The Regulation of Propionate Oxidation in *Prototheca zopfii*

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1. Whole cell suspensions of *Prototheca zopfii* grown on propionate oxidize propionate, acrylate, malonic semialdehyde and acetate immediately, whereas acetate-grown cells only oxidize acrylate or propionate rapidly after a lag of 20–30 min. This adaptation to propionate is slowed down by 8-azaguanine or *p*-fluorophenylalanine, and is not influenced by adding an ammonium salt or an amino acid mixture.
2. The adaptation involves induction of the enzymes of β -oxidation of propionate.
3. A small proportion (5–8%) of the activities of propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase are consistently associated with mitochondria isolated from propionate-grown cells.
4. Such mitochondria will oxidize propionyl-CoA, β -hydroxypropionate and malonic semialdehyde, and the respiration rates with these substrates in the presence of inorganic phosphate are ADP-dependent.
5. Mitochondria from acetate-grown cells do not contain detectable activities of the enzymes of propionate oxidation.

The colourless alga, *Prototheca zopfii*, grown on propionate as sole carbon and energy source assimilates this fatty acid by a pathway of β -oxidation; acetyl-CoA so produced is incorporated by way of the tricarboxylic acid and glyoxylate cycles (Callely & Lloyd, 1964*a,b*; Lloyd & Callely, 1965). Thus the control of propionate assimilation in this system may be partly dependent on the mechanism of regulation of the inducible glyoxylate cycle, which have been extensively studied in organisms growing on acetate as sole carbon source (Kornberg, 1966). The oxidation rates with various tricarboxylic acid-cycle intermediates of mitochondria isolated from *Prototheca* grown on acetate have been shown to be dependent on the availability of ADP (Lloyd, 1965*b*). The present paper reports two other control mechanisms involved in growth on propionate. The β -oxidation of propionate is carried out by an inducible system, which is at least partly mitochondrial. The mitochondrial oxidations of some of the intermediates of the β -hydroxypropionate pathway are coupled to phosphorylation and are thus subject to respiratory control.

MATERIALS AND METHODS

Maintenance and growth of the organism. The organism was that used previously; its growth and maintenance have been described in earlier papers (Callely & Lloyd, 1964*a,b*).

Preparation of extracts. French press extracts were prepared as previously described (Callely & Lloyd, 1964*a,b*) and centrifuged at 4° for 8 min. at 1000*g* to remove remain-

ing intact cells. Such extracts were not further fractionated.

Extracts used for the fractionation of enzyme activities and the preparation of mitochondria were made by grinding a cell paste with an equal volume of Ballotini no. 15 beads in a buffer consisting of sucrose (1.0*M*)-tris (0.02*M*)-EDTA (5*mM*), pH 7.4. The mortar used was chilled in ice and the semi-solid paste was ground by hand for 3 min. The further fractionation of the extract thus produced and the washing of the mitochondria have previously been described in detail (Lloyd, 1966).

Materials. 2,6-Dichlorophenol-indophenol and β -propiolactone were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. ATP was from Sigma Chemical Co., St Louis, Mo., U.S.A., and ADP, nicotinamide nucleotides, 2-mercaptoethanol and CoA were from C. F. Boehringer und Sohne G.m.b.H., Mannheim, Germany. 8-Azaguanine and *p*-fluorophenylalanine were from British Drug Houses Ltd., Poole, Dorset. Chloramphenicol was obtained from Boots Pure Drug Co. Ltd., Nottingham, and actinomycin C from Farbenfabriken Bayer A.-G., Leverkusen, Germany. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and casein hydrolysate was from Difco Laboratories, Detroit, Mich., U.S.A. Sodium acrylate was a gift from Professor D. E. Hughes. Malonic semialdehyde was generated, as required, from the acetal ester as described by Robinson & Coon (1963); β -hydroxypropionate was prepared from β -propiolactone also by the method of these authors. Propionyl-CoA was synthesized from the propionic anhydride and CoA by the method of Stadtman (1957).

Analytical methods. Manometric measurement of oxygen uptake was made in a conventional Warburg apparatus (Umbreit, Burris & Stauffer, 1957). Polarographic measurements of oxygen uptake were made with a Beckman oxygen electrode system (Lloyd, 1965*b*; Lloyd & Brookman, 1967).

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1953).

Enzyme assays. The assays for propionyl-CoA synthase [propionate-CoA ligase (AMP); EC 6.2.1.1] and propionyl-CoA dehydrogenase [acyl-CoA-(acceptor) oxidoreductase; EC 1.3.99.3] were performed exactly as described by Calley & Lloyd (1964b). The high activities of NADH oxidase in extracts containing intact mitochondria, and in mitochondrial fractions, necessitated some modification of the assays for β -hydroxypropionate dehydrogenase (3-hydroxypropionate-NAD oxidoreductase; EC 1.1.1.59) (Calley & Lloyd, 1964b) and malonic semialdehyde dehydrogenase [malonate semialdehyde-NAD(P) oxidoreductase (acylating CoA); EC 1.2.1.18] (Lloyd, 1965a). As the NADH oxidase of mitochondria from *P. zopfii* is rotenone-sensitive (Lloyd, 1966), this inhibitor was always used (20 μ g. in 20 μ l. of ethanol added to each cuvette) in the assays. Controls showed that ethanol itself does not interfere. The spectrophotometric assays were carried out at room temperature (20–22°) with a Cary model 14 recording spectrophotometer.

RESULTS

Whole-cell experiments on propionate oxidation. Washed, non-proliferating suspensions of propionate-grown cells readily oxidized propionate, acrylate, malonic semialdehyde and acetate. No oxygen uptake above the endogenous control was detected with β -hydroxypropionate as substrate at pH 7.0 (Fig. 1).

The oxidation of acetate and propionate by acetate-grown cells is shown in Fig. 2; the rate of oxygen uptake with propionate is initially quite low and parallels the rapid oxidation of acetate only after a lag of about 30 min. The total oxygen uptake with propionate after 10 min. varied between 18% and 23% of that with acetate after a similar time-interval, in six separate experiments. With the oxygen electrode a progressive increase in the rate of oxygen uptake could be followed over the first 10 min. after adding propionate to acetate-grown cells.

Similar curves were obtained with acetate-grown cells adapting to acrylate, and with glucose-grown cells adapting to acrylate or to propionate.

8-Azaguanine (1.7 mM) or *p*-fluorophenylalanine (3.4 mM) when included in the incubation system consistently slowed down the rate of adaptation of acetate-grown cells to the oxidation of propionate, but were not completely inhibitory at these concentrations (Fig. 2). Chloramphenicol (50 μ g./ml.) or actinomycin C (6.5 μ g./ml.) had no inhibitory effect on the rate of increase of propionate oxidation.

The rate of adaptation of cells to the oxidation of propionate in resting cells (harvested from the late log phase) was not increased by the inclusion of 3 mM-ammonium sulphate or an amino acid mixture (7.0 mg. of casein hydrolysate/ml.) in the system. Acetate-grown cells, starved by forced

Fig. 1. Oxidation of intermediates of propionate assimilation by propionate-grown cells. Each flask contained 700 μ moles of KOH in the centre well, 15.0 mM-phosphate buffer (pH 6.0) and 10 μ moles of substrate (as indicated) in the main compartment with washed algal suspension (5 mg. dry wt.) in the side bulb. Total liquid volume, 3.0 ml. Temperature of incubation, 30°. (1) No substrate; (2) β -hydroxypropionate; (3) acetate; (4) acrylate; (5) propionate; (6) malonic semialdehyde.

Fig. 2. Oxidation of acetate and propionate by acetate-grown cells. Each flask contained 700 μ moles of KOH in the centre well, 15.0 mM-phosphate buffer and 1.25 mg. dry wt. of algal suspension in the main compartment together with inhibitor in (Δ) and ($\blacktriangle$). The side arms contained 5 μ moles of substrate. Total volume, 3 ml. Temperature of incubation, 30°. The control flasks contained no substrates in each case; these endogenous rates were subtracted. The results are typical of four experiments. The inhibitors did not affect the rates of acetate oxidation. ●, Acetate; ○, propionate; Δ , propionate + 5 μ moles of 8-azaguanine; $\blacktriangle$, propionate + 10 μ moles of *p*-fluorophenylalanine.

aeration in phosphate buffer (10 days, 30°), still adapted to oxidize propionate, and again the addition of a nitrogen source, either as NH_4^+ ions or amino acids, to the flasks produced no increase in the rate of adaptation.

Induction of key enzymes of propionate oxidation

Experiments with French press extracts. To demonstrate that the adaptation of acetate-grown cells to propionate oxidation involves induced synthesis of enzymes of the β -hydroxypropionate pathway, four of the key enzymes involved were assayed in crude cell-free extracts prepared in the French press. Table 1 indicates that extracts of acetate-grown cells contain low but detectable activities of propionyl-CoA synthase, propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase. The values shown are typical of those obtained in six experiments. Acetate-grown cells were harvested, resuspended, and allowed to grow for 6 hr. (mean generation time on propionate 4.7 hr.) on propionate medium. Extracts of these cells contained all four enzymes at much higher levels of activity. Cells grown on propionate from a small inoculum, through many generations, gave extracts which

contained still higher activities of propionyl-CoA synthase and malonic semialdehyde dehydrogenase.

Increases in the specific activities of β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase that were observed when cells were incubated as non-proliferating suspensions with 20 mM-propionate are shown in Table 2. It is seen that the formation of both these enzymes is decreased in the presence of 3.4 mM-*p*-fluorophenylalanine.

Fractionation of extracts produced by grinding with glass beads. A study was undertaken of the distribution of four of the enzymes of propionate oxidation in extracts produced by grinding with glass beads, as it is known that such extracts contain a proportion of intact mitochondria which may be subsequently isolated by differential centrifugation (Lloyd, 1965*b*, 1966).

Fractionation of such extracts indicated that almost all the activity of propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase was recovered in the supernatant from the centrifugation at 15 000*g* for 15 min. (Table 3). In some experiments this supernatant was further fractionated by centrifuging at 100 000*g* for 1 hr.; the resulting pellet

Table 1. *Effect of growth substrate on activity of enzymes of propionate oxidation in P. zopfii*

Cells were either grown from small inocula in acetate or propionate, or acetate-grown cells were grown for 6 hr. in propionate. Cell-free extracts were prepared with a French press as detailed in the Materials and Methods section.

Enzyme	Specific activity (μ moles/mg. of protein/hr.)		
	Acetate-grown	Propionate-grown	Acetate-grown + propionate medium (6 hr.)
Propionyl-CoA synthase	0.08	0.62	0.22
Propionyl-CoA dehydrogenase	0.07	1.36	1.7
β -Hydroxypropionate dehydrogenase	0.38	10.25	9.7
Malonic semialdehyde dehydrogenase	0.16	3.84	1.6

Table 2. *Induction of enzymes of propionate oxidation in non-growing suspensions of P. zopfii*

Acetate-grown cells were harvested and resuspended in a medium consisting of 0.02 M-propionate in 0.02 M-phosphate buffer, pH 7.2. The concentration of *p*-fluorophenylalanine was 3.4 mM; incubation was for 90 min. at 30° with forced aeration. Cell-free extracts were prepared with a French press as detailed in the Materials and Methods section.

Enzyme	Specific activity (μ moles/mg. of protein/hr.)		
	Acetate-grown	Acetate-grown + propionate	Acetate-grown + propionate + <i>p</i> -fluoro- phenylalanine
β -Hydroxypropionate dehydrogenase	0.31	9.5	6.3
Malonic semialdehyde dehydrogenase	0.21	1.26	0.45

Table 3. *Distribution of propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase, malonic semialdehyde dehydrogenase and cytochrome oxidase in fractionated extracts*

Extracts were prepared and fractionated as detailed in the Materials and Methods section. The mitochondrial pellet was washed once with the isolation buffer, and this pellet wash was assayed separately for the enzymes. The small amounts of total activity found in this fraction (about 1%) have been added to the figures for the final supernatant. The figures presented are typical of those obtained in four separate experiments.

	Propionyl-CoA dehydrogenase		β -Hydroxypropionate dehydrogenase		Malonic semialdehyde dehydrogenase		Cytochrome oxidase	
	Units	% of total	Units	% of total	Units	% of total	Units	% of total
Crude extract	2.86	100	6.25	100	12.0	100	5.83	100
Final supernatant	2.56	89.5	6.16	98.2	11.23	93.4	2.92	50
Mitochondrial fraction	0.17	5.8	0.36	5.8	0.96	8.0	2.57	44.1
Recovery (%)		95.3		104.0		101.4		94.1

Table 4. *Release of enzymes from the mitochondrial suspension by ultrasonic treatment*

Mitochondrial suspension (2ml.) was treated ultrasonically for 2min. at the full output of the MSE 50w sonicator. The resulting suspension was fractionated by centrifuging at 100000g for 1hr. into a pellet and supernatant. The pellet was taken up in 2ml. of isolation buffer and the fractions were assayed for β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase.

	β -Hydroxypropionate dehydrogenase		Malonic semialdehyde dehydrogenase	
	Units	% of total	Units	% of total
Mitochondrial suspension	2.66	100	4.75	100
100000g (1hr.) pellet	0.93	35	2.05	43
100000g (1hr.) supernatant	1.73	65	2.70	57

contained at the most only 1% of the total activity of any of the three enzymes assayed.

The mitochondrial fraction contained a small percentage of the total activity of all three enzymes. This proportion (5–8%) was fairly constant in six separate experiments. Washing this fraction once with the isolation buffer released a barely measurable activity of all three enzymes (less than 1% of the total activities of the crude extract) into the supernatant after resedimenting the mitochondria at 15000g for 15min. Further washing did not release any measurable activity of these enzymes. Sonic treatment of the mitochondrial suspension (full output of the MSE 50w sonicator for 2min.) released between 50% and 70% of the total mitochondrial activities of the enzymes into a fraction which did not sediment on centrifuging at 100000g for 1hr. (Table 4).

All of the activity of the ATP-linked propionyl-CoA synthase system was found in the supernatant from the 15000g for 15min. centrifugation; no activity could be detected in the mitochondrial fraction (before or after sonic treatment for 2min.) by the hydroxamate assay.

We have been unable to detect the key enzymes of propionate oxidation in mitochondria isolated

from cells grown on acetate. Such mitochondria were resuspended together with 15000g supernatant from extracts of propionate-grown cells, centrifuged down after standing for 30min. at 2°, then resuspended in isolation medium. Propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase could not then be detected in the mitochondrial suspension treated in this way (Table 5).

Experiments with isolated mitochondria. To confirm further the presence of some of the enzymes of propionate oxidation in mitochondria prepared from propionate-grown *P. zopfii*, various intermediates of the pathway of propionate oxidation were tested as respiratory substrates for mitochondrial suspensions.

When propionate (final concn. 10mM) was added to mitochondria from propionate-grown cells, no increase in rate of oxygen uptake was detected polarographically. The addition of CoA, or of CoA, ATP and magnesium sulphate, did not produce any increase in rate of oxygen uptake above the endogenous respiration rate of the mitochondria. Propionyl-CoA, β -hydroxypropionate and malonic semialdehyde were all oxidized by the mitochondria, and in the presence of P the addition of ADP was

Table 5. *Specific activities of malonic semialdehyde dehydrogenase and β -hydroxypropionate dehydrogenase in mitochondrial fractions*

Adaptation of acetate-grown cells was for 2.5 hr. in 0.02M-propionate in 0.02M-phosphate buffer, pH 7.2.

Enzyme	Specific activity (μ moles/mg. of protein/hr.)			
	Mitochondria from propionate-grown cells	Mitochondria from acetate-grown cells	Mitochondria from acetate-grown cells adapted to propionate	Mitochondria from acetate-grown cells incubated for 30 min. with supernatant (15000g, 15 min.) from propionate-grown extract
Malonic semialdehyde dehydrogenase	6.3	0	2.1	0
β -Hydroxypropionate dehydrogenase	5.8	0	3.5	0

Fig. 3. Polarographic trace of oxygen uptake with β -hydroxypropionate as substrate. Mitochondria (0.5 mg. of protein in 0.2 ml. of sucrose-tris-EDTA isolation medium) were added to 0.8 ml. of buffer, pH 7.4 [sucrose (0.25M)-EDTA (5 mM)- KH_2PO_4 (14 mM)-bovine serum albumin (0.15%, w/v)], which had been equilibrated with air at 30°. Addition of substrate (10 μ moles) and two additions of ADP (80 μ moles each) were made as indicated. Temperature of incubation, 30°. Figures refer to oxygen uptake rates in $\text{m}\mu\text{moles/mg. of protein/min.}$ above the endogenous value (1). Addition of ADP to mitochondria in state 1 produced no increase in oxygen uptake rate. R.C., respiratory control ratio.

found to stimulate the rates of oxygen uptake with these substrates. Thus it was possible to measure the rates of respiration in the absence of ADP (state 4) and in the active phase of respiration after addition of ADP (state 3) and hence to obtain respiratory control ratios (Chance & Williams, 1955). It was also possible to obtain an indication

of the P:O ratios for these substrates during the oxidative phosphorylation of small known amounts of ADP. A typical oxygen-electrode trace of the oxidation of β -hydroxypropionate is shown in Fig. 3. In this case, the rate of oxygen uptake was the same in the first and second states 3 and 4 measured before and after two separate additions of ADP. This indicates a high degree of intactness of the mitochondria (Ohnishi & Hagihara, 1964). In some preparations lower respiratory control ratios than those cited in Table 6 were obtained and the efficiency of oxidative phosphorylation could not be accurately determined. Often completely uncoupled mitochondria were obtained which still oxidized the intermediates listed but showed no stimulation of respiration after the addition of ADP. The phosphorylating system of the isolated mitochondria was quite unstable and aging of the suspensions at 0° produced a progressive decrease in the degree of respiratory control and P:O ratio. After 4 hr. respiratory control was rarely detected. Some preparations of mitochondria also showed a high rate of endogenous respiration which made accurate measurements of oxygen uptake due to the substrates difficult; however, little or no increase in the endogenous oxygen uptake rate was found on adding ADP to mitochondrial suspensions in the absence of respiratory substrate. Endogenous respiration rates were subtracted throughout and allowed for in the calculations of respiratory control ratios and P:O ratios.

The P:O ratios, determined by this method, for propionyl-CoA were just above 2, whereas those for the two NAD-linked oxidations were sometimes as high as 2.9. The P:O ratios for β -hydroxypropionate and malonic semialdehyde were independent of pH between pH 6.3 and pH 7.4; the highest respiratory control ratios were obtained at pH 6.9. The addition of NAD^+ did not measurably

Table 6. *Respiration rates, respiratory control and P:O ratios obtained with mitochondria from propionate-grown cells*

For conditions of incubation see Fig. 3. Endogenous rates were subtracted. Results are typical of four experiments with different mitochondrial suspensions.

Substrate	Final concn. (mM)	Oxygen uptake (mμmoles/mg. of protein/min.)		Respiratory control ratio	P:O ratio
		State 3	State 4		
Propionyl-CoA	0.6	20	14	1.4	2.2
β-Hydroxypropionate	10.0	21.6	9.7	2.2	2.9
Malonic semialdehyde	1.0	24.2	16.9	1.4	2.7
Succinate	10.0	46	24.5	1.6	2.0

increase the rates of oxygen uptake with either substrate. The addition of 15000g supernatant (1.5mg. of protein) from extracts of propionate-grown cells did not increase the rates of oxidation of propionyl-CoA, malonic semialdehyde or β-hydroxypropionate.

Mitochondria from acetate-grown cells have never been shown to oxidize propionyl-CoA. Occasionally a barely detectable uptake of oxygen was obtained on adding β-hydroxypropionate or malonic semialdehyde (2–5 mμmoles/min./mg. of protein). No stimulation of this respiration was obtained on adding ADP. Often no oxygen uptake was detectable with these substrates although the same mitochondrial suspensions would oxidize succinate or α-oxoglutarate and couple the oxidation of these tricarboxylic acid-cycle intermediates to phosphorylation (Lloyd, 1965b).

DISCUSSION

The data presented in this paper clearly establish the operation of two mechanisms of metabolic control during the growth of *P. zopfii* on propionate apart from the regulatory mechanisms commonly associated with the utilization of acetyl-CoA during the growth of micro-organisms on acetate (Kornberg, 1966) or during the phosphorylation-linked tricarboxylic acid-cycle oxidations (Lloyd, 1965b).

The first of these control mechanisms involves changes in activities of key enzymes of the β-hydroxypropionate pathway in cells adapting to the oxidation of propionate. The second aspect of control is that associated with tightly coupled phosphorylation during the mitochondrial oxidation of intermediates of this pathway.

Cells grown on propionate rapidly oxidize the fatty acid (or its β-oxidation intermediate, malonic semialdehyde) without a lag, but cells grown with glucose or acetate adapt to the oxidation of propionate. During this adaptation propionate oxidation gradually increases until after 30–40 min. it proceeds almost as rapidly as the oxidation of the

growth substrate. Propionate-grown cells will also oxidize acrylate or acetate immediately, although these substrates must be activated to their CoA derivatives, which lie on the pathway of propionate oxidation (Callely & Lloyd, 1964b). β-Hydroxypropionate is not oxidized at pH 7.0; it seems likely that this substrate may not be available to intact cells as *P. zopfii* is impermeable to many organic anions (Anderson, 1945).

The adaptation of acetate-grown cells to propionate oxidation in resting cell suspensions is initially slowed down by both *p*-fluorophenylalanine and 8-azaguanine but is not completely inhibited. The lack of dependence of the process on the presence of a nitrogen source would seem to indicate that the organism has a large amino acid pool even after starvation for 10 days. Cirillo (1956, 1957) has previously demonstrated the adaptation of acetate-grown *Polytoma uvella* to oxidation of higher fatty acids, but in that organism there was a requirement for NH₄⁺ ions. Actinomycin C or chloramphenicol do not slow down the adaptation of *P. zopfii* to propionate; with chloramphenicol it seems likely that the intact organism is impermeable to this antibiotic, as the growth of *P. zopfii* is not inhibited in media containing 3–5 mg. of chloramphenicol/ml., although growth of the related organism *Polytomella caeca* is completely inhibited at this concentration (unpublished results with D. A. Evans).

Experiments with cell-free extracts demonstrate that the activities of four of the key enzymes of propionate oxidation are low in acetate-grown cells, and that these activities are increased on exposure of the cells to propionate. The formation of these enzymes occurs in the absence of growth and is slowed down by *p*-fluorophenylalanine. The activities of the enzymes in acetate-grown cells that have subsequently been grown for one generation on propionate are similar to those found in propionate-grown cells, except for the propionate-activating system, which would seem to constitute the slowest step of the four reactions of the pathway under study. It should be noted, however, that the

figures for β -hydroxypropionate dehydrogenase are those obtained by assaying the enzyme in the direction opposite to that which operates *in vivo*. The implications of the properties of this enzyme have been discussed by Calley & Lloyd (1964a).

The best available method for preparing extracts of *P. zopfii* which contain a proportion of intact mitochondria involves grinding with glass beads (Lloyd, 1966); various other methods (including attempts to find an enzyme which will produce protoplasts of the organism) have not been successful. Thus the intracellular location of enzymes has not been unequivocally established; some of the difficulties involved in this type of work have been reviewed by Dixon & Webb (1964).

There are three possible explanations for the pattern of enzyme distribution found in fractionated extracts. (1) The β -oxidation is entirely mitochondrial and the activity of the supernatant fraction arises during the rupture of mitochondria during the extraction and isolation procedures. (2) The β -oxidation system is extramitochondrial in origin and the activity found in the isolated mitochondrial fraction can be accounted for by contamination with microsomal or soluble enzymes. (3) In the intact cell two systems of β -oxidation of propionyl-CoA are present, one inside and another outside the mitochondria. Alternative (1) seems unlikely in view of the fact that only a proportion of the mitochondrial β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase can be removed by repeated washing or even by treatment with ultrasound. The second possibility seems to be ruled out as mitochondria from acetate-grown cells suspended in supernatant fractions from propionate-grown cells did not adsorb measurable activities of propionyl-CoA dehydrogenase, β -hydroxypropionate dehydrogenase or malonic semialdehyde dehydrogenase. Also, oxidative phosphorylation occurs with various intermediates of β -oxidation in mitochondria from propionate-grown cells. This presumably requires a high degree of organization of the enzymes of β -oxidation on the inner mitochondrial membrane (Chappell & Crofts, 1965). Alternative (3) provides an explanation for the distribution of the enzymes as reported. In animals and higher plants, β -oxidation of fatty acids has been located in mitochondria (Green, 1954; Lynen, 1953; Stumpf & Barber, 1956). However, two discrete pathways (intra- and extra-mitochondrial) for the β -oxidation of long-chain fatty acids have been proposed by Yamada & Stumpf (1965) in castor beans, and in peanut cotyledons by Rebeiz, Castelfranco & Englebrecht (1965); in both these investigations less than 6% of the total activity was recovered in mitochondrial pellets.

In the present investigation, although the main

site for the oxidation of propionyl-CoA seems to be extra-mitochondrial, a small but significant fraction of β -oxidation was located in the particulate fraction. The figures for the activities located in the mitochondrial fraction may well be minimal, as undoubtedly some release of enzymes from ruptured mitochondria occurs during cell disruption. It is evident, however, that the mitochondrial β -oxidation is coupled to phosphorylation and constitutes an energy-producing mechanism. Extra-mitochondrial β -oxidation, if present *in vivo*, would result in the production of NADH and acetyl-CoA in the hyaloplasm; control of utilization of these substrates as energy or carbon sources would then be dependent upon the permeability properties of the mitochondrial membranes. There is some evidence that mitochondria isolated from *P. zopfii* oxidize NADH (Lloyd, 1966) and, as in mitochondria from *Saccharomyces carlsbergensis* (Ohnishi, Kawaguchi & Hagihara, 1966), the rate of oxidation is influenced by ADP; with the mitochondria from *P. zopfii* there was no return to state 4 after adding ADP. The production of propionyl-CoA and acetyl-CoA outside mitochondria also raises the question of the transport of acyl-CoA derivatives across the mitochondrial membranes; in mammalian mitochondria acyl-carnitine is an intermediate in this process (Greville, 1966). The failure to detect any propionyl-CoA synthase activity in the mitochondrial fraction indicates that propionate activation is exclusively extra-mitochondrial; a GTP-linked activation (Rossi, Galzigna & Gibson, 1966) was not detected in any fraction.

The respiratory control ratios are low compared with those found in mammalian systems, or with yeast mitochondria prepared after lysis with snail digestive enzyme (Ohnishi *et al.* 1966), but compare favourably with those obtained with mitochondria produced after mechanical disruption of yeast (Ohnishi & Hagihara, 1964; Duell, Inoue & Utter, 1964). It seems likely that the physiological control operative in the intact cell would be more efficient; such a tight coupling between oxidation and phosphorylation would provide a finely controlled situation in which the rate of propionate oxidation would depend on the availability of ADP, and hence on the rate of utilization of ATP.

The present investigation reveals that a study of microbial mitochondria might provide a convenient system for the study of induced synthesis of enzymes in the pre-existing mitochondria of resting cells, and that such a system might cast light on the controversial subject of the site of synthesis of mitochondrial proteins (Bronsert & Neupert (1966).

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