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Acceleration of Gluconeogenesis from Propionate by DL-Carnitine in the Rat Kidney Cortex

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1. The rate of gluconeogenesis from propionate in rat kidney-cortex slices was stimulated up to 3·5-fold by DL-carnitine and by bicarbonate, and was inhibited by inorganic phosphate or high concentrations of propionate (above 3mM). 2. The stimulatory effect of carnitine was dependent on the bicarbonate concentration and could be replaced at low propionate concentration by addition of 25mM-bicarbonate–carbon dioxide buffer. At low bicarbonate concentration the carnitine concentration can be rate-limiting. 3. All observations are in accordance with the view that the action of carnitine is in principle the same as that established for other fatty acids in other tissues, namely that carnitine promotes the appearance of propionyl-CoA within the mitochondrion by acting as a carrier. 4. The accelerating effects of carnitine and bicarbonate and the inhibitory effect of phosphate can be explained on the basis of the known properties of key enzymes of propionate metabolism, i.e. the reversibility of the reactions leading to the formation of methylmalonyl-CoA from propionyl-CoA. 5. 5mM-Propionate caused a five- to ten-fold fall in the free CoA content of the tissue. This fall can account for the inhibition of respiration and gluconeogenesis caused by high propionate concentration. 6. Relatively large quantities of propionyl-L-carnitine (15% of the propionate removed) were formed when DL-carnitine was present; thus the 'activation' of propionate proceeded at a faster rate than the carboxylation of propionyl-CoA. The metabolism of added propionyl-L-carnitine was accompanied by glucose synthesis. 7. The appearance of radioactivity from [2-^{14}C]propionate in both glucose and carbon dioxide was as expected on account of the randomization of C-2 and C-3 of propionate, i.e. the formation of succinate as an intermediate. 8. The maximum rate of glucose synthesis from propionate (93·3 ± 3·3 μmoles/g. dry wt./hr.) was not affected by dietary changes aimed at varying the rate of caecal volatile fatty acid formation in the rat. 9. Inhibition of gluconeogenesis by high propionate concentration was not found in those species where the rate of caecal or ruminal propionate production is high under normal conditions (rabbit, sheep and cow).

Propionate is known to be a ready gluconeogenic precursor in the mammalian liver and kidney cortex (Ringer, 1912; Eckstein, 1933; Krebs & Yoshida, 1963; Leng & Annison, 1963). During experiments on the metabolism of fatty acids in rat kidney-cortex slices, it was noted (Weidemann & Krebs, 1967) that the rate of removal of propionate is accelerated by DL-carnitine much more effectively than the rate of removal of other fatty acids. The utilization of propionate in rat kidney cortex can also be accelerated by bicarbonate, as observed in sheep rumen epithelium by Pennington (1954) and in sheep liver by Leng & Annison (1963), and inhibited by inorganic phosphate.

This paper is a detailed study of the effects of carnitine, bicarbonate and inorganic phosphate on propionate metabolism in rat kidney-cortex slices. The effects of these substances and their interplay can be satisfactorily explained on the basis of the known properties of key enzymes of propionate metabolism.

EXPERIMENTAL

Incubation procedure. Washed kidney-cortex slices from male rats starved for 48hr. were incubated as described by Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963), either in the phosphate-buffered medium of Krebs & de Gasquet (1964) or in the bicarbonate medium of Krebs & Henseleit (1932). Total respiratory CO_2 was collected and determined manometrically as described by Krebs, Hems, Weidemann & Speake (1966). For the determination of

metabolites the medium was deproteinized with $HClO_4$ as described by Gevers & Krebs (1966).

Reagents. Propionyl-L-carnitine was prepared by the method of Bøhmer & Bremer (1968). Chromatography on thin-layer silicic acid plates eluted with chloroform–methanol–aq. ammonia (sp.gr. 0·88) (25:15:4, by vol.) and assay by the methods of Friedman & Fraenkel (1955) and Pearson & Tubbs (1967) revealed no impurities.

Analytical methods. The metabolites were determined spectrophotometrically by enzymic methods; glucose, L-lactate, pyruvate, malate and α-oxoglutarate as given in Krebs, Dierks & Gascoyne (1964); fumarate and α-glycerophosphate as given in Gevers & Krebs (1966); acetoacetate and β-hydroxybutyrate by the method of Williamson, Mellanby & Krebs (1962); short-chain acyl-L-carnitine by the method of Pearson & Tubbs (1967); phosphoenolpyruvate by the method of Czok & Eckert (1963); succinate by the method of Rodgers (1961). Free CoA was determined by the method of Garland (1964).

Propionate was determined by gas–liquid chromatography as described by Baumgardt (1964) with valeric acid as the internal standard. Propionyl-L-carnitine gave peaks with retention times identical with those of propionic acid and carnitine under these conditions, indicating substantial breakdown of the ester. The values given in this paper for propionate disappearance therefore do not include the fraction of the fatty acid converted into acyl-L-carnitine.

Radiochemical methods. [2-^{14}C]Propionate was obtained from The Radiochemical Centre, Amersham, Bucks., and checked for radiochemical purity by steam-distillation and gas–liquid chromatography. No significant volatile or non-volatile contaminants were revealed.

The radioactivity in glucose and other metabolic products not decomposing on paper was separated by two-dimensional paper chromatography and radioautography as described by Gevers & Krebs (1966) and Crowley, Moses & Ullrich (1963). [2-^{14}C]Propionic acid was recovered after incubation by steam-distillation. Separation from [^{14}C]propionyl-L-carnitine was achieved by this method, as pure samples of propionyl-L-carnitine did not decompose and distil as propionic acid.

The radioactivity incorporated into protein and lipid in the slice was determined by the following method. The slices were washed 12 times with 2% (w/v) $HClO_4$ (4·0ml.) to remove contaminating radioactivity and were then homogenized in water (4·0ml.). Samples (2·0ml.) were treated with 20% (w/v) $HClO_4$ (0·5ml.) and the $HClO_4$-insoluble material was centrifuged and washed six times with 2% $HClO_4$ (2·0ml.). The washed pellet was dissolved in 1·0ml. of 1M-hyamine hydroxide in methanol by heating at 60° for 120min. The volume was readjusted to 1·0ml. and samples (0·5ml.) were taken for liquid-scintillation counting. Total radioactivity in the $HClO_4$-insoluble material of the slice was determined by this method. Radioactivity in the lipid fraction was determined separately by extracting samples (1·0ml.) of the tissue homogenate with heptane as described by Dole & Meinertz (1960).

[2-^{14}C]Propionyl-L-carnitine was separated from other incubation products bearing significant radioactivity by thin-layer silicic acid chromatography (Bøhmer &Bremer, 1968). Samples (10μl.) of incubation media were applied to thin-layer plates and small quantities of unlabelled carrier propionate, glucose, L-carnitine and propionyl-L-carnitine were added. The plates were subsequently developed three times with chloroform–methanol–aq. ammonia (sp.gr. 0·88) (25:15:4, by vol.) and the spots detected with iodine vapour. Approximate R_F values were: [2-^{14}C]propionyl-L-carnitine, 0·12–0·13; [^{14}C]glucose, 0·055; [2-^{14}C]propionate, 0·003; L-carnitine, 0·015; therefore good separation of propionylcarnitine was achieved by triple development. The radioactivities measured were corrected for traces of contaminating radioactivity contained in the [2-^{14}C]-propionate by running control chromatograms on unincubated solutions.

Counting procedure. Radioactivity in the separated products was determined by liquid-scintillation counting in toluene–2-methoxyethanol (3:2, v/v) containing 2-(4-*tert.*-butylphenyl)-5-(4-biphenylyl)-1-oxa-3,4-diazole (6g./l.) and naphthalene (80g./l.). The counting efficiency was 82·5% and all measurements were corrected to 100% efficiency with standard [^{14}C]toluene.

RESULTS

Effect of propionate concentration, DL-carnitine and bicarbonate on oxygen consumption and gluconeogenesis. In the phosphate-buffered saline (with no added bicarbonate) DL-carnitine strikingly increased the rate of gluconeogenesis from propionate (Table 1). The concentration of propionate affected the rates of gluconeogenesis and respiration in an unusual way. Gluconeogenesis was maximal at the lowest concentration tested (1·25mM) and was almost completely abolished at 10mM. The rate of tissue respiration was affected in the presence of DL-carnitine in a similar way, but the percentage changes were smaller. In the absence of added carnitine the respiration was increasingly inhibited by rising concentrations of propionate.

It is already known that bicarbonate accelerates gluconeogenesis from propionate (Krebs, Speake & Hems, 1965). There are thus at least three factors that can control the rate of propionate utilization in kidney, namely the concentrations of propionate,

Table 1. *Effect of propionate concentration on gluconeogenesis by rat kidney cortex-slices*

Kidney-cortex slices were incubated in phosphate-buffered saline containing no added bicarbonate; for other experimental details see the text.

Concn. of added propionate (mM)	O_2 consumption (μmoles/g. dry wt./hr.)		Glucose production (μmoles/g. dry wt./hr.)	
	No further addition	Plus DL-carnitine (1·0mM)	No further addition	Plus DL-carnitine (1·0mM)
0	−961	−1020	+25·0	+25·3
1·25	−911	−1206	+38·9	+68·0
2·5	−870	−1089	+32·0	+67·8
5·0	−822	−1005	+23·6	+49·7
10·0	−695	−851	+20·3	+29·8
20·0	−654	−857	+16·5	+19·7

Table 2. *Effect of* DL-*carnitine and bicarbonate on propionate metabolism in rat kidney-cortex slices*

Kidney-cortex slices were incubated in either phosphate-buffered saline (Krebs & de Gasquet, 1964) or bicarbonate-buffered saline (Krebs & Henseleit, 1932) with phosphate omitted; for other experimental details see the text. The results are given as means ± S.D. for three experiments. In calculating the amounts of propionate accounted for by the products no corrections were made for blanks; glucose was taken to require two propionate molecules and the C_3 compounds one each.

	Metabolic changes (μmoles/g. dry wt./hr.)							
Conditions of incubation ...	Phosphate-buffered saline (6 mM) containing no added bicarbonate				Bicarbonate-buffered saline (25 mM) containing no added phosphate			
Substrates added	No added substrate	DL-Carnitine (4·0 mM)	Propionate (2·5 mM)	Propionate (2·5 mM)+ DL-carnitine (4·0 mM)	No added substrate	DL-Carnitine (4·0 mM)	Propionate (2·5 mM)	Propionate (2·5 mM)+ DL-carnitine (4·0 mM)
O_2 consumption	918±57	886±7	735±64	1182±70	—	—	—	—
Glucose production	15·0±1·1	19·0±0·9	21·4±1·5	72·7±4·8	13·0±3·4	18·9±2·2	72·9±7·1	89·1±4·0
Propionate disappearance	—	—	126±21	235±14	—	—	265±13	268±21
Lactate production	8·7±1·5	10·7±3·4	15·7±0·9	14·8±1·8	9·0±0·4	7·2±0·6	17·8±1·4	15·1±1·9
Pyruvate production	1·8±0·2	1·7±0·2	1·8±0·3	1·5±0·4	2·0±0·8	2·1±0·2	3·5±0·4	3·1±0·9
α-Glycerophosphate production	0·97±0·24	1·3±0·2	1·0±0·5	1·7±0·1	0·17±0·24	0·38±0·06	0·27±0·25	0·37±0·29
Lactate/pyruvate ratio	4·8	6·3	8·7	9·9	4·5	3·4	5·1	4·9
Percentage contribution of propionate oxidation to O_2 consumption			30·8	21·2			—	—
Percentage suppression of endogenous O_2 consumption			44·6	0			—	—
Propionate removed not accounted for			64·7	71·6			97·6	71·2

bicarbonate and DL-carnitine. In the series of experiments recorded in Table 2, the propionate concentration was 2·5mM and the effects of DL-carnitine in phosphate-buffered saline and bicarbonate-buffered saline were compared.

When no carnitine was added, the rates of propionate removal and glucose formation were much greater in the bicarbonate-buffered saline (2·1- and 3·5-fold respectively). In the presence of DL-carnitine (4·0 mM) this difference largely disappeared, as DL-carnitine had stimulating effects on propionate removal and gluconeogenesis that were similar to those of bicarbonate, and the effects of bicarbonate and DL-carnitine were not additive. In phosphate-buffered saline the increased uptake of propionate (110μmoles/g. dry wt./hr.) from the medium caused by DL-carnitine was accompanied by increased glucose synthesis (51μmoles/g. dry wt./hr.), indicating an almost quantitative conversion of the extra propionate into glucose.

Because, in all cases, the amounts of propionate removed greatly exceeded those of glucose formed, tests for other products of propionate metabolism were made (Table 2). There was a small increase in the formation of lactate and, in the bicarbonate buffer, also of pyruvate. Very small quantities of α-glycerophosphate appeared and these were three- to four-fold higher in the phosphate-buffered saline. The products identified, however, did not account for all of the propionate removed. This suggested that some of the propionate underwent complete oxidation. The propionate not accounted for by the formation of glucose, lactate, pyruvate and α-glycerophosphate was sufficient, in fact, to contribute 30·6% of the total respiration when propionate only was added and 21·2% when propionate plus DL-carnitine was added to the phosphate-buffered saline.

Propionate added alone was oxidized in preference to endogenous substrates, whereas in the presence of DL-carnitine the contribution of propionate to the oxygen consumption was exactly equivalent to the amount of propionate used not accounted for by other products (see last row of Table 2).

Effect of phosphate concentration on gluconeogenesis at different concentrations of bicarbonate. The low rate of gluconeogenesis from propionate found in the phosphate-buffered saline raises the question whether this effect is due to the absence of bicarbonate or to an inhibitory effect of phosphate. The effect of phosphate on gluconeogenesis at different bicarbonate concentrations is shown in

Fig. 1. These graphs show the following facts: (1) Propionate at concentrations higher than 2·5 mM always inhibited gluconeogenesis, but the inhibition was much more marked in the absence of added carnitine. (2) DL-Carnitine stimulated gluconeogenesis under all conditions, although the effect was slight at low propionate concentration in bicarbonate-buffered media containing no phosphate. (3) Stimulation of gluconeogenesis by bicarbonate in the absence of carnitine occurred only at low propionate concentration and was counteracted by the addition of inorganic phosphate. (4) Inorganic phosphate at the higher concentration (6·0 mM) always inhibited gluconeogenesis; at the lower concentration (1·0 mM), significant inhibition occurred only at low bicarbonate concentration (2·0 mM) in the absence of added carnitine.

Thus the concentration of inorganic phosphate is a further factor influencing the rate of propionate metabolism under certain conditions.

When both bicarbonate and phosphate were omitted from the medium and it was buffered with 6mM-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid at pH7·4 (containing 100% oxygen in the gas space), results very similar to those in Table 1 were obtained, i.e. a marked stimulation of gluconeogenesis by DL-carnitine at low propionate concentration.

Effect of acetoacetate on gluconeogenesis from propionate. With L-lactate as substrate the yield of glucose is more than doubled and the ratio lactate used/glucose formed falls from 5·5 to values approaching 2·0 when 5·0 mM-acetoacetate is added to kidney-cortex slices (Krebs *et al.* 1966).

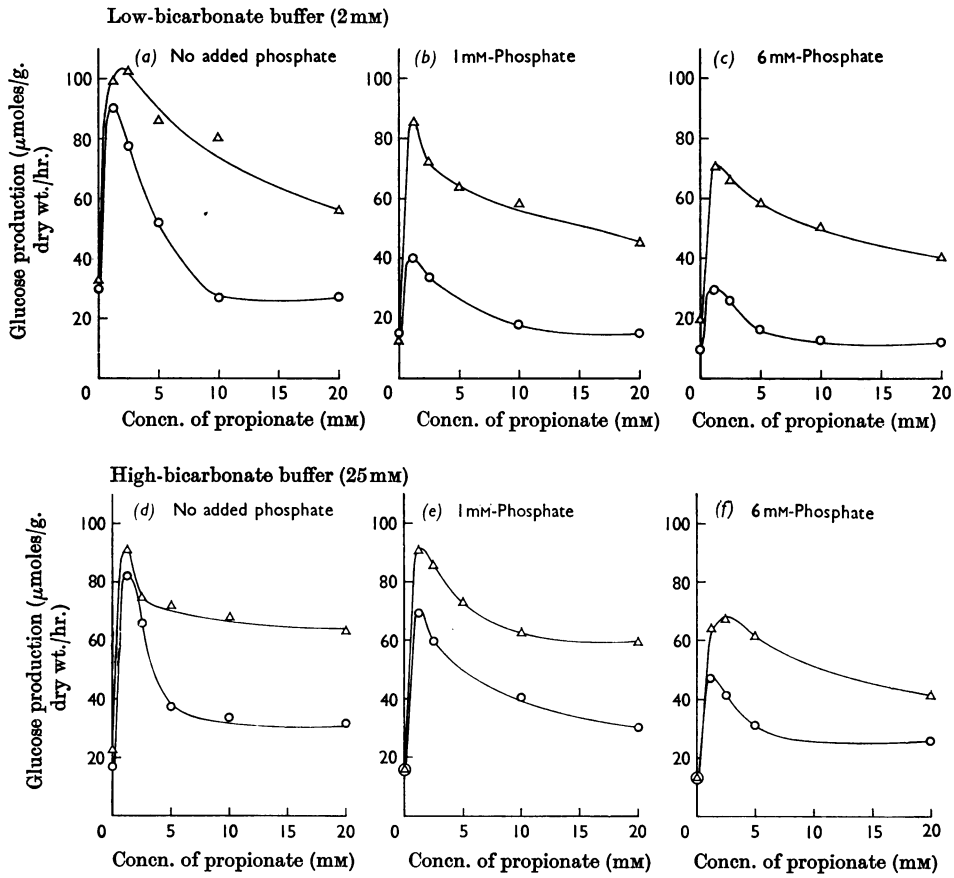

Fig. 1. Effect of inorganic phosphate concentration on gluconeogenesis from propionate with and without added DL-carnitine. (*a*)–(*c*) In buffer containing low bicarbonate concentration (2·0 mM), gassed with 100% O_2; the concentrations of inorganic phosphate were: (*a*) none; (*b*) 1 mM; (*c*) 6 mM. (*d*)–(*f*) In buffer containing high bicarbonate concentration (25 mM), gassed with O_2+CO_2 (95:5); the concentrations of inorganic phosphate were: (*d*) none; (*e*) 1 mM; (*f*) 6 mM. ○, Glucose synthesis in the absence of carnitine; △, glucose synthesis with DL-carnitine (1·0 mM) present.

Table 3. *Effect of* DL-*carnitine on gluconeogenesis from various substrates*

Kidney-cortex slices were incubated in phosphate-buffered saline containing no added bicarbonate. For other experimental details see the text. The results are given as means ± S.D. for four experiments.

Substrate added	O_2 consumption (μmoles/g. dry wt./hr.) No further addition	O_2 consumption (μmoles/g. dry wt./hr.) Plus DL-carnitine (1·0mM)	Stimulation or inhibition due to carnitine (%)	Glucose production (μmoles/g. dry wt./hr.) No further addition	Glucose production (μmoles/g. dry wt./hr.) Plus DL-carnitine (1·0mM)	Stimulation or inhibition due to carnitine (%)
None	929±53	920±60	−1·0	17·5±4·4	20·6±2·9	+17·7
Propionate (5mM)	544±43	1019±98	+87·4	15·5±3·3	44·9±5·3	+189·8
Pyruvate (5mM)	1147±125	1269±147	+10·7	135·5±13·3	138·2±12·4	+2·0
L-Lactate (5mM)	1261±127	1294±94	+2·6	125·7±30·8	132·5±29·4	+5·4
Succinate (10mM)	1701±324	1865±208	+9·6	218·2±27·5	234·4±23·8	+7·4
Fumarate (10mM)	1262±94	1254±153	−0·6	242·7±23·9	251·0±28·3	+3·4
L-Malate (10mM)	1350±142	1331±230	−1·4	273·2±27·8	251·7±55·6	−7·9

With 2·5mM-propionate as substrate, in phosphate-buffered saline, the ratio propionate used/glucose formed fell from 4·5 to 3·6 on addition of 5·0mM-acetoacetate, but neither in the presence nor absence of DL-carnitine (1·0mM) did acetoacetate significantly affect the rate of propionate removal. Gluconeogenesis was slightly increased in both cases, but this increase amounted to no more than 19% and 25% respectively. Similarly, the rates of removal of acetoacetate (and formation of β-hydroxybutyrate) were unaffected by the presence of propionate or propionate plus DL-carnitine. Some mutual inhibition may have been expected, as the metabolism of both acetoacetate and propionate involves an initial activation with CoA. As the effect of acetoacetate addition was essentially negative, the results are not reported in detail.

Effect of DL-*carnitine on gluconeogenesis from other glucose precursors.* A systematic study of the action of DL-carnitine on gluconeogenesis from various substrates showed that its effect on glucose synthesis from propionate was specific. No significant effect of DL-carnitine on either oxygen consumption or glucose synthesis was found with pyruvate, lactate, succinate, fumarate and malate (Table 3). DL-Valine (10mM) and DL-isoleucine (10mM) did not stimulate glucose formation above the blank value, either in the presence or absence of DL-carnitine.

Effect of 10mM-propionate on gluconeogenesis from other glucose precursors. As propionate at concentrations higher than 2·5mM inhibits its own oxidation and conversion into glucose in the absence of added carnitine, the effect of 10mM-propionate on gluconeogenesis from α-oxoglutarate, succinate, malate and lactate was tested to see whether similar inhibitory effects are exerted on glucose formation from other substrates. As shown in Table 4, 10mM-propionate had no inhibitory effects, but it greatly stimulated the formation of glucose from lactate. The extent of this stimulation was not affected by the further addition of DL-carnitine. The stimulation in each case, however,

Table 4. *Effect of propionate on gluconeogenesis from lactate, α-oxoglutarate, succinate and malate in rat kidney-cortex slices*

Kidney-cortex slices were incubated for 60min. in the bicarbonate-buffered saline of Krebs & Henseleit (1932). For other conditions of incubation see the text. In Expt. 1 the rates of lactate removal were 621 μmoles/g. dry wt./hr. with lactate alone and 830 μmoles/g. dry wt./hr. with lactate and propionate.

Expt. no.	Substrates added	Glucose production (μmoles/g. dry wt./hr.) No further addition	Glucose production (μmoles/g. dry wt./hr.) Plus DL-carnitine (1·0mM)
1	None	15	
	Propionate (10mM)	27	61
	L-Lactate (10mM)	120	
	L-Lactate (10mM)+ propionate (10mM)	255	273
2	None	17	
	Propionate (10mM)	40	68
	α-Oxoglutarate (10mM)	230	
	α-Oxoglutarate (10mM)+ propionate (10mM)	260	242
3	None	9	
	Propionate (10mM)	46	64
	Succinate (10mM)	175	
	Succinate (10mM)+ propionate (10mM)	179	198
4	None	16	
	Propionate	38	—
	L-Malate (10mM)	273	
	L-Malate (10mM)+ propionate (10mM)	247	—

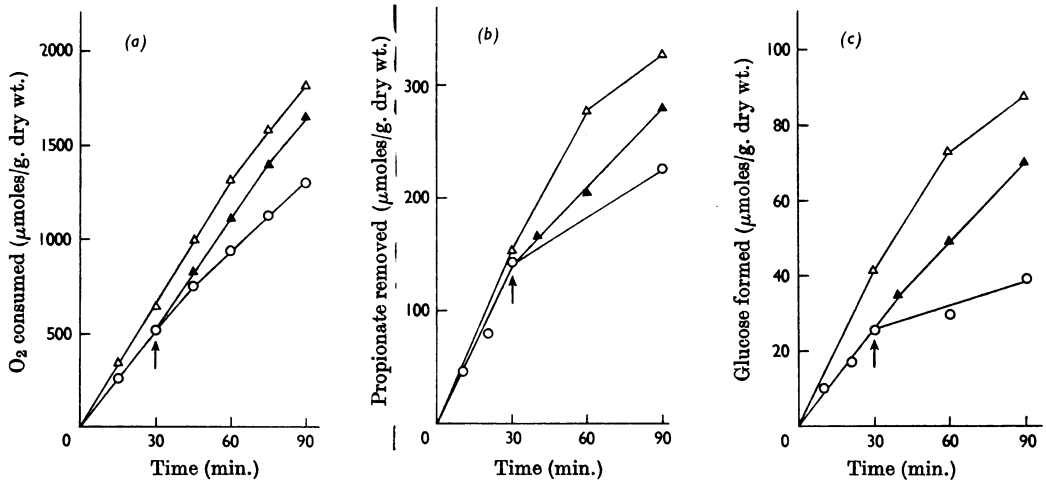

Fig. 2. Effect of DL-carnitine on the time-course of (*a*) oxygen consumption, (*b*) propionate disappearance and (*c*) glucose synthesis by rat kidney-cortex slices. Kidney-cortex slices were incubated in 6 mM-phosphate-buffered saline containing 2 mM-bicarbonate and 2·5 mM-propionate. ○, No added carnitine; △, DL-carnitine (1·0 mM) added at zero time; ▲, DL-carnitine (1·0 mM) added at 30 min., indicated by arrow ↑.

was accompanied by an increase in lactate removal equivalent to the extra glucose formed. The propionate effect is in all probability due to an activation of pyruvate carboxylase, which requires catalytic quantities of acetyl-CoA or propionyl-CoA (Utter & Keech, 1963; Krebs *et al.* 1965). The failure of DL-carnitine to influence the effect may therefore not be surprising.

Time-course of propionate uptake and gluconeogenesis. The rate of propionate uptake during the first 30 min. of incubation was independent of the presence of carnitine (Fig. 2*b*). The rate fell, however, during the next 60 min. unless DL-carnitine was added. Glucose synthesis was lower in the absence of carnitine and the difference increased with time. The addition of DL-carnitine at the beginning of the incubation gave a high rate of gluconeogenesis (more than 1·0 μmole/g. dry wt./min.) that was maintained for 60 min. DL-Carnitine added after 30 min. prevented the sharp fall in glucose synthesis that occurred in its absence (Fig. 2*c*). The stimulatory effect of carnitine on propionate uptake and glucose synthesis was immediate.

Effect of propionate on free CoA content of kidney cortex. The fall in the rate of respiration that occurred on addition of propionate in the absence of carnitine (Table 3 and Fig. 2) can be explained on the assumption that the concentration of free CoA, which may be a factor determining the rate of the tricarboxylic acid cycle and the formation of acetyl-CoA from endogenous long-chain fatty acids, decreases because of an accumulation of propionyl-CoA (see also Pearson & Tubbs, 1967). Accumulation of this intermediate at low concentrations of carnitine or bicarbonate would be expected, because in this situation extramitochondrial propionyl-CoA cannot be further metabolized. The concentration of free CoA in the tissue on addition of propionate was therefore determined. Table 5 shows that, in fact, the concentration of CoA fell on average to one-tenth on incubation with propionate (5·0 mM) and to about one-fifth when both propionate and DL-carnitine were present. A fall in respiration due to low CoA concentration could also account for the decreased rates of gluconeogenesis, since the formation of succinate from propionyl-CoA depends on ATP. Further evidence that propionyl-CoA accumulates under certain conditions is provided by the stimulation of gluconeogenesis from lactate in the presence of 10 mM-propionate (Table 4) and by the accumulation of propionyl-L-carnitine in the incubation medium when DL-carnitine is added (Tables 6, 7 and 8).

Effect of varying the DL-*carnitine concentration.* Ontko (1967) has shown that concentrations of DL-carnitine higher than 1·5 mM inhibited endogenous ketogenesis from fatty acids in rat liver homogenates, and found a sharp optimum of DL-carnitine concentration (1·5 mM) for maximum ketogenesis. The optimum carnitine concentration for gluconeogenesis from propionate in kidney cortex was found to be much broader and to vary with the propionate concentration.

As shown in Fig. 3, more than 80% of the stimulation in glucose synthesis occurred at low

concentrations of DL-carnitine (0·5mM) when the concentration of propionate was 5·0mM or below. Small inhibitory effects, amounting to no more than 25%, were found at higher DL-carnitine concentration (5·0mM). Approximately equimolar concentrations of DL-carnitine and propionate were required for maximum gluconeogenesis when the propionate concentration was high (10–20mM), but the addition of DL-carnitine at optimum concentration was unable to overcome completely the inhibitory effect of the excess of propionate.

Metabolic products of [2-^{14}C]*propionate.* Because the disappearance of propionate is always greater than can be accounted for by the formation of glucose (Table 2), an attempt was made to find the other products of propionate metabolism. Kidney-cortex slices were incubated with [2-^{14}C]propionate in the presence and absence of DL-carnitine. In the experiment shown in Table 6 the ratio propionate used/glucose formed was 4·82 when propionate alone was added and 2·87 when propionate and DL-carnitine were added together.

The distribution of radioactivity at the end of the experiment (Table 7) shows that propionate carbon appeared mainly in glucose and carbon dioxide. Minor quantities appeared in lactate and pyruvate, glycogen, hexose phosphates, protein, lipid, intermediates of the tricarboxylic acid cycle and several amino acids. The presence of DL-carnitine increased the incorporation of radioactivity into glucose 3·6-fold, i.e. to the same extent as it increased net glucose formation. The intermediates related to glucose (glycogen and hexose phosphates) and the

Table 5. *Effect of 5·0mM-propionate on free CoA content of kidney-cortex slices*

Washed kidney-cortex slices from 48hr.-starved rats were incubated for 40min. in phosphate-buffered saline without added bicarbonate. After incubation, slices were frozen in liquid nitrogen, pulverized and mixed with 30% $HClO_4$ (0·5ml.) at 0° and homogenized after the addition of 2·5ml. of water. The protein was removed by centrifugation and the deproteinized extracts were neutralized with 20% KOH. Tissue extracts representing approx. 0·5g. fresh wt. of kidney cortex/cuvette were assayed for free CoA as described in the text. The results are given as means ± S.D. for three experiments.

Substrates added	Free CoA (mμmoles/g. fresh wt.)
None	67±19
Propionate (5·0mM)	6±6
Propionate (5·0mM)+DL-carnitine (4·0mM)	11±2

Table 6. *Net changes on addition of* [2-^{14}C]*propionate to rat kidney-cortex slices in the presence and absence of* DL-*carnitine*

Kidney-cortex slices were incubated for 60min. in phosphate-buffered saline. For full experimental details see the text. The specific radioactivity of [2-^{14}C]propionate was $46·8 \times 10^4$ counts/min./μg.atom of C and the total radioactivity added per cup was 12060×10^3 counts/min. Each cup contained 9–10mg. dry wt. of tissue. The formation of metabolites is indicated by a + sign and disappearance by a − sign.

	Metabolic changes (μmoles/g. dry tissue/hr.)			
			[2-^{14}C]Propionate (2·0mM)	
Substrate added ...	None	DL-Carnitine (4·0mM)	No further addition	Plus DL-carnitine (4·0mM)
O_2	−929	−930	−667	−1213
CO_2	+765	+757	+579	+928
Respiratory quotient	0·83	0·82	0·87	0·77
Glucose	+16·1	+17·9	+22·9	+78·3
Propionate			−110·3	−224·7
Short-chain acyl-carnitine			+2·2	+50·5
Lactate	+8·6	+8·0	+16·7	+12·4
Pyruvate	+1·7	+1·8	+2·4	+1·6
Phosphoenolpyruvate	+1·4	+1·3	+0·7	+1·2
α-Glycerophosphate	+1·2	+1·3	+1·5	+1·7
Succinate+fumarate+malate	+0·7	+0·6	+0·2	+0·8
Percentage contribution of propionate oxidation to O_2 consumption			22·6	14·5
Percentage suppression of endogenous O_2 consumption			44·5	0
Propionate used/glucose formed			4·8	2·9

Table 7. *Isotope distribution on addition of* [2-^{14}C]*propionate with and without* DL-*carnitine to rat kidney-cortex slices at the end of incubation for the experiment recorded in Table* 6

Substrate added	[2-^{14}C]Propionate (2·0 mM)			[2-^{14}C]Propionate (2·0 mM) + DL-carnitine (4·0 mM)		
	10^{-2} × Radioactivity recovered (counts/min.)	Percentage of recovered radioactivity (not including propionate)	10^{-4} × Specific radioactivity (counts/min./ μg.atom of C)	10^{-2} × Radioactivity recovered (counts/min.)	Percentage of recovered radioactivity (not including propionate)	10^{-4} × Specific radioactivity (counts/min./ μg.atom of C)
Propionate	104260		46·04	77522		47·20
Propionyl-L-carnitine	292	2·1	39·43*	5522	14·5	32·75*
CO_2	7006	51·4	10·91	13119	34·6	12·93
Glucose	4279	31·4	29·44	15424	40·6	28·55
Glycogen	22	0·2		121	0·3	
Hexose phosphates	134	1·0		286	0·8	
Aspartate	172	1·3		208	0·6	
Glutamate	191	1·4		278	0·7	
Glutamine + glycine + serine	496	3·6		1513	4·0	
Alanine	178	1·3		356	0·9	
Lactate + pyruvate	458	3·4		462	1·2	
α-Oxoglutarate	36	0·3		94	0·3	
Succinate	16	0·1		52	0·1	
Protein	243	1·8		418	1·1	
Lipid (heptane extract of slice)	129	0·9		143	0·4	
Total radioactivity recovered	117912			115518		
Percentage recovery	97·8			95·8		

* Uncorrected for unlabelled acetyl-L-carnitine formed.

Table 8. *Production and metabolism of propionyl-*L-*carnitine by rat kidney-cortex slices*

Kidney-cortex slices were incubated for 60 min. in phosphate-buffered saline with no added bicarbonate. For full experimental details see the text.

	Metabolic changes (μmoles/g. dry wt./hr.)					
		Propionate (2·0 mM)				
Substrates added ...	None	No further addition	Plus DL-carnitine (4·0 mM)	Plus L-carnitine (2·0 mM)	Plus D-carnitine (2·0 mM)	Propionyl-L-carnitine (2·0 mM)
O_2	−872	−897	−1218	−1202	−646	−1036
Glucose	+13·7	+29·2	+81·8	+75·2	+19·9	+49·2
Propionate	—	−115	−254	−269	−70	—
Propionyl-L-carnitine	+0·6	+3·2	+49·5	+59·2	0	−124
Final concn. of propionyl-L-carnitine in the incubation medium (mM)	0·002	0·008	0·15	0·19	0	1·64

intermediates related to the tricarboxylic acid cycle (α-oxoglutarate, succinate, aspartate, glutamate, glutamine and alanine) all showed an increased radioactivity on addition of carnitine. A relatively large proportion (15%) of the [2-^{14}C]propionate taken up in the presence of DL-carnitine was recovered as [^{14}C]propionyl-L-carnitine. Of the added radioactivity about 98% was recovered when propionate alone was added and 96% when propionate and DL-carnitine were added together.

In the absence of carnitine 51% of the [2-^{14}C]-propionate removed was recovered as $^{14}CO_2$. The absolute amount of radioactivity recovered in this fraction was about twice as high when DL-carnitine was added, but the percentage of the [2-^{14}C]-propionate converted into $^{14}CO_2$ was somewhat lower (35%). From the net values (Table 6) it was calculated that the contribution of propionate to the oxygen consumption of the tissue was 23% in the absence of carnitine and 15% when DL-carnitine was

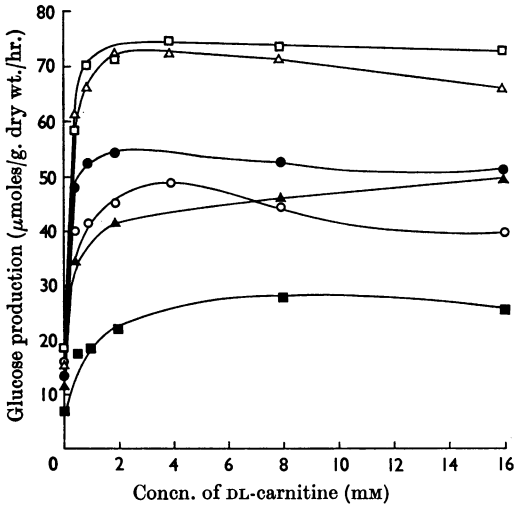

Fig. 3. Effect of DL-carnitine concentration on gluconeogenesis from propionate. Kidney-cortex slices were incubated in 6mM-phosphate buffer. The concentrations of propionate in the incubating medium were: ○, 0·625mM; △, 1·25mM; □, 2·5mM; ●, 5·0mM; ▲, 10·0mM; ■, 20·0mM.

added. The ratio of the specific radioactivity per μg. atom of C of the respiratory carbon dioxide/specific radioactivity of added [2-^{14}C]propionate in the presence of DL-carnitine was distinctly higher (29% instead of 15%) than that calculated simply on the basis of the net changes. However, this is to be expected if the [2-^{14}C]- or [3-^{14}C]-oxaloacetate formed from [2-^{14}C]propionate (Eggerer, Stadtman, Overath & Lynen, 1961) passes through the tricarboxylic acid cycle before giving rise to phosphoenolpyruvate (Krebs *et al.* 1966). If oxaloacetate passes more than once through the cycle some C-2 carbon of propionate appears in carbon dioxide even though there is a quantitative net conversion of propionate into glucose (for details of the reason see Krebs *et al.* 1966).

Formation and utilization of propionyl-L-carnitine by kidney cortex. The assumption that propionyl-L-carnitine is an obligatory intermediate of propionate metabolism is supported by the observation that it accumulates in the medium on incubation of kidney-cortex slices with propionate plus DL- or L-carnitine (Table 8). DL-Carnitine and L-carnitine gave a similar concentration (about 0·2mM), whereas D-carnitine suppressed the small endogenous formation and inhibited tissue respiration. As expected, D-carnitine did not stimulate gluconeogenesis. Added propionyl-L-carnitine (2·0mM) was rapidly removed and gave rise to net glucose synthesis. The rate in this experiment was somewhat lower than the rates of glucose formation observed with propionate (2·0mM) plus DL- or L-carnitine. Further experiments showed that the propionyl-L-carnitine concentration used was suboptimum; at 4·0mM, rates of gluconeogenesis similar to those found with propionate plus L-carnitine were observed.

Effect of light and washing on gluconeogenesis by kidney-cortex slices. Kidney-cortex slices from vitamin B_{12}-deficient rats do not synthesize glucose from propionate, and the uptake of propionate is decreased by 75% compared with tissue from litter mates given a vitamin B_{12} supplement (M. J. Weidemann, H. A. Krebs, D. L. Williams & G. H. Spray, unpublished work). A special problem in studying propionate metabolism in tissue from normal animals is the possibility of loss of activity of methylmalonyl-CoA mutase due to washing out or inactivation of the B_{12}-coenzyme, which is light-sensitive (Weissbach, Toohey & Barker, 1959). To check for loss of B_{12}-coenzyme activity in normal tissue, the effect on gluconeogenesis of high illumination and washing of the slices during preparation was compared with glucose synthesis in control slices. Unwashed slices gave rates of gluconeogenesis 48% higher than those washed for 5min. This effect, however, was eliminated by the addition of acetoacetate (5·0mM). The lower rates in washed slices are therefore ascribed to loss of endogenous substrates rather than to effects on vitamin B_{12} concentrations. Exposure of the slices to high illumination during preparation (150w lamp 4in. above the flask for 5min.) did not affect the rates of glucose synthesis in comparison with slices prepared under Kodak no. 1 (red) Safelight and incubated in total darkness. Addition of dimethylbenzimidazolyl-cobamide (10μg./ml.) to the incubation medium and incubation in total darkness did not increase the rate of glucose synthesis by more than 10%. As with the unresolved holoenzyme from sheep kidney cortex (Lengyel, Mazumder & Ochoa, 1960), methylmalonyl-CoA mutase from rat kidney cortex is apparently not light-sensitive *in situ*.

Effect of diet on maximum rates of gluconeogenesis from propionate. The major source of blood propionate in the rat is the caecal fermentation of material not attacked by the gastric and intestinal enzymes. Propionate concentrations as high as 9mM have been observed in the caecal contents, and concentrations of up to 0·2mM in the portal blood, of the well-fed rat (Keane, 1967). Diets that stimulate or abolish caecal fermentation and diets containing tripropionin would be expected to influence the concentration of propionate in the blood. The occurrence of inducible changes in the enzymes of propionate metabolism in response to diet might then be reflected in alterations in the

Table 9. *Effect of diet on maximum rates of gluconeogenesis from propionate by rat kidney-cortex slices*

The low-carbohydrate diet consisted of casein (75%) and margarine (25%). One group of animals were given 0·5% Neomycin in the drinking water. 25% Tripropionin was used instead of margarine with a further group; this diet contained, in addition, 100 μg. of cyanocobalamin/kg. of diet to avoid toxic effects of tripropionin. The high-roughage diet consisted of rat cubes (5%) and whole bran (95%). With the exception of the tripropionin diet, which was of 14 days duration, all treatments were for 3 days. Kidney-cortex slices were incubated in bicarbonate-buffered saline for 60 min. The cups containing propionate (2·0 mM) always contained, in addition, DL-carnitine (1·0 mM) and acetoacetate (5·0 mM). The results are given as means ± S.D. for three experiments.

Diet	Glucose formed (μmoles/g. dry wt./hr.)		Stimulation over rate for balanced diet (%)
	No added substrate	Propionate (2·0 mM)	
Normal balanced diet	4·6 ± 1·2	76·4 ± 2·2	
95%-Roughage	10·5 ± 1·3	79·7 ± 2·0	4
Low-carbohydrate	7·1 ± 1·5	92·7 ± 5·9	21
Low-carbohydrate (+0·5% Neomycin)	12·1 ± 2·3	95·4 ± 8·4	25
Low-carbohydrate (+25% tripropionin)	14·0 ± 1·4	105·0 ± 10·0	37
Starved (48 hr.)	14·1 ± 0·4	93·3 ± 3·3	22

capacity of kidney-cortex slices to form glucose from propionate.

To test this, gluconeogenesis from propionate was measured with tissue from rats kept on different diets (Table 9). In general, the dietary effects were similar to those described by Krebs *et al.* (1963) for a variety of substrates: gluconeogenesis was stimulated by 48hr. of starvation and by feeding on low-carbohydrate high-protein diets. Neomycin treatment, which decreased the caecal propionate concentration by 70% to 3 mM (Keane, 1967), did not lower the rate of glucose synthesis; conversely, the inclusion of 95% of roughage or 25% of tripropionin in the diet gave no significant stimulation.

The increased gluconeogenesis after 48hr. of starvation was accompanied by a rise of only 10% in the uptake of propionate by slices. Propionate oxidized to carbon dioxide in the well-fed state (contributing up to 43% of the total oxygen consumption) is thus 'spared' for gluconeogenesis in starvation, when the contribution of propionate oxidation to the total oxygen consumption falls to 23%.

Gluconeogenesis from propionate in various species. Krebs & Yoshida (1963) obtained high rates of gluconeogenesis from 10 mM-propionate when kidney-cortex slices from sheep and rabbits were incubated in bicarbonate-buffered saline. The effect of DL-carnitine on gluconeogenesis from propionate over a wide concentration range was investigated in these and other species under similar conditions of incubation (Fig. 4).

The most striking feature is the lack of inhibition of gluconeogenesis by high propionate concentration in herbivorous species normally dependent on high rates of ruminal or caecal volatile fatty acid production for their nutrition (sheep, cattle and rabbit). In these species 10 mM-propionate was near optimum for gluconeogenesis, whereas in tissues from the rat, guinea pig and pig gluconeogenesis fell steeply at concentrations of propionate higher than 2·0 mM. In general terms, DL-carnitine only affected the rates of glucose synthesis in those species where propionate was inhibitory at low concentration.

DISCUSSION

Nature of the action of carnitine on propionate metabolism. All observations reported in this paper on the accelerating action of carnitine on propionate metabolism are in accordance with the view that the mechanism of action of carnitine in this case is analogous to its effect on the metabolism of other fatty acids, namely that it acts as a carrier of acyl groups across the internal mitochondrial membrane. This view is especially supported by the fact that propionyl-L-carnitine accumulated under certain conditions and that propionyl-L-carnitine can also be readily metabolized (Table 8). The step in which carnitine participates is taken to be the readily reversible reaction:

$$\text{L-Carnitine} + \text{propionyl-CoA} \rightleftharpoons \text{propionyl-L-carnitine} + \text{CoA}$$

catalysed by carnitine acetyltransferase (EC 2.3.1.7) (Fritz, Schultz & Srere, 1963).

The stimulating effect of carnitine suggests that at low concentrations of carnitine the formation of propionyl-L-carnitine was limiting the rate of propionate utilization. The accumulation of propionyl-L-carnitine at higher carnitine concentrations (Tables 6, 7 and 8) implies that under these conditions either the mitochondrial step propionyl-

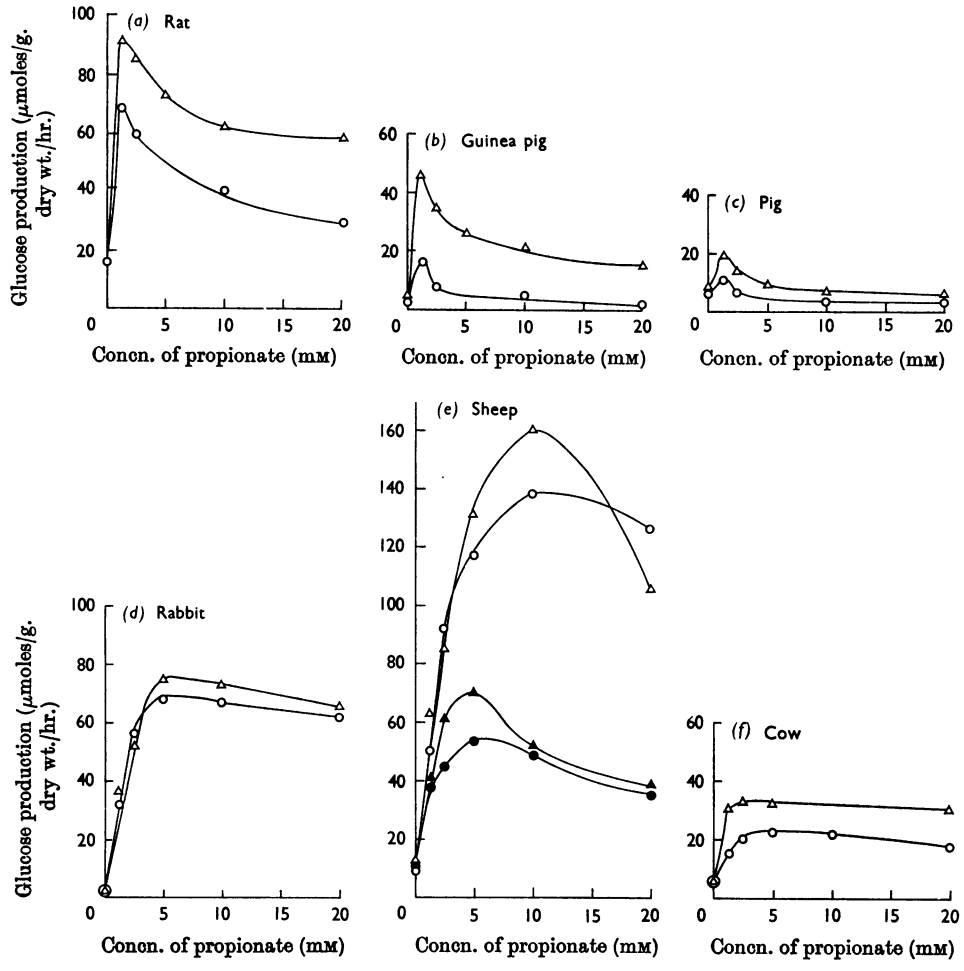

Fig. 4. Effect of propionate concentration and DL-carnitine on gluconeogenesis by kidney-cortex slices from various species: (*a*) rat; (*b*) guinea pig; (*c*) pig; (*d*) rabbit; (*e*) sheep; (*f*) cow. Glucose synthesis from propionate in 25mM-bicarbonate-buffered saline: ○, with no added carnitine; △, with 1·0mM-DL-carnitine. Glucose synthesis from propionate in 6mM-phosphate-buffered saline: ●, with no added carnitine; ▲, with 1·0mM-DL-carnitine.

L-carnitine → propionyl-CoA or the carboxylation of propionyl-CoA was rate-limiting. That the formation of glucose as well as the oxidation of propionate was accelerated by carnitine is due to the fact that both processes are initiated by the same sequence of reactions, leading from propionate to oxaloacetate.

If the basic mechanism of action of carnitine is therefore the same for propionate and other fatty acids, there is a remarkable difference in the magnitude of the effect. This is far greater with propionate than with any other fatty acid studied in the intact tissue under similar conditions. The rate of removal of propionate by kidney-cortex slices was more than doubled by carnitine (Table 2), whereas the rates of removal of acetate, butyrate and oleate were hardly affected (M. J. Weidemann & H. A. Krebs, unpublished work). The stimulatory effect of DL-carnitine on $^{14}CO_2$ production from [1-^{14}C]palmitate by rat kidney-cortex homogenates is only slight (Fritz, 1964).

Effects of the concentrations of bicarbonate, phosphate and carnitine. A striking feature of the action of carnitine is the interplay between the concentrations of carnitine, bicarbonate and phosphate. Under some conditions carnitine can be largely replaced by the addition of bicarbonate, and phosphate antagonizes the action of carnitine. Bicarbonate and inorganic phosphate are reactant and end product respectively of the first step of

the propionyl-CoA carboxylase reaction (Kaziro, Hass, Boyer & Ochoa, 1962) and, as Dr Irving Fritz has suggested to us, it is very likely that the effects of bicarbonate and phosphate described in this paper are connected with their participation in this step. The following considerations show how these effects, and the inhibitory effect of high concentrations of carnitine, can be explained, on the assumption that the reactions leading from propionate to methylmalonyl-CoA are all reversible (Tietz & Ochoa, 1959; Fritz *et al.* 1963). The first three steps of propionate metabolism taking place in the mitochondrion are the following (Kaziro *et al.* 1962):

$$\text{Propionyl-L-carnitine} + \text{CoA} \rightleftharpoons \text{propionyl-CoA} + \text{L-carnitine}$$

$$\text{ATP} + HCO_3^- + \text{enzyme} \rightleftharpoons \text{ADP} + P_i + HCO_3^-\text{–enzyme}$$

$$HCO_3^-\text{–enzyme} + \text{propionyl-CoA} \rightleftharpoons \text{enzyme} + \text{methylmalonyl-CoA}$$

$$\text{Sum:}\quad \text{Propionyl-L-carnitine} + \text{CoA} + \text{ATP} + HCO_3^- \rightleftharpoons \text{L-carnitine} + \text{ADP} + P_i + \text{methylmalonyl-CoA}$$

At equilibrium the following relation holds:

$$K = \frac{[\text{L-carnitine}]}{[\text{propionyl-L-carnitine}]} \times \frac{[\text{ADP}][P_i]}{[\text{ATP}]} \times \frac{[\text{methylmalonyl-CoA}]}{[\text{CoA}][HCO_3^-]}$$

or

$$[\text{Methylmalonyl-CoA}] = K \times \frac{[\text{CoA}][HCO_3^-][\text{ATP}]}{[\text{ADP}][P_i]} \times \frac{[\text{propionyl-L-carnitine}]}{[\text{L-carnitine}]}$$

If the concentration of methylmalonyl-CoA limits the rate of succinate formation from propionate (which is very likely), it would be expected that a rise of $[HCO_3^-]$ can promote the rate of propionate removal, and that a rise of $[P_i]$ and [L-carnitine] can inhibit it. It would also be expected that the extents of these promoting and inhibitory effects depend on the intramitochondrial concentrations of the other reactants occurring in the above expression. All these postulates are in accord with the observed facts.

Inhibition of fatty acid oxidation by phosphate. The preceding considerations are analogous to those put forward by van den Bergh (1966) to explain the inhibition of fatty acid oxidation by phosphate (see also Davis, 1965). This inhibition occurs when fatty acid oxidation in mitochondria is initiated by the GTP-dependent reaction discovered by Rossi & Gibson (1964):

$$\text{GTP} + \text{fatty acid} + \text{CoA} \rightleftharpoons \text{acyl-CoA} + \text{GDP} + P_i$$

If this reaction is reversible, inorganic phosphate would decrease the equilibrium concentration of acyl-CoA, which may determine the rate of fatty acid oxidation.

Explanation of the inhibitory effect of excess of carnitine on the utilization of fatty acids. The acceleration by carnitine of the formation of ketone bodies by liver homogenates shows a sharp optimum concentration at about 1·5 mM-DL-carnitine (Ontko, 1967; W. Feldheim & H. A. Krebs, unpublished work cited by Krebs, 1967). The inhibition by higher concentrations of carnitine can be explained on the basis of the principles discussed in the preceding paragraphs. The inhibitory effect would be expected if: (*a*) the rate of utilization of acyl-CoA rises with the concentration of acyl-CoA; (*b*) equilibrium exists in the mitochondrion in the carnitine acyltransferase systems so that:

$$[\text{Acyl-CoA}] = \frac{K \times [\text{acyl-carnitine}][\text{CoA}]}{[\text{carnitine}]}$$

In this case [acyl-CoA] is inversely proportional to [carnitine]. With acetyl-CoA such an equilibrium exists in rat heart (Pearson & Tubbs, 1967).

At low carnitine concentrations the rate of acyl-CoA formation is limited by the availability of carnitine, so that in this range carnitine stimulates the formation of acyl-CoA.

Compartmentation of methylmalonyl-CoA synthesis. If carnitine in effect transfers propionyl-CoA from one compartment to another, it would appear that propionyl-CoA is first synthesized in a compartment (presumably the cytoplasm) different from that in which it is further metabolized (the mitochondrion). It is feasible that equilibrium conditions of the kind discussed in the preceding paragraphs make it necessary for this compartmentation to exist. If propionyl-CoA were metabolized by reversible reactions in the same com-

partment in which it is synthesized, the following reactions would all occur in one compartment:

$$\begin{aligned} \text{ATP} + \text{propionate} + \text{CoA} &\rightleftharpoons \text{propionyl-CoA} + \text{AMP} + \text{PP}_i \\ \text{ATP} + \text{HCO}_3^- + \text{enzyme} &\rightleftharpoons \text{ADP} + \text{P}_i + \text{HCO}_3^-\text{–enzyme} \\ \text{HCO}_3^-\text{–enzyme} + \text{propionyl-CoA} &\rightleftharpoons \text{enzyme} + \text{methylmalonyl-CoA} \\ \text{PP}_i &\rightleftharpoons 2\text{P}_i \\ \text{AMP} + \text{ATP} &\rightleftharpoons 2\text{ADP} \end{aligned}$$

The combination of these reactions gives:

$$[\text{Methylmalonyl-CoA}] = K \times \frac{[\text{propionate}][\text{CoA}][\text{HCO}_3^-][\text{ATP}]^3}{[\text{ADP}]^3[\text{P}_i]^3}$$

This relation would mean that the concentration of methylmalonyl-CoA would be extremely sensitive to variations in the concentrations of ATP, ADP and phosphate, as the above term includes the cubes of these concentrations. For example, if, in a given situation, the ATP concentration were 3mM, that of ADP 1mM and that of phosphate 2mM, and if, then, one-third of the ATP were converted into ADP and phosphate, the steady-state concentration of methylmalonyl-CoA at equilibrium would decrease by the factor 91·3.

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REFERENCES

Baumgardt, B. R. (1964). *Bull. Dairy Sci. Dep., Univ. Wisconsin*, p. 1.
Bøhmer, T. & Bremer, J. (1968). *Biochim. biophys. Acta*, **152**, 559.
Crowley, G. J., Moses, V. & Ullrich, J. (1963). *J. Chromat.* **12**, 219.
Czok, R. & Eckert, L. (1963). In *Methods of Enzymatic Analysis*, p. 224. Ed. by Bergmeyer, H.-U. New York: Academic Press Inc.
Davis, E. J. (1965). *Biochim. biophys. Acta*, **96**, 217.
Dole, V. P. & Meinertz, H. (1960). *J. biol. Chem.* **235**, 2595.
Eckstein, H. C. (1933). *J. biol. Chem.* **102**, 591.
Eggerer, H., Stadtman, E. R., Overath, P. & Lynen, F. (1961). *Biochem. Z.* **333**, 1.
Friedman, S. & Fraenkel, G. (1955). *Arch. Biochem. Biophys.* **59**, 491.
Fritz, I. B. (1964). *Amer. J. Physiol.* **206**, 1217.
Fritz, I. B., Schultz, S. K. & Srere, P. A. (1963). *J. biol. Chem.* **238**, 2509.
Garland, P. B. (1964). *Biochem. J.* **92**, 10c.
Gevers, W. & Krebs, H. A. (1966). *Biochem. J.* **98**, 720.
Kaziro, Y., Hass, L. F., Boyer, P. D. & Ochoa, S. (1962). *J. biol. Chem.* **237**, 1460.
Keane, D. M. (1967). D.Phil. Thesis: University of Oxford.
Krebs, H. A. (1967). 3. *Konf. Ges. biol. Chemie, Oestrich/Rheingau*, p. 129. Berlin: Springer-Verlag.
Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.
Krebs, H. A. & de Gasquet, P. (1964). *Biochem. J.* **90**, 147.
Krebs, H. A., Dierks, C. & Gascoyne, T. (1964). *Biochem. J.* **93**, 112.
Krebs, H. A., Hems, R., Weidemann, M. J. & Speake, R. N. (1966). *Biochem. J.* **101**, 242.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Krebs, H. A., Speake, R. N. & Hems, R. (1965). *Biochem. J.* **94**, 712.
Krebs, H. A. & Yoshida, T. (1963). *Biochem. J.* **89**, 398.
Leng, R. A. & Annison, E. F. (1963). *Biochem. J.* **86**, 319.
Lengyel, P., Mazumder, R. & Ochoa, S. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1312.
Ontko, J. A. (1967). *Biochim. biophys. Acta*, **137**, 1.
Pearson, D. J. & Tubbs, P. K. (1967). *Biochem. J.* **105**, 953.
Pennington, R. J. (1954). *Biochem. J.* **56**, 410.
Ringer, A. I. (1912). *J. biol. Chem.* **12**, 511.
Rodgers, K. (1961). *Biochem. J.* **80**, 240.
Rossi, C. R. & Gibson, D. M. (1964). *J. biol. Chem.* **239**, 1694.
Tietz, A. & Ochoa, S. (1959). *J. biol. Chem.* **234**, 1394.
Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
van den Bergh, S. G. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 125. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
Weidemann, M. J. & Krebs, H. A. (1967). *Biochem. J.* **104**, 71P.
Weissbach, H., Toohey, J. & Barker, H. A. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 521.
Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962). *Biochem. J.* **82**, 90.