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COMMENTARY

ON THE MECHANISMS OF SOME PHARMACOLOGICAL ACTIONS OF THE HYPOGLYCAEMIC TOXINS HYPOGLYCIN AND PENT-4-ENOIC ACID. A WAY OUT OF THE PRESENT CONFUSION

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Ingestion of the unripe arrilus of the Jamaican ackee fruit, *Blighia sapida*, sometimes causes 'vomiting sickness', an often fatal disease associated with severe disturbances of carbohydrate and lipid metabolism [1], and which has claimed perhaps 5000 deaths [2]. It is characterised by hypoglycaemia, depletion of hepatic glycogen, fatty infiltration of the liver and probably by isovalericacidaemia [3]. There has been a sustained interest in the toxic principle of the ackee, hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid), since its isolation 21 yr ago [4]. Hypoglycin is converted *in vivo* to methylenecyclopropylpyruvic acid (MCPP) by transamination, which is then oxidatively decarboxylated to the CoA ester of methylenecyclopropylacetic acid (MCPA) [5] (Fig. 1). Several synthetic analogues of MCPA were made. Only compounds containing the structure $CH_2{=}C.C.C.COOH$, or those converted *in vivo* to metabolites containing this structure (as their CoA esters), were said to be hypoglycaemic [6, 7] although all were considered too toxic for clinical use. The structurally simplest analogue, pent-4-enoic acid (pent-4-enoate) [6] (Fig. 2) has been extensively investigated because it is much more readily available than hypoglycin, and because it has been generally assumed to have a similar mechanism of action.

Although there is little disagreement about the symptoms of 'vomiting sickness', this harmony does not extend to discussions of its etiology. A similar status applies to discussions of the action of pent-4-enoate. Much of the current disagreement stems from the views of Bressler and his associates that the primary effect of hypoglycin and of pent-4-enoate is to sequester cellular CoA and carnitine as inert-acyl-deri-

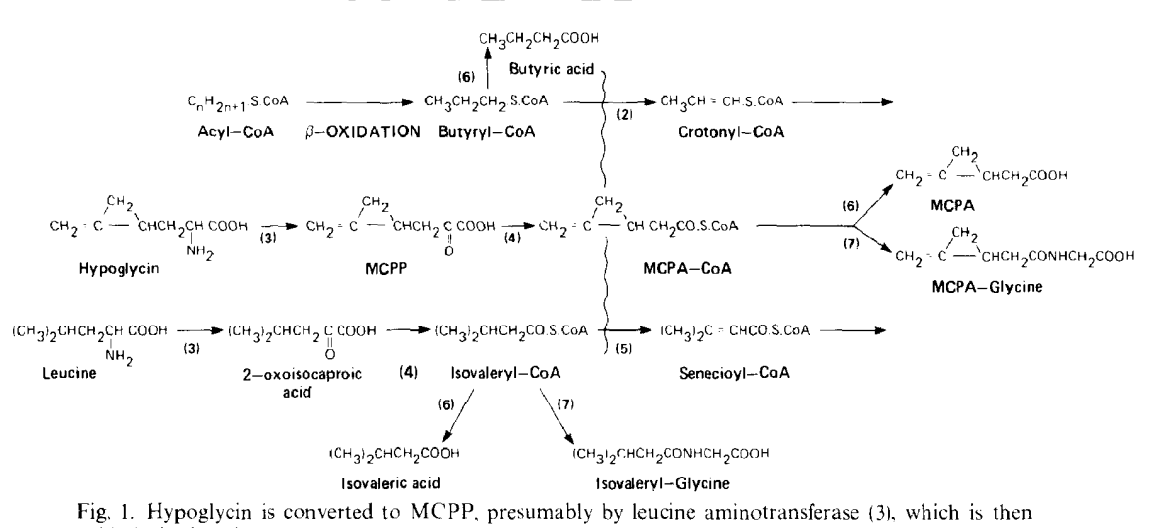

Fig. 1. Hypoglycin is converted to MCPP, presumably by leucine aminotransferase (3), which is then oxidatively decarboxylated to MCPA-CoA, presumably by isooxocaproate dehydrogenase (4). MCPA-CoA is partly deacylated by an acyl-CoA hydrolase to free MCPA (6) and partly conjugated with glycine by glycine acyltransferase (7). The normal β-oxidation sequence is inhibited by MCPA-CoA at butyryl-CoA dehydrogenase (2) and accumulated butyryl-CoA is deacylated (6). Leucine is converted to iso-oxocaproate (3) followed by oxidative decarboxylation to isovaleryl-CoA which is then dehydrogenated to senecioyl-CoA by isovaleryl-CoA dehydrogenase (5). MCPA-CoA inhibits isovaleryl-CoA dehydrogenase causing accumulation of isovaleryl-CoA which is partly deacylated to isovaleric acid (6) and partly conjugated with glycine (7). (Inhibitions are indicated by wavy lines.)

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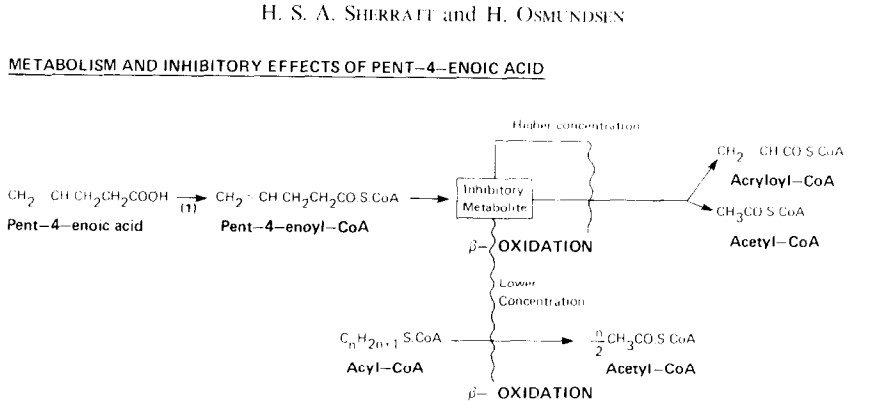

Fig. 2. Pent-4-enoate is converted to pent-4-enoyl-CoA by butyryl-CoA synthetase (1). Acetyl-CoA and acryloyl-CoA are formed from this by β-oxidation: acetyl-CoA (in liver) is mainly converted to ketone-bodies while acryloyl-CoA is mainly deacylated. One of the reactions of the β-oxidation of pent-4-enoyl-CoA is slow, some accumulation of the substrate for this reaction occurs as the preceding reactions are faster. It is proposed that because of the strict product-precursor relationships of the intermediates of β-oxidation (see ref. [21]) a small increase in the concentration of an intermediate will inhibit (inhibitions are indicated by wavy lines) the simultaneous oxidation of a substrate (derived from the oxidation of an even-chained acyl-CoA) which is normally oxidised at a fast rate, by preventing the access of the substrate to the reaction preceding that which is slow (i.e. inhibits the enzyme which also forms the metabolite of pent-4-enoyl-CoA which is subsequently slowly metabolised).

With higher concentrations of pent-4-enoate inhibition of β-oxidation occurs at a reaction distal to that which is slowed down and self-inhibition ensues.

vatives of their metabolites thus impairing fatty acid oxidation [8], and our contention that the primary effects are specific inhibitions of some enzymes of fatty acid oxidation by unique metabolites of these hypoglycaemic compounds [1, 9–11].

We believe that the concept of an essentially complete sequestration of CoA as unusual metabolites of foreign compounds should be questioned from a general point of view. As the cellular content of CoA is small in relation to the magnitude of the metabolic fluxes involving this cofactor, continuous recycling of CoA is essential for normal metabolism. We therefore think it likely that some mechanisms have evolved to avoid complete sequestration of CoA. The short-chain acyl-CoA hydrolases which we and some other groups have recently found in the mitochondrial matrix are particularly important in this respect. For example, butyryl-CoA, hexanoyl-CoA and acryloyl-CoA are deacylated by soluble enzymes with apparent K_m values (at 20° and pH 7·2) of 2 mM, 0·5 mM and 70 μM, respectively [12–16]. The maximum concentration of any acyl-CoA species that could be formed in the matrix of liver mitochondria is about 7 mM [17, 18] while during rapid oxidation of palmitoyl-carnitine the free CoA concentrations are about 0·6 mM [19, 20]. Such hydrolases, therefore, are ideally suited as 'safety valves' for the deacylation of abnormally high concentrations of acyl-CoAs, since intermediates of β-oxidation do not accumulate sufficiently to be deacylated [19, 21].

Both hypoglycin and pent-4-enoate induce hypoglycaemia and ketosis in animals [1], but only hypoglycin causes isovalericacidaemia [3]. It is believed that following administration of hypoglycin or pent-4-

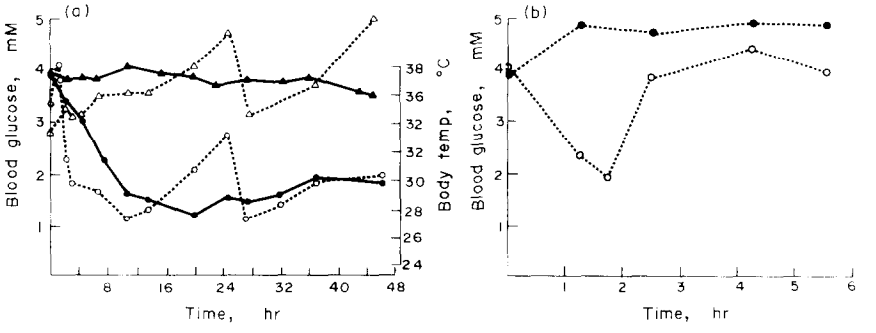

Fig. 3. The effects of hypoglycin and of pent-4-enoate on blood glucose concentrations in rats. Male albino rats (250 g) were starved for 24 hr and food was withheld during the experiment. Glucose was determined in serial 20 μl blood samples from the rail and the rectal temperature was measured [31]. The ambient temperature was 22°. Individual rats were given intraperitoneally at time 0: (a) hypoglycin (75 per cent pure, the main contaminants were leucine and isoleucine) 60 mg/kg body wt. (○) blood glucose concentration, (●) rectal temperature; or 0·14 M NaCl (10 ml/kg body wt). (△) blood glucose, (▲) rectal temperature; (b) pent-4-enoic acid (350 mg/kg body wt. adjusted to pH 7·4 with NaOH), (○) blood glucose; or *n*-pentanoic acid (350 mg/kg body wt. adjusted to pH 7·4 with NaOH) as control, (●) blood glucose.

enoate, gluconeogenesis is impaired secondarily to inhibition of fatty acid oxidation, so that glucose is then only the available major fuel and when glycogen is exhausted hypoglycaemia ensues [9]. This interpretation was first explicitly stated by Senior in 1967 for the hypoglycaemic activity of pent-4-enoate [22]. Others have outlined similar but less precise explanations for the effects of hypoglycin and pent-4-enoate, which also envisage an increased rate of utilisation of glucose [8, 23]. It is not always appreciated, however, that to get hypoglycaemia it is only necessary for glucose to be used at a greater rate than it can be replaced [24]. Although it is now well established that metabolites of both hypoglycin and of pent-4-enoate inhibit fatty acid oxidation and gluconeogenesis *in vitro* [13, 17, 24–27], it has never been directly demonstrated that they do so *in vivo*. Although there are broad similarities in their pharmacological effects we now know that these compounds have different detailed mechanisms of action. Thus, in rats hypoglycaemia is over 4–6 hr after injection of pent-4-enoate, whilst that caused by hypoglycin persists much longer, with a transient but marked increase in blood glucose concentrations after 24 hr (Fig. 3).

The mechanisms of the effects of hypoglycin and of pent-4-enoate on β-oxidation and on other CoA-dependent oxidations, on gluconeogenesis and on metabolism in the whole animal are by no means fully understood. These have been investigated by a multiplicity of techniques and it is perhaps in this multiplicity, and in the failure to remember that different methods may not necessarily give directly comparable results, that the causes of the present misunderstandings concerning the explanation of the pharmacological effects of hypoglycin and of pent-4-enoate lie. We attempt here to resolve some of this confusion. Space does not permit a complete discussion of all aspects of these problems. Further information can be found in reviews [1, 8, 9, 28, 29] and some of our arguments are given in more detail elsewhere [11, 17, 30–33].

1. INTERPRETATION OF MEASUREMENTS OF FATTY ACID OXIDATION

Measurement of fatty acid oxidation and assessment of its modification by drugs *in vitro* or *in vivo* presents many difficulties which are not even now always appreciated. This has been investigated *in vitro*, for example, by collection of $^{14}CO_2$ when ^{14}C-labelled fatty acids were used as substrates, or by determining oxygen uptake manometrically or polarographically. Sometimes formation of ketone-bodies by liver preparations has been measured. Caution is necessary when comparing results obtained with these very different techniques applied to, for example, isolated mitochondria, homogenates, tissue slices or perfused organs, and in their extrapolation to whole animals. Further, in animals difficulties in interpretation are compounded since interference with fatty acid oxidation can occur at any stage between mobilisation of free fatty acids from triglyceride stores to the final release of fatty acid carbon as CO_2 by the citrate cycle.

The first stage of fatty acid catabolism involves β-oxidation which shortens long-chain fatty acyl-CoA derivatives by successive removal of C_2 units as acetyl-CoA. The fate of this acetyl-CoA is complex. It can condense with oxaloacetate to form citrate, which may accumulate or be oxidised further by the citrate cycle to CO_2 or to other products. In liver, formation of acetoacetate also competes very successfully for acetyl-CoA. This, after partial reduction to 3-hydroxybutyrate, is subsequently oxidised by the citrate cycle in extrahepatic tissues. The nature and extent of fatty acid oxidation depends on the availability of free fatty acids, the concentrations of citrate-cycle intermediates and carnitine, the ADP/ATP ratio and the NAD^+/NADH ratio [34–38]. Further, in many *in vitro* experiments the extent of damage to mitochondria caused by some homogenisation procedures, and the use of high concentrations of substrates with detergent properties which may be manifest in the absence of added serum albumin have not been controlled. Meaningful estimation of the flux through β-oxidation is therefore impossible without strictly defined conditions, particularly because of the uncertain extent of the further oxidation of acetyl-CoA. Most measurements of fatty acid oxidation *in vitro* described in the literature have not been made with adequate control of all of these factors, and many are consequently, for whatever purpose, only of qualitative value. Particularly unsatisfactory are measurements of $^{14}CO_2$ release alone from 1-^{14}C-labelled fatty acids in liver homogenates [39], since Ontko has shown that in rat liver homogenates as little as 5 per cent of the added radioactivity of [1-^{14}C]palmitate is recovered as $^{14}CO_2$, with about 40 per cent appearing as ketone-bodies and 50 per cent in complex lipids within 15 min [40]. Inhibition of $^{14}CO_2$ release, therefore, may indicate a shift in the relative proportions of these metabolites (Fig. 4), rather than a direct inhibition of β-oxidation.

Perhaps the least ambiguous method of measuring the flux through β-oxidation is to record polarographically the oxygen uptake by isolated liver mitochondria oxidising small amounts (10–30 μM) of even-chain acyl-carnitines in the presence of an inhibitor of the citrate cycle (fluorcitrate or malonate) and in the absence of a citrate cycle-intermediate, when the maximum flux through the pathway is stimulated by ADP and Pi (state 3 conditions). With these conditions acyl-groups are quantitatively converted to acetoacetate, and the rate of oxygen uptake is a direct measure of the maximum attainable rate of β-oxidation [13, 17].

Estimates of the effects of a drug on the oxidation of [1-^{14}C]-labelled substrates *in vivo* made by collecting expired $^{14}CO_2$ are also ambiguous, particularly when hypoglycaemia is a manifestation of its pharmacological effects [9, 33]. Administration of hypoglycin indeed decreases exhalation of $^{14}CO_2$ by rats given [1-^{14}C]palmitate [23]. However, McKerns pointed out in 1960 that the increased serum concentrations of free fatty acids following administration of hypoglycin 'may dilute the fatty acid pool sufficiently to account for the observed decrease in oxidation of labelled acids' [41]. Indeed, adrenaline and glucagon released in response to hypoglycaemia caused by many different mechanisms will liberate free fatty acids from triglyceride stores. In two studies the absolute rate of production of respiratory CO_2 was decreased by hypoglycin [23, 41]. Some decrease would

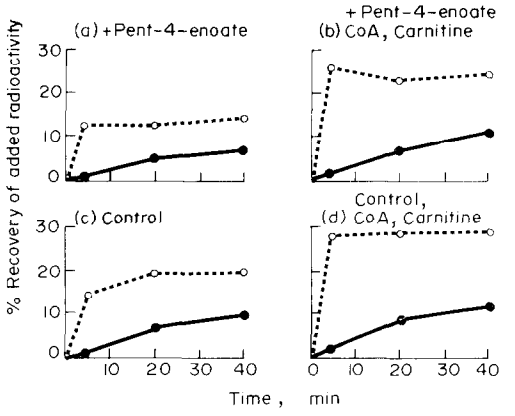

Fig. 4. The effects of carnitine and CoA on the inhibition of the metabolism of [1-^{14}C]palmitate by pent-4-enoate in pigeon liver homogenate. A pigeon was starved for 72 hr and the liver was homogenised in 9 vol of Krebs Ringer phosphate medium [39]. Various additions were made: (a) 1 mM pent-4-enoate, (b) 0·2 mM L-carnitine, 0·1 mM CoA and 1 mM pent-4-enoate, (d), 0·2 mM L-carnitine and 0·1 mM CoA. Homogenate (2·0 ml) was incubated in rubber-capped vials containing a centre well, at 37° with shaking with an atmosphere of O_2. 0·1 mM [1-^{14}C]Palmitate, specific activity 1500 μCi/m-mole was added at time 0. After various times the reaction was stopped by injection of 0·20 ml 3·0 M $HClO_4$, and 0·25 ml β-phenylethylamine toluene–methanol–water (6:8:4:1) was added to the centre wells to absorb $^{14}CO_2$ evolved, and the vials were incubated for a further 40 min at 10°; then the acid-soluble fraction was obtained by centrifuging the acidified homogenate in an Eppendorf microcentrifuge. The $^{14}CO_2$ evolved (●) and the radioactivity in the acid-soluble fraction (○) (ketone-bodies, citrate, etc. [38]) was determined using appropriate quench corrections. Radioactivity incorporated into complex lipids or any unchanged palmitate was present in the acid-insoluble fraction [40]. The flux through β-oxidation is given by the rate of formation of acid-soluble ^{14}C-labelled products plus that of $^{14}CO_2$.

be expected, both due to hypothermia which develops in small animals at ambient temperatures less than about 26°C and to the psychomotor depression, which is perhaps due to narcotic effects of accumulated short-chain fatty acids [42]. It has been claimed that hypoglycin and pent-4-enoate sometimes increase the rate of oxidation of glucose in rats and mice, respectively [23, 43]. Clearly the absolute rate of oxidation of either fatty acids or glucose cannot be deduced from the specific activity of exhaled $^{14}CO_2$, unless the size and specific activities of the pools of substrates *in vivo* are also known. Thus a diminished rate of gluconeogenesis (induced by a drug) would decrease the size and hence increase the specific activity of the glucose pool, thereby causing an increase in the specific activity of expired $^{14}CO_2$ following an injected dose of [U-^{14}C]glucose, even if the actual rate of glucose oxidation was lowered. A much more worthwhile approach for investigating metabolic changes in the intact animal is offered by the kinetic methods of Heath [44]; Katz [45]; Shipley [46].

2. INHIBITION OF β-OXIDATION AND GLUCONEOGENESIS BY HYPOGLYCIN

MCPA was shown several years ago to inhibit the release of $^{14}CO_2$ from ^{14}C-labelled fatty acids in rat liver mitochondria [23] and in guinea-pig skin slices [47]. Such experiments do not necessarily rule out the possibility that the block was at the stage of release of $^{14}CO_2$ by the citrate cycle. However, we have recently shown that MCPA-CoA inhibits β-oxidation directly [13]. Both butyryl-CoA and isovaleryl-CoA dehydrogenases were almost completely inactivated in mitochondria that had been incubated with MCPP or MCPA, or in mitochondria isolated from livers taken from rats that had been given hypoglycin [48, 49]. Surprisingly, neither palmitoyl-CoA dehydrogenase [48] nor any other enzyme of β-oxidation tested was inhibited [49]. Mitochondria from rat heart, kidney and liver are unable to oxidise hexanoylcarnitine or butyryl-carnitine after preincubation with MCPA (10 μM–1 mM) [13, 50]. With long-chain acyl-carnitines the inhibition of the state 3 rate of oxidation increases with increasing chain-length greater than C_{10}, from about 40% inhibition with decanoylcarnitine to about 60% with palmitoyl-carnitine [13]. A similar pattern of inhibition is observed in mitochondria isolated from livers of rats that had been given hypoglycin [50]. Von Holt reported that, by contrast, the oxidation of free long-chain fatty acids was inhibited more strongly by MCPA than those of shorter-chain length [23]. This apparent difference is simply explained by medium- and short-chain fatty acids in high concentrations suppressing the formation of MCPA-CoA in the mitochondrial matrix by competition for butyryl-CoA (medium-chain acyl-CoA) synthetase. Long-chain fatty acids are activated outside the matrix and therefore do not prevent formation of MCPA-CoA [13, 17].

This decrease in the rate of oxidation of long-chain acyl-carnitines is probably due to accumulation of short-chain acyl-CoA in the matrix. The stoichiometry of oxygen uptake during the oxidation of acetyl-carnitines in MCPA-inhibited mitochondria indicates that their oxidation is incomplete and proceeds as far as butyryl-CoA rather than to hexanoyl-CoA as might have been expected [13]. Extensive accumulation of butyryl-CoA would sequester all the intramitochondrial CoA and thus stop β-oxidation. Since MCPA only slows β-oxidation it is necessary that deacylation of short-chain acyl-CoA occurs to allow recycling of CoA. This is achieved in the matrix by short-chain acyl-CoA hydrolases with relatively high K_m's for their substrates. As a consequence of MCPA inhibition, short-chain acyl-CoAs accumulate in the matrix only up to a critical concentration defined by the kinetic properties of the hydrolase, allowing steady state concentrations of free CoA sufficient to maintain a limited rate of β-oxidation. Further, we have detected the expected amount of free butyrate accumulating as a product of palmitoyl-carnitine oxidation in MCPA-inhibited mitochondria [13], and butyrate accumulates in the blood of rats and mice poisoned with hypoglycin [3, 52]. Similarly, deacylation of accumulated isovaleryl-CoA as a result of inhibition of isovaleryl-CoA dehydrogenase accounts for the massive isovalericacidaemia in rats and mice poisoned with hypoglycin [3, 49, 52].

Formation of $^{14}CO_2$ from 0·1 mM [1-^{14}C]palmitate was impaired in homogenates of hearts from mice given hypoglycin (500 mg/kg body wt) and 2 mM carnitine reversed this inhibition [53]. Addition of carni-

tine provides an extra-mitochondrial acyl-sink for some unusual acyl-groups as a result of the action of the carnitine acetyltransferases associated with the inner mitochondrial membrane, thereby liberating an equivalent amount of free CoA in the matrix [32]. Relief of inhibition by carnitine was consequently assumed by Bressler to be evidence for the CoA-sequestration hypothesis [8, 53]. We have repeated this experiment [51]. However, we also found that formation of $^{14}CO_2$ from [16-^{14}C]palmitate, which is also inhibited, is decreased still further by 2 mM carnitine. Formation of acid-soluble ^{14}C-labelled products from [16-^{14}C]palmitate was greatly stimulated by added carnitine (about 10 times accounting for 50 per cent of the added radioactivity, presumably as [4-^{14}C]butyrate and its carnitine ester) and to a lesser extent from [1-^{14}C]palmitate (presumably as [1-^{14}C]acetate [15] and [1-^{14}C]citrate). This indicates that when butyryl-CoA dehydrogenase is inhibited following administration of hypoglycin, chain-shortening of palmitoyl-CoA to butyryl-CoA occurs by removal of carbon atoms 1–12 by 6 cycles of β-oxidation. Added carnitine probably stimulates the rate of this partial oxidation by lowering the concentration of butyryl-CoA in the mitochondrial matrix [13].

It was also reported that injection of carnitine largely prevents induction of hypoglycaemia in an unspecified strain of albino mice by hypoglycin (600 mg/kg body wt) [53]. We completely failed to repeat this using a strain of albino Swiss mice [31]. Recently, by contrast, we found that carnitine may sometimes delay the onset of hypoglycaemia and death in a strain of Balb C mice [52]. The latter result, if confirmed, is consistent with our interpretation of the mechanism of hypoglycin in action. The apparent absence of any effect of carnitine can, however, be caused by factors other than those directly related to β-oxidation (for example, the permeability of cells to carnitine and the rate of its disposal). These discrepancies appear to be due to strain differences, as do the large differences in the reported effective hypoglycaemic doses of hypoglycin in mice which range from 100 to 600 mg/kg body wt [31, 52–54].

The finding of an apparently irreversible inactivation of butyryl-CoA and isovaleryl-CoA dehydrogenases suggests an explanation of the specific protective effect of riboflavin against the chronic toxicity of hypoglycin [55]. This is that *de novo* synthesis of dehydrogenases with flavin prosthetic groups is limited by the dietary supply of riboflavin and that supplementary riboflavin therefore facilitates the replacement of these essential enzymes.

Hypoglycin and its metabolite MCPP inhibit glucose synthesis from several precursors in isolated rat hepatocytes and in kidney slices [26, 27]. It is unlikely that the partial inhibition of β-oxidation completely explains the impairment of gluconeogenesis and two other factors might also contribute. First, butyryl-CoA antagonises the activation of pyruvate carboxylase, a key enzyme for gluconeogenesis from several precursors, by its normal activator acetyl-CoA [56]. Inhibition of β-oxidation by MCPA is probably associated with an increased concentration of butyryl-CoA and with a decreased concentration of acetyl-CoA in the matrix, which would decrease pyruvate carboxylase activity. An inhibitory effect of MCPA-CoA on pyruvate carboxylase during the whole time course of hypoglycaemia is unlikely since MCPA is apparently eliminated from the body while hypoglycaemia still persists [3, 51]. Second, Tanaka has shown that both lysine and tryptophane potentiate the hypoglycaemic effects of hypoglycin. He has suggested that glutaryl-CoA, a metabolite of both these amino acids, accumulates as a result of inhibition of glutaryl-CoA dehydrogenase by MCPA-CoA, and that glutaryl-CoA also contributes to the inhibition of gluconeogenesis [3].

Tanaka also found that rats which had been injected with hypoglycin excreted significant quantities of unsaturated dicarboxylic acids, particularly *cis*-4-decene-1,10-dioate [3]. These could be products of a detoxification mechanism which attempts to dispose of excessive amounts of free long-chain fatty acids that cannot be oxidised or re-esterified to triglycerides.

3. INHIBITION OF β-OXIDATION AND GLUCONEOGENESIS BY PENT-4-ENOATE

Pent-4-enoate causes decreased release of $^{14}CO_2$ from ^{14}C-labelled fatty acids in several systems [30, 39, 47, 57]. Using carefully defined conditions, Senior *et al.* found that pent-4-enoate inhibited β-oxidation more effectively than some control non-hypoglycaemic fatty acids (*n*-pentanoate, pent-2-enoate, cyclopropanecarboxylate and cyclobutanecarboxylate). Of these compounds investigated, *n*-pentanoate and cyclopropanecarboxylate lowered the free CoA concentrations in mitochondria to the same extent as pent-4-enoate [17]. By contrast, most of these compounds were as effective as pent-4-enoate in inhibiting the CoA-dependent oxidations of pyruvate and of 2-oxoglutarate with the experimental conditions used. Cyclobutanecarboxylate, however, did not conform to this pattern as it had little inhibitory effect on the latter two reactions [10]. From this data it was concluded in 1967 that inhibition of β-oxidation is not simply due to sequestration of CoA as acyl-derivatives by metabolites of pent-4-enoate, and that inhibition of pyruvate and 2-oxoglutarate oxidation is caused by the combined effects of an acyl-CoA derivative on the enzymes concerned and by a decreased concentration of CoA in the matrix [10, 30].

The view that inhibition of CoA dependent reactions by pent-4-enoate is caused by sequestration of CoA and carnitine as inert acyl-derivatives, thought to be acryloyl-esters, was mainly based on measurements of $^{14}CO_2$ release from [1-^{14}C]palmitate in pigeon liver homogenates in experiments where some elementary requirements for valid kinetic studies were not met (for example, linearity of $^{14}CO_2$ formation with time was not shown) [8, 39]. Pre-incubation with 0·5 mM pent-4-enoate decreased the total amount of $^{14}CO_2$ evolved during a 15 min period compared with a control incubation. Addition of 0·1 mM CoA and 0·2 mM carnitine, assumed to be extra-mitochondrial acyl-sinks, largely prevented this decrease. However, as Holland and Sherratt pointed out these cofactors may also remove an acyl-CoA with specific inhibitory effects from the matrix [17]. We have done some similar experiments with rat or pigeon liver homogenates, measuring in addition the acid soluble

radioactive products formed to get more complete information about the fate of added [1-^{14}C]palmitate [58]. We sometimes obtained similar results for the effects of CoA and carnitine on the inhibition by pent-4-enoate of the evolution of $^{14}CO_2$, although the results were rather variable (Fig. 4). It is clear that evolution of $^{14}CO_2$ does not remain proportional to the flux through β-oxidation. De-inhibition of $^{14}CO_2$ release by addition of cofactors may be partly explained by removal of a block in the citrate cycle at 2-oxoglutarate dehydrogenase by an acyl-CoA derivative [10] thus allowing an increased rate of oxidation of an accumulated ^{14}C-labelled metabolite, for example of citrate. Although Holland and Sherratt found a self-inhibitory pulse of pent-4-enoyl-carnitine oxidation in isolated rat liver mitochondria [17], we have recently found that low concentrations (up to 200 μM) of pent-4-enoate can be utilised in the presence of 10 mM carnitine [50]. Further, Williamson has shown that perfused rat livers are capable of a sustained oxidation of pent-4-enoate provided that its concentration in the perfusate is kept below a critical concentration [25].

Mitochondria isolated from livers of rats that had been given pent-4-enoate are sometimes unable to oxidise palmitoyl-carnitine (depending on the time after injection) although their ability to oxidise pyruvate may not be impaired [59]. This result clearly discounts CoA depletion as a primary cause of inhibition of β-oxidation. As long as β-oxidation of pent-4-enoate is maintained there may not necessarily be strong inhibition of normal fatty acid oxidation. No excessive accumulation of inert acyl-CoA derivatives occurs during the sustained oxidation of pent-4-enoate [25, 50], but at high concentrations of pent-4-enoate a reaction becomes rate-limiting and an intermediate of its oxidation builds up causing impairment of palmitoyl-carnitine oxidation, and subsequently self-inhibition (Fig. 2). Added carnitine may prevent an accumulation of inhibitory products during the oxidation of low, but not of high, concentrations of pent-4-enoate by buffering the concentrations of some intermediates in the matrix [32]. This explanation can rationalise the apparently conflicting results for the effects of pent-4-enoate on isolated mitochondria, homogenates, perfused livers and whole animals. In animals, sublethal doses of pent-4-enoate may be cleared from the system after a few hours enabling the 'log jam' in its metabolism to resolve, thus permitting recovery.

Finally, we have not detected any convincing irreversible inhibition of any enzymes of β-oxidation in mitochondria pre-incubated with pent-4-enoate, nor in mitochondria isolated from livers of rats injected with pent-4-enoate [59]. In our opinion, all these results can only mean that a metabolite of pent-4-enoate specifically and reversibly inhibits an enzyme of β-oxidation. On these metabolites (pent-4-enoyl-CoA, penta-2,4-dienoyl-CoA, 3-oxopent-4-enoyl-CoA, 3-hydroxypent-4-enoyl-CoA and acryloyl-CoA), we do not think that acryloyl-CoA is involved as we have found that it is rapidly deacylated by soluble enzymes in the matrix. Further, acrylate is a feeble inhibitor of β-oxidation and it does not decrease free CoA concentrations in mitochondria significantly [17], although it is a substrate for butyryl-CoA synthetase [11]; neither did acryloyl-CoA strongly inhibit any enzymes of β-oxidation tested [32].

A powerful and specific inhibition of acetoacetyl-CoA-thiolase by penta-2,4-dienoyl-CoA was observed with the purified enzyme [32] or in soluble extracts of mitochondria [12], but not when intact mitochondria were used [59]. These results appear surprising unless it is remembered that a considerable degree of structural organisation of the enzymes of β-oxidation may exist in the matrix [13, 21] which may afford some protection against inactivation of an enzyme by metabolites that are not analogues of its substrate. This underlines the importance of including organised cellular and subcellular systems in toxicological investigations since inhibitions found with isolated enzymes must also be shown to occur in intact cells if they are to be considered as a cause of any toxic effect.

Williamson has presented extensive evidence that inhibition of gluconeogenesis by pent-4-enoate in perfused rat liver is due largely to a block at pyruvate carboxylase, possibly caused by lack of acetyl-CoA, and to a block in the conversion of 1,3-diphosphoglycerate to triose phosphates as a result of a decreased supply of reducing equivalents from the mitochondria [25]. Both blocks were apparently caused by impaired β-oxidation. These sites of inhibition were located by assay of gluconeogenic intermediates in freeze-clamped livers. Other mechanisms may contribute to inhibition of gluconeogenesis. Thus, metabolites of pent-4-enoate might modify the activity of pyruvate carboxylase [33]. However, recovery of normal blood glucose concentrations in some rats that had been made hypoglycaemic with pent-4-enoate occurred at a time when β-oxidation was about 80 per cent inhibited in liver mitochondria from these animals [51].

Both hypoglycin and pent-4-enoate poisoning are marked by ketosis [60, 61] and this has not been adequately explained. It is unlikely to arise from excessive generation of ketone bodies, but it is more likely to be caused by their decreased peripheral utilisation [60]. Preliminary results indicate that isovalerate and butyrate, which accumulate in hypoglycin poisoning inhibit acetoacetate oxidation by about 50 per cent in isolated rat heart mitochondria at concentrations found in poisoned animals [50]. Further, metabolites of MCPA and pent-4-enoate may inhibit acetoacetate oxidation directly.

4. CONCLUDING REMARKS

We have always rejected the idea that the sequestration of CoA is the major mechanism of action of this group of compounds because of the requirement for the $CH_2{=}C.C.C.COOH$ group for hypoglycaemic activity [6] which indicates that there must be specific inhibition of a few enzymes. It is difficult to explain on this basis, for example, why dihydrohypoglycin is not hypoglycaemic. This derivative would be expected to be metabolised to methylcyclopropylacetyl-CoA which should be equally effective as MCPA-CoA in tying up free CoA [33].

The available data fits the hypothesis that hypoglycaemia caused by hypoglycin or pent-4-enoate is a consequence of impaired gluconeogenesis following

inhibition of fatty acid oxidation, and we are trying to verify this by the sophisticated techniques now available for studying *in vivo* kinetics. A large number of miscellaneous hypoglycaemic compounds inhibit hepatic gluconeogenesis in many different ways, and this emphasises the crucial importance of gluconeogenesis in maintaining normal concentrations of blood glucose [1, 9, 24]. In accordance with the above interpretation, some other fatty acids which do not inhibit fatty acid oxidation or gluconeogenesis strongly *in vitro* are not hypoglycaemic [9, 61]. However, cyclopropanecarboxylate does not fit neatly into this picture and this discrepancy cautions against the application of too simplistic concepts. This compound is not hypoglycaemic in rats or mice, although it is in guinea pigs [28], and it does not inhibit β-oxidation strongly [17, 30] yet it inhibits gluconeogenesis from 10 mM-alanine in rat hepatocytes more powerfully than pent-4-enoate at concentrations below 1 mM [27].

A correct understanding of the problems outlined here is in our opinion essential since questions of fundamental importance to intermediary metabolism and its perturbation by pharmacologically active compounds are involved. A thorough analysis of the actions of hypoglycin and pent-4-enoate will highlight hitherto unrecognised relationships which will aid in future investigations.

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