*STUDIES ON THE MECHANISM OF THE HYPOGLYCEMIC ACTION OF 4-PENTENOIC ACID**

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The hypoglycemic activity of certain intermediate-chain-length carboxylic acids has been described.[1,2] Each of these compounds, methylenecyclopropylacetic acid (I), 3-methylenecyclobutane-1-carboxylic acid (II), and 4-pentenoic acid (III) has a vinyl group separated by two carbon atoms from the carboxyl group.

"Vomiting sickness," a syndrome commonly observed in Jamaica, occurs as a result of ingestion of the unripe fruit of the tropical plant *Blighia sapida* (ackee fruit).[3] The unripe ackee fruit contains L-α-amino-β-(methylenecyclopropyl)-propionic acid (hypoglycin), which was found to be the principle causing vomiting, hypoglycemic convulsions, and coma in man and laboratory animals.[4–7] The studies of DeRenzo[2,8] and Holt[9] suggested that hypoglycin impaired the utilization of lipids. A decreased oxidation of long-chain fatty acids was observed, and it was suggested that hypoglycin caused a shift in substrate utilization resulting in decreased oxidation of fatty acids and an augmented use of carbohydrate.[10] The hypoglycemic effect is consistent with this hypothesis.

The effects of hypoglycin are not demonstrable *in vitro*, but Holt has recently reported that in the liver hypoglycin is converted by transamination to methylenecyclopropyl-pyruvic acid which is oxidatively decarboxylated to methylenecyclopropyl-acetic acid (I).[11] It was shown that the latter is the active compound which depresses long-chain fatty acid oxidation and acetoacetate formation in liver homogenates or mitochondria.

Carnitine mediates oxidation of long-chain fatty acids.[12] Since hypoglycin inhibits the oxidation of palmitate but not that of hexanoate, it was suggested that the active agent might impair this oxidation. The administration of hypoglycin to mice resulted in decreased palmitate oxidation by myocardial homogenates, an effect which preceded the decrease in blood glucose. The addition of carnitine to such homogenates restored palmitate oxidation to normal levels, whereas administration of carnitine to hypoglycin-treated mice prevented both the depression of palmitate oxidation and the hypoglycemia.[13]

In this communication data are presented which show that the administration of 4-pentenoic acid to mice decreased gluconeogenesis from pyruvate and increased $C^{14}O_2$ production from glucose-U-C^{14} and resulted in hypoglycemia. Palmitate oxidation by myocardial homogenates in the presence of 4-pentenoic acid was

decreased to a level comparable to that observed with hypoglycin. The decreased palmitate oxidation was associated with lowered levels of free carnitine and elevated levels of acid-soluble acylcarnitines in both heart and liver. The addition of carnitine to myocardial homogenates restored palmitate oxidation to normal. Administration of carnitine to 4-pentenoic acid-treated mice prevented the depression of palmitate oxidation and gluconeogenesis as well as the hypoglycemia.

Methods.—White male mice, weighing 20–25 gm, were offered a standard diet until 24 hr before use. Where myocardial oxidation of fatty acids was to be determined, the experimental animals received 7.5–15 mg of 4-pentenoic acid in 0.5 ml normal saline intraperitoneally, whereas the control animals received only saline. The hearts were homogenized in 5.0 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4. Protein concentration was measured by a modification of the biuret method as previously described.[14]

In the gluconeogenesis studies, the treated animals received intraperitoneally 100 μmoles of pyruvate-3-C^{14} (9×10^6 cpm) and either 7.5 mg sodium 4-pentenoate, 15 mg hypoglycin, or 50–75 mg sodium acrylate, whereas the controls received only pyruvate-3-C^{14}. In some experiments both groups of animals were also given 100 μmoles of (±)-carnitine. At the start of the experiment and at 15-min intervals thereafter, two 10-μl aliquots of blood were taken from the tail vein. Blood glucose concentration was determined on 1 aliquot using the glucose oxidase method modified for microdeterminations.[15, 16] The second aliquot was deionized on a 10 cm × 0.2-cm^2 mixed-bed resin column, consisting of Dowex-50-X8 (100–200 mesh) in the H^+ form and Dowex-1-X8 (100–200 mesh) in the HCO_3^- form. The sample was eluted with 2 ml of water and the eluate assayed for radioactivity. The eluted material was further characterized by thin-layer chromatography on cellulose plates developed in two different systems.[17a] Ninety-seven per cent of the eluate radioactivity was in a spot which had the *Rf* of glucose.

Glucose utilization was assayed by administration of a tracer dose of glucose-U-C^{14} and measurement of the blood glucose and the radioactivity of the expired CO_2 at 10-min intervals.[17b]

The concentration of free fatty acids (FFA) in the myocardium was determined by the method of Dole as modified by Trout, Estes, and Friedberg.[18]

Assays of palmitate, hexanoate, and glucose oxidation were carried out as previously described.[13] Since the myocardial concentrations of FFA in the treated and control animals were not the same, the specific activities of the substrate pools were determined at the beginning of the incubation period. In the treated group, the FFA were significantly higher (mean 2.9 μmoles/gm protein, SE 0.4) than in the control group (mean 1.3, SE 0.3) ($p < 0.01$). These differences in endogenous FFA and the subsequent differences in specific activity of the substrate resulting from the addition of 0.1 μmole of labeled palmitate to both the treated and control incubations were taken into account in the calculations of the rates of long-chain fatty acid oxidation. In these calculations complete mixing of the endogenous FFA and the added radioactive palmitate was assumed.

The incorporation of palmitate-1-C^{14} into myocardial triglycerides was quantitated as previously described.[14]

Tissue levels of free and bound carnitine were determined by the method of Tubbs, Pearson, and Chase.[19] The acetyl CoA-carnitine acyltransferase used in the carnitine assay was prepared from pigeon breast muscle by the procedure of Chase, Pearson, and Tubbs[20] and had a specific activity of 3.8 (μmoles CoA released from acetyl CoA/min/mg protein at 25°).

Assay of acyl CoA formation was carried out by measuring hydroxamic acid formation[21] and the disappearance of the sulfhydryl group of coenzyme A by the nitroprusside reaction.[22]

The medium-chain acyl CoA synthetase (FAAE, fatty acid-activating enzyme) was prepared from beef liver by the procedure of Mahler, Wakil, and Bock.[23] The specific activity was 14.5 based on the nitroprusside assay of hexanoic acid activation (disappearance of μmoles of coenzyme A sulfhydryl groups/mg protein/min at 25°).

Carnitine and acylcarnitines were separated by thin-layer chromatography on Al_2O_3 plates.[24] (−)-Carnitine and acylcarnitines were prepared by previously described procedures.[24] 4-Pentenoic acid-1-C^{14} was prepared by a Grignard reaction from 4-Bromobutene-2 and $C^{14}O_2$.[17b] 4-Pentenoyl CoA was synthesized chemically by the thiophenol procedure of Wieland and Koeppe.[25] Acrylyl CoA was prepared from acrylic anhydride by the method of Simon and Shemin.[26]

Palmitate-1-C^{14}, hexanoate-1-C^{14}, glucose-U-C^{14}, pyruvate-3-C^{14}, acrylic acid-1-C^{14}, $C^{14}O_2$, and 4-pentenoic acid were all obtained from commercial sources.

Results.—Effect of 4-pentenoic acid on blood glucose and substrate oxidation by myocardial homogenates: The intraperitoneal administration of 15 mg of 4-pentenoic acid to mice resulted in decreases of blood glucose that were maximal at 30 minutes (Table 1). Myocardial homogenates prepared from these animals showed a decreased rate of palmitate oxidation and normal rates of oxidation of both hexanoate and glucose (Table 1).

4-Pentenoic acid and the incorporation of palmitate into triglycerides: The livers from animals treated with 4-pentenoic acid incorporated more than twice as much of the labeled palmitate as did the controls (treated, 0.37 ± 0.06 μmole/gm protein/30 min, controls, 0.17 ± 0.03, $p < 0.01$).

Effect of 4-pentenoic acid and (−)-carnitine on blood glucose levels and myocardial palmitate oxidation: Carnitine had no effect on the blood glucose of control animals, but protected against the hypoglycemic effects of 4-pentenoic acid in the treated animals. The administration of carnitine to control animals resulted in a 25 per cent stimulation of palmitate oxidation by their myocardial homogenates, whereas the stimulation of palmitate oxidation by homogenates of 4-pentenoic acid-treated animals was 4- to 20-fold, comparable to control levels (cf. Table 2).

Activation of hypoglycemic carboxylic acids: In view of the hypoglycemic activity of some short-chain unsaturated fatty acids, but not others, which are chemically related, it was thought that activation of the acids might be necessary for hypoglycemic activity. The data in Table 3 show that methylenecyclopropylacetic

TABLE 1

EFFECT OF 4-PENTENOIC ACID ON BLOOD GLUCOSE AND MYOCARDIAL OXIDATION OF HEXANOATE-1-C^{14}, PALMITATE-1-C^{14}, AND GLUCOSE-U-C^{14}*

Substrate	$C^{14}O_2$ Production (μmoles/gm protein/30 min) 4-Pentenoic Acid		Control
Glucose-U-C^{14}	4.6		4.8
SD	1.1		0.9
p		>0.5	
Hexanoate-1-C^{14}	6.8		6.4
SD	1.2		0.8
p		>0.5	
Palmitate-1-C^{14}	0.93		2.85
SD	0.28		0.51
p		<0.01	
	(mg/100 ml)		
Blood glucose	23.		73.
SD	11.		8.
p		<0.01	

Each reaction mixture contained from 8 to 12 mg of myocardial homogenate in 0.7 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4, and either 10 μmoles D-glucose-U-C^{14} (440,000 cpm), 5 μmoles hexanoate-1-C^{14} (270,000 cpm), or 0.1 μmole palmitate-1-C^{14} (230,000 cpm). Final reaction volumes were 0.9 ml. Incubations were at 20° for 30 min. Blood glucoses were done at 30 min after administration of the 4-pentenoic acid. SD, standard deviation.
* Each group contained 5 animals.

TABLE 2

EFFECT OF 4-PENTENOIC ACID AND (−)-CARNITINE ON BLOOD GLUCOSE AND MYOCARDIAL PALMITATE OXIDATION

Exptl. groups	Palmitate-1-C^{14} oxidation (moles/gm protein/30 min)	Blood glucose (mg/100 ml)
Controls (I)	3.8	76
	4.0	81
	3.6	84
Carnitine (II)	5.2	70
	4.8	91
	5.0	77
4-Pentenoic acid	0.41	18
(III)	0.18	15
	0.83	26
4-Pentenoic acid	2.9	63
+ carnitine	3.2	61
(IV)	3.8	78

Mice were divided into 4 groups of 3 animals. At zero time all 4 groups received intraperitoneal injections of the following: (I) 0.5 ml normal saline, (II) 6 mg of (−)-carnitine in 0.5 ml normal saline, (III) 15 mg of 4-pentenoic acid in 0.5 ml normal saline, (IV) 15 mg of 4-pentenoic acid and 6 mg of (−)-carnitine in 0.5 ml normal saline. Thirty min later the blood glucose was determined and the animals sacrificed. The hearts were removed and used for assay of palmitate oxidation.

Each reaction mixture contained from 6 to 9 mg of myocardial homogenate in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4, and 0.1 μmole palmitate-1-C^{14} (135,000 cpm). Final reaction volumes were 1.1 ml. Incubations were carried out at 30° for 30 min.

TABLE 3

ACTIVATION OF FATTY ACIDS BY THE C_4-C_{12} ACYLTHIOKINASE (FAAE)

Fatty acid	Acyl CoA Formed —SH (μmoles/mg protein/hr)	Hydroxamate
Methylenecyclopropylacetic	2.56	2.40
4-Pentenoic	2.60	2.43
Acrylic	3.48	3.23
Methylenecyclopropanecarbonic	0.28	0.13
2-Methyl acrylic	0.10	0.04

Hydroxamate assay: Each reaction mixture contained 2 μmoles of fatty acid, 5 μmoles potassium ATP, 0.25 μmole coenzyme A, 10 μmoles $MgCl_2$, 50 μmoles Tris·HCl pH 8.0, 70 μg FAAE, and 400 μmoles neutralized hydroxylamine. Final reaction volumes were 1 ml. Incubations were carried out at 35° for 15 min.

Nitroprusside assay: Each reaction mixture contained 2 μmoles of fatty acid, 2 μmoles potassium ATP, 0.3 μmole coenzyme A, 2 μmoles $MgCl_2$, 25 μmoles Tris·HCl pH 8.0, and 35 μg FAAE. Final reaction volumes were 0.33 ml. Incubations were carried out at 35° for 15 min under nitrogen.

acid, 4-pentenoic acid, and acrylic acid, which are all hypoglycemic agents, are activated to acyl CoA derivatives.

Methylenecyclopropanecarbonic acid and 2-methylacrylic acid, which are not hypoglycemic agents, are not activated (data for the hypoglycemic activity of these compounds not given here).

4-Pentenoic acid and tissue carnitine: Because of the stimulatory effect of carnitine on palmitate oxidation by the hearts of 4-pentenoic acid or hypoglycin-treated mice, carnitine levels were assayed in liver and heart. Carnitine was extracted by the method of Pearson and Tubbs,[27] yielding perchloric acid-soluble and -insoluble fractions of carnitine and acyl carnitines. The total acid-soluble fraction contains free carnitine and bound carnitine which is primarily acetylcarnitine, whereas the acid-insoluble fraction contains long-chain fatty acylcarnitines.[27] Table 4 shows that treatment of mice with 4-pentenoic acid resulted in a marked decrease in levels of free carnitine and an increase in bound carnitine in both heart and liver.

4-Pentenoyl CoA and acrylyl CoA as substrates for acetyl CoA-carnitine acyltransferase: Since those short-chain unsaturated fatty acids, which have been found to be hypoglycemic, were all activated to the corresponding CoA derivatives and the β-oxidation of methylenecyclopropylacetyl CoA and 3-methylenecyclobutanecarboxyl CoA would be blocked by the presence of the β-carbon within a ring system, these acyl CoA's might represent a metabolic dead end for oxidation. However, they might serve as substrates for acetyl CoA-carnitine acyltransferase, which would result in a decrease in free and an increase in bound carnitine. In order to assess this possibility, 4-pentenoyl CoA and the product which would result from its β-oxidation, acrylyl CoA, were tested as substrates for acetyl CoA-carnitine acyltransferase. Figure 1 shows that both 4-pentenoyl CoA and acrylyl CoA serve as substrates for the enzyme, although at rates somewhat lower than that observed with acetyl CoA. The data of Table 5 show that while 4-pentenoic acid-1-C^{14} is oxidized by myocardial homogenates, its presumed product of β-oxidation, acrylic acid, is not.

Effect of 4-pentenoic acid, acrylic acid, and hypoglycin on gluconeogenesis: Because of the important role of gluconeogenesis in the maintenance of blood glucose, the effect of these hypoglycemic agents on the conversion of pyruvate-3-C^{14} to C^{14}-glucose was assessed. The administration of pyruvate-3-C^{14} to control animals resulted in a rise in blood glucose which was

TABLE 4

EFFECT OF 4-PENTENOIC ACID ON TISSUE LEVELS OF CARNITINE AND ACID-SOLUBLE ACYLCARNITINES*

Expt.	Carnitine: Free (μmoles/gm protein)	Bound
Control hearts	6.83 ± 0.47	0.78 ± 0.41
4-Pentenoic hearts	3.45 ± 0.53	2.61 ± 0.62
Control livers	2.57 ± 0.48	0.37 ± 0.17
4-Pentenoic livers	1.87 ± 0.23	1.18 ± 0.26

* Each group contained 10 animals.

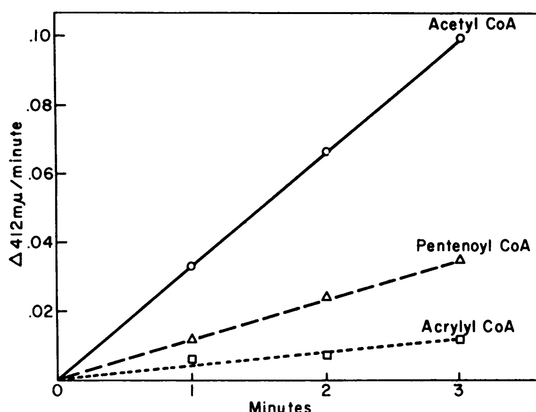

FIG. 1.—4-Pentenoyl CoA and acrylyl CoA as substrates for acetyl CoA-carnitine-acyltransferase. The reaction mixtures contained 200 μmoles Tris·HCl pH 8.0, 200 mμmoles of 5,5'-dithiobis-(2-nitrobenzoic acid), 0.5 μg acetyl CoA-carnitine acyltransferase (sp. act. 1.8), and 100 mμmoles of the indicated acyl CoA's. Reactions were carried out at 30° and absorbance increases recorded.

TABLE 5

OXIDATION OF 4-PENTENOIC ACID-1-C^{14} AND ACRYLIC ACID-1-C^{14} BY MYOCARDIAL HOMOGENATES

Homogenate (mg protein)	$C^{14}O_2$ Production 4-Pentenoic acid-1-C^{14} (cpm/30 min)	Acrylic acid-1-C^{14}
0	43	34
5	4,380	27
10	8,750	41
15	13,540	37
20	22,410	23

Each reaction mixture contained 1 μmole of either 4-pentenoic acid-1-C^{14} (105,000 cpm) or acrylic acid-1-C^{14} (109,000 cpm), and the indicated amounts of myocardial homogenate in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4. Final reaction volumes were 1.1 ml. Incubations were carried out at 20° for 30 min.

maximal at 15–30 minutes, and a conversion to C^{14}-glucose which was maximal at 30 minutes (Figs. 2 and 3). Carnitine had no significant effect on these parameters in control animals. 4-Pentenoic acid markedly inhibited the conversion of pyruvate-3-C^{14} to C^{14}-blood glucose and caused profound hypoglycemia. The administration of carnitine to 4-pentenoic acid-treated mice stimulated gluconeogenesis about sixfold (about two thirds of normal) at 15 minutes and prevented the hypoglycemia (Figs. 2 and 3). However, the protective effect of carnitine in the 4-pentenoic acid-treated animals was transient and began to wear off at 30 minutes. Hypoglycin and acrylic acid also resulted in a depression of gluconeogenesis and a relative hypoglycemia (Figs. 2 and 3). The dose of hypoglycin used was of the same order as that of 4-pentenoic acid, whereas acrylic acid had to be used at 7–10 times this dose to elicit these effects.

Effect of 4-pentenoic acid on glucose oxidation: The effect of 4-pentenoic acid on the conversion of intraveneously administered D-glucose-U-C^{14} to $C^{14}O_2$ is shown in Figure 4. The $C^{14}O_2$ production in the initial ten minutes (5–8% of the injected dose of radioactivity) was used as the standard of comparison for the subsequent ten-minute $C^{14}O_2$ collections. The treated and control animals produced levels of $C^{14}O_2$ which were not significantly different for the first 20 minutes. Thereafter (30- to 110-min period), the treated animals produced levels of $C^{14}O_2$ which were both more elevated and more sustained than those of the controls totaling 33 per cent more during the time period observed. At 100 minutes, between 45 and 60 per cent of the injected radioactivity of glucose-U-C^{14} could be accounted for as $C^{14}O_2$. It is noteworthy that the effect of 4-pentenoic acid on the stimulation of glucose oxidation is manifest at a time when hypoglycemia is profound, depression of fatty acid oxidation marked, and depression of gluconeogenesis well established.

Discussion.—The data presented show that certain short-chain unsaturated fatty acids are hypoglycemic agents and that these compounds have characteristic

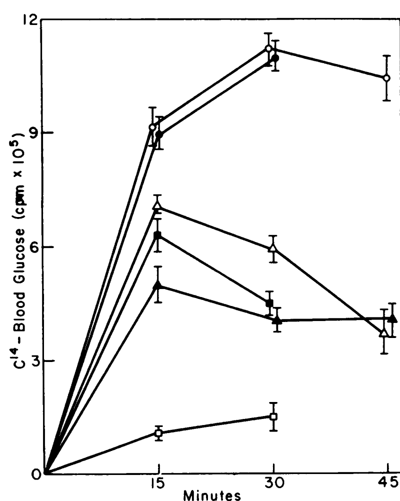

FIG. 2

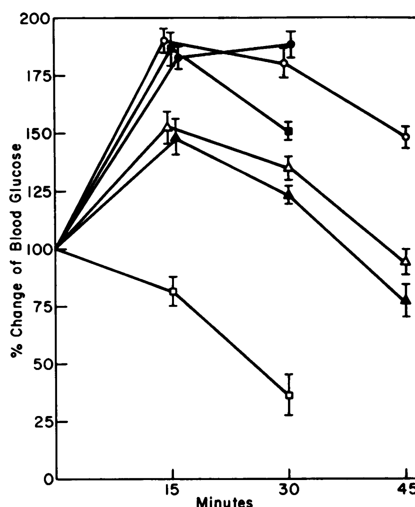

FIG. 3

FIG. 2.—Effect of 4-pentenoate, acrylate, and hypoglycin on gluconeogenesis. The animals all received 100 μmoles of sodium pyruvate-3-C^{14} intraperitoneally and either 7.5 mg sodium 4-pentenoate, 60 mg sodium acrylate, or 7.5 mg hypoglycin. (+)-Carnitine (50 μmoles) was given intraperitoneally at 30 min prior to, and at the start of, the experiment where indicated. Each point represents the mean plus or minus the standard error of separate assays of 10 animals for the acrylate and hypoglycin groups, and 15 animals for the 4-pentenoate and control groups. Controls ○; controls + carnitine, ●; 4-pentenoate □; 4-pentenoate + carnitine, ■; acrylate, △; hypoglycin, ▲.

FIG. 3.—Effect of 4-pentenoate, acrylate, and hypoglycin on the blood glucose. Conditions as per Fig. 2.

metabolic and biochemical effects: (1) They decrease long-chain fatty acid oxidation. (2) They augment rates of glucose utilization while simultaneously decreasing gluconeogenesis. This combination of effects results in a profound hypoglycemia. (3) They decrease tissue levels of free carnitine and increase levels of acid-soluble acylcarnitines. These observations are substantiated by the reversal of the noted effects upon administration of (−)-carnitine. The data is consonant with the hypothesis that these acids are activated to acyl CoA derivatives which cannot be oxidized. In the case of 4-pentenoyl CoA, β-oxidation to acrylyl CoA can occur, but further oxidation does not take place. These acyl CoA's serve as

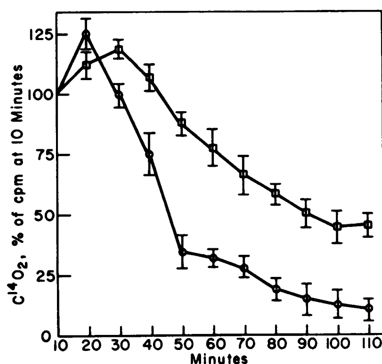

FIG. 4. Effect of 4-pentenoic acid on glucose oxidation. The animals all received 0.028 mg of D-glucose-U-C^{14} (1.4 × 10^6 cpm) intravenously at the start of the experiment, and one group also received 4-pentenoic acid (0.3 gm/kg body weight) intraperitoneally. Respiratory $C^{14}O_2$ was collected in hyamine at 10-min intervals thereafter. Each point represents the mean plus or minus the standard error of 15 animals in each group. Controls, ○; 4-pentenoate-treated, □.

substrates for acetyl CoA-carnitine acyltransferase, which catalyzes the formation of the corresponding acylcarnitines in the presence of carnitine. This results in a decrease in free carnitine and an increase in acid-soluble acylcarnitine. The role of carnitine in long-chain fatty acid oxidation has been attributed to the formation of long-chain fatty acylcarnitines, which, in contrast to the acyl CoA derivatives, can penetrate to mitochondrial sites of fatty acid oxidation.[12] Acylcarnitine formation from acyl CoA is catalyzed by the long-chain acyl CoA-carnitine acyltransferase,[28] which has been shown to be the rate-limiting enzyme in long-chain fatty acid oxidation.[29]

Observations from a number of laboratories have recently shown that increased rates of fatty acid oxidation enhance gluconeogenesis.[30-34] The locus of this stimulatory influence of fatty acid oxidation has been attributed to the generation of acetyl CoA, ATP, and NADH. It has been demonstrated that acetyl CoA and ATP stimulate pyruvic carboxylase which results in increased rates of formation of phosphoenolpyruvate from pyruvate,[30, 35, 36] whereas pyruvic kinase and pyruvic dehydrogenase are inhibited by fatty acids or their products.[37] It has been suggested that NADH might also contribute to enhanced gluconeogenesis by providing the reduction potential necessary for the conversion of phosphoglyceric acid to phosphoglyceraldehyde.[38]

Freinkel and co-workers have shown that carnitine stimulates gluconeogenesis from $C^{14}O_2$, C^{14}-alanine, and C^{14}-lactate in liver slices.[39] Fritz has presented evidence which suggests that the control of long-chain acyl CoA-carnitine acyltransferase may be a key site of regulation of gluconeogenesis.[37, 40] He has shown that (+)-palmitylcarnitine, a competitive inhibitor of long-chain acyl CoA-carnitine acyltransferase, decreases fatty acid oxidation and gluconeogenesis, and stimulates glucose utilization in liver homogenates. Stimulation of the long-chain acyl CoA-carnitine acyltransferase with (−)-acylcarnitines increased gluconeogenesis and decreased glucose utilization. (−)-Carnitine reversed the effects of (+)-palmitylcarnitine on both gluconeogenesis and glucose utilization.[37, 40]

The hypoglycemic short-chain unsaturated fatty acids simulate the effects of (+)-palmitylcarnitine, and their effects are also reversed by carnitine.[13] Since neither the acyl CoA nor the acylcarnitine derivatives of these hypoglycemic acids inhibit palmityl CoA-carnitine acyltransferase (data not given here), the effects on glucose metabolism must be elsewhere. The decrease in the free carnitine fraction in tissues can also result in a depression of long-chain fatty acid oxidation,[41] and the hypoglycemic fatty acids decrease free, and increase bound, tissue carnitine.

The toxicity of the hypoglycin-type compounds resides in part in their capacity to decrease gluconeogenesis, increase glucose utilization, and consequently cause hypoglycemia. The immediate effect may be the synthesis of acylcarnitines of the nonmetabolizable acyl moieties of the short-chain unsaturated fatty acids. The resulting decreased levels of free carnitine impair mitochondrial oxidation of long-chain fatty acids and cause a shift to utilization of glucose as the major energy source. The concomitant failure of glucose homeostasis is not directly explicable, but may be related to the control of pyruvic carboxylase and the levels of reduced pyridine nucleotide. The other key enzymes in gluconeogenesis, fructose diphosphatase, and glucose-6-phosphatase have not yet been studied. The depression of long-chain fatty acid oxidation which results in increased cellular utilization of

glucose underscores the intimate relationship between fatty acid oxidation and gluconeogenesis in maintenance of blood glucose levels.

The hypoglycemic fatty acids may also affect other enzymatic activities which are unrelated to the hypoglycemia. These studies are in progress.

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