# A COMPARATIVE STUDY OF THE LIGHT AND DARK FERMENTATIONS OF ORGANIC ACIDS BY RHODOSPIRILLUM RUBRUM[1]

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Received for publication December 5, 1950

The photosynthetic bacterium, *Rhodospirillum rubrum*, rapidly ferments pyruvic acid and dicarboxylic acids in the light, with the formation of $CO_2$, $H_2$, and other products as yet unidentified (Gest, Kamen, and Bregoff, 1950). Evolution of $H_2$ in the presence of these substrates is a light-dependent reaction that is completely suppressed by molecular nitrogen and ammonium salts. These observations suggest that the photochemically liberated $H_2$ is derived from an intermediate carrier that is also functional as a hydrogen donor in the process of nitrogen reduction.

Molecular hydrogen is a commonly observed product in the fermentative metabolism of various heterotrophic bacteria. In a number of these cases, it appears that formic acid is produced as an intermediate and that $H_2$ is formed from this compound as a result of hydrogenlyase activity. In other instances, formic acid is not attacked by organisms capable of liberating $H_2$ from more complex compounds. This suggests the existence of another mechanism of $H_2$ formation (Stephenson, 1947). Although the evolution of $H_2$ by *R. rubrum* appears to be light-dependent, it is evident that the $H_2$ may be produced by dark reactions from a precursor that is formed only as the result of photochemical activity. It is consequently necessary to determine the nature of the fermentative metabolism of *R. rubrum* in the dark as well as in the light before a comparison of light-dependent and heterotrophic $H_2$ production can be attempted.

In the present studies, the anaerobic decomposition of several organic acids by *R. rubrum* in the light and in the dark has been investigated from this point of view.

## EXPERIMENTAL METHODS

*Growth of bacteria and preparation of resting suspensions.* In order to obtain younger and more active cells of *R. rubrum*, strain SI, the methods of cultivation previously described by Gest and Kamen (1949) have been modified as indicated below. The bacteria were transferred daily using a 3 per cent inoculum in a liquid medium of the following composition:

[1] This investigation was performed under a contract (No. N6-ori-208, T.O.1) administered by the Office of Naval Research for the Atomic Energy Commission.

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*Medium G5*

| | | |
|---|---|---|
| Distilled water | 1 | liter |
| Difco peptone | 5 | g |
| Difco yeast extract | 5 | g |
| L-Glutamic acid | 4 | g |
| DL-Malic acid | 3.5 | g |
| $KH_2PO_4$ | 120 | mg |
| $K_2HPO_4$ | 180 | mg |

pH adjusted to 7 with NaOH before autoclaving.

Fifty-ml stock cultures (in 150-ml bottles) were incubated at a temperature of 30 to 32 C under constant illumination. A bank of 100-watt incandescent lamps, placed approximately 5 cm from the cultures, was employed as a source of light. To compensate for the heat given off by the lamps, a fan was used to circulate the air in the vicinity of the cultures.

Bacteria for metabolic experiments were obtained by inoculating the medium described below:

*Medium G3X*

| | | |
|---|---|---|
| Distilled water | 1 | liter |
| $MgSO_4 \cdot 7H_2O$ | 200 | mg |
| $FeSO_4 \cdot 7H_2O$ | 5 | mg |
| $CaCl_2 \cdot 2H_2O$ | 50 | mg |
| Trace element solution[3] | 1 | ml |
| $KH_2PO_4$ | 600 | mg |
| $K_2HPO_4$ | 900 | mg |
| L-Glutamic acid | 2 | g |
| DL-Malic acid | 6 | g |
| Biotin | 13.7 | $\mu$g |
| Difco yeast extract | 600 | mg |

pH adjusted to 6.7 with NaOH before autoclaving.

To start a culture in the G3X medium, 2.5 ml of a 24- to 36-hour stock culture (G5) were added to 175 ml of medium sterilized in a 300-ml pyrex reagent bottle, and the bottle was then completely filled with sterile medium; aseptic precautions were used. The bottle was closed with an autoclaved glass stopper, and the latter was fastened securely with adhesive tape. Bottle cultures prepared in this manner were incubated in the light as noted above. Luxuriant growth accompanied by the formation of large quantities of $CO_2$ and $H_2$ was obtained in 24 to 36 hours. For the preparation of resting cell suspensions, the cells from one bottle were harvested, washed with 25 ml of 1 per cent KCl, and suspended in 25 ml of 0.05 M phosphate buffer pH 6.8. Cells that were grown in this manner are referred to as "normal cells."

"Dark-grown" cells were also cultivated in the G3X medium. Shallow-layer cultures were incubated in the dark at 30 C and thoroughly aerated by continuous shaking. The organisms were subcultured in this manner at least once before use. For resting suspensions, the cells from 1,200 ml of medium were

[3] Per 100 ml distilled water: $H_3BO_3$, 280 mg; $MnSO_4 \cdot 4H_2O$, 210 mg; $Cu(NO_3)_2 \cdot 3H_2O$, 20 mg; $Na_2MoO_4 \cdot 2H_2O$, 75 mg; $CoCl_2$, 20 mg; $Zn(NO_3)_2 \cdot 6H_2O$, 25 mg.

harvested, washed with 50 ml of 1 per cent KCl, and suspended in 25 ml of 0.05 M phosphate buffer pH 6.8.

*Analytical procedures.* The volatile acid content of growing cultures was determined by steam distillation. A 200-ml aliquot of the culture was adjusted to pH 1 to 2 by the addition of $H_2SO_4$, and the suspension was centrifuged. The volume of the supernatant was reduced to 10 ml by ordinary distillation, and then 10 volumes were distilled over with steam. Carbon dioxide was removed from the distillate by refluxing it for 15 minutes, and the entire solution was titrated with $CO_2$-free 0.05 N NaOH, using phenolphthalein as the indicator.

Identification and estimation of fatty acids produced by resting cells were accomplished by chromatographic separation on celite columns according to the methods developed by Yale and Bueding (1950). Their procedures, which will be published in detail at a later date,[4] were followed in all particulars except for the manner of sample preparation. Samples for analysis were prepared as follows: The acidified suspension from the Warburg vessel was diluted to a known volume (usually 10 ml) and centrifuged. Approximately 2 to 4 ml of supernatant, ordinarily containing 5 to 20 $\mu$M of fatty acids, were made alkaline

TABLE 1

*Recovery of individual acids from a mixture using the column buffered at pH 6*

| ACID* | RECOVERY (%) |
|---|---|
| Butyric | 93 |
| Propionic | 94 |
| Acetic | 88 |

* Approximately 20 $\mu$M of each were present in the mixture.

to thymol blue by the addition of a few drops of 6 N NaOH, and the solution was evaporated to dryness over a hot plate or in an oven at 110 C. After cooling, 2 ml of chloroform (equilibrated with 2 M phosphate buffer) and 1 drop of concentrated $H_2SO_4$ were added to the residue, and the latter was thoroughly rubbed with a stirring rod to facilitate extraction of the fatty acids into the chloroform. The chloroform was decanted and the residue treated in the same manner two additional times, giving a total of 6 ml of chloroform extract. This extract was permitted to flow into a 4-g celite column, and the individual acids were eluted as described by Yale and Bueding (1950). The data of table 1 indicate typical recoveries from a mixture of known acids carried through the procedure described above, with a column buffered at pH 6 (the type of column most frequently used in these experiments). Control columns were run using 20 $\mu$M samples of malate, succinate, fumarate, lactate, and pyruvate; none of these compounds were eluted from the pH 6 column in appreciable quantity during development with the usual amounts of the three solvents. All fatty acid

[4] We wish to thank Doctor Bueding (Department of Pharmacology, Western Reserve University School of Medicine) for his courtesy in making the details available to us at this time.

titers given in the results have been corrected for blank titration values, but not for yield from the column (table 1).

Formic acid was determined by oxidation with HgO to $CO_2$ and also characterized by the partition constant between ether and water as described by Osburn, Wood, and Werkman (1933). Residual pyruvic acid was estimated by the direct method of Friedemann and Haugen (1943). Standard samples of pyruvic acid containing the same concentration of $H_2SO_4$ as the unknowns were analyzed at the same time.

*Manometric techniques.* The fermentations were run in 25-ml Warburg vessels at 30 C with helium as the gas phase. In all experiments, 1.5 to 1.7 ml of cell suspension were placed in each vessel; upon the addition of substrates, etc., the final volume was 2 ml. Light and dark exposures were made simultaneously in a bath separated into two halves by a stainless steel baffle; on the light side vessels were illuminated by a bank of 75-watt reflector flood lamps (General Electric) suspended above the bath.[5] The dark side of the bath was covered with a black cloth. After equilibration in the dark, substrates were tipped in, and the lamps were immediately turned on. Ordinarily, the experiments were terminated by tipping in $H_2SO_4$ from a side arm when the pressure changes had become negligible. $H_2$ was determined as alkali-insoluble gas.

*Preparation of substrates.* Sodium pyruvate was prepared by slowly neutralizing freshly distilled pyruvic acid in the cold with $NaHCO_3$ to a pH of about 5. After removal of excess $CO_2$ by aeration with helium, the pH of the solution was usually between 6 and 7. Before use, the pyruvate solution was quantitatively assayed with yeast carboxylase and by $Ce(SO_4)_2$ oxidation. Both assays gave the same result, indicating that the pyruvate was 100 per cent biologically active. Malate, fumarate, succinate, and lactate were prepared by neutralizing solutions of the free acids to a pH of 7 with NaOH.

## RESULTS

*Volatile acid formation in growing cultures.* The only reported analyses for volatile acids in growing cultures of photosynthetic bacteria are those given by Muller (1933) for various sulfur purple bacteria growing anaerobically in the light in a variety of media. Muller analyzed his cultures only after long incubation periods and found no evidence for volatile acid accumulation, except in the case where pyruvate was the carbon source for growth; in this instance, small but significant quantities of volatile acid were detected. Relatively large amounts of volatile acid have been found to accumulate in cultures of the nonsulfur purple bacterium, *R. rubrum*, growing anaerobically in the G3X medium under the conditions described above. Representative results are given in table 2.

Identification of the volatile acids was made by chromatographic separation. Analysis with the pH 3.5 column indicated the presence of formic acid only.

[5] Because of the heat dissipated by the lamps, it was necessary to have a fan blowing air across the top of the bath during the experiments. Satisfactory temperature control was obtained by this crude arrangement.

This result was substantiated by mercuric oxide oxidation of a larger sample; the $CO_2$ yield was 102.2 per cent of theory. Water-ether partition constants compared favorably with the values obtained for formic acid by Osburn *et al.* (1933).

TABLE 2

*Volatile acid formation in anaerobic cultures of R. rubrum (SI) growing in the light in G3X medium*

| | AGE OF CULTURE | VOLATILE ACID* |
|---|---|---|
| | *hours* | *mM/liter* |
| Experiment A | 33 | 1.5 |
| | 48 | 3.5 |
| | 70 | 6.8 |
| | 94 | 8.9 |
| Experiment B | 26 | 1.1 |
| | 51 | 4.1 |
| | 76 | 6.1 |
| | 97 | 10.4 |
| | 121 | 13.1 |
| | 148 | 13.1 |
| Experiment C | 40 | 1.3 |
| | 68 | 6.5 |
| | 113 | 13.9 |
| | 142 | 15.8 |

* The volatile acid content of control cultures incubated in the dark was usually about 0.4 mM per liter, regardless of age.

TABLE 3

*Influence of the nitrogen source on volatile acid formation in anaerobic cultures of R. rubrum (SI)*

(Millimoles of volatile acid per liter)

| AGE OF CULTURE | LIGHT | | DARK | |
|---|---|---|---|---|
| | $NH_4Cl$* | Glutamic acid† | $NH_4Cl$* | Glutamic acid† |
| *hours* | | | | |
| 42 | 2.0 | — | — | — |
| 71 | 1.5 | — | — | — |
| 91 | 1.9 | 8.4 | — | — |
| 116 | 2.2 | — | 0.4 | 1.1 |

* G3X medium; with 1 g of $NH_4Cl$ per liter substituted for glutamic acid.
† G3X medium.

It is of interest that volatile acid accumulation is greatly decreased when 1 g of $NH_4Cl$ per liter is substituted for the glutamic acid in the G3X medium (table 3). In this modified medium, growth of the organism is rapid and luxuriant, but $H_2$ is not produced in significant quantity. The results of table 3 suggest that Muller's failure to detect volatile acid may have been due to the fact that he used an ammonium salt as the sole source of nitrogen.

*Fermentation of organic acids by "normal" resting cells.* It has been reported (Nakamura, 1937, 1939, 1941) that the nonsulfur purple bacterium *Rhodobacillus palustris* ferments various organic acids in the dark with the production of $H_2$. Previous experiments with *R. rubrum* (Gest, Kamen, and Bregoff, 1950) indicated little or no fermentation of these compounds in the dark, and it was consequently believed that pyruvate, fumarate, etc., can be metabolized by this organism only by means of a light-dependent mechanism. A more careful investigation of this point, however, using younger and more active cells has disclosed that *R. rubrum* can decompose some of these compounds in the dark at relatively slow rates. The results of a typical experiment indicating the relative rates of gas production from pyruvate in the light and in the dark are shown in figure 1.

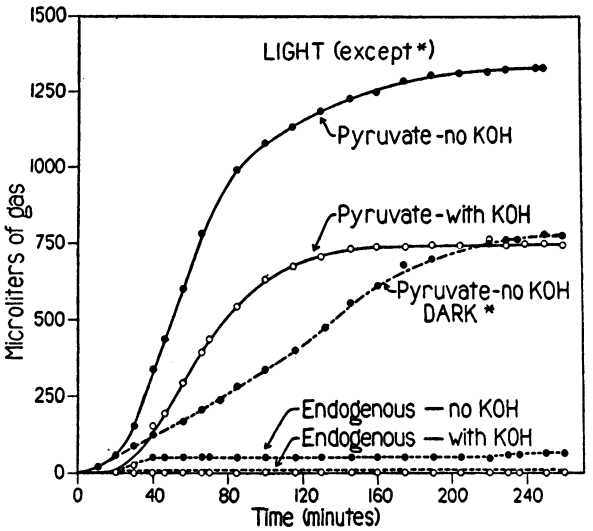


*Figure 1.* Fermentation of pyruvic acid by "normal" resting cells of *R. rubrum* in light and dark. In the dark, endogenous production of gases and the formation of $H_2$ from pyruvate were negligible. For the sake of clarity, these curves have been omitted (table 4).

In the light, there is a rapid evolution of $CO_2$ and $H_2$, usually in approximately equimolar quantities. The total amount of gas produced from a given quantity of pyruvate is somewhat variable, depending upon the age and previous history of the organisms. Decomposition of pyruvate in the dark, on the other hand, is considerably slower, and $CO_2$ is the only gas produced in significant amounts. In addition, considerable quantities of fatty acids are formed by suspensions in the dark. Analytical data for the experiment depicted in figure 1 are given in table 4.

It is evident from the results that the decomposition of pyruvate in the dark by *R. rubrum* is a typical propionic acid fermentation. Removal of $CO_2$ (by the presence of KOH) from the suspension has a marked effect; the utilization of pyruvate is inhibited, and accordingly there is a marked decrease in the amounts of acetic and propionic acids produced. These results suggest that pyruvate is

partially converted by $CO_2$ fixation to a dicarboxylic acid that is a precursor of propionic and acetic acids. Several recent reports lend support to the view that succinic acid, formed by $CO_2$ fixation, may be an intermediate in the production of propionic acid from pyruvate by true propionic acid bacteria. For example, Delwiche (1948) has reported the formation of propionic acid at appreciable rates from succinate by *Propionibacterium pentosaceum*. If dicarboxylic acids were important intermediates in the present instance, it would be expected that these compounds would be decomposed at significant rates in the dark with the formation of the end products noted above. This is clearly not the case, as indicated by the data given in table 5. As compared with the rate of pyruvate fermentation usually observed, the dicarboxylic acids are decomposed in the dark very slowly, if at all, under the conditions of these experiments. It would

TABLE 4

*Fermentation of pyruvic acid by "normal" resting cells of R. rubrum in light and dark*

| CONDITIONS | $CO_2 + H_2$ | $H_2$ | PROPIONIC ACID | ACETIC ACID | RESIDUAL PYRUVIC ACID |
|---|---|---|---|---|---|
| | μM | μM | μM | μM | μM |
| *Light* | | | | | |
| Endogenous; no KOH | 4.9 | — | 0 | 1.9 | — |
| Endogenous; KOH present | — | 0 | 0 | 1.7 | — |
| Pyruvic acid; no KOH | 74.9 | — | 0 | 3.8 | 1.9 |
| Pyruvic acid; KOH present | — | 32.0 | 0 | 3.7 | 2.4 |
| *Dark* | | | | | |
| Endogenous; no KOH | 1.8 | — | 2.0 | 2.9 | — |
| Endogenous; KOH present | — | 0 | 0.7 | 1.1 | — |
| Pyruvic acid; no KOH | 36.4 | — | 13.1 | 23.6 | 0.9 |
| Pyruvic acid; KOH present | — | 2.0 | 3.0 | 9.4 | 19.5 |

To 40-hour-old cells 42.3 μM of Na-pyruvate were supplied under the conditions described in "Experimental Methods." The experiment was terminated after 4.5 hours by the addition of $H_2SO_4$ from the side arms. The pH 6.0 column was used for fatty acid analysis.

appear that the relatively slow fermentation of dicarboxylic acids in the dark is not due to poor permeability since the addition of a hydrogen acceptor such as $K_3Fe(CN)_6$ under these conditions induces rapid decomposition (Gest, 1950*a*).

In contrast to the results observed in the dark, acetic and propionic acids have not been found to accumulate in appreciable quantity as a result of the light-induced fermentations (tables 4 and 5). A further point of difference is found in the fact that utilization of pyruvate in the light is not particularly affected by the $CO_2$ tension (table 4). In this connection it must be emphasized that the $CO_2$ tension existing in the suspension when KOH is present in the system is determined by the balance between the rate of $CO_2$ production and the rate of removal by KOH absorption. The concentration of $CO_2$ in solution will consequently depend on the particular conditions employed (pH, density and activity of cell suspensions, etc.). It is possible that under conditions slightly

different from those used here, the presence of KOH in the system will have a decided effect on the extent or manner in which a substrate such as pyruvate is utilized in the light.[6]

*Fermentation of pyruvic acid by "dark-grown" resting cells.* Several types of nonsulfur purple bacteria can be grown in the absence of light provided the organisms are supplied with oxygen (van Niel, 1944).[7] *R. rubrum* (SI) can be rapidly grown in moderately good yield in this manner in the G3X medium described above. The cells obtained are morphologically similar to those grown anaerobically in the light, but, instead of having the typical deep-red pigmentation, they are light pink in color. Pyruvic acid is fermented by "dark-grown" cells in the light and in the dark as indicated in figure 2.

TABLE 5

*Fermentation of various organic acids by R. rubrum in the light and dark*

| SUBSTRATE | LENGTH OF FERMENTATION | $CO_2 + H_2$* | PROPIONIC ACID | ACETIC ACID |
|---|---|---|---|---|
| | *hours* | *μM* | *μM* | *μM* |
| *Light* | | | | |
| Endogenous | 3.5 | 5.4 | 0 | 0 |
| Na-lactate | 3.5 | 72.1 | 0 | 0 |
| Na-succinate | 2.0 | 55.5 | 0 | 0 |
| Na-fumarate | 2.0 | 43.5 | 0 | 0 |
| Na-malate | 2.0 | 49.0 | 0 | 0 |
| *Dark* | | | | |
| Endogenous | 7.0 | 2.8 | 1.4 | 1.9 |
| Na-lactate | 7.0 | 2.6 | 1.2 | 2.0 |
| Na-succinate | 7.0 | 3.8 | 2.6 | 3.1 |
| Na-fumarate | 7.0 | 10.6 | 7.2 | 5.9 |
| Na-malate | 7.0 | 10.6 | 7.2 | 5.7 |

To 40-hour-old cells 20 μM of each substrate were supplied. Volatile acids were determined by the column buffered at pH 6.

* In the dark, $CO_2$ is the only gas produced.

During the first 1 to 2 hours, the gas production by cells exposed to light is essentially the same as that of cells kept in darkness. After this time, however, the cells produce $CO_2$ and $H_2$ in amounts ordinarily observed with "normal" cells in the light. As is the case for "normal" cells, only negligible quantities of acetic and higher fatty acids are found in the suspension at the end of the experiment (table 4).

It appears then that cells grown completely heterotrophically still retain the capacity to react to light. The resumption of a photosynthetic type of metabolism

[6] Effects of this kind in the light metabolism of photosynthetic bacteria have been recently noted by Siegel and Kaman (1950).

[7] Photosynthetic bacteria have not been successfully cultivated under anaerobic conditions in the dark. We have attempted to grow *R. rubrum* (SI) anaerobically in the dark in media containing hydrogen acceptors such as $K_3Fe(CN)_6$ without success.

after a lag period in the light may be superficially described as an "adaptive" change. There are, however, no data available as yet which indicate that the transition is conditioned by formation of adaptive enzymes.[8]

The analytical data for the experiment described in figure 2 are summarized in table 6. A comparison of these data with those obtained using "normal" cells (table 4) discloses a number of significant differences. In contrast to the "normal" cells, "dark-grown" organisms usually show (a) distinctly higher endogenous gas production; (b) definite formation of $H_2$ in the dark in the presence of pyruvate; and (c) no significant effect of $CO_2$ tension on the extent of pyruvate utilization in the dark. Although, as indicated previously, the absence of a $CO_2$ effect in certain instances may be related to the particular conditions of the experiment, this result was consistently obtained with "dark-

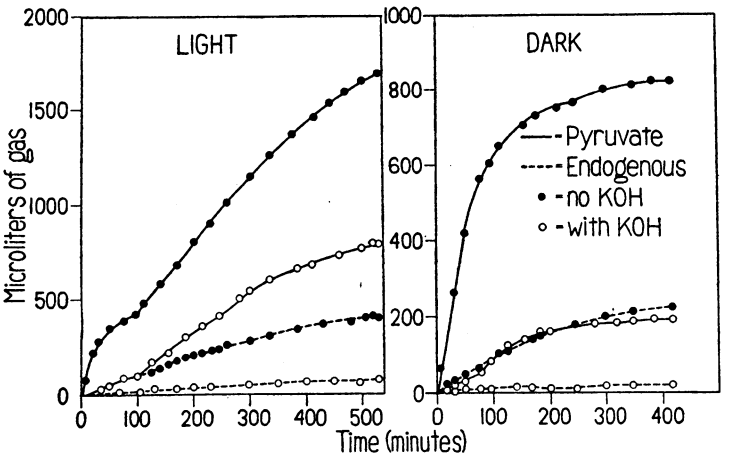


*Figure 2.* Fermentation of pyruvic acid by "dark-grown" resting cells of *R. rubrum* in light and dark (table 6).

grown" cells. Even though there was no effect of $CO_2$ removal on substrate disappearance, the presence or absence of KOH in the system did have a definite influence on the dark production of fatty acids.

In the presence of substrate, removal of $CO_2$ from the suspension results in a diminished yield of propionic acid, whereas the acetic acid titer is relatively unaffected. The absorption of $CO_2$ also results in a marked increase in the amounts of butyric, valeric, and caproic acids formed. It should be noted that "dark-grown" cells display a rather high endogenous acid production as well as gas production. The endogenous production of propionic and higher fatty acids is influenced by $CO_2$ tension in the same way that formation of these acids from pyruvate is affected. Consistent changes in endogenous formation of

[8] It has been observed that the pigmentation of "dark-grown" cells becomes noticeably more intense during anaerobic exposure to light for several hours, particularly in the presence of substrates. Similar phenomena have been noted in various higher plants (Strain, 1949).

acetic acid dependent on $CO_2$ tension, however, have not been observed (e.g., compare tables 6 and 7).

Estimation and identification of the fatty acids produced in the dark were accomplished using columns buffered at pH 6 and pH 7.55. The results are

TABLE 6

*Fermentation of pyruvic acid by "dark-grown" cells of R. rubrum*

| CONDITIONS | $CO_2 + H_2$ | $H_2$ | BUTYRIC, VALERIC, AND CAPROIC ACIDS* | PROPIONIC ACID | ACETIC ACID | RESIDUAL PYRUVIC ACID |
|---|---|---|---|---|---|---|
| | μM | μM | μM | μM | μM | μM |
| *Light* | | | | | | |
| Endogenous; no KOH.... | 21.7 | — | 0 | 0 | 1.4 | — |
| Endogenous; KOH present..... | — | 2.9 | 0 | 0 | 0.7 | — |
| Pyruvic acid; no KOH..... | 92.3 | — | 0 | 0 | 3.4 | 1.9 |
| Pyruvic acid; KOH present..... | — | 36.0 | 0 | 0 | 5.3 | 1.8 |
| *Dark* | | | | | | |
| Endogenous; no KOH..... | 10.8 | — | 3.8 | 13.5 | 12.1 | — |
| Endogenous; KOH present..... | — | 1.3 | 7.3 | 3.1 | 6.8 | — |
| Pyruvic acid; no KOH..... | 35.4 | — | 2.9 | 24.7 | 35.5 | 1.0 |
| Pyruvic acid; KOH present..... | — | 8.2 | 11.3 | 9.4 | 29.7 | 1.1 |

To 40-hour-old cells grown in the dark as described in "Experimental Methods," 37.3 μM of Na-pyruvate were supplied. The experiment was terminated after approximately 9 hours by tipping in $H_2SO_4$ from the side arms.

* Volatile acids were estimated with the column buffered at pH 6, which determines butyric, valeric, and caproic acids as a group.

TABLE 7

*Fatty acid formation by "dark-grown" cells of R. rubrum in the dark*

| CONDITIONS | $CO_2 + H_2$ | $H_2$ | CAPROIC ACID | VALERIC ACID | BUTYRIC ACID | PROPIONIC ACID | ACETIC ACID | RESIDUAL PYRUVIC ACID |
|---|---|---|---|---|---|---|---|---|
| | μM | μM | μM | μM | μM | μM | μM | μM |
| Endogenous; no KOH.. | 17.5 | — | ←——— | 5.2 | ———→ | 21.6 | 6.1 | — |
| Endogenous; KOH present ..... | — | 1.6 | 0.6 | 13.3 | 3.8 | 4.1 | 11.9 | — |
| Pyruvic acid; no KOH.. | 56.0 | — | ←——— | 3.0 | ———→ | 36.4 | 28.2 | 3.7 |
| Pyruvic acid; KOH present..... | — | 6.4 | 3.6 | 16.9 | 8.6 | 10.1 | 30.4 | 4.5 |

To 40-hour-old cells grown in the dark as described in "Experimental Methods," 58 μM of Na-pyruvate were supplied. The experiment was terminated after 7.5 hours by the addition of $H_2SO_4$. A column buffered at pH 7.55 was used to determine butyric, valeric, and caproic acids individually. Propionic and acetic acids were separated on the pH 6 column.

given in table 7. It is evident from the analytical data that $CO_2$ absorption results in a striking increase in the valeric acid titer.

Adequate fermentation balances cannot be obtained from the data given in tables 6 and 7 for several reasons. In the dark fermentations, the amount of

gas and acids produced endogenously is relatively large, and only arbitrary assumptions can be made regarding the effect of pyruvate utilization on the endogenous metabolism. In addition, the relative amounts of $CO_2$ and $H_2$ produced cannot be estimated with confidence by the usual methods employed here because of the effects of $CO_2$-absorption on the course of the fermentation. Similar considerations apply to the light-induced fermentations by both "normal" and "dark-grown" cells, with the added complication that the fate of the substrate carbon not converted to $CO_2$ in the light is still completely obscure. For these reasons, the analytical data must be considered primarily in a qualitative sense.

## DISCUSSION

Several investigators have noted the endogenous production of gases and acidic products by resting cells of various types of purple bacteria incubated in the dark under anaerobic conditions (Gaffron, 1934; Roelofsen, 1935). The acidic products were not identified, and it was claimed that formation of these acids could not be materially enhanced by the addition of substrates. These observations, together with the fact that no one has been able to grow the organisms anaerobically in the dark, led to the view that the fermentative metabolism was of a peculiar nature. It has been suggested that, although growth cannot occur on the basis of these reactions, the latter might be suitable for maintaining the organisms during anaerobic dark periods (Muller, 1933; Roelofsen, 1935; van Niel, 1944).

The experiments of Nakamura (1937, 1939, 1941) and those described here show clearly that certain photosynthetic bacteria can ferment externally supplied substrates anaerobically in the dark. In the case of "normal" cells of *R. rubrum* (SI), the end products of pyruvic acid decomposition are those typically observed in the fermentative growth of propionic acid bacteria, i.e., $CO_2$, and acetic and propionic acids. The utilization of pyruvate is to some extent $CO_2$-dependent and may accordingly proceed partially through the intermediate formation of a dicarboxylic acid.

Cells of *R. rubrum* (SI) grown aerobically in the dark effect a dark fermentation of pyruvic acid with the formation of the products noted above and also of $H_2$ and butyric, valeric, and caproic acids in varying amounts depending on the conditions. Although the utilization of pyruvate by these cells is not significantly affected by $CO_2$ tension, the character of the fermentation is influenced by this factor. It appears that the removal of $CO_2$ favors the formation of higher fatty acids, which are possibly synthesized by condensation reactions analogous to those occurring in the *Clostridia* (Bornstein and Barker, 1948). A likely mechanism for the effect of $CO_2$ on these syntheses cannot be derived from the limited data available.

In view of the fact that the dark fermentative metabolism of *R. rubrum* is similar to that of various heterotrophic bacteria, there is no reason for believing that photosynthetic bacteria cannot be grown under anaerobic conditions in the dark. Although *R. rubrum* does not grow appreciably in the usual media under these conditions, the cells remain viable for long periods, as indicated by the

fact that, when such cultures are transferred to the light, growth is immediately resumed.[9] It is possible that the addition of certain vitamins or cofactors, or both, to media containing substrates fermentable by resting cells in the dark will permit the development of photosynthetic bacteria in the absence of light.

The light-induced fermentation of pyruvic acid differs from the dark fermentation in two major respects: in the light, approximately 1 mole of $H_2$ is produced per mole of substrate decomposed, and only negligible quantities of acetic, propionic, and higher fatty acids accumulate. Several explanations can be offered for the absence of fatty acids under these conditions. It is conceivable that acids are produced by dark reactions and immediately utilized together with $CO_2$ by the photochemical reactions demonstrated by Gaffron (1933, 1935) with resting cell suspensions. The relative yield of $CO_2$ in the light and in the dark and the appearance of large quantities of $H_2$ in the light argue against this interpretation.

It seems more likely that in the light pyruvate is metabolized by a different series of reactions. The magnitude of the $H_2$ production suggests the occurrence of the phosphoroclastic reaction with formic acid or a similar compound being produced as an intermediate. Several observations are in support of the hypothesis that formate is the precursor of the $H_2$ produced. First, formic acid accumulates in cultures actively producing $H_2$ but does not in cultures growing without $H_2$ formation. Second, active cells of *R. rubrum* (SI) obtained from a malate-glutamate medium (G3X) immediately decompose formate in the dark with the production of $CO_2$ and $H_2$ (Gest, 1950*b*). Although these observations are suggestive, more direct evidence is obviously required in order to prove that formic acid, or an active form of formate, is in fact an intermediate.

The results of previous studies (Gest *et al.*, 1950) indicated that pyruvate and formate were probably not intermediates in the light-induced fermentation of dicarboxylic acids. This conclusion was based on the observations that the cell preparations (from 3- to 5-day-old cultures) produced relatively small amounts of $H_2$ from pyruvate in the light and appeared to decompose formate only in the dark after a lengthy "adaptation" period. In view of the present results, obtained with younger and more active suspensions, the possibility that pyruvate and formate are intermediates must be reconsidered.

The products of the decomposition of pyruvate and dicarboxylic acids in the light, aside from $CO_2$ and $H_2$, have not been identified. It is generally assumed that the substrate carbon not liberated as $CO_2$ is assimilated by the cells into a carbohydrate type of storage material. There is no evidence that this is the case, and this point remains to be critically tested using tracer techniques.

ACKNOWLEDGMENTS

The authors are indebted to Mrs. M. A. Lasoski for technical assistance and to Professor L. O. Krampitz for his interest in this investigation.

[9] Similar observations using green algae have been recently reported by Finkle, Appleman, and Fleischer (1950).

SUMMARY

The anaerobic decomposition of pyruvic acid in the dark by "normal" resting cells of the nonsulfur purple photosynthetic bacterium, *Rhodospirillum rubrum*, has been found to be a typical propionic acid fermentation. Cells grown aerobically in the dark also ferment pyruvate with the production of $CO_2$, small quantities of $H_2$, and acetic, propionic, butyric, valeric, and caproic acids. The course of the dark fermentations by both types of cells is affected by the $CO_2$ tension.

The light-induced decomposition of pyruvate by "normal" cells is characterized by the production of approximately equimolar quantities of $CO_2$ and $H_2$ and the absence of fatty acids as end products. After a lag period, "dark-grown" cells exposed to light ferment pyruvate in a similar manner.

"Normal" cells ferment fumarate and malate very slowly in the dark with the production of $CO_2$ and acetic and propionic acids; lactate and succinate are not decomposed at significant rates. All four acids are rapidly fermented in the light with the formation of $CO_2$ and $H_2$.

Formic acid has been found to accumulate in $H_2$-producing cultures growing anaerobically in the light in a malate-glutamate medium. When the nitrogen source for growth is $NH_4Cl$, $H_2$ is not produced, and formic acid does not accumulate.

The significance of the results is discussed in relation to various aspects of the physiology and metabolism of photosynthetic bacteria.

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