Biochem. J. (1968) **110**, 521
Printed in Great Britain

Biochemical Effects of the Hypoglycaemic Compound Pent-4-enoic Acid and Related Non-hypoglycaemic Fatty Acids

CARBOHYDRATE METABOLISM

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(*Received* 17 *May* 1968)

1. The effects of the hypoglycaemic compound, pent-4-enoic acid, and of four structurally related non-hypoglycaemic compounds (pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid), on glycolysis, glucose oxidation and gluconeogenesis in some rat tissues were determined. 2. None of the compounds at low concentrations inhibited glycolysis by particle-free supernatant fractions from rat liver, skeletal muscle and intestinal mucosa, though there was inhibition by cyclopropanecarboxylic acid and cyclobutanecarboxylic acid at 3mM concentration. 3. Pent-4-enoic inhibited the oxidation of [1-^{14}C]palmitate by rat liver slices, but did not increase the oxidation of [U-^{14}C]glucose. 4. Pent-4-enoic acid (0·01 mM) strongly inhibited gluconeogenesis by rat kidney slices from pyruvate or succinate, but none of the other compounds inhibited significantly at low concentrations. 5. There was also some inhibition of gluconeogenesis in kidney slices from rats injected with pent-4-enoic acid. 6. The mechanism of the hypoglycaemic effect of pent-4-enoic acid is discussed; it is suggested that there is an inhibition of fatty acid and ketone-body oxidation and of gluconeogenesis so that glucose reserves become exhausted, leading to hypoglycaemia. 7. The mechanism of the hypoglycaemic action of pent-4-enoic acid appears to be similar to that of hypoglycin.

Some effects of pent-4-enoic acid on carbohydrate metabolism have been investigated. Both pent-4-enoic acid and hypoglycin are hypoglycaemic in starved animals, so this effect cannot be due to inhibition of glycogenolysis (Feng & Patrick, 1958; Senior & Sherratt, 1968*a*). Hypoglycin has no effect on glycolysis *in vitro* (Feng & Patrick, 1958; McKerns, Bird, Kaleita, Coulomb & De Renzo 1960; Patrick, 1962). In intact rats hypoglycin either did not change the rate of glucose oxidation to carbon dioxide (McKerns *et al.* 1960) or decreased it (Holt, Holt & Böhm, 1966). Patrick (1966) reported that hypoglycin at high concentrations (3mM) inhibits gluconeogenesis in rat kidney and liver slices.

Previously suggested mechanisms for the action of hypoglycin invoke inhibition of gluconeogenesis (Holt *et al.* 1966). Here we report the effects of pent-4-enoic acid on glycolysis in supernatant fractions from rat tissues, on glucose and palmitate oxidation in liver slices and on gluconeogenesis in kidney slices. A mechanism for the hypoglycaemic action of pent-4-enoic acid is proposed on the basis of experimental evidence presented here and in the preceding papers (Senior & Sherratt, 1968*b*; Senior, Robson & Sherratt, 1968*b*), and this mechanism is discussed with reference to information about other hypoglycaemic compounds. A preliminary account of this work has appeared (Senior & Sherratt, 1968*a*).

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METHODS

Chemicals. NAD^+ and glucose oxidase kits for glucose determinations were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. [U-^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks.

Enzymes. Lactate dehydrogenase (EC 1.1.1.27) was obtained from C. F. Boehringer und Soehne G.m.b.H. (crystalline suspension, 140 units/mg.).

Miscellaneous. Animals, laboratory equipment and other chemicals have been described in the two preceding papers (Senior & Sherratt, 1968*b*; Senior *et al.* 1968*b*).

Measurement of glycolysis in particle-free supernatants of rat tissues. Glycolysis was measured in particle-free supernatants prepared from homogenates of rat liver, rat skeletal muscle and rat small-intestinal mucosa. Liver homogenates were prepared as described by Senior &

Sherratt (1968*b*). Muscle homogenates were prepared in the same way but with longer homogenization. Mucosal homogenates were prepared as described by Clark & Sherratt (1967). The particle-free supernatant fractions were prepared by centrifugation at 0° in a Spinco model L ultracentrifuge (rotor no. 50) at 45000rev./min. (100000*g*) for 45min.

L-Lactate formation from glucose was measured in an incubation system consisting of $KHCO_3$ (16mM), $(NH_4)_2HPO_4$ (10mM), nicotinamide (33mM), ATP (1mM), NAD^+ (1mM), glucose (25mM), $MgCl_2$ (7mM) [for mucosal incubations ATP (6mM) and $MgCl_2$ (16mM) were used] and particle-free supernatant (approx. 2mg. of protein) in a total volume 1·0ml., pH7·0, in centrifuge tubes at 37°. After 45min., 1·0ml. of 0·3N-$HClO_4$ was added and, after centrifugation to sediment denatured protein, L-lactate was determined enzymically in the supernatant (Hohorst, 1957). The rate of lactate formation was linear during incubation. Trial experiments established that none of the fatty acids had any effect on the lactate assay system even at concentrations (10mM) vastly in excess of those used.

Measurement of oxidation of glucose and palmitate in rat liver slices. Livers were excised from rats (starved for 48hr. to deplete the livers of glycogen) and placed in 0·14M-NaCl at 20°. Slices 0·15mm. thick were cut with a mechanical tissue chopper (McIlwain & Buddle, 1953). About 100mg. of tissue (weighed wet on a torsion balance) was put in the main compartment of each Warburg flask, which also contained phosphate–saline medium, pH7·4 (Krebs & de Gasquet, 1964) and either glucose (20mM; unlabelled, or U-^{14}C-labelled, specific radioactivity 0·1μc/μmole) or palmitate (potassium salt, 1mM; unlabelled, or 1-^{14}C-labelled, specific radioactivity 0·1 or 0·2μc/μmole) or both. The final volume was 2·50ml. The centre wells contained 0·25ml. of 2N-KOH; the side arms contained 0·20ml. of 3·7N-trichloroacetic acid coloured with acid fuchsin. Incubation was for 50min. at 38°, the gas phase was O_2 and the shaking rate 100strokes/min. At the end of incubation acid was tipped in from the side arms and shaking was continued for 10min. $^{14}CO_2$ production was measured as described in the accompanying paper (Senior *et al.* 1968*b*). The O_2 uptake was linear throughout incubation. The dry weights of liver slices were found. These were 24–28% of the wet weight.

Measurement of gluconeogenesis in rat kidney slices. Gluconeogenesis was measured by the technique of Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963) with minor modifications. Rats were starved for 48hr. before experiments. The kidneys were excised and placed in oxygenated 0·14M-NaCl for 5min. at 20° to exhaust the endogenous glucose. These kidneys were then dissected and slices of cortex (0·15mm. thick) were cut. Slices (25–30mg. wet wt.) were placed in the main compartment of each Warburg flask. The main compartments also contained 3·0ml. of bicarbonate–saline, pH7·4 (Krebs & Henseleit, 1932), and 10mM-pyruvate or 10mM-succinate as precursor. The flasks were kept on ice during preparation.

Table 1. *Effect of pent-4-enoic acid and related compounds on glycolysis in particle-free supernatant fractions from rat tissues*

The experimental conditions are given in the Methods section. Results are quoted for two experiments with the fraction from small-intestinal mucosa.

Compound	Concn. of compound (mM)	Rate of L-lactate formation (mμmoles/min./mg. of protein)		
		Liver	Skeletal muscle	Small-intestinal mucosa
None	—	12·7	31·5	19·7, 24·8
Pent-4-enoic acid	1·0	—	34·6	20·0, 24·7
	3·0	—	29·2	21·7, 2·57
	10	12·2	40·0	20·0, 2·48
Pent-2-enoic acid	1·0	—	36·0	20·7, 25·6
	3·0	—	36·0	20·0, 25·6
	10	12·0	33·3	20·0, 20·0
Cyclopropanecarboxylic acid	1·0	—	33·2	20·0, 27·1
	3·0	—	2·0	8·3, 20·0
	10	0·8	0	2·9, 33·3
Cyclobutanecarboxylic acid	1·0	—	37·2	17·9, 23·3
	3·0	—	11·3	9·2, 19·2
	10	2·0	0	0·3, 5·4
Propionic acid	1·0	—	30·7	—
	3·0	—	33·5	—
	10	12·0	31·3	—
Hexanoic acid	1·0	—	32·0	17·9, 25·6
	3·0	—	31·3	18·3, 24·2
	10	14·5	30·0	19·1, 25·6
Octanoic acid	1·0	—	33·6	20·0, 25·2
	3·0	—	36·5	20·0, 25·2
	10	12·8	36·0	17·3, 22·8

Table 2. *Effect of pent-4-enoic acid on the oxidation of* [1-^{14}C]*palmitate and* [U-^{14}C]*glucose by rat liver slices*

Experimental details are given in the Methods section. Values for duplicate determinations are quoted.

Expt. no.	Additions	Concn. of pent-4-enoic acid (mM)	O_2 uptake (mμg.atoms/min./mg. of protein dry wt.)	Total $^{14}CO_2$ produced (counts/min./mg. dry wt.)
1	[U-^{14}C]Glucose and palmitate	—	5·94, 4·23	66·0, 54·0
		0·4	3·58, 4·55	35·4, 46·0
	[1-^{14}C]Palmitate and glucose	—	7·12, 5·68	56·4, 67·0
		0·4	3·78, 4·17	36·5, 36·8
2	[U-^{14}C]Glucose and palmitate	—	4·59, 4·20	376, 318
		0·4	4·27, 4·06	272, —
	[1-^{14}C]Palmitate and glucose	—	3·90, 4·47	48·0, 69·5
		0·4	—	20·2, 44·0
3	[U-^{14}C]Glucose and palmitate	—	6·03, 5·95	504, 453
		0·5	5·05, 6·90	424, —
		1·0	5·90, 5·60	574, —
	[1-^{14}C]Palmitate and glucose	—	6·11, 6·38	90·8, 122
		0·5	7·10, 5·34	101, 78·5
		1·0	6·34, 5·16	58·0, 84·5

Incubation was at 39° for 1·5–2hr., the gas phase was O_2+CO_2 (95:5) and the shaking rate was 100strokes/min. At the end of incubation the flasks were removed, and 2·0ml. of medium was pipetted into 0·20ml. of 3N-$HClO_4$ in centrifuge tubes. After centrifugation to sediment denatured protein the glucose in the supernatant was assayed by the glucose oxidase method. Neither the medium nor the fatty acids used interfered with the glucose analysis. Dry weights of kidney slices were found by drying at 105°. The endogenous glucose in kidney slices was assayed by placing samples of slices directly into a mixture of 0·30ml. of 3N-$HClO_4$ and 3·0ml. of bicarbonate–saline contained in a homogenization tube. The slices were homogenized by hand with a Teflon pestle and glucose was determined in the supernatant after centrifugation.

RESULTS

Effect of pent-4-enoic acid and related compounds on glycolysis. The results are shown in Table 1. Pent-4-enoic acid and pent-2-enoic acid neither stimulated nor inhibited lactate production from glucose. The cyclic compounds cyclobutanecarboxylic acid and cyclopropanecarboxylic acid inhibited glycolysis at concentrations of 3·0 and 10mM, but not at 1·0mM. In a few similar experiments it was also found that pent-4-enoic acid (0·1 and 1·0mM) did not affect lactate production from glucose 6-phosphate by rat liver or rat muscle homogenates. At these concentrations it did not affect the aerobic production of lactate by rings of rat small intestine incubated with oxygen as the gas phase (method given by Sherratt, 1968). Therefore there does not seem to be any inhibition of the sequence of reactions constituting glycolysis by pent-4-enoic acid or pent-2-enoic acid. The inhibition by the cyclic acids was unexpected; however, these concentrations would not be reached in our experiments *in vivo* (Senior, 1967).

Propionate, hexanoate and octanoate were included in these experiments because in recent years there has been much interest in the control of glycolysis and gluconeogenesis, and saturated fatty acids are known to stimulate gluconeogenesis and inhibit glycolytic enzymes in some systems (see Williamson, Kreisberg & Felts, 1966). These compounds had no effect on lactate production (Table 1). Weber, Convery, Lea & Stamm (1966) reported that incubation of octanoate (1–2mM) with rat liver particle-free supernatant causes strong inhibition of the rate-limiting glycolytic enzymes glucokinase (EC 2.7.1.2), hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40). Our results seem unaccountable in terms of the findings of Weber *et al.* (1966).

Effects of pent-4-enoic acid on oxidation of glucose and palmitate by liver slices. Three experiments were done (Table 2). There was some variation, but three points are clear. Pent-4-enoic acid did not consistently decrease the total oxygen uptake. It did not give any acceleration of glucose oxidation to carbon dioxide; indeed, in two experiments it inhibited oxygen uptake slightly. It inhibited $^{14}CO_2$ production from [1-^{14}C]palmitate consistently. The inhibition of palmitate oxidation with liver slices (40–50%) was less than that with these concentrations of pent-4-enoic acid with isolated liver mitochondria (Senior *et al.* 1968*b*). The pent-4-enoate ion may not enter liver cells freely, or it may be bound intracellularly.

Effects of pent-4-enoic acid and related compounds on gluconeogenesis by rat kidney slices. The rate of glucose formation was constant during incubation

Table 3. *Effect of pent-4-enoic acid and related compounds on glucose formation from pyruvate by rat kidney slices*

The rate of glucose formation is given as μmoles/min./g. dry wt. as means ± S.D. with the numbers of experiments in parentheses. *P* indicates significance of difference from the control. Experimental details are given in the Methods section.

Compound	Concn. of compound (mM)	Rate of glucose formation	Inhibition (%)	*P*
None	—	1·86 ± 0·32 (27)	—	—
Pent-4-enoic acid	0·001	1·61 ± 0·22 (4)	14	< 0·2 > 0·1
	0·01	1·36 ± 0·25 (8)	26·8	< 0·01
	0·10	0·87 ± 0·14 (7)	53·3	< 0·001
	1·0	0·65 ± 0·16 (7)	65·3	< 0·001
Pent-2-enoic acid	0·01	2·07 (3)	+11	—
	1·0	2·04 ± 0·11 (4)	+ 9	< 0·3 > 0·2
Pentanoic acid	0·01	1·86 ± 0·17 (7)	0	—
	1·0	1·78 ± 0·36 (6)	4·4	< 0·7 > 0·6
Cyclopropanecarboxylic acid	0·01	2·22 ± 0·36 (4)	+19·0	< 0·1 > 0·05
	1·0	1·63 ± 0·72 (4)	12·5	< 0·3 > 0·2
Cyclobutanecarboxylic acid	0·01	1·68 (2)	9·5	—
	1·0	1·78 (2)	4·4	—

Table 4. *Effect of pent-4-enoic acid and related compounds on glucose formation from succinate in rat kidney slices*

The rate of glucose formation is given as μmoles/min./g. dry wt. as means ± S.D. with the numbers of experiments in parentheses. *P* indicates significance of difference from the control. Experimental details are given in the Methods section.

Compound	Concn. of compound (mM)	Rate of glucose formation	Inhibition (%)	*P*
None	—	1·15 ± 0·24 (23)	—	—
Pent-4-enoic acid	0·001	0·94 ± 0·06 (4)	18·5	< 0·2 > 0·1
	0·01	0·91 ± 0·15 (9)	21·0	< 0·01
	0·10	0·75 ± 0·08 (9)	34·5	< 0·001
	1·0	0·47 ± 0·105 (9)	59·5	< 0·001
Pent-2-enoic acid	0·01	1·09 (3)	5·0	—
	1·0	1·14 (3)	1·0	—
Pentanoic acid	0·01	1·11 ± 0·12 (4)	3·0	< 0·5 > 0·4
	1·0	1·06 ± 0·12 (5)	8·4	< 0·8 > 0·7
Cyclopropanecarboxylic acid	0·01	1·05 (3)	9	—
	1·0	0·64 ± 0·06 (4)	44	< 0·001
Cyclobutanecarboxylic acid	0·01	1·28 (2)	+10	—
	1·0	1·38 (2)	+19	—

and is expressed in the Tables as μmoles of glucose formed/min./g. dry wt. of kidney tissue. The rate of formation of glucose was only one-third of that observed by Krebs & de Gasquet (1964). However, Subrahmanyan, Joseph & Natarajan (1967) found that the environmental temperature may affect rates of gluconeogenesis in rats, and our values agree with their results. The mean rate of formation of glucose from pyruvate was 111·9 ± 19·0 (S.D.) μmoles/hr./g. dry wt. (27 experiments); from succinate it was 69·1 ± 14·4 (S.D.) mμmoles/hr./g. dry wt. (23 experiments). The mean dry weight of kidney slices found here was 21·6 ± 3·9 (S.D.) % of the wet weight (49 observations). The mean endogenous content of glucose of the slices was 2·42 ± 2·24 (S.D.) μmoles/g. dry wt. (17 experiments). This was considered negligible and no correction was made for it.

The effects of pent-4-enoic acid and related compounds on glucose formation are shown in Tables 3 and 4. The significance of differences between control and experimental (pent-4-enoic acid or related compound added) values were assessed by Student's *t* test. In all cases the variance

Table 5. *Gluconeogenesis in kidney slices from rats treated with pent-4-enoic acid*

The rate of glucose formation is given as μmoles/min./g. dry wt. as means ± S.D. with the numbers of determinations in parentheses. *P* indicates significance of difference from the control. Experimental details are given in the Methods section.

Rat no.	Gluconeogenetic precursor	Dose of pent-4-enoic acid, time of injection and time of killing	Rate of glucose formation	Inhibition (%)	*P*
1	Pyruvate	Control	1·98 ± 0·33 (4)	—	—
2	Pyruvate	170 mg./kg. at time 0, killed at 60 min.	1·41 ± 0·25 (4)	29	> 0·05
3	Pyruvate	170 mg./kg. at time 0, 170 mg./kg. at 90 min., killed at 150 min.	1·68 ± 0·23 (4)	15	< 0·3 > 0·2
4	Succinate	Control	1·92 ± 0·08 (4)	—	—
5	Succinate	300 mg./kg. at time 0, killed at 100 min.	1·45 ± 0·10 (4)	25	< 0·001
6	Succinate	200 mg./kg. at time 0, 100 mg./kg. at 60 min., killed at 135 min.	1·80 ± 0·04 (4)	6	< 0·1 > 0·05

ratio was satisfactory ($F < 5\%$), so the *t* test was valid and Bessel's correction for small samples was applied. Pent-4-enoic acid inhibited gluconeogensis from pyruvate and succinate, the inhibitions being significant at 0·01 mM and at higher concentrations. Cyclopropanecarboxylic acid stimulated gluconeogenesis from pyruvate at 0·01 mM, and inhibited it from succinate at 1·0 mM. No other significant effect was observed. Pent-2-enoic acid, cyclobutanecarboxylic acid and pentanoic acid had no significant effects.

Some inhibition of gluconeogenesis from pyruvate and from succinate was also shown in kidney slices prepared from rats pre-injected with pent-4-enoic acid (Table 5). The concentration of pent-4-enoic acid during incubation was probably much less than *in vivo* since the slices were suspended in an excess of medium.

Krebs, Speake & Hems (1965) found that short-chain fatty acids and ketone bodies stimulate gluconeogenesis markedly, by sparing gluconeogenetic precursors from oxidation and by supplying acetyl-CoA for the activation of pyruvate carboxylase. Thus the fact that the fatty acids used here had no stimulatory effect (except 0·01 mM-cyclopropanecarboxylic acid) supports other evidence that they are not oxidized significantly (Senior & Sherratt, 1968*b*). Patrick (1966) has shown that hypoglycin at high concentration (3·0 mM) or 2,4-dinitrophenol (0·12 mM) inhibits gluconeogenesis and lowers ATP concentrations by 30–50% in kidney slices and in liver slices.

DISCUSSION

Pent-4-enoic acid is the only one of the compounds tested that was found to inhibit gluconeogenesis strongly in kidney slices. This inhibition is significant, since the kidney may contribute substantially to total gluconeogenesis in the body (Krebs, 1963, 1964). It is also reasonable to assume that pent-4-enoic acid also inhibits hepatic gluconeogenesis, since this is essentially the same process in liver and kidney (Krebs, 1963, 1964).

The mechanism causing the decrease in gluconeogenesis was not investigated, but two possible causes merit discussion. First, gluconeogenesis from all precursors requires ATP. Synthesis of one molecule of glucose from two of pyruvate requires six molecules of ATP, and for synthesis of one molecule of glucose from two of succinate four ATP molecules are necessary. Pent-4-enoic acid partly uncouples oxidative phosphorylation and inhibits long-chain fatty acid oxidation by isolated liver mitochondria (Senior & Sherratt, 1968*b*; Senior *et al.* 1968*b*). Similar inhibition of long-chain fatty acid oxidation by pent-4-enoic acid has been reported in skin (Yardley, 1964; Yardley & Godfrey, 1967) and hypoglycin is inhibitory in mouse heart homogenate (Entman & Bressler, 1967), so this effect may be assumed to be widespread and not limited to liver. Accumulation of fatty acids might occur *in vivo* and this may cause additional uncoupling of oxidative phosphorylation. Further, indirect evidence (Senior, 1967; Senior, Reay & Sherratt, 1968*a*) suggests that in peripheral tissues oxidation of ketone bodies is inhibited by pent-4-enoic acid. Long-chain fatty acids and ketone bodies are normally major respiratory fuels in intact animals (Krebs, 1966). Therefore it is very likely that gluconeogenesis is decreased by lack of ATP. Secondly, pyruvate carboxylase (EC 6.4.1.1) requires acetyl-CoA for activation (Utter, Keech & Scrutton, 1964). Gluconeogenesis from pyruvate would be inhibited if intramitochondrial acetyl-CoA concentrations were much decreased by pent-4-enoic

acid. There is no information whether other key enzymes of gluconeogenesis are inhibited.

Glycolysis and the oxidation of glucose are not significantly enhanced or impaired by pent-4-enoic acid. Pent-4-enoic acid readily causes hypoglycaemia in starved animals and this cannot therefore be due to decreased glycogenolysis. If glucose utilization is not increased, hypoglycaemia must be due to decreased synthesis of glucose. It is therefore proposed that after administration of pent-4-enoic acid the animal continues to utilize the only major respiratory fuel left to it, i.e. glucose. This cannot be replaced at a sufficient rate because of impaired gluconeogenesis, and hypoglycaemia occurs (Senior, 1967).

This proposed explanation is supported by available data on other hypoglycaemic compounds. Fructose given to patients with hereditary fructose intolerance causes hypoglycaemia (Black & Simpson, 1967). This disease is characterized by a deficiency of fructose 1-phosphate aldolase (EC 4.1.2.7) in the liver. According to Zalitis & Oliver (1967) gluconeogenesis is inhibited after fructose administration when accumulated fructose 1-phosphate competitively inhibits glucose phosphate isomerase (EC 5.3.1.9) in the gluconeogenetic direction only. Ethanol may cause hypoglycaemia when there is little hepatic glycogen, and there is evidence that ethanol decreases hepatic gluconeogenesis by an unknown mechanism (Lochner, Wulff & Madison, 1967). Several hypoglycaemic guanidine derivatives appear to act mainly by preventing ATP synthesis (Hollunger, 1955). Substituted guanidines inhibit respiratory-chain phosphorylation at site I similarly to oligomycin (Pressman, 1963; Chappell, 1963; Chance & Hollunger, 1963; Haas, 1964). Sythalin A inhibits at site III but not at site I (Guillory & Slater, 1965). The hypoglycaemic effects of the biguanides and sulphonylureas may be largely dependent on the presence of insulin (Duncan & Clarke, 1965), but the metabolic effects of the biguanides could also contribute since they inhibit both phosphorylation at site II (Pressman, 1963; Schäfer, 1964) and gluconeogenesis *in vitro* (Patrick, 1966; Jangaard, 1967; Meyer, Ipakchi & Clauser, 1967). Similarly some hypoglycaemic sulphonylureas can uncouple oxidative phosphorylation (Pentillä, 1966; De Beer & De Schepper, 1967) and there is indirect evidence that they inhibit gluconeogenesis *in vivo* (Goto & Lukens, 1961; De Meutter & Shreeve, 1963). Atractyloside from *Atractylis gummifera* is a potent hypoglycaemic agent (Santi, 1958) and this compound blocks respiration by inhibiting oxidative phosphorylation (Bruni, 1966). All these compounds have effects independent of insulin and they either inhibit gluconeogenesis directly or inhibit ATP synthesis.

A similar explanation has been given for the mechanism of action of hypoglycin by Holt *et al.* (1966), who suggested that methylenecyclopropylacetic acid inhibits gluconeogenesis, but they did not give any direct experimental confirmation. They state that 'the hypoglycaemia could . . . be the result of a relative increase of carbohydrate consumption (i.e. relative to fat consumption) with a consequent depletion of the glycogen stores which cannot be replenished *inter alia* due to a diminished ATP production in the cell'. Yet in the same paper they showed that hypoglycin decreased net glucose oxidation in the intact animal. An enhancement of glucose utilization is not a necessary prerequisite for hypoglycaemia and a relative increase in carbohydrate consumption is not a sufficient cause. It is the net balance between glucose utilization and gluconeogenesis that determines the quantity of glucose in the blood.

An early report by Hassall, Reyle & Feng (1954) that plasma urea concentrations are doubled after hypoglycin treatment suggests that glucogenic amino acids are deaminated to supply more gluconeogenetic precursor. Alternatively it may be that the animal, unable to use long-chain fatty acids or ketone bodies as fuel, begins to oxidize amino acids.

Several of the effects of pent-4-enoic acid found (Senior & Sherratt, 1968*b*) do not appear to contribute substantially to the hypoglycaemia; indeed, inhibition of pyruvate oxidation may tend to prevent it. Hypoglycin metabolites do not inhibit pyruvate metabolism strongly (Holt *et al.* 1966) and this may be one reason why hypoglycin is more potent in rats than pent-4-enoic acid. It is unlikely that the ketosis caused in rats by pent-4-enoic acid and by hypoglycin contributes directly to their hypoglycaemic effects (Senior 1967). Injection of L-leucine (100–400mg./kg.) causes a small fall in blood glucose concentrations in rats (Cochrane, 1960). Cyclopropane derivatives, such as hypoglycin and methylenecyclopropylpyruvic acid, inhibit leucine metabolism in rat liver slices and *Escherichia coli*; although transamination of leucine is inhibited the major block is at the stage of oxidation of isovaleryl-CoA (Harding & Deshazo, 1967; Posner & Raben, 1967). An accumulation of leucine, because of an inhibition of its metabolism, might also contribute to the hypoglycaemic effect of hypoglycin and partly explain why in rats it is two to four times as potent as (and also more toxic than) pent-4-enioc acid (Feng & Patrick, 1958) even though pent-4-enoic acid may inhibit long-chain fatty acid oxidation more strongly than hypoglycin (Holt *et al.* 1966; Senior *et al.* 1968*b*). Some hypoglycaemic sulphonylureas cause an accumulation of leucine in rat diaphragm (De Schepper, 1967).

Since this work was completed a report on the hypoglycaemic effect of pent-4-enoic acid has appeared (Corredor, Brendel & Bressler, 1967). These workers observed an inhibition of long-chain fatty acid oxidation in heart homogenates from mice pretreated with pent-4-enoic acid and that its hypoglycaemic effect was antagonized by L-carnitine. They also deduced that there is an inhibition of gluconeogenesis by pent-4-enoic acid from the inhibition of the incorporation of [3-^{14}C]-pyruvate into blood glucose and they reported that pent-4-enoic acid also caused a stimulation of [U-^{14}C]glucose oxidation.

We are most grateful to Mr C. G. Chambers for excellent technical assistance. This work was supported by a grant from the Medical Research Council and A.E.S. was in receipt of a Science Research Council training grant.

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