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Regulation of carnitine palmitoyltransferase activity by malonyl-CoA in mitochondria from sheep liver, a tissue with a low capacity for fatty acid synthesis

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1. The characteristics of inhibition of carnitine palmitoyltransferase (CPT) I by malonyl-CoA were studied for the enzyme in mitochondria isolated from sheep liver, a tissue with a very low rate of fatty acid synthesis. 2. Malonyl-CoA was as potent in inhibiting the sheep liver enzyme as in inhibiting the enzyme in rat liver mitochondria. CPT I in guinea-pig liver mitochondria was also similarly inhibited. The inhibition showed the same time-dependent characteristics previously established for the rat liver enzyme. 3. Methylmalonyl-CoA was as effective an inhibitor of CPT I as malonyl-CoA in sheep liver mitochondria, but did not affect CPT I activity in mitochondria from rat or guinea-pig liver. 4. The concentrations of malonyl-CoA required to inhibit CPT I in sheep liver mitochondria *in vitro* were similar to those found in freeze-clamped sheep liver samples (about 7 nmol of malonyl-CoA/g wet wt.). 5. In sheep liver cells the content of malonyl-CoA was only one-tenth of that observed *in vivo* when glucose only was added to the incubation medium. Inclusion of acetate and/or insulin increased the malonyl-CoA content about 10-fold, to values similar to those observed *in vivo*. 6. The rate of fatty acid synthesis in sheep liver cells was about 1% of that observed in rat liver, but was correlated with the concentrations of malonyl-CoA in the cells under various incubation conditions. 7. These observations are discussed in relation to (i) the regulatory role of malonyl-CoA in tissues that have a low capacity for fatty acid synthesis, and (ii) the utilization by sheep liver of propionate as a gluconeogenic precursor.

INTRODUCTION

The inhibition of overt carnitine palmitoyltransferase (CPT I) of mitochondria by malonyl-CoA was discovered by McGarry *et al.* (1977), using homogenates of rat liver. It has provided the rationale for a mechanism whereby, in a tissue that is capable of substantial rates of both fatty acid synthesis and oxidation, the operation of the two pathways does not occur simultaneously. However, it has become evident that the sensitivity of CPT I to malonyl-CoA inhibition is a widespread property of the enzyme, found even in tissues that have negligible capacity for fatty acid synthesis and hence for malonyl-CoA as intermediate (e.g. cardiac and skeletal muscle: McGarry *et al.*, 1978*a*; Saggerson & Carpenter, 1981; Mills *et al.*, 1983).

Not all mammalian species have high capacities for hepatic fatty acid synthesis. For example, in the liver of ruminants and the guinea pig the rate is only about 1% of that in the rat on a unit-wet-weight basis (Ingle *et al.*, 1972; Patel & Hanson, 1979; Liepa *et al.*, 1978). However, in these animals the requirement for regulation of the rate of hepatic fatty acid oxidation and of CPT I activity is just as pertinent as in the rat (for review see Zammit, 1983). This is evident from the high rates of ketone-body formation that occur in the liver of these species under conditions of limited glucose availability. Consequently, with respect to the involvement of malonyl-CoA in the regulation of the rate of fatty acid oxidation, the situation in these livers is similar to that which obtains in rat cardiac and skeletal muscle, namely that the activity of CPT I still has to be modulated in response to the changing physiological requirements of the tissue, despite a low rate of turnover of malonyl-CoA in fatty acid synthesis. This situation may appear paradoxical if one generalizes from the original observation that in rat liver the rate of fatty acid synthesis is proportional to the hepatic content of malonyl-CoA (Guynn *et al.*, 1972), such that high concentrations of malonyl-CoA only occur during high rates of fatty acid synthesis.

We were therefore interested in establishing (i) whether, in a tissue with a low rate of fatty acid synthesis, proportionality between this rate and malonyl-CoA concentration exists, and (ii) the range of malonyl-CoA concentrations in intact cells over which such proportionality is displayed. Isolated liver cells can be readily prepared from sheep (Donaldson *et al.*, 1979) (cf. preparation of cells from other tissues with a low capacity for fatty acid synthesis, e.g. rat heart or skeletal muscle), so that we have chosen these cells for studies of the above relationship. We have previously reported that CPT I in mitochondria isolated from sheep liver is sensitive to inhibition by malonyl-CoA (see Lomax *et al.*, 1983; Zammit, 1983). This system therefore also permitted us to test quantitatively whether CPT I of mitochondria

Abbreviation used: CPT, carnitine palmitoyltransferase (EC 2.3.2.21).

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from sheep liver exhibits inhibition by malonyl-CoA with the same characteristics as those shown by the enzyme in rat liver mitochondria. In addition we could test the action of other metabolites characteristic of ruminant liver metabolism on the activity of CPT I. We have therefore made a comparative study of the potency of several short-chain acyl-CoA esters as inhibitors of CPT I in mitochondria from the livers of rats, sheep and guinea pigs. The guinea pig was chosen because this species exhibits a low capacity for hepatic fatty acid synthesis, but differs from ruminants in that this is not accompanied by permanently high rates of gluconeogenesis from propionate. A preliminary account of some of the results presented in this paper has been presented elsewhere (Brindle *et al.*, 1985).

MATERIALS AND METHODS

Animals

Rats were females of the Wistar strain. Their source and maintenance were as described previously (Zammit, 1980). Sheep were 7–9-month-old males of the Fin × Dorset Horn strain, and they were fed on 300 g of cereal mix per day, with access to hay and water *ad libitum*. Guinea pigs were females of the Dunkin–Hartley strain.

Preparation of isolated sheep liver cells

Sheep were anaesthetized by an intrajugular infusion of sodium pentabarbitone (25 mg/kg body wt.) through a cannula which was introduced into the blood vessel 24 h before induction of anaesthesia. The abdominal cavity was quickly opened and the caudate lobe of the liver excised and immediately perfused with medium (Krebs & Henseleit, 1932) at 37 °C. Isolated liver cells were prepared as described by Lomax *et al.* (1983) except that 1 mM-$CaCl_2$ and 20 mM-D-glucose were included in the perfusion medium and that, 2 min before the end of the perfusion, Ca^{2+} was sequestered by addition of 1 mM-EGTA to the medium. After isolation, the cells were preincubated for 15 min without albumin. This procedure was found to increase both the proportion of viable cells obtained and their hormone-sensitivity.

Incubation of liver cells

Cells were incubated at 37 °C (40–50 mg dry wt./flask) in 4 ml of medium (Krebs & Henseleit, 1932) containing 2% (w/v) defatted albumin (Chen, 1967) and 5 mM-glucose in silicone-treated glass conical flasks (Kontes, Vineland, NJ, U.S.A.). These were gassed, stoppered and shaken at 100 cycles/min. After 15 min substrates and hormones and, where appropriate, ^3H_2O (1 mCi) were added. When rates of fatty acid synthesis were being measured, incubations were terminated by centrifugation (500 *g* for 30 s) and lipid was extracted from the homogenized cells by the method of Folch *et al.* (1957). Saponifiable and non-saponifiable lipids were separated as described by Brunengraber *et al.* (1973), and incorporation of ^3H into fatty acids was quantified by liquid-scintillation counting. In experiments in which cellular malonyl-CoA concentrations was measured, incubations were terminated by rapid separation of cells from the medium by centrifugation in plastic tubes, followed by freezing of the pellets in liquid N_2. Malonyl-CoA was measured in neutralized supernatants of $HClO_4$ extracts (Zammit, 1981).

Preparation of mitochondria and assay of CPT I activity

These were performed as described previously for rat liver mitochondria (Zammit, 1980; Robinson & Zammit, 1982).

Freeze-clamping of sheep liver

A portion of the left lateral lobe of the liver exposed after opening of the abdominal cavity was frozen *in situ* between aluminium blocks cooled in liquid N_2 (Wollenberger *et al.*, 1960). Samples of frozen tissue were pulverized in a stainless-steel mortar cooled with liquid N_2; extracts in 6% (v/v) $HClO_4$ were prepared using a Polytron tissue disintegrator (Kinematica, Basle, Switzerland).

Protein was measured by the method of Lowry *et al.* (1951).

Chemicals

The source of these was as described previously (Zammit, 1980). In addition crystalline pig insulin was from Sigma (Poole, Dorset, U.K.) and collagenase was from BCL Chemicals (Lewes, East Sussex, U.K.).

RESULTS

Concentration of malonyl-CoA in freeze-clamped sheep liver and in isolated hepatocytes

The concentration of malonyl-CoA in freeze-clamped sheep liver was 7.08 ± 1.35 nmol/g wet wt. (mean ± S.E.M. for four determinations). This value is comparable with the range of 3–12 nmol/g reported for livers from fed rats (see, e.g., Lynen, 1979; Zammit, 1981). In sheep liver cells incubated with glucose (5 mM) in the absence of added hormones, the steady-state concentration of malonyl-CoA attained between 15 and 45 min of cell incubation was 3.1 ± 0.3 nmol/g dry wt., equivalent to 0.84 nmol/g wet wt. This was almost an order of magnitude lower than the value observed *in vivo*. However, when cells were incubated either with acetate (plus glucose) or with insulin in the presence of glucose, the content of malonyl-CoA was increased to 8.6 and 17.5 nmol/g dry wt. respectively.

Relationship between the rate of fatty acid synthesis and the concentration of malonyl-CoA in isolated hepatocytes

The two parameters were measured for the 30 min incubation period between 15 and 45 min of cell incubation. The rate of fatty acid synthesis was reproducibly linear over this interval, and malonyl-CoA concentrations also reached steady-state values. The rates of fatty acid synthesis observed were very low compared with those obtained for rat hepatocytes (McGarry *et al.*, 1978*b*). In the presence of glucose the rate of ^3H incorporation into fatty acids was 0.81 μg-atom of ^3H/h per g wet wt. Inclusion of lactate had no effect, but acetate, butyrate or ketone bodies increased the rate by about 20%. Insulin when added either alone or in combination with acetate increased the rate of fatty acid synthesis even higher (Table 1). Glucagon inhibited the rate observed in the presence of acetate to the value obtained with glucose alone.

A linear relationship was observed between the rate of fatty acid synthesis obtained under the different conditions described above and the content of malonyl-CoA in the liver cells (Fig. 1). This relationship was

Table 1. Effects of different substrates and hormones on the rate of fatty acid synthesis by isolated sheep liver cells

Glucose (5 mM) was present in all cell incubations, and other substrates were added as shown. When added, insulin and glucagon were present at 1.5 munits/ml and 0.1 μM respectively. The numbers of determinations performed on separate hepatocyte preparations are shown in parentheses. Values are means ± S.E.M. Those that are significantly different (t test, $P < 0.05$) from the rate observed with glucose only are denoted by an asterisk.

Substrate(s) in addition to glucose	Hormone	Rate of fatty acid synthesis (μg-atoms of ^3H/g wet wt. per h)
None	—	0.81 ± 0.04 (4)
	Insulin	1.12 ± 0.04 (3)*
Acetate (10 mM)	—	0.99 ± 0.10 (3)*
	Insulin	1.26, 1.34 (2)
	Glucagon	0.79 ± 0.04 (3)
Butyrate (10 mM)	—	1.00 ± 0.02 (3)*
Acetoacetate (1 mM) plus L-3-hydroxybutyrate (10 mM)	—	0.97 ± 0.06 (3)*
Lactate (10 mM) plus pyruvate (1 mM)	—	0.75 ± 0.01 (3)

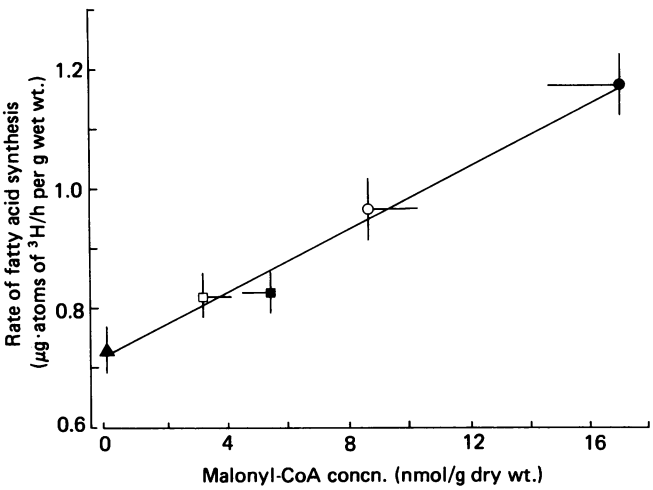

Fig. 1. Relationship between the rate of fatty acid synthesis and the content of malonyl-CoA in sheep liver cells

Liver cells were incubated in the presence of 5 mM-glucose only (□) or in the additional presence of 0.1 μM-glucagon (▲), glucagon plus 10 mM-acetate (■), acetate only (○) or 1.5 munits of insulin/ml (●). Values are means (± S.E.M.) for three determinations on separate preparations.

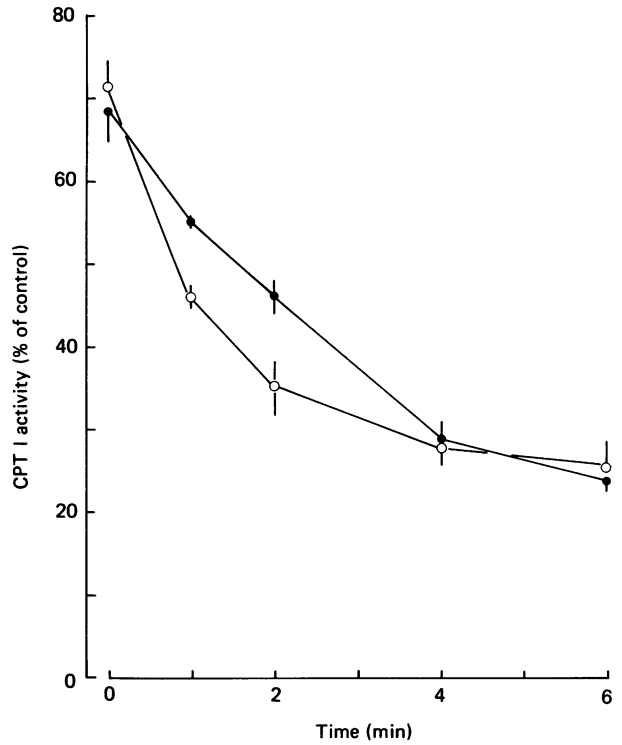

Fig. 2. Inhibition of CPT I in mitochondria from sheep (○) and rat (●) liver by malonyl-CoA after different periods of incubation

Mitochondria were incubated with 10 μM-malonyl-CoA for the length of time shown, and the activity of CPT I was then assayed over a 15 s period (see the Materials and methods section). Values are means (± S.E.M.) for three determinations on separate preparations.

similar to that observed for rat liver hepatocytes (McGarry *et al.*, 1978*b*; Beynen, 1981), although the rates of lipogenesis were very markedly lower in sheep liver cells. The apparent synthesis of fatty acids in the absence of detectable amounts of malonyl-CoA was a phenomenon similar to that observed by Beynen (1981).

Inhibition of CPT I by malonyl-CoA in mitochondria from livers of sheep, guinea pig and rat

CPT I activity in mitochondria from all three species was equally sensitive to malonyl-CoA inhibition. In addition, the inhibition of the enzyme in mitochondria from sheep and rat liver showed the same time-dependence (Fig. 2) previously demonstrated for the enzyme from rat liver mitochondria (Zammit, 1984). When the concentration-dependence of this inhibition was studied, curvilinear Dixon plots similar to those obtained for rat CPT I (Fig. 3*a*; see also Zammit, 1984) were obtained for CPT I in sheep liver mitochondria (Fig. 3*b*). For both sheep and rat CPT I, increased duration of incubation of the mitochondria with malonyl-CoA resulted in increased potency of malonyl-CoA as an inhibitor (Fig. 3).

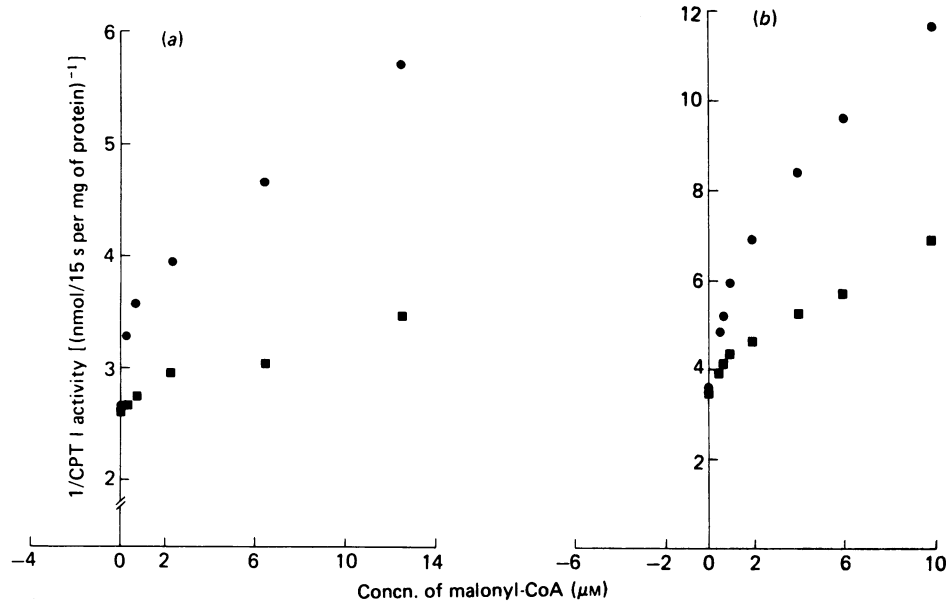

Fig. 3. Dixon plots for the concentration-dependence of malonyl-CoA inhibition of CPT I in rat (*a*) and sheep (*b*) liver mitochondria

Mitochondria were either added directly to the assay medium to start the reaction (■) or incubated with malonyl-CoA for 1 min (●) before CPT I activity was assayed. Experiments representative of three similar sets of results are presented.

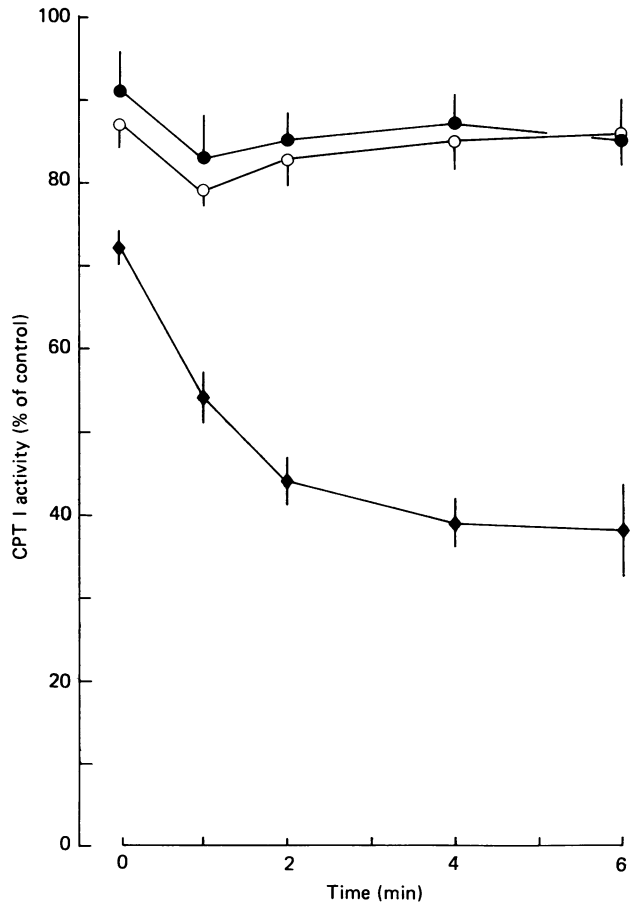

Fig. 4. Effect of methylmalonyl-CoA (10 μM on CPT I activity in mitochondria from sheep (◆), rat (●) and guinea-pig (○) liver

Experimental details are given in the legend to Fig. 2.

Effect of methylmalonyl-CoA, propionyl-CoA and succinyl-CoA on CPT I activity

The effects of three intermediates of propionate metabolism, propionyl-CoA, methylmalonyl-CoA and succinyl-CoA (3-carboxypropionyl-CoA), on the activity of CPT I in mitochondria isolated from the three animal species were studied. Methylmalonyl-CoA inhibited CPT I in sheep liver mitochondria in a manner similar to that seen with malonyl-CoA (Fig. 4), but had only a minor effect on CPT I in mitochondria from the other species (cf. McGarry *et al.*, 1983). Propionyl-CoA and succinyl-CoA had little effect on CPT I in mitochondria from all three species (results not shown).

DISCUSSION

The present results have important implications with respect both to the understanding of the role of malonyl-CoA as a regulatory metabolite and to the comparative aspects of the regulation of hepatic fatty acid oxidation.

The demonstration of the presence of micromolar concentrations of malonyl-CoA in freeze-clamped samples of sheep liver and in isolated sheep liver cells suggests that a low rate of fatty acid synthesis (about 1% of that found in rat liver) is not necessarily accompanied by low concentrations of malonyl-CoA in a tissue. This presumably arises from the fact that, although the absolute activities of acetyl-CoA carboxylase and fatty acid synthetase in sheep liver are about two orders of magnitude lower than those found in rat liver, their relative activities in both tissues are very similar (about 1:6: see Burch *et al.*, 1982; Zammit & Corstorphine, 1982). Consequently, if the affinity of sheep liver fatty acid synthetase for malonyl-CoA is also similar to that in rat liver, similar concentrations of malonyl-CoA will be

maintained in both tissues irrespective of the relative flux through the fatty-acid-synthesizing pathway. Therefore, the potential for the expression of the regulatory role of malonyl-CoA in the control of the rate of hepatic fatty acid oxidation is unimpaired in sheep and guinea-pig liver. Because the concentration of malonyl-CoA is capable of modulation by insulin and glucagon (Table 1, Fig. 1), changes in hepatic malonyl-CoA concentration *in vivo* could respond to different nutritional and physiological conditions.

In sheep and guinea-pig liver, as in muscle tissue of the rat, the amounts of fatty acids synthesized are largely unimportant in terms of the total fatty acid requirement of the tissue (e.g. heart) or animal (sheep liver), but the presence of substantial concentrations of malonyl-CoA in these tissues (McGarry *et al.*, 1983; the present work) can play a major regulatory role.

The comparative implications of the inhibitory effects of methylmalonyl-CoA on CPT I in mitochondria from sheep liver, but not from rat or guinea-pig liver, are noteworthy. Although both sheep and guinea pig have low rates of hepatic fatty acid synthesis, only in the sheep (and other ruminants) is there a continuous requirement for gluconeogenesis (see, e.g., Baird, 1977). Indeed, the gluconeogenic flux in sheep liver is highest in the fed state (Ingle *et al.*, 1972), and the major gluconeogenic substrate under these conditions is propionate. Methylmalonyl-CoA is an intermediate of propionate metabolism. It can be formed through carboxylation of propionyl-CoA either in the mitochondrial matrix or in the cytosol through the action of propionyl-CoA carboxylase and acetyl-CoA carboxylase (Buckner & Kolattukudy, 1975) respectively. Translocation of the propionyl and methylmalonyl moieties across the mitochondrial inner membrane can be achieved through the formation and transport of their respective carnitine esters. Therefore the finding that methylmalonyl-CoA is a potent inhibitor of CPT I in sheep liver mitochondria suggests that the availability of propionate for hepatic gluconeogenesis may inhibit the oxidation of long-chain non-esterified fatty acids in the fed ruminant through the effect of this metabolite. Such a mechanism would have the added advantage of signalling to the fatty-acid-oxidizing pathway the type of gluconeogenic substrate available to the liver. Thus, during starvation, when delivery of propionate to the liver is markedly curtailed, the major gluconeogenic precursors are likely to be lactate, glycerol and alanine (Wolff & Bergman, 1972). Because gluconeogenesis from lactate does not involve the formation of methylmalonyl-CoA, a significant gluconeogenic flux can be maintained without inhibition of CPT I. The rate of fatty acid oxidation and ketogenesis would therefore be differentially sensitive to gluconeogenesis from endogenous and exogenous precursors.

If the activity of acetyl-CoA carboxylase varies (within its limited capacity) in sheep liver depending on the physiological state of the animal (e.g. in response to insulin or glucagon; see Table 1), CPT I may be under dual control by both malonyl-CoA and methylmalonyl-CoA (which can also be formed by propionyl-CoA carboxylase; see above). The two metabolites could act in concert to amplify the effect on CPT I to a greater extent than if either metabolite acted alone. Such amplification may account for the severe ketosis that is precipitated in episodes of relative glucose deficiency accompanied by decreased food intake, such as occur in pregnancy toxaemia in sheep (see Baird, 1977). Such conditions would be characterized by decreased insulin/glucagon molar ratios in the circulation, resulting in decreased activity of acetyl-CoA carboxylase, and consequently of hepatic malonyl-CoA concentration. Decreased food intake would exacerbate this effect by interrupting the supply of propionate substrate for methylmalonyl-CoA formation in the liver. Therefore the anti-ketogenic effect of propionate (Bergman & Kon, 1964) may derive both from its stimulation of insulin secretion (Machlin *et al.*, 1966) and from its ability to generate a potent inhibitor of CPT I in the liver of ruminants.

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