodospirillum* sp. assimilating CO_2 in the presence of alcohols failed to show measurable acid or alkali production. This supports the previously described experiment

from which it was concluded that during the oxidation of primary alcohols fatty acids do not accumulate as oxidation products.

ADAPTIVE ENZYME FORMATION

When *Rhodospirillum* sp. was cultivated in malate medium instead of the alcohol media used previously, and the cell suspensions obtained therefrom allowed to act on various alcohols in the Warburg apparatus, curves typified by those in figure 4 were obtained. A pronounced lag was always observed which is characterized by a slow but gradual increase in the rate of CO_2 assimilation, i.e. utilization of the alcohol, from an imperceptible rate to a maximum repre-

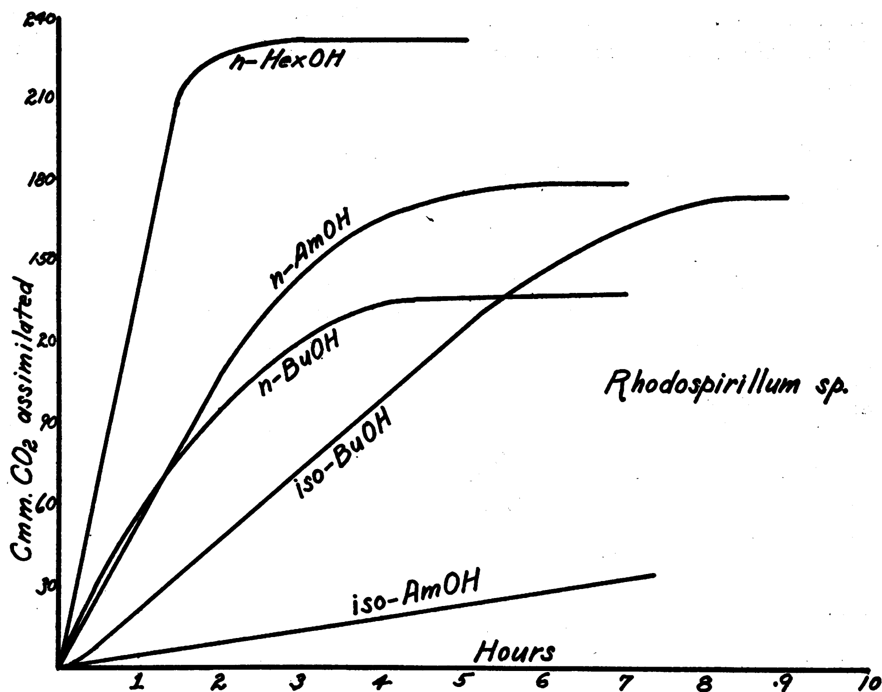


FIG. 3. PHOTOSYNTHETIC REDUCTION OF CO_2 BY RHODOSPIRILLUM SP. WITH HIGHER ALCOHOLS AS SUBSTRATES

sented by the straight line portion of the curves. These suspensions appeared inert towards the alcohols for a period as long as 2 hours before oxidation commenced. Indeed, several previous experiments had been terminated after the first hour or so, due to the failure to observe appreciable activity of the cells towards the alcohols as measured by CO_2 assimilation. Since it is a common experience to encounter feebly active suspensions of these photosynthetic bacteria, such results were initially ascribed to physiological damage to the organism before or during the preparation of the suspension (see also Gaffron, 1935). However, the new observations suggested a different explanation.

The initial period of apparent inactivity followed by rapidly increasing rate of action up to a maximum is characteristic of bacterial systems involving adaptive enzyme formation (Karström, 1930, 1937; Stephenson, 1939). Under

the conditions of these experiments where dense suspensions of cells are used in the absence of assimilable nitrogen and minerals, i.e., "resting suspensions," the increased activity cannot readily be attributed to increase in cell numbers during this time, but to a gradual change in the physiological properties of the organisms, induced by the presence of the particular substrate, i.e. the formation of adaptive enzymes.

The experimental results show that cells of *Rhodospirillum* sp. grown in malate, either lack completely the ability to attack alcohols or possess it to such an insignificant extent that it remains undetectable for a more or less considerable period of time. Since the decomposition of the alcohols can assume a normal, high rate after a prolonged period of exposure of the cells to these substrates,

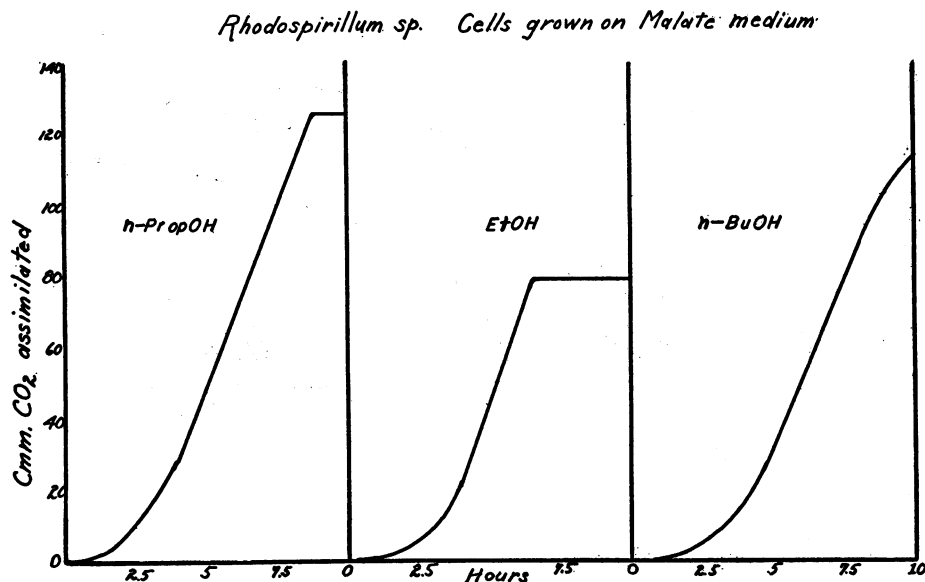


Fig. 4. Alcohol Oxidation by Cells of Rhodospirillum sp. Grown in the Absence of Alcohol

such findings imply the formation of adaptive enzymes for alcohol decomposition. Furthermore, the response of a cell suspension to ethanol, *n*-propanol, and *n*-butanol is essentially the same as regards both the length of the lag period and the duration of the adaptation process. In view of the virtual certainty that the decomposition of the alcohols proceeds by way of a dehydrogenation, the conclusion may be drawn that the adaptive enzyme involved is one concerned with the dehydrogenation of the primary alcohol group. These experiments furnish the first reported example of adaptive formation of an alcohol dehydrogenase, nay, even of an oxidative enzyme system.

A direct comparison of alcohol oxidation with cells grown in the absence and in the presence of alcohol was now made. Regular 0.1% *n*-butanol and 0.2% Na malate media, respectively, were used to cultivate the cells, each receiving inoculum from the same source. After three days' incubation in the light, the

cells were collected by centrifugation, washed once with sterile tap water and resuspended in 0.05% $NaHCO_3$ for use in the respirometers. In each case 0.005 mM of ethanol and *n*-butanol were supplied as substrates, respectively. Figure 5 demonstrates that cells grown in alcohol commence to oxidize both alcohols immediately and at the maximum rate from the beginning. Cells grown in the absence of alcohol failed to oxidize perceptibly the same alcohols for a period of one hour, and after that with gradually increasing rate up to a maximum; the former were completely adapted to alcohol, whereas the latter went through the adaptation process in the respirometer.

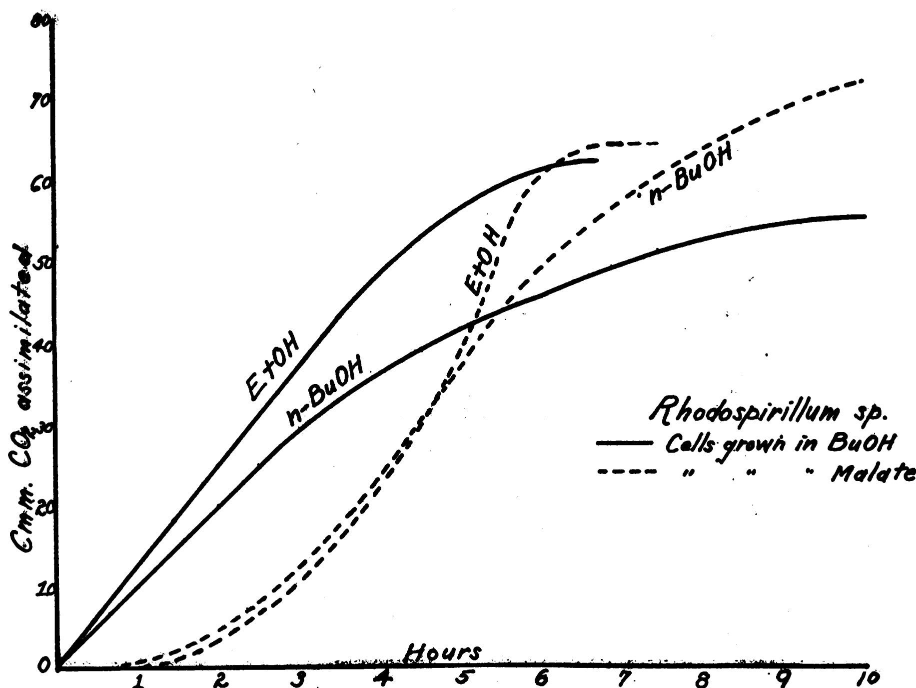


FIG. 5. ALCOHOL OXIDATION BY CELLS OF RHODOSPIRILLUM SP. GROWN IN THE PRESENCE OF ALCOHOL (ADAPTED) AND IN THE ABSENCE OF ALCOHOL (UNADAPTED)

HYDROGENASE ADAPTATION

A striking example of adaptive enzyme formation in *Rhodospirillum* sp. is shown in its behavior toward hydrogen gas. The photosynthetic assimilation of carbon dioxide with molecular hydrogen by purple bacteria is known from the work of Roelofson (1935) with sulfur purples and from that of Gaffron (1935) with non-sulfur purples. In none of the previously described experiments (see also French, 1937; Wessler and French, 1939; van Niel, 1936, unpublished) with different purple bacteria utilizing molecular hydrogen was the adaptation phenomenon recorded. French (1937) observed that ". . . . The marked increase in rate with time. . . . " (referring to figure 2, p. 715) may well have been due to adaptive enzyme formation.

Malate-grown cells suspended in 0.05 per cent $NaHCO_3$ solution were exposed in a respirometer to a gas phase consisting of 5 per cent CO_2 in H_2. For two hours there was no detectable hydrogenase activity and for 4.5 hours after that the action was barely measurable. Only after a total exposure time of about 8 hours had enzymatic adaptation to hydrogen gas definitely commenced. It increased rapidly thereafter up to a maximum attained after 10 hours, as shown in figure 6. In this experiment the adaptation was complete about 6 hours after the first signs of its commencement.

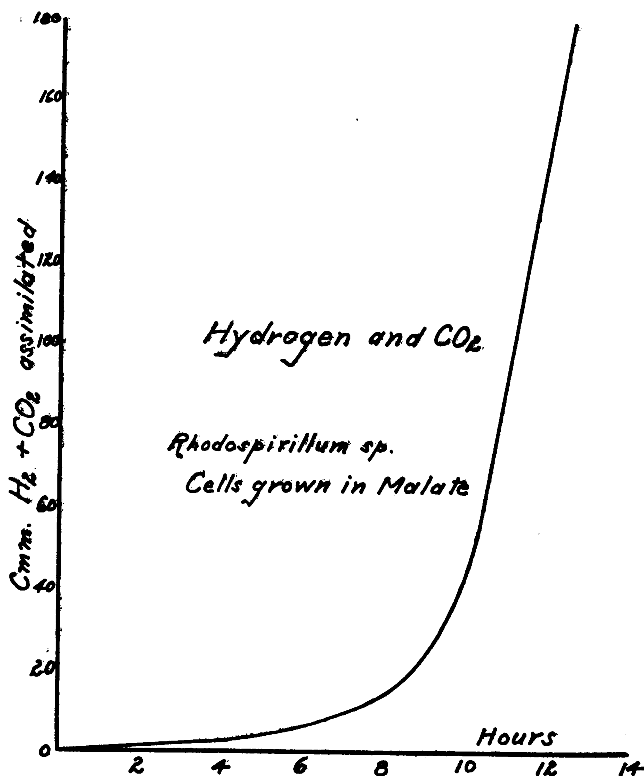


FIG. 6. ADAPTIVE FORMATION OF HYDROGENASE BY RHODOSPIRILLUM SP.

DISCUSSION

Any new instance of rapid formation of enzyme in the presence of a specific substrate carries with it the responsibility for supplying absolute proof that the phenomenon in question is truly an example of adaptive enzyme formation in the sense of Karström and not one of "selection" of a small portion of the total population capable of responding to the specific substrate. This idea has been adequately expanded elsewhere (Lwoff and Audureau, 1941; Stephenson, 1939; Yudkin, 1932). Decisive proof may not always be obtainable; indeed, the only approach apparently available is an indirect one, e.g., proof of the first alternative by eliminating the possibility of the second. Often this is done by demonstrating

the absence of significant growth during the adaptation period (Stephenson and Yudkin, 1936; Stephenson and Stickland, 1933). This latter demonstration is contingent upon establishing the absence of increase in cell material during the adaptation process, else the phenomenon may be viewed as an ordinary manifestation of growing cells.

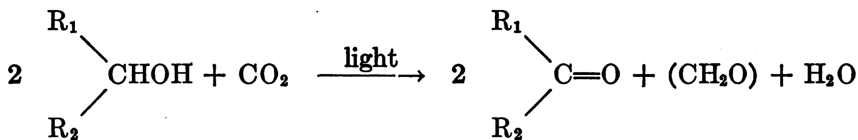
The following consideration makes it highly improbable that the experiments expressed in figures 4, 5 and 6 could be due to selection. The selection idea connotes that in any large bacterial population there always exist a small number of individuals which differ from the masses by their ability to effect a particular function, in this case, to dehydrogenate alcohols. In the presence of a specific substrate, e.g., alcohols, only these few individuals are able to proliferate, whereas the majority of the cells present are unable to attack the alcohol. Multiplication of these few is, therefore, favored by the available substrate and is accompanied by a corresponding increase in rate of metabolic change, manifested in this case by CO_2 assimilation. Since the rate at the beginning of the adaptation is virtually negligible, it must be concluded that the numbers of cells initially able to dehydrogenate alcohols must have been relatively negligible. And yet, if the selection theory were to apply, in the short space of a few hours these few cells must have become numerous enough to attack the alcohol at the same rate as the suspension already fully adapted as a result of having been harvested from alcohol-containing media. This means the number of alcohol dehydrogenating cells in the former instance must finally have been approximately equal to that in the suspension prepared from alcohol medium. This would have had to take place in the absence of other available minerals and nitrogen supply, and although it is conceivable that dead and autolyzing cells could be the source for these, it is extremely unlikely that more than very small amounts would be liberated in available form under the above conditions. The assumption that such an astonishing rate of proliferation would take place within a few hours is, of course, wholly inconsistent with our fundamental concepts of bacteriology. The remaining possibility is that all the living cells which initially were unable to attack the alcohol became capable of doing so as a result of exposure to the alcohol for a few hours, thus fulfilling the definition of adaptive enzyme formation.

Not to be overlooked in this discussion are van Niel's (1936) experiments in which a similar induction period was observed during photosynthetic utilization of salts of fatty acids by a species of sulfur purple bacteria, viz., *Chromatium* sp. This lag period could be eliminated by the addition of suitable concentrations (approximately M/400) of any one of a number of inorganic salts, i.e., K_2SO_4, NaCl, or $NaHCO_3$. The effect was non-specific. These findings call for a cautious evaluation of the alcohol adaptation results since it is conceivable that some similar, as yet entirely unappreciated, non-specific influence may account for the observed results. However, this possibility seems remote in the experiments reported herein: K_2SO_4 had no effect on the adaptation to alcohol; no induction period was observed for fatty acid oxidation by *Rhodospirillum* sp.; different concentrations of $NaHCO_3$ affected the oxidation rate only insofar as the CO_2 concentration limited the rate of photosynthesis; and, finally, the

adaptation is a consequence only of exposure to alcohol, all other factors (washings, $NaHCO_3$ concentrations, etc.) being identical; hence, the adaptation would appear to be interpretable only as a relation between the cells and the alcohols directly. The ideas suggested by van Niel as possible explanations of the salt effect on fatty acid decomposition by *Chromatium* center around the overcoming of toxic effects of whatever free fatty acid was existent as a result of dissociation at acid pH's. This could not, of course, apply to the alcohols which are non-dissociable. Finally, van Niel's discussion (1936, p. 343) indicates that the peculiar results he observed were not obtained with non-sulfur purple bacteria, in which category *Rhodospirillum* sp. lies.

SUMMARY

Seventy strains of non-sulfur purple photosynthetic bacteria all capable of utilizing various alcohols for the photosynthetic reduction of CO_2 were isolated by specific enrichment culture techniques. One strain, resembling *Streptococcus varians*, oxidizes a number of secondary alcohols to the corresponding ketones, meanwhile stoichiometrically assimilating CO_2 in the light, according to the following general equation:



$$2\ \begin{matrix}R_1 \\ \quad\diagdown \\ \quad\ \ CHOH \\ \quad\diagup \\ R_2\end{matrix} + CO_2 \xrightarrow{\text{light}} 2\ \begin{matrix}R_1 \\ \quad\diagdown \\ \quad\ \ C{=}O \\ \quad\diagup \\ R_2\end{matrix} + (CH_2O) + H_2O$$

Another strain, *Rhodospirillum* sp., oxidizes only primary alcohols and does not attack secondary alcohols. This organism requires the calcium ion for normal growth and motility, and its peculiar cultural characteristics in the absence of added calcium are described. Alcohol oxidation by *Rhodospirillum* sp. occurs only after adaptive enzyme formation takes place when the organism has been cultivated in the absence of alcohol. Cells grown in the presence of alcohol attack alcohol immediately and at maximum rate. Hydrogenase also is an adaptive enzyme in *Rhodospirillum* sp.

I wish to record here my extreme gratitude to Professor van Niel for the inspiration resulting from my association with him, for his unfailing counsel during these investigations, and for constructive aid in the preparation of the manuscript.

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