Synthesis of Phosphoenolpyruvate from Propionate in Sheep Liver

By R. M. SMITH AND W. S. OSBORNE-WHITE

*Commonwealth Scientific and Industrial Research Organization, Division of Nutritional Biochemistry,
Kintore Avenue, Adelaide, S. Austral. 5000, Australia*

(Received 31 March 1971)

1. Utilization of propionate by sheep liver mitochondria was stimulated equally by pyruvate or α -oxoglutarate, with formation predominantly of malate. Pyruvate increased conversion of propionate carbon into citrate, whereas α -oxoglutarate increased formation of phosphoenolpyruvate. The fraction of metabolized propionate converted into phosphoenolpyruvate was about 17% in the presence or absence of α -oxoglutarate and about 7% in the presence of pyruvate. Pyruvate consumption was inhibited by 80% by 5mM-propionate. 2. Compared with rat liver, sheep liver was characterized by very high activities of phosphoenolpyruvate carboxykinase and moderately high activities of aconitase in the mitochondria and by low activities of 'malic' enzyme, pyruvate kinase and lactate dehydrogenase in the cytosol. Activities of phosphoenolpyruvate carboxykinase were similar in liver cytosol from rats and sheep. Activities of malate dehydrogenase and NADP-linked isocitrate dehydrogenase in sheep liver were about half those in rat liver. 3. The phosphate-dicarboxylate antiport was active in sheep liver mitochondria, but compared with rat liver mitochondria the citrate-malate antiport showed only low activity and mitochondrial aconitase was relatively inaccessible to external citrate. The rate of swelling of mitochondria induced by phosphate in solutions of ammonium malate was inversely related to the concentration of malate. 4. The results are discussed in relation to gluconeogenesis from propionate in sheep liver. It is proposed that propionate is converted into malate by the mitochondria and the malate is converted into phosphoenolpyruvate by enzymes in the cytosol. In this way sufficient NADH would be generated in the cytosol to convert the phosphoenolpyruvate into glucose.

Propionate produced by fermentation in the rumen is a major precursor of the glucose synthesized in ruminant tissues (Bergman, Roe & Kon, 1966; Leng, Steel & Luick, 1967; Annison, Brown, Leng, Lindsay & West, 1967). Intravenous injection of sodium propionate into sheep leads to a rapid rise in the concentrations of blood glucose, blood lactate and blood pyruvate and evidence based on the rate of clearance of propionate suggests that part, at least, of these increases is the result of direct and rapid synthesis from propionate (Smith & Marston, 1971). Annison, Leng, Lindsay & White (1963) found considerable labelling of blood lactate and blood glucose after injecting radioactively labelled propionate into sheep.

During synthesis of glucose from most of its common precursors the first specifically gluconeogenic intermediate is phosphoenolpyruvate, formed from oxaloacetate and GTP by the action of phosphoenolpyruvate carboxykinase. Metabolism of

propionate in sheep liver homogenates occurs only in the mitochondria and only by the methylmalonate pathway (Smith, Osborne-White & Russell, 1967). Propionate thus enters the pool of common metabolic intermediates as intramitochondrial succinyl-CoA, a potential source both of GTP from the substrate-level phosphorylation and of oxaloacetate from oxidation of the generated succinate. The present work, however, shows that the major product of propionate metabolism by sheep liver mitochondria was malate. Less than 20% of the propionate metabolized by mitochondria was converted into phosphoenolpyruvate despite a high mitochondrial activity of phosphoenolpyruvate carboxykinase. The present paper seeks to explain these results and examines their significance in relation to gluconeogenesis from propionate. A preliminary report of some of the findings has been given (Smith & Osborne-White, 1969).

MATERIALS AND METHODS

Animals. Australian Merino ewes (2–4 years old) and wethers (1 year old) were killed and liver homogenates prepared in 0.25 M-sucrose as described by Smith, Osborne-White & Russell (1965). Hooded Norwegian (Wistar) rats (8–32 weeks old) were killed by a blow on the head and homogenates prepared similarly from the livers of groups of four or five rats of the same age and sex. Centrifugal fractionation of the homogenates was carried out in the cold by the method of Hogeboom, Schneider & Pallade (1948) and mitochondria were washed twice in 0.25 M-sucrose.

Incubation of mitochondria. Mitochondria were incubated in 6 ml volumes in Pyrex tubes with side arms, in the medium and under the conditions described by Smith *et al.* (1965). The gas phase was $O_2 + CO_2$ (95:5). Incubations were stopped and protein was denatured and removed either with tungstic acid for determination of residual volatile fatty acids (Smith *et al.* 1965) or with $HClO_4$ (final concn. 2% w/v) for determination of pyruvate, malate and phosphoenolpyruvate. Duplicate incubations were performed for each kind of protein-free supernatant required. Propionate consumption was determined as described by Smith & Osborne-White (1965). Pyruvate was determined by the method of Bücher, Czok, Lamprecht & Latzko (1965), malate by the method of Hohorst (1965) and phosphoenolpyruvate by the method of Czok & Eckert (1965).

Labelled products of [2- ^{14}C]propionate metabolism. Mitochondria (0.40 mg of protein) were incubated in pre-weighed conical tubes, in final volumes of 0.3 ml, for 36 min at 37°C with 5 mM-[2- ^{14}C]propionate (0.66 $\mu Ci/\mu mol$), 20 mM-potassium phosphate buffer, pH 7.4, 25 mM- $NaHCO_3$, 65 mM-KCl, 5 mM- $MgCl_2$, 86 mM-sucrose, 11 μM -cytochrome *c* and 1.3 mM-ATP in equilibrium with $O_2 + CO_2$ (95:5) at pH 7.4. The [2- ^{14}C]propionate with or without pyruvate or α -oxoglutarate (final concentrations 1.67 mM) was added in 0.1 ml of water after 1 min at 37°C. The reactions were stopped by adding 0.1 ml of 5 M-KOH. After incubation for a further 30 min at 37°C to hydrolyse CoA thioesters, 5 ml of 0.12 M-HCl was added and the tubes were heated for 5 min in a boiling-water bath, cooled, weighed and centrifuged to sediment protein. Weighed samples of the supernatant were used to determine the total ^{14}C fixed and the distribution of radioactivity in the products. Incubations were performed in duplicate and the total ^{14}C fixed was determined in duplicate 0.1 ml samples after repeated evaporation with unlabelled propionic acid (Smith & Russell, 1967b). The radioactivity-counting efficiency in 5 ml of Bray's (1960) scintillation fluid was 67.2%, determined by using an internal standard with a Packard Tri-Carb model 3001 liquid-scintillation spectrometer. The distribution of ^{14}C among non-volatile products was determined on samples of 0.1–1.0 ml (after repeated evaporation with unlabelled propionic acid) by paper chromatography in the presence of carrier acids, followed by radioactive strip-scanning as described by Smith & Russell (1967b). The total areas of the strip-scanner peaks for the individual samples were compared with the corresponding measurements of the total ^{14}C fixed. The correlation coefficient was 0.924 ($P < 0.001$) and so the radioactivity in each peak was calculated from the total fixation for the corresponding

sample by fractional distribution of the peak areas. The results shown are means of duplicate chromatograms on duplicate incubated samples.

Enzyme assays. In general, enzyme activities were determined alternately in rats and sheep. Malate dehydrogenase was determined by the method of Ochoa (1955a); 'malic' enzyme was determined by the method of Ochoa (1955b); aconitase was determined by the method of Ochoa (1948) with a final concentration of 0.5 mM-NADP⁺; NADP-linked isocitrate dehydrogenase was determined by the method of Siebert, Dubuc, Warner & Plaut (1957), with 0.56 mM-KCN present; lactate dehydrogenase was determined by the method of Bergmeyer, Bernt & Hess (1965); and pyruvate kinase was determined by the method of Bücher & Pfeleiderer (1955). Phosphoenolpyruvate carboxykinase was determined, in the direction of phosphoenolpyruvate synthesis, by the method of Holten & Nordlie (1965) but with 2-mercaptoethanol (10 mM) present both in the incubation medium and in the sucrose solution used in preparing the homogenate (Gevers, 1967). Incubations were for 1, 2 and 3 min at 30°C and the phosphoenolpyruvate formed was determined by the method of Czok & Eckert (1965). All enzyme assays other than phosphoenolpyruvate carboxykinase were performed at 25°C, and the E_{340} was measured by using either a Unicam SP.800 spectrophotometer with a scale-expansion accessory (Unicam Instruments Ltd., Cambridge, U.K.) or a Zeiss PMQII spectrophotometer (Carl Zeiss, Oberkochen, Germany) fitted with a Polycamp point recorder (Hartmann & Braun A.G., Frankfurt, Germany). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Anion permeability measurements. Light-scattering by mitochondria suspended in the media described in the legends of the relevant figures was estimated from the residual light transmission at 610 nm (Chappell & Haarhoff, 1967). Measurements were made in 1 cm square glass cells in 2 ml volume at 25°C with a Zeiss PMQII spectrophotometer programmed to record the extinction in each of the five test cells at an interval of 32.8 s. Osmolality of the media was determined by measuring the depression of freezing point with a Fiske model H62 osmometer (Fiske Associates Inc., Bethel, Conn., U.S.A.).

Materials. Sodium [2- ^{14}C]propionate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and purified as described by Smith & Russell (1967b). Malate dehydrogenase, lactate dehydrogenase and pyruvate kinase were obtained from C.F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. The NADP-linked isocitrate dehydrogenase, NADP⁺, NADH, ATP, ADP, ITP and potassium pyruvate (dimer-free) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The monocyclohexylammonium salt of phosphoenolpyruvate, prepared by the method of Clark & Kirby (1963), was kindly given by M. F. Hopgood. Other materials were as previously described (Smith *et al.* 1967) or were reagent-grade chemicals.

RESULTS

Synthesis of phosphoenolpyruvate from propionate, pyruvate and α -oxoglutarate by sheep liver mitochondria. The effects of pyruvate and α -oxo-

glutarate on production of malate from propionate are shown in Table 1. Propionate utilization was stimulated equally by either of the ancillary substrates and the proportion of metabolized propionate appearing as malate increased from 35% to more than 60% with either. Consumption of pyruvate was small.

The effects of pyruvate and α -oxoglutarate on the fate of propionate carbon were studied in more detail with $[2-^{14}\text{C}]$ propionate. The results are summarized in Fig. 1. In this experiment pyruvate or α -oxoglutarate stimulated fixation of ^{14}C by more than 400% and the major effect of either was to increase by about 20 times the amount of ^{14}C fixed into malate. However, whereas with pyruvate ^{14}C fixed into citrate was increased by

280%, with α -oxoglutarate ^{14}C in citrate was increased by only 50%. The relatively high incorporation of ^{14}C from propionate into citrate in the presence of unlabelled pyruvate indicates a greatly increased conversion into citrate of oxaloacetate arising from propionate.

Table 2 shows the interactions between propionate and pyruvate or α -oxoglutarate in the production of phosphoenolpyruvate. Propionate consumption was stimulated by about 60% by either α -oxoglutarate ($P < 0.01$ by the t test) or pyruvate ($P < 0.01$ by the t test), but whereas phosphoenolpyruvate production from propionate was stimulated by 67% by α -oxoglutarate ($P < 0.01$ by the t test) it was inhibited by 35% by pyruvate ($P = 0.1$, not significant). The fraction of metabolized propionate found as phosphoenolpyruvate was therefore not altered by α -oxoglutarate (16–17%) but was markedly decreased by pyruvate (to about 7%).

The mitochondria metabolized pyruvate fairly rapidly in the absence of propionate, but the presence of 5 mM-propionate inhibited pyruvate consumption by 80% ($P < 0.01$ by the t test).

Intracellular distribution and activities of enzymes in sheep liver and rat liver. The intracellular distributions of several enzymes directly or indirectly involved in the metabolism of propionate carbon were examined in sheep liver and, for comparison with a non-ruminant, in rat liver. Results for ewes are shown in Table 3 and for male rats in Table 4. Enzyme activities in freshly prepared mitochondria were compared after suspending them in 0.25 M-sucrose, water or 0.2% (w/v) Triton X-100 for 15 min before adding them to the various assay media. Maximum activity of all the enzymes

Table 1. *Effects of pyruvate and α -oxoglutarate on production of malate from propionate*

Mitochondria (12.3 mg of protein) were incubated for 30 min at 37°C with 5 mM-propionate in the presence or absence of 1.67 mM-pyruvate or 1.67 mM- α -oxoglutarate. Utilization of propionate and pyruvate and production of malate were measured. Results are the means of duplicate incubations.

Substrate	Δ Propionate (μmol)	Δ Pyruvate (μmol)	Δ Malate (μmol)
Propionate	-9.4	+0.04	+3.3
Propionate	-12.3	-2.16	+8.1
+ pyruvate			
Propionate	-11.4	+0.11	+8.8
+ α -oxoglutarate

Fig. 1. Effects of pyruvate and α -oxoglutarate on the fate of propionate carbon. Mitochondria (0.4 mg of protein) were incubated in 0.3 ml volumes for 36 min at 37°C with 5 mM- $[2-^{14}\text{C}]$ propionate (0.66 $\mu\text{Ci}/\mu\text{mol}$) alone or with 1.67 mM-pyruvate or α -oxoglutarate. The amount of radioactivity in fixation products was determined as described in the text. The bars in the Figure represent ^{14}C found in the separated products: O, origin, containing labelled glutamate, aspartate and phosphoenolpyruvate; S, succinate; F, fumarate; M, malate; C, citrate plus isocitrate. Results are the means of duplicate chromatograms on duplicate incubated flasks.

Table 2. *Effects of pyruvate and α -oxoglutarate on phosphoenolpyruvate synthesis from propionate in sheep liver mitochondria*

Mitochondria (7.2–10 mg of protein) were incubated for 30 min at 37°C in the presence of the substrates shown (all 5 mM). Substrates were added 2 min after placing the tubes in the bath. The medium contained (final concentrations) K_2HPO_4 (16.4 mM), KH_2PO_4 (3.6 mM), $NaHCO_3$ (25 mM), KCl (85 mM), $MgCl_2$ (5 mM), sucrose (42 mM), ATP (1.3 mM) and cytochrome *c* (11 μ M) and was at pH 7.4 in equilibrium with $O_2 + CO_2$ (95:5). Mean rates of consumption of substrates and appearance of products are shown together with the s.e.m. derived from an analysis of variance (residual variance after removing treatment effects and variation between experiments). The numbers of observations are shown in parentheses.

Substrate	Δ Propionate (nmol/min per mg of protein)	Δ Pyruvate (nmol/min per mg of protein)	Δ Phosphoenolpyruvate (nmol/min per mg of protein)
Pyruvate	—	-53.3 ± 6.7 (3)	$+3.42 \pm 0.52$ (5)
Propionate	-36.5 ± 2.5 (5)	-10.5 ± 6.7 (3)	$+2.40 \pm 0.52$ (5)
+ pyruvate			
Propionate	-22.5 ± 2.5 (5)	$< +1.0$ (3)	$+3.68 \pm 0.52$ (5)
Propionate	-36.0 ± 2.5 (5)	$< +1.0$ (3)	$+6.13 \pm 0.52$ (5)
+ α -oxoglutarate			
α -Oxoglutarate	—	$< +1.0$ (3)	$+3.32 \pm 0.52$ (5)

studied was obtained by suspending the mitochondria in a 0.2% solution of the detergent Triton X-100. It was noted, however, that sheep liver mitochondria were more readily disrupted by osmotic shock than were rat liver mitochondria.

In mitochondria from both rat liver and sheep liver lactate dehydrogenase and pyruvate kinase showed relatively low activity and no increase in activity occurred on disruption of the mitochondrial membranes. This is consistent with the idea that these enzymes are present in the mitochondrial fractions as contaminants from the cytosol. The specific activities of lactate dehydrogenase and pyruvate kinase in sheep liver cytosol were 12% and 26% respectively of their activities in rat liver cytosol.

A negligible activity of 'malic' enzyme in ruminant liver was reported by Somasundaram, Hibbs & Conrad (1965). The values for 'malic' enzyme in sheep liver cytosol given in Table 3 are about 10 times greater than those reported by Hanson & Ballard (1967) but are similar to the values for bovine liver found by Ballard, Hanson, Kronfeld & Raggi (1968) and by Young, Thorp & de Lumen (1969).

Brdiczka & Pette (1971) found no 'malic' enzyme activity in three out of four bovine livers, however, and observed a several-fold greater activity in rat liver than that shown in Table 4. They reported that about 5% of the 'malic' enzyme activity in the livers of several species (including rat) was present in the mitochondria but the fractional extraction technique used may have overestimated the activity in this fraction. With isolated mitochondria from rat liver Brdiczka & Pette (1971) found that 80%

of the mitochondrial 'malic' enzyme activity was released on treatment with digitonin. This fraction of the mitochondrial enzyme was electrophoretically similar to the 'malic' enzyme in the cytosol but differed in mobility from the residual 'malic' enzyme in the matrix. It was found (Table 4) that less than 1% of the 'malic' enzyme in rat liver was associated with the mitochondria.

The specific activities of malate dehydrogenase in both mitochondria and cytosol of sheep liver were a little under half those in the corresponding fractions of rat liver. About 13% of the combined mitochondrial and soluble activities of malate dehydrogenase was present in the mitochondria in sheep liver and about 8% in rat liver.

Isocitrate dehydrogenase activities in both mitochondria and cytosol of sheep liver were slightly more than half those in the corresponding fractions of rat liver. In both species the mitochondria contained less than 10% of the combined activities of the two fractions.

Activity of aconitase in sheep liver cytosol was 37% of that in rat liver cytosol but activity in sheep liver mitochondria was 31% greater than that in rat liver mitochondria. Of the combined activities of aconitase in mitochondria and cytosol the mitochondria accounted for 28% in sheep liver, but only 6% in rat liver. With sheep liver mitochondria no aconitase activity could be detected unless the mitochondria were pretreated to disrupt the membranes. Rat liver mitochondria, however, exhibited 90% of the maximum activity without pretreatment.

Phosphoenolpyruvate carboxykinase activity was high in both mitochondria and cytosol from

Table 3. *Distribution and activities of enzymes in sheep liver*

The values are for ewes, 2-4 years old. Liver samples from freshly killed animals were homogenized in 0.25 M-sucrose and fractionated by the method of Hogeboom *et al.* (1948). Activities in mitochondria were obtained after treatment for 15 min in 0.2% (w/v) Triton X-100. Enzyme activities were assayed at 25°C, except for phosphoenolpyruvate carboxykinase, which was assayed at 30°C. The table shows the means $\pm$ s.e.m. for the number of preparations given in parentheses. Values for the first four enzymes listed were also measured in similar fractions prepared from the livers of six yearling wethers. The values for wethers did not differ significantly from those given for ewes.

Homogenate fraction	Protein recovered in fraction (mg/g of fresh liver)	Enzyme activities (nmol/min per mg of protein)						
		Malate dehydrogenase	'Malic' enzyme	Aconitase	Isocitrate dehydrogenase (NADP)	Lactate dehydrogenase	Pyruvate kinase	Phosphoenolpyruvate carboxykinase
Whole homogenate	186.2 $\pm$ 2.6 (6)	777 $\pm$ 47 (3)	<0.2 (3)	4.3 $\pm$ 0.2 (3)	60 $\pm$ 3 (3)	229 $\pm$ 23 (4)	28 $\pm$ 3 (4)	9.5 $\pm$ 0.5 (6)
Nuclear fraction	65.3 $\pm$ 2.5 (6)	300 $\pm$ 35 (3)	<0.2 (3)	4.0 $\pm$ 1.0 (3)	20 $\pm$ 3 (3)	68 $\pm$ 3 (4)	24 $\pm$ 2 (4)	7.3 $\pm$ 0.5 (6)
Washed mitochondria	23.0 $\pm$ 1.5 (6)	669 $\pm$ 84 (6)	<0.2 (6)	7.6 $\pm$ 0.6 (6)	43 $\pm$ 4 (6)	6 $\pm$ 1 (4)	6 $\pm$ 1 (4)	81.0 $\pm$ 3.3 (6)
Cytosol	62.8 $\pm$ 1.5 (6)	1710 $\pm$ 300 (3)	0.7 $\pm$ 0.5 (3)	7.3 $\pm$ 0.5 (3)	170 $\pm$ 13 (3)	591 $\pm$ 58 (4)	67 $\pm$ 3 (4)	23.0 $\pm$ 2.0 (6)
Microsomal fraction	36.7 $\pm$ 0.9 (6)	133 $\pm$ 7 (3)	<0.2 (3)	0.5 $\pm$ 0.3 (3)	10 $\pm$ 2 (3)	30 $\pm$ 3 (4)	8 $\pm$ 1 (4)	3.8 $\pm$ 0.5 (6)

Table 4. *Distribution and activities of enzymes in rat liver*

The values are for male rats, 8-32 weeks old. Fractions were prepared and mitochondrial enzymes exposed and assayed as described in Table 2. Values are the means $\pm$ S.E.M. for the numbers of preparations in parentheses.

Homogenate fraction	Protein recovered in fraction (mg/g of fresh liver)	Enzyme activities (nmol/min per mg of protein)					
		Malate dehydrogenase	'Malic' enzyme	Aconitase	Isocitrate dehydrogenase (NADP)	Lactate dehydrogenase	Pyruvate kinase
Whole homogenate	198.9 ± 3.9 (17)	988 ± 49 (4)	<0.2 (4)	7.5 ± 0.1 (4)	97.7 ± 11.9 (4)	1937 ± 132 (9)	67.2 ± 4.6 (9)
Nuclear fraction	89.2 ± 2.7 (17)	363 ± 14 (4)	<0.2 (4)	6.8 ± 0.8 (4)	38.5 ± 0.4 (4)	763 ± 50 (9)	17.5 ± 2.4 (9)
Washed mitochondria	15.4 ± 0.7 (17)	1460 ± 160 (6)	<0.2 (4)	5.8 ± 0.6 (6)	78.2 ± 8.8 (6)	455 ± 30 (9)	8.8 ± 0.6 (9)
Cytosol	71.1 ± 1.1 (17)	3450 ± 500 (4)	6.3 ± 1.9 (4)	19.5 ± 0.7 (4)	283 ± 19 (4)	4977 ± 387 (9)	259 ± 22 (9)
Microsomal fraction	31.1 ± 0.6 (17)	310 ± 21 (4)	<0.2 (4)	<0.2 (4)	15.2 ± 0.6 (4)	707 ± 54 (9)	19.5 ± 1.7 (9)
							Phosphoenolpyruvate carboxykinase
							6.7 ± 0.7 (13)
							3.5 ± 0.4 (13)
							4.3 ± 0.3 (13)
							25.0 ± 0.8 (13)
							4.0 ± 0.8 (13)

sheep liver; the mitochondrial activity accounted for 56% of the combined activity recovered in the two fractions. In rat liver only 4% of the combined activity of phosphoenolpyruvate carboxykinase in mitochondria and cytosol was present in the mitochondria, in agreement with other work (Nordlie & Lardy, 1963).

Permeability of sheep liver mitochondria to anions. Gluconeogenesis from propionate in liver involves enzymes from both mitochondria and cytosol and includes the operation of part of the tricarboxylic acid cycle. Observed differences in anion permeability of mitochondria from different sources (Chappell, 1968) prompted a study of anion translocation in sheep liver mitochondria in relation to this process. The procedure used was that of Chappell & Haarhoff (1967), in which graded concentrations of solutes are used to test the adequacy of the spectrophotometric method in relation to the osmometer model for mitochondrial volume changes. The results for sheep liver mitochondria were generally in agreement with those of Chappell & Haarhoff (1967) obtained with rat liver mitochondria, but the extent of phosphate-induced swelling found at high osmotic strengths of ammonium succinate was different.

As shown in Figs. 2 and 3 suspension of individual preparations of sheep liver mitochondria in solutions of solutes that could not pass through the inner membrane led to stable equilibrium volumes ($1/E_{610}$) inversely proportional to the osmolality and generally independent of the solute present. Suspension in solutions of permeable salts (ammonium acetate or ammonium propionate) was accompanied by rapid and extensive swelling with little residual effect of osmotic strength. Addition of 2mM-potassium phosphate (pH 7.4) caused swelling in ammonium succinate or ammonium malate, but not in the case of the other solutes tested.

When phosphate was added to mitochondria suspended in ammonium malate or ammonium succinate the rate of change of extinction with time was described by the function $E = E_0 + k \log t$, where E_0 is the extinction 1 min after addition of phosphate, k is a negative regression coefficient and E is the extinction t min later. The mean correlation coefficient between the above function and observed extinction values was 0.986 (five preparations of sheep liver mitochondria observed for up to 38 min at each of five concentrations of ammonium malate). The function derived does not approach a limit, but phosphate-induced swelling was very slow at high concentrations of malate or succinate and was barely detectable after 30 min at any of the concentrations used. This aspect of the results appears to differ from the findings of Chappell & Haarhoff (1967), who indicate that

Fig. 2. Effect of osmolality on swelling of sheep liver mitochondria. Sheep liver mitochondria (0.45 mg of protein) were suspended at 25°C in 2 ml volumes of medium containing 0.5 μ g of antimycin/ml, 6 mM-tris-chloride buffer (pH 7.4), 0.33 mM-EDTA and test solutes at concentrations between 17 mM and 267 mM at pH 7.4. Extinction in 1 cm cells was recorded at 610 nm. Baseline extinction became steady within 7 min of adding the mitochondria when potassium phosphate (pH 7.4) was added to a concentration of 2 mM. The graph shows reciprocal extinction against reciprocal osmolality at 7 min for: $\circ$, potassium chloride; Δ , sucrose; $\square$, ammonium succinate; and $\bullet$, ammonium acetate. Extinction for ammonium succinate plus phosphate ($\blacksquare$) is shown 18 min after phosphate was added. There was no change in extinction on addition of phosphate to the other solutes.

there are limits to the phosphate-induced swelling of rat liver mitochondria in ammonium succinate, with greater swelling at higher concentrations of succinate.

On differentiating the reciprocal function with respect to time the equation

$$\frac{dE^{-1}}{dt} = \frac{-k}{t(E_0 - k \log t)^2}$$

is obtained, which simplifies to

$$\frac{dE^{-1}}{dt} = \frac{-k}{E_0^2}$$

at $t = 1$ min for initial rates of swelling. Fig. 4 shows these rates as a function of $1/[\text{malate}]$ for liver mitochondria from rats and sheep. The rate of swelling was inversely related to the malate concentration with mitochondria from both species. Swelling rates were not increased by doubling the phosphate concentration and the results are consistent with a rate-limiting transport of malate (accompanied by sufficient water to maintain isotonicity with the medium) into the mitochondria. Rates of swelling so derived are in

Fig. 3. Effect of osmolality on swelling of sheep liver mitochondria and rat liver mitochondria. Sheep liver mitochondria (0.43 mg of protein) (a) and rat liver mitochondria (0.47 mg of protein) (b) were suspended at 25°C in 2 ml of medium containing 0.5 μ g of antimycin/ml, 0.33 mM-EGTA [ethanedioxybis(ethylamine)-tetra-acetate], 6 mM-tris-chloride (pH 7.4) and test solutes at graded concentrations between 17 mM and 267 mM at pH 7.4. The time-course of extinction (1 cm cells) at 610 nm was measured and potassium phosphate (2 mM) was added after 7 min. Reciprocal extinction values shown for ammonium propionate ($\square$) were obtained after 1 min in (a) and 7 min in (b). Values for ammonium L-malate (Δ) were obtained after 7 min and values for ammonium malate plus phosphate ($\blacktriangle$) were obtained at 17 min after addition of phosphate in both (a) and (b). Values for ammonium citrate ($\circ$) were obtained after 7 min in both (a) and (b). Addition of phosphate at 7 min did not alter the extinction in the presence of citrate but further addition of 2 mM-potassium L-malate to mitochondria in 16.7 mM-ammonium citrate-2 mM-phosphate caused the time-course of decrease in extinction (—) shown in the insets for (a) and (b). For comparison, the time-course of decrease in extinction in 66.7 mM-L-malate on addition of phosphate is shown (----). The reciprocal extinctions for ammonium citrate-phosphate-malate ($\bullet$) were obtained 15 min after addition of malate in both (a) and (b).

arbitrary units for each mitochondrial preparation and cannot be assumed to be comparable in different preparations, but the similarities in the form of the relationships (Fig. 4) for liver mitochondria from rats and sheep indicate that similar processes were occurring in both.

In agreement with the results for rat liver mitochondria (Chappell & Haarhoff, 1967) swelling of sheep liver mitochondria in ammonium citrate was found to depend on both phosphate and malate. With mitochondria from either species, however, swelling was slight and was detected only at low concentrations of citrate (Fig. 3). Brierley, Jurkowitz, Scott & Merola (1970) indicate that this process is favoured by high pH in rat liver mitochondria and they show that it does not occur at all in bovine heart mitochondria. As shown in the insets of Fig. 3 the initial rate of swelling of rat liver mitochondria in 16.7 mM-citrate in the presence of phosphate and malate was about equal to that in 66.7 mM-malate in the presence of phosphate. In sheep liver mitochondria under the same conditions swelling in 16.7 mM-citrate was much slower

than that in 66.7 mM-malate. The evidence for a relatively slow citrate-malate antiport in sheep liver mitochondria is consistent with the relative inaccessibility of mitochondrial aconitase to external citrate.

The results suggest that sheep liver mitochondria may take up propionate and lose succinate or malate without serious hindrance but that citrate formed inside the mitochondria may not be so readily discharged.

DISCUSSION

Both pyruvate and α -oxoglutarate increased the utilization of propionate to an equal extent and the predominant effect of either was to increase the conversion of propionate into malate. With pyruvate this was accompanied by increased conversion of propionate carbon into citrate, and with α -oxoglutarate by increased formation of phosphoenolpyruvate. Both agents therefore led to active removal of intramitochondrial oxaloacetate accompanied by increased oxidation of generated

Fig. 4. Initial rates of swelling on addition of 2mm-phosphate to mitochondria suspended in ammonium malate as a function of $1/[\text{malate}]$. The values are for the experiment shown in Fig. 2. Regression coefficients of extinction with log time were obtained by computer and the initial rates of swelling were calculated as described in the text. ○, Sheep liver mitochondria; ●, rat liver mitochondria.

succinate. These results are consistent with previous evidence that propionate utilization by sheep liver mitochondria is limited by succinate oxidase activity (Smith & Russell, 1967b). De-inhibition of succinate oxidase by agents that deplete intramitochondrial oxaloacetate has been extensively studied (Wojtczak, 1969; Papa, Lofrumento, Paradies & Quagliariello, 1969; Oestreicher, van den Bergh & Slater, 1969).

Sheep liver mitochondria contain high activities of phosphoenolpyruvate carboxykinase and during metabolism of propionate should have the capacity to produce both GTP by deacylation of succinyl-CoA and oxaloacetate by oxidation of succinate. Nevertheless only about one-sixth of the propionate consumed was converted into phosphoenolpyruvate and this fraction was not altered by α -oxoglutarate. Preferential use of intramitochondrial GTP for transphosphorylation of the AMP produced after activation of propionate could explain a stoichiometric consumption of the GTP arising on deacylation of the generated succinyl-CoA (Smith, 1971), so leading to the requirement for an additional source of GTP for production of phosphoenolpyruvate. This interpretation implies that formation of phosphoenolpyruvate was limited by the supply of oxaloacetate when α -oxoglutarate was present.

Utilization of propionate is stimulated by catalytic amounts of pyruvate or α -oxoglutarate, but

not at all by malate (Smith & Russell, 1967a). The appearance of malate as the chief product during rapid metabolism of propionate is consistent with a net requirement for two rather than three terminal phosphate bonds of ATP for conversion of propionate into succinate and hence with the proposed stoichiometric consumption of GTP.

Formation of phosphoenolpyruvate from propionate plus pyruvate was less than that from either substrate alone and accounted for only 7% of the propionate consumed. This effect was probably the result of lowered intramitochondrial concentrations of oxaloacetate. Methylmalonyl-CoA formed during propionate metabolism is known to be a potent inhibitor of pyruvate carboxylase activity (Scrutton & Utter, 1967) and the small amount of oxaloacetate arising from propionate appeared to be diverted into citrate when pyruvate was present.

Although the severe inhibition of pyruvate utilization by propionate is consistent with decreased conversion of pyruvate into oxaloacetate, formation of acetyl-CoA from pyruvate may also be decreased by propionate. Utilization of acetate (Smith, 1971) as well as that of pyruvate is inhibited by propionate in sheep liver mitochondria, and Pearson & Tubbs (1967) have shown that propionate markedly decreases the concentration of acetyl-CoA in rat hearts perfused with either acetate or pyruvate. The mechanism of these effects has not been resolved.

Extension of the present findings to formation *in vivo* of glucose from propionate in the liver of sheep makes it unlikely that the mitochondria are primarily responsible for the intermediate formation of phosphoenolpyruvate. Metabolism of propionate by the whole sheep is accompanied by increased blood concentrations of lactate and pyruvate (Jarrett & Potter, 1957; Smith & Marston, 1971), and liver mitochondria presumably metabolize propionate *in vivo* in the presence of pyruvate. The major product of propionate metabolism by mitochondria stimulated by pyruvate was malate, which in the presence of phosphate was found to penetrate mitochondria readily. Most of the malate produced from propionate by sheep liver mitochondria is recovered outside the mitochondria (R. M. Smith & G. R. Russell, unpublished work). The activities of malate dehydrogenase and phosphoenolpyruvate carboxykinase in the cytosol (Table 3) are adequate to account for conversion into phosphoenolpyruvate of all the propionate metabolized by the mitochondria (Table 2) and it is proposed that this spatial route is the one used mainly during conversion of propionate carbon into glucose in the intact liver. An obvious advantage of this intracellular distribution of activities is the provision in the cytosol of stoichiometric

amounts of NADH for reduction of 1,3-diphosphoglycerate to glyceraldehyde 3-phosphate.

The observed pattern of enzyme activities in sheep liver may be viewed as an adaptation of the ruminant to an uncertain supply of carbohydrate. Thus the very low activity of 'malic' enzyme and the relatively low activity of pyruvate kinase in the cytosol would tend to minimize conversion of propionate carbon into pyruvate, whereas the inhibition by propionate of mitochondrial oxidation of pyruvate would tend to maintain pyruvate concentrations and so ensure the rapid and efficient conversion of propionate into malate by the mitochondria.

The relative impermeability of the mitochondrial membranes to citrate may be correlated with the very low activities of ATP citrate lyase in ruminant liver cytosol (Hardwick, 1966; Hanson & Ballard, 1967; Young *et al.* 1969) and with the relatively high activity of mitochondrial aconitase. Citrate produced in the mitochondria from propionate or pyruvate may thus be limited to that required for ATP production via the tricarboxylic acid cycle, so avoiding the diversion of potential sources of glucose to other biosynthetic routes.

We thank Mr R. B. Cunningham, Commonwealth Scientific and Industrial Research Organization, Division of Mathematical Statistics, for the statistical analyses and for mathematical advice. Thanks are due to Mr M. T. Mano and Mr R. A. King for skilled technical assistance.

REFERENCES

- Annisson, E. F., Brown, R. E., Leng, R. A., Lindsay, D. B. & West, C. E. (1967). *Biochem. J.* **104**, 135.
- Annisson, E. F., Leng, R. A., Lindsay, D. B. & White, R. R. (1963). *Biochem. J.* **88**, 248.
- Ballard, F. J., Hanson, R. W., Kronfeld, D. S. & Raggi, F. (1968). *J. Nutr.* **95**, 160.
- Bergman, E. N., Roe, W. E. & Kon, K. (1966). *Am. J. Physiol.* **211**, 793.
- Bergmeyer, H. U., Bernt, E. & Hess, B. (1965). In *Methods of Enzymatic Analysis*, p. 736. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Brdiczka, D. & Pette, D. (1971). *Eur. J. Biochem.* **19**, 546.
- Brierley, G. P., Jurkowitz, M., Scott, K. M. & Merola, J. (1970). *J. biol. Chem.* **245**, 5404.
- Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1965). In *Methods of Enzymatic Analysis*, p. 253. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Bücher, T. & Pfeleiderer, G. (1955). In *Methods in Enzymology*, vol. 1, p. 435. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Chappell, J. B. (1968). *Br. med. Bull.* **24**, 150.
- Chappell, J. B. & Haarhoff, K. N. (1967). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. New York: Academic Press Inc.
- Clark, V. M. & Kirby, A. J. (1963). *Biochim. biophys. Acta*, **78**, 732.
- Czok, R. & Eckert, L. (1965). In *Methods of Enzymatic Analysis*, p. 229. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Gevers, W. (1967). *Biochem. J.* **103**, 141.
- Hanson, R. W. & Ballard, F. J. (1967). *Biochem. J.* **105**, 529.
- Hardwick, D. C. (1966). *Biochem. J.* **99**, 228.
- Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
- Hohorst, H. J. (1965). In *Methods of Enzymatic Analysis*, p. 328. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Holten, D. D. & Nordlie, R. C. (1965). *Biochemistry, Easton*, **4**, 723.
- Jarrett, I. G. & Potter, B. J. (1957). *Aust. J. exp. Biol. med. Sci.* **35**, 103.
- Leng, R. A., Steel, J. W. & Luick, J. R. (1967). *Biochem. J.* **103**, 785.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Nordlie, R. C. & Lardy, H. A. (1963). *J. biol. Chem.* **238**, 2259.
- Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
- Ochoa, S. (1955a). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Ochoa, S. (1955b). In *Methods in Enzymology*, vol. 1, p. 739. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Oestreicher, A. B., van den Bergh, S. G. & Slater, E. C. (1969). *Biochim. biophys. Acta*, **180**, 45.
- Papa, S., Lofrumento, N. E., Paradies, G. & Quagliariello, E. (1969). *Biochim. biophys. Acta*, **180**, 35.
- Pearson, D. J. & Tubbs, P. K. (1967). *Biochem. J.* **105**, 953.
- Serutton, M. C. & Utter, M. F. (1967). *J. biol. Chem.* **242**, 1723.
- Siebert, G., Dubuc, J., Warner, R. C. & Plaut, G. W. E. (1957). *J. biol. Chem.* **226**, 965.
- Smith, R. M. (1971). *Biochem. J.* **124**, 877.
- Smith, R. M. & Marston, H. R. (1971). *Br. J. Nutr.* **26**, 41.
- Smith, R. M. & Osborne-White, W. S. (1965). *Biochem. J.* **95**, 411.
- Smith, R. M. & Osborne-White, W. S. (1969). *Proc. Aust. biochem. Soc.* **2**, 72.
- Smith, R. M., Osborne-White, W. S. & Russell, G. R. (1965). *Biochem. J.* **95**, 423.
- Smith, R. M., Osborne-White, W. S. & Russell, G. R. (1967). *Biochem. J.* **104**, 441.
- Smith, R. M. & Russell, G. R. (1967a). *Biochem. J.* **104**, 450.
- Smith, R. M. & Russell, G. R. (1967b). *Biochem. J.* **104**, 460.
- Somasundaram, A., Hibbs, J. W. & Conrad, H. R. (1965). *J. Dairy Sci.* **48**, 751.
- Wojtczak, A. B. (1969). *Biochim. biophys. Acta*, **172**, 52.
- Young, J. W., Thorp, S. L. & de Lumen, H. Z. (1969). *Biochem. J.* **114**, 83.