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MECHANISMS OF THE METABOLIC DISTURBANCES CAUSED BY HYPOGLYCIN AND BY PENT-4-ENOIC ACID *IN VIVO* STUDIES

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Abstract—1. It was confirmed that administration of either hypoglycin or pent-4-enoate to rats caused severe hypoglycaemia and hypothermia. Hypothermia was prevented by keeping the animals in a thermoneutral environment (30°). Hypoglycaemia caused by hypoglycin lasted longer than that caused by pent-4-enoate. 2. Administration of hypoglycin to rats caused several volatile fatty acids to accumulate in the plasma. By contrast, the only volatile fatty acid found in plasma following administration of pent-4-enoate to rats was pent-4-enoate itself. 3. Several short-chain acyl–CoA dehydrogenase activities were irreversibly inactivated in extracts of mitochondria isolated from livers taken from rats after administration of hypoglycin; no inhibitions were found following administration of pent-4-enoate. 4. Evidence is presented that some of the branched-chain acyl–CoA esters are not, as often assumed, substrates for the butyryl–CoA dehydrogenase of β-oxidation, and that there are some specific branched-chain acyl–CoA dehydrogenases. 5. Mitochondria isolated from livers of hypoglycin-treated rats had their ability to oxidize acyl–carnitines severely impaired, and the O_2 consumption was consistent with the incomplete oxidation of the substrate as far as butyrate. Pyruvate oxidation was uninhibited in these mitochondria. 6. Mitochondria isolated from livers of pent-4-enoate-treated rats had their ability to oxidize acyl–carnitines impaired but the O_2 consumption was consistent with the complete oxidation of the substrate to acetoacetate. Pyruvate oxidation was also inhibited. During recovery, pyruvate oxidation was restored before that of palmitoyl–carnitine indicating that sequestration of mitochondrial CoASH is not the mechanism by which pent-4-enoate inhibits β-oxidation. 7. A working model is proposed to explain the *in vivo* effects of these compounds.

Hypoglycin and the structurally related compound pent-4-enoate, cause profound hypoglycaemia in many species. In the preceding paper some effects of MCPA (the active metabolite of hypoglycin), and of pent-4-enoate, on CoA-requiring reactions in isolated mitochondria were described, and these compounds were shown to inhibit β-oxidation in different ways [1]. In addition, the hypoglycaemia caused by hypoglycin lasts longer than that caused by pent-4-enoate [2, 3]. Hypoglycin poisoning, but not that caused by pent-4-enoate, is also associated with disturbances of branched-chain fatty acid metabolism [4, 5] and a massive excretion of some unsaturated dicarboxylic acids [6]. This paper describes the effects of hypoglycin and of pent-4-enoate on the activities of some tissue enzymes, and attempts to correlate these with their effects in whole animals. The known *in vitro* effects of hypoglycin and of pent-4-enoate were used to construct a working model to explain the hypoglycaemic activities of these compounds.

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Abbreviations—MCPP methylenecyclopropylpyruvate; MCPA methylenecyclopropylacetate; CoA coenzyme A (esterified form); CoASH coenzyme A (free form); PEG-A polyethyleneglycol-adipate; HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

MATERIALS AND METHODS

Animals. Albino, male Wistar rats (250–400 g) and albino, male Balb c mice (25 g), maintained on a standard laboratory diet, were used throughout. Food was withheld from the animals 24 hr prior to and during the experiments. Experiments were conducted in a temperature controlled room when necessary.

Chemicals. The sources of most materials are given in the preceding paper [1] except that PEG-A and diatomite C (60–80 mesh) were obtained from J. J. (Chromatography) Ltd., Kings Lynn, Norfolk, U.K.

Methods. Hypoglycin was injected intraperitoneally as a 2 per cent (w/v) solution in 0.14 M NaCl; control animals were injected with a similar volume of 0.14 M NaCl. Pent-4-enoate was similarly administered as a 2 per cent (w/v) solution in 0.14 M NaCl adjusted to pH 7.4 with NaOH; control animals were injected with the chemically similar, but non-hypoglycaemic fatty acid n-pentanoate [7, 8]. Doses of pent-4-enoate and n-pentanoate are expressed in terms of the undissociated acid.

Glucose concentrations in serial 20 μl blood samples taken from the tail vein of rats or mice were assayed by the glucose oxidase (EC 1.1.3.4) method [9] using test kits supplied by Boehringer Corp. Ltd., London W5 2TZ, U.K. Animals were put in a restriction cage and their rectal temperatures were measured with a thermistor probe connected to a digital thermometer [3].

Gas–liquid chromatography of volatile fatty acids. Volatile fatty acids were separated by a method developed in collaboration with Dr. K. Hutton and Dr. I. Ross, both formerly of the Department of Agricultural Biochemistry, University of Newcastle upon Tyne. Glass columns (2.1 m long, 4 mm internal diameter) were packed with 10 per cent (w/w) PEG-A on acid-washed diatomite C. N_2 was used as carrier gas after first bubbling through water. Water vapour appears to saturate polar sites in the stationary phase and thus decreases trailing of the peaks. Solutions of volatile fatty acids in water were used as standards. It was not possible to separate isovaleric and 2-methylbutyric acids.

Plasma samples were prepared for gas–liquid chromatography of their volatile fatty acid content essentially as described by Remesy and Demigne [10]. Crotonate (20 μl of a 10 mM solution) was added to 0.2 ml of plasma as an internal standard. Absolute ethanol (1.0 ml) was then added and after mixing and centrifugation for 1 min in an Eppendorf microcentrifuge, the supernatant was transferred to a 1 × 2.5 cm tube, made alkaline with 20 μl of 0.2 M NaOH, and then evaporated to dryness in a slow current of air at 20°. The residue was dissolved in 35 μl of water, and immediately before analysis, 5 μl of 25 per cent (v/v) H_3PO_4 was added and 2 μl samples applied to the column. Since equimolar amounts of different fatty acids gave different peak areas, appropriate correction factors were used to calculate their plasma concentrations. Peak areas were measured by triangulation and a computer program was used to calculate the concentration of each fatty acid.

Enzyme assays. These were performed as described in the preceding paper [1]. In addition, malate dehydrogenase (EC 1.1.1.37) was assayed at 20° and pH 9.5 in the direction of malate oxidation [11].

Other methods are described in the preceding paper [1] or in the legends of Tables and Figures where appropriate.

RESULTS AND DISCUSSION

The best known pharmacological effect of both hypoglycin and pent-4-enoate is hypoglycaemia in many, but not all, species [12]. Administration of these compounds to rats and mice at ambient temperatures less than 25° also causes marked hypothermia [2, 3]. This may be due to narcotic effects either of pent-4-enoate *per se*, or of volatile fatty acids accumulated during hypoglycin poisoning [13], or to the impairment of oxidative metabolism caused by these compounds [1]. However, the time course of hypoglycaemia caused by hypoglycin is longer than that caused by pent-4-enoate (see Fig. 1), suggesting differences in their molecular mechanisms of action.

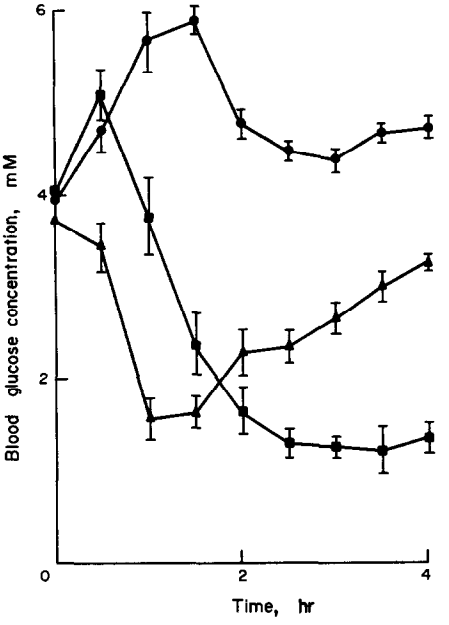


Fig. 1. Effect of hypoglycin and of pent-4-enoate on blood glucose concentrations in mice. Starved mice were injected with either hypoglycin (200 mg/kg body wt) (■), pent-4-enoate (300 mg/kg body wt) (▲) or 0.14 M NaCl (●). Glucose concentrations were determined in serial 20 μl blood samples taken from the tail. Values are means for 6 animals ± S.E.M.

Effects of hypoglycin and pent-4-enoate on rectal temperatures, blood glucose and plasma volatile fatty acid concentrations in rats

Figure 2 shows the hypoglycaemic and hypothermic effects of hypoglycin (80 mg/kg body wt) in two groups of 4 rats kept in an ambient temperature of either 20 or 30°. Rats kept at 20° developed marked hypothermia (Fig. 2a) and died between 28–40 hr after administration of hypoglycin, whereas those rats kept at 30° maintained their body temperature (Fig. 2b) and only 1 animal died between 8–12 hr after injection. With rats kept at 30° the extent of hypoglycaemia was less and its onset faster than in those rats kept at 20° (Fig. 2).

Administration of hypoglycin to rats kept at 20° caused butyrate (derived from the partial β-oxidation of fatty acids) and MCPA (derived from the metabolism of hypoglycin) to accumulate to maximum concentrations of 0.33 mM and 0.49 mM respectively after 7hr (Fig. 3). The combined concentration of isovalerate and 2-methylbutyrate (derived from the metabolism of leucine and isoleucine respectively) reached a maximum of 6 mM after 24 hr and hexanoate peaked at a concentration of 0.14 mM (Fig. 3). Plasma acetate concentrations were unaffected by hypoglycin administration. Acetate (0.2 mM) and hexanoate (0.03 mM) were the only volatile fatty acids detected in the plasma of control rats. At the time of death, the concentration of MCPA had substantially declined and the impairment of fatty acid oxidation appeared to be partly reversed as indicated by the decline in the concentrations of butyrate and hexanoate from their peak values. However, high branched-chain fatty acid concentrations and severe hypoglycaemia still persisted (Fig. 3).

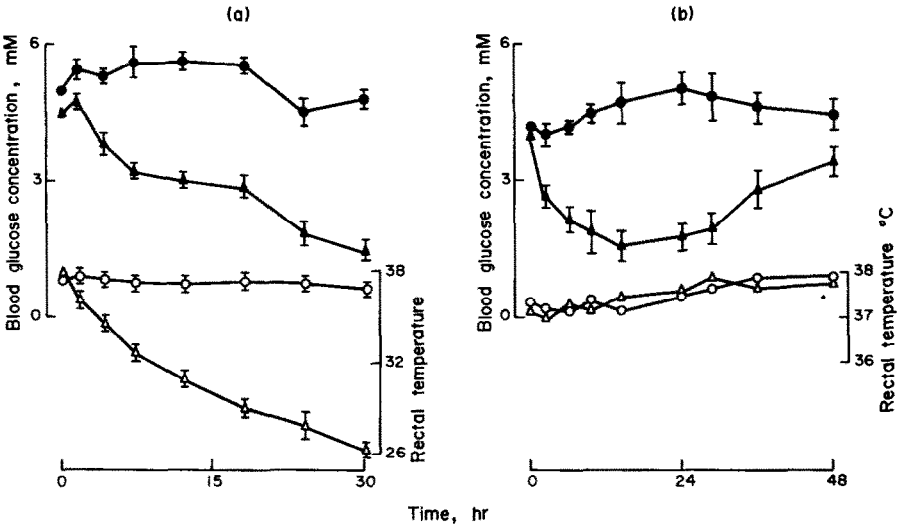


Fig. 2. Effect of hypoglycin on blood glucose concentrations and rectal temperatures in rats. Starved rats were injected with either hypoglycin (80 mg/kg body wt) or 0.14 M NaCl and kept in an ambient temperature of either (a) 20°, or (b) 30°. Blood glucose concentrations (▲) and rectal temperatures (△) in hypoglycin-treated rats. Blood glucose concentrations (●) and rectal temperatures (○) in control rats. Values are means for 4 animals ± S.E.M.

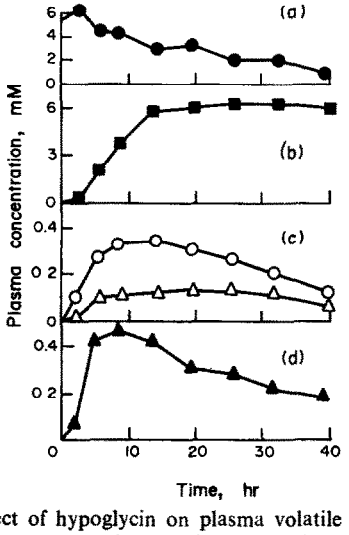


Fig. 3. Effect of hypoglycin on plasma volatile fatty acid concentrations in rats kept at 20°. Starved rats were injected with hypoglycin (80 mg/kg body wt) and kept in an ambient temperature of 20°. Serial blood samples (0.5 ml) were taken from the tail and plasma was obtained by centrifugation at 14,000 *g* for 1 min. (a) Glucose concentrations (●); (b) combined isovalerate and 2-methylbutyrate concentrations (■); (c) butyrate (○) and hexanoate (△) concentrations; (d) MCPA concentrations (▲). Values from one animal are shown; similar results were obtained with several other animals.

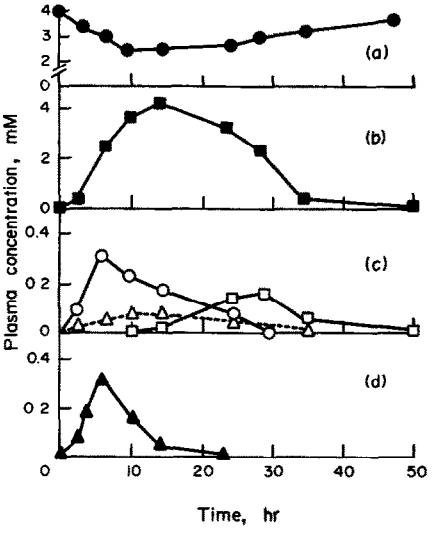


Fig. 4. Effect of hypoglycin on plasma volatile fatty acid concentrations in rats kept at 30°. The experimental conditions were as described in the legend to Fig. 3 except that rats were kept in an ambient temperature of 30°. (a) Glucose concentrations (●); (b) combined isovalerate and 2-methylbutyrate concentrations (■); (c) butyrate (○), hexanoate (△) and propionate (□) concentrations; (d) MCPA concentrations (▲). Values from one animal are shown; similar results were obtained with several other animals.

When rats were kept at 30° the combined concentration of isovalerate and 2-methylbutyrate reached a maximum (4 mM) 14 hr after the administration of hypoglycin (Fig. 4). MCPA, butyrate and hexanoate peaked at concentrations of 0.34 mM, 0.32 mM and 0.1 mM respectively (Fig. 4). These concentrations are lower than those in rats kept at 20° (Fig. 3). Propionate was only detected in those 3 animals that recovered, and then only 15 hr after hypoglycin administration (Fig. 4). The appearance of propionate may be linked to the disappearance of 2-methyl butyrate since propionyl–CoA is a product of the oxidation of 2-methylbutyrate and is presumably deacylated when its disposal by other pathways is inhibited or rate limiting.

Tanaka *et al.* have also reported that administration of hypoglycin causes an accumulation of MCPA and of isovalerate and 2-methylbutyrate

(distinguished by mass spectrographic methods) in rat plasma [5]. Lower amounts of isovalerate and 2-methylbutyrate (1.7 mM) and relatively high concentrations of butyrate (1.9 mM) occur in the plasma of mice 6 hr after the administration of hypoglycin (500 mg/kg body wt) [14]. Some volatile fatty acids are absorbed from the lower intestine of ruminants [15, 16] and the possibility remains that some of the volatile fatty acids found in plasma during hypoglycin poisoning may have been formed by microbial action in the intestine.

Pent-4-enoate (350 mg/kg body wt), when administered to rats kept at 20°, caused maximum hypoglycaemia and hypothermia after 1–2 hr with recovery after 4–6 hr (Fig. 5). By contrast, n-pentanoate (350 mg/kg body wt) did not lower blood glucose concentrations or body temperature (Fig. 5). The only fatty acids found in plasma 1 hr after adminstration of pent-4-enoate were pent-4-enoate (0.4 mM), acetate (0.2 mM) and hexanoate (0.03 mM). Acrylate, hydroxyacrylate or propionate, possible metabolites of pent-4-enoate [8, 17], were not detected. n-Pentanoate (0.25 mM) was found in the plasma of control (n-pentanoate-treated) rats at this time. Both pent-4-enoate and n-pentanoate were no longer detectable in plasma 2 hr after their administration. Injected doses of 350 mg/kg body wt would give a concentration of 5 mM if uniformly distributed solely in the body water, assuming no metabolism or excretion occurred. Rats given a higher dose of pent-4-enoate (500 mg/kg body wt) died after 30–45 min, and the maximum plasma concentration of pent-4-enoate found was approximately 1 mM after 30 min.

The accumulation of volatile fatty acids following hypoglycin administration presumably results from deacylation of their respective acyl–CoA species [1], which themselves accumulate following the inhibition of butyryl–CoA (short-chain) dehydrogenase (EC 1.3.2.1) and branched-chain acyl–CoA dehydrogenases [18–20]. Volatile fatty acids containing three to eight carbon atoms produce coma and electroencephalographic changes in rats and rabbits [21, 22]. Teychenne *et al.* have shown that when the concentration of isovalerate reached 3.0–3.5 mM in the cerebrospinal fluid of rabbits, brain electrical activity was decreased and coma ensued [13]. Also, Rizzoli and Galzigna have suggested that butyrate induces coma by the formation of complexes with the chemical transmitters serotonin and dopamine in the central nervous system [23]. It is reasonable, therefore, to suggest that the volatile fatty acids which accumulate in rats and mice following hypoglycin administration can cross the blood–brain barrier and, either by the formation of similar complexes or by a direct action, produce severe central depression which contributes to the hypothermia, coma and eventual death.

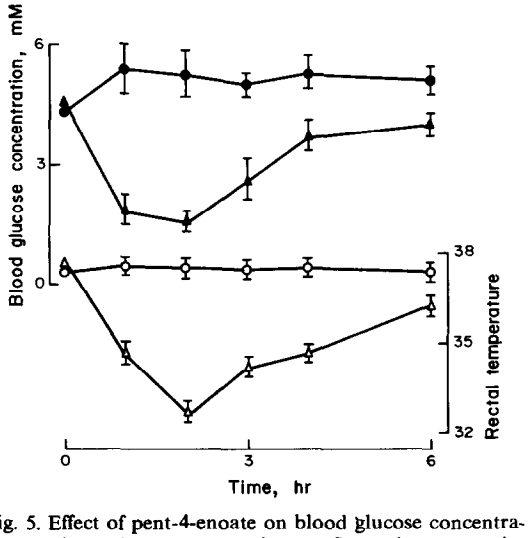


Fig. 5. Effect of pent-4-enoate on blood glucose concentrations and rectal temperatures in rats. Starved rats were injected with either pent-4-enoate or n-pentanoate (both 350 mg/kg body wt) and kept in an ambient temperature of 20°. Blood glucose concentrations (▲) and rectal temperatures (△) in pent-4-enoate treated rats. Blood glucose concentrations (●) and rectal temperatures (○) in n-pentanoate treated rats. Values are means for 4 animals ± S.E.M.

Assuming that the concentration of pent-4-enoate in the cerebrospinal fluid is the same as in plasma (maximum 1 mM), this is probably insufficient to cause hypothermia and coma as a result of its properties as a C_5 fatty acid, unless it has a more specific action. Indeed, the depressant effects of C_5 fatty acids are lessened by the presence of a double bond [13].

Effects of hypoglycin and pent-4-enoate on acyl–CoA dehydrogenase activities

Malate dehydrogenase, which was not inhibited, was equally active in mitochondrial extracts from both control and hypoglycin treated rats and was used as an internal control. The activity of butyryl–CoA (short-chain) dehydrogenase (EC 1.3.2.1) was low or absent in extracts of mitochondria isolated from livers taken from rats 24 hr after the administration of hypoglycin (Table 1). Neither palmitoyl–CoA (long-chain) dehydrogenase (EC 1.3.2.2) nor acetoacetyl–CoA thiolase (EC 2.3.1.9) were inhibited (Table 1). Hexanoyl–CoA dehydrogenase activity was inhibited, although to a lesser extent than butyryl–CoA dehydrogenase (Table 1). This may be because hexanoyl–CoA is also a substrate for palmitoyl–CoA dehydrogenase [19]. Both isovaleryl–CoA and 2-methylbutyryl–CoA dehydrogenase activities were inhibited by 50–60 per cent (Table 1).

Dialysis of inhibited mitochondrial extracts for 18 hr against 20 mM $KHCO_3$, 10 mM HEPES, 1 mM mercaptoethanol, pH 7.0 at 4° did not relieve the inhibition of any acyl–CoA dehydrogenase activity; the activities in control and inhibited extracts decayed at the same rate. Inhibitions also persisted after removal of low molecular weight compounds by passage through a column of Sephadex G-25. Therefore, an inhibitory metabolite of hypoglycin, probably MCPA–CoA [1], binds very tightly or irreversibly to some acyl–CoA dehydrogenases.

In contrast, there was no impairment of any acyl–CoA dehydrogenase activity in either extracts of mitochondria isolated from livers of rats 2 hr after administration of pent-4-enoate (350 mg/kg body wt), or, in extracts of mitochondria incubated *in vitro* with 1.0 mM pent-4-enoate. This agrees with the absence of accumulation of volatile fatty acids in

Table 1. Acyl–CoA dehydrogenase activities in mitochondria isolated from livers taken from rats after administration of hypoglycin

Enzyme activity	Time after injection 24 hr	48 hr	Control activity
Butyryl–CoA dehydrogenase	84.8 ± 5.9 (5)	0 (2)	18.3 ± 5.6 (5)
Isovaleryl–CoA dehydrogenase	56.6 ± 8.0 (4)	0 (2)	8.4 ± 3.8 (4)
2-Methylbutyryl–CoA dehydrogenase	45.0 (1)	0 (1)	9.0 (1)
Hexanoyl–CoA dehydrogenase	54.0 ± 20.1 (5)	0 (2)	12.4 ± 8.8 (5)
Palmitoyl–CoA dehydrogenase	0 (3)	0 (2)	15.1 ± 8.7 (3)
Acetoacetyl–CoA thiolase	0 (3)	0 (2)	143 ± 50 (3)

Starved rats were injected intraperitoneally with hypoglycin (80 mg/kg body wt) or 0.14 M NaCl and kept in an ambient temperature of 30°. Rats were sacrificed either 24 hr or 48 hr after injection and mitochondria isolated from livers. Mitochondrial suspensions were diluted with 10 mM potassium phosphate, pH 7.2 to a protein concentration of approximately 5 mg/ml and Triton-X100 added to a final concentration of 0.5 per cent (v/v). After centrifugation at 130,000 *g* and 4° for 60 min, enzyme activities were assayed in supernatants as described previously [1]. The results are expressed as percentage decreases of the activity of controls and are means ± S.D. with the number of observations in parentheses. Control activities are given in nmol/min/mg of protein.

rat plasma after administration of pent-4-enoate. Penta-2,4-dienoyl–CoA, a metabolite of pent-4-enoate [8], inhibits purified acetoacetyl–CoA thiolase [17]. However, unexpectedly, there was no inhibition of acetoacetyl–CoA thiolase activity in any extract of pent-4-enoate inhibited mitochondria.

Isovalericacidaemia is an hereditary metabolic disorder in man in which large amounts of isovalerate accumulate in the blood [24]. Since butyrate does not accumulate in this disease, Tanaka and Isselbacher [25] proposed that isovaleryl–CoA is not, as often assumed, a substrate for the butyryl–CoA dehydrogenase normally associated with β-oxidation [26, 27], and that there is a specific isovaleryl–CoA dehydrogenase. In support of this proposal, purified ox liver butyryl–CoA dehydrogenase is devoid of detectable activity towards branched-chain acyl–CoA esters [17] and isovaleryl–CoA dehydrogenase activity has been concentrated relative to butyryl–CoA dehydrogenase activity in extracts of rat liver mitochondria [28]. The differing extents of inhibition of butyryl–CoA dehydrogenase and isovaleryl–CoA dehydrogenase (Table 1), and the different time courses of accumulation of butyrate and isovalerate (Figs. 3 and 4), following hypoglycin administration to rats, supports the proposal for the existence of at least two separate acyl–CoA dehydrogenases. In human isovalericacidaemia, 2-methylbutyrate does not accumulate [24, 25], suggesting that in man there are at least two branched-chain acyl–CoA dehydrogenases. Further, the absence of isobutyrate (derived from valine metabolism) in rat plasma following hypoglycin administration, suggests that there is a separate dehydrogenase for isobutyryl–CoA in rat, which is not inhibited by MCPA–CoA.

Our results do not support the alternative proposal that these effects can be explained in terms of different affinities of one enzyme for acyl–CoA esters and inhibitors [29]. This proposal requires that the inhibitions are reversible and our results, together with those of Kean [30], indicate that MCPA–CoA irreversibly inhibits acyl–CoA dehydrogenases. Therefore, *de novo* enzyme synthesis may be required for recovery from hypoglycin poisoning, and this could explain the observation that riboflavin phosphate protects against the chronic effects of hypoglycin [31].

β-Oxidation and pyruvate oxidation in mitochondria isolated from rats given hypoglycin

We have previously reported the decreased rate and extent of β-oxidation of several acyl–carnitines by rat liver mitochondria incubated with MCPA in state 3 conditions [1, 18]. The state 3 rate of oxidation [1] of 10 μM palmitoyl–carnitine by liver mitochondria isolated from rats 4 hr and 24 hr after administration of hypoglycin (80 mg/kg body wt) was inhibited by 55 ± 12% and 51 ± 6% (mean value for 4 experiments ± S.E.M.) respectively. Addition of 1.0 mM L-carnitine had no effect on these inhibitions. The state 3 rate of oxidation of 10 mM succinate (which was not inhibited) was used as an internal standard. The state 3 rate of oxidation of 10 mM pyruvate was not inhibited. The rate of palmitoyl–carnitine oxidation returned to normal 48 hr after administration of hypoglycin, coinciding with the return to normal blood glucose concentrations and the disappearance of plasma volatile fatty acids (Fig. 4). The concentrations of MCPA and butyrate in plasma had substantially declined after 24 hr (Figs. 3 and 4) whilst there was no reversal of the inhibition of palmitoyl–carnitine oxidation. It is possible that at this time the rate of disposal of these volatile fatty acids (as their glycine conjugates) exceeded their rate of accumulation, or, the mobilisation of fatty acids for β-oxidation was diminished.

To examine more closely the *in vivo* effects of hypoglycin on mitochondrial oxidations, liver mitochondria were isolated 18 hr after administration of hypoglycin (100 mg/kg body wt). The rate of oxidation of 10 μM palmitoyl–carnitine was inhibited by 50 per cent in these mitochondria (Fig. 6). With de-

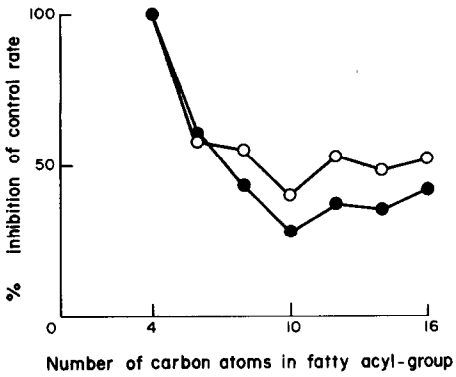


Fig. 6. The percentage inhibition of control rates of acyl-carnitine oxidations by mitochondria isolated from livers of hypoglycin-treated rats. Mitochondria were isolated from the livers of rats 18 hr after the administration of either hypoglycin (100 mg/kg body wt), or, in the case of controls, 0.14 M NaCl. The state 3 or 3u rate of oxidation of 10 μM acyl-carnitines were measured as described previously [1]. The results are expressed as the percentage inhibition of control rates of acyl-carnitine oxidation with either state 3 (O) or state 3u (●) conditions.

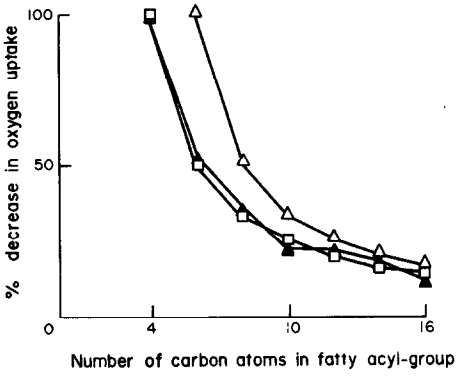


Fig. 8. The observed and theoretical percentage decreases in the oxygen uptake for the state 3 oxidation of 20 μM acyl-carnitines by mitochondria isolated from the livers of rats 18 hr after the administration of hypoglycin (100 mg/kg body wt). Oxygen uptake was measured as described in the text; observed values (▲), and theoretical values for oxidation as far as butyryl-CoA (□) or as far as hexanoyl-CoA (△).

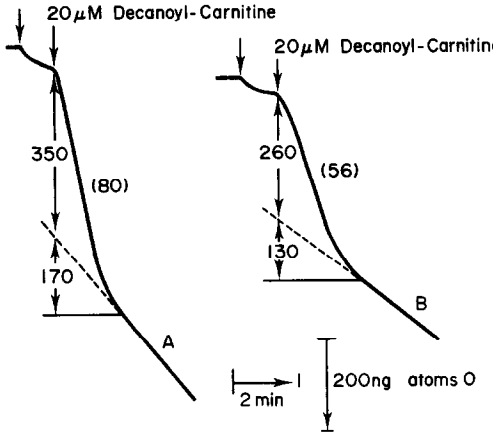


Fig. 7. The state 3 oxidation of decanoyl-carnitine by mitochondria isolated from the livers of (A) control rats, and, (B) rats 18 hr after the administration of hypoglycin (100 mg/kg body wt). Mitochondria (5 mg of protein per incubation) were added where indicated (by unlabelled arrows) followed by 20 μM decanoyl-carnitine. Rates of oxygen uptake (ng atoms O/min/mg of protein) are given in parentheses. The amount of oxygen consumed (ng atoms O) is indicated by the vertical arrows; the upper one for each pulse gives the apparent oxygen consumption and the lower one the apparent contribution of endogenous respiration.

creasing acyl-carnitine chain-length, the inhibition of the rate of oxidation decreased to 25–35 per cent with decanoyl-carnitine and then increased to 100 per cent with butyryl-carnitine (Fig. 6). Similar patterns of inhibition were found using either state 3 or 3u conditions (Fig. 6). Osmundsen and Sherratt found a similar pattern of inhibition in MCPA-treated mitochondria, except that hexanoyl-carnitine oxidation was also completely inhibited [18].

The oxidation of decanoyl-carnitine by mitochondria from control and hypoglycin treated rats is shown in Fig. 7. The stoichiometry of oxygen uptake suggested that endogenous respiration was suppressed during acyl-carnitine oxidation, and that the true amount of oxygen consumed was the sum of the apparent oxygen consumption and the apparent contribution of endogenous respiration (see Fig. 7). Assuming this, the percentage decrease in oxygen uptake during the oxidation of acyl-carnitines of various chain-lengths by mitochondria isolated from hypoglycin-treated rats agrees with the theoretical decrease in oxygen consumption if β-oxidation only proceeds to the level of butyrate (Fig. 8). For example,

$$\text{palmitoyl-CoA} + 6O_2 \rightarrow 3 \text{ acetoacetate} + \text{butyryl-CoA}$$

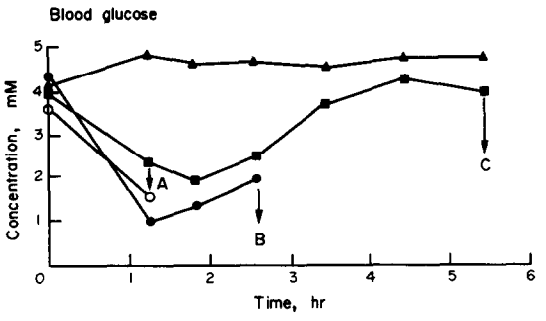
compared to its complete oxidation by mitochondria from control animals,

$$\text{palmitoyl-CoA} + 7O_2 \rightarrow 4 \text{ acetoacetate} + \text{CoASH}.$$

The amount of acyl-carnitine undergoing partial oxidation (60 nmol) is much greater than the total CoASH content of the mitochondria (2–3 nmol/mg of protein [1]) so that repeated recycling of CoASH must occur. This is achieved by an acyl-CoA hydrolase (EC 3.1.2.–) which is located in the matrix and has a high apparent K_m (2 mM) for butyryl-CoA [1]. Therefore, the state 3 rate of partial β-oxidation could be limited either by the rate of hydrolysis of butyryl-CoA, or, by direct inhibition of a stage of β-oxidation by MCPA-CoA or accumulated butyryl-CoA. Since inhibited mitochondria contain significant concentrations of CoASH [1], sequestration of mitochondrial CoASH as inert metabolites (as claimed by Bressler *et al.* [32]) is not the cause of inhibition of β-oxidation.

β-Oxidation and pyruvate oxidation in mitochondria isolated from rats given pent-4-enoate

Pent-4-enoate (0.1–1.0 mM) inhibits palmitoyl-carnitine oxidation [8] and pyruvate oxidation [33]



Rates of substrate oxidation by liver mitochondria expressed as % of control rates

	A	B	C
10mM pyruvate	57	68	100
20μM palmitoyl-carnitine	15	19	20

Percentage change of rate of palmitoyl-carnitine oxidation on addition of 1mM L-carnitine

Inhibited	0	22	50
Control	-10	-14	-8

Fig. 9. The hypoglycaemia and inhibition of palmitoyl–carnitine and pyruvate oxidation after administration of pent-4-enoate to rats. Rats were injected with either pent-4-enoate or n-pentanoate (both 350 mg/kg body wt). The animals were killed at different times (indicated by arrows A, B and C), liver mitochondria isolated, and their state 3 rate of respiration measured with reference to the state 3 rate of oxidation of 10 mM succinate as internal standard. Blood glucose concentrations were measured up to the time of death; control (▲), animals given pent-4-enoate (○, ●, ■).

by 70–90 per cent in isolated mitochondria. Similar inhibitions were found in mitochondria isolated from livers of rats given pent-4-enoate. At various times after administration of pent-4-enoate, their ability to oxidize palmitoyl–carnitine was decreased by 80–85 per cent compared with mitochondria isolated from livers of rats given n-pentanoate; the extent of impairment of oxidation of 10 mM pyruvate was variable (Fig. 9). With mitochondria from pent-4-enoate-treated rats, the addition of 1.0 mM L-carnitine partly restored (by up to 50 per cent) the rate of oxidation of palmitoyl–carnitine (Figs. 9 and 10). During recovery from hypoglycaemia there was a progressive relief of the impairment of pyruvate oxidation, which was paralleled by an increased extent of restoration of palmitoyl–carnitine oxidation by added L-carnitine (Figs. 9 and 10). This indicates that the inhibitions are not due to depletion of CoASH concentrations by accumulation of unusual acyl–CoA species in the matrix as claimed by Bressler *et al.* [32]. However, the oxidation of palmitoyl–carnitine remained powerfully inhibited in the absence of L-carnitine for some time after blood glucose concentrations returned to normal (Figs. 9 and 10). This suggests that *in vivo* most inhibitory metabolites of pent-4-enoate were cleared from the matrix at this time, although sufficient remained to inhibit β-oxidation. The relatively short duration of hypoglycaemia confirmed that some inhibitions of mitochondrial oxidations are readily reversible [1, 7, 34].

The partial restoration of the rate of palmitoyl–carnitine oxidation by added L-carnitine contrasts with results *in vitro* where 1.0 mM L-carnitine did not relieve inhibition of β-oxidation, although there was a partial relief of inhibition of pyruvate oxidation [8]. Addition of L-carnitine to mitochondria inhibited *in vivo* presumably removes some of the inhibitors from the matrix, whereas, with incubations *in vitro*, the presence of pent-4-enoate in the entire incubation medium enables the continuous formation of inhibitory acyl–CoA species.

There was no decrease in the oxygen uptake during a pulse of acyl–carnitine oxidation by either mitochondria isolated from livers taken from rats made hypoglycaemic by injection of pent-4-enoate (Fig. 11), or by mitochondria inhibited *in vitro* with pent-4-enoate (not shown). Thus, although the rate of oxidation of acyl–carnitines is severely impaired by pent-4-enoate, their oxidation to acetoacetate is complete.

CONCLUSIONS

Both hypoglycin and pent-4-enoate cause extensive disturbances of intermediary metabolism in animals. However, there has been much confusion in the investigation and interpretation of these effects [7].

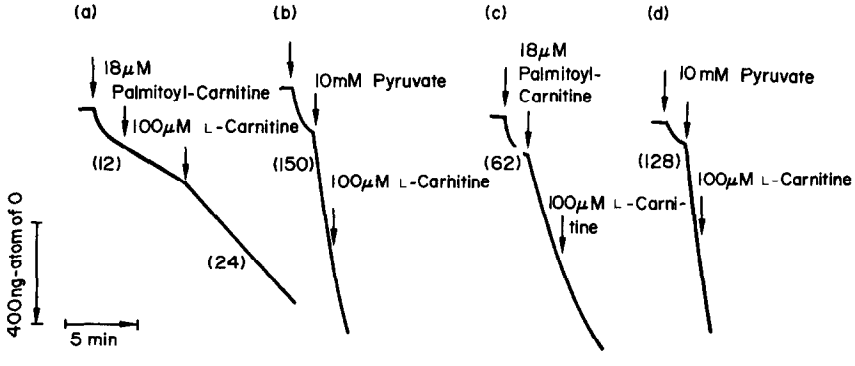


Fig. 10. The state 3 oxidation of palmitoyl–carnitine and pyruvate by mitochondria isolated from livers of rats after administration of either pent-4-enoate or n-pentanoate. Mitochondria were isolated 5 hr after administration of either pent-4-enoate (a, b) or n-pentanoate (c, d) (both 350 mg/kg body wt). Mitochondria (about 5 mg of protein per incubation) were added where indicated (by unlabelled arrows) and palmitoyl–carnitine, pyruvate and L-carnitine were then added as shown. In this experiment the inhibition of palmitoyl–carnitine only required a low concentration (100 μM) of L-carnitine for partial reversal (a).

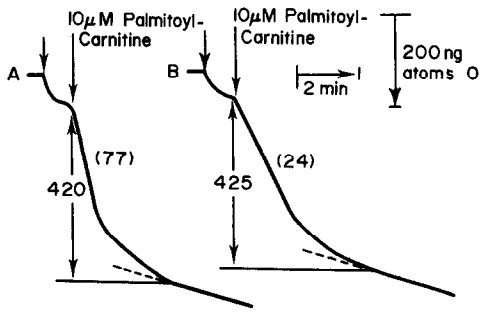


Fig. 11. The state 3 oxidation of palmitoyl-carnitine by mitochondria isolated from livers taken from rats 2 hr after administration of (A) n-pentanoate, or, (B) pent-4-enoate (both 350 mg/kg body wt). Mitochondria (about 5 mg of protein per incubation) were added where indicated (by unlabelled arrows) followed by μM palmitoyl-carnitine. Rates of oxygen uptake (ng atoms O/min/mg of protein) are given in parentheses and the amount of oxygen consumed (ng atoms O) is indicated by the vertical arrows.

Both compounds interfere with β-oxidation and gluconeogenesis in a variety of *in vitro* systems [8, 17–20, 33, 35–38] but the analogies between them must not be extended too far since their mechanisms of action are clearly different [1, 7, 34].

We originally suggested that hypoglycin and pent-4-enoate impair gluconeogenesis secondary to inhibition of fatty acid oxidation, such that glucose is the only major metabolic fuel, and when glycogen is exhausted, hypoglycaemia ensues [39]. Other workers outlined similar explanations which also envisaged increased rates of glucose utilization [32, 40]. It is not always appreciated, however, that to get hypoglycaemia it is only necessary for glucose to be used at a faster rate than it can be replaced [39]. Indeed, we have recently shown that both gluconeogenesis and the rate of glucose utilization are severely inhibited at least between 16 to 21 hr after the administration of hypoglycin to rats [41, 42]. There is, however, no quantitative information about the effects of hypoglycin or of pent-4-enoate on fatty acid oxidation *in vivo*, nor on the effects of pent-4-enoate on glucose metabolism *in vivo* [42].

An increased concentration of plasma free fatty acids is found 4 hr after administration of hypoglycin to rats [43]. A similar increase is found after acute administration of hypoglycaemic doses of pent-4-enoate, but not after chronic administration of smaller doses [44]. Hypoglycin, and probably pent-4-enoate, poisoning is usually associated with fatty livers [45], when presumably free fatty acids are supplied from adipose stores in excess of the amounts that are oxidised to CO_2 and ketone bodies, and are therefore converted to triglycerides. It must not be assumed that this accumulation is necessarily only due to impaired fatty acid oxidation. It may also be caused by excessive lipolysis resulting from the physiological stress of hypoglycaemia. This will cause the concentration of long-chain acyl–CoA esters in the cytosol to increase up to the limit set by the total amount of available CoASH in this compartment (perhaps 40 μM [46]) and by the kinetic properties of the ubiquitous acyl–CoA hydrolases.

Tanaka found that large amounts of some medium-chain, unsaturated dicarboxylic acids were excreted in the urine of rats given hypoglycin [6] and in two cases of human ackee poisoning [47]. An increased concentration of extra-mitochondrial long-chain acyl–CoA esters may result in some ω-oxidation to give mono-CoA esters of some dicarboxylic acids; these may then be transferred into the mitochondrial matrix by the action of the carnitine acyltransferases associated with the inner mitochondrial membrane, where they may undergo limited β-oxidation from the —CO.S.CoA end of the molecule to an extent limited by the inactivation of butyryl–CoA dehydrogenase (i.e. to yield compounds of chain length C_5). Deacylation of the resulting mono-acyl–CoA esters would give dicarboxylic acids such as suberic acid.

The ketosis found in hypoglycin and in pent-4-enoate poisoning has been attributed to a greater inhibition of the peripheral utilization of ketone bodies than of their hepatic production [44, 48]. However, no deductions can be made about their rates of either generation or utilization from blood concentrations alone, and higher concentrations may merely represent another steady state. The lower 3-hydroxybutyrate/acetoacetate ratio indicates that the mitochondrial matrix is more oxidized during hypoglycin or pent-4-enoate poisoning [44, 48], and this is probably due to the decreased rate of dehydrogenation of respiratory substrates. Also, the more oxidised state of the cytosol in rat livers perfused with pent-4-enoate [37] indicates that there is a decreased supply of reducing equivalents from the matrix.

MCPA–CoA inhibits the maximum rate of oxidative generation of acetyl–CoA from palmitoyl–carnitine by isolated rat liver mitochondria incubated in state 3 conditions by 50 per cent [19]. MCPA–CoA will clearly cause a smaller percentage inhibition if the rate of flux through β-oxidation is not maximal. It cannot be assumed that the limited and partial flux through hepatic β-oxidation is always insufficient to maintain gluconeogenesis and hence blood glucose concentrations. Similarly, it cannot be assumed that, although fatty acid oxidation may stimulate gluconeogenesis [49], these processes are always coupled. ATP, NADH and acetyl–CoA required for gluconeogenesis can be generated by oxidation of the carbon skeletons of most amino acids derived from the breakdown of tissue proteins. Further, some of the gluconeogenic precursors may also be oxidized, and metabolites of hypoglycin and of pent-4-enoate will interfere with the oxidation of such alternative energy stores in unpredictable ways. It is therefore likely that the accumulation of high concentrations of several acyl–CoA esters in the mitochondrial matrix will interfere with gluconeogenesis by inhibiting the activation of pyruvate carboxylase (EC 6.4.1.1) (a key gluconeogenic enzyme) by acetyl–CoA [1]. Whether any particular acyl–CoA ester accumulates sufficiently to inhibit depends on the resultant of its rates of formation and of the various reactions that it can undergo [1]. Indeed, Williamson *et al.* [37] and Toews *et al.* [50] found evidence for a block at pyruvate carboxylase from analyses of the conentrations of metabolites in perfused rat livers in which gluconeogenesis was inhibited by pent-4-enoate.

Maximum hypoglycaemia occurred 2 hr after ad-

ministration of pent-4-enoate to rats (Fig. 5), although at this time plasma concentrations of the acid had substantially declined. Further, rats had largely recovered from hypoglycaemia 5 hr after pent-4-enoate administration and yet, at this time, mitochondria isolated from the livers of these animals had their ability to oxidize palmitoyl–carnitine, but not pyruvate, severely impaired (Figs. 9 and 10). This indicates that some unusual acyl–CoA esters derived from pent-4-enoate persist in the matrix and specifically inhibit β-oxidation, but their concentrations are not sufficient to strongly inhibit either pyruvate dehydrogenase (EC 1.2.4.1) or pyruvate carboxylase, or to acylate more than a small proportion of the available CoASH.

MCPA–CoA (derived from the metabolism of hypoglycin) and short-chain acyl–CoA esters (which accumulate as a result of inhibition of some short-chain acyl–CoA dehydrogenases) are disposed of in the urine as the free acid after deacylation and as the glycine conjugate [5, 6, 47]. Conjugation with glycine only occurs in liver and kidney so that MCPA and isovalerate produced elsewhere must be transported in the blood to these tissues. Butyryl–CoA synthetase (EC 6.2.1.2) catalyses the formation of their CoA-esters in the matrix of liver mitochondria [51]. Since glycine *N*-acylase (EC 2.3.1.13) is also located in the mitochondrial matrix [52] and has a lower affinity for glycine than for most of its acyl–CoA esters [53], the rates of conjugation will be dependent on the matrix concentration of glycine rather than on the total quantity in the body. In accordance with these considerations, administration of large amounts of glycine has been reported to protect rats against the toxic effects of hypoglycin [54] and to decrease the acidaemia in a case of hereditary isovalericacidaemia [55].

It is difficult to explain how tryptophan or lysine potentiate the hypoglycaemia and the urinary excretion of glutarate seen in hypoglycin poisoning in rats [56]. Dehydrogenation of glutaryl–CoA is common to the metabolism of both these amino acids suggesting that glutaryl–CoA dehydrogenase is inhibited by hypoglycin metabolites [56]. Since very high concentrations (1–4 mM) of glutaryl–CoA were claimed to inhibit malate uptake by rat liver mitochondria, Tanaka and Kerley also suggested that glutaryl–CoA accumulates in the liver and may inhibit malate efflux, and hence transport of reducing equivalents, out of the mitochondrion [56]. Cytoplasmic NADH is essential for gluconeogensis from pyruvate [57] and malate transport is rate limiting in the early stages of gluconeogenesis [58]. However, although glutaryl–CoA dehydrogenase is associated with the mitochondrial fraction of rat liver [59], its sub-mitochondrial localisation is unknown and some may even occur in the peroxisomes. Further, there is no information on the effects of glutaryl–CoA on other mitochondrial processes, on its rate of hydrolysis by acyl–CoA hydrolases or whether it has any effect on pyruvate carboxylase. Finally, tryptophan or lysine may cause insulin release from the pancreas directly [60, 61].

All our results support the view that the hypoglycaemia caused by hypoglcyin and by pent-4-enoate in starved animals is due to inhibition of gluconeogenesis, with inhibition of pyruvate carboxylase as a major factor. The complex sequence of metabolic events following administration of these compounds to either death or recovery is only partly understood. Further, in most investigations, it has not been appreciated that hypoglycin and pent-4-enoate cause a profound hypothermia in small animals that are not kept in thermoneutral conditions, which will decrease the absolute rates of metabolism compared with control animals. There are, as yet, no measurements on the effects of hypoglycin or of pent-4-enoate on O_2 consumption in animals maintained in thermoneutral conditions.

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REFERENCES

1. D. Billington, H. Osmundsen and H. S. A. Sherratt, *Biochem. Pharmac.* **27**, 2879 (1978).
2. H. S. A. Sherratt, P. C. Holland, J. Marley and A. E. Senior, in *Symposium on Mechanisms of Toxicity* (Ed. W. N. Aldridge) p. 205. Macmillan, London (1971).
3. J. Marley and H. S. A. Sherratt, *Biochem. Pharmac.* **22**, 281 (1973).
4. K. Tanaka, E. M. Miller and K. J. Isselbacher, *Proc. natn. Acad. Sci., U.S.A.* **68**, 20 (1971).
5. K. Tanaka, K. J. Isselbacher and V. E. Shih, *Science, N.Y.* **175**, 69 (1972).
6. K. Tanaka, *J. biol. Chem.* **247**, 7465 (1972).
7. H. S. A. Sherratt and H. Osmundsen, *Biochem. Pharmac.* **25**, 743 (1976).
8. P. C. Holland and H. S. A. Sherratt, *Biochem. J.* **136**, 157 (1973).
9. A. G. Huggett and D. A. Nixon, *Lancet* **1**, 368 (1957).
10. C. Remesy and C. Demigne, *Biochem. J.* **141**, 85 (1974).
11. S. Englard and L. Siegel, *Meth. Enzym.* **13**, 99 (1969).
12. H. S. A. Sherratt, *Br. med. Bull.* **25**, 250 (1969).
13. P. F. Teychenne, I. Walters, L. E. Claveria, D. B. Calne, J. Price, B. B. Macgillivary and D. Gompertz, *Clin. Sci. Molec. Med.* **50**, 463 (1976).
14. S. S. Al-Bassam, unpublished work (1976).
15. A. W. Boyne, R. M. Campbell, J. Davidson and D. R. Cuthburtson, *Br. J. Nutr.* **10**, 325 (1956).
16. A. M. Badawy, R. M. Campbell, D. R. Cuthbertson and W. S. Mackie, *Br. J. Nutr.* **12**, 384 (1958).
17. P. C. Holland, A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **136**, 172 (1973).
18. D. Billington, E. A. Kean, H. Osmundsen and H. S. A. Sherratt, *IRCS (Research on Biochemistry: Pharmacology)* **2**, 1712 (1974).
19. H. Osmundsen and H. S. A. Sherratt, *FEBS Lett.* **55**, 38 (1975).
20. H. Osmundsen, D. Billington and H. S. A. Sherratt, *Biochem. Soc. Trans.* **3**, 331 (1975).
21. A. P. White and F. E. Samson, *Am. J. Physiol.* **186**, 271 (1956).
22. F. E. Samson, N. Dahl and D. R. Dahl, *J. Clin. Invest.* **35**, 1291 (1956).
23. A. A. Rizzoli and L. Galzigna, *Biochem. Pharmac.* **19**, 2727 (1970).
24. K. Tanaka, M. L. E. Budd and K. J. Isselbacher, *Proc. natn. Acad. Sci., U.S.A.* **56**, 236 (1966).
25. K. Tanaka and K. J. Isselbacher, *J. biol. Chem.* **242**, 2966 (1967).
26. D. E. Green, S. Mii, H. R. Mahler and R. M. Bock, *J. biol. Chem.* **206**, 1 (1954).

27. B. K. Bachawart, W. G. Robinson and M. J. Coon, *J. biol. Chem.* **219**, 539 (1956).
28. H. Osmundsen, D. Billington and H. S. A. Sherratt, *Biochem. Soc. Trans.* **2**, 1286 (1974).
29. P. C. Engel, *Nature, Lond.* **248**, 140 (1974).
30. E. A. Kean, *Biochim. biophys. Acta* **422**, 8 (1976).
31. L. von Holt and C. von Holt, *Biochem. Z.***331**, 422 (1959).
32. R. Bressler, C. F. Corredor and K. Brendel, *Pharmac. Rev.* **21**, 105 (1969).
33. A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **110**, 499 (1968).
34. H. S. A. Sherratt, P. C. Holland, H. Osmundsen and A. E. Senior, in *A Sympsoium on Hypoglycin* (Ed. E. A. Kean), p. 127. Academic Press, New York (1976).
35. C. Corredor, K. Brendel and R. Bressler, *J. biol. Chem.* **244**, 1212 (1969).
36. A. E. Senior, B. Robson and H. S. A. Sherratt, *Biochem. J.* **110**, 511 (1968).
37. J. R. Williamson, S. G. Rognstad and M. J. Peterson, *J. biol. Chem.* **245**, 3242 (1970).
38. E. A. Kean and I. J. Rainford, *Biochim. biophys. Acta* **320**, 557 (1973).
39. A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **110**, 521 (1968).
40. C. von Holt, L. von Holt and H. Bohm, *Biochim. biophys. Acta* **125**, 11 (1966).
41. D. Billington, H. Osmundsen, J. R. Taylor and H. S. A. Sherratt, *Biochem. Soc. Trans.* **4**, 1035 (1976).
42. H. Osmundsen, D. Billington, J. R. Taylor and H. S. A. Sherratt, *Biochem. J.* **170**, 377 (1978).
43. E. C. DeRenzo, K. W. McKerns, H. H. Bird, W. P. Cekleniak, B. Coulomb and E. Kaleita, *Biochem. Pharmac.* **1**, 236 (1958).
44. A. E. Senior and H. S. A. Sherratt, *J. Pharm. Pharmac.* **21**, 85 (1969).
45. K. R. Hill, G. Brass and K. P. Clearkin, *West Ind. Med. J.* **4**, 91 (1955).
46. P. B. Garland, D. Sheperd and D. W. Yates, *Biochem. J.* **97**, 567 (1965).
47. K. Tanaka, E. A. Kean and B. Johnson, *New Engl. Med. J.* **295**, 461 (1976).
48. D. H. Williamson and M. B. Wilson, *Biochem. J.* **94**, 19c (1965).
49. J. H. Exton, *Metabolism* **21**, 945 (1972).
50. C. J. Toews, C. Lowry and N. B. Ruderman, *J. biol. Chem.* **245**, 818 (1970).
51. P. B. Garland, D. W. Yates and B. A. Hadock, *Biochem. J.* **119**, 553 (1970).
52. S. J. Gatley and H. S. A. Sherratt, *Biochem. J.* **166**, 39 (1977).
53. K. Bartlett and D. Gompertz, *Biochem. Med.* **10**, 15 (1974).
54. H. S. A. Sherratt and S. S. Al-Bassam, *Lancet* **2**, 1243 (1976).
55. I. Krieger and K. Tanaka, *Pediat. Res.* **10**, 25 (1976).
56. K. Tanaka and R. Kerley, in *A Symposium on Hypoglycin* (Ed. E. A. Kean) p. 163. Academic Press, New York (1976).
57. J. R. Williamson, A. J. Meijer and K. Ohkawa, in *Regulation of Hepatic Metabolism* (Eds F. Lundquist and N. Tygstrup) p. 457. Munksgaard Ltd., Copenhagen (1974).
58. J. B. Chappel, *Br. med. Bull.* **24**, 150 (1968).
59. A. Besrat, C. E. Polan and L. M. Henderson, *J. biol. Chem.* **244**, 1461 (1969).
60. M. E. Krahl, *A. Rev. Physiol.* **36**, 331 (1974).
61. S. A. Smith and C. I. Pogson, *Biochem. J.* **168**, 495 (1977).