

## The Fermentation of Lactic Acid by a Gram-negative Coccus

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(Received 19 May 1958)

A number of types of bacteria convert lactate into propionate anaerobically and the mechanism of this reaction has been widely studied. Johns (1951*a*), using washed cells of *Veillonella gazogenes* (*Micrococcus lactilyticus*), found that lactate was fermented only in the presence of carbon dioxide and that the amount of propionate formed increased with the carbon dioxide tension in the medium. When lactate was fermented in the presence of  $^{13}\text{CO}_2$ , [1- $^{13}\text{C}$ ]propionate was produced. Fumarate and L-malate were both attacked by *V. gazogenes*, giving a mixture of propionate and acetate; succinate was quantitatively decarboxylated to propionate and carbon dioxide. Johns concluded that in this organism the synthesis of propionate involves the formation of a dicarboxylic acid with four carbon atoms by a carbon dioxide-fixation reaction, and that this acid is reduced to succinate which is then decarboxylated to propionate. Whiteley (1953*a-c*) studied the decarboxylation reaction in more detail, using cell-free extracts of *V. gazogenes*. She found that before decarboxylation succinate is converted into succinyl-coenzyme A. The succinyl-coenzyme A is then decarboxylated, yielding carbon dioxide and propionyl-coenzyme A. Transfer of coenzyme A from the latter to succinate yields propionate and succinyl-coenzyme A, thus enabling the reaction to proceed.

A similar mechanism may operate in *Propionibacterium* sp. (Delwiche, 1948; Delwiche, Phares & Carson, 1953, 1954; Johns, 1951*b*; Leaver, Wood & Stjernholm, 1955), but evidence has been presented (Wood, Stjernholm & Leaver, 1955) that it may not be the sole pathway of propionate formation in these bacteria.

By contrast, *Clostridium propionicum* appears to form propionate by a more direct mechanism without production of intermediary dicarboxylic acids. This organism differs from both *V. gazogenes* and *Propionibacterium* sp. in so far as it ferments acrylate. Johns (1952) showed that washed cells of *C. propionicum* grown on lactate neither decarboxylated succinate nor fermented DL-malate and fumarate; further, the fermentation of lactate was not influenced by the tension of carbon dioxide and the propionate formed from lactate in the presence of  $^{13}\text{CO}_2$  was unlabelled.

Leaver *et al.* (1955) showed that *C. propionicum* fermented [3- $^{14}\text{C}$ ]lactate to [3- $^{14}\text{C}$ ]propionate and [2- $^{14}\text{C}$ ]acetate, indicating a direct reduction and oxidation of the lactate. Succinate behaves as a symmetrical molecule in biological systems, and thus if it is an intermediate in the formation of propionate an equimolar mixture of [3- $^{14}\text{C}$ ]- and [2- $^{14}\text{C}$ ]-propionate would have been produced.

The rumen organism LC (Elsden, Volcani, Gilchrist & Lewis, 1956) ferments DL-lactate with the formation of hydrogen, carbon dioxide, acetate, propionate, *n*-butyrate, *n*-valerate and small amounts of *n*-caproate; it also ferments acrylate (Elsden & Lewis, 1953). Elsdén *et al.* (1956) found that neither washed-cell suspensions nor growing cultures decarboxylated succinate and they suggested that lactate is converted into propionate by the direct route with acrylate or a derivative of acrylate as an intermediate.

The fermentation of [2- $^{14}\text{C}$ ]lactate by washed-cell suspensions and cell-free extracts of LC has now been investigated and the results given below support the view that lactate is converted directly into propionate.

### MATERIALS AND METHODS

*Growth media and preparation of washed suspensions.* The strain LC1 was used in all experiments and was maintained on the following medium: 2% (v/v) of 70% DL-sodium lactate, 0.4% (w/v) of Difco-yeast extract, 0.03% (w/v) of thioglycolic acid and 0.1% (w/v) of Davis New Zealand agar (J. W. Towers and Co. Ltd., Manchester) in tap water. The pH was adjusted to 7.0 before autoclaving.

For experiments with washed cells an overnight tube culture of LC in the above medium was inoculated into 500 ml. of the following liquid medium: 2% (v/v) of 70% DL-sodium lactate, 0.4% (w/v) of Difco-yeast extract and 0.02% of sodium dithionite in tap water. The dithionite was sterilized separately by Seitz filtration and added to the rest of the medium immediately before inoculation. The inoculated medium was incubated for 20–24 hr. at 37° under  $\text{H}_2$ . The cells were harvested and washed by the procedure described by Elsdén & Lewis (1953).

*Preparation of cell-free extracts.* Two types of extract were used, one prepared from freshly harvested washed cells, the other from dried cells. A paste of washed cells was crushed in the Hughes press previously cooled to approx. -20° (Hughes, 1951), and the debris thoroughly mixed with freshly boiled 0.1M-phosphate buffer (a  $\text{KH}_2\text{PO}_4$ -

$K_2HPO_4$  mixture was used throughout), pH 6.5, and containing in addition 0.02% (w/v) of  $Na_2S_2O_8 \cdot 9H_2O$ ; 1 ml. of the buffer mixture was used/g. wet wt. of cells. The suspension was centrifuged at 2° in the high-speed head of an International refrigerated centrifuge for 30 min. at 14 000 g and the supernatant, which contained the activity, was stored under air or  $N_2$  at -20°. These extracts contained approx. 50 mg. of protein/ml., as estimated by the method of Stadtman, Novelli & Lipmann (1951).

Dried cells, prepared as described by Walker (1958), were extracted as follows: a 10% (w/v) suspension of dried cells in water was incubated under  $H_2$  for 1 hr. at 37°. The suspension was then centrifuged at 14 000 g for 30 min. and the supernatant, which contained the activity, was stored under air or  $N_2$  at -20°. These extracts contained approx. 20 mg. of protein/ml.

*Fermentation procedure.* Unless otherwise stated, the fermentations were carried out in 100 ml. flasks similar to those used by Lewis & Elsdon (1955), except that one of the side arms was fitted with a stopper carrying a stop-cock. The suspension was placed in the main compartment and the substrate in one of the side arms; the other side arm contained 0.7 ml. of 10N- $H_2SO_4$ . The flasks were gassed with  $O_2$ -free  $N_2$  and were then shaken in a water bath at 37°. After equilibration, the pressure within the flasks was released by momentarily opening the main cock. The substrate was then tipped from the side arm. At the end of the experiment the contents were acidified by mixing with the  $H_2SO_4$ .

*Assay of radioactive carbon dioxide.* A  $CO_2$ -absorption tube (Sakami, 1955) containing 2N-NaOH soln. ( $CO_2$ -free) was fitted to the reaction vessel and the  $CO_2$  produced swept out in a stream of  $CO_2$ -free air. The total  $CO_2$  formed was measured manometrically as follows: duplicate samples (1 ml.) from the absorber were pipetted into Warburg manometer cups and the  $CO_2$  was liberated by acidification with 0.5 ml. of 5N- $H_2SO_4$ . Further samples of NaOH soln. were taken from the absorber, and diluted with water, and carrier  $Na_2CO_3$  was added and barium carbonate plates were prepared and assayed for radioactivity as described by Ormerod (1956). The radioactivity was measured with a Geiger-Müller end-window counter. The activity was corrected for background and self-absorption and the results were expressed as specific activities (counts/min./ $\mu$ mole) at infinite thinness.

*Volatile fatty acids.* After removal of the  $CO_2$  from the reaction vessel the contents were centrifuged and samples of the supernatant were steam-distilled in a Markham apparatus. The distillate was aerated with  $CO_2$ -free air for 2-3 min. and the total acids were determined by titration with standard NaOH soln. (approx. 0.01N). The neutralized solution was evaporated to dryness in a boiling-water bath and the residue dissolved with scratching in 2-3 ml. of an acidified 5% (v/v) solution of butanol in chloroform (2 drops of conc.  $H_2SO_4$  in 50 ml. of solution). The individual fatty acids were separated on Celite columns buffered at either pH 6.5 or pH 7.6, according to Bueding & Yale (1951). Fractions (5 ml.) were collected and titrated with standard NaOH soln. (approx. 4mN) in a stream of  $CO_2$ -free air, with phenol red as indicator. The fractions containing the individual neutralized acids were pooled and evaporated to a final volume, usually 10 ml., and samples taken for assay of the radioactivity.

Where the total radioactivity of the fatty acid was

required, a sample to which sufficient carrier acid had been added to bring the total carbon content to 0.5 mg.-atom was dried in a combustion flask and the residue oxidized to  $CO_2$  (Ormerod, 1956). The  $CO_2$  produced was assayed for radioactivity as described above. Since the combustion procedure is quantitative, the amount of  $CO_2$  produced was not measured and specific activities were calculated from the  $\mu$ moles of acid oxidized.

Fatty acids were degraded by the method of Phares (1951) as modified by Sakami (1955). This procedure is based on the Schmidt reaction. In this reaction, the carboxyl of the fatty acid is converted into  $CO_2$  and the remainder of the carbon chain is converted into an amine which, on oxidation with permanganate, is converted into a fatty acid containing one less carbon atom.

To a sample of fatty acid contained in a reaction tube, sufficient carrier acid was added to bring the total to 0.5 m-mole. The mixture was then taken to dryness and solid sodium azide, activated according to Sakami (1955), was added; the vessel was thoroughly cooled in ice and the  $H_2SO_4$  added. Sakami recommends the gradual addition of the azide after the addition of the acid, but this was found inconvenient and the recoveries of  $CO_2$  by our method were similar to those given by Sakami. The amine resulting from the final decarboxylation was recovered by distillation and oxidized as the sulphate.

Unlike results with the method for complete combustion, there were significant losses at each step in the degradation (up to 25% during the conversion of the amine into the carboxylic acid). The amount of  $CO_2$  produced by decarboxylation and by combustion of the final amine was measured by direct weighing. If  $x$  counts/min. is the radioactivity of the sample counted (corrected for background and self-absorption) then the radioactivity of the starting material was:  $x$  (moles of acid taken after addition of carrier acid/moles of  $CO_2$  produced by decarboxylation or combustion). When the amount of fatty acid in the sample before addition of carrier is known, the specific activity may be calculated.

*Pyruvate.* Where the specific activity of pyruvate was required the following procedure was adopted. At the end of the incubation period, the reaction mixture was acidified. Samples were deproteinized with trichloroacetic acid (final concn. 5%, w/v), centrifuged and 10 ml. of saturated 2:4-dinitrophenylhydrazine in 2N-HCl was added to the supernatant. After 24 hr. at 2°, the precipitated pyruvic acid 2:4-dinitrophenylhydrazone was filtered off, washed with 2N-HCl and dissolved in 20 ml. of ethyl acetate. The acid 2:4-dinitrophenylhydrazone was extracted into 30 ml. of 10% (w/v)  $Na_2CO_3$  soln., from which it was precipitated by the dropwise addition of conc.  $H_2SO_4$ . After it had stood at 2° for 24 hr. the precipitate was filtered off, dissolved in ethyl acetate and transferred to a tared container. The ethyl acetate was evaporated and the 2:4-dinitrophenylhydrazone dried *in vacuo* and weighed. To determine its radioactivity the derivative was dissolved in a measured volume of ethyl acetate and samples, after drying, were oxidized in the usual manner.

Where the specific activity of the pyruvate carboxyl group was required, samples of the acidified reaction mixture, adjusted to pH 5.0, were placed in Warburg cups with two side bulbs, in one of which was placed 0.5 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl and in the other 0.2 ml. of yeast carboxylase.

Table 1. *Fermentation of DL-[2-<sup>14</sup>C]lactate by washed cells of LC*

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (150 mg. dry wt. of cell material) in 0.1M-phosphate buffer, pH 6.5, containing 0.02% of  $\text{Na}_2\text{S}_2\text{O}_8 \cdot 9\text{H}_2\text{O}$ ; 175.5  $\mu\text{moles}$  of DL-[2-<sup>14</sup>C]lactate [specific activity (s.a.) 4190 counts/min./ $\mu\text{mole}$ ] in 1.25 ml. of water, or 1.25 ml. of water in the control, added from one side bulb after equilibration. Gas phase  $\text{N}_2$ ; temp. 37°. Reaction time 0.75 hr.

Product	Amount produced ( $\mu\text{moles}$ )		Specific activity (counts/min./ $\mu\text{mole}$ )		s.a. of product (corr.) s.a. of lactate
	Uncorr.	Corr.	Uncorr.	Corr.	
Carbon dioxide	170	120	6.8	9.6	0.0
Caproate	6.5	6.5	8800	8800	2.1
Valerate	52.5	42.1	6750	8390	2.0
Butyrate	31.1	24.7	5990	7540	1.8
Propionate	11.8	5.9	2700	5420	1.3
Acetate	21.0	6.9	1380	4180	1.0

Carbon recovered 95.5%; activity recovered 94.5%.

After equilibration for 10 min., the carboxylase was added and when gas evolution had ceased the pressure was noted and the 2:4-dinitrophenylhydrazine tipped into the main compartment to fix the aldehyde produced. The  $\text{CO}_2$  was then transferred to an absorber in a stream of  $\text{CO}_2$ -free air, converted into barium carbonate and counted as described above.

**Substrates.** Sodium DL-lactate was prepared by neutralizing a boiling solution of A.R. lactic acid with dilute NaOH soln. and standardized by the method of Elsdon & Gibson (1954). Sodium acrylate was prepared by steam-distilling an acidified solution of 60% acrylic acid (Light and Co., Colnbrook, Bucks) and titrating with standard alkali in a stream of  $\text{CO}_2$ -free air. The stock solution was standardized by hydrogenation with colloidal palladium (Harrison, 1939). Crystalline sodium pyruvate was prepared from redistilled pyruvic acid (Light and Co.) and standardized manometrically with yeast carboxylase (Westerkamp, 1933). The sodium salts of the fatty acids were obtained by neutralizing solutions of the redistilled acids. Sodium [<sup>14</sup>C]carbonate and sodium DL[2-<sup>14</sup>C]lactate were obtained from The Radiochemical Centre, Amersham, Bucks. A radioautograph, prepared from a chromatogram of the radioactive lactate run in the solvent butanol-acetic acid-water (4:1:5, by vol.), gave one spot with an  $R_f$  value (0.72) identical with that of A.R. lactic acid.

## RESULTS

**Fermentation of DL-[2-<sup>14</sup>C]lactate.** The yields and specific activity of the products of DL-[2-<sup>14</sup>C]lactate fermentation by washed cells of LC are shown in Table 1. The relative proportions of the fatty acids formed varied markedly with different batches of cells. In this particular experiment the suspension had a high synthetic activity and the major proportion of the total fatty acids formed consisted of valeric and butyric acids. The  $\text{CO}_2$  and fatty acids produced, corrected for endogenous formation, accounted for 95.5% of the carbon added as lactate.

The  $\text{CO}_2$  was unlabelled. The corrected specific activities of acetate and propionate were each approximately equal to the specific activity of the

Table 2. *Distribution of <sup>14</sup>C in acetate and propionate produced from the fermentation of DL-[2-<sup>14</sup>C]lactate by washed cells of LC*

Experimental conditions were the same as described for Table 1.

Compound or group	Specific activity (counts/min./ $\mu\text{mole}$ )	Percentage of total activity
Acetate	4180	1000
- $\text{CO}_2\text{H}$	3885	93.0
$\text{CH}_3^-$	27	0.7
Propionate	5420	100
- $\text{CO}_2\text{H}$	121	2.2
- $\text{CH}_2^-$	4680	86.5
$\text{CH}_3^-$	44	0.8

Table 3. *Distribution of <sup>14</sup>C in butyrate, valerate and caproate produced from the fermentation of DL-[2-<sup>14</sup>C]lactate by washed cells of LC*

Experimental conditions were the same as described for Table 1.

Compound or group	Specific activity (counts/min./ $\mu\text{mole}$ )	Percentage of total activity
Butyrate	7540	100
- $\text{CO}_2\text{H}$	3870	51.4
$\text{CH}_3 \cdot [\text{CH}_2]_2^-$	4270	56.6
Valerate	8390	100
- $\text{CO}_2\text{H}$	3760	44.7
$\text{CH}_3 \cdot [\text{CH}_2]_3^-$	3880	46.3
Caproate	8800	100
- $\text{CO}_2\text{H}$	2880	32.8
$\text{CH}_3 \cdot [\text{CH}_2]_4^-$	5350	60.9

added lactate and the corrected specific activities of caproate, valerate and butyrate were each approximately twice that of the lactate. The results suggest that both acetate and propionate were produced directly from lactate and that caproate, valerate and butyrate were each synthesized from two molecules of lactate. Evidence that this was not so for caproate will be presented later (Table 3).

Table 4. Fermentation of DL-[2-<sup>14</sup>C]lactate by cell-free extracts of LC

Experiments were carried out in manometer cups containing 1.6 ml. of cell-free extract (13.6 mg. of bacterial protein) in 0.1 M-phosphate buffer, pH 6.5, containing 0.01% of Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>.H<sub>2</sub>O; 44.2 μmoles of DL-[2-<sup>14</sup>C]lactate [specific activity (s.a.) 6650 counts/min./μmole] in 0.6 ml. of water, or 0.6 ml. of water in the control, were added from the side bulb. Gas phase N<sub>2</sub>; temp. 37°. Reaction time 2.5 hr. Reaction was stopped by addition of 0.5 ml. of 5N-H<sub>2</sub>SO<sub>4</sub>. Bacterial extract was prepared from crushed cells.

Product	Amount produced (μmoles)		Specific activity (counts/min./μmole)		s.a. of product (corr.) s.a. of lactate	Percentage of total activity
	Uncorr.	Corr.	Uncorr.	Corr.		
Acetate	19.7	17.5	5340	6010	0.91	100
-CO <sub>2</sub> H	—	—	5160	5800	—	96.6
CH <sub>3</sub> -	—	—	38	43	—	0.7
Propionate	25.6	24.7	5890	6100	0.92	100
-CO <sub>2</sub> H	—	—	30	31	—	0.5
-CH <sub>2</sub> -	—	—	5430	5630	—	92.4
CH <sub>3</sub> -	—	—	57	59	—	1.0

The distribution of radioactivity in the acetate and propionate molecules is shown in Table 2. The acetate was labelled exclusively in the carboxyl group. Propionate was labelled almost entirely in the methylene group. There are two possible explanations for the very slight activity of the propionate carboxyl group: (a) during the degradation there may have been a very small carry-over of methylene carbon in the initial decarboxylation; (b) the propionate, separated by the chromatography procedure, may have contained traces of acetate, the carboxyl group of which would degrade with the carboxyl group of propionate. The methyl group of propionate was not significantly labelled.

The specific activities of the carboxyl group and the remainder of the molecule were determined for butyrate, valerate and caproate and compared with the specific activity of the complete molecule respectively (Table 3). The specific activity of butyrate was twice that of the butyrate carboxyl; the specific activity of valerate was twice that of the valerate carboxyl and the specific activity of caproate was three times that of the caproate carboxyl.

The fermentation of DL-[2-<sup>14</sup>C]lactate was repeated with cell-free extracts of LC (Table 4). Cell-free extracts ferment lactate to CO<sub>2</sub>, H<sub>2</sub>, propionate and acetate. In this experiment, only the acetate and propionate formed were isolated and estimated; the activity of the CO<sub>2</sub> evolved was not measured. The results confirm those obtained with whole cells. Again the acetate was labelled exclusively in the carboxyl group and the propionate labelled exclusively in the methylene group. The specific activity of the acetate and propionate were each approximately equal to the specific activity of the added lactate.

The mechanism of valerate synthesis was studied more closely. Elsdén & Lewis (1953) observed that washed-cell suspensions of LC synthesized appreci-

Table 5. Distribution of <sup>14</sup>C in valerate produced from the fermentation of pyruvate plus [1-<sup>14</sup>C]propionate by washed cells of LC

Experiments were carried out in 100 ml. fermentation flasks containing 3 ml. of bacterial suspension (71 mg. dry wt. of cell material) in 0.1 M-phosphate buffer, pH 6.5, containing 0.02% of Na<sub>2</sub>S<sub>2</sub>O<sub>8</sub>.H<sub>2</sub>O; 150 μmoles of pyruvate plus 110 μmoles of [1-<sup>14</sup>C]propionate (specific activity 550 counts/min./μmole) in 2.1 ml. of water were added from one side bulb after equilibration. Gas phase H<sub>2</sub>; temp. 37°. Reaction time 1.5 hr.

Compound or group	Specific activity (counts/min./μmole)	Percentage of total activity
Valerate	466	100
-CO <sub>2</sub> H	15	3.5
CH <sub>3</sub> -[CH <sub>2</sub> ] <sub>3</sub> -	456	98.0

able amounts of valerate when pyruvate was fermented under H<sub>2</sub> in the presence of added propionate. Valerate, isolated from the fermentation products of pyruvate plus [1-<sup>14</sup>C]propionate, was decarboxylated and the specific activities of both the carboxyl group and the butylamine resulting from the decarboxylation were compared with the specific activity of the total molecule (Table 5). The carboxyl group was unlabelled.

To test whether CO<sub>2</sub> played a part in the formation of propionate (cf. fermentation by *V. gazogenes*) lactate was fermented in the presence of <sup>14</sup>CO<sub>2</sub> and the amount of the isotope incorporated into the fatty acids measured. The results are shown in Table 6. It will be seen that, whereas the acetate and butyrate contained practically no <sup>14</sup>C, the specific activities of both propionate and valerate were approximately 6% of that of the final CO<sub>2</sub>. All the activity of the propionate was found in the carboxyl group.

The experiment was repeated with a different batch of cells and with acrylate as substrate. Determination of the specific activities of propionate and acetate showed no significant incorporation of activity into either acid (Table 7).

To account for the slight labelling of the propionate formed from lactate fermented in the presence of  $^{14}\text{CO}_2$ , extracts of LC were tested for their ability to catalyse an exchange reaction between pyruvate and  $^{14}\text{CO}_2$  (Table 8). Such extracts break down pyruvate quantitatively to  $\text{CO}_2$ ,  $\text{H}_2$  and acetate. Although the pyruvate acquired some of the  $^{14}\text{C}$  label, equilibration with the  $^{14}\text{CO}_2$  was incomplete even after incubation for 60 min., when one-half of the added pyruvate had been decomposed. All the activity of the pyruvate resided in the carboxyl group. A control with boiled extract showed no exchange between pyruvate and  $\text{CO}_2$ .

### DISCUSSION

Two mechanisms have been described for the formation of propionate from lactate and, if  $[2\text{-}^{14}\text{C}]\text{lactate}$  is the substrate, then the distribution of the isotope in the propionate formed by these two mechanisms will be that shown in Fig. 1. The experiments described above show that in LC the conversion of lactate into propionate proceeds by mechanism A. The evidence for this contention is that fermentation of DL- $[2\text{-}^{14}\text{C}]\text{lactate}$  both by whole cells and by cell-free extracts yields  $[2\text{-}^{14}\text{C}]\text{propionate}$ , as predicted by mechanism A. Had the propionate been formed via succinate as in *V. gazogenes* and *Propionibacterium* sp. then  $[2\text{-}^{14}\text{C}]\text{propionate}$  and  $[3\text{-}^{14}\text{C}]\text{propionate}$  would have been formed and this mixture would degrade as  $^{14}\text{CH}_3\cdot^{14}\text{CH}_2\cdot\text{CO}_2\text{H}$ , as was found by Leaver *et al.* (1955).

Table 6. Incorporation of  $^{14}\text{CO}_2$  into products of lactate fermentation by washed cells of LC

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (50 mg. dry wt. of cell material) in 0.1 M-phosphate buffer, pH 6.5, containing 0.02% of  $\text{Na}_2\text{S}_2\text{O}_8$ ; 194  $\mu\text{moles}$  of DL-lactate plus 32  $\mu\text{moles}$  of  $[^{14}\text{C}]\text{-carbonate}$  in 1.05 ml. of water, or 32  $\mu\text{moles}$  of  $[^{14}\text{C}]\text{-carbonate}$  in 1.05 ml. of water in the control, were added from one side bulb after equilibration. Gas phase  $\text{N}_2$ ; temp. 37°. Reaction time 1.5 hr.

Product	Amount produced ( $\mu\text{moles}$ )	Specific activity (counts/min./ $\mu\text{mole}$ )	Equilibration (%)
Carbon dioxide	186	37 300	—
Valerate	26.4	2 120	5.7
Butyrate	20.3	169	0.5
Propionate	51.8	2 340	6.3
$-\text{CO}_2\text{H}$	—	2 510	—
$\text{CH}_3\cdot\text{CH}_2-$	—	35	—
Acetate	45.0	112	0.3

Table 7. Investigation of incorporation of  $^{14}\text{CO}_2$  into products of acrylate fermentation by washed cells of LC

Experiments were carried out in 100 ml. fermentation flasks containing 5 ml. of bacterial suspension (62.5 mg. dry wt. of cell material) in 0.1 M-phosphate buffer, pH 6.5, containing 0.02% of  $\text{Na}_2\text{S}_2\text{O}_8$ ; 148  $\mu\text{moles}$  of acrylate plus 32  $\mu\text{moles}$  of  $[^{14}\text{C}]\text{-carbonate}$  in 1.05 ml. of water, or 32  $\mu\text{moles}$  of  $[^{14}\text{C}]\text{-carbonate}$  in 1.05 ml. of water in the control, were added from one side bulb after equilibration. Gas phase  $\text{N}_2$ ; temp. 37°. Reaction time 1 hr.

Product	Amount produced ( $\mu\text{moles}$ )	Specific activity (counts/min./ $\mu\text{mole}$ )	Equilibration (%)
Carbon dioxide	125	60 500	—
Propionate	86.1	71	0.11
Acetate	40.7	44	0.07

Table 8. Incorporation of  $^{14}\text{CO}_2$  into pyruvate during its fermentation by cell-free extracts of LC

Manometer cups with double side bulbs contained 0.3 ml. of cell-free extract (6.6 mg. of bacterial protein) in water, 0.5 ml. of 0.1 M-phosphate buffer, pH 6.5, and 0.3 ml. of water. One side bulb, fitted with a turnover-type rubber stopper (Ormerod, 1956), contained 0.2 ml. of 0.5 M- $[^{14}\text{C}]\text{-carbonate}$ ; the other side bulb contained 0.5 ml. of 0.4 M-pyruvate. Gas phase  $\text{N}_2$ ; temp. 37°. After equilibration, 0.2 ml. of 5N- $\text{H}_2\text{SO}_4$  was injected into the side bulb containing carbonate. Pyruvate was tipped into the main compartment after equilibration for a further 15 min. After incubation for 15, 30 and 60 min. periods, acid was tipped from the side arm. The control contained boiled extract and was incubated for 60 min. The extract was prepared from dried cells.

Equilibration time ...	Specific activity (counts/min./ $\mu\text{mole}$ )			Control 60 min.
	Reaction			
Compound or group	15 min.	30 min.	60 min.	
Carbon dioxide	580	510	508	1330
Pyruvate	282	376	382	0
$-\text{CO}_2\text{H}$	—	—	394	0
Equilibration (%)	48.6	73.6	75.4	0

It is implicit in mechanism *B* that when lactate is fermented in the presence of labelled carbon dioxide, the carboxyl of the propionate will be labelled and, if  $^{14}\text{CO}_2$  is used, then the specific activity of the propionate carboxyl will be at least 50 % that of the final carbon dioxide, assuming, of course, that the external carbon dioxide is in equilibrium with the internal carbon dioxide. If the decarboxylation of succinate is reversible, then the specific activity of the propionate will approximate to that of the final carbon dioxide. When lactate was fermented by LC in the presence of  $^{14}\text{CO}_2$ , a minute amount of the isotope was found in the carboxyl groups of acetate and butyrate and the specific activity of the propionate carboxyl group was 6.3 % of that of the final carbon dioxide. The specific activity of the valerate was of the same order as that of the propionate. This value is much lower than that required by theory. Nonetheless, the appearance of even a small amount of carbon dioxide in the propionate has to be explained. Wolfe & O'Kane (1955) have demonstrated a rapid exchange reaction between carbon dioxide and the carboxyl group of pyruvate in extracts of *Clostridium butyricum* (equation 1).

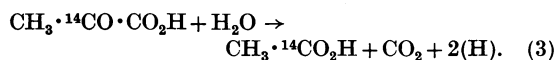
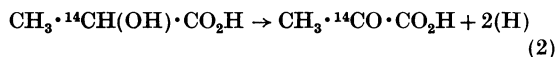
$$\text{CH}_3 \cdot \text{CO} \cdot \text{CO}_2\text{H} + ^{14}\text{CO}_2 \rightleftharpoons \text{CH}_3 \cdot \text{CO} \cdot ^{14}\text{CO}_2\text{H} + \text{CO}_2. \quad (1)$$

This reaction has now been demonstrated in extracts of LC (Table 8). D. J. Walker & J. N. Ladd (in preparation) have shown that in the fermentation of lactate by extracts of LC, part of the lactate is oxidized to pyruvate and, since this reaction is reversible, incorporation of carbon dioxide into the pyruvate by the exchange reaction would lead to the formation of some  $[1-^{14}\text{C}]$ lactate and hence  $[1-^{14}\text{C}]$ propionate. When acrylate was the substrate only a minute amount of  $^{14}\text{CO}_2$  was incorporated into the propionate carboxyl group. No explanation is offered for this difference between the fermentations of lactate and acrylate.

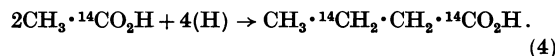
Finally there is no evidence for the decarboxylation of succinate by this organism. Elsdon *et al.* (1956) have observed that cells of LC growing on lactate do not utilize succinate added to the medium. Cell-free extracts which fermented lactate rapidly did not decarboxylate succinate even in the presence of adenosine triphosphate and

coenzyme A. Nor was it possible to demonstrate an exchange reaction between propionate and carbon dioxide.

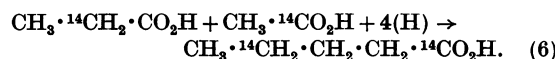
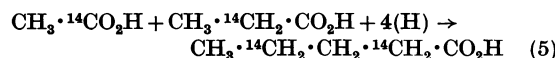
These experiments also provide information on the mechanism of the formation of the other volatile fatty acids. The formation of acetate from lactate by the organism LC may be explained by equations 2 and 3. The exclusive labelling of the acetate carboxyl group and the inactivity of the carbon dioxide indicate that they are formed by a direct oxidation of the lactate via pyruvate.



The formation of butyrate from lactate by the organism LC may be explained by equation (4):



The specific activity of valerate, formed from the fermentation of  $[2-^{14}\text{C}]$ lactate by washed cells of LC, is twice the specific activity of the added lactate and equal to the sum of the specific activities of the acetate and propionate produced. Valerate, labelled in two ways, theoretically may be formed (equations 5 and 6):



If valerate is synthesized according to equation (5) then the valerate carboxyl group should be unlabelled; if formed according to equation (6) the valerate carboxyl group should be labelled to the extent of 50 % of the total activity of the molecule. Decarboxylation of the valerate shows that the carboxyl group is labelled. The degree of labelling suggests that the acetate carboxyl group contributes exclusively to the valerate carboxyl group. This is confirmed by decarboxylation of the valerate produced by the fermentation of pyruvate plus  $[1-^{14}\text{C}]$ propionate. The fact that the valerate carboxyl group is now unlabelled shows that none is

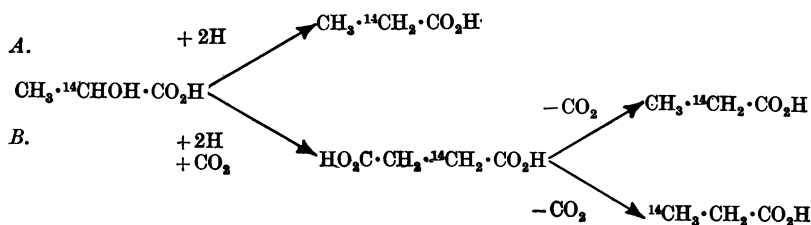
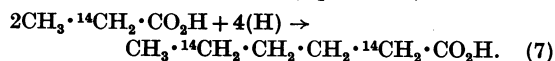


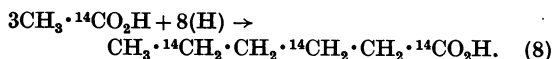
Fig. 1. Formation of labelled propionate from  $[2-^{14}\text{C}]$ -lactate.

derived from the propionate carboxyl group. These findings are in agreement with those of Stadtman, Stadtman & Barker (1949) working with *Clostridium kluyveri*.

The specific activity of caproate produced from [2-<sup>14</sup>C]lactate is twice that of the added lactate, suggesting that it is formed by the condensation of two propionate molecules (equation 7):



If so, the carboxyl group of the caproate should be unlabelled. Decarboxylation of the caproate shows in fact that the carboxyl group is labelled and that it contains approximately 33% of the activity of the total molecule. These results contradict the specific-activity measurements and suggest that caproate is formed by the condensation of three acetate units (equation 8):



This agrees well both with the observations of Elsdén & Lewis (1953) that caproate synthesis is increased markedly by the addition of butyrate to washed cells of LC fermenting pyruvate under hydrogen, and with the isotopic data of Stadtman *et al.* (1949) working with *C. kluyveri*. If equation 8 does represent the mechanism, the specific activity of the caproate formed from the fermentation of [2-<sup>14</sup>C]lactate (Table 1) is too low. This might well be the case, for the yield of caproate was very small (6.5 μmoles in 123 μmoles of total volatile acid) and small absolute errors in the estimation could lead to large relative errors in calculating the specific activity.

### SUMMARY

1. The relative specific activities and distribution of label within the products of [2-<sup>14</sup>C]lactate fermentation by washed cells of the rumen organism LC have been measured. The carbon dioxide produced is unlabelled. The acetate formed is carboxyl-labelled and the propionate is labelled exclusively in the methylene position. The specific activity of the butyrate is twice that of the butyrate carboxyl group; the specific activity of valerate is twice that of the valerate carboxyl group and the specific activity of caproate is three times that of the caproate carboxyl group.

2. The distribution of label within propionate and acetate formed from [2-<sup>14</sup>C]lactate has been confirmed with cell-free extracts.

3. The specific activity of propionate, formed by the fermentation of lactate in the presence of <sup>14</sup>CO<sub>2</sub>

by washed cells of LC, is only 6% of that of the final carbon dioxide. The propionate formed from acrylate under similar conditions is not significantly labelled.

4. Cell-free extracts of LC catalyse an exchange reaction between the pyruvate carboxyl group and carbon dioxide.

5. The mechanisms of formation of fatty acids from lactate by LC are discussed.

The author would like to express his gratitude to Dr S. R. Elsdén for suggesting this problem and for his constant encouragement and advice. Thanks are also due to the Commonwealth Scientific and Industrial Research Organization, Australia, for the award of an Overseas Research Studentship. The work was supported in part by a grant from the Rockefeller Foundation.

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