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HYPOGLYCIN AND HYPOGLYCIN-LIKE COMPOUNDS[1]

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I. INTRODUCTION

The hypoglycemic activity of a number of carboxylic acids of intermediate chain-length has been studied by several investigators during the past decade. These compounds are related to hypoglycin, an amino acid in the seeds and unripe fruit of the ackee tree that is responsible for the *Jamaican vomiting sickness*. Each of these hypoglycemic compounds has a vinyl group separated by two carbon atoms from an actual or potential carboxyl group.

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The mechanism of action of this group of compounds has been recently elucidated. The work that led to this has provided fresh insights into the biochemical mechanisms capable of rapidly changing from one type of metabolic fuel to another. Plasma glucose levels are regulated in the normal state by the release of glucose by the liver and to a lesser extent by the kidney. Acutely enhanced utilization of glucose is compensated by rapid breakdown of glycogen stores. However, the chronic supply of glucose in these organs is produced by gluconeogenesis, a metabolic process that converts amino acids and glycerol to glucose. Gluconeogenesis is enhanced at times when dietary glucose supplies are limited or the metabolism of available glucose is impaired, and when there is increased mobilization and oxidation of fat, as in fasting and diabetes mellitus. It has been recognized that there is a direct relationship between enhanced fatty acid oxidation and enhanced gluconeogenesis in these states. Indeed, the work with these hypoglycemic agents has provided evidence that fatty acid oxidation not only enhances gluconeogenesis, but is essential for the normal operation of this pathway.

This review will summarize the work of investigators on the hypoglycemic carboxylic acids of intermediate chain-length and provide a survey of the subject. A section on the mechanism of action of 4-pentenoic acid will be drawn from our own studies.

II. JAMAICAN VOMITING SICKNESS

A. Historical survey

For many years, the poor people of Jamaica have been subject to an illness, characterized by a sudden onset of vomiting, usually 2 to 3 hr after a meal. This most conspicuous characteristic and the early lack of clues as to its etiology earned this type of food poisoning the descriptive name of "vomiting sickness of Jamaica." A review of the vomiting sickness by Hill (63) summarized knowledge on the subject up to 1952, and it was enriched by his personal observations of many patients. Hassall and Reyle (59) later reviewed the literature from 1952 to 1955.

Cases of vomiting sickness were observed as early as 1875 (63). Many case reports appeared since the beginning of this century; and in 1913, even though the disease was geographically localized to Jamaica, it caused sufficient interest in England to merit the dispatch of an investigating group by the Liverpool School of Tropical Medicine to study the disease. The etiology and treatment of the disease, however, remained obscure until the early 1950's. Both popular folklore and medical literature (101), however, associated the vomiting sickness with the ingestion of unripe ackees, which are fruits from a tree that grows in Jamaica and other Antilles.

The ackee tree, indigenous to West Africa, where it is called Ankye or Ishin,was introduced to Jamaica in 1778 by Thomas Clarke, the island's first botanist (63). Legend, however, attributes the deed to Captain Bligh of *Mutiny on the Bounty* fame, who introduced the breadfruit tree to the West Indies. Koenig perpetuated this belief by naming the plant *Blighia sapida* (63). The fruit of the ackee tree weighs about 100 g (50). The arillus is edible and mainly because of the ubiquitous

distribution of the tree in Jamaica and the cheapness of the fruit, it is a staple in the diet of the poor of that country. Even though the ackee tree is not geographically circumscribed within the western hemisphere to Jamaica—it grows in other Antilles, Central America and the southern part of Florida (63, 76)—the fruit is not commonly eaten outside of Jamaica (63).

It is popular knowledge in both West Africa and Jamaica that the ackee fruit may be poisonous during certain stages of its development (63), and consistent with this belief, the vomiting sickness is seasonal, with outbreaks reaching epidemic proportions during the colder months of the year when food is scarce and the ackees are still unripe. Early studies on the poisonous nature of the ackee fruit and its probable relationship to the vomiting sickness (63, 68) showed that the unripe fruits contain a toxic, water-soluble principle, which is not found in water extracts after the fruit reached maturity. In later studies Hassal *et al.* (58–60) confirmed this relationship and also succeeded in isolating the toxic principle from the ackee (section III).

B. Clinical manifestations

The vomiting sickness is characterized by a sudden onset of vomiting and violent retching, which is preceded by generalized epigastric discomfort, starting from 2 hr to up to 3 days after ingestion of a meal containing ackee. After a period of prostration, which may last up to 18 hr [average 10 hr (65)], a second bout of vomiting may occur, and this is usually followed by convulsions, coma, and death. Actual vomiting may not occur in some cases, but may be replaced by foaming at the mouth, followed by the other symptoms described above. The symptomatology of the disease does not include fever or diarrhea. Blood and urine are acidotic but not ketotic, in a manner reminiscent of lactic acidosis. The most striking finding is a profound hypoglycemia with blood glucose levels as low as 3 mg per 100 ml (35, 59, 64, 65). Liver biopsies show depletion of glycogen stores (35). Portmortem examination reveals fatty metamorphosis of the liver, kidney, and other organs (63) and absence of liver glycogen (35). Generalized hyperemia, edema of connective tissue, swelling of lymph nodes, and necrobiosis of pancreatic, renal, and liver epithelia have also been described (63).

The nutritional state of the patient seems to be a very important factor in ackee fruit poisoning. Well-nurtured persons have not been observed to become victims of the disease. On the other hand, case reports of vomiting sickness usually note that patients exhibited manifestations of chronic malnutrition and vitamin deficiency. There is a slightly higher incidence of poisoning in women than in men (34). Children between the ages of 2 and 5 years represent the largest proportions of reported cases. This age group is closely followed in this respect by that between 5 and 10 years of age. The disease, however, may attack older people, too.

Before the development of treatment, mortality reached 80% (63). Even though it was claimed that treatment with thiamine, riboflavin, or vitamin B complex was beneficial in ackee poisoning (63), after the observation that hypoglycemia was a constant accompaniment of the disease, Jeliffe and Stuart (65) and Hill *et al.* (64) used intravenous glucose as treatment. Their experiences

showed that, provided that hypoglycemia had not already resulted in irreversible shock, intravenous glucose administration resulted in recovery of the patients.

III. THE TOXIC PRINCIPLE OF *BLIGHIA SAPIDA*

A. Isolation of the toxins

The suspected relationship of the ackee fruit to the Jamaican vomiting sickness was established by Jordan and Burrows in 1937 (68). They showed the presence of a water-soluble, toxic material in the seeds and pods of the ackee fruit and suggested that the material was a glycoside. In 1938, Evans and Arnold prepared a similar water extract and found it to be lethal for kittens and guinea pigs (63). They proposed that the substance was a saponin. While the early reports on the toxic principle of *Blighia sapida* were based on mere speculation in respect to the chemical nature of the toxin, Hassall *et al.* (58–60) in 1954 were the first to isolate two toxic compounds in crystalline form. In view of their hypoglycemic activity, these compounds were called hypoglycin A and B. The isolation procedure involved alcohol-water extraction of the unripe seeds, which have the highest concentration of the toxins, followed by a series of chromatographic separations on ion exchange resins and cellulose powder. Hypoglycin A, but not B, occurs also in the arillus of the ackee, the contents being greater in the unripe fruit (58).

B. Properties and structure of hypoglycin

Hassall, von Holt, Patrick, and their coworkers improved the isolation procedure in the following years and determined some of the physical properties of both compounds (58, 59, 74, 83, 119, 122). These properties are summarized in table 1.

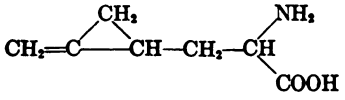
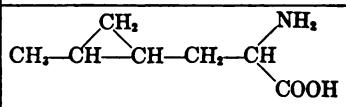
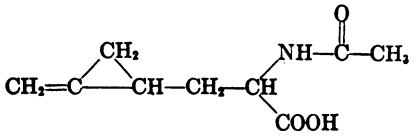
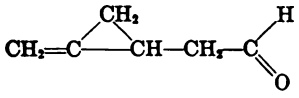
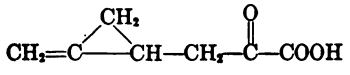
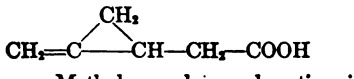
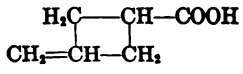
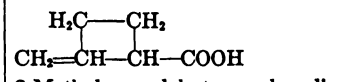
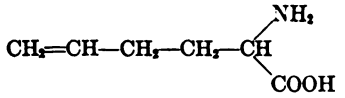
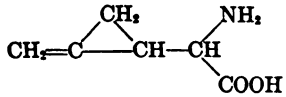
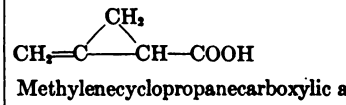
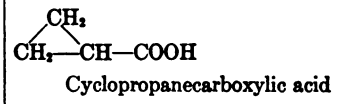
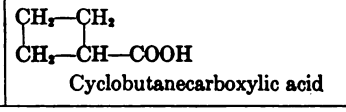
After the initial isolation of the two toxic substances by Hassall, Reyle and Feng, the search for the chemical structure of the hypoglycins was pursued simultaneously in a number of laboratories. It was suggested that both hypoglycin A and B were peptides, since both produced ninhydrin-positive spots on paper chromatography after hydrolysis with HCl (58, 59). In 1956, von Holt and Leppla (74, 119) confirmed the peptide nature of the compounds and established the close chemical relationship between the two, hypoglycin B being a glutamyl derivative of hypoglycin A. Von Holt also suggested that hypoglycin A was probably not a peptide but a new, acid-labile amino acid. Then followed almost simultaneously reports from sx laboratories on the structure of hypoglycin [Anderson *et al.* (2), Ellington *et al.* (27), Renner *et al.* (92), de Ropp *et al.* (95), von Holt and Leppla (120, 121), and Wilkinson (128)], which was shown to be (L)-α-amino-β-

TABLE 1

	Hypoglycin A	Hypoglycin B
Melting point	280°–284°	194°–195°, 200°–206°
Optical rotation	$[\alpha]_D^{20} + 9.2 \pm 2°$	$[\alpha]_D^{22} + 9.6 \pm 2°$
Molecular formula	$C_7H_{11}NO_2$	$C_{12}H_{18}N_2O_5$
Molecular weight by titration	141	270

TABLE 2

Compounds related to hypoglycin

Hypoglycemic	Reference	Nonhypoglycemic	Reference
 Methylenecyclopropyl alanine hypoglycin	(58–60)	 Dihydrohypoglycin	(2)
 Acetyl and isoacetyl hypoglycin	(95)		
 Methylenecyclopropyl acetaldehyde	(2)		
 Methylenecyclopropyl pyruvic acid	(78,93, 117,119)		
 Methylenecyclopropyl acetic acid	(78,93, 117,119)		
 3-Methylenecyclobutanecarboxylic acid	(1)	 2-Methylenecyclobutanecarboxylic acid	(2)
$CH_2=CH-CH_2-CH_2-COOH$ 4-Pentenoic acid	(2–78)	$CH_3-CH_2-CH=CH-COOH$ 2-Pentenoic acid	(104)
		$CH_3-CH_2-CH_2-CH_2-COOH$ Pentanoic acid (valeric)	(104,139)
		$CH_2=CH-CH_2-COOH$ 3-Butenoic acid	(139)
 2-Amino-5-hexenoic acid	(2)	$CH_3-CH_2-CH_2-CH_2-CH_2-COOH$ Hexanoic acid	
$CH_2=CH-COOH$ Acrylic acid			
 Methylenecyclopropyl glycine	(55)	 Methylenecyclopropanecarboxylic acid	
		 Cyclopropanecarboxylic acid	(104)
		 Cyclobutanecarboxylic acid	(104)

(methylene-cyclopropyl)-propionic acid, that is, methylenecyclopropylalanine (table 2). The absolute configuration was suggested and later proved to be 2-(S); 4-(S) (7, 8, 95). The structure of hypoglycin B was then shown to be (56, 57, 66, 67) γ-(L)-glutamyl-hypoglycin.

Hassal and John (57) have proposed that hypoglycin A be referred to simply as hypoglycin, and hypoglycin B as γ-L-glutamyl hypoglycin. We will follow this practice. Hydrogenation and identification of the newly produced amino acids (28, 95), identification of products of acid hydrolysis (28), periodate oxidation (2), and infrared spectroscopy were the major tools in the elucidation of structure. Final proof of structure was given by the chemical synthesis of hypoglycin by Carbon *et al.* (21) and by Black and Landor (7, 8). Although two diastereoisomers could have originated from the synthesis, only the natural isomer was obtained. Hypoglycin B was also synthesized from hypoglycin A and glutamic acid (57).

γ-L-Glutamyl hypoglycin is hydrolyzed in good yield to hypoglycin and glutamic acid by 50% formic acid (122). More severe acid treatment breaks down both hypoglycin and γ-L-glutamyl hypoglycin into several ninhydrin-reactive compounds (121, 122). Neither hypoglycin is stable to hydrogenolysis, both yielding 4 or 5 new amino acids (2, 27). Acetylation of hypoglycin yields two epimeric derivatives (95); periodate or permanganate oxidation leads to destruction of the molecule (2, 95). Ninhydrin degradation as well as treatment with hypochlorite leads to the next lower aldehyde (2). This aldehyde can be oxidized with either freshly precipitated Ag_2O or SeO_2 and H_2O_2 to form methylenecyclopropylacetic acid (2, 116). Methylenecyclopropylpyruvic acid is obtained by oxidative deamination in presence of L-amino acid oxidase and catalase (116).

IV. PHARMACOLOGICAL PROPERTIES AND BIOCHEMICAL EFFECTS OF HYPOGLYCIN

A. Effects in vivo

After a latent period, which can be as long as 4 hr, hypoglycin-treated experimental animals exhibit various evidence of impaired metabolism (ataxia, prostration, slowed ventilation, *etc.*). The animals become hypoglycemic, often vomit, and, depending on the dose and the species, death or complete recovery occurs after various periods of time. The most striking biochemical indication of the metabolic abnormality, discovered by Feng and Patrick (33), is that oxygen consumption and carbon dioxide production decrease in hypoglycin-treated animals.

The general toxicity of hypoglycin [as shown by Hassall *et al.* (58–60) in rats; by Chen *et al.* (22) in mice, pigs, rabbits, and monkeys; by Feng and Patrick (33) and Hassall *et al.* (60) in kittens and guinea pigs] seems to be caused by a multiple-site block of intermediary metabolism restricting the availability of energy sources. The varying amounts of hypoglycin that are toxic in the different animals may correlate with the varying metabolic rates characteristic for each individual animal species. The LD50 for rats is 90 to 100 mg/kg body weight, whereas most mice survive with doses as high as 160 mg/kg body weight. Rabbits and monkeys are particularly susceptible to the toxicity of hypoglycin; rabbits died after intravenous injections of 10 to 20 mg/kg body weight, and monkeys died within 5 days after a single intravenous injection of 20 to 40 mg/kg body weight

of hypoglycin. Toxicity in rats was augmented when fasted rats were used (58–60). The LD50 in fed rats was also determined by Feng and Patrick (33) to be approximately 100 mg/kg body weight, independent of whether the toxin was administered orally or intraperitoneally; but Chen *et al.* (22) observed that in the fasted rat subcutaneous injection of hypoglycin was slightly more toxic than intravenous administration.

The ataxia in rats (58–60) and cats (22), the prostration of all animals except rabbits, and the shallow respiration in rats (58–60) and kittens (33, 60) can be explained by the assumption of a decreased energy supply, especially since repeated intravenous injections of hypoglycin (20 to 150 mg/kg body weight) in kittens anesthetized with pentobarbital did not cause any immediate changes in either the rate and depth of respiration or the blood pressure (33, 60).

Chemical indications in favor of this explanation are supplied by the low blood glucose level, higher glucose utilization, decreased glycogen stores in the liver, fatty metamorphosis of liver and kidney, and augmented acetoacetate and urea levels in the blood.

Blood glucose levels in rats treated with hypoglycin dropped to 25 mg per 100 ml (22) and lower (58–60). Other animals such as cats, rabbits, monkeys (22), and guinea pigs (33, 60) also became markedly hypoglycemic. Entman and Bressler (29) injected hypoglycin (500 to 750 mg/kg body weight) intravenously into mice and hypoglycemia occurred 1 hr after injection. The lowest value of 30 to 40 mg per 100 ml was reached in 90 min, but the blood glucose levels returned to normal or above 3 hr after injection. Chen *et al.* (22) found hypoglycemia to be absent in 75% of pigeons that received 25 to 200 mg/kg body weight hypoglycin by the alar vein. Intravenous injection of glucose could prevent hypoglycemia in rabbits that had received intravenous injections of 10 to 20 mg/kg body weight hypoglycin, even though a single injection of 2 g of glucose did not prevent the later recurrence of hypoglycemia, convulsions, and eventual death.

Von Holt *et al.* (117, 119, 126) administered hypoglycin to alloxan-diabetic rats and showed that the hypoglycemic effect was not mediated by an increased insulin secretion. Chen *et al.* (22) found that the effect of hypoglycin in lowering blood glucose in alloxan-diabetic rats was dependent upon the administered dose. Small doses of 35 mg/kg body weight given subcutaneously or orally were without effect on blood sugar. A single dose of 100 mg/kg body weight reduced blood sugar levels to control values within 6 hr, and the same dose given the following day duplicated the effect. If 35 mg/kg or 50 mg/kg body weight of hypoglycin were given twice daily, the toxin had little effect on the blood glucose level. Glycosuria in these animals, however, was reduced by the administration of 0.1 the dose normally lethal to rats, and the alloxan-diabetic rats were able to tolerate doses of toxin that were lethal to normal animals.

Pulmonary edema was experienced in 34% of rats given lethal doses of hypoglycin (22). Lachrymation and secretion from the nose and mouth occurred in kittens given doses of 110 to 500 mg/kg body weight of hypoglycin (33, 60) and dilation of the pupils of rabbits given 10 to 20 mg/kg body weight of hypoglycin was noted.

De Renzo *et al.* (78, 93) administered to rats by intramuscular injection 150 mg/kg body weight of hypoglycin and then immediately after the first injection, administered 1 g/kg body weight of glucose-U-C^{14}. No major differences were found in the radioactivity incorporated into the expired carbon dioxide when the latter was collected 2 and 4 hr after the injections. Although under similar conlditions, von Holt and Benedict (117) obtained similar glucose oxidation, von Holt *et al.* (125) found a significantly higher rate of glucose oxidation when the injection of glucose-U-C^{14} was delayed for 4 hr after administration of 100 mg/kg body weight of hypoglycin. Thus, if one allows for a latent period of hypoglycin activity, the conversion of glucose to carbon dioxide is increased. De Renzo's group (78) also demonstrated that simultaneous injections of hypoglycin and acetate-1-C^{14} did not influence its oxidation to labeled carbon dioxide. This experiment has not been repeated with a delayed injection of acetate-1-C^{14}. Patrick's earlier findings that the glucose tolerance is impaired in a manner that resembles the impaired glucose uptake of diabetes (33) seems to be contradictory to the reported high level of glucose metabolism. It has also been found that there is no significant change in the plasma concentrations of lactate, pyruvate (33), cholesterol, or chloride (58), even though the plasma glucose level is depressed.

Histological (58–60) and chemical evidence indicates that a reduction in the concentration of liver glycogen occurs before the fall in blood glucose level (33). In the experiments of de Renzo *et al.* (78, 93), hypoglycin had the same effects on glucose-U-C^{14} incorporation into liver and muscle glycogen as did insulin. No radioactivity from labeled glucose was found in liver glycogen, even though up to twice as many counts were obtained in muscle glycogen when compared to the controls. When Feng and Patrick (33) injected lethal doses of hypoglyin (250 mg/kg body weight) into fasted and fed rats, the amount of glycogen in the livers of the experimental animals was depleted 6 hr after injection, while the glycogen stores in skeletal muscle were only insignificantly decreased under both feeding conditions. There was also a significant depletion of heart glycogen in both fasted and fed animals, even though fasting almost doubled the glycogen content of the heart in the control animals. The liver glycogen content was also lower in hypoglycin-treated mice and rabbits. Although the capacity to synthesize liver glycogen has been reported to be unimpaired in hypoglycin-treated animals (22, 31, 93), injections of 30% dextrose to fasted rats treated with hypoglycin did not result in glycogen deposition, even though the injections slowed down the decrease in blood glucose level (82).

Although von Holt *et al.* (117, 119, 126) demonstrated that hypoglycin's effect on glucose metabolism was not mediated by insulin, many similarities between the actions of the two agents exist. The effects on the various glycogen stores and blood glucose levels are the better-investigated parameters.

De Ropp and Snedeker (94) found that hypoglycin had the same effect on the γ-aminobutyric acid level in the brain as does insulin. Male rats given intraperitoneal injections of hypoglycin (140 mg/kg body weight) or insulin (100 U/kg body weight) had almost doubled their brain γ-aminobutyric acid levels 3 hr after injection as compared to controls. Since the hypoglycemic agents did not alter glutamate levels, the workers suggested that the agents partially block transami-

nation of γ-aminobutyric acid to succinic semialdehyde. This finding agrees with the early observation that thiamine administration is beneficial in treatment of ackee poisoning (63).

When de Renzo *et al.* (78, 93) injected glucose-U-C^{14} and hypoglycin at the same time into rats, the radioactivity incorporated into liver lipids was less than in controls while total lipid content was increased. This suggests decreased triglyceride formation in the liver and increased mobilization of lipids from the periphery to the liver. The fatty metamorphosis of the liver in 75% and fatty metamorphosis of the kidney in 35% of rats given lethal doses of hypoglycin also supports a lipid redistribution (22). Although 90% of the mice given injections of as much as 160 mg/kg body weight of hypoglycin survived, fatty metamorphosis of the liver and kidney was still seen in 25% of the animals, (22). Fatty infiltration of the liver and kidney was also observed in monkeys and pigeons that received injections of hypoglycin (22). In contrast, rabbits given injections of 10 to 20 mg/kg body weight of hypoglycin did not show fatty infiltration, even though they did show a loss of liver glycogen (22). An increased level of plasma nonesterified fatty acids has been demonstrated in hypoglycin-treated rats (78, 93).

Williamson and Wilson (129) demonstrated an augmented acetoacetate and a higher acetoacetate/β-hydroxybutyrate ratio in the blood after administration of hypoglycin. At low glucose levels an increase in the acetoacetate level is a generally observed phenomenon. Transamination and oxidation of amino acids will also become of major importance in the hypoglycemic state. In consonance with this is the elevated level of blood urea found in hypoglycin-treated rats just before death (58). This increase in amino acid catabolism might also be involved in hypoglycin's antitumor activity as demonstrated by Gaskin and Persaud (53). A very low, daily dose of 4 mg/kg body weight of hypoglycin for 4 days inhibited tumor growth of sarcoma-180 cells in male mice by 45%. Simultaneous injection of leucine abolished hypoglycin's ability to prevent this neoplastic growth. The action of hypoglycin on amino acid catabolism may also explain Persaud's (88–90) observations of hypoglycin's teratogenic effects in rats, or these effects could be due to a direct effect of hypoglycin as, for instance, an antimetabolite of β-hydroxy-β-methyl glutaryl CoA as suggested by von Holt *et al.* (125).When hypoglycin is administered in a single oral dose of 30 mg/kg body weight during the first 5 days of gestation, a significant incidence of malformed fetuses is found. The incidence of syndactyle was 87% and encephalocele 89%. Failure to gain weight and to grow during gestation was observed in all cases. The incidence of fetal resorption was 6% as compared to 1.8% in the controls. Administration of only two doses of 15 mg/kg body weight on days 3 and 6 of gestation resulted in retardation of growth in 97% of the fetuses and in 5.8% fetal resorption. Fetal malformation, however, was present in only 8.7% of the fetuses at this dose. Histological examination of the fetuses from hypoglycin-treated rats showed arrest of osteogenesis, absence of normal hepatic architecture, absence of portal triads, and large nuclei in parenchymal cells with a prominent nuclear membrane and nucleoli. There was renal glomerular agenesis and poor development of the myocardium and the ventricular septum.

The necrosis of thymic lymphocytes in 60% and of splenic lymphocytes in

30% of rats given lethal doses of hypoglycin (22) may be an early indication of reduced growth and maintenance of the animal due to insufficient energy supply.

Other tissues are also damaged rather early in hypoglycin poisoning. Although von Holt and Leppla (74, 119) found no damage to the α-cells of the pancreas, Feng and Kean (32) observed a reduction in the number of both α-cells and the granules present in these cells after injection of lethal doses of hypoglycin. Feng and Kean did not observe a concomitant effect on the β-cells. Although this may indicate a lowered glucagon level, the authors felt this effect was not responsible for the hypoglycemia, since cobalt chloride does even more damage to the α-cells without producing hypoglycemia.

Under a condition of stress, one might expect an increased adrenal cortical steroid output; however, de Renzo's laboratory (78, 93) showed that hypoglycin injected into rats had no effect on the glucocorticoid level. Von Holt *et al.* (117, 119, 126) demonstrated that an adrenalectomy of rats did not prevent the hypoglycemic effect of the toxin and Chen *et al.* (22) published similar results in mice.

The effects of various experimental diets on the activity of hypoglycin is also indicative of some of the effects this toxin has on basic metabolic rates. Feng and Kean (31) fed rats three diets of different composition. Diet A, which simulated the diet of the Jamaican poor class, was 85% carbohydrate; in Diet B, the brown sugar in Diet A (12.5%) was replaced in the same proportion by casein; and Diet C was a stock diet. The carbohydrate Diet A significantly enhanced the toxicity of hypoglycin. All the rats fed Diet A were killed by 40 mg/kg body weight hypoglycin, whereas doses of 130 to 150 mg/kg body weight were necessary to kill rats fed Diets B or C. In the control animals the incomplete Diets A and B hindered growth, and those fed Diet A had liver glycogen well above those animals eating Diets B or C.

Since Diets A and B had the same effect on growth in the control animals, the difference in toxicity could not be ascribed to the smaller weights of rats fed Diet A. The authors concluded that the susceptibility of animals to hypoglycin is influenced by the carbohydrate/protein ratio in their diet. In contradiction to this, Fox and Miller (38) showed that addition of protein to a diet containing ackee beans did not prevent the decreased growth rate and mortality of rats, while riboflavin addition prevented these effects. Von Holt and von Holt (124, 126) also have reported that the lethal effects and fat accumulation resulting from hypoglycin administration were prevented by riboflavin phosphate. These findings suggest that hypoglycin may interact with a site in the electron transfer chain.

B. Effects in vitro

De Renzo *et al.* (93) suggested that hypoglycin interfered primarily with fat metabolism, and von Holt *et al.* (117, 118) independently came to the same conclusion. While there are other effects such as decreased gluconeogenesis, the block in fatty acid oxidation is one of the major effects of hypoglycin. The metabolic rates of glucose and amino acids are shifted towards catabolism.

Although incubation of rat liver homogenates with increasing amounts of

hypoglycin did not affect the oxidation of exogenous palmitate (123), Entman and Bressler (24) have shown that myocardial homogenates from hypoglycin-treated mice had an impaired capacity to oxidize palmitate, while their ability to oxidize hexanoate was unimpaired. The decreased ability to oxidize palmitate preceded the onset of hypoglycemia (29). In consonance with evidence that the endogenous substrate for skin respiration is triglyceride (5, 25, 137), respiration of skin slices was inhibited by hypoglycin (138). Skin respiration was not inhibited by hypoglycin, however, when glucose was concomitantly added as a respiratory substrate (138).

Although Patrick (83) found that hypoglycin did not significantly influence the rate of glycogen disappearance in rat liver slices, he did demonstrate that conversion of added glucose to glycogen was decreased. Phosphorylase activity in liver homogenate from hypoglycin-treated animals was also not found to be affected (33, 83). Patrick also noted that even though the conversion of exogenous glucose to pyruvate was stimulated, there was a decrease in formation of fatty acids from glucose (83). The activity of hexokinase in liver homogenates from hypoglycin-treated animals was not altered up to 6 hr after injection (33, 83).

Gluconeogenesis from pyruvate was decreased to 65% of the control levels when liver slices were incubated with hypoglycin. Patrick suggested that the hypoglycin effect on gluconeogenesis could be due to its influence on the ATP/AMP ratio. The ATP level was decreased after incubation of liver slices with hypoglycin (85). However, no inhibiton of fructose-1,6-diphosphatase or of glucose-6-phosphatase could be demonstrated (83, 85). In other experiments, it was found that mitochondria prepared from hypoglycin-treated rats showed an impaired capacity to form high-energy phosphate bonds when pyruvate or malate were the oxidation substrates. The fact that there was no impairment of oxidative phosphorylation when hypoglycin was added directly to mitochondria (78) might be due to the latency of hypoglycin activity.

The effects of hypoglycin on glucose metabolism in tissues other than liver have also been studied. Entman and Bressler (29) have shown that the capacity of myocardial homogenates from hypoglycin-treated mice to oxidize glucose was not impaired. No effect on glucose metabolism was found in rat fat pads incubated with hypoglycin (83). There are conflicting reports on the effect of hypoglycin in isolated diaphragm. De Renzo *et al.* (93) reported an inhibition of net glycogen formation when hypoglycin was administered *in vitro*. Patrick (83), however, showed that hypoglycin enhanced the incorporation of the label from glucose-U-C^{14} into diaphragm glycogen. Glucose uptake by diaphragm was not affected by hypoglycin (78, 93).

Patrick's (84) finding that leucine transamination by liver was inhibited by 34% in the presence of hypoglycin and 28% in the presence of glutamate prompted him to suggest that hypoglycin might be a competitive inhibitor of liver transamination. Hypoglycin also inhibited glutamate transamination by 6%. Posner and Raben (91) indicated, however, that this inhibition of transamination was not responsible for the inhibition of leucine oxidation. They showed that, while the decarboxylation of leucine-1-C^{14} which occurs after transamination

was not inhibited, the oxidation of leucine-2-C^{14} to $C^{14}O_2$ was inhibited by hypoglycin. Patrick and Stuart (86) showed that although the conversion of leucine to fatty acids, cholesterol, and carbon dioxide was decreased in the presence of hypoglycin, the incorporation of label from leucine into ketone bodies was not. Liver acetoacetate production, however, was inhibited by hypoglycin (118), as was cholesterol formation from acetate (29). Hypoglycin incubated with liver slices interfered with the conversion of alanine and glutamate into fatty acids (86). These experimental findings indicate a general shift of the metabolism of amino acids toward energy formation, while hypoglycin did not affect the incorporation of amino acids into protein (86).

V. METABOLISM OF HYPOGLYCIN

The latent period of hypoglycin activity and the fact that hypoglycin was inactive *in vitro* under certain conditions suggested to von Holt *et al.* (124, 126) and de Renzo *et al.* (78, 93) that a metabolite of hypoglycin, rather than hypoglycin itself was the active hypoglycemic agent. Von Holt *et al.* (116, 118) showed that hypoglycin was metabolized; the products of its metabolism were isolated from rat liver homogenates and found to be hypoglycemic. Methylenecyclopropylpyruvic acid, the first of the metabolites, is formed by transamination with pyruvate in the presence of pyridoxal phosphate and magnesium ion. The second metabolite, methylenecyclopropylacetic acid is then formed by oxidative decarboxylation in the presence of thiamine pyrophosphate and coenzyme A. Fig. 1 presents a scheme showing these metabolic transformations. A deamination step in the metabolism of hypoglycin was indicated to Yardley and Godfrey (138) by the augmentation of ammonia evolution on hypoglycin addition to skin slice incubations.

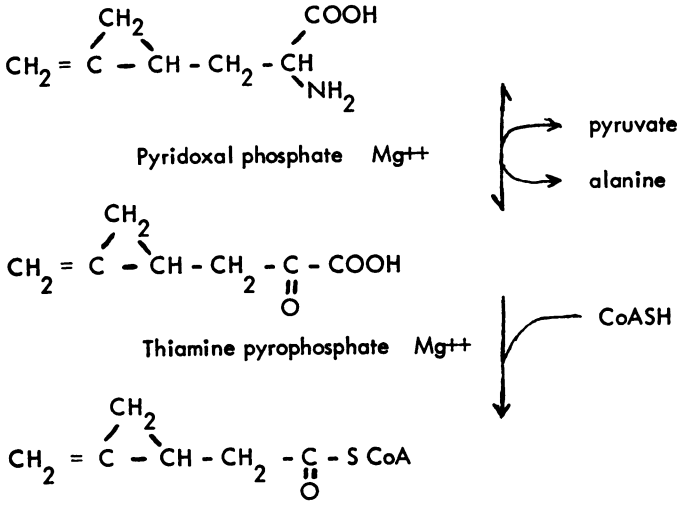


FIG. 1.

VI. OTHER HYPOGLYCIN-LIKE COMPOUNDS

γ-L-Glutamyl hypoglycin, another toxic principle of the ackee fruit, had the same *in vivo* effects in rats as hypoglycin, although about twice the concentrations of it were required (22, 33, 58–60). Similarly, the epimeric pair of N-acetyl derivatives of hypoglycin was also capable of producing hypoglycemia (95). Anderson *et al.* (2), who synthesized a number of hypoglycemic compounds structurally related to hypoglycin, proposed as a rule that an agent in his group of substances must possess a carbon-carbon double bond separated by two carbon atoms from an actual or potential carboxyl group in order to show hypoglycin-like effects. Acrylic acid does not conform with these criteria, but it is also a hypoglycemic agent (23, 55). In table 2, the structures of hypoglycemic compounds are compared with chemically related substances that do not have such activity. Further study of the hypoglycin-like compounds has given support to the proposal that the active agent is a metabolite of hypoglycin and has advanced the understanding of hypoglycin's effects on metabolism.

Glucose conversion to carbon dioxide by intact mice is enhanced by injection of 4-pentenoate (23). Although 4-pentenoate has no effect on the formation of carbon dioxide from exogenous glucose by homogenates of pigeon heart or skeletal muscle, it decreases this conversion in pigeon liver homogenates (24). This inhibition is believed to occur at the level of pyruvate decarboxylation, since glucose conversion to lactate is not affected, but pyruvate oxidation to carbon dioxide is markedly depressed (24, 106). It has also been reported that although methylenecyclopropylpyruvic acid slightly inhibits pyruvate decarboxylation in liver homogenates, methylenecyclopropylacetic acid does not influence it (125). The conversion of pyruvic-2-C^{14} to radioactive carbon dioxide is also unaffected in liver homogenates by methylenecyclopropylacetic acid. 4-Pentenoic acid, however, inhibits the oxidation of acetate to carbon dioxide (78), if adequate time is allowed for the acid to effect a metabolic block.

4-Pentenoic acid inhibited gluconeogenesis from 3-carbon precursors in mice (23), rat liver perfusates (99), and pigeon liver homogenates. The conversion of pyruvate to glucose in intact mice was decreased. In perfused rat liver, glucose formation from alanine was markedly inhibited. Although gluconeogenesis with lactate as precursor was lowered by 4-pentenoic acid in pigeon liver homogenates, glycerol conversion to glucose was not affected.

The two major effects of hypoglycin-like compounds on fatty acid metabolism were the shunting of fatty acids to triglycerides as evidenced by the pale livers from animals treated with methylenecyclopropyl glycine (55), and the inhibition of fatty acid oxidation. Von Holt *et al.* (125) found that in liver homogenates methylenecyclopropylacetic acid inhibited the oxidation of long-chain fatty acids (C_{12} to C_{16}), but not the oxidation of medium-chain fatty acids (C_4-C_{10}). It was also demonstrated that the oxidation of palmitate, but not that of hexanoate, by myocardial homogenates prepared from mice treated with 4-pentenoic acid was much less than that of the controls (23). Brendel *et al.* (17) have shown, however, that the inhibition of fatty acid oxidation in pigeon liver homogenate and mito-

chondria by 4-pentenoic acid depends upon the conditions of incubation. Without preincubation of pigeon liver homogenates with 4-pentenoic acid (addition of 4-pentenoic acid at the start of the incubation), palmitate oxidation was slightly depressed; whereas no inhibitory effects were found on the oxidation of palmitylcarnitine or octanoic acid (16), nor was the oxidation of short-chain fatty acids inhibited by 4-pentenoate in non-preincubated rat liver mitochondria (104). On the other hand, after 20 min preincubation of the liver preparations with 4-pentenoic acid, the oxidations of palmitate, palmitylcarnitine, and octanoate were equally and markedly inhibited (16). Methylenecyclopropylacetic acid and 4-pentenoic acid inhibit endogenous mitochondrial respiration (20, 106), in which the substrates are fatty acids (13, 70). 2-Amino-5-hexenoic acid, methylenecyclopropylglycine, and 4-pentenoic acid interfere both with endogenous respiration and palmitic oxidation of skin slices (135, 136, 138). Although 4-pentenoic acid inhibited palmitate-1-C^{14} oxidation to $C^{14}O_2$ by rat liver mitochondria, valeric acid (the saturated analogue of 4-pentenoic acid), 2-pentenoic acid (an isomer of 4-pentenoic acid), cyclopropanecarboxylic acid (a possible metabolite of methylenecyclopropyl glycine), and cyclobutanecarboxylic acid are ineffective.

Neither 4-pentenoic acid nor 4-pentenoyl CoA inhibit the general acyl CoA dehydrogenase, crotonase, or β-hydroxyacyl CoA dehydrogenase (16). In consonance with the observation that the activity of the long-chain acyl CoA: carnitine acyltransferase is impaired by hypoglycin (29), intraperitoneal injection of (−)-carnitine concomitantly with 4-pentenoic acid prevented the depression of palmitate oxidation by myocardial homogenates. 4-Pentenoic acid decreased free carnitine levels in both heart and liver and increased tissue levels of short-chain acylcarnitines (23). The excess acylcarnitines have been identified as a mixture of 4-pentenoyl and acrylyl carnitine (16, 24). These carnitine derivatives are most likely in equilibrium with the CoA derivatives, since both pentenoyl CoA and acrylyl CoA are substrates for the acetyl CoA: carnitine-O-acetyl transferase (EC2.3.1.7) (23). Carnitine addition *in vitro* did not prevent the 4-pentenoate inhibition of palmitate oxidation by rat liver mitochondria (104), pigeon liver homogenates, or pigeon liver mitochondria (16). The inhibition was, however, prevented if adequate amounts of both CoA and carnitine are added to the 4-pentenoate inhibition reaction mixtures (16). A decreased effect of 4-pentenoic acid on gluconeogenesis in pigeon liver homogenates was obtained by addition of either short-chain fatty acids or palmitylcarnitine. Although carnitine addition had no effect on the inhibition of gluconeogenesis induced by 4-pentenoate in the perfused liver and only a minor effect in reversing the inhibition in pigeon liver homogenates, carnitine administration to mice treated with 4-pentenoic acid alleviated the defect in gluconeogenesis. This protective effect of carnitine in intact mice was transient and proceeded to wear off after 30 min (23, 24). The addition of adequate amounts of both CoA and carnitine completely prevented the inhibition of gluconeogenesis by 4-pentenoic acid in pigeon liver homogenates (24).

Although methylenecyclopropylacetic acid (20), 4-pentenoic acid, and valeric acid (103, 106) uncouple oxidative phosphorylation, methylenecyclopropylacetic acid does not uncouple digitonin submitochondrial particles (20). For this reason

it was suggested that methylenecyclopropylacetic acid does not directly affect oxidative phosphorylation. Although succinate oxidation is not affected in isolated rat liver mitochondria by 4-pentenoate, Sherratt (106) demonstrated a slight inhibition of α-ketoglutarate and citrate oxidations. The accumulation of alanine and the decrease in tissue levels of glutamic and aspartic acids in the presence of 4-pentenoic acid could be explained by a decreased function of the TCA cycle (24). The conversion of acetate to acetoacetate or cholesterol was inhibited by methylenecyclopropylacetic acid in liver homogenates, even though the pathway from mevalonate to cholesterol was not blocked (125). The production of acetoacetate directly from leucine seems to be important in animals poisoned with hypoglycin-like compounds. This principle might be related to the inhibition of growth of seedlings of *Phaseolus aureus* by methylenecyclopropyl glycine, which can be reversed by leucine, but not by isoleucine (36).

VII. MECHANISM OF ACTION OF THE HYPOGLYCIN-LIKE COMPOUNDS

A. Hypoglycemia

The most striking finding in patients with vomiting disease is a severe hypoglycemia (63). The patients may recover if intravenous glucose is given before any permanent damage pursuant to the low blood glucose levels has occurred (35, 64, 65). This led to the proposal that the hypoglycemia was due to a blockage of some enzyme in the gluconeogenetic pathway (65).

This condition could have been due to interference of the toxin responsible for the vomiting sickness with one of the coenzymes derived from the B group of vitamins. Indeed, Johnston reported that all his cases of vomiting sickness were cured with thiamine (63), while Hill (63) emphasized that some of his patients either had manifestations of vitamin deficiency at the time of the onset of the disease or developed them thereafter. After Hassall had isolated hypoglycin from ackee fruits and showed that the toxin was responsible for the hypoglycemia seen in the vomiting sickness (58–60), von Holt showed that the lethal effects of hypoglycin in mice and the lowering of the blood sugar levels in both normal and alloxan-diabetic rats could be prevented by the oral administration of riboflavin. Riboflavin reversed the hypoglycemia, even when given after hypoglycin (124, 126).

Von Holt *et al.* (117, 118, 125, 126) proposed that the primary effect of hypoglycin was interference with the metabolism of fatty acids and not a direct effect on glucose metabolism. The bases for this proposal may be summarized:

a) Riboflavin administration prevented the effects of hypoglycin (124, 126);
b) Hypoglycin increased glucose oxidation in the intact animal (117, 124, 125);
c) Liver glycogen was depleted in patients with vomiting sickness (35, 59, 64, 65) and animals treated with hypoglycin (22, 33, 82), while liver glycogen synthesis (22, 31, 93) or breakdown were unaffected (83);
d) There was fatty infiltration of the liver in patients with vomiting sickness (34, 35, 63–65) and in animals treated with hypoglycin (22, 78, 126) and;
e) Serum nonesterified fatty acids increased after hypoglycin administration (93).

McKerns *et al.* (78) showed that administration of hypoglycin *in vivo* inhibited

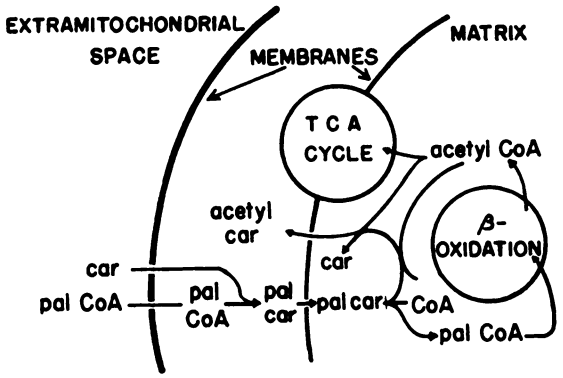


FIG. 2.

the conversion of butyrate, palmitate, or stearate to CO_2. Hypoglycin or its active metabolites decreased palmitate oxidation in liver (118, 125) and in skin (134, 138). Work in Senior and Sherratt's (104, 106), Yardley and Godfrey's (135, 138), and in our own laboratories (16, 23, 24) have shown that 4-pentenoic acid is also an inhibitor of fatty acid oxidation. It seems, therefore, that the hypoglycemia observed in animals treated with hypoglycin or 4-pentenoate is the result of a relative increase of carbohydrate consumption combined with the consequent depletion of the liver glycogen stores and inhibition of gluconeogenesis (section VII C) (23, 104, 125).

B. Inhibition of fatty acid oxidation

The biochemical mechanism by which hypoglycin and the simplest of the hypoglycin-like compounds, 4-pentenoic acid, inhibit fatty acid oxidation, and this inhibition, in turn, results in hypoglycemia, has been extensively studied in this laboratory. The aspects of long-chain fatty acid oxidation and of glucose metabolism relevant to the discussion of the mechanism of action of hypoglycin-like compounds will be briefly reviewed.

1. Factors affecting fatty acid oxidation. THE ROLE OF CARNITINE. Fig. 2 presents a scheme showing how long-chain fatty acids are oxidized in mitochondria. The presence of carnitine in most tissues has been demonstrated (19, 39, 41, 77) and the important role it plays in fatty acid oxidation (11, 14, 40, 43, 44, 49, 133, 139) has been shown. The action of carnitine in fatty acid oxidation is related to its ability to shuttle activated long-chain fatty acids from the extramitochondrial sites of their activation to the mitochondrial inner membrane-matrix where the enzymes of β-oxidation are located (3). The activation of the fatty acid to its acyl CoA is the first step necessary for its further metabolism. This activation occurs both in the cytoplasmic reticulum and in the mitochondria, and the predominance of one site of activation over the other one depends on the tissue and the chain length of the acid. Acyl CoA formation in muscle occurs both in the microsomal fraction and in the outer mitochondrial membrane (1, 69). In either case, the activation occurs outside a barrier to acyl CoA's, as shown by the fact

that long-chain fatty acids cannot be oxidized by muscle preparations from a number of animal species, while their respective acylcarnitines are (69). In liver, the enzyme activating fatty acids is located mainly in the endoplasmic reticulum but activity is found also in the mitochondria (12, 30). Most of the mitochondrial enzyme activity is in the outer membrane fraction (81). Two other activating systems, one GTP-dependent and the other ATP-dependent, have been described in intact liver and kidney mitochondria. Since fatty acid oxidation in these preparations is independent of carnitine, these activating systems are thought to be located in the inner mitochondrial compartment (6, 96, 97, 140). Short-chain fatty acids, which do not require carnitine for their oxidation (18, 45), seem to be activated by these intramitochondrial enzymes (14, 98). However, the bulk of the fatty acid oxidation *in vivo* seems to be carnitine-dependent, as shown by the observation that carnitine depletion in heart leads to increased triglyceride synthesis and decreased fatty acid oxidation (18).

The shuttle mechanism for the entry of acyl CoA's into the mitochondrion is mediated by the formation of acylcarnitines. The mitochondrial membrane is permeable to carnitine esters (11, 69), while it is impermeable to acyl CoA's, free CoA, and free carnitine (51). The transacylation of the CoA and carnitine esters in the reaction

$$\text{AcylCoA} + \text{carnitine} \leftrightharpoons \text{acylcarnitine} + \text{CoA}$$

is catalyzed by two separate and distinct enzymes: acetyl CoA: carnitine acetyl transferase (EC 2.3.1.7), which is specific for acetyl CoA and short chain acyl CoA's (less than C_{10}) (42, 48, 51), and palmityl CoA:carnitine palmityl transferase (EC 2.3.1.—), which is specific for long-chain fatty acyl CoA's (C_{10} to C_{18}) (80). These reactions are freely reversible (48, 80) and mitochondria contain sufficient enzyme levels to permit equilibrium or near equilibrium between the substrate pairs acyl CoA/CoA and acylcarnitine/carnitine *in vivo* (15). This determines that the extramitochondrial acyl CoA's and acylcarnitines (and free CoA and carnitine as well) vary in parallel (9, 10, 69, 87). It has been shown that the formation of the acylcarnitines is the rate-limiting step in the overall process of long-chain fatty acid oxidation (105).

THE ROLE OF CoA. The role of CoA in both the activation and transacylation reactions necessitates an adequate supply of both intra- and extramitochondrial free CoA. Fritz *et al.* (45) have shown that even though heart mitochondria have adequate amounts of CoA for normal rates of fatty acid oxidation, the maximal rate of oxidation resulting from the addition of carnitine could not be obtained without addition of exogenous CoA. Bremer (14) has postulated that availability of free intramitochondrial CoA may be a key regulatory mechanism in fatty acid oxidation. He has also shown that succinate inhibits octanoyl carnitine oxidation, whereas palmitylcarnitine inhibits pyruvate oxidation (14). These observations show that some substrates compete better than others for available CoA. This may be interpreted as indicative of an intramitochondrial compartmentation of CoA, as suggested by Garland (51).

ENZYMES OF FATTY ACID OXIDATION. The first step in the intramitochondrial

oxidation of the long-chain fatty acyl CoA is one of dehydrogenation, catalyzed by a flavin requiring dehydrogenase. At least three acyl CoA dehydrogenase have been described, with specificity for either long- or short-chain acyl CoA's. Reactions that follow this initial dehydrogenation, *i.e.*, hydration to the β-hydroxy acyl CoA, dehydrogenation to the β-ketoacyl CoA, and cleavage to acetyl CoA and a 2-carbon shorter acyl CoA, seem to be catalyzed by single enzymes (for review see 109).

2. Hypoglycin-like compounds and fatty acid oxidation. HYPOGLYCIN EFFECT AND FATTY ACID CHAIN LENGTH. Von Holt *et al.* (125) proposed that the site of action of hypoglycin is located at a point in the fatty acid oxidation pathway where more than one enzyme catalyzes a given reaction, depending on whether the substrates are short-chain or long-chain fatty acids. He discarded the activation step as a possible site, since methylenecyclopropylacetic acid was a substrate of the reaction, and methylenecyclopropaneacetyl CoA was formed from hypoglycin. This leaves only two other possible sites: translocation into the mitochondria and dehydrogenation of the acyl CoA's. He chose the acyl CoA dehydrogenase step as the most plausible site. He based his choice on observations that riboflavin prevented the effects of hypoglycin and that methylenecyclopropylacetic acid did not inhibit the oxidation of fatty acids shorter than C-10 *in vitro* (125). McKerns *et al.* (78), however, have shown that the oxidation of butyrate was inhibited when the acid was injected into rats 4 hr after hypoglycin treatment. They also showed that 4-pentenoic acid inhibited the oxidation of acetate to CO_2; and Brendel *et al.* (16) have likewise found that 4-pentenoate inhibited oxidation of short-chain fatty acids by liver homogenates if adequate time was allowed for the acid to effect a metabolic block of fatty acid oxidation.

3. Effect on isolated enzyme systems. Corredor *et al.* (16, 23), working with partially purified enzyme systems, have established that 4-pentenoate is activated to its acyl CoA and metabolized further to CO_2 and acrylyl CoA. Both pentenoyl CoA and acrylyl CoA are substrates of the acetyl CoA:carnitine acetyl transferase (23). Neither 4-pentenoic acid nor 4-pentenoyl CoA inhibits the general acyl CoA dehydrogenase, crotonase, or β-hydroxyacyl CoA dehydrogenase (16). These observations do not support a direct inhibitory action of 4-pentenoate on these enzymes.

4. Effect on carnitine- and CoA-dependent reactions. Earlier observations from Bressler's laboratory (29) showed an impairment in the activity of the long-chain acyl CoA:carnitine acyltransferase. It was later shown that the levels of free carnitine were reduced in liver and heart of mice given 4-pentenoate (23). With pigeon liver homogenates and mitochondria, Brendel *et al.* (16) have, however, shown that carnitine by itself does not reverse the 4-pentenoate-induced inhibition of fatty acid oxidation. Nevertheless, this inhibition is completely reversed if adequate amounts of both CoA and carnitine are added to the reaction mixtures inhibited by 4-pentenoate (16). There is also an accumulation of pentenoylcarnitine and acrylylcarnitine (16, 24), which are presumed to be in equilibrium with their respective acyl CoA's. Since in higher animals acrylyl CoA represents an oxidative bottleneck (23), the formation of the acrylic acid

esters represents a lethal synthesis, by reducing the amount of available free CoA and free carnitine and so inhibiting the processes that require CoA and free carnitine, such as fatty acid activation, translocation and oxidation.

Other CoA-requiring processes would also be affected, as shown by the inhibition of pyruvate (24) and α-ketoglutarate oxidations (106).

C. Effects on glucose metabolism

1. Factors affecting gluconeogenesis. The intimate relationship between fatty acid oxidation and gluconeogenesis has been recently documented (26, 61, 71, 130, 131). Krebs *et al.* (71) showed that addition of free fatty acids to kidney slices increased glucose formation from lactate. An enhancement of gluconeogenesis by increased free fatty acid levels has been observed in different types of liver preparation (61, 62, 71, 72, 79, 107, 108, 111, 131). This effect has also been obtained *in vitro* after adding carnitine to rabbit liver and kidney slices (4). Increased glucose formation may be the result of augmented glucose production from amino acids, decreased glucose catabolism to pyruvate, or both. The metabolic regulation of these processes may be exerted at any one of the several enzymatic steps involved. However, the most likely sites for such regulation are at the level of those reactions catalyzed by enzymes unique to the glycolytic or gluconeogenetic pathways (102). Such reactions are:

$$\text{Fructose-6-P} + \text{ATP} \rightarrow \text{Fructose-1,6-diphosphate} + \text{ADP} \qquad (1)$$

This reaction is catalyzed by phosphofructokinase, an enzyme activated by fructose-6-phosphate, fructose diphosphate, P_i, and AMP (75) and inhibited by both citrate (102) and free fatty acids (73, 102, 127). The reverse reaction is catalyzed by a different enzyme, fructose diphosphatase, which is allosterically inhibited by AMP (54).

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP} \qquad (2)$$

This reaction is catalyzed by pyruvate kinase, which, along with glucokinase and phosphofructokinase, is inhibited by free fatty acids (73, 127). Pyruvate kinase is also inhibited by NADH, a product of fatty acid oxidation (73) and by ATP and GTP (102, 110). Unlike the phosphofructokinase reaction, the reversal of the pyruvate kinase reaction is catalyzed by several enzymes and requires the expenditure of energy.

$$\text{Pyruvate} + CO_2 \xrightarrow{\text{ATP}} \text{Oxaloacetate} \qquad (3)$$

Reaction 3 is catalyzed by pyruvate carboxylase, an enzyme that has an absolute requirement of acetyl CoA for activation (102, 113) and is inhibited by malonyl CoA (102, 114, 132).

$$\text{Oxaloacetate} \xrightarrow{\text{GTP}} \text{Phosphoenolypyruvate} + CO_2 \qquad (4)$$

Reaction 4 is catalyzed by phosphoenolpyruvate carboxykinase; this enzyme is inhibited by AMP (54).

$$\text{1,3-Diphosphoglyceric acid} + \text{NADH} \rightleftharpoons \text{Glyceraldehyde-3-P} + \text{NAD} + P_i . \qquad (5)$$

Apart from the physiologically irreversible reactions discussed above, Reaction 5 may be also a site for the control of glucose metabolism. The direction of this reaction will be determined by the cytoplasmatic NAD/NADH ratio. When the ratio is high, the glycolytic pathway will predominate over the gluconeogenetic one and *vice versa*. Therefore, a more reduced state in the cytoplasm will favor glucose formation (102).

The role of fatty acids in gluconeogenesis may be ascribed to 1) their ability to inhibit some enzymes in the glycolytic pathway, and 2) the formation of acetyl CoA and NADH as products of their oxidation.

Besides its obligatory role as an activator of pyruvate carboxylase (113), acetyl CoA also acts as inhibitor of pyruvate decarboxylation (52). Krebs *et al.* (71) have pointed out that the role of acetyl CoA is not solely that of an activator. Acetyl CoA is the immediate precursor of most energy-yielding reactions in the mitochondria. Therefore, a steady-state level of acetyl CoA sufficient to supply cell requirements and still maintain amounts necessary to activate the carboxylase is required. This excess is provided by the oxidation of fatty acids, which in this sense have a "sparing action" on carbohydrate reserves. Increased acetyl CoA oxidation will have effects on glucose metabolism, independent of the ones just discussed. In this connection, Underwood and Newsholme (112) have shown that fatty acids enhance glucose formation from substances that enter the gluconeogenetic pathway at the level of the triosephosphates. This enhancement cannot be mediated through an activation of pyruvate carboxylases. They suggest, that at least in part, the effect of fatty acids on gluconeogenesis is mediated through increased citrate levels, which would, in turn, inhibit phosphofructokinase.

The relative importance of acetyl CoA and NADH in gluconeogenesis has not been elucidated. However, Söling *et al.* (107), working with perfused liver from nonfasted rats, have shown that addition of small amounts of caproate, while enhancing gluconeogenesis, do not alter the cytoplasmic NAD^+/NADH ratio. At the same time, the levels of acetyl CoA are increased. Increasing the amount of caproate does not increase the acetyl CoA levels, but leads to a lowered NAD^+/NADH ratio and a further enhancement of gluconeogenesis. Their data suggest that the primary effect of the fatty acid oxidation is to activate the pyruvate carboxylase maximally while inhibiting the pyruvate dehydrogenase *via* augmented levels of acetyl CoA. Only when the carboxylase has been maximally stimulated does the reduction state of the cytoplasma play a part in gluconeogenesis.

Fatty acid oxidation not only enhances gluconeogenesis but also is actually necessary for glucose formation. It has been shown that acyl esters of (+)-carnitine (unnatural isomers), which reversibly inhibit fatty acid oxidation (46, 47), also inhibit gluconeogenesis (26, 130).

2. Hypoglycin-like compounds and gluconeogenesis. Since hypoglycin also inhibits fatty acid oxidation, its addition would be expected to inhibit glucose formation from alanine. This has been shown to occur in both liver and kidney slices (85). Reports from Bressler's laboratory have shown that 4-pentenoate in-

hibits glucose formation from pyruvate in the intact mouse and from lactate in pigeon liver homogenates (23, 24). 4-Pentenoate does not inhibit the accumulation of glucose in liver homogenates when glycerol is the gluconeogenetic precursor (24). Ruderman *et al.* (99, 100) have shown that 4-pentenoate inhibition of gluconeogenesis from alanine is also exerted in the perfused rat liver. Short-chain fatty acids or palmitylcarnitine added to the pigeon liver homogenates inhibited by 4-pentenoate only partially reversed the inhibition of gluconeogenesis, but addition of adequate amounts of CoA and carnitine completely reversed the inhibition (24). In those systems having lactate as a gluconeogenetic precursor, NADH production is not rate-limiting since the reduced nucleotide is a product of the lactic dehydrogenase reaction. In such a preparation, addition of an NADH-generating system did not affect the inhibition caused by 4-pentenoate (24). Addition of ethanol, which provides a supply of NADH, to liver perfusates inhibited by 4-pentenoate resulted in a partial reversal of the inhibition of gluconeogenesis from alanine (99, 100).

3. Effect on glucose oxidation. Glucose oxidation, which is enhanced by either hypoglycin or 4-pentenoate injection in the intact animal (sections IV and VI), is inhibited in liver homogenates. Since glucose oxidation to lactate was not impaired, while pyruvate oxidation to CO_2 was (24), it would seem that the inhibition of liver glucose oxidation is at the level of acetyl CoA formation from pyruvate. This step requires CoA and is inhibited by excess acetyl CoA. Since 4-pentenoate inhibits acetyl CoA production from fatty acids, the reaction should actually be enhanced. The observation that concomitant addition of CoA and carnitine to the homogenates inhibited by 4-pentenoate reverses the inhibition of pyruvate oxidation to a great extent (24) shows that this inhibition is caused, at least in part, by the lowered levels of available intramitochondrial CoA. This is consonant with the observation that 4-pentenoate addition, under certain circumstances, will also inhibit α-ketoglutarate decarboxylation, as reported by Sherratt (106).

4. Effect on cholesterol synthesis. Hypoglycin inhibits liver acetoacetate production at lower concentrations than those necessary to inhibit palmitate oxidation to CO_2 (118). The toxin also inhibits cholesterol formation from acetate, but not from mevalonate (86). Von Holt *et al.* (125) showed that methylenecyclopropylacetic acid has the same action, and suggested that the latter acts by inhibiting the formation or the further metabolism of β-hydroxy-β-methylglutaryl CoA, the metabolic precursor of both free acetoacetate and mevalonic acid (115). The mechanism of action of hypoglycin at this level is not clear yet, but it does not seem likely that this reaction could be inhibited by a lack of CoA. Indeed, formation of β-hydroxy-β-methyl glutaryl CoA from acetoacetyl CoA and acetyl CoA liberates free CoA. It is possible, however, that under conditions in which a higher demand of acetyl CoA for energy production is created by the inhibition of the oxidation of both pyruvate and fatty acids, there is not enough mitochondrial CoA available for the formation of β-hydroxy-β-methylglutaryl CoA.

5. Effect on leucine oxidation. The mechanism of action of hypoglycin on leucine metabolism (84, 86, 91) is also unclear. It would now seem that an inhibition

of the transamination of leucine to α-ketoisocaproic acid is not involved (91). It could be postulated, however, that lack of available CoA could inhibit the further decarboxylation of α-ketoisocaproic acid to isovaleryl CoA. The latter has not yet been demonstrated.

IX. DISCUSSION

From the evidence reported, it seems that the hypoglycemic effect of hypoglycin and related compounds is a consequence of inhibited fatty acid oxidation, which increases the utilization of glucose in peripheral tissues, perhaps, in order to meet the energy needs of the organism. The hypoglycemia is magnified by a block of gluconeogenesis, secondary to the decrease in fatty acid oxidation.

The inhibition of fatty acid oxidation is caused by the synthesis of certain derivatives of acyl CoA and acylcarnitine. They may be slowly metabolized but they cannot be oxidized, and so they accumulate in tissues, lowering the levels of CoA and carnitine. The effects of CoA and carnitine insufficiency are reversible, as shown by the fact that addition of these cofactors *in vitro* completely prevents the effects of 4-pentenoate. Animals given sublethal doses of hypoglycin or 4-pentenoic acid recover completely, and patients who survive attacks of vomiting sickness show no after-effects. In these cases, it may be postulated that the body restores the supply of CoA and carnitine by the slow liberation of the cofactors from the esters. The latter liberation, by hydrolysis of the unsaturated esters, may, however, be complicated by the possible polymerization of accumulated esters in tissues. This possibility is substantiated by the observations in this laboratory that acrylyl carnitine polymerizes readily and rapidly in water solution. Such a polymer, if formed, would hardly be metabolized by the cell.

Even though the capability of these hypoglycemic compounds to inhibit fatty acid oxidation would make them possible therapeutic agents in hyperglycemic states, their use in this connection would be severely handicapped by their ability to decrease available CoA in the cell, an effect that results in severe toxicity.

The study of the hypoglycin-like compounds has provided an insight into the mechanism by which fatty acid oxidation controls gluconeogenesis. It has been shown that, independent of the relative overall importance of acetyl CoA and NADH in enhancing gluconeogenesis, the reduction of acetyl CoA levels resulting from the inhibition of fatty acid oxidation actually inhibits glucose formation from gluconeogenetic precursors, even in the presence of available NADH.

X. SUMMARY

Hypoglycin and hypoglycin-like compounds cause profound hypoglycemia which may be largely attributed to their effects on gluconeogenesis. The toxicity of these agents is due to their capacity to become activated to acyl CoA derivatives whose further oxidation is impaired. Because the activated compounds serve as substrates for the carnitine acetyltransferase, tissue levels of both free carnitine and coenzyme A are depressed. The depression of these cofactors results in a decrease in long-chain fatty acid oxidation, whose products (acetyl

CoA, NADH, ATP) are necessary for gluconeogenesis. Moreover, the decreased oxidation of long-chain fatty acids may also result in augmented glycolysis due to relief of the inhibitory effects of long-chain fatty acid oxidation on glycolysis. Replacement of coenzyme A and carnitine prevent inhibition of long-chain fatty acid oxidation and glyconeogenesis by 4-pentenoate. Addition of noncarnitine-dependent fatty acids or palmitylcarnitine as substrates also partially prevent the 4-pentenoate depression of gluconeogenesis.

The inability to completely reverse the toxic effects of these compounds *in vitro* and *in vivo* suggests that they may be exerting as yet poorly established effects on oxidative phosphorylation and the electron transport system.

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