Isovaleric and α-Methylbutyric Acidemias Induced by Hypoglycin A: Mechanism of Jamaican Vomiting Sickness

Abstract. *Hypoglycin A, the causative agent of the Jamaican vomiting sickness, produced a marked increase in concentration of isovaleric acid in the plasma of rats, when administered in a single dose. α-Methylbutyric acid, a position isomer, also accumulated. The use of hypoglycin A reproduced some features of human isovaleric acidemia. Accumulation of these branched pentanoic acids may be another factor contributing to the pathogenesis of the Jamaican vomiting sickness.*

We have described isovaleric acidemia, an inborn error of leucine metabolism, in which isovaleric acid accumulates in blood because of the genetic absence of isovaleryl coenzyme A (CoA) dehydrogenase (*1, 2*). Other short-chain fatty acids, including isobutyric, *n*-buytric, and *n*-hexanoic acids, do not accumulate in this disease. The studies on this disorder also indicated that, contrary to previous belief, isovaleryl CoA must be dehydrogenated by a specific enzyme, isovaleryl CoA dehydrogenase, which is distinct from green acyl CoA dehydrogenase (*3*).

Subsequently, in biochemical studies on isovaleryl CoA metabolism, it was shown that hypoglycin A and its metabolites specifically inhibit isovaleryl CoA dehydrogenase both in vitro and in vivo (*4*). Dehydrogenation of α-methylbutyrate was also inhibited. However, the degree of inhibition was much lower (*4*). Hypoglycin A is a plant toxin extracted from unripe ackee fruits; its chemical structure is α-aminomethylenecyclopropylpropionic acid. It has been identified as the cause of the vomiting sickness of Jamaica (*5*) but must be converted to methylenecyclopropylacetic acid (MCPA) in order to exert toxic effects (*6*). The mechanism of this highly fatal disease has in the past been attributed solely to the hypoglycin-induced hypoglycemia, in which glucose concentrations in the blood may be as low as 10 mg per 100 ml (*7*). However, our observations on the inhibition of isovaleryl CoA dehydrogenase by hypoglycin A raised the possibility that an increased serum isovaleric acid level might be another factor contributing to the pathogenesis of this disease (*4*).

The concept that isovaleric acid might be involved in the vomiting sickness was suggested by the fact that the symptoms of this disease are to a certain extent similar to those of isovaleric acidemia, especially the severe vomiting, prostration, lethargy, and coma; in addition, isovaleric acid, like other short-chain fatty acids, is neurotoxic (*8*). Chen *et al.* observed that, when cats, dogs and pigeons were given hypoglycin A, they manifested symptoms similar to those in isovaleric acidemia (depression, vomiting, and ataxia) but without any significant hypoglycemia (*9*). Also some patients with the vomiting sickness fail to respond or recover in spite of massive infusions of glucose (*5*). We were thus anxious to determine to what extent isovaleric acid might accumulate in the serum following hypoglycin A administration, and to determine whether such elevations could occur without severe hypoglycemia.

Male Sprague-Dawley rats, weighing about 150 g, were used. Short-chain fatty acids were analyzed by the gas chromatographic method previously described (*1*). Glucose was measured with a Glucostat (Worthington) by the glucose oxidase method, and plasma amino acid analyses were done by a Technicon amino acid analyzer. Urinary *N*-isovalerylglycine was measured as described previously (*10*). Rats were given a single dose of 10 mg of hypoglycin A per 100 g of body weight by intramuscular injection with light ether anesthesia after 17 hours of fasting. Food was withheld until blood was removed by heart puncture under ether anesthesia. Most of the animals survived for 24 hours under these conditions. Control animals were fasted for the same period.

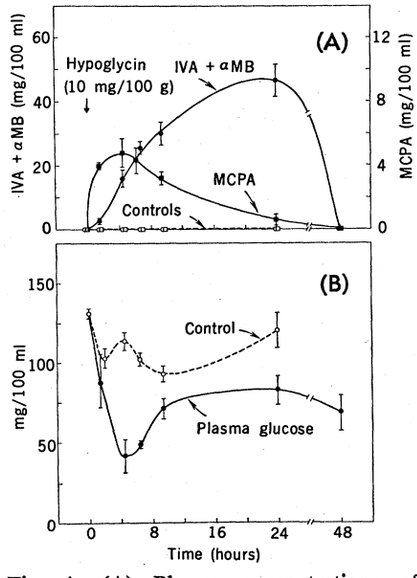

Fig. 1. (A) Plasma concentrations of isovalerate (*IVA*) + α-methylbutyrate (*α-MB*), and MCPA in rats after hypoglycin A administration. Hypoglycin A was injected in a single dose (10 mg/100 g). Each point represents the mean of four experiments ± the standard error. (B) Plasma glucose concentrations after hypoglycin A administration. These determinations were made on the samples from the same group of rats as shown in A.

After the injection of hypoglycin A, plasma concentrations of MCPA increased to a maximum (4.8 ± 0.9 mg/100 ml) within 4.5 hours and then decreased gradually (Fig. 1A). A small amount of MCPA (0.6 ± 0.3 mg/100 ml) was still detected in plasma 24 hours later. Plasma concentrations of branched-chain pentanoic acids (isovaleric and α-methylbutyric acids) increased *300 times* (that is, 15.9 ± 2.4 mg/100 ml, compared to the control value of 0.05 mg per 100 ml), 4.5 hours after hypoglycin injection. Even after the decrease of plasma MCPA, concentrations of branched pentanoic acids rose, reaching a maximum 24 hours after injection; at this time the amounts in plasma were 46.5 ± 4.9 mg per 100 ml, an amount about *1000 times* that of the control and about 1.5 times greater than the highest level we observed in human isovaleric acidemia (*1*). Since these two branched pentanoic acids could not be separated by gas-liquid chromatography (GLC), they were quantitated as methyl esters with the use of a GLC-mass spectrometer (*11*).

Extracts of the 12 samples taken between 6.5 and 24 hours after hypoglycin injection were combined and methylated and then tested by mass spectrometry. Unlike human isovaleric acidemia, where only isovalerate was found (*1*), 55 percent of the branched-chain pentanoic acids were isovalerate and 45 percent were α-methylbutyrate in rats treated with hypoglycin. However, the plasmas taken 2.5 hours after injection contained isovalerate only (*4*).

Blood glucose was the lowest (42 mg per 100 ml) 4.5 hours after hypoglycin injection and then gradually increased so that the concentrations were near normal by 24 hours (Fig. 1B).

Concentrations of leucine and isoleucine in the plasma were essentially unchanged from control values, in

Table 1. Effects of hypoglycin A on leucine and its metabolites, and glutarate in blood and urine. Hypoglycin A was injected in a single dose (10 mg/100 g) after 17 hours of fasting. Control and hypoglycin groups were fasted after injection. The hypoglycin plus sucrose group was given 30 percent sucrose in drinking water. Urine was collected from individual animals housed in metabolic cages for a 24-hour period after injection. Blood was obtained 24 hours after injection. Numbers of the experiments are in parentheses. Results are expressed as mean ± standard error.

Treatment	Time after injection (hours)	Serum concentration (mg/100 ml)		Urinary excretion (mg/day)	
		Isovalerate + α-methylbutyrate	Leucine	*N*-isovalerylglycine	Glutarate
None (4)	24	< 0.05	2.9 ± 0.1	< 0.2	< 0.1
Hypoglycin (4)	24	46.5 ± 4.9	3.2 ± 0.2	8.4 ± 1.8	68.8 ± 5.3
Hypoglycin + sucrose (12)	24	4.5*		11.4 ± 3.4	44.6 ± 17.3

* Mean of eight experiments. Distribution was not Gaussian type; therefore, standard error was not calculated. Only two animals showed increases.

spite of the high elevations of plasma isovalerate and α-methylbutyrate (Table 1). This is consonant with the observation in human isovaleric acidemia where no elevation of plasma leucine was observed. However, some amino acids in plasma, such as lysine, histidine, tyrosine, glutamic acid, ornithine, and citrulline were increased two- to threefold. When L-leucine (60 mg/100 g) was given 30 minutes after the hypoglycin injections, concentrations of plasma isovaleric acid were 20 to 30 mg per 100 ml higher than those in the hypoglycin-fasted group at 4.5 and 6.5 hours after hypoglycin. Most of the animals given L-leucine and hypoglycin died less than 24 hours after injection.

To examine the effect of carbohydrate on hypoglycin toxicity we gave 12 rats 30 percent sucrose in drinking water after hypoglycin administration. Only two of the eight rats in which short-chain fatty acids in the plasma were measured showed increased concentrations of isovaleric and α-methylbutyric acid (16 and 21 mg per 100 ml) 24 hours later. In these two animals blood glucose was low—32 and 60 mg per 100 ml, respectively; and plasma MCPA was also detectable. Six other animals had normal amounts of short-chain fatty acids and blood glucose (122 ± 12 mg per 100 ml) in the plasma; but MCPA could not be detected in the plasma of these animals.

Urine specimens from hypoglycin-treated rats were analyzed for *N*-isovalerylglycine (*10*) and β-hydroxyisovaleric acid (*12*). These unusual leucine metabolites are excreted in the urine of patients with isovaleric acidemia because of an alternate pathway of isovaleryl CoA metabolism (*10, 12*). As in patients with isovaleric acidemia, large amounts of *N*-isovalerylglycine were found in the urine of hypoglycin-treated rats (Table 1). Although quantitative analyses were not done, a large amount of β-hydroxyisovaleric acid was also detected. However, unlike isovaleric acidemia, six additional unknown compounds were found in the urine in large amounts. Five of these were identified as unusual dicarboxylic acids (glutarate, adipate, *cis*-4-decene-1,10-dioate, *cis,cis*-4,7-decadiene-1,10-dioate, and *cis*-4-octene-1,8-dioate) (*13*). The other compound was identified as *N*-MCPA-glycine, the final product of hypoglycin A metabolism (*13*). Excretion of *N*-isovalerylglycine did not decrease with sucrose administration, although as indicated above, concentrations of isovaleric acid in the serum were low in six of eight animals (Table 1).

Our results indicate that isovaleric acidemia may be induced in animals with hypoglycin A and that such animals serve as an experimental model of the human genetic disorder. Although hypoglycin A inhibits biochemical reactions other than dehydrogenation of isovaleryl CoA, it produces the abnormal leucine metabolism seen in isovaleric acidemia. On the basis of earlier studies, we proposed that *N*-isovalerylglycine formation might be the basis of the episodic nature of this disease, since this compound is excreted in the urine in large amounts during remission (when serum isovaleric acid concentrations are not increased), as well as during ketoacidosis (*10*). Just as in the genetic disorder, rats of the hypoglycin-fasted group showed both high concentrations of isovaleric acid in the plasma and excessive amounts of *N*-isovalerylglycine in the urine. In contrast, most of the hypoglycin-sucrose animals did not develop elevated plasma isovaleric acid levels but did excrete increased amounts of the glycine conjugate. This finding is most probably explained by the fact that sucrose administration reduced body protein catabolism resulting in a low endogenous production of leucine, and that the amount of isovaleryl CoA derived from leucine could be effectively detoxified by the glycine conjugating system. Thus, just as in patients with isovaleric acidemia in remission, excessive free isovaleric acid was not produced. However, when the isovaleryl CoA load exceeded the capacity of this conjugating system, isovaleryl CoA was hydrolyzed and liberated into the body fluids as free isovaleric acid.

Our studies also provide for the first time a new insight into the pathogenesis of the clinical features seen in the vomiting sickness of Jamaica. The results obtained with hypoglycin A suggest that isovaleric and α-methylbutyric acids may accumulate in the plasma of patients with this disease. Both of the branched-chain pentanoic acids, like other short-chain fatty acids, are effective central nervous system depressants, and, in preliminary experiments, we have been able to produce coma in 50 percent of rats treated with isovaleric acid in the same dose range as that used to produce coma with *n*-butyric acid (*8*).

Our findings also suggest that elevations of isovaleric acid in serum may occur in the vomiting sickness in the absence of profound hypoglycemia. At present this sickness is diagnosed only indirectly by the presence of extreme symptoms, such as coma and convulsions, together with laboratory evidence of profound hypoglycemia. However, it is quite possible that there may be patients who have an elevation of serum isovaleric acid but who have not yet developed profound hypoglycemia. Furthermore, it is possible that there may be people among the ackee-eating population of Jamaica who have neither hypoglycemia nor an accumulation of serum isovaleric acid and who may not be clinically ill, but who excrete in their urines unusual metabolites such as *N*-isovalerylglycine and medium-chain dicarboxylic acids.

Since the discovery of hypoglycin A by Hassal and Reyle (*14*), the incidence of the vomiting sickness has greatly declined. However, ripe ackee fruit is still a common food in Jamaica and

these fruits apparently still contain small amounts of hypoglycin (*14*). As is shown in our studies, hypoglycin stays in the body as long as 24 hours. It also interacts with isovaleryl CoA dehydrogenase (*4*), glutaryl CoA dehydrogenase (*13*), and CoA and (−)-carnitine (*5*). Therefore, one has to raise the likelihood that small amounts of hypoglycin A ingested over a long period of time may cause cellular injury and possibly liver damage. A number of chronic liver diseases of unknown etiology and of an endemic nature (such as veno-occlusive disease) occur in Jamaica. From our findings, it would seem to us important to explore the possible role of hypoglycin and deranged isovaleric acid metabolism as factors in the pathogenesis of these forms of liver disease.

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Circadian Rhythm in Uptake of Tritiated Thymidine by Kidney, Parotid, and Duodenum of Isoproterenol-Treated Mice

Abstract. *The total uptake of [^{3}H]thymidine by the mouse parotid gland, kidney, and duodenum exhibits a circadian rhythm. A single injection of isoproterenol changes the phasing and amplitude of these rhythms. Depending on the organ, there are certain points in the circadian time structure when isoproterenol stimulates or inhibits the uptake of thymidine; at other time points there is no difference between the responses to isoproterenol and to saline.*

The stimulation of increased DNA biosynthesis in the parotid gland and kidney of mice by a single intraperitoneal injection of isoproterenol (IPR) is of current research importance (*1–3*). Our study demonstrates that the total uptake of [^{3}H]thymidine (TdR) by parotid gland, kidney, and duodenum, as well as the effect that IPR has on this uptake, is dependent on the mouse circadian time structure.

Seventy-two inbred male BDF_1 mice (18 to 24 g) were maintained under rigidly standardized conditions consisting of freely available food and water and a controlled light-dark cycle (light from 0600 to 1800 hours C.S.T.) for 2 weeks before the initiation of the experiment. Beginning at 0900 hours and continuing at 4-hour intervals (1300, 1700, 2100, 0100, and 0500 hours) two subgroups of six mice each were injected intraperitoneally at each time point with either 0.75 ml of saline or an equal volume of distilled water containing 7.5 mg of freshly dissolved isoproterenol hydrochloride. All mice were killed exactly 28 hours after injection of either saline or IPR. Thirty minutes before being killed by cervical dislocation each mouse received a subcutaneous injection of 10 μc of [^{3}H]TdR (22 c/mmole) (*4*). Samples of parotid gland, kidney, and duodenum were removed and fixed in 10 percent buffered formalin. Subsequently each piece was transferred to 70 percent ethyl alcohol for several days, dried overnight in a desiccator, weighed to the nearest 0.001 mg, and digested with 2 ml of hyamine hydroxide at 55°C. The radioactivity was measured in a Nuclear-Chicago Mark I liquid scintillation spectrometer. In one experiment, in addition to the protocol above, pieces of kidney were weighed, homogenized in 1 percent sodium lauryl sarcosinate, and centrifuged at 1000*g*. The supernatant was precipitated with 10 percent trichloroacetic acid (5 percent final concentration) and centrifuged. The precipitate was dissolved in 0.1*N* KOH, spotted on filter paper disks (*5*), and counted.

The uptake of TdR into the parotid gland is increased by IPR (Fig. 1) when compared to the saline controls ($P <$ .001 at all points except 0100 hours where it was $<$ .02). There is a circadian rhythm in the uptake of TdR into the parotid gland in both the saline- and IPR-treated animals. In the IPR-treated animals there is a 92 percent variation between the lowest (at 0100 hours) and highest (at 1300 hours) means recorded. The difference between these means is significant ($P < .05$). The highest (at 2100 hours) and lowest (at 1700 hours) means for the controls differ by 215 percent ($P < .05$). The occurrence of the peak in TdR uptake advanced from 2100 hours in the controls to 1300 hours in the IPR group.

Unlike the parotid gland which responds maximally 28 hours after injection of IPR, the kidney does not re-

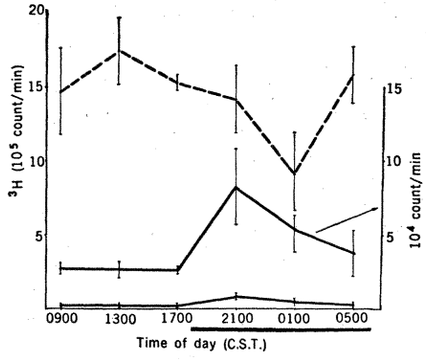

Fig. 1. Circadian pattern in the uptake of [^{3}H]thymidine (TdR) into the mouse parotid gland (solid line) and the effect of a single intraperitoneal injection of isoproterenol (dashed line) administered 28 hours previously. For example, mice injected at 0900 hours were killed at 1300 hours, or 28 hours later. On the abscissa is the time of day with darkness from 1800 to 0600 hours C.S.T. The time of killing is plotted against the uptake of [^{3}H]-TdR (10^5 count/min per gram of dry weight) for the top dashed line and the bottom solid line. (The middle solid line has an expanded scale for the saline-treated animals, the unit being 10^4 count/min per gram.) All animals received TdR 30 minutes prior to killing. Each point is the mean for six animals ± the standard error of the mean.