Metabolism of Propionate by Sheep-Liver Mitochondria

EVIDENCE FOR RATE CONTROL BY A SPECIFIC SUCCINATE OXIDASE

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1. Metabolism of propionate by sheep-liver mitochondria was stimulated catalytically by α -oxoglutarate, pyruvate, citrate and isocitrate. Succinate was stimulatory at higher concentrations, but fumarate and malate were inert. These effects were all independent of the presence of ATP, succinate being less effective when ATP was present. 2. Compared with the metabolism of added succinate, propionate metabolism was resistant to malonate inhibition, but only in the presence of added ATP. In the absence of ATP propionate metabolism was more sensitive to malonate inhibition than was the metabolism of succinate. 3. In the absence of malonate, and at malonate concentrations in the range 5–100 mM, α -oxoglutarate increased the rate of fixation of [2- 14 C]propionate by about 50% without altering the nature of the fixation products. 4. Metabolism of [1- 14 C]propionate in the presence of 50 mM-malonate was accompanied by accumulation of about half the propionate consumed as succinate. When α -oxoglutarate was present in addition part of the α -oxoglutarate was metabolized and the rate of propionate consumption was increased. The total succinate that accumulated corresponded to the α -oxoglutarate consumed plus about half the propionate metabolized. 5. When [1- 14 C]propionate was metabolized in the absence of malonate about 70% of the generated succinate was oxidized to fumarate or beyond. The addition of malonate decreased the rate of propionate metabolism, and decreased to about half the fraction of generated succinate oxidized. 6. When propionate and 10 mM-succinate were metabolized together, the total oxidation of succinate was greater than that with 10 mM-succinate alone. The increment in succinate oxidation corresponded to about half the propionate metabolized in the presence or absence of malonate or ATP. 7. It is suggested that the metabolism of propionate is specifically limited by the rate of oxidation of the generated succinate, and that the succinate oxidase concerned is distinct from that responsible for the oxidation of added succinate. 8. The results are discussed in terms of the mode of action of certain stimulants and inhibitors of propionate metabolism. It is suggested that many of these act by stimulation or inhibition of the specific succinate oxidase that limits propionate metabolism.

The metabolism of propionate by rat-liver mitochondria was shown by Friedberg, Adler & Lardy (1956) to exhibit an absolute requirement for an oxidizable ancillary substrate to provide ATP for the initial energy-requiring steps. Oxidation of β -hydroxybutyrate to acetoacetate was able to fulfil this function.

Sheep-liver mitochondria, on the other hand (Smith & Russell, 1967), were able to metabolize propionate in the absence of ancillary substrate, although the rate of metabolism was increased by the presence of certain members of the tricarboxylic acid cycle. It was clear that the oxidation of succinate generated from propionate proceeded

sufficiently far to support the consumption of propionate, but the effects of the ancillary substrates at very low concentrations were anomalous. They appeared to increase the rate of supply of ATP from the oxidation of propionate itself.

To obtain direct evidence of the extent to which consumption of propionate could be driven by the oxidative step from α -oxoglutarate to succinate, experiments were planned in which oxidation of succinate arising from both sources would be prevented by malonate. Simultaneous measurement of the consumption of both propionate and α -oxoglutarate should then have allowed the efficiency of energy utilization to be assessed.

Preliminary experiments, designed to determine the concentrations of malonate necessary to stop propionate metabolism showed, however, not only that the metabolism of propionate was surprisingly resistant to inhibition by malonate, but also that the extra propionate consumption brought about by α -oxoglutarate was inhibited by malonate as well. Experiments were therefore performed to examine these effects in more detail, and these were extended to the effects of ATP and malonate on the extent of oxidation of the succinate generated during propionate metabolism. The present paper reports these findings.

MATERIALS AND METHODS

Materials. [1,4- ^{14}C]Succinic acid, [2,3- ^{14}C]succinic acid, sodium [1- ^{14}C]propionate and sodium [2- ^{14}C]propionate were obtained from The Radiochemical Centre, Amersham, Bucks. The samples of labelled succinic acid were diluted appropriately with unlabelled succinic acid and recrystallized three times from water. Labelled sodium propionate was diluted with unlabelled sodium propionate to the required specific activity, and radioactive contaminants were destroyed by oxidation with alkaline permanganate. Solutions containing up to 1m-mole of diluted sodium [^{14}C]propionate were heated for 15 min. with 5 ml. of N-KMnO_4 and 1.5 ml. of N-NaOH in a sealed Pyrex tube in a boiling-water bath. After acidification the labelled propionic acid was recovered by steam-distillation, titrated to pH 7.4 with KOH and concentrated *in vacuo* to a convenient volume. Final specific activities of labelled propionate and succinate were determined as described by Smith, Osborne-White & Russell (1967).

Malonic acid was obtained from E. Merck A.-G., Darmstadt, Germany. It was employed as the potassium salt (pH 7.4) either without purification or after crystallization from water as the monopotassium salt hemihydrate. Other materials were as described by Smith, Osborne-White & Russell (1965b, 1967). All solvents were redistilled and glass-distilled water was used throughout.

Mitochondria were prepared from the livers of freshly killed Australian Merino ewes, and washed twice in 0.25 M-sucrose as described by Smith, Osborne-White & Russell (1965a).

Estimation of propionate consumption. In the absence of malonate, propionate consumption was determined by distillation and titration of residual volatile acid (Smith & Osborne-White, 1965). When malonate was present this procedure could not be used because of conversion of about 5% of the malonic acid present into acetic acid during the distillation. In experiments involving malonate [^{14}C]propionate was employed as substrate and uptakes were calculated from residual volatile ^{14}C (Smith *et al.* 1967).

Estimation of ^{14}C . Radioactivity was determined by scintillation counting in Bray's (1960) liquid-scintillator solution (Smith *et al.* 1967), or after combustion to $^{14}\text{CO}_2$ (Smith & Osborne-White, 1965).

Total ^{14}C present as succinate in flask contents was determined after recrystallization from water of known amounts of added carrier succinate. The carrier was extracted and recrystallized five times from water. Weighed samples of the fourth and fifth crystals were dissolved in

Bray's (1960) fluid and counted to determine specific activity. Constant specific activity was uniformly achieved and the mean value used to calculate total ^{14}C in succinate from the weight of carrier added.

Distribution of ^{14}C among the products of labelled propionate or succinate metabolism was determined after paper chromatography of samples, by use of a recording strip-scanner. Samples were chromatographed (descending) for 16 hr. on Whatman no. 1 paper in the presence of 50 μg . quantities of unlabelled citric acid, malic acid, succinic acid, methylmalonic acid and fumaric acid. When labelled propionate was present, this was first removed by repeated evaporation with unlabelled propionic acid (Smith & Russell, 1967). The solvent system was 3-methylbutan-1-ol saturated with 4 N-formic acid (Flavin & Ochoa, 1957). After being dried, papers were sprayed lightly with indicator to locate carrier acid spots, and scanned with a thin end-window Geiger-Müller tube (General Electric Co. EHM2) linked through a rate-meter (Ekco Electronics Pty. Ltd. type N522C) to a Murday recorder (Evershed and Vignoles Ltd.). The recorder chart and paper drive were synchronized at 6 in./hr. The areas under radioactive peaks were determined after tracing on to draughtsman's tracing sheet (Admel Permatrace) by cutting these out and weighing them. The limits of detection were about 0.2 m μC and the error of estimation of 3.5 m μC about $\pm 5\%$. The response of peak area to added radioactivity was linear in the range 0.4–4 m μC . The fraction of ^{14}C in each labelled product was calculated from these data and applied to the value for total fixation products determined as described by Smith & Russell (1967).

Incubation medium. The standard medium contained (final concentrations): K_2HPO_4 (16.4 mm), KH_2PO_4 (3.6 mm), NaHCO_3 (25.0 mm), KCl (60 mm or 85 mm in 0.3 ml. or 6.0 ml. final volumes respectively), sucrose (83 mm or 42 mm in 0.3 ml. or 6.0 ml. final volumes respectively), MgCl_2 (5.0 mm), cytochrome *c* (11 μM) and mitochondria as indicated in individual experiments. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5), the medium being kept saturated with gas both before and during incubation as described by Smith & Russell (1967). The final pH at 37° was pH 7.4. Incubations were for 10 min. or 36 min. in the presence of substrate. The concentrations of substrates and ancillary substrates (potassium salts) are given in the legends to Tables and Fig. 1. In all experiments racks of tubes (Smith & Russell, 1967) were shaken at 120 7 cm. oscillation cycles/min. and gas mixture flowed continuously through each tube at about 200 ml./min.

Effects of ATP and ancillary substrates on propionate metabolism. Reactions in 6 ml. volumes of standard medium with or without ATP (1.3 mm) were started by adding propionate (5 mm) and ancillary substrates from the side arms 2 min. after placing in the 37° bath. Mitochondria (1.53 mg. of N) from 0.33 g. of liver were present in each tube. After 36 min. incubation with substrate, reactions were stopped by placing racks of tubes into an ice-water bath, shaking rapidly for 1 min. to chill and immediately adding 1 ml. of 10% (w/v) $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ and 1 ml. of 0.66 N- H_2SO_4 . After the addition of 12 ml. of water, protein was centrifuged off and residual volatile acid was determined. Uptakes were determined from control tubes in which protein was precipitated at the time of tipping. Results (Fig. 1) are the means of duplicate estimations on duplicate incubations.

Effect of malonate and α -oxoglutarate on the fixation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{propionate}$. Reactions in 0.3 ml. final volumes of standard medium with ATP (1.3 mm) were started by adding mitochondria (0.1 ml. with 0.079 mg. of N) to pre-gassed medium containing all other components at 0° , then transferring rapidly to the 37° bath and incubating with re-established gas flow. During transfer from the cold room, flasks were sealed with rubber stoppers to maintain the gas atmosphere. Tubes were incubated at 37° for 10 min., and then racks were chilled rapidly in an ice-water bath and 0.1 ml. of 5N-NaOH was added immediately to each tube. After the mixture had been kept at room temperature for 1 hr. to hydrolyse any CoA thio esters present, 0.1 ml. of 6N-HCl and 4.5 ml. of water were added and the tubes were heated in a boiling-water bath to precipitate protein. The tubes, previously tared, were then centrifuged, weighed to determine total contents, and 0.1 ml. samples used to determine total ^{14}C fixed by scintillation counting. Residual $[2\text{-}^{14}\text{C}]\text{propionic}$ acid was first removed by repeated evaporation with unlabelled propionic acid (Smith & Russell, 1967). Results given in Table 1 are means of estimates on duplicate incubations, which consistently agreed to within about 5%.

Samples for paper chromatography were prepared similarly by repeated evaporation with unlabelled propionic acid. Sufficient sample was taken for each chromatogram to provide about 3.5 μC of fixed ^{14}C . Chromatograms were run with carrier acids, and the fractional distribution of ^{14}C among the total fixation products was determined as described under 'Estimation of ^{14}C '.

Radioactive products of metabolism of $[2,3\text{-}^{14}\text{C}_2]\text{succinate}$. Incubations were performed in 6 ml. volumes of standard medium with ATP (1.3 mm), containing mitochondria (2.26 mg. of N) from 0.5 g. of liver, and where appropriate potassium malonate (50 mm). Substrate ($[2,3\text{-}^{14}\text{C}_2]\text{succinate}$, specific activity 11.0 $\mu\text{C}/\mu\text{mole}$) was added from the side arm to a final concentration of 5 mm 2 min. after placing tubes in the bath. Tubes were incubated for 36 min., then chilled as before, and 2 ml. of 2.5N-HCl was added to each. After centrifuging to sediment the protein, 5 ml. of supernatant was mixed with 1 ml. of carrier acids, and 50 and 100 μl . samples were chromatographed. Chromatograms were scanned for radioactivity as described under 'Estimation of ^{14}C '. The results given in the text are means of the results from two chromatograms on each of two replicate incubations.

Accumulation of succinate in the presence of 50 mm-malonate when $[1\text{-}^{14}\text{C}]\text{propionate}$ was metabolized in the presence and absence of α -oxoglutarate. Incubations were carried out in 100 ml. conical flasks fitted with side arms, with constant gassing ($\text{O}_2 + \text{CO}_2$, 95:5) as before. Flasks contained 20 ml. of standard medium with ATP (1.3 mm), malonate (50 mm) and mitochondria from 1.67 g. of liver. Substrates (2 ml.) were added from the side arms 2 min. after placing in the 37° bath, and the mixtures incubated for 36 min. Flasks were then chilled rapidly in ice-water and samples immediately removed by pipette for precipitation of protein. Consumption of $[1\text{-}^{14}\text{C}]\text{propionate}$ was determined from residual volatile ^{14}C after treatment of a 6 ml. sample with tungstic acid and dilution to 20 ml. Duplicate samples were distilled and duplicate samples of distillate counted. Total ^{14}C in succinate was determined in 4 ml. samples of the tungstic acid supernatant by measuring the specific activity of recovered carrier succinic acid (250 μC)

added to the sample. The carrier succinic acid was extracted overnight with diethyl ether in a liquid/liquid extractor, the ether evaporated and the residue recrystallized for measurement of specific activity. Consumption of α -oxoglutarate was determined by the method of Friedemann (1957), 6 ml. samples of flask contents being added to 14 ml. of 4.3% metaphosphoric acid and residual α -oxoglutaric acid determined in duplicate.

The specific activity of the succinate produced from $[1\text{-}^{14}\text{C}]\text{propionate}$ was determined from the specific activity of succinylhydroxamic acid. Samples (6 ml.) of flask contents were added to 2 ml. of 12% (w/v) metaphosphoric acid and protein was centrifuged off. Before recovery of succinic acid, α -oxoglutaric acid was removed as the DNP-hydrazone. Samples (5 ml.) of the protein-free supernatant were incubated at 25° for 30 min. with 5 ml. of 0.1% DNP-hydrazine in 2N-HCl. The DNP-hydrazone was extracted successively with 3 ml. and 2 ml. of diethyl ether and discarded. The residual solution was made alkaline (pH above 10) with 6N-NH₃, and excess of DNP-hydrazine was extracted with three 5 ml. lots of diethyl ether. The residual aqueous solution was acidified with HCl (pH below 2), and succinic acid together with malonic acid was extracted overnight with diethyl ether in a liquid/liquid extractor. After evaporation of the ether the residue was transferred in ethanol to a small (10 mm. $\times$ 75 mm.) Pyrex test tube and the ethanol evaporated at 80° in a stream of N₂. The residue was dissolved in 0.1 ml. of 0.1N-HCl, and the tube sealed and heated for 2 hr. at 140° in an oil bath to convert the malonic acid into acetic acid. The seal was broken and the acetic acid and HCl were removed in a vacuum desiccator (mechanical pump) over CaCl₂ and NaOH. Acetic anhydride (0.1 ml.) was then added, and the tube resealed and heated in a boiling-water bath for 1 hr. The seal was broken and most of the acetic anhydride and acetic acid removed in 30 min. *in vacuo* as above. Neutral (pH 6.7) hydroxylamine was freshly prepared by adding excess (5 g.) of moist Dowex 1 (OH⁻ form) to 5 ml. of 14% (w/v) hydroxylamine hydrochloride and filtering. A 0.1 ml. sample of this solution was added to the succinic anhydride in the tube and left for 30 min. to form succinylhydroxamic acid. Triplicate 10 μl . or 20 μl . samples were then chromatographed on Whatman no. 1 paper [prewashed with 10% (w/v) acetic acid] with butan-1-ol-acetic acid-water (4:1:5, by vol.) as solvent (Thompson, 1951) and estimated by the method of Stadtman & Barker (1950) except that the succinylhydroxamic acid spots were macerated with 1 ml. of 0.5% (w/v) FeCl₃·6H₂O in 0.05N-HCl and filtered. The coloured solution was read (Beckman model DU spectrophotometer) within 30 min. at 540 μm against a reagent blank similarly prepared from a blank area of the chromatogram. Duplicate 0.2 ml. samples of this solution were used to determine ^{14}C by combustion in the presence of about 5 mg. of filter paper (as carrier carbon source) and counting as $^{14}\text{CO}_2$. (Heavy and erratic quenching by this solution prevented estimation of ^{14}C by scintillation counting.) The total succinic acid in flask contents was determined from the specific activity of the succinylhydroxamic acid and the total ^{14}C present as succinate. Recovery of $[^{14}\text{C}]\text{succinate}$ as the hydroxamic acid varied from 28% to 55%, but this does not affect the validity of the measurement of specific activity. The method of measuring specific activity was standardized with $[2,3\text{-}^{14}\text{C}_2]\text{succinic}$ acid (11.0 $\mu\text{C}/\mu\text{mole}$) and the molar extinction coefficient of the succinylhydroxamic acid-FeCl₃

complex [$\epsilon 1.02 \times 10^3$ l. mole⁻¹ cm.⁻¹ (mean of eight observations)] calculated from the ¹⁴C present in the chromatogram eluates. Replicate estimations of 10.7 μ moles of [2,3-¹⁴C₂]-succinate (11.0 μ mc/ μ mole) added to 6 ml. samples of the contents of flasks that were not incubated yielded a value of 10.4 ± 0.6 μ moles (s.e.m., four observations). The specific activity of the added succinate did not change during recovery, indicating the absence of succinic acid (less than 0.3 μ mole in 20 ml. of flask contents in Expt. 2) from the mitochondria. The specific activity of succinylhydroxamic acid in replicate chromatograms showed good agreement, the maximum deviation of individual values from the mean of three replicates in any of the samples measured being 6%.

Consumption of [1-¹⁴C]propionate and α -oxoglutarate was determined by difference from duplicate initial samples prepared after 2 min. in the bath without substrate.

Effect of malonate and ATP on the metabolism of [1-¹⁴C]-propionate and [1,4-¹⁴C₂]succinate. Three experiments were performed, one in 6 ml. final volumes of standard medium containing mitochondria with 1.28 mg. of N, and two in 0.3 ml. final volumes of standard medium containing mitochondria with 0.104 and 0.108 mg. of N respectively.

When 0.3 ml. final volumes were used reactions were started by adding 0.1 ml. of mitochondria at 0° at 30 sec. intervals to tubes of continuously gassed medium at 37° containing all other components. Tubes were incubated with shaking for 36 min. Final concentrations of 10 mm.-potassium propionate (unlabelled or 1-¹⁴C-labelled with specific activities of 271 and 353 μ mc/ μ mole in the two experiments respectively), 10 mm.-potassium succinate (unlabelled or 1,4-¹⁴C₂-labelled with specific activity 35.8 μ mc/ μ mole in both experiments), 10 mm.- or 50 mm.-potassium malonate and 1.3 mm.-ATP were employed. Reactions were stopped in the 37° bath by adding 3.0 ml. of 0.33 N-NaOH containing 200 mg. of succinic acid as the sodium salt and 5 μ moles of potassium propionate as carriers. Gas flow to each tube was discontinued on adding NaOH.

When 6 ml. final volumes were used reactions were started by adding substrates (propionate and succinate) from the side arms 2 min. after placing in the bath. In this experiment reactions were stopped 36 min. after adding substrate by chilling in ice-water for 1 min. before adding 5 ml. of N-NaOH containing 1.00 g. of carrier succinic acid as the sodium salt. Final concentrations of 5 mm.-potassium propionate (unlabelled or 1-¹⁴C-labelled with specific activity 40.7 μ mc/ μ mole), 10 mm.-potassium succinate (unlabelled or 1,4-¹⁴C₂-labelled with specific activity 5.41 μ mc/ μ mole), 10 mm.- or 50 mm.-potassium malonate and 1.3 mm.-ATP were used.

In all experiments consumption of [1-¹⁴C]propionate was determined as described above by distillation of the entire flask contents after acidification. Duplicate samples of the distillate were counted by scintillation counting, and uptakes determined from control tubes that were not incubated. Results were the means of duplicate estimates on duplicate incubations.

¹⁴C present as succinate was determined from the specific activity of the carrier succinate recovered. Where [1-¹⁴C]propionate had been present, this was removed in estimating the uptake as described above, and labelled succinate was recovered from the distillation residue. Where no labelled propionate had been present, carrier succinate was recovered directly from the flask contents after acidification. In all cases most of the carrier succinate

(70–80%) was recovered from the acidified solution (pH less than 2 with 10 N-H₂SO₄) by extraction with three 5 ml. or 10 ml. lots of butan-1-ol-chloroform (1:1, v/v). After evaporation of the solvent *in vacuo*, the residual succinic acid was recrystallized five times and the specific activity determined. Both [¹⁴C]succinate production from [1-¹⁴C]-propionate and [1,4-¹⁴C₂]succinate consumption were determined in this way, the latter from initial concentrations determined in control tubes that were not incubated.

RESULTS

Effects of ATP and tricarboxylic acid-cycle intermediates on propionate metabolism by mitochondria. The effectiveness of certain members of the tricarboxylic acid cycle as stimulants of propionate metabolism was previously demonstrated with mitochondria that had been aged at 37° for 19 min. without substrate (Smith *et al.* 1965b). This work was repeated with mitochondria that had not been aged and was extended to determine whether the effects were dependent on ATP. The results of this experiment are shown in Fig. 1, from which it is clear that a similar pattern of stimulation applied to fresh as to aged mitochondria. Marked stimulation was obtained with catalytic concentrations (0.167 mm) of α -oxoglutarate, pyruvate, citrate and isocitrate, succinate was much less effective, and fumarate and malate were inert. Further, with the exception of succinate, the effects of the stimulants were not significantly modified by ATP (1.3 mm) (see also Table 3).

These results therefore differ from those of Lehninger (1945), who studied the effects of some tricarboxylic acid-cycle intermediates on the rate of oxidation of octanoate by rat-liver mitochondria. Stimulatory effects of all tricarboxylic acid-cycle intermediates tested were noted, but α -oxoglutarate was unique in that it alone did not require the presence of AMP to be effective.

Effects of malonate on the rate and products of fixation of [2-¹⁴C]propionate, with and without α -oxoglutarate. The effects of malonate on metabolism of propionate have been examined by Pennington & Sutherland (1956) with slices of sheep rumen epithelium, and by Willmer & Gutfreund (1963) with slices of guinea-pig mammary gland. Both studies showed a resistance of propionate metabolism to inhibition by malonate. The results given in Table 1 demonstrate that this was also true of sheep-liver mitochondria in the presence of 1.3 mm-ATP. A concentration of 100 mm-malonate inhibited fixation of [2-¹⁴C]propionate by only 77%.

Also shown in Table 1 are the rates of fixation of [2-¹⁴C]propionate in the presence of 1.67 mm- α -oxoglutarate. In the absence of malonate, and at all concentrations of malonate tested, the rate in the presence of α -oxoglutarate was about 50% faster than the rate in its absence [mean ratio 1.47 ± 0.03].

Fig. 1. Effects of ATP and tricarboxylic acid-cycle intermediates on the rate of consumption of propionate by sheep-liver mitochondria. Mitochondria (1.53 mg. of N) were incubated for 36 min. at 37° with propionate (5 mM) and the initial concentrations of the tricarboxylic acid-cycle intermediates shown. Final volumes of 6 ml. of standard medium (○) or standard medium with ATP (1.3 mM) (●) were used, substrates being added 2 min. after placing in the bath. The gas phase was O₂ + CO₂ (95:5). The concentrations of D,L(+)-isocitrate shown are half those of the DL-isocitrate actually used. Results are the means of duplicate estimates on duplicate incubations.

Table 1. *Effect of malonate on the fixation of ¹⁴C from [2-¹⁴C]propionate by sheep-liver mitochondria in the presence and absence of α-oxoglutarate*

Tubes containing 0.3 ml. of standard medium with ATP (1.3 mM), [2-¹⁴C]propionate (5 mM, 0.50 μC/μmole) and mitochondria with 0.079 mg. of N were incubated for 10 min. at 37° in a gas phase of O₂ + CO₂ (95:5) together with the concentrations of potassium malonate shown, and where appropriate α-oxoglutarate (1.67 mM). Fixation of ¹⁴C was determined after removal of residual labelled propionate as described in the Materials and Methods section. Results are the means of duplicate incubations.

Concn. of malonate (mM)	[2- ¹⁴ C]Propionate		[2- ¹⁴ C]Propionate + α-oxoglutarate		Ratio b/a
	¹⁴ C fixed (μC/mg. of N/hr.) (a)	Inhibition by malonate (%)	¹⁴ C fixed (μC/mg. of N/hr.) (b)	Inhibition by malonate (%)	
0	6.13	—	8.76	—	1.43
5	3.29	46	4.90	44	1.49
10	2.88	53	4.14	53	1.44
20	2.36	62	3.74	57	1.59
50	2.11	66	3.16	64	1.50
100	1.42	77	1.97	78	1.38

Table 2. *Products of [2-¹⁴C]propionate metabolism in the presence and absence of malonate and α -oxoglutarate*

The results refer to the same experiment as Table 1. The approximate distribution of total ¹⁴C fixed among products was determined after paper chromatography with carrier acids (except glutamic acid and aspartic acid), by measurement of the relative areas under radioactive peaks on a strip-scanner record as described in the Materials and Methods section. Radioactive propionic acid was removed before chromatography by repeated evaporation of the samples with unlabelled propionic acid. All chromatograms contained about 3.5 m μ C of total ¹⁴C, and results are the means of chromatograms on duplicate incubations. Zero values represent apparent formation of less than 0.2 μ C of radioactive product/mg. of N/hr. At low rates, however, chromatograms contained higher amounts of inorganic acids and particularly malonic acid; such chromatograms were less satisfactory and errors may have been higher.

Distribution of ¹⁴C in products (μ C/mg. of N/hr.)

Concn. of malonate (mM)	Concn. of α -oxoglutarate (mM)	Succinate	Fumarate	Malate	Citrate	Origin (glutamate + aspartate)
0	0	2.6	0.4	2.2	0.3	0.7
5	0	3.3	0	0	0	0
10	0	2.9	0	0	0	0
20	0	2.4	0	0	0	0
50	0	2.1	0	0	0	0
100	0	1.4	0	0	0	0
0	1.67	1.8	0.4	5.3	0.4	0.9
5	1.67	4.1	0	0	0.2	0.6
10	1.67	3.7	0	0	0	0.4
20	1.67	3.3	0	0	0	0.4
50	1.67	3.2	0	0	0	0
100	1.67	2.0	0	0	0	0

(s.e.m.)], i.e. propionate metabolism was inhibited by malonate to the same extent whether α -oxoglutarate was present or not.

At high concentrations of malonate (and low rates of [2-¹⁴C]propionate consumption) [¹⁴C]-succinate was the only radioactive product detected on scanning of chromatograms (Table 2). However, it is shown below (Tables 3 and 4) that at a concentration of 50 mM-malonate a substantial fraction of the succinate produced from [1-¹⁴C]propionate failed to accumulate. The accumulation predominantly of labelled succinate from [2-¹⁴C]-propionate shown in Table 2 is best interpreted as due to cycling back to [¹⁴C]succinate of a major part of the [2,3-¹⁴C₂]fumarate formed when the rate of formation of fumarate was low. Such cycling has been observed to occur (Smith *et al.* 1967) and with [2,3-¹⁴C₂]fumarate would not be accompanied by loss of label. This interpretation is consistent with the persistence of label in other products at intermediate concentrations of malonate, and particularly that in amino acids, since these would have become labelled by exchange.

Production of succinate from α -oxoglutarate and propionate in the presence of 50 mM-malonate. The results of two experiments where succinate production from [1-¹⁴C]propionate in the presence of 50 mM-malonate was measured in the presence and absence of α -oxoglutarate are shown in Table 3.

Metabolism of propionate was accompanied by oxidation of succinate whether α -oxoglutarate was present or not. When α -oxoglutarate was present consumption of propionate was increased and more succinate accumulated. If the contribution of succinate from the α -oxoglutarate metabolized is subtracted, then the accumulated succinate represents about half the propionate metabolized. In the absence of α -oxoglutarate the accumulated succinate represents 34% and 61% of the propionate metabolized in the two experiments. There was no apparent correlation between α -oxoglutarate metabolized and either total propionate or net extra propionate consumed.

These results do not permit a close assessment of any correlation between propionate metabolized and succinate oxidized because it is not known to what extent the fumarate formed was oxidized back to succinate (2 mol. of fumarate + 3 mol. of oxygen $\rightarrow$ 1 mol. of succinate). That some such cycling occurred is indicated by the specific activity of the succinate produced. In the absence of α -oxoglutarate this was 67% and 60% that of the substrate [1-¹⁴C]propionate in Expts. 1 and 2 respectively.

Nevertheless these results do support an interpretation of the results of Table 1, that propionate consumption was linked to the oxidation of succinate and that the effect of α -oxoglutarate was

Table 3. *Production of succinate by mitochondria in the presence of 50mM-malonate*

Mitochondria with 7.53 and 9.09 mg. of N (Expts. 1 and 2 respectively) were incubated for 36 min. at 37° with [1-¹⁴C]propionate (5mM, specific activity 108.5 and 78.8 mμC/μmole in Expts. 1 and 2 respectively). All flasks contained malonate (50mM) and ATP (1.3mM), α-oxoglutarate when present (1.67mM in Expt. 1 and 2.5mM in Expt. 2) being added with [1-¹⁴C]propionate from the side arms after 2 min. in the bath at 37°. The total fluid volume was 20 ml. and the gas phase was O₂ + CO₂ (95:5). Consumption of propionate and α-oxoglutarate and production of succinate were measured as described in the text. Results are the means of duplicate incubations.

Substrate	Expt. no.	Propionate consumed (μmoles)	α-Oxoglutarate consumed (μmoles)	Succinate produced (μmoles)	Sp. activity of succinate (mμC/μmole)
[1- ¹⁴ C]Propionate	1	12.1	—	4.1	73.2
	2	5.4	—	3.3	47.6
[1- ¹⁴ C]Propionate + α-oxoglutarate	1	18.2	10.2	19.4	34.6
	2	11.2	20.9	26.5	32.5

due, not to the direct provision of energy during its oxidation to succinate, but to stimulation of the rate at which succinate was oxidized.

Metabolism of propionate and succinate in the presence and absence of malonate and ATP. The effects of succinate on propionate metabolism differed from those of the other stimulatory members of the tricarboxylic acid cycle in that concentrations of succinate about fivefold higher were required to give comparable effects.

The fate of metabolized succinate in the presence and absence of 50mM-malonate was examined by incubating [2,3-¹⁴C₂]succinate with mitochondria for 36 min. in the standard medium (with 1.3mM-ATP) and examining the labelled products. The experiment is described in the Materials and Methods section. The distribution of ¹⁴C among the products in the absence of malonate was as follows: succinate, 20%; fumarate, 12%; malate, 51%; citrate plus isocitrate, 10%; origin (presumed to be glutamate and aspartate, which remain at the origin, but which were not added as carriers), 8%. Total recovery was 101% of the ¹⁴C recovered in the succinate peak of control flasks to which acid was added at the time of addition of substrate [2,3-¹⁴C₂]succinate. In the presence of 50mM-malonate, the incubated [2,3-¹⁴C₂]succinate was completely recovered (103%) in the succinate peak.

This distribution of ¹⁴C is consistent with a rapid oxidation of succinate to fumarate and malate, together with a limited formation of citrate via oxaloacetate and pyruvate. The results do not exclude some complete cycling of succinate (2 mol. of succinate + 3.5 mol. of oxygen → 1 mol. of succinate), since no label would be lost by this process. The extent of such cycling must have been small, however, because any further cycling of the re-formed succinate (now labelled in all carbon atoms) would have led to loss of label as ¹⁴CO₂.

In assessing the results of further experiments

in which both labelled succinate and labelled propionate were employed, three assumptions have been made. These are (1) that propionate was metabolized in mitochondria solely via succinate, (2) that succinate was metabolized solely via fumarate and (3) that no significant dilution of [1,4-¹⁴C₂]succinate occurred from unlabelled succinate produced from it via the tricarboxylic acid cycle. The evidence for the first of these assumptions is substantial (Smith *et al.* 1967). The second and third assumptions are supported by the distribution of label from metabolized [2,3-¹⁴C₂]succinate described above, although from Table 3 dilution by cycling did become significant in the presence of 50mM-malonate when the rate of formation of [¹⁴C]succinate from [1-¹⁴C]propionate was low.

In the experiments referred to in Tables 3 and 4 two kinds of measurement were made: the rate of disappearance of ¹⁴C in [1-¹⁴C]propionate, and the rate of appearance or disappearance of label in [¹⁴C]succinate when [1-¹⁴C]propionate or [1,4-¹⁴C₂]succinate were the respective labelled substrates. When both substrates were present together these were [1-¹⁴C]propionate plus unlabelled succinate, and in otherwise identical tubes [1,4-¹⁴C₂]succinate and unlabelled propionate. The 1-¹⁴C-labelled substrates were used to avoid any return of label to succinate by complete cycling of labelled fumarate, and hence to permit estimation of the rate at which labelled succinate traversed succinate oxidase.

The rate of disappearance of label from [1-¹⁴C]propionate is a valid measure of the molar rate of metabolism of propionate in the presence or absence of unlabelled succinate. The rate of disappearance of label from [1,4-¹⁴C₂]succinate, however, is a valid measure of the molar rate of metabolism of added succinate only in the absence of succinate generated from unlabelled propionate. The simultaneous

metabolism of unlabelled propionate could affect this rate in several ways. First, if there is a distinction within the mitochondria between added and generated succinate, the measured rate of loss of added $[1,4-^{14}\text{C}_2]$ succinate will depend partly on the rate of exchange of label between the two classes of succinate molecules, and partly on dilution of the labelled succinate by any unoxidized fraction of the generated succinate. If there is no distinction then the effect will depend only on dilution of labelled succinate with unlabelled succinate. Secondly, the rate of loss of label will be modified by any real effect of the occurrence of propionate metabolism on the rate of metabolism of added succinate. In any event the measured rate will truly represent the rate of conversion of actual molecules of $[1,4-^{14}\text{C}_2]$ succinate into $[1,4-^{14}\text{C}_2]$ fumarate.

Similarly, in the absence of added succinate, the rate at which ^{14}C from metabolized $[1-^{14}\text{C}]$ propionate fails to appear as ^{14}C succinate will validly represent the molar rate of conversion of synthesized succinate into fumarate. In the presence of unlabelled succinate, the difference between $[1-^{14}\text{C}]$ propionate consumed and ^{14}C succinate produced will represent only the rate of conversion of actual molecules of $[1-^{14}\text{C}]$ propionate via $[1,4-^{14}\text{C}_2]$ succinate into $[1,4-^{14}\text{C}_2]$ fumarate, and not necessarily the molar rate of this sequence.

Since there are only two sources of succinate molecules (neglecting endogenous substrate as small) the sum of the two quantities ' $[1,4-^{14}\text{C}_2]$ succinate substrate lost' and ' $[1-^{14}\text{C}]$ propionate lost in excess of ^{14}C succinate produced from it' expressed in molar terms ($\text{m}\mu\text{C} \div \text{specific activity of substrate}$) will validly represent the total molar conversion of succinate into fumarate from both sources. However, it will not be possible to assign significance in terms of molar conversion rates to either of these quantities alone.

Table 4 sets out the mean rates and standard errors of three experiments in which the effects of ATP (1.3mm) and malonate (10mm and 50mm) on the metabolism of $[1-^{14}\text{C}]$ propionate and $[1,4-^{14}\text{C}_2]$ succinate were measured. These results are direct observations and involve no assumptions. For the reasons given above they are expressed in terms of radioactivity, corrected to a common basis by application of the specific activities of the substrates.

The following points emerge.

(1) The rate of metabolism of $[1,4-^{14}\text{C}_2]$ succinate alone was several times faster than that of $[1-^{14}\text{C}]$ propionate alone in the absence of ATP, and more than twice as fast in the presence of ATP. ATP stimulated the rate of consumption of $[1-^{14}\text{C}]$ propionate about threefold but only slightly increased the rate of metabolism of $[1,4-^{14}\text{C}_2]$ succinate.

(2) The rate of metabolism of $[1-^{14}\text{C}]$ propionate

was increased by 10mm-succinate, but little further increase was caused by ATP.

(3) In the absence of ATP, $[1-^{14}\text{C}]$ propionate metabolism was virtually abolished by 10mm-malonate either in the presence or absence of succinate.

(4) In the absence of ATP, $[1-^{14}\text{C}]$ propionate metabolism was relatively more sensitive to inhibition by malonate, and in the presence of ATP relatively less sensitive to inhibition by malonate, than was the metabolism of $[1,4-^{14}\text{C}_2]$ succinate.

(5) In the absence of succinate the fraction of ^{14}C from metabolized $[1-^{14}\text{C}]$ propionate that was recovered in succinate was not altered by the presence of ATP. It was increased by malonate, but even in the presence of 50mm-malonate only about 56% was recovered as succinate.

(6) The fraction of ^{14}C from metabolized $[1-^{14}\text{C}]$ propionate that was recovered in succinate was increased by the presence of an unlabelled succinate pool. This fraction was not affected by the presence of ATP and was not significantly further increased by malonate.

These results show that, though the effects of ATP and malonate on the metabolism of $[1-^{14}\text{C}]$ propionate and $[1,4-^{14}\text{C}_2]$ succinate were qualitatively similar, quantitatively they were quite distinct. The findings are consistent with the metabolism of $[1-^{14}\text{C}]$ propionate having occurred by way of succinate, but they suggest that the succinate oxidase involved was not identical with that responsible for metabolism of added $[1,4-^{14}\text{C}_2]$ succinate. Of particular significance is the observation that, in the absence of ATP and the presence of succinate, negligible metabolism of $[1-^{14}\text{C}]$ propionate occurred with 10mm-malonate, despite the appreciable metabolism of $[1,4-^{14}\text{C}_2]$ succinate detected in the control incubations. Oxidation of added succinate was apparently unable to supply the energy to bring about consumption of propionate.

In Table 5 are shown the quantities derived from the data summarized in Table 4, by assuming that the ^{14}C from metabolized $[1-^{14}\text{C}]$ propionate that failed to appear as labelled succinate represented generated labelled succinate that had been oxidized. With $[1-^{14}\text{C}]$ propionate alone, the calculation was direct and involved measurement of two quantities in the same incubation. With propionate plus succinate the calculation was less direct and involved four measurements in three different incubations. The 'succinate increment' was defined as the quantity by which the total metabolism of succinate, with both succinate and propionate present, exceeded the metabolism of succinate that occurred in the absence of propionate. Subject to the assumptions discussed above, the results may be expressed as molar conversion rates. The existence

Table 4. *Metabolism of [1-¹⁴C]propionate and [1,4-¹⁴C₂]succinate by sheep-liver mitochondria in the presence and absence of malonate and ATP*

Concn. of malonate (mM)	Results of three experiments are shown in which [1^{14}C]propionate, either alone or in the presence of unlabelled succinate, and [$1,4^{14}\text{C}_2$]succinate, either alone or in the presence of unlabelled propionate, were incubated for 36 min. at 37° with sheep-liver mitochondria. The standard medium was used and the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). The conditions and procedures used are described in the Materials and Methods section. Where the Table shows that both labelled substrates were present, this represents the combined results of parallel incubations each with one labelled substrate. Expressed in the terms used in the Table the results of all three experiments were similar in all respects and have been combined. The significance of the unit of measurement ($\text{m}\mu\text{C}/\text{mg. of N/hr.} \div \text{specific activity of substrate}$) is discussed in the text. For [1^{14}C]propionate in all cases and for [$1,4^{14}\text{C}_2$]succinate alone it is equivalent to $\mu\text{moles of substrate consumed}/\text{mg. of N/hr.}$, but for [$1,4^{14}\text{C}_2$]succinate in the presence of unlabelled propionate the significance is more restricted. The results for each experiment, both for propionate and succinate, were the means of duplicate estimates of ^{14}C on duplicate incubations. The results given are the means $\pm$ s.e.m. of the three experiments.																
	Propionate alone						Propionate + succinate						Succinate alone				
	No ATP			1.3 mM-ATP			No ATP			1.3 mM-ATP			No ATP		1.3 mM-ATP		
	[1^{14}C]-			[1^{14}C]-			[1^{14}C]-			[$1,4^{14}\text{C}_2$]-			[$1,4^{14}\text{C}_2$]-			[$1,4^{14}\text{C}_2$]-	
	Propionate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Propionate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Propionate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)	Fraction of ^{14}C from consumed	Succinate consumed ($\text{m}\mu\text{C}/\text{mg. of N/hr.}$)
$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	found in succinate (%)	$\div$ sp. activity of substrate (%)	$\div$ sp. activity of substrate (%)	$\div$ sp. activity of substrate (%)	
0	2.2 $\pm$ 0.5	28 $\pm$ 7	6.9 $\pm$ 0.6	30 $\pm$ 2	10.3 $\pm$ 0.5	67 $\pm$ 7	18.5 $\pm$ 1.3	12.4 $\pm$ 1.7	62 $\pm$ 5	19.8 $\pm$ 1.6	5.0 $\pm$ 0.5	15.9 $\pm$ 2.1	17.9 $\pm$ 1.9				
10	0.5 $\pm$ 0.5	< 50	2.4 $\pm$ 0.1	40 $\pm$ 11	0.5 $\pm$ 0.1	< 50	4.6 $\pm$ 0.2	4.1 $\pm$ 0.7	77 $\pm$ 7	5.0 $\pm$ 0.5	77 $\pm$ 7	4.2 $\pm$ 0.6	4.7 $\pm$ 0.3				
50	0.6 $\pm$ 0.2	< 50	2.0 $\pm$ 0.1	56 $\pm$ 13	0.5 $\pm$ 0.4	< 50	0.9 $\pm$ 0.1	2.8 $\pm$ 1.1	55 $\pm$ 20	1.3 $\pm$ 0.1	55 $\pm$ 20	0.5 $\pm$ 0.1	1.0 $\pm$ 0.2				

Results of three experiments are shown in which [1-¹⁴C]propionate, either alone or in the presence of unlabelled succinate, and [1,4-¹⁴C₂]succinate, either alone or in the presence of unlabelled propionate, were incubated for 36 min. at 37° with sheep-liver mitochondria. The standard medium was used and the gas phase was O₂ + CO₂ (95:5). The conditions and procedures used are described in the Materials and Methods section. Where the Table shows that both labelled substrates were present, this represents the combined results of parallel incubations each with one labelled substrate. Expressed in the terms used in the Table the results of all three experiments were similar in all respects and have been combined. The significance of the unit of measurement (mμC/mg. of N/hr. ÷ specific activity of substrate) is discussed in the text. For [1-¹⁴C]propionate in all cases and for [1,4-¹⁴C₂]succinate alone it is equivalent to μmoles of substrate consumed/mg. of N/hr, but for [1,4-¹⁴C₂]succinate in the presence of unlabelled propionate the significance is more restricted. The results for each experiment, both for propionate and succinate, were the means of duplicate estimates of ¹⁴C on duplicate incubations. The results given are the means ± s.e.m. of the three experiments.

Table 5. *Correlation between the rate of metabolism of [1-¹⁴C]propionate and the rate of oxidation of the generated succinate in the presence and absence of ATP, malonate and a succinate pool*

The quantities and ratios shown were derived from the data of the three experiments summarized in Table 4, and represent the means $\pm$ s.e.m. of the three experiments. The estimated rate of succinate oxidation accompanying the metabolism of propionate alone represents that fraction of metabolized ¹⁴C from [1-¹⁴C]propionate that was not recovered as labelled succinate. The succinate increment represents the additional succinate metabolized as a direct result of the occurrence of propionate metabolism. It was calculated by subtracting the rate of metabolism of [1,4-¹⁴C₂]succinate alone from the total rate of metabolism of succinate that occurred when both substrates were present (at the appropriate concentrations of ATP and malonate). The latter quantities were first derived as the sum of two observed rates: the rate of consumption of [1,4-¹⁴C₂]succinate in the presence of unlabelled propionate, and the rate at which metabolized [1-¹⁴C]propionate failed to appear as labelled succinate. The assumptions involved in making these calculations and the evidence obtained to justify them are discussed in the text relating to the experiments. The ratios given are the means $\pm$ s.e.m. of the individual ratios derived from the three experiments, and not the ratios of the mean rates quoted in the Table.

Concn. of malonate (mm)	Propionate alone						Propionate + succinate					
	No ATP			1.3 mm.-ATP			No ATP			1.3 mm.-ATP		
	Rate of propionate consump- tion (μ moles/ mg. of N/hr.)	Estimated rate of succinate oxidation (μ moles/ mg. of N/hr.)	Ratio propionate consumed/ succinate oxidized	Rate of propionate consump- tion (μ moles/ mg. of N/hr.)	Estimated rate of succinate oxidation (μ moles/ mg. of N/hr.)	Ratio propionate consumed/ succinate oxidized	Rate of propionate consump- tion (μ moles/ mg. of N/hr.)	Estimated rate of succinate oxidation (μ moles/ mg. of N/hr.)	Ratio propionate consumed/ succinate increment	Rate of propionate consump- tion (μ moles/ mg. of N/hr.)	Estimated rate of succinate oxidation (μ moles/ mg. of N/hr.)	Ratio propionate consumed/ succinate increment
0	2.2 $\pm$ 0.5	1.6 $\pm$ 0.2	1.4 $\pm$ 0.2	6.9 $\pm$ 0.6	4.8 $\pm$ 0.5	1.4 $\pm$ 0.1	10.3 $\pm$ 0.5	6.1 $\pm$ 1.5	1.9 $\pm$ 0.5	12.4 $\pm$ 1.7	6.4 $\pm$ 1.0	2.0 $\pm$ 0.4
10	<1	—	—	2.4 $\pm$ 0.1	1.5 $\pm$ 0.3	1.8 $\pm$ 0.4	<1	—	—	4.1 $\pm$ 0.7	1.3 $\pm$ 0.3	3.2 $\pm$ 0.8
50	<1	—	—	2.0 $\pm$ 0.1	0.9 $\pm$ 0.2	3.0 $\pm$ 1.2	<1	—	—	2.8 $\pm$ 1.1	1.9 $\pm$ 0.9	1.6 $\pm$ 0.4

of the 'succinate increment' was directly attributable to the occurrence of concurrent propionate metabolism.

In the absence of succinate or malonate the relationship between propionate consumed and generated succinate oxidized was independent of the presence of ATP, despite the increase in rate brought about by ATP. The addition of malonate caused the ratio to increase, while decreasing the rate. In the presence of succinate and the absence of malonate, the relationship between propionate consumed and the 'succinate increment' corresponded to about 2mol. of propionate consumed/mol. of extra succinate oxidized in the presence or absence of ATP. This ratio was not significantly increased by malonate, although the errors involved in the low rates observed make such a test of significance insensitive. There was no coherent relationship between propionate metabolized and total succinate oxidized when both substrates were present.

DISCUSSION

It has been shown that metabolism of propionate by sheep-liver mitochondria occurs solely by the methylmalonate pathway, with oxidation of the succinate formed by way of pyruvate and the tricarboxylic acid cycle (Smith *et al.* 1967). Substantial evidence has also been obtained that the rate of propionate metabolism is limited by the rate of supply of ATP by oxidative phosphorylation within the mitochondria. This rate may be increased in two ways: by the addition of catalytic concentrations (0.5mM) of ATP to the medium, or by the addition of catalytic concentrations (0.167mM) of certain members of the tricarboxylic acid cycle (Smith & Russell, 1967). The results in Fig. 1 show that these two effects were independent.

The catalytic effects of the organic acids can probably be attributed to the effects of pyruvate and α -oxoglutarate, since citrate and isocitrate could form α -oxoglutarate within the mitochondria. The effects of α -oxoglutarate and particularly of pyruvate on propionate consumption are greater than can reasonably be attributed to energy supplied by their own oxidation (Smith & Russell, 1967), and it must be concluded that they increase catalytically the rate of supply of ATP from the oxidation of propionate itself. Oxidation of propionate, however, consists in oxidation of succinate via pyruvate, and pyruvate cannot be supposed to have a catalytic effect either on its own rate of oxidation or on the efficiency of oxidative phosphorylation linked to its own oxidation. It therefore appears that in the oxidation of propionate there is a rate-limiting step between succinate and pyruvate that controls the rate of supply of ATP, limiting propionate consumption, and that is

affected catalytically by α -oxoglutarate or pyruvate. The results discussed below suggest that this step is the oxidation of succinate to fumarate.

Table 1 shows that increasing concentrations of malonate increasingly inhibited fixation of [2- 14 C]-propionate, consistent with an inhibition of succinate oxidase. In the absence of malonate, and at all concentrations of malonate tested, 1.67mM- α -oxoglutarate increased the fixation of [2- 14 C]propionate by about 50%, consistent with its having increased a limiting activity of succinate oxidase by this amount. In the absence of malonate the effect of α -oxoglutarate was to increase the conversion of [2- 14 C]propionate into [14 C]malate, also consistent with an enhanced activity of succinate oxidase. If the step α -oxoglutarate $\rightarrow$ succinate had contributed materially to the energy requirements of the rate-limiting step of propionate consumption, then this increment should not have been inhibited by malonate, unless malonate also depressed the efficiency of energy transfer. The latter possibility is not borne out by the results of Table 5, where malonate did not decrease the ratio propionate consumed/succinate oxidized. The inference that oxidation of α -oxoglutarate to succinate did not contribute energy for the rate-limiting step of propionate consumption is supported by the results for production of succinate from α -oxoglutarate and propionate. Metabolism of propionate was accompanied by oxidation of about the same fraction of generated succinate whether α -oxoglutarate was present or not, whereas no relationship was apparent between propionate consumed and α -oxoglutarate converted into succinate. Considered together these results suggest that the rate of propionate consumption was specifically determined by the rate of oxidation of succinate even when an alternative source of energy appeared to be available.

The experiments summarized in Tables 4 and 5 were designed to discover whether there was a quantitative relationship between the rate of oxidation of succinate and the rate of consumption of propionate. The results show, however, that a distinction must first be made between the oxidation of added and generated succinate. The oxidation of added succinate did not appear to have any effect on the metabolism of propionate, although the presence of relatively high concentrations of succinate stimulated propionate consumption, and limited exchange of label occurred between added and generated succinate. It is possible that the stimulatory effect of succinate was due to formation of succinyl-CoA, as was suggested for the effect of α -oxoglutarate (Smith & Russell, 1967).

Oxidation of propionate is accompanied by accumulation of part of the generated succinate. The accumulating fraction is presumably available for oxidation by the 'external' succinate oxidase

responsible for oxidation of added succinate. When this oxidation was limited either by addition of malonate or by the presence of a succinate pool intended to saturate the 'external' succinate oxidase, then the ratios propionate consumed/succinate oxidized and propionate consumed/'succinate increment' both achieved maximum values of about 2mol. of propionate consumed/mol. of generated succinate oxidized.

The extent to which the generated succinate must be oxidized to supply the total energy for propionate consumption (3 terminal phosphate bonds of ATP/mol. of propionate) is not known, but the complete failure of added fumarate or malate to stimulate propionate consumption under conditions of limiting energy supply suggests that the oxidation of succinate to fumarate controls a rate-limiting step. It was shown that oxidation of added succinate proceeded at least as far as citrate, and, unless a malate dehydrogenase unavailable either to external malate or to malate generated from added succinate was present, then oxidation of malate cannot have contributed energy to the rate-limiting step of propionate consumption.

Oxidation to fumarate of half the succinate generated from propionate would supply, at a P/O ratio 2, sufficient ATP for the carboxylation of propionyl-CoA by propionyl-CoA carboxylase (EC 6.4.1.3), but not for the activation of propionate by acetate-CoA ligase (AMP) (EC 6.2.1.1), and certainly not for both. The possibility therefore exists that only the carboxylation of propionyl-CoA is rate-limiting by reason of restricted energy supply, and that activation of propionate consumes energy from less specific sources. This is in agreement with the accumulation chiefly of [^{14}C]malate as a result of stimulation of [$2\text{-}^{14}\text{C}$]propionate metabolism with α -oxoglutarate, and is suggested by the work of Yates & Garland (1966) with carnitine palmitoyl-transferase in rat-liver mitochondria. Palmitoyl-CoA produced on one side of a mitochondrial membrane was said to be oxidized on the other side, penetration of the membrane by the palmitoyl group depending on the prior formation of palmitoyl-carnitine.

The stabilizing effect of ATP on propionate metabolism appears to be associated in part with an effect on mitochondrial structure (Smith & Russell, 1967), and the resistance of propionate metabolism to inhibition by malonate, only in the presence of ATP, suggests the erection by ATP of permeability barriers to the approach of malonate to the succinate oxidase concerned with its oxidation. It is possible that this succinate oxidase occupies an inner mitochondrial compartment and that it operates only when a suitable energy-acceptor system (in this case propionyl-CoA) is present. In such a case there need be no essential difference between added and

generated succinate. Added succinate would not be oxidized in the absence of an acceptor system, and when propionyl-CoA was present succinate would be generated in excess. This would be consistent with the observed exchange of label between added and generated succinate.

The effects of a number of the stimulants and inhibitors of propionate metabolism studied may be explained in terms of their effects on the activity of succinate oxidase. Mitochondrial succinate oxidase is inhibited by oxaloacetate generated within the mitochondria (Williams, 1961; Chance & Hagihara, 1962), but added oxaloacetate does not inhibit unless mitochondrial permeability is increased or oxidative phosphorylation uncoupled (Jones & Gutfreund, 1963). Added oxaloacetate has been found to inhibit propionate metabolism in aged mitochondria (Smith *et al.* 1965b).

The effects of stimulants of mitochondrial succinate oxidase are frequently attributed to the removal of internally generated oxaloacetate, but only a few such stimulants (glutamate, cysteine-sulphinate) may be shown to bring about a measurable depletion of mitochondrial oxaloacetate (Schollmeyer & Klingenberg, 1961). Labelled oxaloacetate has been found to accumulate to some extent during the metabolism of [$2\text{-}^{14}\text{C}$]propionate (Smith *et al.* 1967), and the stimulatory effect of pyruvate may be due in part to the removal of oxaloacetate to form citrate. R. M. Smith & W. S. Osborne-White (unpublished work) have shown that pyruvate and α -oxoglutarate, in stimulating the metabolism of [$2\text{-}^{14}\text{C}$]propionate, gave rise to similar distributions of labelled products. In both cases the major product formed as a result of the stimulation was [^{14}C]malate. Pyruvate differed from α -oxoglutarate, however, in that it also substantially increased the formation of labelled citrate.

The stimulation and stabilization of propionate metabolism by ATP is probably complex, and associated in part with the maintenance of mitochondrial structure. Added ATP, however, is well known to increase the activity of mitochondrial succinate oxidase (Tyler, 1955; Schollmeyer & Klingenberg, 1961), although it is not clear that oxaloacetate is involved (Slater & Hülsmann, 1961; Azzone & Ernster, 1961c; Gregolin & Scaella, 1965). The effect of added ATP on propionate metabolism was clearly distinct from that of α -oxoglutarate or pyruvate, but it did increase slightly the rate of oxidation of added succinate (Table 4). The effect of added ATP is therefore consistent with an effect on succinate oxidase, but it may have had other functions as well.

Stimulation of mitochondrial succinate oxidase by α -oxoglutarate has been observed (Klingenberg & Schollmeyer, 1961) and was attributed to the

generation of ATP from the substrate-level phosphorylation accompanying oxidation of α -oxoglutarate. The inhibition of propionate metabolism by AMP and its partial reversal by α -oxoglutarate, as well as the catalytic effect of α -oxoglutarate itself (Smith & Russell, 1967), are consistent with Azzone & Ernster's (1961c) hypothesis relating the maintenance of mitochondrial succinate-oxidase activity to a particular pool of mitochondrial ATP. This ATP pool was shown to be sustained by the substrate-level phosphorylation accompanying oxidation of α -oxoglutarate. It was not depleted by external ADP, but was accessible for depletion by external AMP (Azzone & Ernster, 1961a,b).

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