71. BIOLOGICAL SYNTHESIS OF OXALOACETIC ACID FROM PYRUVIC ACID AND CARBON DIOXIDE

2. THE MECHANISM OF CARBON DIOXIDE FIXATION IN PROPIONIC ACID BACTERIA[1]

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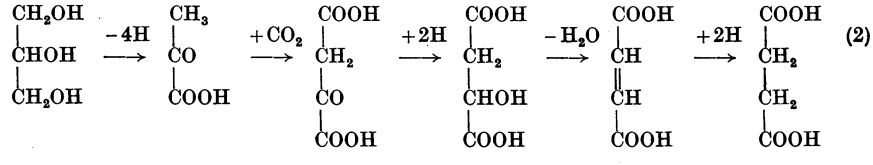
WOOD & WERKMAN [1936, 1; 1938] found that propionic acid bacteria utilize CO_2 when glycerol is present and that the CO_2 fixation is coupled with the formation of an equivalent amount of succinic acid. These observations are summarized by equation (1):

$$CH_2OH.CHOH.CH_2OH + CO_2 = COOH.CH_2.CH_2.COOH + H_2O. \quad (1)$$

Recently, Wood & Werkman [1940] suggested the following mechanisms for this reaction:



(2)

This scheme is supported by several experimental observations. Wood *et al.* [1937] found that glycerol in accordance with (2) can be converted into pyruvic acid. Wood *et al.* [1940] showed, with the help of the isotope C^{13}, that the fixed CO_2 appears in fact in the carboxyl groups of succinic acid. The experiments reported in this paper were mainly undertaken to supply further evidence in support of scheme (2).

The organism

A culture of *Bacterium acidi propioni* obtained from the National Collection of Type Cultures (No. 4759) showed a very feeble fermenting capacity. The organism[2] grew vigorously under aerobic conditions (on the surface of agar), but it failed to grow anaerobically on glucose-lactate-yeast water, or on whey. Suspensions of washed bacteria, grown on agar, did not attack glucose, lactate or glycerol under anaerobic conditions; pyruvate was slowly decomposed. Attempts to recover the original fermenting power by transferring the organism to liquid media (glucose-lactate-yeast water, whey) were unsuccessful. Even after 2 months no growth or fermentation was perceptible. The Curator of the Collection informed us that the organism had been cultivated for 5 years on agar slopes. This may account for the inability to ferment, for Jensen [1931,

[1] A preliminary report appeared in *Chem. and Ind.* **59**, 849 (1940).

[2] This is the organism recently used by Quastel & Webley [1939; 1941]. We can confirm the effects of vitamin B_1 described by Quastel & Webley with this atypical bacterium, but we obtained no vitamin effects with the six strains of propionic acid bacterium used in the present paper.

p. 47] states 'when propionic acid bacteria have become accustomed to aerobic conditions, their fermentative powers are gradually weakened'.

The organism used in the greater part of this work was isolated from Gruyère cheese obtained at a local shop. Material from the 'eye' of the cheese was spread over an agar plate of lactate-yeast water and incubated at 25° in an anaerobic jar filled with CO_2. Among several organisms a typical propionic acid bacterium was obtained. Its properties are as follows:

(1) Very short, non-motile, gram-positive rods, non-spore forming, assuming involution forms in old cultures, or when grown aerobically at 37°. Agar stab culture: abundant stab growth, moderate surface growth.

(2) Lactate is rapidly fermented under anaerobic conditions, yielding 25–30 % CO_2 and 90–100 % fixed acids. Among these propionic acid was identified as mercurous salt [Musicant & Kaszuba, 1939].

(3) Glycerol is slowly fermented with the fixation of CO_2 and formation of succinate, as described by Wood & Werkman.

(4) Acid is produced from glucose, lactose, galactose, mannose, fructose, arabinose.

(5) Neither acid nor gas is produced from starch, sucrose, xylose.

(6) A powerful catalase is present.

The properties listed under (1), (2) and (3) characterize the organism as a *Propionibacterium*. The other properties are identical with those of the species *Propionibacterium Shermanii* [Sherman, 1920; van Niel, 1928; Werkman & Brown, 1933].

During the later stages of this work we received five strains of propionic acid bacteria of the American Collection of Type Cultures through the courtesy of Dr J. G. Davis, National Institute for Research in Dairying, University of Reading. They were *P. Freudenreichii*, *P. Shermanii*, *P. Jensenii*, *P. Thönii* and *P. rubrum*. Some experiments were repeated with these organisms.

Most of the experiments reported in this paper were carried out with suspensions of washed cells. The medium used for the large scale preparation of bacterial suspensions contained the following ingredients:

200 ml. yeast water (200 g. dried brewer's yeast and 800 ml. tap water) heated in the autoclave (120°, 30 min.) and filtered. The liquid contained about 3·5 % dry matter.

200 ml. phosphate buffer (0·1 *M*, *p*H 7·4).

25 ml. tryptic casein digest [Gladstone & Fildes, 1940].

40 ml. 50 % sodium lactate.

500 ml. tap water.

25 g. agar.

Sterile medium was poured into large sterile petri dishes (15 cm. diameter) and these were inoculated with 3–4 ml. of a liquid culture (the above medium without agar). The petri dishes were placed in a vacuum jar. The air pressure in the jar was reduced on the water pump to 15–20 mm. Hg and 400–500 mm. CO_2 were then admitted. The temperature of the incubator was 34°. After 4–5 days the cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. This 'stock suspension' was kept in the refrigerator. In the case of *P. Shermanii* the yield was about 200–250 mg. dry cells per petri dish. For metabolic experiments the stock suspension was diluted with solutions of $NaHCO_3$ or phosphate. The concentrations used are stated in the tables.

The manometric and analytical methods were the same as those described in previous papers from this laboratory. All experiments were carried out at 30°.

Metabolism of oxaloacetate in Propionibacterium Shermanii

The metabolism of oxaloacetate was investigated in order to see if oxaloacetate can react to form malate, fumarate and succinate as predicted by scheme (2). The suspension contained 14 mg. cells per ml. 0·031 *M* $NaHCO_3$ solution. Four conical Warburg cups were used and 4 ml. suspension were measured into each cup. The side-arms contained 0·5 ml. 0·2 *M* oxaloacetate, the centre chamber yellow phosphorus and the gas space CO_2. The CO_2 production was measured over a period of 22 hr. Two cups were removed from the bath after 3 hr. for the determination of succinate and of fumarate and malate [Krebs, Smyth & Evans, 1940]. The remaining cups were left in the bath until the CO_2 production had almost stopped and succinate, fumarate and malate were then determined. The results are shown in Table 1.

Table 1. *Metabolism of oxaloacetate in* Propionibacterium Shermanii

For experimental conditions see text; the data refer to 4 ml. suspension.

Time hr.	μl. CO_2 formed	μl. succinate formed	μl. fumarate + malate formed
1	335	—	—
2	585	—	—
3	920	654	322
4	1024	—	—
5	1118	—	—
21	1640	—	—
22	1655	908	0

Table 2. *Formation of bicarbonate from oxaloacetate in* Propionibacterium Shermanii

3 ml. suspension containing 38 mg. bacteria; 5% CO_2 in N_2; initial amount of bicarbonate in 3 ml.: 878 μl.; concentration of oxaloacetate: 0·0061 *M*; period of incubation 6 hr.

	μl.
Free CO_2 evolved	239
Final amount of bicarbonate	1030
Increase in bicarbonate	152

The quantity of free CO_2 (not including HCO_3') evolved amounted to 74% of the oxaloacetate added. Fumarate and malate appeared in the earlier stages, but disappeared later. The amount of succinate increased with time and reached about 40% of the added oxaloacetate. It should be remembered that the decarboxylation of oxaloacetate does not cause an increase in CO_2 pressure since in this reaction bicarbonate is formed.

The formation of bicarbonate was measured in another experiment [for methods see Krebs, Eggleston, Kleinzeller & Smyth, 1940] and it was found (Table 2) that the quantities of bicarbonate formed are somewhat smaller than those of free CO_2.

The data observed agree approximately with the following scheme of oxaloacetate fermentation:

$$3 \text{ oxaloacetate} = 1 \text{ succinate} + 2 \text{ acetate} + 2HCO_3' + 2CO_2.$$

The yields of succinate and of free CO_2 are greater than expected (40·5% succinate instead of 33·3%; 74% CO_2 instead of 66·6%), while the yield of bicarbonate is below the calculated quantity. These discrepancies are probably due to other reactions of oxaloacetate. In the above scheme the conversion of

2 mol. of oxaloacetate into acetate (via pyruvate) is the equivalent of the reduction of a third oxaloacetate molecule to succinate. Various experiments suggest that oxaloacetate can also be reduced at the expense of other oxidative processes in which either intracellular substances, or acetate (see later) undergo oxidation and this would account for the experimental deviations from the theory.

Experiments with mesoxalate and α-ketoglutarate showed that these nearest homologues of oxaloacetate are not metabolized by washed suspensions of *P. Shermanii*. The organism thus possesses a highly active enzyme system which specifically ferments oxaloacetate. The existence of this system strongly suggests that oxaloacetate is a normal metabolite in *P. Shermanii*.

Metabolism of fumarate and malate in Propionibacterium Shermanii

(1) *Presence of fumarase.* Fumarate is rapidly converted into *l*(−)-malate as shown in the following polarimetric experiment: 50 ml. 0·1 *M* fumarate (=5 m.mol.), 20 ml. 0·1 *M* phosphate buffer, *p*H 6·8; 14 ml. bacterial suspension containing 331 mg. dry cells and 2·5 ml. propyl alcohol were incubated at 30°. At various intervals 15 ml. were removed, mixed with 1 g. uranyl acetate and 0·5 ml. glacial acetic acid and heated for 10 min. in a boiling water bath. After cooling and filtering, the rotation of the solution was determined in a 1 dm. tube. The readings were: after 60 min. −0·59°; 120 min. −1·40°; 360 min. −2·47°. Experiments with pure *l*(−)-malic acid showed that the specific rotation was about 485° under the conditions used. The quantity of *l*(−)-malic acid formed (m.mol.) was therefore after 60 min. 0·813; 120 min. 1·93; 360 min. 3·40. In terms of the usual metabolic quotients the rate of malic acid formation is for the first hour 55 μl. per mg. cells per hour. The occurrence of fumarase in propionic acid bacteria has also been reported by Erkama [1938].

(2) *Reduction to succinate.* A suspension containing 9 mg. cells per ml. 0·04 *M* $NaHCO_3$ solution was used and 0·25 ml. 0·2 *M* fumarate (=1120 μl.) was added to 4 ml. suspension. Otherwise the conditions were the same as those described in the analogous experiments with oxaloacetate. The evolution of CO_2 (including CO_2 liberated from $NaHCO_3$) was 99 μl. in 1 hr.; 184 μl. in 2 hr.; 475 μl. in 18 hr. After 18 hr. the solution contained 1032 μl. succinate. Thus the yield of succinate, in terms of added fumarate was 95%. The nature of the oxidative equivalent for reduction of fumarate has not been investigated in detail. Obviously intracellular substances serve as substrates for the oxidative reactions. That oxidizable substances are present is shown by the O_2 uptake of washed cells in the absence of added substrate (Table 3).

Table 3. *Rate of respiration of* P. Shermanii

The data refer to 3 ml. suspension containing 14·4 mg. bacteria. Medium 0·04 *M* phosphate buffer, *p*H 6·8; gas, air; 30°. Substrate concentration: 0·02 *M*; no increase was observed on addition of glycollate, oxalate, formaldehyde, ethyl alcohol.

Substrate added	...	...	*d*(−)-Lactate	Glycerol	Succinate	*l*(−)-Malate	Acetate	Propionate	Formate	Glucose
μl. O_2 absorbed after										
20 min.	56		122	113	68	55	85	86	88	118
40 min.	86		330	260	115	93	167	182	182	232
60 min.	124		506	412	158	138	240	285	255	358
Q_{O_2}	8·6		35·2	28·6	10·9	9·6	16·7	19·8	17·7	24·8

(3) *Experiments with* l(−)-*malate.* Fumarate and *l*(−)-malate behaved identically in *P. Shermanii*, as may be expected if a powerful fumarase is present;

d(+)-malate was not metabolized. This is worthy of note since the stereoisomerides of lactate were both fermented at the same rate.

Rate of succinate formation from various substrates

If pyruvate, oxaloacetate and fumarate are intermediates in the formation of succinate from glycerol, as suggested by scheme (2), the succinate formation from these substrates should be at least as rapid as the succinate formation from glycerol. Table 4 shows that such is the case. Although the yield of succinate is much lower when lactate and pyruvate are the substrates, the *rate* of succinate formation from these substances is of the same order as that from glycerol.

Table 4. *Rate of succinate formation from various substrates*

39 mg. bacteria in 4 ml. 0·03 *M* $NaHCO_3$. Initial substrate concentration 0·02 *M*; gas phase CO_2; period of incubation (1 hr.).

Substrate ...	Glycerol	*dl*-Lactate	Pyruvate	Oxaloacetate	Fumarate or *l*(−)-malate
µl. CO_2 and acid	96	676	532	352	131
µl. succinate	73	95	83	177	372

Fermentation of glycerol

(1) *Formation of fumarate.* In the case of *Bact. coli* it has been possible to demonstrate a simultaneous formation of succinate and fumarate from pyruvate [Krebs & Eggleston, 1940]. In experiments with *P. Shermanii*, using glycerol as a substrate, we obtained similar results when an excess of glycerol was present. Details of the experimental conditions are given in Table 5. The yields of fumarate

Table 5. *Formation of fumarate from glycerol in* P. Shermanii

64 mg. bacteria in 6 ml. 0·04 *M* $NaHCO_3$; 5% CO_2+N_2; 2% glycerol; 21 hr.

	Exp. I	Exp. II
µl. CO_2 formed	1540	1380
µl. succinate formed	586	690
µl. fumarate formed	64	62

are rather low, presumably because of the rapid reduction of fumarate in the presence of glycerol (see the following paragraph). The simultaneous formation of fumarate and succinate can be taken as evidence in support of scheme (2) [see Krebs & Eggleston, 1940].

(2) *Effects of fumarate and oxaloacetate on the fermentation of glycerol.* When fumarate and glycerol are added together to washed suspensions of *P. Shermanii* the rate of acid and CO_2 production is 2–3 times greater than expected from experiments in which the substrates are added alone (Table 6). The rate of

Table 6. *Effect of glycerol and fumarate on the acid and CO_2 production by* Propionibacterium Shermanii

36 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.) ...	None	Fumarate (0·02 *M*)	Glycerol (0·02 *M*)	Fumarate (0·02 *M*), glycerol (0·02 *M*)
µl. CO_2 evolved after 20 min.	13	47	30	87
40 min.	30	93	65	212
80 min.	39	142	98	440
200 min.	49	228	236	1385
280 min.	83	444	314	1980
µl. succinate formed (280 min.)	—	1556	346	2008

succinate formation is also increased and the data suggest that the following reaction occurs in the presence of glycerol and fumarate:

glycerol + fumarate → oxidation products of glycerol + succinate.

This scheme, however, does not account for all the observations. When small quantities of fumarate are added the increased CO_2 output and succinate formation is equivalent to a multiple of the fumarate added (Table 7). Thus 112 μl.

Table 7. *Effect of oxaloacetate and fumarate on the succinate formation in the presence of glycerol*

26 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.)	0·1 *M* glycerol	0·1 *M* glycerol, 0·00125 *M* oxaloacetate (=112 μl.)	0·1 *M* glycerol, 0·00125 *M* fumarate (=112 μl.)
μl. CO_2 formed after 100 min.	106	322	280
280 min.	302	730	562
22 hr.	1100	1756	1488
μl. succinate formed	374	814	714
Extra succinate (due to addition of oxaloacetate or fumarate), μl.	—	440	340

fumarate cause an increase in CO_2 output of 388 μl. and in succinate formation of 340 μl. The effect of fumarate, therefore, is catalytic. From what is known about the metabolism of fumarate in other cells it is very probable that the catalytic effect is associated with hydrogen transport; it might be due either to the system succinate ⇌ fumarate, or the system fumarate ⇌ oxaloacetate. The first possibility, however, can be excluded, for if succinate were reversibly dehydrogenated it should be possible to replace fumarate by succinate, and this is not the case. Addition of succinate has no effect on the rate of acid production from glycerol and the added succinate is quantitatively recovered from the solution (Table 10). These results show that the system succinate ⇌ fumarate is not a hydrogen carrier under *anaerobic* conditions. Apparently O_2 cannot be replaced by other physiological oxidizing agents. On the other hand oxaloacetate can replace fumarate (Table 7) and the catalytic effect with this substance is even slightly greater than with fumarate. This supports the view that the system oxaloacetate ⇌ fumarate acts catalytically. Experiments with radioactive carbon [Carson & Ruben, 1940] lead to the same conclusion (see below).

(3) *Adaptation to glycerol.* Figures given in Tables 6 and 7 show that the fermentation of glycerol is relatively slow in washed cells grown on a lactate-containing medium. When the lactate of the culture medium is replaced by glycerol, cells are obtained which ferment glycerol much more rapidly (Table 8). The enzyme systems attacking glycerol are thus partly adaptive.

Table 8. *Adaptation to glycerol*

30 mg. washed cells in 3 ml. 0·05 *M* $NaHCO_3$; gas phase, CO_2.

Culture medium	Substrate added	μl. CO_2 formed 4 hr.	μl. CO_2 formed 20 hr.	μl. succinate formed 20 hr.
Lactate yeast-water	—	41	177	102
,,	0·1 *M* glycerol	115	694	150
Glycerol yeast-water	—	217	631	248
,,	0·1 *M* glycerol	735	1870	732

Effects of oxaloacetate on the fermentation of other substrates

Effects of oxaloacetate similar to that on the fermentation of glycerol were found when mannitol, inositol, arabinose or erythritol were the substrates. The rate of acid formation from these substrates was increased by the addition of oxaloacetate (Table 9). No effects were observed when glucose, lactate or pyruvate were the substrates.

Table 9. *Effect of oxaloacetate on the fermentation of various substrates in* P. Shermanii (*strain of the American Collection of Type Cultures*)

30 mg. cells in 3 ml. 0·05 *M* $NaHCO_3$; CO_2; 5 hr.

Substrates added (final conc.)	μl. acid and CO_2 formed	Extra acid and CO_2 formed from added polyhydric alcohol: Without oxaloacetate	With oxaloacetate
None	171	—	—
0·00625 *M* oxaloacetate	375	—	—
Mannitol	291	120	275
Mannitol; 0·0065 *M* oxaloacetate	650		
Inositol	357	186	471
Inositol; 0·00625 *M* oxaloacetate	846		
Arabinose	274	103	171
Arabinose; 0·00625 *M* oxaloacetate	546		
Erythritol	191	20	71
Erythritol; 0·00625 *M* oxaloacetate	446		

Anaerobic metabolism of succinate

Previous investigators [Wood *et al.* 1937] have discussed the formation of propionic acid by decarboxylation of succinic acid and it has been stated that succinate disappears [Wood *et al.* 1937; Fromageot & Bost, 1938] when added together with glucose to suspensions of *P. pentosaceum*. In *P. Shermanii* succinate is not metabolized under anaerobic conditions, either when present alone, or when added together with glucose, glycerol or pyruvate (Table 10). (Aerobically, however, succinate can undergo oxidation.)

Table 10. *Anaerobic metabolism of succinate in* Propionibacterium Shermanii

3 ml. suspension; 27 mg. bacteria; 0·07 *M* $NaHCO_3$; gas phase, CO_2; 370 min.

Substrates added	μl. CO_2 found	μl. succinate found
0·4 ml. 0·1 *M* succinate (=896 μl.)	71	882
0·4 ml. 0·1 *M* glucose	1462	26
0·4 ml. 0·1 *M* glucose; 0·4 ml. 0·1 *M* succinate	1448	942
0·4 ml. *M* glycerol	133	96
0·4 ml. *M* glycerol; 0·2 ml. 0·1 *M* succinate (=448 μl.)	139	536

Alternative mechanisms of succinate synthesis

A second mechanism of succinate synthesis has been suggested by Wood & Werkman [1938; 1940] who assume that a dehydrogenation and condensation of 2 mol. of acetate might lead to succinate:

$$2CH_3.COOH \xrightarrow{-2H} COOH.CH_2.CH_2.COOH. \quad (3)$$

They arrived at this view from the following considerations. The conversion of pyruvate into acetate and CO_2 is the only defined reaction so far known to yield

CO_2 in propionic acid bacteria under anaerobic conditions. If this reaction were the only source of CO_2, acetate and CO_2 should appear in equimolecular quantities. In fact less acetate than CO_2 is formed under certain conditions [Wood & Werkman, 1936, 2]. To explain this Wood & Werkman assumed that some acetate disappears through reaction (3).

This hypothesis is not supported by direct evidence and we do not regard it as satisfactory. In a number of Wood & Werkman's experiments [1936, 2, p. 619, Table II, exps. 1 and 2] the amounts of succinate formed do not make up the deficit in acetate production, even if it is assumed that all the succinate was formed through reaction (3). Thus the hypothesis does not account for the low yield of acetate. However, we accept Wood & Werkman's idea of a further oxidation of acetate, whilst differing from their views on the course of this oxidation. The data on CO_2 production published by Wood & Werkman and by van Niel [1928] suggest that acetate is oxidized to form CO_2. Since succinate cannot be oxidized under anaerobic conditions, it cannot be an intermediate in the oxidation of acetate. The mechanism of acetate oxidation is still completely obscure.

The ability of acetate to undergo oxidation has already been demonstrated by Stone *et al.* [1936] (see also Table 3). The formation of CO_2 from acetate under aerobic conditions is shown in Table 11. Anaerobically addition of acetate

Table 11. *Oxidation and CO_2 formation in the presence of acetate*

P. Shermanii; 10 mg. cells in 3 ml. 0·01 *M* phosphate buffer, *p*H 7·1. Method of Warburg & Yabusoe [1924]; period of incubation 105 min.

Substrate	—	0·03 *M* acetate
μl. O_2 used	135	250
μl. CO_2 (including HCO_3') formed	151	289

Table 12. *Effect of acetate on the fermentation of oxaloacetate*

30 mg. cells, *P. Shermanii*, in 3 ml. 0·04 *M* $NaHCO_3$; 5 % CO_2 in N_2; period of incubation 21 hr. The bacteria were shaken before the experiment for 18 hr. in the presence of O_2 to remove preformed intracellular substrates.

Added substrates	0·5 ml. 0·1 *M* oxaloacetate	0·5 ml. 0·1 *M* oxaloacetate, 0·5 ml. 0·5 *M* acetate
μl. succinate formed	352	446

causes an increase of the yield of succinate from oxaloacetate (Table 12). We take this as an indication of a reduction of oxaloacetate at the expense of an oxidation of acetate. The hypothesis of the oxidation of acetate also explains the high values for the ratio propionate/acetate which have often been found. According to the original concept of propionic acid fermentation [Fitz, 1878] 3 triose equivalents are assumed to form 2 mol. of propionic acid and 1 mol. of acetic acid and of CO_2. These quantities have been found in many experiments but there are also many cases where the yield of acetate is low. Wood & Werkman [1936, 2] found the ratio propionate/acetate to be between 2·13 and 14·72. These deviations from the expected value 2 are explained by the assumption that acetate undergoes decomposition to CO_2. The reduction equivalent for this oxidation may be the formation of either propionate or of succinate.

Experiments with other strains of propionic acid bacteria

Further experiments were undertaken to see whether the reduction of oxaloacetate and the catalytic effects of oxaloacetate on the fermentation of glycerol

Table 13. *Fermentation of oxaloacetate and glycerol in various strains of propionic acid bacteria*

30°; 0·045 *M* $NaHCO_3$; gas phase CO_2; the data refer to 3 ml. suspension.

Strain	mg. cells in 3 ml. suspension	Substrates added (final conc.)	Period of incubation min.	μl. CO_2 formed (free CO_2 from substrate plus CO_2 from bicarbonate)	μl. succinate formed
P. Freudenreichii	28	—	120	56	—
	28	0·0133 *M* oxaloacetate	120	215	220
	28	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	120	675	251
	28	0·1 *M* glycerol	120	120	77
P. Jensenii	40	—	160	102	59
	40	0·0133 *M* oxaloacetate	160	561	571
	40	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	160	1380	846
	40	0·1 *M* glycerol	160	301	164
P. Shermanii	30	—	240	147	33
	30	0·0133 *M* oxaloacetate	240	458	440
	30	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	1960	1150
	30	0·1 *M* glycerol	240	227	61
P. Thönii	27	—	240	155	—
	27	0·0133 *M* oxaloacetate	240	257	49
	27	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	970	96
	27	0·1 *M* glycerol	240	407	76
P. rubrum	27	—	240	116	—
	27	0·0133 *M* oxaloacetate	240	319	25
	27	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	1025	81
	27	0·1 *M* glycerol	240	312	87

occur generally in propionic acid bacteria. Five strains of the American Collection of Type Cultures were tested and on the whole the results (Table 13) are similar to those obtained with *P. Shermanii*. In each case oxaloacetate is reduced to succinate, and glycerol increases the rate of succinate formation. The rate of acid production from glycerol was also increased by the addition of oxaloacetate. In the case of *P. Thönii* and *P. rubrum* the yield of succinate was low, and a further investigation of this difference showed that fumarate and malate appear instead of succinate in these strains (Table 14).

Table 14. *Fermentation of oxaloacetate and glycerol in* P. Thönii

39 mg. cells in 3 ml. 0·05 *M* $NaHCO_3$; CO_2; 5 hr. For the calculation of fumarate + *l*(−)-malate it was assumed that the ratio $\frac{l(-)\text{-malate}}{\text{fumarate}} = 3{\cdot}54$ for temp. 30°.

Substrates added	0·027 *M* oxaloacetate	0·027 *M* oxaloacetate 0·1 *M* glycerol	0·1 *M* glycerol
Total CO_2 evolved (μl.)	629	2360	700
Succinate formed (μl.)	45	78	119
Fumarate formed (μl.)	78	141	89
Fumarate + *l*(−)-malate (calc.)	354	640	404

Discussion

The evidence in support of scheme (2). The most important part of the evidence in favour of scheme (2) is the simultaneous formation of fumarate and succinate from glycerol (Tables 5 and 14). Since the reaction succinate → fumarate does not occur under anaerobic conditions (Table 10), fumarate formed anaerobically cannot have arisen from succinate: if the formation of succinate by reduction is rejected, the improbable assumption of two separate mechanisms for the formation of succinate and of fumarate must be made.

The fact that all the reactions postulated by (2) can occur, lends much further support to the scheme, for if an added substance is readily metabolized, it is very likely to be a normal metabolite.

Biological significance of the synthesis of oxaloacetate. As shown by Wood & Werkman [1938] glycerol is fermented in propionic acid bacteria in two ways:

$$CH_2OH.CHOH.CH_2OH \longrightarrow CH_3.CH_2.COOH + H_2O,$$
$$CH_2OH.CHOH.CH_2OH + CO_2 \longrightarrow COOH.CH_2.CH_2.COOH + H_2O.$$

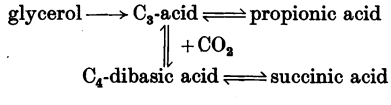
The significance of the second form of glycerol fermentation lies probably in the fact that it produces the oxaloacetate and fumarate required as hydrogen carriers.

It would be of interest to compare the changes of free energy in the two fermentations, but it appears that the necessary data are not yet available.

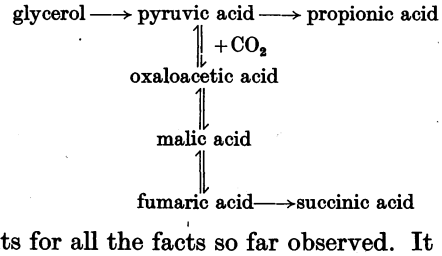
The experiments of Carson & Ruben with radioactive carbon. Carson & Ruben [1940] allowed propionic acid bacteria to ferment glycerol in the presence of CO_2 containing the radioactive isotope C^{11}. The isotope was found to be present in succinic acid and in propionic acid. Its presence in propionic acid was somewhat unexpected and the authors first thought it might be due to the following reversible reaction:

$$CH_3.CH_2.COOH + CO_2 \rightleftharpoons COOH.CH_2.CH_2.COOH.$$

However Carson & Ruben, like ourselves, were unable to find evidence in support of this reaction and they proposed another scheme which explains all the experimental observations:



If 'pyruvic acid' is substituted for 'C_3 acid' and 'fumaric acid' for 'C_4-dibasic acid' the scheme of Carson & Ruben becomes almost identical with the following scheme comprising only reactions which have been shown to occur:



This scheme accounts for all the facts so far observed. It should be noted that the presence of radioactive carbon in propionic acid is explained if the C_4-

dibasic acid is fumaric acid, but it is not explained if it is malic acid. For malic acid, formed by reaction (2), would on breakdown yield a pyruvic acid containing no isotope. On the other hand, fumaric acid containing an active carbon atom in the carboxyl group would be converted into two different malic acids, viz. *COOH.CHOH.CH_2.COOH and COOH.CHOH.CH_2.*COOH (the asterisk denotes the isotope). On breakdown the first would yield a pyruvic acid (and on reduction of the latter a propionic acid) containing the isotope, whilst the second would lose the isotope.

The appearance of the isotope in the propionic acid formed from glycerol may be taken as further evidence in support of the view that the system oxaloacetate $\rightleftharpoons$ fumarate acts as a hydrogen carrier in the fermentation of glycerol. It is direct proof of the reversibility, under 'normal' conditions, of the reactions of the scheme (2) leading from pyruvate and CO_2 to fumarate.

Summary

1. Propionic acid bacteria readily reduce oxaloacetate to succinate. Six different strains were examined. Fumarate and *l*(−)-malate are formed as intermediate metabolites. The hydrogen required for the reduction of oxaloacetate can be supplied by several reactions, e.g. the conversion of pyruvate into acetate.

2. Fumarate and *l*(−)-malate are readily reduced to succinate in *P. Shermanii*.

3. A powerful fumarase is present in *P. Shermanii*.

4. The rates of succinate formation from oxaloacetate, fumarate and malate are sufficiently high to allow the assumption that these acids are intermediates in the formation of succinate from glycerol, in accordance with scheme (2).

5. Fumarate is shown to be formed when glycerol is anaerobically fermented by *P. Shermanii*. This is considered as evidence in support of (2).

6. Fumarate, malate or oxaloacetate catalytically accelerate the fermentation of glycerol. It is concluded that these acids act as intermediary hydrogen carriers in the fermentation of glycerol. It is pointed out that the experiments of Carson & Ruben with radioactive carbon support this view.

7. Similar catalytic effects of oxaloacetate were observed when mannitol, inositol, arabinose or erythritol were the substrates of fermentation.

8. The view that succinate is synthesized in propionic acid bacteria from 2 mol. of acetate is rejected. Acetate can be oxidized aerobically, and probably anaerobically, to form CO_2. The oxidative conversion of acetate into CO_2 explains the low yields of acetate observed by previous investigators.

9. The facts concerning the synthesis of succinate in propionic acid bacteria are all explained by scheme (2), according to which the synthesis of oxaloacetate from pyruvate and CO_2 is the primary step in the synthesis of the 4 carbon chain.

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