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The Effects of Hypoglycin on Glucose Metabolism in the Rat

A KINETIC STUDY *IN VIVO* WITH [U-^{14}C,2-^{3}H]GLUCOSE

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1. The kinetics of glucose metabolism were evaluated in rats deprived of food 15–21 h after the administration of hypoglycaemic doses of hypoglycin (100 mg/kg body wt.) by following changes in the specific radioactivities of ^{14}C and ^{3}H in blood glucose after an intravenous dose of [U-^{14}C,2-^{3}H]glucose [Katz, Rostami & Dunn (1974) *Biochem. J.* **142**, 161–170]. 2. During this time, recycling of glucose through the Cori cycle was virtually abolished, the rate of irreversible disposal of glucose and its total body mass were both decreased by about 70%, whereas there was little effect on the mean transit time for glucose. 3. It was concluded that hypoglycaemia is due to inhibition of gluconeogenesis.

Hypoglycaemia is a major feature in several species, including man, of poisoning by hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid), the toxic principle of the Jamaican ackee fruit *Blighia sapida* (Hill, 1952; Feng & Patrick, 1958; Sherratt, 1969). Hypoglycin inhibits gluconeogenesis from pyruvate, lactate and alanine in rat liver and kidney slices (Patrick, 1966; Kean & Rainford, 1973) and in isolated rat hepatocytes (Billington, 1976). A plausible hypothesis, therefore, is that hypoglycaemia is due to inhibition of gluconeogenesis, which is a consequence of the known impairment of fatty acid oxidation by metabolites of hypoglycin (von Holt *et al.*, 1966; Senior, 1967; Bressler *et al.*, 1969; Sherratt *et al.*, 1971, 1975; Osmundsen & Sherratt, 1975; Sherratt & Osmundsen, 1976).

It has never been directly demonstrated that hypoglycin inhibits gluconeogenesis in the intact animal (see Sherratt & Osmundsen, 1976). We have used the methods of Katz *et al.* (1974*a*) to determine the effects of hypoglycin on the kinetics of glucose metabolism in rats deprived of food. This uses the rate of fall of the specific radioactivities of ^{3}H and of ^{14}C in blood glucose after an intravenous dose of [U-^{14}C,2-^{3}H]glucose to determine the rate of irreversible disposal of glucose, the percentage of glucose carbon recycled back to glucose and the total body mass of glucose. A preliminary account of this work has already appeared (Billington *et al.*, 1976*b*).

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Materials and Methods

Materials

[1-^{14}C]Pyruvate (11.7 μCi/mmol), [2-^{3}H]glucose (500 μCi/mmol) and [U-^{14}C]glucose (2.8 μCi/mmol) were from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Kits for assaying glucose were from Boehringer Corp. (London), London W5 2TZ, U.K. Dowex ion-exchange resins, toluene, Triton X-100 and polyvinylpyrrolidone were from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Hopkin and Williams, Chadwell Heath, Essex RM1 4HA, U.K. Sagatal (pentobarbitone) was from May and Baker, Dagenham, Essex RM10 7XS, U.K., and heparin was from the Boots Pure Drug Co., Nottingham NG2 3AA, U.K.

Ackee seeds were obtained through the courtesy of the Scientific Research Council, Kingston, Jamaica, West Indies, and the Tropical Products Institute, London W.C.1, U.K. Hypoglycin was isolated from seeds essentially as described by Kean (1974). Our preparation contained 15% of contaminating leucine and isoleucine detected by the method of Fincham (1975). It was shown in control experiments with pure hypoglycin, prepared by the

hydrolysis of hypoglycin B as described by Fowden (1975), that these impurities did not qualitatively alter the effects of hypoglycin in rats.

Methods

Cannulation of the rats. The right jugular veins of male albino rats (400–450 g) from a local inbred Wistar strain were cannulated under Sagatal anaesthesia essentially as described by Upton (1974) and Popovic & Popovic (1960), and the cannula (polythene tubing, internal diameter no. 20 gauge) was exteriorized at the back of the head between the ears. The cannula was filled with sterile 0.14M-NaCl containing 10 units of heparin/ml and then washed out daily with this solution. The animals were kept in separate cages and used 4 days after the operation.

Experimental design. When rats are given hypoglycin (100 mg/kg body wt.) their blood glucose concentrations fall to about 2–3 mM after 12–15 h and remain approximately constant for another 6 h (see Fig. 1) before they start to return to normal (5–6 mM) (Billington *et al.*, 1976*a*; Sherratt & Osmundsen, 1976). Rats were deprived of food for 9 h before they were injected intraperitoneally with either hypoglycin [100 mg/kg body wt. as a 2% (w/v) solution in 0.14M-NaCl] or with an equivalent volume of 0.14M-NaCl; they were then kept without food overnight for a further 15 h with an ambient temperature of 22°C, before administration of intravenous [2-^{3}H]glucose (2.5×10^7 d.p.m.) and [U-^{14}C]glucose (6×10^6 d.p.m.) through a smaller-diameter polythene tube fed down the indwelling cannula. Blood samples (0.3 ml) were removed through the indwelling cannula at increasing time intervals (Fig. 2) after administration of the labelled glucose; during the whole sampling period the animals were lightly anaesthetized with Sagatal and both the control and hypoglycin-treated rats were in an approximately steady state with respect to blood glucose concentrations during this time (Fig. 1). The blood volume was maintained by replacing the samples with an equal volume of 0.14M-NaCl containing 4 mg of polyvinylpyrrolidone/ml. Body temperatures were kept constant at 37°C by keeping the animals on a thermostatically controlled operating table and their rectal temperatures were monitored with a thermistor probe and a digital thermometer. Adequate respiration was maintained during sampling as judged by lack of cyanosis.

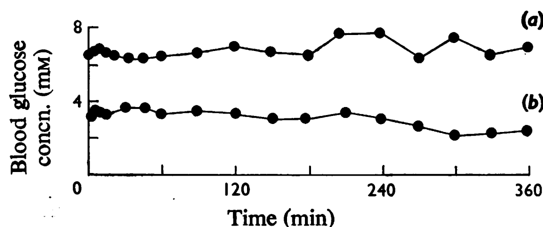

Fig. 1. *Blood glucose concentrations in representative control (a) and hypoglycin-treated (b) rats during the period when blood samples were taken*

Hypoglycin or 0.14M-NaCl was given 15 h before the commencement of sampling. Other details are given in the text.

Determination of the specific activities of blood glucose. Blood samples were treated essentially as described by Katz *et al.* (1974*b*). Blood (0.3 ml) was deproteinized by adding 2.7 ml of 0.33 M-$HClO_4$ and centrifugation. Glucose was determined in 50 μl samples of the supernatant by using glucose oxidase (EC 1.1.3.4) kits. The remainder of the supernatant was adjusted to pH 7.0–7.5 with 10M-KOH. Precipitated $KClO_4$ was removed by centrifugation and 2.0 ml of the supernatant was freeze-dried to remove ^3H_2O. The residue was dissolved in 0.3 ml of water and 0.1 ml was passed through two ion-exchange columns in series; these were Dowex-50 (H^+ form, 20–50 U.S. mesh) and then Dowex-1 (acetate form, 20–50 U.S. mesh), each in disposable 1 ml plastic syringes. The columns were eluted with water, the first 20 drops were discarded and the next 100 (about 3 ml) collected in scintillation vials and mixed with 8 ml of scintillation fluid [toluene/Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.2 g/l)], to form a gel. It was assumed that glucose was the only radioactively labelled compound in the eluate (see Katz *et al.*, 1974*b*). Heath *et al.* (1977*b*) reported after this work was completed that 10% of the radioactivity may be contributed by other compounds after 60 min. In control experiments the percentage recovery in the eluates of [U-^{14}C]glucose added to the columns was greater than 97%, whereas [1-^{14}C]pyruvate was not eluted in the first 120 drops. The radioactivities of ^{14}C and of ^{3}H were measured by twin-channel counting with an Intertechnique ABAC SL40 scintillation spectrometer (with an efficiency of 20% of ^{14}C and of 25% for ^{3}H) programmed to calculate the d.p.m. for each isotope after appropriate quench corrections.

Parameters of glucose metabolism. From the rates of change of the specific radioactivity of ^{3}H in glucose in the blood of an animal deprived of food (in a steady state with respect to glucose metabolism), given an intravenous dose of [2-^{3}H]glucose, it is possible to estimate several parameters of glucose metabolism as defined by Katz *et al.* (1974*a*).

1. The rate of irreversible disposal of glucose, which is essentially equal to the rate of glucose phosphorylation by hexokinase (EC 2.7.1.1) and glucokinase (EC2 .7.1.2), or, since steady-state conditions are maintained, to the replacement of glucose by the hydrolysis of glucose 6-phosphate by glucose 6-phosphatase (EC 2.1.3.9).

2. The mass of the sampling pool, which is the mass of glucose in the circulation and those pools in rapid equilibrium with it.

3. The total body mass of glucose.

4. The minimum transit time of glucose, which is the transit time if the catabolism occurs solely in the sampling pool.

5. The maximum transit time, which is the transit time if the catabolism of glucose occurs in a region removed as far as possible from the sampling pool; 'far' referring to the path requiring the longest transit time.

It is also possible to estimate the extent of reincorporation of ^{14}C-labelled metabolites of glucose into glucose. The major ^{3}H-labelled metabolite of [2-^{3}H]glucose is $^{3}H_2O$, so loss of ^{3}H from glucose will give the 'true' rate of irreversible disposal of glucose. However, metabolism of [U-^{14}C]glucose gives several labelled products and its 'apparent' rate of irreversible disposal does not allow for reincorporation of ^{14}C into glucose by gluconeogenesis. The percentage of ^{14}C derived from [U-^{14}C]glucose recycling back to glucose may be defined (Katz *et al.*, 1974*a*, 1976) as:

$$\frac{R_t - R_a}{R_t} \cdot 100$$

or as

$$\frac{A_a - A_t}{A_t} \cdot 100$$

where R_t is the 'true' rate of irreversible disposal of glucose and R_a is the 'apparent' rate. R_t is inversely proportional to A_t, the area under the curve for the change in the specific radioactivity of ^{3}H in blood glucose against time, and R_a is inversely proportional to A_a, the area under the curve for the change in specific radioactivity of ^{14}C in blood glucose against time.

Calculation of the parameters of glucose metabolism. The evaluation of various kinetic parameters *in vivo* has been discussed by Shipley & Clark (1972), Heath & Barton (1973) and Katz *et al.* (1974*a*). The ^{14}C and ^{3}H specific radioactivities of glucose were normalized to an injected dose of 10^6 d.p.m./kg body wt. The resultant data were fitted to a biexponential function using a non-linear regression program (Atkins, 1971), and the computed values for the exponents and coefficients used to calculate the various kinetic parameters of glucose metabolism (H. Osmundsen, unpublished work). Starting estimates for the coefficients and exponents of the two exponential terms were obtained graphically from semi-logarithmic plots of specific radioactivity against time (see Fig. 2). Finally, the computed function and the experimental data were printed by a data-plotter for checking visually.

Although there are well-known uncertainties adhering to this approach (Shipley & Clark, 1972; Heath & Barton, 1973), the values of certain key parameters of glucose metabolism, for example the rate of irreversible disposal and transit times, are not critically dependent on the numerical value of any one coefficient or exponent provided that they together give a good fit to the data. Values computed in this way agree well with those obtained graphically as described by Katz *et al.* (1974*b*) (H. Osmundsen, unpublished work).

Results

Previous workers, with the exceptions of Katz *et al.* (1974*b*) and Heath *et al.* (1977*a*), have estimated rates of irreversible disposal in rats by using [^{14}C]glucose. Our control rates of irreversible disposal of glucose (about 60 μmol/min per kg body wt.) (Table 1) were higher than those reported for unanaesthetized

Table 1. *Effects of hypoglycin on some parameters of glucose metabolism*

Experimental details are given in the text, all parameters were computed from 10–17 data points. The results quoted were obtained by using [2-^{3}H]glucose, except for the 'apparent' rate of irreversible disposal, which was obtained by using [U-^{14}C]glucose. Values are means ± S.E.M. with the numbers of observations in parentheses. Significances were assayed by using an unpaired Student's *t* test. Values for all parameters were also computed by using the data for [U-^{14}C]glucose (not shown), and these were in reasonable agreement with those obtained by using [2-^{3}H]glucose.

Parameter of glucose metabolism	Control rats (5)	Hypoglycin-treated rats (4)	Decrease in relation to control value (%)	Significance
Rate of irreversible disposal (μmol/min per kg body wt.)				
^{3}H	101 ± 3.7	35.3 ± 3.0	65	$P<0.001$
^{14}C	59.1 ± 4.3	33.2 ± 3.6	45	$0.02>P>0.01$
Mass of sampling pool (μmol/kg body wt.)	3660 ± 215	1060 ± 198	71	$P<0.01$
Total body mass (μmol/kg body wt.)	7660 ± 1000	2000 ± 120	74	$P<0.01$
Minimum transit time (min)	59.3 ± 6.4	52.1 ± 5.3	12	$P>0.3$
Maximum transit time (min)	92.4 ± 7.0	71.3 ± 4.8	21	$P>0.05$
Recycling of glucose carbon (%)	40.2 ± 4.4	6.2 ± 6.0	85	$P<0.01$

rats by Katz *et al.* (1974*b*) (about 40 μmol/min per kg body wt.) or by Heath & Coreny (1973) and Heath *et al.* (1977*a*) (about 26–35 μmol/min per kg body wt.). This discrepancy may be partly related to the fact that we used very large rats (400–450 g).

Administration of hypoglycin to rats caused large differences in the values of the parameters of glucose metabolism when compared with the controls (Table 1, Fig. 2). The mass of glucose in the sampling pool was decreased by about 70%, and similarly the total mass of body glucose was also decreased by about 70% (Table 1). Recycling of glucose carbon was essentially abolished in hypoglycin-treated rats (Table 1, Fig. 2), consistent with profound inhibition of gluconeogenesis and also severe hypoglycaemia. In both groups the mass of glucose in the sampling pool was one-half to one-third of the total body mass of glucose (Table 1). Hypoglycin caused a 65% decrease in the rate of irreversible disposal of glucose (Table 1). That only a small change occurred in the estimated values for the maximum and minimum transit times of glucose during hypoglycaemia (Table 1) is explained by similar decreases in both the rate of glucose disposal and in the total body mass.

Discussion

Validity of the techniques used

In this investigation the techniques of Katz *et al.* (1974*a*,*b*) have been successfully applied to evaluate the effects of hypoglycin on some parameters of glucose metabolism. This was possible because of clear-cut differences between treated and control animals, and because it can be assumed that both groups were in an approximately steady state (Fig. 1). These results, of course, are only applicable during the period of sampling and they give no information about transitions between normal and hypoglycaemic states.

The use of doubly labelled glucose to study the kinetics of glucose metabolism *in vivo* has been discussed in detail by Katz *et al.* (1974*a*,*b*, 1976) and by Clark *et al.* (1975). The more rapid fall in the specific radioactivity of [2-^{3}H]glucose than of [U-^{14}C]glucose in blood (see Fig. 2*a*) is due to recycling through the Cori cycle (glucose → lactate → glucose), and to a futile cycle in the liver (glucose → glucose 6-phosphate → fructose 6-phosphate → glucose 6-phosphate → glucose), where the carbon

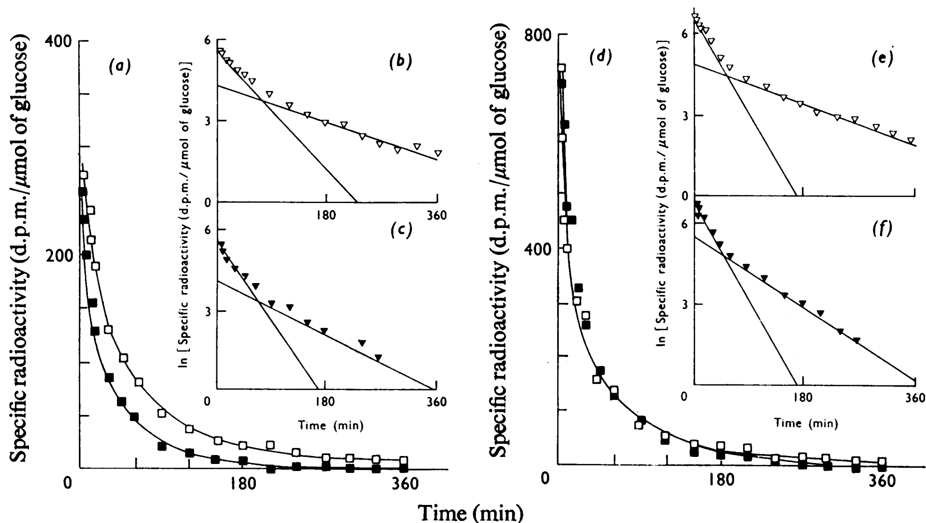

Fig. 2. *Effects of hypoglycin on the time course of the radioactivity in blood due to* ^{14}*C and to* ^{3}*H after intravenous administration of* [*U*-^{14}*C*,2-^{3}*H*]*glucose to rats*

Experimental details are given in the text. Specific radioactivities of ^{14}C and ^{3}H in freeze-dried deproteinized deionized blood are expressed in terms of μmol of glucose present. (*a*) Control rat: □, ^{14}C radioactivity; ■, ^{3}H radioactivity; insets (*b*) and (*c*): data from (*a*) plotted semi-logarithmically: ▽, ^{14}C specific radioactivity [specific radioactivity (at time t) = $210e^{-0.03t}+70e^{-0.007t}$; ▼, ^{3}H specific radioactivity [specific radioactivity (at time t) = $200e^{0.067t}+70e^{-0.012t}$]. (*d*) Hypoglycin-treated rat: □, ^{14}C radioactivity; ■, ^{3}H radioactivity; insets (*e*) and (*f*): data from (*d*) plotted semi-logarithmically: ▽, ^{14}C specific radioactivity [specific radioactivity (at time t) = $500e^{-0.093t}+280e^{-0.014t}$]; ▼, ^{3}H specific radioactivity [specific radioactivity (at time t) = $550e^{-0.029t}+140e^{-0.02t}$]. The S.D. of the coefficients and exponents in these experiments were all less than 5%.

skeleton is conserved while ^3H is shed (Rognstad *et al.*, 1973; Hue & Hers, 1974; Clark *et al.*, 1975). Clark *et al.* (1975) and Katz *et al.* (1976) considered that only 10% of ^3H formed from [2-^3H]glucose in the rat is due to futile recycling in the liver, and if our values for the rates of irreversible disposal (R_t) (Table 1) are decreased by this amount our estimate of the extent of glucose carbon recycled will be decreased by 25%. In retrospect, it would have been better to have used glucose labelled with ^3H in the 6-position as an irreversible tracer for glucose (see Clark *et al.*, 1975; Katz *et al.*, 1976) which gives rather lower values for R_t than glucose labelled in other positions. Similarly, measurements of recycling from ^{14}C from [U-^{14}C]glucose will overestimate the apparent rate of irreversible disposal (R_a), because the ^{14}C of pyruvate, derived from [U-^{14}C]glucose, exchanges with unlabelled CO_2 by the conversion pyruvate → oxaloacetate → phosphoenolpyruvate, and because of exchange of labelled oxaloacetate carbon with unlabelled acetyl-CoA by the citrate cycle (Krebs *et al.*, 1966). This dilution may result in an overestimation of R_a of up to 20%, giving an error in the estimation of recycling of 33% (Katz *et al.*, 1976), although large overestimates of both parameters would give a smaller overestimate of the percentage recycling. These errors would not, however, alter our conclusion that recycling of glucose carbon is severely inhibited during hypoglycin poisoning. The use of [6-^3H]glucose rather than [2-^3H]glucose would, however, have caused smaller differences in the estimation of the other parameters of glucose metabolism (Table 1).

Our results contrast with claims that hypoglycin increases the rate (or relative rate to that of fatty acid oxidation) of glucose oxidation, based only on measurements of total radioactivity of exhaled $^{14}CO_2$ after administration of [U-^{14}C]glucose to rats or mice (von Holt & Benedict, 1959; von Holt *et al.*, 1966; Corredor *et al.*, 1967). However, as was pointed out by Sherratt *et al.* (1971), if the pool size of available glucose is decreased to a greater extent than its rate of irreversible disposal, and there is also profound inhibition of gluconeogenesis, then a net increase in the rate of production of $^{14}CO_2$ from [U-^{14}C]glucose might be obtained. Indeed, the smaller glucose pool after giving hypoglycin is shown by the higher specific radioactivities of blood glucose (Fig. 2). Similarly, the experiments reported by Corredor *et al.* (1967) on the decreased incorporation of ^{14}C into total blood glucose from [1-^{14}C]pyruvate cannot be regarded as valid evidence for the inhibition of gluconeogenesis *in vivo* by hypoglycin.

Mechanism of the hypoglycaemic action of hypoglycin

The finding that gluconeogenesis and glucose utilization are greatly impaired in hypoglycin poisoning reinforces the conclusion that it is the net balance between glucose disposal and gluconeogenesis that is important, and that an increased rate of glucose utilization alone is not a sufficient cause of hypoglycaemia (Senior, 1967; Senior & Sherratt, 1968). However, we cannot simply explain why the steady-state concentrations of glucose (2–3mM) were maintained during hypoglycin poisoning, since glucose is still being utilized at a finite rate, whereas recycling of glucose carbon is apparently suppressed (Table 1), unless this paradox is due to errors in estimating R_a and R_t. van Hoof *et al.* (1972) reported a similar apparent inhibition of recycling in patients with Type-1 glycogen-storage disease given [U-^{14}C,2-^3H]glucose, which is characterized by a genetic defect in glucose 6-phosphatase. However, our results cannot be explained by inhibition of glucose 6-phosphatase, since glucose synthesis by isolated rat hepatocytes from 10mM-dihydroxyacetone is not inhibited by 4mM-hypoglycin, which inhibits glucose synthesis from 10mM-pyruvate, 10mM-lactate or 10mM-alanine by 60% (Billington, 1976).

Impaired gluconeogenesis may be a consequence of the effects of hypoglycin metabolites on β-oxidation, and on the metabolism of the branched-chain fatty acids, leucine and isoleucine (Senior *et al.*, 1975; Sherratt & Osmundsen, 1976). β-Oxidation of long-chain acyl-CoA esters only proceeds as far as butyryl-CoA, because of the inhibition of butyryl-CoA dehydrogenase (EC 1.3.99.2) by methylenecyclopropylacetyl-CoA, derived from the metabolism of hypoglycin (Osmundsen & Sherratt, 1975). The dehydrogenation of isovaleryl-CoA and 2-methylbutyryl-CoA is also inhibited (Billington *et al.*, 1974, 1976*a*,*b*). Activation of pig liver pyruvate carboxylase (EC 6.4.1.1) by acetyl-CoA is competitively inhibited by butyryl-CoA (apparent K_i 20μM) and by isovaleryl-CoA (H. Osmundsen, unpublished work), and accumulation of these compounds in the matrix of liver mitochondria during hypoglycin poisoning may contribute to inhibition of gluconeogenesis from pyruvate and those precursors that have first to be converted into pyruvate (Sherratt & Osmundsen, 1976). Further hypoglycin poisoning in rats is also marked by ketosis when the blood 3-hydroxybutyrate/acetoacetate ratio is lowered from about 3 to 1, indicating a more oxidized state of tissue mitochondria than in control animals (Williamson & Wilson, 1965). This suggests that gluconeogenesis from some presursors may also be limited by a decreased supply of reducing equivalents from the mitochondria (see Senior *et al.*, 1975). The mechanism by which glucose utilization is decreased during hypoglycin poisoning is not clear, although it may be partly related to the fact that the rate of disposal of glucose is a function of the blood glucose concentration (Heath & Corney, 1973; Heath *et al.*, 1977*a*).

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