AN INVESTIGATION OF PROPIONATE METABOLISM OF RHODOSPIRILLUM RUBRUM USING C^{14}.

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CAR	CARBON
PRO	PROPRIONATE
RHO	RHODOSPIRILLUM-RUBRUM

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PREFACE

The investigation reported herein was done in the physics laboratory of the U.S. Naval Postgraduate School, Monterey, California in the spring of 1956 for the purpose of assisting in the research studies of Dr. Roderick K. Clayton.

The writers wish to express their most sincere appreciation to their advisor, Dr. Clayton, for his encouragement and invaluable aid during the course of this investigation. It was his suggestions, inspiration and editorial assistance that made this project successful.

Appreciation is due Dr. Ernest Dobson of the University of California for consenting to be the "second reader" of this paper, to Mr. Milton K. Andrews, OMC Robert C. Moeller, and Mr. Thomas Burson for their aid in obtaining necessary equipment, and to Mrs. Alice Robertson for her assistance in preparing the manuscript.

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CHAPTER I

INTRODUCTION

Metabolism has been defined as the sum total of the chemical reactions that occur within cells or within the organism. In higher organisms these reactions are quite difficult to study due to the complexity and the differentiation of the many cells which comprise an individual. Unicellular plants and animals, on the other hand, are much easier to study, and as a result, much of what is known today of metabolic reactions is based on studies of these organisms. Metabolic studies are aimed to investigate how certain materials are utilized by the cell. These involve quite complex thermodynamic, kinetic, and reactive mechanisms. For many years the facilities available to the investigator were limited. Today, however, with advances in instrumentation, radiochemistry, and analytical procedures, many of the theories and hypotheses made in the past have been confirmed or rejected.

Of particular interest in these studies are the photosynthetic bacteria which are similar to members of both the plant and animal kingdoms. One species is Rhodospirillum rubrum, a spiral-shaped, non-sulphur, purple bacterium belonging to the family Athiorhodaceae (13). For a detailed description of the activities of Rh. rubrum the reader is invited to van Niel's investigation (17).

For the past few years Clayton (5) and his students have conducted extensive studies on the metabolic rates of Rh. rubrum under varying conditions of light intensity and oxygen concentration.

A curious effect noted in the course of these investigations was the phototactic response of Rh. rubrum in the presence of propionate as a substrate. Clayton (5) reported that "the ratio of saturating intensity for phototaxis to that for photosynthesis depends markedly upon what substance serves as hydrogen donor for the photosynthetic process". He found that this ratio was unity for acetate and four for propionate and suggested that this striking effect of propionate was important in the mediation of phototaxis. A connection between early metabolic pathways and tactic responses has been established by Clayton (5) and by Manten (11).

Rh. rubrum is an organism which can adapt to either an aerobic or anaerobic metabolism. In the light this organism grows anaerobically, but in the dark, it requires oxygen. Light and oxygen are thus interchangeable in the metabolism of these bacteria and van Niel (18) concludes "the degradation of the oxidizable substrate, whether in photosynthesis or in dark metabolism, must be accomplished by the same enzyme systems". This present investigation of propionate metabolism was undertaken in order that the mediation of tactic responses might be better understood.

Ellingson and Shaw (7) in their investigation of oxidative metabolism showed that there was no appreciable utilization of propionate aerobically and in the dark, unless CO_2 was introduced in some manner (the amount of CO_2 necessary was extremely small). They also found that after an initial addition of CO_2, the metabolism became self sustaining at a high rate even under CO_2-free conditions. While these results suggest that propionate is carboxylated directly to succinate,

the exact mechanism of propionate incorporation into the metabolic cycle of Rh. rubrum and its terminal oxidation is not well known. Bell (2) has surveyed the literature quite extensively to find how propionate is metabolized by various microorganisms and has found conflicting reports. Some of these reports are summarized below.

Larsen (10) found that illuminated resting cells of Chlorobium thiosulphatophilum produce succinic acid in the presence of propionic acid and CO_2. No conversion takes place in the dark. Barban and Ajl (1) present data implicating a direct reversibility among succinate, propionate and CO_2 in Propionibacterium pentosaceum:

$$\text{propionic acid} + CO_2 \longrightarrow \text{succinic acid}$$

In their classic papers, Wood and Workman (16) showed with propionic bacteria that as succinate was formed the proportion of propionic acid decreased. They also showed that in the absence of CO_2, little or no succinic acid is formed. Stadtman and Stadtman (15) in their review reported different mechanisms of propionate conversion. Research with Clostridium propionicum did not involve the succinate pathway, but

$$\text{propionate} \longrightarrow \text{acrylate} \longrightarrow \text{lactate.}$$

Experiments with Viellonella gazogenes and Propionibacterium pentosus indicated a carboxylation of propionate:

$$\text{propionate} + CO_2 \longrightarrow \text{succinate.}$$

On the other hand in Propionibacterium arabinosum propionate must arise from other sources in addition to succinate. Van Niel (18) in commenting on work by Carlson and Ruben (4) which favors the mechanism

$$\text{propionate} + CO_2 \longrightarrow \text{succinate}$$

inferred that a logical test of this mechanism was to suspend propionic bacteria in either propionate or succinate, in the presence of labeled CO_2. Both labeled propionate and succinate should result. However, special experiments to test this point failed. Van Niel felt however that the "Szent Gyorgyi Cycle"

propionic → pyruvic → oxaloacetic → malic → fumaric → succinic

could account for the observed facts.

In this investigation of propionate metabolism involving Rh. rubrum, a basic assumption is that the Krebs cycle is operative in terminal oxidations of metabolic products. The basis of this assumption is the work of many investigators. Eisenberg (6) found in Rh. rubrum the presence of all the enzymes required for the oxidation of the Krebs cycle members. Eisenberg showed that the TCA cycle is probably the pathway for the terminal oxidation of acetic and pyruvic acids. Glover, Kamen and Van Genderen (8) found in C^{14} investigations that dark metabolism of acetate and carbonate involved labeling of the various Krebs cycle components.

With the above assumption established, methods by which propionate enters the Krebs cycle in oxidative metabolism were hypothesized taking into account various mechanisms mentioned in the above discussion.

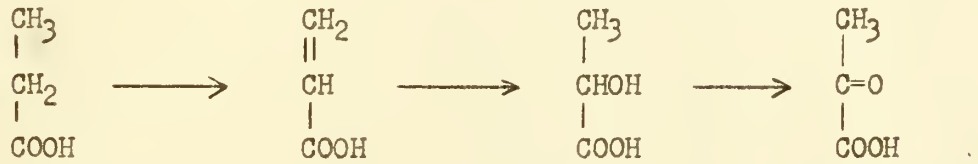
The first hypothesis was that propionate is carboxylated directly to form succinic acid

$$CH_3CH_2COOH + CO_2 \longrightarrow COOHCH_2CH_2COOH.$$

Investigation using 1-C^{14} labeled propionate in a mixture of propionate, pyruvate, and succinate showed no conversion of propionate

to succinate by Rh. rubrum but an appreciable conversion to pyruvate. This hypothesis can now be rejected.

The second hypothesis was that propionate is converted to pyruvate by dehydrogenation, hydration, and oxidation as follows:



PROPIONIC ACID ACRYLIC ACID LACTIC ACID PYRUVIC ACID

Investigation using 1-C^{14} and 2-C^{14} labeled propionate in a propionate, pyruvate, and lactate mixture showed appreciable conversion to pyruvate and lactate. This hypothesis now seems valid but confirmation of these results are in order and studies with acrylate carrier are suggested for further validation.

The specific activity of the cells increased with time in all cases, indicating that propionate was being incorporated into the cells.

In order to determine the specific activity of the various organic acids involved, a method to separate them qualitatively had to be developed. As a result of many trials of known methods of separation, partition chromatography using a silicic acid column proved most satisfactory.

CHAPTER II

PROCEDURE

The experiments involved in this investigation required the separation of propionic, pyruvic, succinic and lactic acids from the experimental bacteria culture. Methods of separation by precipitation and solvent extraction were first attempted but at an early point it became apparent that these methods were not suitable because of poor resolution of the acids and inconsistent recovery.

The partition chromatography procedures described by Marvel and Rands (12); Bulen, Varner and Burrell (3); and Kinnary, Takeda and Greenberg (9) were investigated and employed in preliminary experiments. A chromatographic system incorporating parts of each of the three foregoing methods was developed. The system proved to be highly satisfactory for the requirements of this investigation.

Silica gel was prepared from Mallinckrodt's silicic acid (specially prepared for chromatographic analysis) which had been treated according to Bulen, et al, (3). Eight grams of the silicic acid were mixed and ground thoroughly with 4.5 ml. of 0.05 N H_2SO_4 until a finely divided powder was obtained. Fifty ml. of CP chloroform were added in several portions and the grinding continued until a smooth, lump-free slurry was obtained. The slurry was poured into a 50 ml. burette (1.1 cm. in diameter) provided with glass fiber filter disks at the bottom. The tube was then rotated between the hands or tapped to remove any air bubbles which might have been trapped between particles of the silica gel. The excess chloroform

was forced through the column by nitrogen under a pressure of 30 to 50 cm. of mercury. When the last drop of chloroform had passed into the column, the pressure was removed and the burette stop-cock closed. (The column should not be allowed to become dry, since it is apt to crack and must be re-slurried.) Any silica gel remaining on the sides of the burette was removed by suction with a glass tube.

The column was then ready to receive the bacteria culture from which the organic acids were to be separated. A 0.5 ml. or 1.0 ml. sample of the experimental culture was obtained. The bacteria were killed at the proper time by the addition of 0.1 ml. of 1.0 normal H_2SO_4 per ml. of sample. This solution was mixed thoroughly with one to two grams of dry silicic acid in a ten ml. beaker. The resulting free-running powder was added to the top of the column. However, to insure that this dry powder did not absorb any chloroform from the column, two ml. of chloroform were added prior to the introduction of the powdered mixture. A disk of filter paper was then pushed down the burette onto the top of the sample. This operation served to wipe down any powder adhering to the tube and also prevented disturbance of the column during addition of the eluting solvents.

Eluting solvents were prepared from CP chloroform and n-butanol. Each solvent was saturated with .05 N H_2SO_4. Two hundred ml. of eluant were used. The first 100 ml. contained 5 per cent butanol and the second 100 ml. contained 15 per cent butanol. The solvents were forced through the column under a pressure of 30 to 50 cm. of mercury. The pressure was adjusted by a screw-clamp in the line to the tube so

that a uniform flow rate of two to three ml. per minute was obtained. During the final experiments, five columns were run simultaneously, each of which could be individually regulated. The effluent fractions were titrated with 0.01 N CO_2-free NaOH using a 0.02 per cent phenol red solution as indicator. Prior to titration, a drop of two per cent Dreft detergent solution was added to the fraction.

The results of a typical separation of propionic, pyruvic and succinic acids are shown in the chromatogram of Figure 1. In this type of "calibration" run, five ml. fractions were collected and titrated. However, in the final experiments, five cumulative fractions were collected. The sizes of these fractions are indicated in Figure 1. No calibration run was made for lactic acid since the references on chromatographic separation of organic acids indicate that the lactic acid fraction comes over very near the succinic acid fraction.

When the titration was completed, 0.1 ml. of 0.5 N NaOH plus enough H_2O were added to make the aqueous phase exactly two ml. One ml. aqueous phase was carefully withdrawn and placed on a 3.1 cm. stainless steel planchet. This solution was completely dried in a vacuum dessicator and later counted.

Rh. rubrum (strain S-1, VAN NIEL) were cultured and prepared according to the method used by Ellingson and Shaw (7). The bacteria were then adapted to propionate for about 24 hours. A bacterial density of two according to Petrie (14) was used for each run.

Substrates consisted of sodium propionate, 10 μmoles/ml. with a specific activity of 1 μc/ml; sodium pyruvate, 10 μmoles/ml; sodium succinate, 10 μmoles/ml; and sodium lactate, 10 μmoles/ml. At the

start of the experiment, the bacteria were added to the substrate, and placed in a light-free container (7). Air containing 5% CO_2 was bubbled through this solution at the rate of approximately 300 ml/ minute. Samples were withdrawn at specified times for chromatographic analysis.

At the same time that the bacteria sample was withdrawn for the column, the pyruvate concentration was determined by the method described by Ellingson and Shaw (7).

The C^{14} activity of the cells was determined by withdrawing 0.5 ml. samples and centrifuging with 10 ml. 0.1 N H_2SO_4. The cells were washed once with distilled water and centrifuged again. 0.6 ml. water was then mixed with the cells and 0.4 ml. of this mixture put on a planchet for counting.

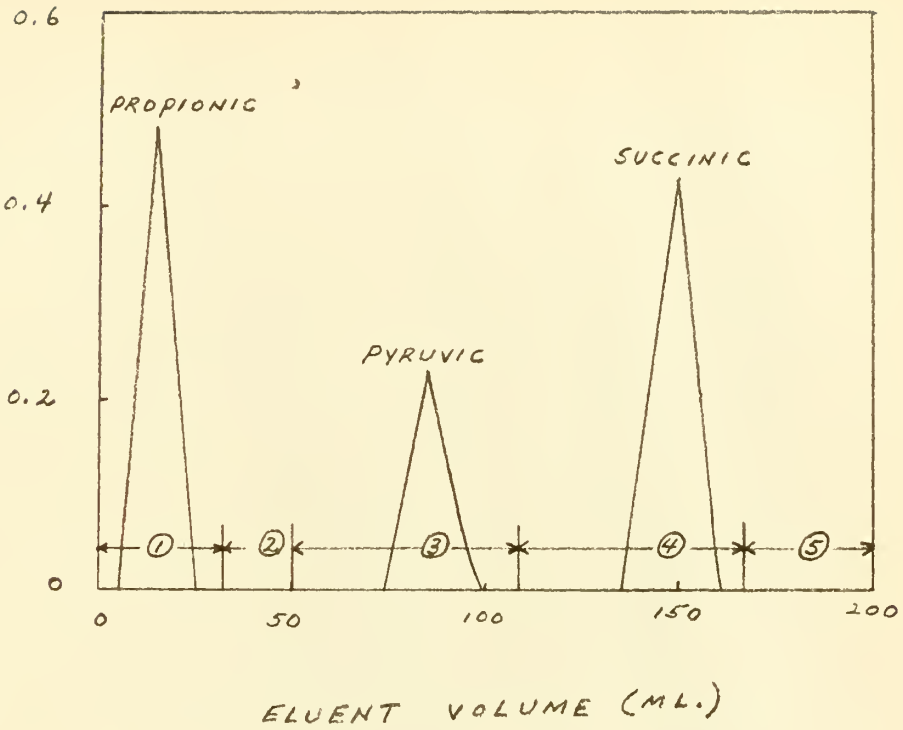


Figure 1. Typical Chromatogram Showing the Separation of Propionic, Pyruvic and Succinic Acids. (The encircled figures show the location and size of the cumulative fractions which were collected during the final experiments.)

CHAPTER III

DISCUSSION OF RESULTS

Appendix I contains the data for the three runs made during the course of this investigation. It should be mentioned at this time that, although trials are carried out under theoretically identical conditions, biological organisms fluctuate in their activities. This will account, to some degree, for the spread in the data obtained. In order to get reliable average values, a number of runs would have to be made. Interpretations of any fluctuations could then be made with some degree of accuracy. It was felt that plotting the results would be meaningless with the limited amount of data available.

All trials resulted in an increase in cell activity which indicated that the propionate or some reactive product of propionate was being assimilated by the organism.

The propionate activities were too inconsistent to be of any value. Propionic acid, being highly volatile, could have been lost to varying degrees during its titration. It is felt this was the reason for the results obtained. The titration values were also inconclusive, although a trend could be noted of decreased propionate as the experiment progressed. It is suggested that more accurate procedures be found for titrating the propionic acid in chloroform solution. Time did not permit the investigation of this difficulty.

A succinate substrate was used in only one run. The titrations of the succinate fractions indicated that succinate was not being utilized to any measureable degree by the organism. The activity of the

succinate was negligible and remained constant within a small statistical variation during the entire experiment. This appeared to prove that no labeled propionate was converted directly to succinate. If the experiment had been extended, it was felt that the labeled carbon would have found its way into the succinate via the Krebs cycle.

The pyruvate activity increased consistently during each run indicating conversion of propionate to pyruvate by the organism. Previous experiments (Dr. Clayton in private communication) have shown that the 1-C^{14} and 2-C^{14} are not exchanged between propionate and pyruvate in the absence of cells, and an increase in activity by such means may be discounted. The titrations in the first two runs indicated little if any pyruvate conversion but the third run showed a considerable decrease in pyruvate present. Colorimetric assay of the pyruvate indicated a decrease of about 25% in all three runs.

Two runs which contained lactate in place of succinate showed appreciable increases in lactate activity during the course of each run. It will be noted however that the activities of the lactate are much smaller than the pyruvate which seems to contradict the pathway of propionate as suggested in the second hypothesis. This can be accounted for if the kinetic rate constants between (1) lactate in the cell and pyruvate in the cell, (2) lactate in the cell and lactate in the substrate, and (3) pyruvate in the cell and pyruvate in the substrate are assumed to be such as to cause more labeled pyruvate to be carried over the substrate than lactate.

In summary, the foregoing experiments indicate that propionate is converted to lactate and to pyruvate (probably by the pathway suggested on page 5); a direct carboxylation of propionate to succinate appears to be ruled out.

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APPENDIX I

RUN DATA

Run Number 1.

100 μmoles sodium propionate (1-C^{14} = 10 μcuries)
100 μmoles sodium pyruvate
100 μmoles sodium succinate
Rh. rubrum - density two
Total volume 10 ml.

1.0 ml. samples withdrawn for analysis at 0, 30, 60 and 120 minutes.

RESULTS

TIME (minutes)	0	30	60	120	120
μmoles propionate recovered	8.8	10.0	9.8	8.0	8.6
propionate activity (counts/min.)	15886	14184	10900	11950	8840
μmoles pyruvate recovered	7.8	7.4	6.9	6.4	6.6
pyruvate activity (counts/min.)	98	173	213	300	341
μmoles succinate recovered	6.2	6.3	6.1	6.1	6.0
succinate activity (counts/min.)	37	39	59	38	46
pyruvate analysis μmoles/ml.	10	8.6	7.4	8.2	
cell activity (counts/min.)	11	91	152	308	

Run Number 2.

80 μmoles sodium propionate (1-C^{14} = 8 μcuries)
80 μmoles sodium pyruvate
80 μmoles sodium lactate
Rh. rubrum - density two
Total volume 8.0 ml.

0.5 ml. samples withdrawn for analysis at 0, 30, 60, 120 and 240 minutes

RESULTS

TIME (minutes)	0	30	60	120	240
μmoles propionate recovered	4.7	4.1	4.6	4.2	3.2
propionate activity (counts/min.)	1563	1500	3020	2570	1250
μmoles pyruvate recovered	3.6	3.8	3.7	3.4	3.3
pyruvate activity (counts/min.)	18	63	104	114	219
μmoles lactate recovered	4.9	4.7	4.8	4.6	4.8
lactate activity (counts/min.)	5	21	16	48	86
pyruvate analysis μmoles/ml.	10	9.0	8.9	8.5	7.8
cell activity (counts/min.)	0	36	68	115	283

Run Number 3.

80 μmoles sodium propionate (2-C^{14} = 8 μcuries)
80 μmoles sodium pyruvate
80 μmoles sodium lactate
Rh. rubrum - density two
Total volume 8.0 ml.

0.5 ml. samples withdrawn for analysis at 0, 60, 120 and 240 minutes

RESULTS

TIME (minutes)	0	60	120	240	240
μmoles propionate recovered	5.9*	4.6	4.5	2.5	2.5
propionate activity (counts/min.)	2753	1753	944	1375	1945
μmoles pyruvate recovered	4.1	3.7	3.5	2.3	2.5
pyruvate activity (counts/min.)	40	162	363	588	538
μmoles lactate recovered	5.2	4.9	4.7	4.0	4.1
lactate activity (counts/min.)	8	52	104	155	148
pyruvate analysis μmoles/ml.	10	9.6	9.1	7.7	7.8
cell activity (counts/min.)	0	205	443	1276	1358

* overran titration

Thesis
D455 Dettmer
An investigation of propionate metabolism of rhodospirillum rubrum using C^{14}.