Metabolism of Propionate by Sheep-Liver Mitochondria

EFFECTS OF α -OXOGLUTARATE, ADENOSINE TRIPHOSPHATE, SODIUM CHLORIDE AND POTASSIUM CHLORIDE

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1. A study has been made of the effects of ATP and α -oxoglutarate on the rate of metabolism of propionate by whole mitochondria from sheep liver, and by mitochondria disrupted with ultrasonic energy or by freezing and thawing. Whole mitochondria metabolized propionate aerobically; the rate was increased and stabilized by 0.5 mM-ATP, and increased at least a further 50% by 1.67 mM- α -oxoglutarate. 2. Anaerobically, externally added ATP at high concentrations permitted slow consumption of propionate. 3. In the presence of 1.3 mM-ATP, but in the absence of α -oxoglutarate, there was no significant lag phase in the removal of propionate by whole mitochondria, and the rate declined at concentrations below 2 mM. In the additional presence of 1.67 mM- α -oxoglutarate or -glutamate, propionate was removed at linear rates until the residual propionate concentration was about 0.1 mM. 4. Maximum rates of metabolism of propionate by whole mitochondria with 1.3 mM-ATP occurred with alkali-metal chloride concentrations of 65–95 mM and with K^+/Na^+ ratios 5–10, both in the presence and absence of α -oxoglutarate. 5. With disrupted mitochondria stimulatory effects of α -oxoglutarate were obtained only aerobically, only with propionate and not propionyl-CoA as substrate, and only when sufficient mitochondrial structure remained to permit unsupplemented metabolism of propionate to occur. 6. In the presence of ATP and CoA, disrupted mitochondria fixed $[2-^{14}C]$ propionate at a rate adequate to explain the rate with whole mitochondria stimulated with ATP and α -oxoglutarate. 7. With both whole and partially disrupted mitochondria in the absence of ATP, the rate of metabolism of propionate was inhibited by about 80% by 3.3 mM-AMP. The inhibition was partly overcome by α -oxoglutarate plus CoA. 8. It is concluded that the ultimate effect of α -oxoglutarate was to increase the rate of supply of ATP within the mitochondria. Reasons are given why it is premature to conclude that the extra ATP arose entirely from the oxidation of α -oxoglutarate itself.

Isolated sheep-liver mitochondria have been shown to metabolize propionate more slowly than the nuclear-free homogenates from which they were derived, and to be more susceptible to loss of activity on aging at 37° without added substrate (Smith & Osborne-White, 1965). This effect was shown (Smith, Osborne-White & Russell, 1965a,b) to be mainly due to the L-glutamate present in the cell sap, which at low concentrations not only stimulated the rate of propionate metabolism in unaged mitochondria, but was also capable both of protecting the mitochondria against loss of activity on aging without propionate by its presence during aging and of restoring much of the lost activity when added after aging. An additional protective

effect was conferred by a protein factor from the cell sap that, however, was not necessary for glutamate to act.

The stimulatory effect of low concentrations (1 mM) of glutamate appeared to be exerted during its oxidation via α -oxoglutarate to aspartate. Glutamate could be replaced by α -oxoglutarate, pyruvate, citrate or isocitrate at low concentrations (1 mM), but not by fumarate, malate, oxaloacetate or aspartate. Succinate was stimulatory at higher concentrations, whereas oxaloacetate was inhibitory.

These effects could be explained in several ways (Smith *et al.* 1965b), and the present work was undertaken to define more closely the nature of the

effect of α -oxoglutarate on mitochondrial propionate metabolism. α -Oxoglutarate was chosen because of the absence of a catalytic effect of succinate, and the consequent indication that the effect was exerted either directly by α -oxoglutarate, or else during its oxidation to succinate. With the possible exception of pyruvate, the effects of the other stimulants could be attributed to their conversion into α -oxoglutarate by the mitochondria.

The effects of sodium chloride and potassium chloride and the K^+/Na^+ ratio were examined chiefly because of the finding by Leng & Annison (1963) that sheep-liver slices metabolized propionate more rapidly in a high- Na^+ medium than in a high- K^+ medium. The present results show that propionate metabolism by sheep-liver mitochondria proceeds faster in a high- K^+ medium, and that there is an optimum range of the K^+/Na^+ ratio. This work has been included here because it seems to bear directly on the question of the mode of action of α -oxoglutarate.

The results obtained indicate that the increased rate of propionate metabolism caused by α -oxoglutarate is due to an increased rate of supply of ATP within the mitochondria. The stoichiometry of the effect suggests, however, that α -oxoglutarate may not be supplying this ATP directly by its own oxidation, but rather by stimulating the rate of supply of ATP from the oxidation of propionate itself.

MATERIALS AND METHODS

Materials. Unless otherwise stated materials were as described by Smith, Osborne-White & Russell (1965b, 1967). CoA, ADP and AMP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. $[2-^{14}C]$ Propionyl-CoA was prepared from $[2-^{14}C]$ propionic anhydride by the method of Simon & Shemin (1953), and its concentration determined by the hydroxamic acid method of Lipmann & Tuttle (1945). $[2-^{14}C]$ Propionic anhydride was prepared from sodium $[2-^{14}C]$ propionate by reaction with $POCl_3$. A Pyrex test tube (160 mm. $\times$ 14 mm. diam.) was fitted with a side arm normal to the tube and 60 mm. from the bottom. A solution of 76 μ moles of sodium $[2-^{14}C]$ propionate was evaporated to dryness in the bottom of the tube and further dried by fusion at 280° for 30 min. The tube was cooled while being flushed with dry N_2 , the neck constricted in a flame and the tube stored overnight over P_2O_5 . With the entire tube supported in an atmosphere of dry N_2 , 10 μ moles (1.75 μ l.) of redistilled $POCl_3$ was introduced from a dry micro-pipette, and the tube rapidly sealed with a flame and immersed in an oil bath at 140° for 90 min. $[2-^{14}C]$ Propionic anhydride was collected in the side arm by submerging the tip of this in liquid N_2 for 1 hr. while warming the remainder of the tube with an infrared lamp. The yield of anhydride was about 60% and of $[2-^{14}C]$ propionyl-CoA 21% by the method of Lipmann & Tuttle (1945).

Mitochondria were prepared from the livers of freshly killed Australian Merino ewes, 2–5 years of age, as described

by Smith *et al.* (1965a). They were washed twice and finally suspended in 0.25 M-sucrose.

Mitochondria in 0.25 M-sucrose (about 0.8 mg. of N/ml.) were disrupted by either of two procedures. Disintegration with ultrasonic energy was performed with a Dawe type 1130A Soniprobe (Dawe Instruments Ltd., London, W.3) operated at 60 w capacity for 1 min. at 20 kcyc./sec. Samples (5 ml.) were treated in test tubes (14 mm. diam.) held in an ice-water bath. Disruption by freezing and thawing consisted of freezing 2–10 ml. samples in polystyrene vials by placing in a domestic freezer at -15° to -20° for periods varying from 2 to 14 days, and thawing by shaking under running tap water. The nitrogen content of mitochondrial preparations was determined by the Kjeldahl procedure of McKenzie & Wallace (1954).

Medium. The standard medium in which whole or disrupted mitochondria were incubated comprised (final concentrations): K_2HPO_4 (16.4 mm), KH_2PO_4 (3.6 mm), $NaHCO_3$ (25.0 mm), KCl (65–90 mm, except for Table 3), $MgCl_2$ (5.0 mm), sucrose (42 or 83 mm, indicated in legends), cytochrome *c* (11 μ M), sodium propionate or potassium propionate (5.0–10.0 mm, indicated in legends), and whole or disrupted mitochondria as described, in equilibrium at 37° with $O_2 + CO_2$ (95:5) or $N_2 + CO_2$ (95:5) at pH 7.4. Further additions to the medium, incubation volumes and incubation times are shown in the legends to Tables and Figures.

Incubations. Incubations in 6 ml. volumes were performed as described by Smith *et al.* (1965a) in racks of 25 tubes with side arms, and with constant gassing. In one experiment (Table 3) two racks of similar design but each carrying 50 tubes were used, other conditions being the same. Reactions were stopped by shaking rapidly for 1 min. in an ice bath, followed by the immediate addition of 1 ml. of 10% (w/v) $Na_2WO_4 \cdot 2H_2O$ and 1 ml. of 0.66 N- H_2SO_4 . Water was added to a volume of 20 ml., protein sedimented by centrifuging and the supernatant stored at -20° in glass stoppered bottles.

Incubations in 0.3 ml. volumes were performed in tubes of about 25 ml. capacity without side arms and with conical tips, with gassing as above at 200 ml./min. Mitochondria (0.1 ml.) were added last, through the gassing vent, to the reaction medium saturated with gas mixture at 0°. Tubes were then stoppered, transferred rapidly to the incubation bath at 37° and gassing was restarted. This procedure led to about 0.5–1 min. time lag in reaching temperature equilibrium.

Reactions were stopped at selected time-intervals by adding the appropriate quantity of NaOH solution through the gassing vent and then discontinuing the gas supply. When large numbers of reactions were stopped together the rack was transferred to an ice-water bath and chilled for 1 min. before adding NaOH solution.

In all experiments great care was taken to ensure gas saturation of the medium before and after addition of mitochondria, and to maintain rapid shaking rates (120–140 cyc./min.) of tubes during incubation.

Rate measurements. Measurements were made either of the rate of consumption of propionate or of the rate of conversion of $[2-^{14}C]$ propionate or $[2-^{14}C]$ propionyl-CoA into radioactive non-volatile products.

The rates of consumption of propionate shown in Tables 1, 2 and 3 and in Fig. 1 were determined by titration of residual steam-volatile acid as described by Smith & Osborne-White

(1965). In these experiments 6 ml. reaction volumes were used and protein was precipitated with tungstic acid. Propionate consumption in the experiment referred to in Fig. 2 was determined by the disappearance of steam-volatile radioactivity on incubating [$2\text{-}^{14}\text{C}$]propionate with mitochondria in 0.3 ml. final volumes. Reactions were stopped by adding 1 ml. of N-NaOH containing 5 μmoles of sodium propionate as carrier. Entire tube contents were acidified and volatile acid was distilled as above, 100 ml. of distillate being collected. Replicate samples (0.5 ml.) of distillate were measured by scintillation counting as described by Smith *et al.* (1967). Specific activity was determined in a similar way on samples of original substrate (from which carrier was omitted), by titration of part (50 ml.) of the distillate. Consumption of ^{14}C , and hence, by application of the specific activity, consumption of propionate, was determined by difference from initial samples that were not incubated.

In the experiments with whole and disrupted mitochondria referred to in Tables 4, 5 and 6, the rate of formation of non-volatile radioactive products from [$2\text{-}^{14}\text{C}$]propionate or [$2\text{-}^{14}\text{C}$]propionyl-CoA was measured. Incubations were carried out in tared tubes in 0.3 ml. final volumes, and after addition of NaOH the tubes were kept for 1 hr. at room temperature to hydrolyse CoA thio esters. After the addition of 0.1 ml. of 6N-HCl and 4.5 ml. of water, tubes were heated for 5 min. in a boiling-water bath, cooled and centrifuged to sediment protein. Tube contents were determined by weight, and 0.1 ml. samples used to measure ^{14}C fixed after removal of residual [$2\text{-}^{14}\text{C}$]propionic acid by repeated evaporation with unlabelled propionic acid. The samples for measurement were placed in small (10 ml.) flat-bottomed glass vials, 1 drop of 5N-HCl and 0.1 ml. of 10% (v/v) propionic acid added and the tubes evaporated just to dryness *in vacuo* over CaCl_2 and NaOH . The residue was immediately dissolved in a further 0.1 ml. of 10% (v/v) propionic acid and the evaporation repeated. The latter step was repeated a further three times, although it was shown that three such evaporations sufficed to remove the [$2\text{-}^{14}\text{C}$]propionic acid present. The residue was finally dissolved in 0.1 ml. of water, 5 ml. of Bray's (1960) liquid-scintillator solution was added and ^{14}C was measured by scintillation counting as described by Smith *et al.* (1967). Counting efficiency was 75% as determined with standard [$1,4\text{-}^{14}\text{C}_2$]succinic acid. The preparation and measurement of up to 50 samples was performed in a single day, since it was found that leaving the dried samples overnight in the vacuum desiccator led to blackening and some loss (about 10%) of radioactivity. When performed as described replicate measurements from the same sample were reproducible to within 2–3%, and replicate incubations to within about 5%. Blank values were determined from control samples to which NaOH was added before incubation. These were negligible with [$2\text{-}^{14}\text{C}$]propionate, but measurable non-volatile ^{14}C was found with [$2\text{-}^{14}\text{C}$]propionyl-CoA. This has been subtracted from the values quoted.

RESULTS

Effect of α -oxoglutarate on propionate metabolism by mitochondria with and without aging. The results of a number of experiments where α -oxoglutarate was used to stimulate mitochondrial propionate metabolism are shown in Table 1, which summarizes

Table 1. *Effects of α -oxoglutarate on the rate of metabolism of propionate by mitochondria with and without aging*

Mitochondria were aged for the stated time at 37° in standard medium containing ATP (1.3 mM). Substrate was then added and the incubation continued in 6 ml. final volumes for 36 min. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5). Conditions of incubation and the estimation of propionate consumption are described in the Materials and Methods section. Individual values for volatile acid consumption are the means of duplicate estimates of duplicate incubations. Results show the means $\pm$ s.e.m. for the numbers of different mitochondrial preparations indicated. Flasks contained mitochondria (0.88–2.13 mg. of N) derived from 0.17–0.50 g. of liver.

Substrate	Rate of consumption of propionate ($\mu\text{moles/mg. of N/hr.}$)	
	Substrate added at 2 min.	Substrate added at 19 or 20 min.
Propionate (5 mM)	10.5 $\pm$ 1.0 (10)	6.6 $\pm$ 0.9 (14)
Propionate (5 mM) + α -oxoglutarate (1.67 mM)	17.5 $\pm$ 0.7 (8)	17.2 $\pm$ 1.9 (4)

the effects with and without aging of the mitochondria. In all cases the reaction mixture contained ATP (1.3 mM).

The results show that with fresh mitochondria stable rates of propionate metabolism occurred in the presence of ATP (1.3 mM) and that a higher rate, also stable, resulted from the additional presence of 1.67 mM- α -oxoglutarate. When the mitochondria were aged for 19 min. (in the presence of ATP) a loss of activity occurred. If α -oxoglutarate (1.67 mM) was added with the propionate after aging, the rate of propionate metabolism was largely restored to the rate obtained in the presence of α -oxoglutarate without aging.

Catalytic effect of α -oxoglutarate and pyruvate on propionate metabolism in the presence and absence of ATP. Table 2 shows the results of two experiments where the effects on propionate consumption of the addition of α -oxoglutarate or pyruvate (each 0.167 mM) were measured in the presence and absence of ATP. For comparison concentrations of 1.0 mM or 1.67 mM were also included, concentrations of about 1 mM having previously been shown to achieve maximum stimulatory effect with aged mitochondria (Smith *et al.* 1965b).

In the presence of ATP up to 5.5 μmoles of additional propionate was consumed as a result of the presence of 1 μmole of α -oxoglutarate, and up to 9.1 μmoles of additional propionate with 1 μmole of pyruvate. In the absence of ATP the effects were slightly greater.

At concentrations of 1.67 mM the effects of pyru-

Table 2. *Effects of low concentrations of α -oxoglutarate and pyruvate on mitochondrial propionate metabolism*

Mitochondria (1.41 and 1.53 mg. of N in Expts. 1 and 2 respectively) were incubated at 37° in standard medium with and without ATP (1.3 mM) for 2 min. before adding propionate (5.0 mM) and the ancillary substrates shown. Incubations were continued in 6 ml. final volumes for 36 min. The gas phase was O₂ + CO₂ (95:5). Reactions were stopped and propionate consumption was determined as described in the Materials and Methods section. Values shown are the means of duplicate estimates on duplicate incubations.

Additions to standard medium	Expt. no.	Propionate consumed in 36 min. (μ moles)	
		No ATP	1.3 mM-ATP
None	1	3.4	7.5
	2	1.2	6.1
α -Oxoglutarate (0.167 mM)	1	9.2	13.0
	2	7.0	11.6
α -Oxoglutarate (1.67 mM)	1	8.1	11.3
α -Oxoglutarate (1.0 mM)	2	9.5	14.0
Pyruvate (0.167 mM)	1	13.4	16.6
	2	8.3	11.9
Pyruvate (1.67 mM)	1	12.6	15.9
Pyruvate (1.0 mM)	2	15.3	19.4
α -Oxoglutarate (1.67 mM) + pyruvate (1.67 mM)	1	7.4	11.1

vate and α -oxoglutarate were not additive and the rate was less than that with pyruvate alone.

Effect of ATP on propionate metabolism by whole mitochondria. Fig. 1 shows the effects of increasing concentrations of ATP on the rate of propionate metabolism by mitochondria under aerobic and anaerobic conditions. With oxygen present a maximum rate was obtained with 0.5 mM-ATP, higher concentrations having no further effect. In the absence of oxygen, little or no effect was obtained below 1.5 mM-ATP, but higher concentrations led to some consumption of propionate.

Time-course of propionate removal in the presence and absence of α -oxoglutarate and glutamate. Smith *et al.* (1965b) suggested that the pronounced stimulation by catalytic concentrations (0.167 mM) of α -oxoglutarate on the rate of metabolism of propionate by sheep-liver mitochondria may have been a 'sparking' effect, α -oxoglutarate providing the ATP to initiate propionate metabolism, which then became self-sustaining as the concentration of oxidizable intermediates increased. Such a mechanism might be expected to lead to increasing rates of consumption of propionate with time in the absence of α -oxoglutarate. Fig. 2 indicates that this was not the case. Apart from a slight (0.5 min.) lag in all flasks brought about by starting the reaction at 0°, the rates in the presence and absence

of ancillary substrate (α -oxoglutarate or glutamate) were linear, and in the later stages of incubation the rate in the absence of α -oxoglutarate or glutamate declined rather than increased. When either α -oxoglutarate or glutamate was present, propionate continued to be removed at linear rates until 97–98% of the propionate had been consumed, leaving a residual concentration of about 0.1 mM. In the absence of ancillary substrate the rate declined at substrate concentrations below about 2 mM. These effects were measured in the presence of 1.3 mM-ATP.

Effects of sodium chloride and potassium chloride with and without α -oxoglutarate. Leng & Annison (1963) found that both the rate of consumption of [¹⁴C]propionate and the rate of incorporation of label into glucose by sheep-liver slices were sensitive to the K⁺/Na⁺ ratio of the medium, maximum rates occurring in media with high Na⁺ concentrations. The metabolism of propionate, however, is dependent on oxidative phosphorylation (Smith & Osborne-White, 1965), which in turn has been

Fig. 1. Effect of added ATP on the rate of consumption of propionate by mitochondria in aerobic and anaerobic systems. Mitochondria (1.37 mg. of N) from 0.33 g. of liver were incubated with propionate (10 mM) in 6 ml. final volumes of standard medium containing KCl (85 mM) and sucrose (42 mM) for 36 min. at 37°. Substrate was added 2 min. after placing in the bath. The gas phase was O₂ + CO₂ (95:5) (aerobic) or N₂ + CO₂ (95:5) (anaerobic). Final concentrations of added ATP were 0, 0.1, 0.5, 1.0, 1.5, 3.0, 5.0 and 10.0 mM, propionate consumption being determined as described in the Materials and Methods section, from initial values determined at the time of addition of substrate. Results are the means of duplicate estimations on duplicate incubations, the Figure showing propionate consumed under both aerobic (●) and anaerobic (○) conditions.

Fig. 2. Time-course of removal of [2-¹⁴C]propionate by whole mitochondria in the presence and absence of α -oxoglutarate or glutamate. Mitochondria (0.21 mg. of N) from 25 mg. of liver were incubated for 5, 10, 20 or 40 min. in final volumes of 0.3 ml. of standard medium with KCl (67 mM), sucrose (83 mM) and ATP (1.3 mM). Initial amount of [2-¹⁴C]propionate was 1.5 μ moles (0.33 μ C/ μ mole), and propionate consumption was determined from residual steam-volatile ¹⁴C as described in the Materials and Methods section. Glutamate and α -oxoglutarate concentrations were 1.67 mM. The gas phase was O₂ + CO₂ (95:5) and the temperature was 37°. Reactions were started by adding mitochondria (0.1 ml.) at 0° to the pregassed medium at 0° and then transferring rapidly to the bath. Zero time was taken as the time of entry into the bath with the result that about a 0.5 min. lag has occurred in the consumption of propionate in all flasks. Each point on the graph is the mean of four replicate estimates of ¹⁴C on each of two replicate incubations. Results shown are: propionate alone (▲); propionate + α -oxoglutarate (●); propionate + glutamate (○).

shown in some cases to be dependent on K⁺ (Blond & Whittam, 1965). Since it was suspected that the effect of α -oxoglutarate might lie in its capacity to act as substrate for oxidative phosphorylation, the effects of both the K⁺/Na⁺ ratio and the total alkali-metal chloride concentration on the rate of metabolism of propionate with and without α -oxoglutarate (1.67 mM) were tested. All flasks contained ATP (1.3 mM). The results are given in Table 3, where two effects are apparent.

First, a general salt effect occurred in which the rate rose with increasing added alkali-metal chloride, reaching a maximum between 55 and 85 mM and declining slightly at 105 mM. This appeared to be an effect of the osmolality of the medium, as it was observed both with the all-Na⁺ and the all-K⁺ media. Secondly, a marked effect of the K⁺/Na⁺ ratio was evident, maximum rates both with and without α -oxoglutarate occurring with K⁺/Na⁺ ratios 5–10.

Combination of these two ranges produced the maximum observed rates of propionate metabolism both with and without α -oxoglutarate. Under ionic conditions where the rate of metabolism of propionate alone was near the maximum (12.0 ± 2.0 μ moles consumed) the mean stimulation by α -oxoglutarate was 55% [$55 \pm 5\%$ (S.E.M.), 11 observations], but the stimulation increased rapidly with lower rates of unsupplemented propionate metabolism. Thus the rate of metabolism of propionate was less susceptible to variation of the K⁺/Na⁺ ratio and to the total alkali-metal chloride concentration when

Table 3. *Effect of sodium chloride and potassium chloride on the rate of metabolism of propionate and the response to α -oxoglutarate*

Mitochondria (1.88 mg. of N) derived from 0.5 g. of liver were incubated for 36 min. at 37° after the addition of propionate after a 2 min. equilibration period in the bath. The gas phase was O₂ + CO₂ (95:5). The media were based on the standard medium but with the concentrations of alkali-metal chlorides and the K⁺/Na⁺ ratios shown. The various K⁺/Na⁺ ratios were achieved by mixing appropriate quantities of all-Na⁺ and all-K⁺ media. These media contained (final concentrations): NaHCO₃ or KHCO₃ (25 mM), sodium phosphate or potassium phosphate buffer, pH 7.4 (20 mM), sodium propionate or potassium propionate (10 mM), sodium ATP or potassium ATP (1.3 mM), MgCl₂ (5 mM), cytochrome *c* (11 μ M), sucrose (42 mM) and the concentrations of added NaCl or KCl shown. Media containing no added alkali-metal chloride thus contained 10 mM-Cl⁻ added as MgCl₂ and about 70 mM-alkali-metal ion added as phosphate, bicarbonate, propionate and neutralized ATP. The standard medium contains 85 mM-alkali-metal chloride with a K⁺/Na⁺ ratio 5. Consumption of propionate was determined as described in the Materials and Methods section from residual volatile acid, from initial values determined at the time of addition of substrate. The results are the means of duplicate or triplicate analyses on single incubations.

Added alkali-metal chloride (mM)	Propionate consumed (μ moles)											
			No α -oxoglutarate					1.67 mM- α -oxoglutarate				
	Total Na ⁺ + K ⁺	Total Cl ⁻										
	(mM)	(mM)	K ⁺ /Na ⁺ <0.01	K ⁺ /Na ⁺ 1	K ⁺ /Na ⁺ 5	K ⁺ /Na ⁺ 10	K ⁺ /Na ⁺ >100	K ⁺ /Na ⁺ <0.01	K ⁺ /Na ⁺ 1	K ⁺ /Na ⁺ 5	K ⁺ /Na ⁺ 10	K ⁺ /Na ⁺ >100
0	70	10	0.0	3.8	4.3	3.6	3.3	4.1	11.7	12.7	10.7	11.1
30	100	40	0.7	7.9	10.1	11.3	9.5	6.6	14.8	15.8	15.5	14.8
55	125	65	2.1	11.1	12.4	13.2	10.2	7.6	17.3	17.6	16.5	15.6
85	155	95	2.1	10.4	11.1	11.4	10.1	9.8	18.0	18.6	18.8	16.9
105	175	115	1.6	9.4	—	11.9	7.5	8.8	17.0	19.4	18.7	16.9

Table 4. *Effect of disruption of mitochondria with ultrasonic energy on metabolism of propionate and its inhibition by AMP*

Mitochondria in 0.25M-sucrose were disrupted by treatment for 1 min. at 0° with an ultrasonic oscillator operating at 20kcyc./sec. Incubations were for 10 min. at 37° in standard medium containing KCl (87mM) and sucrose (83mM), with the additions indicated. Final concentrations of the additions were: AMP (3.3mM), ATP (3.3mM), CoA (1.0mM), α -oxoglutarate (1.67mM) and [2-¹⁴C]propionate (6.7mM, 0.5 μ C/ μ mole). The gas phase was O₂+CO₂ (95:5), and whole mitochondria or mitochondria exposed to ultrasonic energy with 0.079mg. of N were used in 0.3ml. final volumes. Reactions were stopped with 0.1 ml. of 5N-NaOH and fixation products determined as described in the text. Results are the means of duplicate incubations.

Addition to standard medium	Whole mitochondria		Mitochondria exposed to ultrasonic energy	
	Rate of fixation of [2- ¹⁴ C]propionate in the absence of AMP (μ C/mg. of N/hr.)	Inhibition by 3.3mM-AMP (%)	Rate of fixation of [2- ¹⁴ C]propionate in the absence of AMP (μ C/mg. of N/hr.)	Inhibition by 3.3mM-AMP (%)
None	4.6	85	1.9	76
CoA	5.5	79	2.4	72
α -Oxoglutarate	9.5	50	4.9	50
α -Oxoglutarate + CoA	10.9	33	6.2	37
ATP	7.3	—	2.4	—
ATP + α -oxoglutarate	9.3	—	3.5	—
ATP + CoA	6.0	—	5.8	—
ATP + CoA + α -oxoglutarate	10.2	—	7.5	—

α -oxoglutarate was present than when it was absent.

A stimulation of about 50% of propionate metabolism by mitochondria (with 1.3mM-ATP) incubated in the presence of α -oxoglutarate or glutamate (each 1.67mM) has been frequently observed. This is a minimum value, applying to mitochondria that have not been aged. Thus in eight observations of mitochondria aged without added substrate for 19 min. at 37° (in the standard medium with 1.3mM-ATP), glutamate or α -oxoglutarate stimulated propionate metabolism by an average of 114% (range 50–270%). When the mitochondria were not aged (substrate added after 2 min. at 37°) the mean stimulation by glutamate or α -oxoglutarate in eight observations was 63% (range 40–120%; six of the eight values lay between 40% and 60%). The higher stimulation with aged mitochondria reflects the lowered rate of metabolism of propionate alone under these conditions, and its restoration to near maximum rates by α -oxoglutarate or glutamate.

Studies with disrupted mitochondria. Preliminary work showed that 1 min. exposure to ultrasonic energy of fresh mitochondria suspended in 0.25M-sucrose at 0° diminished, but did not destroy, the capacity of the preparation to metabolize propionate alone or to respond to α -oxoglutarate or ATP. The results of one such experiment are shown in Table 4, where the accumulation of non-volatile radioactive products from the metabolism of [2-¹⁴C]propionate has been measured.

With whole mitochondria the predominant stimulatory effect was that of α -oxoglutarate (1.67mM), although ATP (3.3mM) was also effective. There was little effect of CoA (1.0mM) in the presence or absence of the other factors. With mitochondria exposed to ultrasonic energy some capacity for unsupplemented propionate metabolism remained, and a marked stimulation by α -oxoglutarate occurred. Although neither ATP nor CoA had much effect alone, the combination increased fixation more effectively than did α -oxoglutarate, and when α -oxoglutarate was present in addition a maximum rate was obtained. This rate was lower than that with whole mitochondria stimulated to maximum rate with ATP plus α -oxoglutarate.

Also shown in Table 4 is the effect of AMP (3.3mM) on propionate metabolism with and without α -oxoglutarate (1.67mM) and CoA (1.0mM). AMP was a potent inhibitor of propionate metabolism: the inhibition was partially overcome by α -oxoglutarate, and more effectively by α -oxoglutarate plus CoA. CoA alone had no effect. The effects of AMP were equivalent in both whole mitochondria and mitochondria exposed to ultrasonic energy.

A more extensive experiment with whole mitochondria, mitochondria exposed to ultrasonic energy and frozen-and-thawed mitochondria is reported in Tables 5 and 6. For clarity of presentation some of the data in Table 5 have been repeated in Table 6.

Table 5. *Fixation of [2-¹⁴C]propionate or [2-¹⁴C]propionyl-CoA by mitochondria exposed to ultrasonic energy in aerobic and anaerobic systems*

Mitochondria in 0.25 M-sucrose were disrupted by treatment for 1 min. at 0° with an ultrasonic oscillator operating at 20 kc/sec. Disrupted mitochondria (0.077 mg. of N) were incubated in final volumes of 0.3 ml. for 10 min. at 37° in standard medium containing KCl (67 mM) and sucrose (83 mM). The gas phase was either O₂ + CO₂ (95:5) (aerobic) or N₂ + CO₂ (95:5) (anaerobic). The final concentrations of the additions to the medium were: α -oxoglutarate (1.67 mM), CoA (1.0 mM), ADP (3.3 mM), ATP (3.3 mM), [2-¹⁴C]propionate (6.67 mM, 0.5 μ C/ μ mole) and [2-¹⁴C]propionyl-CoA (1.64 mM, 0.5 μ C/ μ mole). Reactions were begun and terminated and fixation products estimated as described in the Materials and Methods section. The results with [2-¹⁴C]propionyl-CoA have been corrected for a non-volatile radioactive residue detected in control flasks that were not incubated, and equivalent to 1.4 μ C/mg. of N/hr. There was no blank when [2-¹⁴C]propionate was substrate. The experiment was performed without replicate incubations and the results were statistically analysed. The findings are reported in the text.

Additions to standard medium	¹⁴ C fixed (μ C/mg. of N/hr.)			
	Aerobic (O ₂ + CO ₂ , 95:5)		Anaerobic (N ₂ + CO ₂ , 95:5)	
	[2- ¹⁴ C]Propionate	[2- ¹⁴ C]Propionyl-CoA	[2- ¹⁴ C]Propionate	[2- ¹⁴ C]Propionyl-CoA
None	0.5	0.4	0.2	0.3
α -Oxoglutarate	1.3	0.5	0.3	0.1
α -Oxoglutarate + CoA	0.9	0.3	0.3	0.1
ADP	0.8	3.0	0.4	3.7
ADP + α -oxoglutarate	1.3	3.3	0.8	3.1
ADP + CoA	2.5	3.5	1.7	3.5
ADP + α -oxoglutarate + CoA	2.7	2.8	1.6	3.5
ATP	1.8	5.8	1.0	4.6
ATP + α -oxoglutarate	2.4	5.4	1.1	5.3
ATP + CoA	4.3	4.1	3.4	4.6
ATP + α -oxoglutarate + CoA	5.0	3.3	3.4	5.9

The data in Table 5 were obtained without replicate incubations, but the factorial arrangement of the experiment permitted a statistical analysis from which the effects of the individual additions and some of their interactions were estimated. After estimating these effects the residual variation provided an estimate of error variance (Davies, 1954). Before analysis the data were transformed to logarithms, because the range of responses was large and the effects were best represented on this scale. The discussion of results that follows is in terms of the transformed scale.

With [2-¹⁴C]propionate as substrate significantly higher ($P < 0.01$ by the t test) rates were obtained aerobically than anaerobically. The stimulatory effect of α -oxoglutarate was significant ($P < 0.05$ by the t test) only in the aerobic system. Significant stimulatory effects were obtained with ATP ($P < 0.001$ by the t test) and ADP ($P < 0.001$ by the t test) both aerobically and anaerobically, the effect of ATP being significantly greater ($P < 0.01$ by the t test) than that of ADP in both cases, and the effects of ATP being significantly greater ($P < 0.05$ by the t test) in the aerobic than the anaerobic system. The effect of CoA was not significant in the absence of ATP or ADP, but was significant ($P < 0.01$ by the t test) when either of these was present in both aerobic and anaerobic systems.

With [2-¹⁴C]propionyl-CoA as substrate the only significant effects were those of ATP ($P < 0.001$ by the t test) and ADP ($P < 0.001$ by the t test), ATP being significantly more effective ($P < 0.05$ by the t test) than ADP. On the logarithmic scale used there was no significant difference between aerobic and anaerobic systems with [2-¹⁴C]propionyl-CoA and no significant effects of CoA or α -oxoglutarate.

Table 6 sets out that part of the experiment concerned with the correlation of the rates of fixation of propionate and propionyl-CoA with the extent of disruption of the mitochondria, and the influence of disruption on the responses to α -oxoglutarate, ATP and CoA. It is clear that progressive destruction of the capacity of the mitochondria to metabolize [2-¹⁴C]propionate alone has occurred on treatment with the ultrasonic oscillator and freezing and thawing, the latter treatment having entirely abolished the formation of labelled non-volatile products. The stimulatory effect of α -oxoglutarate was similarly affected. At the same time the rate of formation of non-volatile radioactive products from [2-¹⁴C]propionate supplemented with ATP plus CoA, or from [2-¹⁴C]propionyl-CoA supplemented with ATP, increased, and in frozen-and-thawed material the two rates were equivalent and approached the rates of metabolism of propionate by whole mitochondria in the presence of ATP (1.3 mM)

Table 6. *Effects of exposure to ultrasonic energy and freezing and thawing of mitochondria on metabolism of propionate and propionyl-CoA in an aerobic system*

The same mitochondrial preparation was used as in Table 5 and for comparison the appropriate results with mitochondria treated with ultrasonic energy are repeated. Mitochondria were frozen in 0.25 M-sucrose and thawed 10 days later as described in the Materials and Methods section. The conditions of incubation and measurement and the composition of the medium were as described in the legend to Table 5, except that the results for whole mitochondria and frozen-and-thawed mitochondria are the means of duplicate incubations. The gas phase was $O_2 + CO_2$ (95:5).

Addition to standard medium	^{14}C fixed ($\mu C/mg.$ of N/hr.)					
	[2- ^{14}C]Propionate			[2- ^{14}C]Propionyl-CoA		
	Whole mitochondria	Mitochondria exposed to ultrasonic energy	Frozen-and- thawed mitochondria	Whole mitochondria	Mitochondria exposed to ultrasonic energy	Frozen-and- thawed mitochondria
None	1.4	0.5	0.1	0.2	0.4	0.0
ATP	—	1.8	0.4	—	5.8	7.7
ATP + α -oxoglutarate	—	2.4	0.3	—	5.4	6.5
ATP + CoA	—	4.3	8.1	—	4.1	7.7

plus α -oxoglutarate (1.67 mM). The rates represent fixation of $15.4 \mu moles$ of propionate and $16.2 \mu moles$ of propionyl-CoA/mg. of N/hr. respectively, neglecting any loss of ^{14}C as $^{14}CO_2$. From Table 1 the rate for whole mitochondria with ATP and α -oxoglutarate was $17.5 \pm 0.7 \mu moles/mg.$ of N/hr. The nature of the radioactive products in this experiment was examined by paper chromatography of samples (0.3 ml.) of the protein-free supernatant in the presence of carrier acids, followed by strip-scanning of the chromatograms (Smith & Russell, 1967). Both with [2- ^{14}C]propionate and [2- ^{14}C]propionyl-CoA the only labelled products detected were methylmalonate, succinate and malate, with traces of labelled fumarate. The results of Tables 5 and 6 demonstrate the adequacy of both propionyl-CoA synthetase (presumably EC 6.2.1.1) and of propionyl-CoA carboxylase (EC 6.4.1.3) to explain the rates of propionate metabolism with whole mitochondria stimulated with ATP and α -oxoglutarate.

The stimulatory activity of α -oxoglutarate appeared to require a comparable degree of structural integrity of the mitochondria, as was demanded by the capacity to metabolize propionate without ATP or ancillary substrate.

DISCUSSION

Friedberg, Adler & Lardy (1956) observed that rat-liver mitochondria metabolized propionate with fixation of $^{14}CO_2$ from [^{14}C]bicarbonate in the presence of 2 mM-ATP, only if an oxidizable ancillary substrate (β -hydroxybutyrate, succinate or α -oxoglutarate) were also present. No second substrate was required by ox- or guinea-pig-liver mito-

chondria. The effect of the ancillary substrate on rat-liver mitochondria was abolished by 2,4-dinitrophenol or by a glucose-hexokinase trap, and it was concluded that its function was to provide a high ATP/ADP ratio to drive the reactions leading to fixation of $^{14}CO_2$. Fixation of $^{14}CO_2$ by mitochondria in the presence of propionate could be brought about anaerobically by 2 mM-ATP supplemented with creatine phosphate and creatine phosphokinase, but ATP alone led to little fixation.

The present results show that sheep-liver mitochondria metabolized propionate in the absence of either external ATP or an oxidizable ancillary substrate. The rates obtained under these circumstances, however, have been found to be variable, and to be stabilized by the presence of 1.3 mM-ATP (cf. Tables 1 and 2). Different preparations of sheep-liver mitochondria have given results varying from almost complete dependence on added ATP to almost complete independence. The observation (Fig. 1) that anaerobically there was little or no consumption of propionate with below about 1.5 mM-ATP, whereas aerobically a maximum stable rate was obtained with 0.5 mM-ATP, suggests that the function of ATP in the aerobic system was not that of a direct source of energy for the initial steps of propionate metabolism. The absence of further stimulation aerobically by concentrations of ATP higher than 0.5 mM indicates the inability of external ATP to substitute for α -oxoglutarate in fully stimulating aerobic metabolism of propionate.

It is clear that external ATP was not fully available for the energy-requiring steps of propionate metabolism unless mitochondrial structure was disrupted. Since both the metabolism of propionate and its stimulation by α -oxoglutarate could proceed

in the absence of added ATP it is concluded that the function of external ATP was not solely that of an intermediate carrier in oxidative phosphorylation. The ATP driving propionate consumption appeared to be both generated and consumed within the mitochondria. The results are consistent with an effect of added ATP on the stability or structural integrity of the mitochondrial system responsible for propionate metabolism. The stabilizing effect of ATP on mitochondrial structure is well known (Lehninger, 1960; Dallam & Stoler, 1966).

The results with disrupted mitochondria support the view that the rate of metabolism of propionate in whole mitochondria was determined by the rate of supply of ATP within the mitochondrial space where propionate activation and carboxylation occurred. This space did not appear to be accessible to externally added CoA or propionyl-CoA, and to only a limited extent to external ATP. Partial disruption of the mitochondria by exposure to ultrasonic energy and more complete disruption by freezing and thawing led to progressive exposure of the enzymes responsible for propionate consumption with added ATP and CoA, in the presence of which the activity approached the mitochondrial rates.

The effects of α -oxoglutarate were obtained only aerobically, only with propionate (not propionyl-CoA) as substrate and only when sufficient mitochondrial structures remained to permit unsupplemented propionate metabolism to occur. The progressive loss of these functions on exposure to ultrasonic energy and freezing and thawing is consistent with the loss of the capacity of mitochondria for oxidative phosphorylation with these treatments. Thus McMurray, Maley & Lardy (1958) and Kielley & Bronk (1958) observed that brief exposure to ultrasonic energy, though it caused extensive disruption of mitochondria, did not destroy the capacity for oxidative phosphorylation. Treatment by freezing and thawing, however, has been generally reported to be more severe. It almost invariably lowered (Privitera, Greiff, Strength, Anglin & Pinkerton, 1958; Weinbach & Garbus, 1959) and in some cases destroyed (Cooper & Lehninger, 1956) the capacity of mitochondria for oxidative phosphorylation.

The effects of sodium chloride and potassium chloride and of the K^+/Na^+ ratio on the rate of metabolism of propionate and its response to α -oxoglutarate are also consistent with a rate-determining effect of oxidative phosphorylation. Maximum rates of propionate metabolism with or without α -oxoglutarate were obtained with alkali-metal chloride concentrations of 65–95 mM and with K^+/Na^+ ratios 5–10. Both these variables have been reported to affect the efficiency or extent of oxidative phosphorylation. The importance of the

osmolarity of the medium on demonstrating oxidative phosphorylation was stressed by Lehninger (1953). Stimulatory effects of K^+ have been observed by Pressman & Lardy (1955) on respiratory rates and by Krall, Wagner & Gozansky (1964) on respiratory control of mitochondria. Effects of the K^+/Na^+ ratio on both the respiratory rate and the P/O ratio of rat-kidney mitochondria were demonstrated by Blond & Whittam (1965).

The results discussed above leave little doubt that the rate of sheep-liver mitochondrial propionate metabolism was dependent on the rate of supply of ATP by oxidative phosphorylation within the mitochondria. α -Oxoglutarate appeared to increase the supply of ATP. Three ways can be conceived by which this might occur: (a) by providing a direct substrate for oxidative phosphorylation, α -oxoglutarate oxidized in sufficient quantity might account directly for the additional consumption of propionate; (b) by providing 'sparker' amounts of ATP, α -oxoglutarate could initiate propionate metabolism to the point where it became self-sustaining; or (c) α -oxoglutarate or one of its oxidation products might exert a catalytic effect on the rate of supply of ATP from the oxidation of propionate itself. The available evidence favours the third alternative.

The first alternative is made unlikely by the magnitude of the catalytic effect. With fresh mitochondria in the presence of ATP, 1 μ mole of α -oxoglutarate has been found to increase propionate consumption by up to 5.5 μ moles (Table 2). Although the extent of oxidation of α -oxoglutarate and the efficiency of oxidative phosphorylation necessary to achieve this result are conceivable (69% oxidation at P/O ratio 3, assuming a requirement of 3 terminal phosphate bonds of ATP/mol. of propionate), this is not consistent with the limited oxidation and apparent inefficiency of energy transfer observed with propionate alone (Smith *et al.* 1967). With glutamate, which is oxidized only to aspartate (Smith *et al.* 1965*a,b*), even more stringent arguments apply, and the stimulatory effect of pyruvate (Table 2) is well beyond its capacity to supply energy directly.

The second alternative, that of a 'sparker' effect, is not consistent with the results given in Fig. 2, where the rate of metabolism of propionate alone decreased rather than increased with time.

The third alternative remains. It is supported by more recent work with malonate inhibition reported in an accompanying paper (Smith & Russell, 1967).

That the effect of α -oxoglutarate may have been concerned with the maintenance of an intramitochondrial ATP pool is suggested by the effect of AMP on whole mitochondria and mitochondria exposed to ultrasonic energy. Unlike ADP, 3.3 mM-AMP strongly inhibited propionate metabolism

and its effects were at least partially overcome by α -oxoglutarate, with an additional effect of added CoA, even with whole mitochondria. Azzone & Ernster (1961) have developed the concept of several mitochondrial ATP pools. According to these workers one pool of ATP arises from the substrate-level phosphorylation accompanying the deacylation of the succinyl-CoA formed from α -oxoglutarate. This ATP was said to be unavailable for depletion by external ADP, but it could be depleted by external AMP. The results obtained with AMP and α -oxoglutarate are consistent with such a pool of ATP having a catalytic rate-controlling function on the metabolism of propionate by sheep-liver mitochondria. Against this view, however, must be placed the fact that succinyl-CoA may also be formed from propionate. In cases where marked stimulation of the rate of metabolism of propionate was obtained with catalytic concentrations of α -oxoglutarate, the flux of succinyl-CoA from α -oxoglutarate must have been quite small compared with that from propionate itself. It is possible that the succinyl-CoA arising from propionate was formed in a mitochondrial compartment different from that arising from α -oxoglutarate. Such an interpretation receives some support from the observation (Table 4) that externally added CoA, which had no effect on the rate of metabolism of propionate by whole mitochondria, increased the effectiveness of α -oxoglutarate in overcoming the inhibition by AMP.

Thus, although the stimulation of the rate of propionate metabolism by α -oxoglutarate appears to be brought about by an increased rate of supply of ATP from oxidative phosphorylation within the mitochondria, the conclusion that this ATP arose from the oxidation of α -oxoglutarate itself may not be entirely valid. The evidence is more consistent with a catalytic role of α -oxoglutarate, perhaps via the substrate-level phosphorylation and compartmented ATP, in stimulating the rate of supply of ATP from the oxidation of propionate itself.

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