THE PATHWAY OF PROPIONATE OXIDATION*, **

by

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Propionate is generally believed to arise as the terminal three-carbon fragment in the oxidation of odd-carbon fatty acids[1,3,11-14]. The complete oxidation of propionate to carbon dioxide and water has been the topic of two recent communications from this laboratory[1,2]. On the basis of the evidence presented, the pathway in Fig. 1 appeared to account for all the effects observed. All of the enzymes responsible for this sequence of reactions are contained in the mitochondrial particles[2,4,12] with the exception of a soluble racemase, obtained from the supernatant fraction, which interconverts the lactate isomers[5].

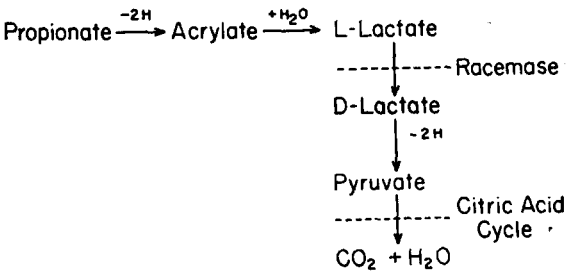

Fig. 1. Pathway of propionate oxidation.

It appeared desirable to supplement the manometric data previously presented with isotopic tracer experiments. These experiments, reported in the present communication, are based upon the oxidation of propionate-1-^{14}C in the presence of pools of unlabelled postulated intermediates.

EXPERIMENTAL

Enzyme preparations

Rabbit liver cyclophorase, rabbit kidney cyclophorase, and racemase (factor) preparations were prepared as previously described[1,2,5].

The soluble lactic *oxidase* from yeast was prepared by the unpublished method of Mr. L. HYNDMAN in this laboratory. This preparation is purified from an extract of acetone-dried brewer's yeast by means of adsorption on calcium phosphate gel and ammonium sulfate fractionation. As shown

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in Table I, the enzyme is capable of oxidizing L-lactate quantitatively to pyruvate in the presence of oxygen, a carrier dye, such as methylene blue or pyocyanine, and cyanide as a trapping agent. D-lactate, DL- and L-malate, DL-*iso*citrate, citrate, *cis*-aconitate, α-ketoglutarate, succinic semialdehyde, succinate, fumarate, oxalacetate and pyruvate are not oxidized by the enzyme, under conditions which lead to a quantitative conversion of L-lactate to pyruvate. Oxidation by means of this preparation can therefore be considered to be relatively specific for L-lactate*.

TABLE I

OXIDATION OF L-LACTATE BY YEAST L-LACTATE OXIDASE

	With lactate	*Without lactate*	*Δ*	*per cent. of theory*
L-Lactate				
Oxygen uptake (μ atoms)	115.4	21.8	93.6	94
Pyruvate found (μmoles)			94.0	94
D-Lactate				
Oxygen uptake (μ atoms)	22.0	22.2	0	0

The flasks contained 8 mg of purified yeast enzyme, 50 μmoles phosphate buffer, pH 6.4, 0.30 mg pyocyanine, 400 μmoles KCN, 100 μmoles L- or D-lactate (where indicated) and water to make 3.0 ml.

Oxygen in the gas phase; temperature 38°; 5 minutes thermal equilibration; duration of experiment: 3 hours.

Pyruvate was estimated by the method of STRAUB[6].

Materials and methods

The labelled sodium propionate used was a commercial preparation**, shown to be homogenous by filter paper chromatography and radio-autographs[7]. The material used had an activity of $2.79 \cdot 10^4$ counts per micromole in the counting arrangement used, as determined both by direct counting and by counting after combustion to CO_2.

In all the experiments to be described two radioactive fractions were isolated and counted: (a) carbon dioxide—obtained from the center wells of the Warburg flasks and precipitated as barium carbonate[8]; and (b) the added "pool"—the pyruvate "pool" was isolated and counted directly as the 2,4-dinitrophenylhydrazone, whereas L-lactate was first oxidized enzymically to pyruvate and then treated as the previous case.

Pyruvate was isolated as the 2,4-dinitrophenylhydrazone according to the following procedure: after deproteinization with trichloracetic acid (final concentration 5%) and removal of the precipitated protein by centrifugation, the acid supernatant was added to 25 ml of an ice-cold, saturated solution of dinitrophenylhydrazine reagent in 2 *N* HCl. If cyanide had been present in the original reaction mixture, the acid supernatant was heated briefly to the boiling point, in order to decompose the pyruvate cyanohydrin, prior to its addition to the hydrazine reagent. After 24 hours at 0°, the precipitate of pyruvate-2, 4-dinitrophenylhydrazone was filtered, washed thoroughly with dilute hydrochloric acid and water and dried *in vacuo* over calcium chloride (recovery 87 to 90%). After one recrystallization from aqueous acetic acid the derivatives gave melting points of 218°, identical to that reported[9], and did not depress the melting point of an authentic sample of pyruvate-2, 4-dinitrophenylhydrazone.

The hydrazones were further purified by (a) recrystallization from aqueous ethanol to constant specific activity and (b) adsorption chromatography.

For the latter, the crystalline phenylhydrazones were dissolved in petroleum ether containing a small amount of diethyl ether to facilitate solution in a relatively small volume (approximately 1.0 ml/mg). The solutions were then allowed to percolate through columns of Florisil***. The chromatograms were developed in order with chloroform, chloroform-butanol (3:1 and 1:3) and butanol. This treatment will develop and elute the bands of all carbonyl compounds tested, as well as those of various aldehydes and keto acids of physiological importance, including the derivatives of oxal-

* The letters D- and L- as applied to lactate isomers refer to absolute configurations, not rotations.

** Obtained from the Texas Research Foundation, Renner, Texas, on allocation from the Atomic Energy Commission.

*** A magnesium silicate adsorbent manufactured by the Floridin Co., Warren, Pennsylvania.

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acetate, α-ketoglutarate, oxalosuccinate, succinic semialdehyde, acetoacetate, acetone and acetaldehyde. The phenylhydrazone of pyruvate, which is held very tightly at the top of the column as a brilliant, golden band during these washing procedures, was finally eluted by the use of butanol/glacial acetic acid, reprecipitated by removal of the solvents, recrystallized and once again counted. In all the cases tested only *one* band was observed, and the specific activity at the end of the chromatographic treatment compared favorably with that determined on the original sample. Typical values from one of the experimental runs are presented in Table II. All 2,4-dinitrophenylhydrazones were counted directly on the aluminum planchets after deposition from a light petroleum ether slurry.

TABLE II

SPECIFIC ACTIVITY OF PYRUVATE 2,4-DINITROPHENYLHYDRAZONE AFTER PURIFICATION

Treatment	*Specific activity (cts/min/mg)*
Original crystals	306
1 × recr. from acetic acid-H_2O	361
2 × recr. from acetic acid-H_2O	372
1 × recr. from ethanol-H_2O	370
2 × recr. from ethanol-H_2O	370
Chromatographed	360

All plates (both barium carbonate and phenylhydrazones) were prepared using thin aluminum planchets (active area 3.7 cm^2) and depositing the material to be counted from slurry cups[8]. Counts were taken using a "Q-gas" counter* of approximately 2 π geometry, for a length of time sufficient to reduce the probable error to less than one per cent. Corrections for coincidence, self-absorption and back-scattering were made, and all counts are thus normalized to infinite thickness of barium carbonate.

RESULTS

Pyruvate as an intermediate

In the first experiment labelled propionate was oxidized in the presence of a large "pool" of unlabelled pyruvate. Experimental conditions and results are outlined in Table III. Data are reported for the oxidation of 10 μmoles of propionate in the presence (column A) and absence (column B) of 200 μmoles of pyruvate. Each of these results

TABLE III

OXIDATION OF LABELLED PROPIONATE IN THE PRESENCE OF UNLABELLED PYRUVATE

Measurement	*Addition*	
	(A) Pyruvate	*(B) No pyruvate*
1. Propionate oxidized (μmoles)	2.62	2.25
2. Total activity* (expected in products)	72,500	61,500
3. Activity as pyruvate	39,700	1,180
4. Activity as CO_2	40,200	56,800
Sum (3) + (4)	79,900	57,980

Each flask contained 1.0 ml of R_3L cyclophorase, 0.7 ml of racemase, 3 μmoles of adenylic acid, 20 μmoles of phosphate buffer (pH 7.5), 4 μmoles of $MgCl_2$, 5 μmoles of citrate as a sparker, 10.8 μmoles of sodium propionate-1-^{14}C, containing $3.02 \cdot 10^5$ counts/min, and 200 μmoles of pyruvate (where indicated). All other conditions similar to Table I.

* All activities are expressed as cts./min.

* Nuclear Instrument and Chemical Co., Chicago, Illinois.

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is corrected for appropriate blanks without substrates. Line (1), the amount of *propionate* oxidized in each case, is calculated from the oxygen uptake, while line (2) is the activity expected in the products (pyruvate and CO_2) and is based upon the specific activity of the propionate which was oxidized. The main findings are presented in lines (3) and (4) and may be summarized as follows: In the presence of a large pool of added, unlabelled pyruvate (column A), about 50% of the activity to be accounted for is found in the pool, the remaining 50% going to carbon dioxide. In the absence of the trapping intermediate (column B) essentially all the activity is found in the carbon dioxide. In both cases, the agreement between the total activity to be accounted for (line 2) and the activity found experimentally (sum (3) and (4)), as well as the general agreement of lines (1), (2) and (4) between columns (A) and (B), appear to be satisfactory. The results of this experiment show clearly that pyruvate must be on the pathway of propionate oxidation, although it does not indicate the intervening intermediates.

L-lactate as an intermediate

The second experiment was designed to test the hypothesis that the L-lactate is on the pathway of propionate oxidation. It was carried out under conditions similar to the preceding experiment, except that labelled propionate was oxidized in the presence of 100 μmoles of L-lactate, instead of pyruvate, as the "trap". At the conclusion of the experiment, the L-lactate pool was not isolated as such, but was converted to pyruvate by means of the specific L-lactic oxidase and the pyruvate isolated, purified and counted as the dinitrophenylhydrazone. This procedure avoids any chemical racemization of L-lactate during isolation and insures quantitative recoveries.

The contents of the Warburg flasks at the end of the oxidative runs were deproteinized with trichloracetic acid, the protein removed, the bulk of the acid removed by ether extraction, the filtrate reneutralized and concentrated to a small volume by lyophilization. The L-lactate was then oxidized by the specific L-lactic oxidase and the pyruvate isolated and counted as in the previous case. Experimental findings are summarized in Table IV. A more active enzyme preparation in this case resulted in the oxidation of larger amounts of propionate and correspondingly larger amounts of label in each fraction. However, the distribution of label is remarkably similar to that

TABLE IV

OXIDATION OF LABELLED PROPIONATE IN THE PRESENCE OF UNLABELLED L-LACTATE

Measurement	*Addition*	
	(A) L-*lactate*	*(B)* *No* L-*lactate*
1. Propionate oxidized (μmoles)	9.3	9.7
2. Total activity (cts./min)	260,000	280,000
3. Activity in L-lactate (isolated as pyruvate)	46,000	1,000
4. Activity as CO_2	151,200	198,000
Sum (3) + (4)	197,200	199,000

All conditions and additions as in Table III except that 100 μmoles of L-lactate replaced the pyruvate.

At the end of the experiment, samples were deproteinized and subjected to oxidation by the yeast L-lactic oxidase, as described in the text.

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found in the case of the pyruvate trap, and on this basis it would appear that L-lactate is likewise on the pathway of propionate oxidation.

D-lactate as an intermediate

The unavailability of a purified D-lactic oxidase prevented the analagous experiment in which a D-lactate pool could be analyzed by enzymic conversion to pyruvate. It was possible, however, to carry out the experiment of oxidizing labelled propionate in the presence of 100 μmoles of unlabelled D-lactate, and then to analyze for the amount of label in the pool by an *indirect* method. Since in all of these experiments the oxidation of propionate requires the participation of a soluble, α-hydroxy acid racemase, in addition to the particulate enzyme fraction, it follows that during the above experiment at least a small portion of the D-lactate pool must have been enzymically racemized to L-lactate. Therefore, if after deproteinization the samples are treated with the L-*lactic* oxidase and the resulting pyruvate isolated, any label in this pyruvate must be referable to L-lactate which, in turn, must have arisen from the D-lactate pool. Any endogenous L-lactate, which would normally be produced during the oxidation of propionate, could be corrected for in the experiment identical to the one above except for the omission of the D-lactate pool.

Table V presents the data obtained in such experiments, utilizing a D-lactate "trap". In experiments A and B the soluble L-lactic oxidase was used to convert any L-lactate present at the end of the experiment, whereas in experiment C omitting the enzymic oxidation caused only the endogenous pyruvate to be isolated. As can be seen in experiment A, a significant amount of label appears in the pyruvate, and, moreover, this label is not due to pre-existent pyruvate but to L-lactate which had been converted to pyruvate, since in experiment C much less label is found in the pyruvate isolated. This appearance of label in L-lactate must have been due to a conversion of some of the D-isomer into the L-form, *i.e.*, to the operation of the racemase during the oxidation of propionate. It can not have been due to trapping by endogenous L-lactate initially since similar results were not observed in experiment B, in which no D-lactate pool was present during the oxidation of propionate.

These results, although not obtained as unambiguously as in the previous two cases, provide positive evidence, nonetheless, that D-lactate is likewise on the pathway of propionate oxidation.

TABLE V

OXIDATION OF LABELLED PROPIONATE IN THE PRESENCE OF UNLABELLED D-LACTATE

Measurement	*Experiment*		
	A	*B*	*C*
1. Propionate oxidized (μmoles)	4.40	2.25	4.0
2. Total activity (cts./min)	148,000	61,500	138,000
3. Activity as Pyruvate (found)	9,080	0	1,000
4. Activity as CO_2	76,200	58,600	130,000
Sum (3) + (4)	85,280	58,600	131,000

Additions and conditions as in Table IV except that 100 μmoles of D-lactate were added in Exps. A and C.

Exp. C was *not* subjected to oxidation by the L-lactic enzyme prior to phenylhydrazone precipitation.

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DISCUSSION

By means of the experiments just described, pyruvate and the two isomers of lactate have been implicated in propionate oxidation and thus a considerable portion of the scheme proposed initially has received substantiation. Because of its toxicity to enzyme systems[1,2] acrylate could not be employed similarly in high concentrations as a trap. It is also apparent that the experiments just described leave open certain questions. The appearance of a considerable portion of the activity in the carbon dioxide naturally leads to a consideration of its origin. This may lie in alternate pathways of propionate oxidation, to be discussed subsequently, or else in the fact that the compounds employed as "traps" are not in rapid equilibrium with the actual intermediates. Such incomplete equilibration between a large pool of a postulated intermediate and its metabolically active form, produced enzymically, is not unknown and in more extreme cases may even lead to complete non-appearance of label in a postulated intermediate, as for instance in STADTMAN AND BARKER's experiments on butyrate synthesis by *Clostridium kluyveri*[10].

It is of interest to compare the metabolic pathway of propionate proposed in the present and preceding papers from this laboratory[2] with the results of other investigators.

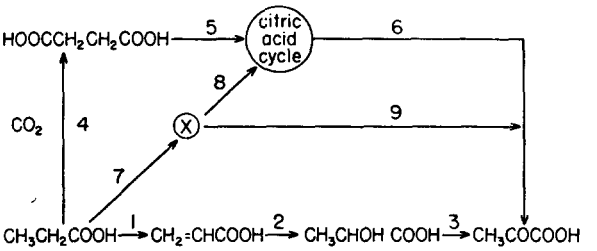

Fig. 2. Alternate pathways of propionate oxidation.

From feeding experiments propionate has long been known to be glycogenic[15,16] and this fact has been amply substantiated by investigations with labelled compounds[17–19]. Valine-β-^{14}C similarly leads to labelled glycogen and from the distribution of label within the glucose molecule it was concluded that propionate must be a precursor of pyruvate[20]. Other evidence along this line was furnished by HAAN AND HAARMANN[21] who noted an increase of pyruvate in a muscle mince which had been incubated with propionate. Contrary to these results, BLOCH AND RITTENBERG[22,23] found that deuterium-labelled alanine gave a higher level of tracer in acetyl groups than deuterium-labelled propionate. Assuming reasonably that alanine will reflect the behaviour of pyruvate, an assumption later verified by ANKER[24], they concluded that propionate is not metabolized *via* pyruvate. More recently, however, SHREEVE[19] has reinvestigated this problem and has pointed out that deuterium-labelled propionate may undergo enolization with subsequent loss of label. Using ^{14}C-labelled propionate, he has confirmed the earlier results[18].

A possible pathway from propionate to pyruvate is that of *α-oxidation*, shown previously in Fig. 1 and as reactions 1–3 in Fig. 2, which was suggested first by WALKER AND COPPOCK[25] and HAAN AND HAARMANN[21]. Further evidence for this pathway was supplied by HUENNEKENS *et al.*[2], who found that a soluble α-hydroxy acid racemase, necessary to interconvert the lactic isomers, was required as a supplement to rabbit

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liver mitochondria for propionate oxidation. More recently the properties of the D-specific lactic oxidase in rabbit liver and kidney mitochondria have been documented in more detail[26]. The tracer experiments reported in the present paper, in addition to implicating both lactate isomers and pyruvate in propionate oxidation, also provide additional evidence for the participation of the racemase. This pathway has been further confirmed by the experiments of KINNORY AND GREENBERG[27].

A second pathway, that of β-oxidation of propionate leading to malonate, has been shown to be untenable by the tracer experiments of LORBER *et al.*[18].

A third pathway, proposed by WOOD and his colleagues[17–19] is that propionate is converted to a symmetrical intermediate, either *via* the citric acid cycle (reactions 4, 5 and 6) (Fig. 2) or to an unspecified intermediate, X (reaction 7) which in turn may go through the citric acid cycle (reactions 8 and 6), or directly to pyruvate (reaction 9). This pathway has been invoked to explain the finding that either α- or β-labelled propionate gives rise to completely equivalent α, β-labelled pyruvate (as indicated by the labelling in glucose) while only a partialr andomization occurs in the case of α-labelled lactate[28]. That this randomization in the α, β positions occurs between propionate and

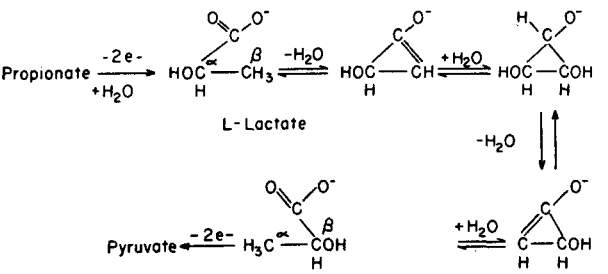

Fig. 3. Randomization and racemase action.

pyruvate has been confirmed and extended by the findings of SHREEVE[19] and PETERSON *et al.*[20], and has been further localized by DAUS *et al.*[29] as occurring between propionate and lactate. Furthermore, when labelled propionate is used to propionylate an amine[19] or is converted to hydroxyvalerate[29] no randomization occurs, indicating that propionate *per se* is not randomized. Reaction 4 has been demonstrated in the case of certain bacteria[30–32] and recently in animal tissues[33] while the structure of the intermediate, X, is unknown.

A possible explanation of the randomization experiments while still retaining the α-oxidation pathway and the racemase action is illustrated schematically in Fig. 3. Such a formulation would require further that carboxylate ion be bound to the enzyme so that complete randomization between all three carbon atoms would be prohibited. If the racemase action should require a symmetrical intermediate, as suggested in the diagram, then complete randomization between the α- and β-carbons would result in the case of propionate where racemization is obligatory. In the case of lactate, however, the use of the DL-substrate in all other investigations would only result in a partial randomization since the withdrawal of the D-isomer *via* pyruvate and the citric acid cycle would compete with the route leading to its inversion. The validity of such an explanation might be tested by determining the degree of α, β-randomization when D- and L-lactate (labelled in the α-position) were metabolized separately, since this theory would predict a much greater randomization in the case of the L-isomer.

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The loss of deuterium label from propionate by means of enolization is likewise readily accounted for by inspection of the structure of the hypothetical intermediates. On the other hand, the above mechanism still does not offer an explanation for the obligatory requirement of oxidative phosphorylation during racemase action[5].

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It is a pleasure to thank Dr. DAVID E. GREEN for his encouragement of this investigation.

SUMMARY

1. The oxidation of labelled propionate in the presence of large pools of unlabelled L-lactate, D-lactate and pyruvate gives rise to the appearance of label in these pools. The appearance of label suggests that the compounds mentioned are in equilibrium with the actual intermediates. Some evidence for incomplete equilibration has been presented.

2. The modified α-oxidation pathway for propionate, supported by the above evidence is shown to be compatible with the randomization of label within propionate, if an α, β-symmetrical intermediate is assumed to occur during racemase action.

RÉSUMÉ

1. L'oxydation de propionate marqué en présence de pools importants de L-lactate, D-lactate et pyruvate non marqués, provoque l'apparition du marqueur dans ces pools. L'apparition du marqueur suggère que les corps cités sont en équilibre avec les intermédiaires réels. La preuve que l'équilibre est incomplet est donnée en partie.

2. Les auteurs montrent que le mécanisme de l'α-oxydation du propionate, modifié en fonction des résultats précédents, est compatible avec un marquage au hasard du propionate, si l'on suppose qu'un intermédiaire α, β-symétrique apparaît au cours de l'action de la racémase.

ZUSAMMENFASSUNG

1. Die Oxydation markierten Propionats bei Gegenwart einer grossen Menge von gespeicherten, nicht markierten L-Lactat, D-Lactat und bernsteinsaurem Salz verursacht das Auftreten der Kennzeichen in dieser gespeicherten Menge. Dieses Auftreten der Kennzeichen lässt vermuten, dass die erwähnten Verbindungen sich mit den vorhandenen Zwischenverbindungen im Gleichgewicht befinden. Es wurden einige Tatsachen angeführt, aus denen sich schliessen lässt, dass das Gleichgewicht nicht vollständig ist.

2. Es wurde gezeigt, dass der durch die obige Klarlegung unterstützte, modifizierte Reaktionsweg der α-Oxydation von Propionat mit der zufälligen Verteilung der Kennzeichen im Propionat vereinbar ist, wenn das Auftreten einer α, β-symmetrischen Zwischenverbindung während der Racemasewirkung angenommen wird.

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