BIOCHEMISTRY OF HYPOGLYCIN

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1. Origin

Hypoglycin A, β-(methylenecyclopropyl)alanine, is a non-protein amino acid first isolated [1] from the unripe fruit of the ackee, *Blighia sapida*, a tree much cultivated in Jamaica where the fleshy aril constitutes an important item of diet, particularly of the lower income groups. The unripe ackee aril may contain around 1 mg of hypoglycin per g wet weight and the seeds two to three times as much, but as the fruit ripens the content of the aril decreases to less than 0.01% of the amino acid [2]. Hypoglycin is found in the seeds both as the free amino acid and the γ-glutamyl dipeptide conjugate, hypoglycin B [3]. It is also found in ripe sycamore fruits (*Acer pseudoplatanus*) [4] and as the major component of the free amino acid pool in the seeds of *Billia hippocastanum* of Costa Rica [5].

Hypoglycin possesses both a cyclopropane ring and a methylene group, neither of which are found in any protein amino acids. Fruits of species related to ackee and some others contain several varieties of non-protein amino acids with cyclopropane rings, e.g. α-(methylenecyclopropyl)glycine from *Litchi chinensis* and *Acer* genus as well as *B. sapida* [3, 6, 7], β-(methylenecyclopropyl)-β-methylalanine from *Aesculus californica* [8] and *cis*- and *trans*-α-(carboxycyclopropyl)glycine from the seeds of *A. parviflora* and *A. pavia* and *B. sapida* [9]. Methylene groups in non-protein amino acids are also quite widespread in the plant world [10].

Little is known of the pathway of hypoglycin synthesis. [U-^{14}C]Acetate and [methyl-^{14}C]methionine have been found to act as precursors in sycamore and ackee fruits [7]. It is possible that a linear C_6 skeleton is first formed and the methyl C of methionine becomes the third C of the cyclopropane ring.

2. Toxicity

Hypoglycin was first isolated and gained prominence as the toxic, and probably causative, agent involved in Jamaican 'vomiting sickness' (reviewed in [11, 12]). This condition is characterised by a sudden onset of vomiting several hours after a meal containing unripe ackees, and development of a profound hypoglycaemia which may lead eventually to convulsions, coma and death. Malnourished individuals and children appear to be the most susceptible victims. Fatalities are still occasionally reported, though, with improved educational and nutritional standards, the incidence has declined in the years since the last major outbreak in Jamaica in 1951. Treatment with thiamine, riboflavin or B vitamin complex is reportedly beneficial – in fact ackees have been described as an antimetabolite with respect to riboflavin [13]. Intravenous glucose relieves the hypoglycaemia. In the cases that the author has seen reported both ackees and rum have been taken. It is an interesting question (section 6.2.) whether alcohol may aggravate the effects of hypoglycin. Although many of the clinical symptoms of ackee poisoning can be repro-

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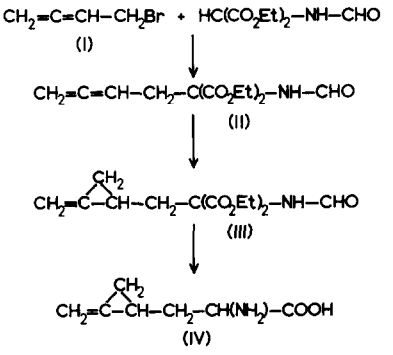

Fig. 1.

duced in experimental animals – rats, monkeys, guinea pigs, cats, dogs, rabbits, mice, there are no reported tests of hypoglycin action in man.

3. Chemical synthesis

Complete chemical synthesis of hypoglycin was first reported by Carbon et al. [14] in a six stage procedure starting from 2-bromopropene, forming the cyclopropane ring by treatment of this with ethyl diazoacetate. Subsequently Black and Landor [15, 16] introduced a three stage synthesis (fig. 1) starting from 1-bromobuta-2,3-diene (I) and diethyl 1-formylaminomalonate which condense in the presence of sodium hydride to give diethyl buta-2,3-dienylformylaminomalonate (II). Treatment of this with di-iodomethane and a Zn/Cu couple resulted in addition of methylene to the non-terminal double bond to give the crude cyclopropane (III), which was hydrolysed in alkali, then decarboxylated in acid to yield hypoglycin (IV), whose configuration was established to be that of the natural product. Virtually all work on the metabolic actions of hypoglycin has been carried out with material from natural sources.

Von Holt [17] prepared [1-^{14}C]hypoglycin by treating methylenecyclopropane-acetaldehyde with [^{14}C]cyanide and ammonium carbonate to form the corresponding hydantoin which was subsequently saponified to the amino acid.

4. Purification

One of the inevitable problems about use of the natural product is its purity. The usual procedure for extraction of hypoglycin is by boiling ground seeds or ackee arils with water or aqueous ethanol followed by isolation of the neutral amino acids on ion exchange resins [1, 2]. On concentration, hypoglycin as the most abundant and a poorly soluble acid, crystallises out and can be purified by recrystallisation. It seems clear however that hypoglycin prepared by these means is far from pure and there may be contamination by other amino acids. These can be recognised by paper chromatography or, more accurately, by the usual Moore and Stein type amino acid analyser. Samples analysed in this manner have been shown to be contaminated by valine, isoleucine, phenylalanine, tyrosine, methionine and other unidentified products [18]. However one of the most likely contaminants of hypoglycin is recognised to be leucine which is particularly difficult to resolve – either to recognise in the presence of hypoglycin or to separate. Patrick [19] reported the impossibility of resolving leucine and hypoglycin on paper in 17 different solvent systems tested, and with the standard citrate buffers on ion exchange columns hypoglycin and leucine appear to run with identical mobilities under conditions in which valine, isoleucine, norleucine and leucine are all resolvable [18, 20, 21]. It may be that with lithium citrate buffers, which are claimed to improve resolution of certain amino acid overlaps [22], or with an older elution procedure using HCl [23], a separation of leucine and hypoglycin is possible. Although there are claims for procedures by which leucine and hypoglycin can be separated and separately identified [24, 25], the surest way at present of preparing hypoglycin free of leucine appears to be to make it from hypoglycin B, which can be unequivocally separated from leucine, when the hypoglycin liberated on hydrolysis is readily separable from the glutamate [20, 26]. However, only a limited amount of hypoglycin has been prepared by this means to date.

The methylene group of hypoglycin is, of course, readily reduced, e.g. by sodium borohydride, giving a variety of products. It is also attacked by halogens. Patrick [19] found that on iodination hypoglycin was converted to a derivative now readily separable from leucine by paper chromatography, thus allowing him to estimate the low extent of contamination of his hypoglycin preparation by leucine. More recently, Fincham [21] has developed a procedure using the auto-analyser for measuring hypoglycin and leucine in

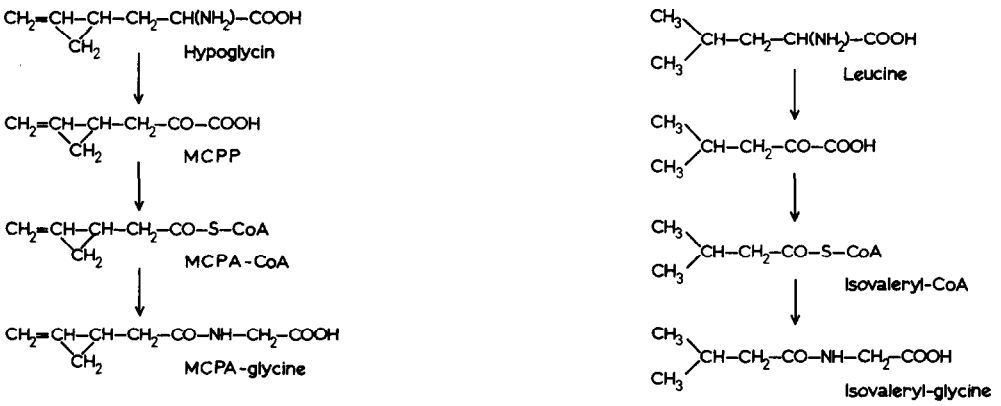

Fig. 2.

the presence of each other. This involves running two samples – the first estimates leucine plus hypoglycin. The second sample is first treated under acid conditions with bromine after which analysis of the remaining leucine is made, with the difference being hypoglycin. Some hypoglycin samples so analysed have contained up to 20% leucine [18]. Unequivocal detection and estimation of hypoglycin in extracts of ackee has recently become a problem of commercial importance in Jamaica since a lucrative export trade of canned ackees to Europe and North America is threatened by an American FDA requirement that the hypoglycin content of the canned product be stated.

5. Degradation

Hypoglycin, as its name implies, produces hypoglycaemia in responsive species, but there is usually a delay before the blood sugar level begins to fall. This suggests that it may be a metabolite of hypoglycin that exerts the major toxic actions.

When injected into rats hypoglycin is rapidly distributed to the various organs and it is not obviously concentrated in any one [27], a pattern such as one might expect for a branched chain amino acid. There is some loss of hypoglycin in the urine but most of it is metabolised. Whether the pattern of distribution is the same when ingested as opposed to being injected has not been investigated.

Degradation of hypoglycin in rats, and presumably man, follows the pattern of catabolism of the branched chain amino acids. It is first deaminated by transamination to yield methylenecyclopropylpyruvate (MCPP) which then undergoes oxidative decarboxylation to form methylenecyclopropylacetyl-CoA (fig. 2). Rats injected with [1-^{14}C]hypoglycin excrete 60% of the label within 2 hours as $^{14}Co_2$ [17]. Presumably hydrolysis of the ester is possible and Tanaka et al. [28] have shown that, after a single dose of hypoglycin, plasma concentrations of methylenecyclopropylacetate (MCPA) rise to a maximum by 4–5 hr and then slowly decline. It is not known what proportion of hypoglycin degradation normally occurs in the liver – skin for example appears to be able to metabolise it [29] and extrahepatic tissues are a major site of branced chair amino acid catabolism. Whether MCPA can be degraded further has not been shown. A major portion of it is excreted as the glycine conjugate MCPA–glycine [28].

6. Metabolic actions

The toxic effects of hypoglycin can result from actions either of the amino acid itself or of any of its degradation products. Early work (reviewed in [11] and [30]) suggested that hypoglycin interferes with utilisation of long chain fatty acids. Their oxidation is inhibited and the blood concentration rises, as does the fat content of the liver. A decrease in fatty acid metabolism will lead to increased utilisation of glucose. As a result of a disturbance of hepatic mitochondrial function [31, 32] gluconeogenesis may be decreased. There is therefore rapid depletion of glycogen reserves and hypoglycaemia follows. The marked

effects of hypoglycin on the blood sugar level and on fat metabolism thus strongly suggest an inhibition of β-oxidation of fatty acids by MCPP or MCPA as an important site of action. More recently it has become evident that the effects of hypoglycin may be more diverse.

6.1. *Effects of hypoglycin itself*

As an analogue of leucine hypoglycin might be expected to compete with leucine in various reactions. Fowden has found [33] that the lower homologue of hypoglycin, methylenecyclopropylglycine, inhibits growth of seedlings through antagonising utilisation of leucine. Anderson and Fowden [26] also found that hypoglycin would interact with leucyl-tRNA synthetase from *A. hippocastanum* as judged by pyrophosphate exchange. The K_m of the enzyme towards hypoglycin was roughly 80-fold higher and the V_{max} about half that obtainable with leucine. Moreover hypoglycin in 100-fold excess depressed the activity observable with leucine alone. Hypoglycin did not affect activity of phenylalanyl-tRNA synthetase from the same source. As Anderson and Fowden concluded, a study of the substrate specificity of mammalian leucyl-tRNA synthetase with respect to hypoglycin would be of interest. This has not been done. However the present author has looked at the influence of hypoglycin on the incorporation of leucine into protein by ribosomes isolated from rat liver. The results indicate how important it is to know the purity of the hypoglycin samples employed in study of its actions, for all samples of hypoglycin tested except one showed marked inhibition of leucine incorporation and some also inhibited incorporation of phenylalanine. The only sample that at first appeared to have no inhibitory influence on incorporation of either amino acid when present even at concentration ratio of 100 : 1 was one kindly provided by Professor L. Fowden and which had been prepared by hydrolysis of hypoglycin B (section 4). The inhibitory influence of the other samples thus seems likely to be a result of impurities – a point confirmed by amino acid analysis. The above experiments however were carried out under conditions in which the concentration of activating enzymes were not rate limiting in amino acid incorporation. Further results suggest that if the quantity of cell sap fraction is reduced such that it is limiting, then an inhibitory influence of hypoglycin, as of other amino acids, does become apparent. The molar ratios of hypoglycin to leucine required of course are far in excess of anything likely to be encountered in the cell and the effect is unlikely to be of physiological significance.

6.2. *Inhibition of fatty acid oxidation*

Von Holt et al. [31] proposed that a major action of hypoglycin is to interfere with oxidation of fatty acids. The circumstantial evidence for this proposal was 1) the rise in the serum free fatty acids and fatty infiltration of the liver after hypoglycin administration, 2) hypoglycin increased glucose oxidation in the intact animal, 3) liver glycogen was depleted after hypoglycin treatment though its synthesis or breakdown appeared unaffected, 4) riboflavin administration prevented the effects of hypoglycin.

More direct evidence for the site of action of hypoglycin and its derivatives is that production of CO_2 by hypoglycin-poisoned rats is diminished as a result of reduction in fatty acid oxidation [31]. MCPA inhibits oxidation of $C_{12}-C_{18}$ fatty acids by isolated mitochondria, though not that of shorter chain acids. Acetoacetate production from palmitate is reduced, as also is acetate incorporation into cholesterol. It seems possible that MCPA as the CoA derivative inhibits an enzyme within the fatty acid oxidation spiral, most probably a flavin-nucleotide-dependent-acyl-CoA dehydrogenase. The capacity of riboflavin to prevent development of hypoglycin poisoning would be consistent with this, though the precise manner in which it would relieve inhibition of an enzyme has yet to be established.

Further understanding of possible ways in which MCPA might influence fatty acid oxidation has been gained by the use of 4-pentenoate. This short chain acid is the simplest of a series of unsaturated hypoglycaemic fatty acids related to hypoglycin, and likewise inhibits fatty acid oxidation (discussed in refs. [34] and [35]). It seems clear that formation of pentenoyl-CoA is necessary for inhibition, but Bressler et al. [11] did not find pentenoyl-CoA to be an inhibitor of acyl-CoA dehydrogenase. A possible mechanism by which MCPA (and pentenoate) might inhibit fatty acid oxidation is in effect by a lethal synthesis – formation of MCPA-CoA which is only slowly, if at

all, further metabolised and depletes available CoA resources. Alternatively an interaction with acyl-CoA carnitine transferase would lead to accumulation of the MCPA-carnitine ester, which in turn would have the effect of depleting available carnitine. This action might interfere with entry of long chain, though not of the shorter chain, fatty acids into the mitochondrion and so explain why oxidation of longer chain acids is more susceptible to inhibition than is that of the shorter (though an alternative explanation of this phenomenon is that the shorter chain fatty acids compete effectively with the inhibitor for the acyl-CoA ligase [35]).

Bressler and coworkers [36] observed that addition of CoA and carnitine together would overcome inhibition of fatty acid oxidation produced in pigeon liver homogenates and mitochondria by pentenoate, and under these conditions there was accumulation of pentenoyl-carnitine and acrylyl-carnitine. Similar results have not been published for MCPA. Sherratt [29, 34, 35] has questioned the validity of this 'sequestration' hypothesis since a number of non-hypoglycaemic compounds can also affect intramitochondrial CoA concentrations. He has been unable to overcome hypoglycaemic effects of either hypoglycin or pentenoic acid in mice with carnitine, and has stressed the point that hypoglycaemia may result from or be aggravated by the hypothermia induced by both acids [34, 37]. He finds [38] that 2,4-pentadienoyl-CoA formed from 4-pentenoate is a strong inhibitor of β-ketoacyl-CoA thiolase. Pentenoic acid and a number of other fatty acids with ring-containing structures have been studied as possible oral hypoglycaemic agents [30], but they are all, not surprisingly, too toxic for clinical use.

Inhibition of utilisation of fatty acids is to be expected to produce in vivo a greater demand for glucose. This demand can in part be supplied by gluconeogenesis. An inhibition by hypoglycin of this process [39] would aggravate hypoglycaemic effects, particularly in fasting. As with fatty acid metabolism MCPP and MCPA are likely to be toxic agents that could interfere with glucose production and this has been demonstrated recently with kidney cortex slices [40]. Pentenoate also reduces glucose formation by kidney slices [41] and markedly depresses gluconeogenesis from alanine and pyruvate in the perfused rat liver [42, 43]. This inhibition seems, in the liver, to be the consequence of diminution of the cytoplasmic NADH concentration resulting from decreased fatty acid oxidation, which leads to a fall in triose phosphate formation [42, 43]. A marked decrease in acetyl-CoA concentrations, with consequent diminished activation of pyruvate carboxylase, also occurs. Addition of ethanol restored glucose formation by raising the NADH level of the cytoplasm [42]. It would be interesting to know how pentenoate affects gluconeogenesis from lactate which itself generates NADH. Moreover ethanol in the malnourished inhibits its oxidation of lactate and can promote hypoglycaemia [44]. It is therefore an interesting question whether alcohol, as rum or otherwise, taken with unripe ackees may aggravate or ameliorate the ensuing hypoglycin poisoning.

Despite inhibiting hepatic ketone body production [31], intramuscular administration of hypoglycin appears to raise blood levels of ketones and particularly of acetoacetate [45]. The most likely explanation of this phenomenon is that hypoglycin, like leucine, is degraded extrahepatically as well as in the liver and that MCPA so formed can interfere with acetoacetate utilisation, possibly through sequestration of CoA.

6.3. *Inhibition of leucine catabolism*

In addition to their effects on fatty acid metabolism the catabolic products of hypoglycin have more recently been implicated as inhibitors of the normal metabolism of leucine and lysine. Leucine catabolism involves first deamination to form α-ketoisocaproate followed by oxidative decarboxylation to yield isovaleryl-CoA (fig. 2). This then undergoes α,β-dehydrogenation to form β-methylcrotonyl-CoA. There is an inborn error of metabolism, isovaleric acidaemia, in which isovaleric acid accumulates in blood in large amounts. This disorder (which is analogous to another inborn error of leucine catabolism, the maple syrup urine disorder) is thought to arise through a deficiency of the specific flavin-containing dehydrogenase, namely isovaleryl-CoA dehydrogenase, required to dehydrogenate isovaleryl-CoA [46]. Tanaka et al. [47] find that MCPP inhibits oxidation of leucine by liver slices. They have proposed that inhibition of isovaleryl-CoA dehydrogenase, presumably by the derivative MCPA-CoA acting as a competitive inhibitor, is a major site of action of hypoglycin.

Administration of hypoglycin causes a very marked

rise in blood levels of isovalerate (and α-methylbutyrate – a structural isomer) and also a marked increase in the urinary excretion of *N*-isovalerylglycine [48, 49], a situation similar to that encountered in hereditary isovaleric acidaemia characterised by lack of hepatic isovaleryl-CoA dehydrogenase. By comparison with leucine, hypoglycin has little effect on oxidation of valine and isoleucine [47, 50]. Isovaleric acid, like other short chain fatty acids, is neurotoxic [51, 52] and can produce symptoms of depression, vomiting and ataxia [28]. Tanaka and colleagues [28] suggest that it is isovaleric acid poisoning rather than interference with β-oxidation that is responsible for many of the symptoms of hypoglycin poisoning.

Administration of hypoglycin to rats is also found to promote excretion of glutarate, adipate and a number of longer chain dicarboxylic acids [47]. Glutarate is an intermediate of lysine breakdown and is normally metabolised by glutaryl-CoA dehydrogenase which is presumably subject to inhibition like isovaleryl-CoA dehydrogenase by MCPA-CoA. Glutarate is nephrotoxic [53, 54] and may be the cause of histological changes observed in kidneys of patients suffering from vomiting sickness. The origin of the adipate and other acids is less clear.

7. Teratogenic effects

The capacity of hypoglycin to produce nephro- and particularly neurotoxic agents may well be part explanation of the teratogenic effects of the amino acid noted by Persaud [55]. Hypoglycin given to pregnant rats from the first to the sixth day induced a significant incidence of congenital anomalies and foetal resorption, an effect that was markedly aggravated when leucine was given simultaneously. In fact administration of leucine alone was teratogenic. Whilst a hypoglycaemia produced by hypoglycin in the mother may well have an effect on the foetal metabolism, it seems unlikely that hypoglycin would be affecting foetal fat metabolism directly, if only because foetal tissue is characterised by a high glycolytic dependence. However, the presence of an abnormally high plasma concentration of either MCPP or MCPA or of isovaleric acid might well be expected to have a deleterious influence on embryonic development. The simultaneous administration of carnitine with the hypoglycin did not diminish foetal malformation. Administration of riboflavin however did. On the other hand riboflavin had no effect on leucine induced abnormalities. It is interesting to note that the mouse by comparison with the rat is less sensitive to hypoglycin both as regards hypoglycaemia and teratogenic effects [11, 56]. It would be of value to know whether there are significant differences between the two species in the circulating levels of isovaleric acid in response to the various agents.

8. Conclusions

Clearly the sites of action of hypoglycin are multiple and it is the metabolites that exert the major toxic actions. However the precise details of how fatty acid oxidation is inhibited remain to be worked out. There is also scope for explanation of species differences in sensitivity and response. Maybe further work on hypoglycin will lead to clearer understanding of the reason for the remarkably similar chromatographic properties of leucine and hypoglycin, and to more detailed knowledge of the enzymology of β-oxidation and intermediary metabolism in various species.

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