OXIDATION OF PROPIONIC ACID BY *NOCARDIA CORALLINA*

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Two pathways have been postulated for the conversion of propionic acid to pyruvic acid by liver mitochondria. Mahler and Huennekens (1953) suggested that propionic acid was oxidized to acrylic acid which was converted to pyruvic acid through L- and D-lactic acids. The alternative pathway involves a direct carboxylation of propionic acid to succinic acid (Lardy and Adler, 1956; Flavin *et al.*, 1955).

An enzyme system which catalyzes the carboxylation of propionic acid has been demonstrated in *Propionibacterium pentosaceum* (Barban and Ajl, 1951), *Chlorobium thiosulfatophilum* (Larsen, 1951), *Micrococcus lactilyticus* (Whiteley, 1953) and *Veillonella gazogenes* (Delwiche *et al.*, 1954).

Cells of a thiamin deficient *Nocardia corallina* oxidized propionic acid to pyruvic acid which accumulated in the system. This communication includes results which suggest that two pathways operate in *N. corallina* for the oxidation of propionic acid.

METHODS

N. corallina strain S was isolated by Batt and Woods (1951) and a growth requirement for thiamin was shown for the organism by Martin and Batt (1957).

Thiamin deficient cells were obtained by growing the organism in a medium containing a vitamin free casein hydrolyzate (General Biochemicals Ltd.), 0.05 per cent; KH_2PO_4 , 0.05 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 per cent; sodium propionate, 0.04 M; thiamin, 0.5 $\mu\text{g}/\text{ml}$, and phosphate buffer, 0.01 M; medium pH 7.0. For the production of thiamin enriched cells (i.e., cells grown under conditions where the thiamin concentration in the growth medium was not limiting the growth rate) either yeast extract (0.01 per cent) or thiamin (1.0 $\mu\text{g}/\text{ml}$) was added to the medium. Growth media were distributed in Erlenmeyer flasks (200 ml/500 ml flask) and autoclaved (121 C/15 min).

The inoculum for growing deficient cells was prepared by preincubating cells from a glucose

(0.5 per cent)-yeast extract (2.5 per cent)-agar slope (incubation for 36 hr at 30 C) in a medium (pH 7.2) containing sodium acetate (0.04 M), ammonium sulphate (0.005 M) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.02 per cent) for 24 hr. The inoculum was used at a level of 1 ml/200 ml medium. The media for growing enriched cells was inoculated directly from a glucose-yeast extract agar slope (incubation for 36 hr). An inoculated medium was incubated aerobically at 30 C with slow mechanical shaking for 3 to 5 days.

The cells were harvested by centrifugation (2,000 $\times$ G for 20 min), washed once with phosphate buffer (0.1 M; pH 7.0) and suspended in this buffer (10 mg dry wt cells/ml).

Experiments with cell suspensions were carried out manometrically using conventional procedures (Umbreit *et al.*, 1949). Each manometer cup contained cells equivalent to 10 mg dry wt and phosphate buffer (pH 7.0; final concentration 0.05 M); total volume, 3.0 ml. The substrates were added at a level of 10 $\mu\text{mole}/\text{cup}$ and all experiments were carried out at 30 C. The details of manometric experiments using C-14 labelled substrates are given in table 1.

Analytical methods. Estimations of α -ketonic acids were made colorimetrically (Lu, 1939). The quantitative analyses of mixtures of α -ketonic acids were carried out by the method of Cavallini and Frontali (1954).

Isolation of products. In one instance (Experiment A, table 1) pyruvic acid was isolated as the 2:4-dinitrophenylhydrazone and purified by solution in the minimal volume of 0.1 N NaOH, and acidification of the filtered solution with 0.1 N HCl. The precipitate obtained was washed with distilled water and dried under vacuum. From a supernatant containing 0.6 mM pyruvic acid (colorimetric estimation), 0.38 mM of purified pyruvic 2:4-dinitrophenylhydrazone was prepared. The mother liquor remaining after the removal of the crude 2:4-dinitrophenylhydrazone was steam distilled and the propionic acid in the distillate was purified by chromatography on a celite column (Swim and Krampitz, 1954).

TABLE 1
Manometric conditions and products formed in experiments using C¹⁴-labelled substrates

	Experiment			
	A	B	C	
Manometric conditions				
System volume (ml).....	30	30	3	
Phosphate buffer, 0.2 M, pH 7.0 (ml).....	10	10		
KH ₂ PO ₄ 0.2 M (ml).....			1	
CO ₂ -Absorber: 10% KOH (ml).....	1	nil	nil	
Gas phase.....	air	air	air	
Cells (mg dry wt).....	500	500	20	
Substrates			(a)	(b)
3-C ¹⁴ -propionate.....	2 mm	1.5 mm		10 μ moles
Propionate.....			10 μ moles	
NaHC ¹⁴ O ₃			100 μ moles	
NaHCO ₃				100 μ moles
Incubation time.....	12 hr	5.5 hr	50 min	50 min
O ₂ -Uptake.....	0.9 mm	0.7 mm	10 μ moles	10 μ moles
Compounds isolated				
Propionate.....	0.52 mm	0.33 mm		
Pyruvate.....	0.6 mm	0.4 mm	5 μ moles	7 μ moles
Succinate.....	0.02 mm	0.12 mm	—	—

The distribution of C¹⁴ in the products of these experiments are given in tables 2 and 3 (table 2, experiments A and B; table 3, experiment C).

The following procedure was used in the other experiments. The cells were centrifuged from the incubation system and the supernatant lyophilized at pH 7.0. The acids in the residue were separated on a celite column. The propionic acid from the column was steam distilled, the distillate titrated and evaporated to dryness. The succinic acid fraction was oxidized with acid permanganate to remove any lactic acid present and then extracted continuously for 2 days with diethyl ether. The succinic acid was finally sublimed at 115 C under vacuum. The pyruvic acid fraction after titration was extracted with water and the aqueous extract used directly in the degradation reactions.

Degradations of products. Pyruvic 2:4-dinitrophenylhydrazone, dissolved in 0.1 N NaOH, was added slowly to a solution containing 30 ml KMnO₄ (1 N) and 15 ml catalyst (150 g MnSO₄ and 125 ml H₃PO₄/L). The carbon dioxide evolved was collected in 3 N NaOH. The manganese oxides were removed by centrifugation and the excess KMnO₄ was decomposed with oxalic acid. The acetic acid formed during the oxidation was recovered by steam distillation, purified on a celite column and again steam distilled. The titrated distillate was evaporated to dryness. This

method of degrading pyruvic 2:4-dinitrophenylhydrazone gave a nitrophenol fraction on the celite column indicating incomplete oxidation of the aromatic nucleus. Accordingly the dilution of the carbon dioxide from the carboxyl carbon of the pyruvic acid was estimated from the yield of acetic acid.

Pyruvic acid obtained by chromatographic separation on a celite column was degraded to acetic acid and carbon dioxide by treating a solution of the acid with a suspension of cerium sulphate (0.1 M in 0.1 N H₂SO₄) at room temperature.

The degradation of acetic acid was carried out by a Schmidt degradation using a modification of the procedure described by Phares (1951). Solid sodium acetate (0.6 mm) was treated with sodium azide (100 mg) and concentrated sulphuric acid (0.5 ml; sp gr 1.84). After completion of the reaction sodium hydroxide (23 per cent wt/vol) was added and the methylamine distilled into 0.5 N H₂SO₄ (3.5 ml). The methylamine sulphate solution was evaporated to a small volume (0.5 ml) in an air oven at 100 C and the residue oxidized to carbon dioxide by a wet combustion with a chromic acid solution (Van Slyke and Folch, 1940). This procedure gave excellent re-

coveries of each of the carbons of the acetate as carbon dioxide.

Succinic acid was converted to propionic acid and carbon dioxide with a suspension of *Micrococcus lactilyticus* as described by Wood *et al.* (1956).

The propionic acid was distilled in steam, purified on a celite column and degraded to acetic acid by the Schmidt reaction and alkaline permanganate oxidation of the ethylamine formed.

The accuracy of each degradation was checked by comparing the sum of the activities of individual carbons with the total activity of the compound determined following a wet combustion.

The carbon dioxide samples were plated as BaCO_3 on filter paper discs and counted with an end window Geiger-Muller counter. The counts recorded were corrected for background and self-absorption.

Sodium propionate (3-C^{14}) was supplied by Professor H. G. Wood; $\text{NaHC}^{14}\text{O}_3$ was obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Propionate oxidation by thiamin enriched and thiamin deficient cell suspensions. The O_2 -uptake and CO_2 -production curves obtained when thiamin enriched and thiamin deficient cell suspensions were incubated with propionate are shown in figure 1. With the enriched cells, no ketonic acids accumulated during the oxidation. When the O_2 -uptake with the deficient cells acting on propionate had reached $1.08 \mu\text{mole}/\mu\text{mole}$ propionate added, $1.1 \mu\text{mole}$ pyruvic acid/ μmole propionate had accumulated in the incubation system. A slow rate of removal of the pyruvic acid was observed and no pyruvic acid remained after 12 hr incubation with the cells. The O_2 -uptake curves for propionate and pyruvate with deficient cells are given in figure 2.

Effect of carbon dioxide on propionate oxidation. A relationship between CO_2 tension and rate of propionate oxidation was suggested from experiments with deficient cells. The rate of O_2 -uptake was greater in flasks without a CO_2 -absorber than in flasks in which any CO_2 produced was absorbed in 10 per cent KOH. The effect was enhanced if the incubation systems were buffered at pH 5.0. The results obtained when the oxidation of propionate by deficient cells was measured

Figure 1. The O_2 -uptake (A; C) and CO_2 -production (B; D) curves for thiamin enriched (A; B) and thiamin deficient (C; D) cell suspensions acting on propionate ($5 \mu\text{mole}/\text{manometer cup}$). Values corrected for endogenous respiration.

Figure 2. Oxygen-uptake curves obtained from the incubation of thiamin deficient cell suspensions with propionate and pyruvate. Substrates; $10 \mu\text{mole}/\text{manometer cup}$. All values corrected for endogenous respiration.

in flasks containing an atmosphere of either 100 per cent O_2 or 95 per cent O_2 -5 per cent CO_2 are shown in figure 3.

Deficient cells grown in the presence of propionate rapidly oxidized succinate to pyruvate,

the conversion being almost quantitative on the basis of the reaction

The deficient cells slowly oxidized lactate to pyruvate after several hours.

Experiments with C^{14} -labelled substrates. The oxidation of propionate by thiamin deficient cell suspensions was carried out under three sets of conditions which differed with respect to the amount of carbon dioxide present in the incubation system (table 1).

In the first experiment (A, table 1), 3- C^{14} -propionate was oxidized to pyruvic acid in a large

Figure 3. The oxidation of propionate by thiamin deficient cell suspensions in the presence of different concentrations of carbon dioxide at pH 5.0. Gas phase: A, 95% O_2 -5% CO_2 ; B and C, 100% O_2 . A CO_2 -absorber was used in the manometer cup in one instance (Curve C). All values are corrected for endogenous respiration.

manometer flask (210 ml) which contained a CO_2 -absorber (1 ml 10 per cent KOH). The reaction was stopped by adding 3 N H_2SO_4 (2 ml) when approximately 25 per cent of the initial propionate remained in the system. The pyruvate (isolated as the 2:4 dinitrophenylhydrazone) and residual propionate were degraded, the distribution of C^{14} in these compounds being given in table 2. Only a very small amount of succinate (0.02 mm) was obtained by ether extraction of the supernatant following removal of the propionate.

In the second experiment (B, table 1) no CO_2 -absorber was used in the manometer flask (210 ml). The pyruvic acid was degraded to acetic acid and CO_2 using cerium sulphate. A relatively large amount of succinate accumulated under these conditions; it was degraded and the C^{14} distribution in this compound and in the pyruvate and residual propionate are given in table 2.

The accumulation of sufficient pyruvate for isolation and purification required prolonged manometric experiments. To determine the ex-

TABLE 3
Distribution of C^{14} in the pyruvate formed during the oxidation of propionate in the presence of sodium bicarbonate

System	Specific Activity (cpm/mmmole C)		
	Pyruvate		
	CH_3	CO	COOH
Na propionate-3- C^{14} + $\text{NaH}^{14}\text{CO}_3$	975,000	470,000	53,400
Na propionate- C^{14} + $\text{NaHC}^{14}\text{O}_3$	0	0	30,200

Specific activity of (a) propionate added = 3,200,000 cpm/mmmole C in 3 position and (b) sodium bicarbonate = 128,500 cpm/mmmole C.

TABLE 2
Distribution of C^{14} in the products of oxidation of 3- C^{14} propionate and in the residual propionate

	Specific Activity (cpm/mmmole C)							
	Residual propionate			Pyruvate			Succinate	
	CH_3	CH_2	COOH	CH_3	CO	COOH	CH_2	COOH
A, Incubation with a CO_2 -absorber.....	258,500	13,870	2,360	121,280	113,200	27,500		
B, Incubation with no CO_2 -absorber.....	256,000	15,000	1,900	149,000	95,600	13,600	81,800	29,200

Specific activity of the propionate added = 316,200 cpm/mmmole C in the 3 position.

tent to which CO_2 (from $\text{NaHC}^{14}\text{O}_3$) was incorporated into the pyruvate during propionate oxidation, experiments of short duration were carried out. A small amount of propionate (10 μ moles) was added to the thiamin deficient cell suspension and the pyruvate formed was diluted with a large amount of carrier (1.0 mM) (C, table 1). The distributions of C^{14} in the pyruvate formed are given in table 3.

DISCUSSION

The stimulation of propionate oxidation by carbon dioxide with cell suspensions of *N. corallina* suggested the possibility that succinate was an intermediate in the reaction sequence.

The results obtained from a study of the oxidation of 3- C^{14} -propionate to pyruvate by thiamin deficient cell suspensions were consistent with the proposal that succinate was an intermediate in the conversion. Under conditions where any carbon dioxide formed during the oxidation of propionate was absorbed in alkali, the pyruvate isolated showed almost equal labelling in the 2 and 3 positions (table 2). The observed distribution of C^{14} in the pyruvate would be expected if the conversion of propionate to pyruvate involved a symmetrical intermediate such as succinate. In addition, succinate accumulated in relatively large amounts during the oxidation of propionate when any carbon dioxide produced in the incubation system was not removed. Under these conditions, the pyruvate formed was found to have a considerable incorporation of C^{14} into the 2 position (table 2). The succinate had a high specific activity relative to the activity of the initial propionate and the activity was high in the methylene carbons of the succinate. This suggested that the succinate was formed directly from the propionate.

Although the results are in agreement with the suggestion that a symmetrical intermediate is present in the propionate oxidation pathway, evidence was obtained for a second pathway which contained no symmetrical intermediate. The ratio of activity in the 3 and 2 positions of the pyruvate formed from 3- C^{14} -propionate under different conditions were as follows:

Activity ratio: C-3/C-2

Carbon dioxide absorbed from the system..	1.07
Carbon dioxide not absorbed from the system.....	1.57
Sodium bicarbonate added to the system...	2.10

The ratio of activity in the 3 and 2 positions of pyruvate should be 1.0 if the only pathway operating contained a symmetrical intermediate. Apparently carbon dioxide increased the amount of propionate being oxidized through the asymmetrical pathway.

The factors affecting the conversion of propionate to pyruvate are complex as indicated by the results of an experiment carried out under the conditions of experiment A (table 1) with the modifications that 120 hr old cells were used and 100 per cent O_2 was substituted for air as the gas phase. Under these conditions (CO_2 -absorbed) the ratio of activity in the 3 and 2 positions of the pyruvate formed was 3.22, i. e., factors other than CO_2 can presumably influence the extent to which propionate is converted to pyruvate by a symmetrical or an asymmetrical pathway.

The formation of pyruvate from propionate is reversible, at least partially, as shown by the presence of C^{14} in the 1 and 2 positions of the residual propionate in the incubation systems. The presence of C^{14} in the carboxyl group of pyruvate when carbon labelled sodium bicarbonate was incubated with propionate indicates that carbon dioxide fixation occurs during the oxidation of propionate.

SUMMARY

The oxidation of propionic acid by cell suspensions of *Nocardia corallina* is stimulated by carbon dioxide. Thiamin deficient cells convert propionate to pyruvate with a simultaneous accumulation of succinic acid. Using 3- C^{14} -propionate, evidence was obtained for two oxidation pathways to pyruvate, only one of which included a symmetrical intermediate (probably succinate).

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