THE FERMENTATION OF THREE CARBON SUBSTRATES BY *CLOSTRIDIUM PROPIONICUM* AND *PROPIONIBACTERIUM*[1]

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Fitz (1878) was the first to determine the quantitative relationship of products formed by species of the genus *Propionibacterium* and formulated what is known as the Fitz equation: 3 lactate $\rightarrow$ 2 propionate + 1 acetate + 1 CO_2. It was considered that one mole of lactic acid was oxidized to CO_2 and acetate and that the hydrogens produced from this oxidation reduced two moles of lactic acid to propionic acid. Recently there has been increasing evidence that propionic acid may not be produced by direct reduction but rather may arise by an indirect pathway involving the dicarboxylic acids. This hypothesis was based upon the following observations: Erb (1934) demonstrated that the bacteria were capable of decarboxylating succinate to form propionate and CO_2. Wood and coworkers (1941*a*, 1941*b*) found that labeled CO_2 was fixed in the carboxyl carbons of both propionate and succinate, and Krampitz *et al.* (1943) using *Micrococcus lysodeikticus* showed that CO_2 is fixed in oxalacetate. Delwiche (1948) and Johns (1951) investigated the decarboxylation of succinate with propionic acid bacteria and concluded that the reaction was rapid enough to be the sole pathway for the formation of propionic acid.

The mechanism may be diagrammed as shown in figure 1. This mechanism accounts for the fixation of labeled CO_2 in the carboxyl carbons of propionate and succinate and also for the formation of succinate in these fermentations. However, Wood and Leaver (1953) have shown that the turnover of CO_2 is frequently low in the propionic acid fermentation. They fermented several substrates in the presence of a pool of $C^{14}O_2$ and from the dilution of the C^{14} in the final CO_2 they calculated the amount of $C^{12}O_2$ produced from the substrate. If propionate were formed as shown in figure 1, 50 mM of $C^{12}O_2$ would be produced for every 100 mM of propionate formed. In some experiments the amount of $C^{12}O_2$ produced was much below this value. Furthermore, in some fermentations there was very little $C^{14}O_2$ fixed in the propionate and succinate. They concluded therefore that it is unlikely that CO_2 is an essential intermediate in the production of propionate. They also concluded that if succinate is an intermediate, a C_1 is formed in the decarboxylation which is not in complete equilibrium with the CO_2.

In contrast to the production of propionate by propionibacteria, Cardon and Barker (1947) have presented evidence that *Clostridium propionicum* may reduce lactate directly to propionate, possibly via acrylate. Non-volatile acids are not produced by these bacteria and the only products of this fermentation are propionate, acetate, and CO_2. Johns (1952) found that the C^{14} of $C^{14}O_2$ was not incorporated into the propionate.

In the present study we have fermented C^{14}-labeled lactate, pyruvate, and glycerol to evaluate the possible mechanisms for the formation of propionic acid by *Propionibacterium arabinosum* and *Clostridium propionicum*. If propionate is formed by the direct reduction of the lactate, then propionate-3-C^{14} and acetate-2-C^{14} should be formed from lactate-3-C^{14}. If propionate is formed via a symmetrical 4-carbon intermediate, such as succinate, the propionate should be equally labeled in the 2- and 3-carbons. It has been found with *C. propionicum* that propionate and acetate labeled almost exclusively in the methyl carbons are formed from lactate-3-C^{14}, suggesting the direct reduction and oxidation of the lactate. On the other hand *P. arabinosum* produces propionate labeled almost equally in the 2- and 3-carbons, which suggests that succinate may be an intermediate. However, it will be shown by Wood, Leaver and Stjernholm (*in

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preparation) that propionate is not a stable end product of the fermentation. It therefore is possible that the propionate is formed by direct reduction by *Propionibacterium* but the C^{14} is randomized in C-2 and C-3[2] by a secondary change which is not an obligatory reaction during the formation of propionate.

METHODS

The methods employed for the separation of products of the fermentation and the degradation of the propionate and succinate were the same as those given by Wood and Leaver (1953). In some of the early experiments the acetate was degraded by pyrolysis of the barium salt; in later experiments the propionate and acetate were degraded by the Schmidt reaction according to the method outlined by Phares (1951). The succinate in these latter experiments was decarboxylated by *Micrococcus lactilyticus* and the resulting propionate after separation on a celite column was degraded by the Schmidt reaction. Johns (1951) has shown that these bacteria readily decarboxylate succinate to give propionate and CO_2, and H. E. Swim (personal communication) has demonstrated that carboxyl-labeled succinate gives rise to carboxyl-labeled propionate and 2,3-labeled succinate to 2,3-labeled propionate. Degradation of lactic acid was accomplished by oxidation with potassium permanganate as described by Wood *et al.* (1941*b*).

The lactate-3-C^{14} was prepared from propionate-3-C^{14} by bromination and treatment with potassium carbonate (Wood and Leaver, 1953). The benzoic acid was removed by steam distillation and the lactic acid was removed from the inorganic salts by ether extraction. The propionate-3-C^{14} was synthesized by condensing $C^{14}H_3I$ with ethyl malonate. The ester was saponified, acidified and refluxed to give propionate-3-C^{14} which was purified by steam distillation followed by chromatography on a silica gel column as described by Elsden (1946). The lactic acid-1-C^{14} was made from propionate-1-C^{14}. The propionate-1-C^{14} was synthesized by the Grignard reaction. The pyruvate-2-C^{14} was prepared from acetate-1-C^{14} as described by Anker (1948). The glycerol-C^{14} used in these experiments was a generous gift by M. L. Karnovsky of the Harvard Medical School Biophysics Laboratory. The pyruvate-1-C^{14} was a gift of M. F. Utter of the Western Reserve University School of Medicine.

All the experiments were conducted with cell suspensions under anaerobic conditions. The conditions used in each experiment are described in the tables.

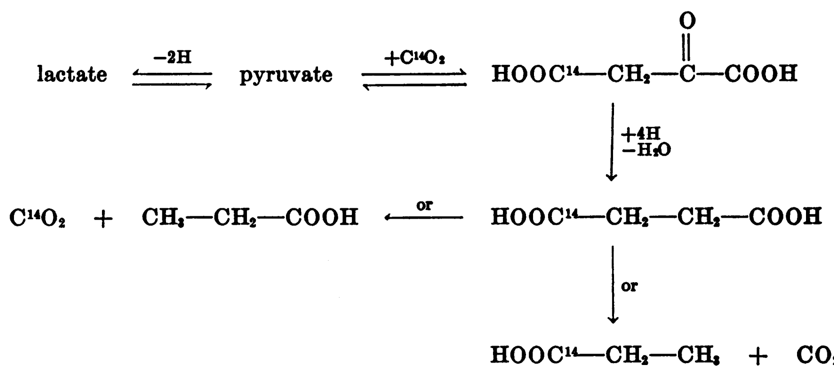


Figure 1. Formation of propionate via a C_4-dicarboxylic acid.

Conversion of lactate-3-C^{14} to propionate and acetate by Clostridium propionicum. Cardon and Barker (1947) have shown that this organism ferments lactate in almost quantitative agreement with the Fitz equation. This fact was confirmed in our present experiments using resting cells. The distribution of C^{14} in the products from lactate-3-C^{14} is shown in table 1. The values are the per cent of the carbon which was derived from the β carbon of lactate and were obtained by dividing the specific activity of the carbon in the product by the specific activity

[2] C-3 is used as an abbreviation for carbon three of a compound; C_3 as an abbreviation for a three-carbon compound.

of the labeled carbon of the substrate and multiplying by 100. For example, C-3 of lactate = 61.0 cpm/μM of C and of propionate = 57.4 cpm/μM C, therefore the activity in the C-3 of propionate is $57.4 \div 61.0 \times 100 = 94.1$. As a control on the reliability of the degradation each compound was oxidized to CO_2 and the specific activity of the CO_2 was determined and multiplied by the number of carbons in the compound. The sum of the activities of the individual carbons as determined by degradation was compared with the activity obtained from the oxidized carbon (for propionate 95.7 as compared to the 98.0 found by oxidation).

The demonstration that 94 per cent of the β carbon of the propionate was from the β carbon of lactate and that no other position had appreciable labeling supports Cardon and Barker's suggestion that lactate is reduced directly to propionate by *C. propionicum*. It is noted that the acetate is labeled solely in the methyl carbon showing that lactate was converted to acetate without any randomization of the label. The somewhat low activity of the methyl carbon of the acetate may be due to dilution by endogenous acetate. It should also be noted that very little C^{14} was found in the CO_2.

TABLE 1

Distribution of C^{14} in products from fermentation of lactate-3-C^{14} by Clostridium propionicum

Values are specific activity per μM of C expressed in per cent of the activity of the C-3 of lactate (61.0 cmp per μM).

Propionate				Acetate			
CH_3—	CH_2—	COOH	Oxidized C x 3	CH_3—	COOH	Oxidized C x 2	CO_2
94.1	1.6	0	98.5	78.0	0	78.8	0.03

The reaction mixture was added to a 300-ml flask which had been evacuated. 0.5 mM of CO_2 were added, then 57 ml of a 3 per cent suspension of the bacteria in a mixture of 0.05 per cent sodium thioglycolate and 0.1 M phosphate buffer at pH 7.0 together with the lactate-3-C^{14} in 3 ml were added by means of a dropping funnel. The lactate consisted of 0.312 mM of D,L-lactate containing 48,500 cpm and 0.242 mM of unlabeled L-lactate. It therefore contained 0.398 mM of L-lactate with a specific activity of 61.0 cpm per μM and .156 mM of D-lactate with 155.0 cpm per μM. The calculations are on the basis of only L-lactate being fermented. The temperature was 38 C and reaction time 5 hr.

The bacteria were grown 1 day at 38 C in the lactate, peptone, yeast, extract medium of Cardon and Barker (1946) and were washed under anaerobic conditions with 0.05 per cent sodium thioglycolate by excluding the air with nitrogen.

Fermentation of labeled lactate, pyruvate and glycerol by Propionibacterium arabinosum. Similar studies were undertaken with labeled lactate and *P. arabinosum*. The bacteria were grown on a lactate-glucose medium since it was found that growth on this medium increased their activity on lactate as compared to cells grown on glycerol or glucose. In these experiments from 1 to 7 mM of succinate were produced per 100 mM of lactate fermented. Usually more propionate and CO_2 but less acetate was formed than would be expected on the basis of the Fitz equation. The data showing the distribution of C^{14} are presented in table 2. It is obvious that this distribution is strikingly different from that found with *C. propionicum*. With 2- or 3-labeled lactate all the products were labeled in all positions, with carboxyl-labeled lactate the C^{14} was confined almost entirely to the carboxyl carbons.

The labeling in the propionate will be considered first. The concentration of C^{14} was approximately equal in the 2- and 3-positions of propionate but the labeling in the propionate was not always identical with that of the succinate; particularly in fermentation 1 where the center carbons of succinate had less C^{14} than C-2 and C-3 of the propionate. If succinate were the precursor of propionate it would be expected that the labeling in the succinate and propionate would be the same. However, differences in labeling between succinate and propionate could occur if during the fermentation the metabolism of the lactic acid changed in such a manner that the C^{14} distribution was altered in the succinate. For example, if succinate with one type of labeling were formed early in the fermentation which was largely converted to propionate, and later a different type of labeled succinate were formed which accumulated, the labeling in the succinate and propionate would differ yet the succinate could have been the source of all the propionate.

In table 3 are presented data on the distribution of C^{14} from the fermentations of labeled pyruvate and glycerol. The propionate from pyruvate-2-C^{14} or glycerol-2-C^{14} had a C^{14} pattern

TABLE 2

Distribution of C^{14} in products from fermentation of lactate-C^{14} by Propionibacterium arabinosum, 34W

Values for products are specific activity in per cent of the specific activity of the labeled position of the lactate.

No.	Substrate	Propionate				Succinate			Acetate			CO_2	cmp/μM Labeled C of Lactate
		CH_3—	CH_2—	COOH	Oxidized C x 3	(CH_2—	$COOH_2)_2$	Oxidized C x 4	CH_3—	COOH	Oxidized C x 2		
1	Lactate-3-C^{14}	40.7	43.9	3.62	87.2	35.6	2.54	76.6				0	5.90
2a	Lactate-3-C^{14}	41.5	51.0	9.44	99.6	43.1	7.4	101	43.1	30.6	74.3	2.4	25.6
2b	Lactate-2-C^{13}	40.0	44.3	7.85	85.8				26.7	56.6	83.5	4.3	1.4*
3	Lactate-3-C^{14}	47.6	45.5	4.91	93.6								14.3
4	Lactate-1-C^{14}†	0.60	0.74	98.3	93.1	0.79	72	167					11.6
5	Lactate-1-C^{14}	1.1	1.1	85.4	91.5	1.39	63	133					11.6

* Atoms per cent excess C^{13}.

† No. 4, the bacterium used in this experiment was *Propionibacterium pentosaceum*, 49W.

Nos. 1, 3, 4, and 5 were in an evacuated 300-ml flask to which the following components were added: 5 per cent suspension of washed cells, 0.075 M potassium phosphate buffer pH 5.9, 0.125 M $NaHCO_3$, plus substrate as indicated below.

No. 1, total volume 60 ml, lactate was 0.074 M.

Nos. 3, 4, and 5 total volume 180 ml, lactate was 0.109 M.

No. 2 was in a 1,000-ml Erlenmeyer flask containing 100 ml of the following medium: 0.0,566 M lactate, 0.3 M potassium phosphate buffer pH 7.0, 5 per cent washed cells. The flask was flushed with 5 per cent CO_2-95 per cent N_2 and the pressure reduced to 48 cm of Hg.

The cells were grown on 1 per cent lactate, 0.1 per cent glucose, 0.5 per cent yeast extract for 3 days at 30 C. Cells were centrifuged and washed twice with distilled water.

very similar to that of the lactate-2-C^{14} or lactate-3-C^{14}. It should be noted that in the fermentation of pyruvate-2-C^{14} and glycerol-2-C^{14} (nos. 6 and 9), the 2-position of the propionate had a significantly higher specific activity than the 3-position. This observation might be construed as evidence for at least a partial formation of propionate by direct reduction. However, this interpretation is weakened by the observation made in fermentation no. 2, table 2, in which the lactate was doubly labeled (2-C^{13}, 3-C^{14}). It is noted that the C-2 of the lactate gave rise to propionate with higher labeling in the 2-position than the 3-position, but the C-3 also gave the same type of distribution. This latter result is the opposite of that expected from direct reduction. It is of interest that in this fermentation and in most of the others the sum of the activities of the C-1 and C-3 of propionate nearly equalled the activity of C-2 (nos. 1, 2, 3, 6, 9, 10). At present there is no satisfactory explanation of the C^{14} distribution. One possibility is the reversible conversion of propionate or its C_3 precursor to a symmetrical C_3 compound. In this manner the 3-position would be randomized into the 1-position and the activity in the 2-position would remain unchanged (see Leaver and Wood, 1953, for an illustration). Thus a sequence in which the 2- or 3-labeled substrate was converted first to succinate, next to propionate or its precursor and then was partially equilibrated with a symmetrical C_3 compound would yield 50 per cent of the activity in C-2 and the remaining 50 per cent in C-3 and C-1. With some exceptions this is approximately the distribution which was found with the 2- or 3-labeled substrates, i.e., C-1 plus C-3 equalled C-2. The relationship of the 3- and 1-positions is believed not to be due to "cross contamination" during the degradation. When chemically synthesized propionate-3-C^{14} was degraded less than 1 per cent of the activity was in the 1- and 2-positions and 97 to 100 per cent of the activity was found in the 3-position. The question of the conversion of propionate to a symmetrical C_3 compound is considered further by Wood, Leaver and Stjernholm (1955).

The propionate and succinate from fermentations of carboxyl-labeled lactate and pyruvate (nos. 4, 5 and 7) were of particular interest. In fermentations of glucose-3,4-C^{14} it has been observed (Leaver and Wood, 1953, and Wood, Stjernholm and Leaver, 1955) that approximately

TABLE 3

Distribution of C^{14} in products from fermentation of labeled pyruvate and glycerol by Propionibacterium arabinosum, 34W

Values for products are specific activity in per cent of labeled position of the substrate.

No.	Substrate	Propionate				Succinate			Acetate			CO_2	cpm/µM of Labeled C in Substrate*
		CH_3—	CH_2—	COOH	Oxidized C x 3	(CH_2—	COOH)$_2$	Oxidized C x 4	CH_3—	COOH	Oxidized C x 2		
6	Pyruvate-2-C^{14}	36.8	46.4	12.1	95.4				35.4	63.5	98.3		8.5
7	Pyruvate-1-C^{14}	1.6	1.3	69.8	73.5			71.9			2.31	81.5	26.0
8	Glycerol-1,3-C^{14}	36.0	37.2	62.4	137.8	38.2	52.2	183.6	37.6	17.5	55.4	12.5	13.0†
9	Glycerol-2-C^{14}	32.5	45.0	3.68	79.9	45.5	1.99	94.4	26.0	35.5	62.0	2.1	10.8
10	Glycerol-2-C^{14}*	48.5	46.2	0.90	100*	—	—	—	—	—	—	—	11.3

See table 5 for yields of fermentation products.

* The activities of no. 10 are expressed in per cent of the total activity of the propionate.

† 26 cpm per µM of glycerol.

The cells were grown as follows: A culture was grown for 3 days at 30 C on the following medium: 0.3 per cent i-erythritol, 0.05 per cent adonitol, 0.05 per cent mannitol, 0.5 per cent yeast extract, and 0.01 M K-phosphate pH 6.8. These cells were used to inoculate the following medium: 0.3 per cent glycerol, 0.05 per cent D-mannitol, 0.05 per cent adonitol, 0.05 per cent i-erythritol, 0.5 per cent yeast extract, and 0.01 M KPO_4 pH 6.8, except no. 9 which was grown out on 0.5 per cent yeast extract, 0.5 per cent glycerol, and 0.05 M K-phosphate 6.9. All growth was for 3 days at 30 C. The cells were centrifuged and washed twice with distilled water.

Fermentation nos. 6, 8 and 9 were in a 1000-ml Erlenmeyer flask. Volume of medium was 100 ml. The flasks were gassed with N_2 and partially evacuated, then the substrate, bacteria and $NaHCO_3$ were added. Fermentation no. 7 was in a 125-ml. Warburg vessel containing 50 ml of medium. Gas phase N_2. Fermentation no. 10 was in a 300-ml evacuated vessel to which 100 ml of medium were added. All flasks contained 5 per cent suspension of washed cells. Temperature of all fermentations was 30 C.

No. 6 contained 0.3 M K-phosphate buffer pH 7.0, 0.0178 M $NaHCO_3$, 0.0495 M pyruvate and was fermented for 24.5 hrs.

No. 7 contained 0.3 M K-phosphate buffer pH 7.0, 0.0872 M pyruvate and was fermented for 30.5 hrs.

No. 8 contained 0.3 M K-phosphate buffer pH 7.0, 0.0455 M $NaHCO_3$, 0.0828 M glycerol and was fermented for 40 hrs.

No. 9 contained 0.3 M K-phosphate buffer pH 7.0, 0.0376 M $NaHCO_3$, 0.0617 M glycerol and was fermented for 23 hrs.

No. 10 contained 0.3 M K-phosphate buffer pH 6.6, 0.015 M $NaHCO_3$, 0.0433 M glycerol, 8.88×10^{-4} M HCHO and was fermented for 6.3 hrs.

6 per cent of the carbon in the α- and β-positions of the propionate and succinate were derived from the 3- and 4-carbons of glucose. These results have been interpreted as evidence that glucose is metabolized to an appreciable extent by some mechanism other than the Meyerhof scheme. In making this interpretation it is assumed that carboxyl-labeled lactate or pyruvate, which would be formed from glucose-3,4-C^{14} by the Embden-Meyerhof reactions, does not yield α,β-labeled propionate or succinate. The present results show the assumption is correct since very little of the carboxyl carbon of lactate or pyruvate was converted to the non-carboxyl carbons of propionate and succinate.

The C^{14} distribution in the acetate is likewise of interest and differs from that found in the *C. propionicum* fermentation. In the fermentation by *P. arabinosum* the C^{14} was found in both positions. With lactate-3-C^{14} and glycerol-1,3-C^{14} the activity was highest in the methyl group. With lactate-2-C^{14}, pyruvate-2-C^{14}, and glycerol-2-C^{14} the highest activity was in the carboxyl group. This distribution is in accord with the suggestion that the acetate is formed in part prior to randomization of the C^{14} and in part after randomization. The randomization of C^{14} in acetate could be explained by assuming that pyruvate is the precursor and that pyruvate is reversibly converted to succinate.

TABLE 4

Distribution of C^{14} in the fermentation of glycerol-2-C^{14} Ferment no. 9 of table 3

Compound Isolated	mM of Compound per 100 Ml of Fermentation	Cpm per μM of C (CO_2 from Total Oxidation)	Total Counts in Compound	Per Cent of Total Counts of Fermented Glycerol*
Propionate...	3.58	2.87	30,800	56.4
Acetate.......	0.77	3.35	5,160	9.4
Formate......	0.03			
Succinate.....	0.68	2.55	6,840	12.5
Propyl alcohol	0.22	2.53†	1,690†	3.1†
CO_2..........	3.116	0.227	720	1.3
Cells.........	26.5	0.197	5,220	9.5
Total.......			50,430	92.2

99.5 per cent C recovery, O/R = 1.08.

* 5.08 mM of glycerol were fermented. Specific activity was 10.78 cpm per μM or 54,700 counts were metabolized.

† The activity of the propyl alcohol is assumed to be the same as the propionate.

$$C_1 + \text{pyruvate} \rightleftarrows \text{oxalacetate} \rightleftarrows \text{succinate} \quad (1)$$

$$\text{pyruvate} \rightarrow \text{acetate} + CO_2 \quad (2)$$

Another possible mechanism of randomization has been advanced by Mahler and Hunnekens, 1953.

Balance studies of the products from pyruvate and glycerol. There are a number of observations which indicate that the fermentation of C_3 compounds is more complex than is generally indicated in schemes for the propionic acid bacteria. If the formation of propionate, succinate and acetate occurred solely as illustrated in figure 1 and equations 1 and 2, then 2- or 3-labeled lactate, pyruvate or glycerol would yield succinate, propionate and acetate containing a full equivalent of labeled carbon, providing there was no dilution of C^{14} by the endogenous metabolism of the cells. The C-1-labeled substrates would yield unlabeled acetate and since carboxyl labeled succinate and propionate would be formed there most likely would be some loss of C^{14} by CO_2 exchange with the $NaHCO_3$ buffer.

It is of interest that most of the products from the fermentations given in tables 2 and 3 had a lower specific activity than the substrates which were fermented. If the products contained one equivalent of labeled carbon, the values listed under oxidized C would be 100. The only products with more than one equivalent were the succinate from lactate-1-C^{14} and the propionate and succinate from glycerol-1,3-C^{14}. The latter is to be expected because of the double labeling in the substrate. The presence of more than 1 equivalent of labeled carbon (167 and 133) in the succinate from lactate-1-C^{14} is evidence that the carboxyl group of lactate is a source of carbon for both carboxyls of the succinate, there being very little C^{14} in the non-carboxyl carbons. It is likely that a "C_1" is formed from the carboxyl group of lactate and contributes to the second carboxyl of the succinate.

The question of C^{14} dilution by endogenous metabolism and by CO_2 exchange is of importance in consideration of the specific activity of the C^{14} in the products. In table 4 the distribution of the C^{14} in the products and in the cells is given for fermentation no. 9, glycerol-2-C^{14}. In this fermentation very little C^{14} (1.3 per cent) was converted to CO_2, so the loss of C^{14} by exchange

TABLE 5

Carbon balance of products of fermentations of table 3

No.	Substrate	mM Fermented	Products per 100 mM Substrate Fermented: Propionate	Acetate	Succinate	Propyl alcohol	CO_2	Formate	Per Cent Recovery: C^{14}	C	O/R
6	Pyruvate-2-C^{14}	3.00	35.3	49.6	5.3*	0	75.5	0.9	92.5	100.9	1.16
7	Pyruvate-1-C^{14}	4.36	40.0	40.0	2.5*	0	77.4	2.2	95.2	96.5	1.14
8	Glycerol-1,3-C^{14}	3.85	92.2	7.54	6.24	1.56	−2.0	2.34	87.5†	107	1.03
9	Glycerol-2-C^{14}	5.08	70.5	15.2	13.6	4.33	−11.8	0.59	92.2†	99.5	1.06

See table 3 for isotope distribution in the products and conditions of fermentation.

† Included in these values is the amount of C^{14} incorporated in the cells. 8.2 per cent of the total activity was found in the cells of experiment no. 8 and 9.5 per cent in experiment no. 9.

* These values represent the total non-volatile acid produced.

of CO_2 by the fixation reaction was small. However, 9.5 per cent of the C^{14} of the fermented glycerol was converted to cellular material as determined by oxidation of the washed cells. It is evident therefore that considerable dilution of C^{14} could occur by turnover of cellular material. It is by no means certain, however, that all the C^{14} uptake by cells is accompanied by a simultaneous contribution of C^{12} to the end products by turnover since some of the C^{14} in the cells may result from net synthesis of a cellular component. The endogenous dilution is thus an unknown factor, but the dilution probably is less than 10 per cent in the non-carboxyl carbons of the products.

Another indication that the fermentation is more complex than is indicated in the schemes of figure 1 and equations 1 and 2 is the fact that carbons 2 and 3 of lactate contribute to the CO_2 (Fermentation 2a, 2b, table 2). There was 5 per cent $C^{12}O_2$ in the gas phase of this fermentation, hence the C^{14} converted to CO_2 was diluted. The actual per cent of the CO_2 derived from labeled carbon was probably two or three times larger than is indicated by the uncorrected specific activities of the CO_2.

Examination of the yields of products also points to the complexity of the fermentation. In table 5 are presented the mM of products per 100 mM of substrate metabolized in the fermentations described in table 3. The carbon and C^{14} recoveries as well as the O/R balances are given.

The variation from the predicted values is indicated by the following considerations. The fermentation of pyruvate to propionate, acetate and CO_2 is usually represented as follows:

$$3CH_3\cdot CO\cdot COOH + H_2O \rightarrow CH_3\cdot CH_2\cdot COOH + 2CH_3\cdot COOH + 2CO_2. \quad (3)$$

The fact that propionate formation may involve a CO_2 fixation followed by decarboxylation does not alter the above stoichiometry. In fermentation 7, there was 2.5 mM of succinate produced. By the mechanism of figure 1 this would take place by CO_2 fixation and may be represented as follows:

$$2.5CO_2 + 2.5CH_3\cdot CO\cdot COOH \rightarrow 2.5COOH\cdot CH_2\cdot CO\cdot COOH \xrightarrow{+10H} 2.5COOH\cdot CH_2CH_2COOH.$$

For the above reduction 5 mM of pyruvate would be required.

$$5CH_3\cdot CO\cdot COOH + 5H_2O \rightarrow 5CH_3\cdot COOH + 5CO_2 + 10H.$$

By equation 3, the remaining 92.5 mM would yield

$$92.5CH_3COCOOH \rightarrow 30.8CH_3CH_2COOH + 61.6CH_3COOH + 61.6CO_2.$$

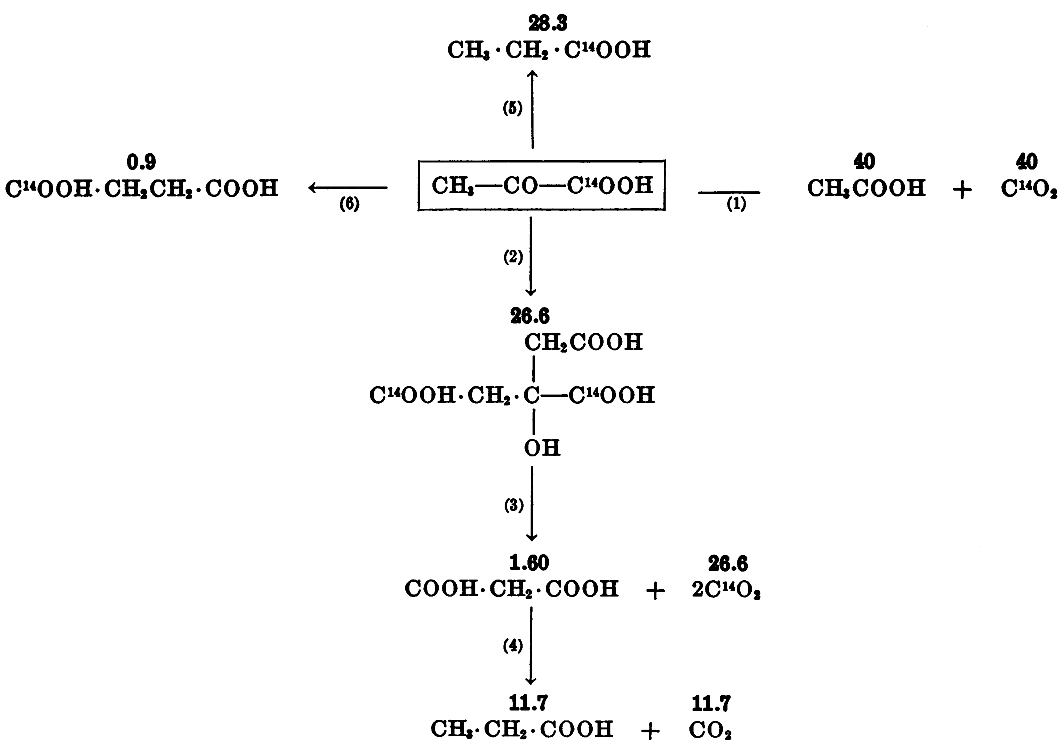
The net of these reactions is: succinate = 2.5, propionate = 30.8, acetate = 66.6, and CO_2 = 64.1. The actual yields of fermentation 7 were quite different from these values, i.e., propionate 40.0, acetate 40.0, and CO_2 77.4. Since the O/R balance for the determined products was 1.14, it is obvious that there cannot be a perfect fit between the observed and theoretical values, but the discrepancy appears to be beyond that of errors indicated by the O/R balance. Similar results were obtained in balance studies of glucose fermentations by Wood and Werkman (1936). They proposed that there was formation of succinate by a synthesis possibly involving condensation of two molecules of acetate and that succinate was broken down to propionate and CO_2. These proposals were made to account for the high yields of CO_2 and propionate accompanied by low yields of acetate.

Carson and Delwiche (1952) and Delwiche and Carson (1953) have presented evidence for the occurrence of citrate and α-ketoglutarate in the propionic acid fermentation and have considered the possibility of the occurrence of part of the Krebs cycle in the fermentation. Figure 2 shows the data of fermentation 7 as adapted to the above suggestions. The scheme is presented in skeleton form and it is not intended to imply that the actual mechanism occurs as pictured. The condensation may occur, of course, in other ways than via citrate, for example by condensation of two C_2 units to give succinate. The amount of acetate has been used as the starting point for the calculation, 40 mM of acetate were produced in the fermentation and this would account for 40 mM of CO_2 via reaction 1. It has been assumed that all the remaining CO_2 is formed via reactions 2, 3, and 4 and that succinate is formed by two routes: one by C_3 and C_1 condensation, reaction 6, and the other via citrate, reaction 3. The amount of succinate arising by each route has been calculated from the

C^{14} content of the succinate. The succinate formed by reaction 6 would contain two equivalents of labeled carbon, that by reaction 3 none. The succinate isolated from the fermentation contained 71.9 equivalents of C^{14}; therefore 71.9/2 = 36 per cent of the succinate or 2.5 × 0.36 = 0.90 mM formed by the fixation reaction. By the non-labeling process there would be 2.5 − 0.90 = 1.60 mM. In the fixation reaction 6, 0.90 mM of "C_1" would be used. The total C_1 would be the observed CO_2 plus the C_1 fixed in the succinate or 77.4 + 0.90 = 78.3. The CO_2 produced in reactions 3 and 4 is therefore 78.3 − 40.0 = 38.3 (the 40.0 being the CO_2 formed along with the acetate). Of these 38.3 mM of CO_2, 2 × 1.60 = 3.20 would be formed along with the 1.60 mM of succinate in reaction 3 and the remainder, 38.3 − 3.2 = 35.1, would arise in the formation of propionate via reactions 3 and 4. Since 3 mM of CO_2 are formed per mM of propionate in this conversion, 35.1/3 = 11.7, the mM of propionate which would be formed via Reactions 3 and 4. 26.6 mM of pyruvate would be required for reactions 3 and 4; i.e., (2 × 11.7) + (2 × 1.6) = 26.6.

40.0 mM of propionate were formed in the fermentation so that 40.0 − 11.7 = 28.3, the mM of propionate which would be formed via reaction 5. It is seen that the total pyruvate required would be 95.8 mM (28.3 via reaction 5, 0.9 via reaction 6, 40.0 via reaction 1, and 26.6 via reaction 2). The recovery of carbon was 96.5 per cent in the products.

In these calculations the C^{14} content has been utilized only in calculating the relative amounts of succinate which would be formed by the different pathways. Therefore, the isotope content of the other products may be used as an independent indication of whether or not the scheme is in agreement with the observations. It is seen from figure 2 that 66.6 mM of CO_2 out of 77.4 mM of CO_2 arose from a carboxyl group labeled with C^{14}, whereas 11.7 mM arose from non-labeled carbon. The per cent from the labeled positions



Values in parentheses are numbers of the reaction, bold face numbers are mM.

Figure 2. Agreement of C^{14} data with formation of succinate by condensation and the conversion of succinate to propionate and CO_2.

is therefore (66.6/78.3)100 = 85.1. It is seen that the per cent equivalent of carboxyl positions that arose from the labeled position (table 3) was 81.5.

In the case of propionate, 28.3 of the 40 mM are calculated to arise from a labeled source and 11.7 mM from a non-labeled source. Therefore (28.3/40)100 = 70.8 per cent of the carboxyls are from labeled carbon. The observed value for the per cent equivalents of the labeled position in the carboxyl group was 69.8. The agreement of the calculated C^{14} concentrations with the observed values is therefore remarkably good. The exchange reactions of CO_2 with the carboxyl groups would not affect the results very much in this fermentation since there was not a large difference between the C^{14} concentration of the CO_2 and the carboxyl groups of the acids. It is not intended to indicate that only the succinate formed by reaction 3 would be decarboxylated; this was done only to simplify the calculations.

A similar fitting of the data of experiment 6 to the scheme would be of interest but it was not possible because the C^{14} distribution in the succinate was not obtained. It would be valuable to have a complete balance on such a fermentation and to conduct similar calculations.

There are obvious inadequacies in the above explanation of the C^{14} results. It does not account for the observed occurrence of less than 1 equivalent of labeled carbon in the acetate from the lactate-2-C^{13}, 3-C^{14} of fermentation 2a, b, nor of glycerol-2-C^{14} of fermentation 9. In the latter case, since a small amount of acetate is formed, the endogenous acetate may have a large dilution effect. The fact that the C-3 of propionate has less activity than does C-2 is not considered. The conversion of the C-3 and C-2 of the substrate to the carboxyls of propionate and succinate could be accounted for by this mechanism.

In the glycerol fermentations the relationship of the total "C_1" to the succinate is of interest. Wood and Werkman (1938) found with proliferating cells that the acetate plus CO_2-utilized equalled the succinate. It was assumed that the formation of acetate was accompanied by formation of a "C_1" and this together with CO_2 provided the "C_1" for the $C_3 + C_1$ condensation. It is to be noted in fermentation 9 (table 5) that this relationship does not hold; 15.2 mM of acetate were formed and 11.8 mM of CO_2 were utilized. The sum is 28.0 and only 13.6 mM of succinate were formed. The present results only serve to emphasize the large variability in the quantitative relationship of the end products that are obtained in the propionic acid fermentation. They indicate that the traditional schemes which have been proposed to represent the fermentation are not adequate to account for the total results.

SUMMARY

It has been found that *Clostridium propionicum* reduces lactate-3-C^{14} to propionate-3-C^{14}. Acetate is labeled only in the methyl carbon. Such a distribution of labeling suggests the direct reduction of lactate to propionate by *C. propionicum*. When lactate-3-C^{14} is fermented by *Propionibacterium arabinosum* the propionate is labeled mainly in the 2- and 3-carbons but some tracer is found in the carboxyl carbon and the CO_2. Both carbons of the acetate are labeled, the methyl carbon higher than the carboxyl carbon. A similar distribution of tracer in the propionate was found when lactate, pyruvate, or glycerol was labeled in the 2-carbon. In these latter fermentations the acetate was labeled highest in the carboxyl group. The distribution of tracer in propionate is suggestive that succinate may be its precursor; however, C^{14} in the propionate did not always mirror that of the succinate, as might be expected if propionate were formed solely from succinate (Wood, Stjernholm, and Leaver, 1955).

The quantitative relationship of the end products and the C^{14} data indicate that the succinate and propionate may be formed in part by a condensation reaction, perhaps via citrate.

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