Factors that Prevent the Premature Appearance of Glucokinase in Neonatal Rat Liver

Michael J. O. WAKELAM, M. Bruce ALLEN and Deryck G. WALKER

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(Received 13 August 1979)

1. The physiological factors that prevent the precocious appearance of glucokinase activity in the 13-day-old rat that can be induced by oral glucose administration were explored. 2. Evidence is presented that the galactose component of milk sugar is inhibitory. In the absence of this inhibitory galactose, the amount of glucose necessary to effect appreciable induction is greater than that present in milk. 3. The induction is prevented both by administration of mannoheptulose, which inhibits insulin release, and by excess insulin; the amount of insulin available therefore seems to be critical. 4. The inhibition of induction by galactose does not appear to be via competition with glucose but by enhancing insulin release and thereby making this excessive. The relative amounts of glucose and insulin appear to be important in regulating glucokinase induction. 5. The precocious induction of glucokinase by glucose is inhibited by simultaneous treatment with appropriate amounts of adrenaline, glucagon, dibutyryl cyclic AMP or isoprenaline but not by vasopressin or angiotensin II. 6. No single cause of glucokinase induction in neonatal rat liver can be recognized. The process is subject to regulation by many factors at a time subsequent to when competence to synthesize the enzyme has been established.

The high- K_m glucose-phosphorylating enzyme glucokinase (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2) can first be detected in rat liver about 16 days after birth (Walker & Holland, 1965; Jamdar & Greengard, 1970). Several attempts in this laboratory to induce precocious appearance of the enzyme by glucose administration resulted in marginal effects only (Walker & Holland, 1965; Walker & Eaton, 1967). Reports that treatment with glucose plus cortisol resulted in glucokinase activity being detectable around the 10th postnatal day (Jamdar & Greengard, 1970) or even earlier after the administration of corticotropin (Adelman & Freeman, 1972) have not been confirmed (Partridge *et al.*, 1975; Allen *et al.*, 1978).

Partridge *et al.* (1975) reported that treatment of neonatal rats with tri-iodothyronine plus glucose resulted in the precocious development of glucokinase. Wakelam *et al.* (1979) confirmed this observation, but presented evidence that, contrary to the suggestion by Partridge *et al.* (1975), the normal increases in the amounts of thyroid hormones circulating in the blood around weaning are not an essential feature for glucokinase activity to be inducible precociously by means of glucose administration. Knowing now conditions under which precocious induction of the enzyme *in vivo* can be

effected, we report the results of studies designed to explore the physiological regulation of glucokinase development. The main approach has been to examine possible factors that prevent the precocious appearance of the enzyme resulting from oral glucose intubation in 13-day-old rats. The term induction will be used to describe this appearance of glucokinase activity, but it is not intended to have any implication about any specific mechanism for the effect of glucose. A preliminary account of some of this work has been given (Walker *et al.*, 1978).

Materials and Methods

Chemicals

The sources of most materials have been given (Holroyde *et al.*, 1976). Adrenaline was from BDH Chemicals, Poole, Dorset, U.K. Angiotensin II, *NO*-dibutyryl cyclic AMP, D-mannoheptulose and vasopressin (grade V) were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Glucagon was from Novo Industri A/S, Copenhagen, Denmark. Insulin, B.P. (20 i.u./ml) and isoprenaline were from The Wellcome Foundation, London, U.K. Oxytocin was from Sandoz Products, Feltham, Middx., U.K. The GOD-Perid kits for glucose assays were purchased from Boehringer

Corp. (London) Ltd., London W5 2TZ, U.K., and the radioimmunoassay kits from The Radiochemical Centre, Amersham, Bucks., U.K. The standard rat insulin was from Novo Research Institute, Bagsvaerd, Denmark.

Animals and treatments

Rats were from our departmental strain of specific-pathogen-free Wistar-derived animals maintained on Modified Rat Breeding Diet (Heygate and Sons, Northampton, U.K.) and a 14h light/10h dark lighting schedule at 22°C. Times of birth of litters were known within 8h, and very large litters were culled to ten animals. Litters of less than eight animals were not used.

Neonatal animals removed from their mothers were maintained in a humidicrib at 30°C. Intra-peritoneal injections were made with a 30-gauge needle and oral intubation was by means of a ball-tipped needle made in our workshop as described by Miller & Dymsha (1963).

Rat milk was obtained from 13-day-lactating animals that had been separated from their litters for 30–45 min, injected with 1 i.u. of oxytocin and then lightly anaesthetized with diethyl ether. The milk was expressed manually from all the nipples and collected by mild suction into a tube as described by Carrick (1979). Milk was dialysed against 0.02 M-KCl/0.009 M-NaCl/0.11 M-CaCl₂ (Macy *et al.*, 1953). The milk lipid fraction was obtained by low-speed centrifugation of the milk; the lipid fraction was resuspended in the above dialysis medium.

The artificial milk used in many of the experiments was prepared by addition of the appropriate sugar(s) to a basal mixture that had carbohydrate-free Galactomin 19 as the base powder. Galactomin 19 is a proprietary synthetic milk powder low in lactose content and marketed for the feeding of humans requiring extreme restriction of lactose in the diet. We are grateful to Mrs. R. C. Richards, Senior Nutritionist, Cow & Gate Baby Foods, Trowbridge, Wilts., U.K., for supplying us with the base powder of this formulation (before the normal addition of fructose). Our artificial milk was made by dissolving 15 g of the above base powder, 1 g of a vitamin mix (Walker & Eaton, 1967), 62.3 mg of inositol, 5.25 mg of ascorbic acid and carbohydrate, as specified in the details of each experiment, in 100 g of water. As a result, the milk contained, in addition to this carbohydrate, 5.07 g of total fat (a mixture of coconut and maize oil), 7.82 g of protein (casein) and appropriate salts and vitamins per 100 ml.

Determinations on blood

Animals were killed by decapitation and blood was collected in heparinized plastic centrifuge tubes

for the preparations of plasma for glucose measurements, which used the GOD-Perid reagent (Boehringer) and an Autoanalyser. Plasma insulin concentrations were determined by radioimmunoassay. Plasma samples had to be diluted 5 or 10 times when the insulin concentrations were high. A rat insulin standard was employed.

Enzyme assays

Our assay of glucokinase activity (Walker & Rao, 1964) has been re-evaluated and discussed (Wakelam *et al.*, 1979) with special reference to the problems of measuring low activities (Walker & Holland, 1965; Weinhouse, 1976). Briefly, assays were performed on the supernatant fraction of 20% (w/v) homogenates of rat livers. The coupled enzyme assay included applying corrections for low- K_m hexokinase (EC 2.7.1.1) and glucose dehydrogenase activities. The addition of 6-phosphogluconate dehydrogenase together with the usual excess of glucose 6-phosphate dehydrogenase (Storer & Cornish-Bowden, 1974) did not increase the rate of NADPH production in the coupled assay system; the amount of NADPH formed was therefore halved to give glucose phosphorylated. We recognize that low glucokinase activities, especially those of less than about 0.1 unit/g of liver, are subject to considerable error. For this reason we report both glucokinase and hexokinase activities in all our experiments; 1 unit of these activities is that catalysing the phosphorylation of 1 μ mol of glucose/min at 32°C. That the low glucokinase activities recorded were indeed due to the presence of this enzyme was checked regularly by staining for high- K_m glucose-phosphorylating activity electrophoretograms prepared in agarose gel (Wakelam *et al.*, 1979); this procedure permitted the separation of glucokinase from the other low- K_m hexokinases (Grossbard & Schimke, 1966) within 1 h, during which time essentially no glucokinase activity was lost. Visual inspection of electrophoretograms of preparations from treated and control animals always corroborated the spectrophotometric findings.

Enzyme activities were also calculated on other bases (per whole liver, per g body wt. and per mg of supernatant protein), but these data have not been included in the Tables because no additional conclusions can be drawn from them. Results are given as means $\pm$ S.E.M. and the numbers of separate observations recorded. The statistical significance of differences was calculated by Student's *t* test.

Results

Rats at 13 days of age were employed for most of our experiments. At this age the neonatal rat is

physically much more mature than a few days earlier, yet another 3 days are to elapse before glucokinase is normally detectable (see the introduction). It should be noted therefore that in all the experiments reported the normal control animal has zero glucokinase activity.

Dietary effects

The effect of oral glucose administration is apparent 4 h later (Table 1, Expt. 1a), as reported previously (Wakelam *et al.*, 1979), provided that the animals have been removed from their mothers. In those animals that remained with their mothers and imbibed milk during the 4 h period, as revealed by visual inspection of their stomach contents at the time of killing, no glucokinase activity appeared. This effect of milk ingestion was not observed in animals given the same amount of glucose intraperitoneally (Table 1, Expt. 1b). The administration

of either mannose or galactose instead of glucose under the conditions of Expt. 1(a) resulted in no induction of glucokinase activity.

Table 2 presents results suggesting that a dialysable component of the milk is responsible for the inhibition of glucokinase induction after oral glucose administration, because the concurrent intubation of dialysed milk was without effect (Expt. 2a). Again, no such effects were observed if the glucose was injected intraperitoneally (Expt. 2b).

A dialysable component of milk likely to be exerting such effects is lactose. Experiments were therefore performed to see whether the oral intubation of galactose or lactose together with an inducing dose of glucose was inhibitory. In Expt. 3(a) (Table 3) the sugars were added to dialysed rat milk. Galactose and lactose both inhibited glucokinase induction, but, as a control, the addition of mannose did not. Pure sugar solutions were intubated orally

Table 1. *Effect of route of administration and milk feeding upon precocious induction of glucokinase by glucose*
All animals were 13 days old and either remained with or were removed from their mothers as indicated. Glucose (5 mg/g body wt.) was administered either orally or intraperitoneally. The animals were killed 4 h later when the enzyme activities were determined as described in the text. Results are given as means $\pm$ S.E.M.

Expt.	Route of glucose administration	Status of animals	No. of animals	Enzyme activities (unit/g of liver)	
				Glucokinase	Hexokinase
1(a)	Oral	Removed from mother 1 h previously	4	0.18 $\pm$ 0.03	0.23 $\pm$ 0.03
	Oral	Removed from mother 4 h previously	3	0.23 $\pm$ 0.03	0.24 $\pm$ 0.01
	Oral	Remained with mother	3	0	0.18 $\pm$ 0.03
1(b)	Intraperitoneal	Removed from mother 1 h previously	9	0.15 $\pm$ 0.02	0.25 $\pm$ 0.03
	Intraperitoneal	Remained with mother	9	0.15 $\pm$ 0.02	0.31 $\pm$ 0.03

Table 2. *Effect of milk and milk fractions upon precocious induction of glucokinase by glucose*
Animals were 13 days old and were removed from their mothers 1 h before oral intubation of glucose (5 mg/g body wt.) dissolved in 0.25 ml of the milk or milk substitute (see the Materials and Methods section) as indicated. Animals were killed 4 h later; other details were as in legend to Table 1. *Significance of difference from control, $P < 0.05$.

Expt.	Route of glucose administration	Additional oral treatment	No. of animals	Enzyme activities (unit/g of liver)	
				Glucokinase	Hexokinase
2(a)	Oral	None	4	0.14 $\pm$ 0.05	0.25 $\pm$ 0.04
	Oral	Whole milk	4	0.02 $\pm$ 0.01*	0.24 $\pm$ 0.01
	Oral	Dialysed milk	4	0.17 $\pm$ 0.05	0.26 $\pm$ 0.02
	Oral	Lipid fraction	4	0.11 $\pm$ 0.05	0.27 $\pm$ 0.05
2(b)	Intraperitoneal	None	5	0.23 $\pm$ 0.03	0.30 $\pm$ 0.03
	Intraperitoneal	Whole milk	5	0.20 $\pm$ 0.01	0.34 $\pm$ 0.01
	Intraperitoneal	Dialysed milk	4	0.23 $\pm$ 0.04	0.32 $\pm$ 0.02
	Intraperitoneal	Dialysed milk + lactose	4	0.20 $\pm$ 0.03	0.25 $\pm$ 0.01

in Expt. 3(b), and again significant inhibition of induction by glucose was observed with galactose and lactose. Because some form of competition between glucose and galactose might be the reason for these effects and because lactose on hydrolysis yields glucose as well as galactose, it was considered important to examine the concentration-dependence of the inhibitory effects. Using a similar experimental protocol to that used in Table 3 the inhibitory effect of galactose was found to be fully effective when it represented only 20% of sugar intubated (Table 4, Expt. 4a). The inhibitory action of lactose required a higher amount for maximum effect; yet higher concentrations of lactose reversed the inhibition (Table

4, Expt. 4b). This latter result has been confirmed in another separate experiment (not shown).

This apparent interplay between the relative amounts of glucose and galactose or lactose was explored further in experiments performed over a 2-day period during which time the various sugars were included in an artificial milk diet (Table 5). When the amount of glucose included in the milk corresponded to the normal physiological concentration (Walker & Eaton, 1967), the induction seen with glucose in the absence of galactose was significant but very small; the addition of equimolar galactose prevented the induction by glucose (Expt. 5a). Fig. 1 shows the effect of glucose concentration

Table 3. *Effects of other sugars upon precocious induction of glucokinase by glucose*

All animals were 13 days old and were removed from their mothers 1 h before oral intubation of glucose (5 mg/g body wt.) contained in 0.25 ml of dialysed rat milk (Expt. 3a) or 0.25 ml of water (Expt. 3b). Other sugars were included as indicated in the following amounts: in Expt. 3(a), galactose or mannose (0.45 mg/g body wt.) or lactose (0.9 mg/g body wt.); in Expt. 3(b), galactose or lactose (5 mg/g body wt.). Animals were killed 4 h later; other details were as in legend to Table 1. Significance of difference from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Expt.	Treatment	No. of animals	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
3(a)	Dialysed milk + glucose	4	0.23 ± 0.03	0.21 ± 0.03
	Dialysed milk + glucose + galactose	5	$0.04 \pm 0.01^{***}$	0.26 ± 0.01
	Dialysed milk + glucose + lactose	5	$0.07 \pm 0.04^*$	0.31 ± 0.01
	Dialysed milk + glucose + mannose	4	0.18 ± 0.02	0.21 ± 0.03
3(b)	Glucose	7	0.35 ± 0.06	0.28 ± 0.03
	Glucose + galactose	8	$0.04 \pm 0.02^{***}$	0.34 ± 0.04
	Glucose + lactose	7	$0.08 \pm 0.03^{**}$	0.31 ± 0.02

Table 4. *Effects of various amounts of galactose and lactose upon precocious induction of glucokinase by glucose*

All animals were 13 days old and were removed from their mothers 1 h before oral intubation of glucose (5 mg/g body wt.) together with various amounts of either galactose or lactose as indicated, all dissolved in 0.5 ml of water. Animals were killed 4 h later; other details are as in legends to Tables 1 and 3.

Expt.	Amount of galactose or lactose (mg/g body wt.)	No. of animals	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
4(a)	0	11	0.31 ± 0.04	0.30 ± 0.02
	Galactose 1.25	5	$0.01 \pm 0.01^{***}$	0.31 ± 0.01
	2.5	9	$0.03 \pm 0.01^{***}$	0.33 ± 0.01
	5.0	8	$0.04 \pm 0.02^{***}$	0.34 ± 0.02
	10.0	4	$0.05 \pm 0.02^{**}$	0.29 ± 0.02
	15.0	3	$0.04 \pm 0.03^{**}$	0.32 ± 0.01
4(b)	0	7	0.35 ± 0.06	0.28 ± 0.03
	Lactose 2.5	3	0.18 ± 0.04	0.30 ± 0.02
	3.5	5	$0.03 \pm 0.02^{***}$	0.36 ± 0.05
	5.0	7	$0.08 \pm 0.03^{***}$	0.31 ± 0.02
	7.5	5	$0.12 \pm 0.04^*$	0.35 ± 0.10
	10.0	3	0.47 ± 0.18	0.32 ± 0.02

Table 5. *Effects of various amounts of glucose and other sugars upon precocious induction of glucokinase during a 2-day period*

All animals were 11 days old and were removed from their mothers 1 h before oral intubation every 4 h from time 0 to 48 h of 0.5 ml of an artificial milk diet containing milk powder base (see the text) and the various sugars as indicated. In Expt. 5(a) the normal (equivalent to physiological) concentration of each individual sugar was 1.8 g/100 ml so that, in Expts. 5(b) and 5(c), 3 × normal concentrations represented 5.4 g of individual sugar/100 ml. Animals were killed 4 h after the last feed when 13 days old; other details are as in legend to Table 3.

Expt.		No. of animals	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
5(a)	Base + glucose and mannose each at normal concentration	9	0.04 ± 0.01	0.25 ± 0.02
	Base + glucose and galactose each at normal concentration	8	0**	0.28 ± 0.01
5(b)	Base + glucose and mannose each at 3 × normal concentration	9	0.37 ± 0.04	0.20 ± 0.01
	Base + glucose and galactose each at 3 × normal concentration	9	0.03 ± 0.01***	0.26 ± 0.01
5(c)	Base + glucose and mannose each at 3 × normal concentration	6	0.51 ± 0.10	0.33 ± 0.02
	Base + lactose at 3 × normal concentration	6	0.03 ± 0.02***	0.31 ± 0.02

Fig. 1. *Effect of amount of glucose on precocious induction of glucokinase*

Animals at 11 days old were removed from their mothers and fed every 4 h from 0 to 48 h with 0.5 ml of an artificial milk diet containing milk base powder (see the text) and glucose at the concentration indicated. The animals were 13 days old when killed. The lines join the data obtained in two separate experiments by procedures as described in the text. Each point represents the mean ± S.E.M. for six observations

amounts of galactose (Expt. 5b) or lactose (Expt. 5c). We have previously shown (Wakelam *et al.*, 1979) that glucokinase activity can be induced within 4 h in younger neonatal rats intubated with glucose. In an experiment similar in all respects to Expt. 5(c) above, except that the artificial milk diet was fed (volume of each feed, 0.25 ml) from postnatal day 6 and the animals were killed when 8-days-old, the glucokinase activity with glucose as the only sugar component was 0.36 ± 0.03 unit/g wet wt., whereas the added presence of lactose resulted in the significantly lower activity of 0.04 ± 0.02 unit/g wet wt. (*n* = 4 in each case, *P* < 0.001). Practical difficulties prevented us from doing similar experiments on even younger animals.

Involvement of insulin

There is a wealth of evidence that both glucose and insulin are necessary for the induction of glucokinase in, for example, an adult rat that has been either starved or fed on a carbohydrate-free diet and is then given glucose (reviewed by Niemeyer *et al.*, 1975; Weinhouse, 1976). This is also true for the normal development of glucokinase in the rat's third postnatal week of life (Walker & Holland, 1965; Walker & Eaton, 1967). Table 6 shows that when mannoheptulose, an inhibitor of insulin release (Simon *et al.*, 1961) and which therefore evokes a marked hyperglycaemia, is injected at the same time as the intubation of glucose, a complete inhibition of induction of glucokinase activity is apparent 4 h later. The simultaneous additional administration of an appropriate amount of soluble insulin (such that only a mild hypoglycaemia was observed after 4 h) permitted induction by glucose to proceed (Table 6).

in artificial milk upon induction of glucokinase over a 48 h period. Glucose at 3 times the normal concentration resulted in substantial induction of glucokinase activity, which was prevented by high

In other experiments (not shown) in which insulin was administered at a range of concentrations but without glucose intubation no induction occurred. Higher doses of insulin resulted, of course, in gross hypoglycaemia and death.

On the other hand, too much insulin associated with normoglycaemia seems to inhibit glucokinase induction. Table 7 shows that injection of increasing amounts of insulin progressively inhibits induction. This effect is observed even when the animals were normoglycaemic at the time of death. A tentative interpretation of all these results is that it is the relative concentrations of glucose and insulin that regulate the induction of glucokinase activity. It is thus possible that the inhibitory effects of other sugars such as galactose might also operate by disturbing the insulin/glucose balance.

In preliminary experiments performed before testing this possibility, we determined the amount of mannoheptulose that needed to be injected at the time of glucose intubation in order to prevent glucokinase induction without an associated gross hyperglycaemia. This amount of mannoheptulose was used in the experiment shown in Table 8 (see line 2 of results). Intubation of galactose along with glucose (line 3) resulted in inhibition of induction, as expected from earlier results (Tables 3 and 4). Intubation of galactose to animals receiving the same dose of mannoheptulose (line 4, Table 8) permitted normal induction to occur. Under these circumstances the galactose appears to counter-balance the effect of mannoheptulose in inhibiting insulin release.

That intubation of galactose does result in

Table 6. *Effects of mannoheptulose and of insulin upon precocious induction of glucokinase by glucose*

All animals were 13 days old and were removed from their mothers 1 h before oral intubation of glucose (5 mg/g body wt.). Some animals, as indicated, were injected intraperitoneally at the same time with mannoheptulose (3 mg/g body wt., dissolved in 0.25 ml of 0.9% NaCl) and soluble insulin (5 m-i.u./g body wt.). Animals were killed 4 h later; other details are as in legend to Table 3.

Treatment	No. of animals	Blood glucose (mM)	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
Glucose only	6	6.4 ± 0.4	0.24 ± 0.02	0.32 ± 0.01
Glucose + mannoheptulose	6	29.4 ± 0.4	0.01 ± 0.01	0.35 ± 0.02
Glucose + mannoheptulose + insulin	6	3.9 ± 0.3	0.32 ± 0.06	0.34 ± 0.02

Table 7. *Dose-dependent effect of insulin in inhibiting the precocious induction of glucokinase by glucose*

All animals were 13 days old and were removed from their mothers 1 h before oral intubation of glucose (5 mg/g body wt.). The animals were also injected intraperitoneally at the same time with soluble insulin as indicated. They were killed 4 h later; other details are as in legend to Table 3.

Dose of insulin (m-i.u./g body wt.)	No. of animals	Blood glucose (mM)	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
—	8	5.5 ± 0.2	0.32 ± 0.04	0.39 ± 0.03
0.1	4	5.9 ± 0.4	0.20 ± 0.04	0.31 ± 0.02
0.5	4	5.5 ± 0.4	0.16 ± 0.10	0.30 ± 0.01
1.0	8	5.6 ± 0.3	0.08 ± 0.01	0.39 ± 0.03
2.5	4	5.1 ± 0.7	0.06 ± 0.03	0.40 ± 0.02
5.0	4	3.3 ± 0.6	0.01 ± 0.01	0.40 ± 0.04

Table 8. *Effects of galactose and/or mannoheptulose upon precocious induction of glucokinase by glucose*

Animals were 13 days old and were removed from their mother 1 h before oral intubation of glucose (5 mg/g body wt.). Some animals also received galactose (5 mg/g body wt.) orally and some mannoheptulose (0.3 mg/g body wt., dissolved in 0.9% NaCl) intraperitoneally, as indicated. They were killed 4 h later; other details are as in legend to Table 3.

Treatment	No. of animals	Blood glucose (mM)	Enzyme activities (unit/g of liver)	
			Glucokinase	Hexokinase
Glucose only	4	5.3 ± 0.3	0.51 ± 0.05	0.54 ± 0.02
Glucose + mannoheptulose	4	9.6 ± 1.3	0.04 ± 0.02***	0.49 ± 0.03
Glucose + galactose	4	6.9 ± 0.1	0.06 ± 0.05***	0.41 ± 0.04
Glucose + galactose + mannoheptulose	4	9.5 ± 0.7	0.45 ± 0.03	0.40 ± 0.01

enhanced insulin secretion was demonstrated in a further series of experiments in which animals were killed and blood samples taken over a range of times after oral intubation of various sugars (Table 9).

Because serial blood sampling cannot be performed on such small animals, a large number of animals have to be used and plasma samples pooled to obtain these types of data. Expt. 9(a) involved the

Table 9. *Plasma glucose and insulin concentrations after intubation of sugars*

Rats (13-day-old) were removed from their mothers 1 h before oral intubation of sugars as indicated. Animals were killed at various time intervals later and blood was collected for the subsequent assay of plasma glucose and insulin by procedures given in the Materials and Methods section. Results are given as means $\pm$ s.e.m. of duplicate determinations on five animals in each case. The zero time concentrations were: plasma glucose, 6.4 ± 0.1 mM; plasma insulin 3.7 ± 0.4 ng/ml.

Expt.	Sugar(s) intubated	Time after intubation						
		15 min	30 min	45 min	60 min	90 min	2 h	4 h
9(a)	5 mg of glucose/g body wt.							
	Plasma glucose (mM)	17.5 ± 0.2	11.6 ± 0.3	10.6 ± 0.3	10.1 ± 0.7	—	10.1 ± 0.1	6.0 ± 0.4
	Plasma insulin (ng/ml)	8.0 ± 0.4	10.9 ± 1.5	5.9 ± 0.5	6.4 ± 0.3	—	4.8 ± 0.7	3.9 ± 1.0
9(b)	10 mg of glucose/g body wt.							
	Plasma glucose (mM)	22.9 ± 1.9	23.4 ± 2.1	19.9 ± 2.5	18.1 ± 2.3	12.1 ± 1.1	9.4 ± 0.8	7.7 ± 0.3
	Plasma insulin (ng/ml)	16.7 ± 1.2	28	27.2 ± 1.1	28	20.9 ± 1.4	8.8 ± 0.7	4.5 ± 0.1
9(c)	5 mg of glucose and 5 mg of mannose/g body wt.							
	Plasma glucose (mM)	17.2 ± 3.2	17.5 ± 2.7	15.5 ± 2.6	15.9 ± 3.7	10.5 ± 0.6	8.3 ± 1.5	6.4 ± 0.2
	Plasma insulin (ng/ml)	11.8 ± 0.6	24.7 ± 1.2	14.6 ± 0.7	28	12.7 ± 1.7	6.3 ± 1.1	4.3 ± 0.4
9(d)	5 mg of glucose and 5 mg of galactose/g body wt.							
	Plasma glucose (mM)	18.7 ± 0.2	12.0 ± 0.7	13.0 ± 0.5	9.5 ± 0.5	9.3 ± 0.1	9.1 ± 0.3	6.8 ± 0.3
	Plasma insulin (ng/ml)	18.2 ± 0.4	28	14.1 ± 1.1	19.2 ± 2.0	16.7 ± 0.8	6.2 ± 0.6	4.6 ± 0.3

Table 10. *Effects of various hormones and other agents upon precocious induction of glucokinase by glucose*

All animals were 13 days old and were removed from their mother 1 h before oral intubation of glucose (5 mg/g body wt.). Some animals were injected intraperitoneally at the same time with a hormone or other agent as indicated. Control animals were injected with an equal volume of the carrier. All were killed 4 h later; other details are as in legend to Table 3.

Expt.	Agent (wt./g body wt.)	No. of animals	Enzyme activity (unit/g of liver)	
			Glucokinase	Hexokinase
10(a)	None	4	0.28 ± 0.08	0.30 ± 0.02
	Adrenaline (0.5 μ g)	4	0***	0.34 ± 0.02
10(b)	None	4	0.36 ± 0.07	0.35 ± 0.01
	Dibutyryl cyclic AMP (100 μ g)	3	0***	0.39 ± 0.04
	Isoprenaline (0.2 μ g)	5	0.08 ± 0.04 **	0.48 ± 0.04
10(c)	None	4	0.21 ± 0.04	0.26 ± 0.02
	Glucagon (0.25 μ g)	4	0.11 ± 0.03	0.38 ± 0.02
	(0.50 μ g)	4	0.07 ± 0.03 *	0.40 ± 0.04
	(1.0 μ g)	4	0.02 ± 0.01 **	0.32 ± 0.02
10(d)	None	4	0.44 ± 0.03	0.43 ± 0.03
	Glucagon (0.35 μ g)	4	0.09 ± 0.04 ***	0.49 ± 0.02
	(0.50 μ g)	4	0.08 ± 0.03 ***	0.48 ± 0.01
	(0.75 μ g)	4	0.03 ± 0.01 ***	0.47 ± 0.02
10(e)	None	4	0.42 ± 0.04	0.43 ± 0.04
	Vasopressin (21.5 μ g)	4	0.34 ± 0.10	0.44 ± 0.04
	(43 μ g)	4	0.25 ± 0.05 *	0.43 ± 0.03
10(f)	None	4	0.44 ± 0.09	0.35 ± 0.02
	Angiotensin II (0.23 ng)	4	0.29 ± 0.02	0.36 ± 0.02
	(0.46 ng)	4	0.36 ± 0.03	0.34 ± 0.02

standard glucokinase-inducing protocol (e.g. in Table 4). In Expt. 9(b) twice as much glucose was intubated. High plasma glucose concentrations were recorded over the first hour and the plasma insulin concentrations rose to very high values. The value at 45 min was perhaps lower than at 30 and 60 min after glucose intubation, suggesting a biphasic response. A biphasic response in plasma insulin values is seen more clearly in Expts. 9(c) and 9(d). We do not wish to make detailed conclusions from these data, but draw attention to the fact that, upon comparison of the results from Expts. 9(a) and 9(d), whereas plasma glucose concentrations varied very similarly, the addition of galactose in Expt. 9(d) resulted in much higher insulin values during the first hour after intubation.

Other hormonal factors

Weinhouse (1976) summarized the evidence reported by Ureta *et al.* (1970) and Pilgis (1970) indicating that the restoration of glucokinase activity that follows glucose re-feeding of starved rats is inhibited by simultaneous treatment with adrenaline, glucagon and 3':5'-cyclic AMP. The precocious appearance of glucokinase in the livers of 13-day-old rats evoked by glucose intubation is also inhibited by adrenaline, dibutyryl cyclic AMP, glucagon and the β -adrenergic blocker isoprenaline (Table 10, Expts. 10a, b, c and d). On the other hand, only a very high concentration of vasopressin caused a small inhibition of the induction (Expt. 10e), and injection of angiotensin II (Expt. 10f) resulted in no significant inhibition.

Discussion

The association between the normal first appearance of glucokinase activity in neonatal liver and the change of diet with weaning is well recognized (Walker & Holland, 1965; Vernon & Walker, 1968), although neither an increase in carbohydrate content of the diet (Walker & Eaton, 1967) nor the intake of solid food (Walker *et al.*, 1974) is essential for low glucokinase activity to appear, so that hormonal factors must also be involved (Green-gard, 1971). An experimental system that results in the precocious appearance of glucokinase activity after oral intubation of glucose (Wakelam *et al.*, 1979) has permitted us to identify several factors that can limit the normal appearance of significant amounts of activity until 16 days or more after birth.

The results in Table 1 indicate that a milk component, perhaps galactose, might be involved in the inhibition of the induction of glucokinase activity. This conclusion is supported by the experiments showing inhibition of induction reported in Tables 3 and 4. The need to remove the animals from their mothers [and thereby deprive them of

further intake of lactose (Table 1)] for precocious induction to occur and the fact that dialysed milk is not inhibitory (Table 2) is thereby also explained. These effects are observed provided that the inducing dose of glucose is given orally but not if given intraperitoneally (Table 2). This latter result points to the route of sugar absorption being a second factor regulating the level of induction. Mannose, which is not actively absorbed, does not inhibit glucokinase induction (Expts. 3a and 5b and c). Competition between galactose and glucose for one or more active-absorption or transport system is a possibility. The precise nature of these systems remains to be elucidated [see McMichael (1975) and Dahlqvist (1978) for reviews] and their development has not been examined, although marked adaptations are seen histologically during the weaning period (Höhn *et al.*, 1977). If the intubation of galactose results in a lower rate of absorption of glucose, then a smaller response of the pancreatic β -cells might result if glucose was the only secretagogue. The amount of insulin released appears, however, to be enhanced by galactose (Table 9).

We suggest that the regulation of the induction of glucokinase activity depends in some manner upon the relative amounts of glucose and insulin available at the site where regulation of induction occurs. That there is an insulin requirement is not in doubt and is confirmed by our results in Tables 6 and 8, but, in reviewing the evidence for this, Weinhouse (1976) has pointed out that the actual amounts of insulin that have been used in the experimental studies reported from a number of laboratories have varied considerably and have often been what may be regarded as large. Too much insulin would produce hypoglycaemia which might counter the inducing influence of the insulin. That too much insulin associated with normoglycaemia inhibits induction of glucokinase is clearly shown in Table 7. The inhibitory effect of galactose, rather than being via competition with glucose, may be therefore due, at least in part, to the release of too much insulin in relation to the amount of glucose present. A possible mechanism whereby galactose exerts this effect is via gastrointestinal hormones. Ross & Dupre (1978) have shown that, although it has little direct effect upon insulin secretion, galactose evokes secretion of the gastric inhibitory polypeptide hormone. Marks & Turner (1977) reviewed the evidence that gastrointestinal hormones might regulate insulin secretion, and Brown & Otte (1978) and Pederson & Brown (1978) have shown that gastric inhibitory polypeptide is particularly important in this respect. Complex and differing relationships between the effect of this hormone and the glucose concentration upon the release of both insulin and glucagon from the rat pancreas have been demonstrated (Pederson & Brown, 1978), and Morgan *et al.*

(1979) report that in man oral galactose releases gastric inhibitory polypeptide, which is powerfully insulinotropic in the presence of moderate hyperglycaemia.

The effects of galactose that we report may be compared with the results of experiments in which mannose was intubated. The simultaneous administration of mannose with glucose did not increase the insulin/glucose ratio to such a great extent as did galactose (cf. Expts. 9c and 9d) and did not inhibit induction of glucokinase (Table 5). This supports our hypothesis that it is the concentration of insulin relative to glucose that is critical in this control.

The livers of 13-day-old rats that had been fed on artificial galactose-free milk for 48 h possessed only very low glucokinase activity (Table 5, Expt. 5a). This experiment does indicate, however, that, in the absence of equimolar galactose, the small amount of glucose potentially available in normal rat milk could induce glucokinase. A galactose-free diet containing glucose at 3 times the normal (physiological) glucose concentration resulted in significantly more activity (Expts. 5b and 5c). This result and that in Fig. 1 points to the amount of inducing carbohydrate being important. Both the amount of glucose available and the amount of insulin released will be affected. There is, of course, no need for the neonatal rat to possess glucokinase activity until the amount of carbohydrate ingested increases as solid food is eaten. The amount of glucose necessary to effect substantial induction is clearly greater than that present in milk.

Because the natural rise in glucokinase activity extends over a long period (Walker & Holland, 1965), it would be difficult to devise a controlled experiment designed to demonstrate the inhibition of that natural rise by galactose. However, galactose-containing diets fed to adult rats do result in lowered glucokinase activity compared with suitable controls (Walker *et al.*, 1965/66).

In addition to the relative amounts of glucose and insulin themselves, results in Table 10 support the view (Ureta *et al.*, 1970; Pilkis, 1970) that cyclic AMP in some way regulates the synthesis of glucokinase. Those workers studied the response to glucose feeding of carbohydrate-deprived adult rats. Our study is most analogous to that of Ureta *et al.* (1970), who started with adult rats fed on a carbohydrate-free high-fat diet. The 13-day-old rat feeds only on milk in which the carbohydrate content is low (Dymsza *et al.*, 1964). The animal's plasma insulin concentration is low (Table 9; Blazquez *et al.*, 1970, 1972; Beaudry *et al.*, 1977), but the circulating glucagon concentration is high at this age (Blazquez *et al.*, 1972; Girard *et al.*, 1975; Beaudry *et al.*, 1977). As a result, the concentration of liver cyclic AMP in the neonatal period is high

(Novak *et al.*, 1972; Christofferson *et al.*, 1973; Beaudry *et al.*, 1977; DiMarco & Oliver, 1978). The high insulin concentrations that follow glucose intubation (Table 9) presumably lower the hepatic cyclic AMP concentrations and this in turn in some way permits synthesis of glucokinase to occur. On this hypothesis, the injection of glucagon (cf. Niemeyer *et al.*, 1966), adrenaline, isoprenaline or dibutyryl cyclic AMP (cf. Ureta *et al.*, 1970) counteracts this effect of insulin and maintains the hepatic cyclic AMP concentrations, thus inhibiting induction of glucokinase. On the other hand, only an exceptionally high concentration of vasopressin showed a small inhibition of induction (Table 1) and a relevant dose of angiotensin II was without effect. The latter two hormones are not believed to exert their effects upon the liver via adenylate cyclase and cyclic AMP (Kirk *et al.*, 1979; Keppens & De Wulf, 1976). The results in Table 10 therefore support the notion that cyclic AMP affects glucokinase synthesis in some way (see also Seitz *et al.*, 1979). There is no evidence for inactivation of glucokinase activity by phosphorylation via a cyclic AMP-dependent protein kinase (Weinhouse, 1976).

Several factors thus emerge from this study that can play a role in preventing precocious appearance of glucokinase activity. All these interact *in vivo*, as studied here. The competence of the liver to be able to make glucokinase at younger ages having been established (Partridge *et al.*, 1975; Wakelam *et al.*, 1979), the normal appearance of glucokinase activity around day 16 in the neonatal rat requires a number of variables to be of the correct magnitude. No one event can be designated as the cause of this developmental phenomenon. This conclusion supports the comments made by Wright (1979) on 'cause-effect' relationships in biological systems. If, as seems likely, differential gene expression is being observed, then regulation by many factors appears to be involved. Further exploration of these controls and their detailed mechanism of regulating glucokinase synthesis must await studies on simpler systems, where, for example, the truly hepatic events can be separated from extrahepatic changes. Isolated hepatocytes in culture may prove to be a suitable experimental system for such studies, as shown to be possible for adult rat hepatocytes (Katz *et al.*, 1979; Schudt, 1979). In both of these papers a permissive effect of glucocorticoids on glucokinase induction *in vitro* has been described. Our experiments *in vivo* have been performed on neonatal rats at an age when circulating glucocorticoids are low (Greengard, 1975), although the cytosolic receptor protein for glucocorticoids appeared and hormone responsiveness was acquired during gestation (Oliver, 1979). The role of glucocorticoids in the neonatal development of glucokinase needs further investigation.

This work was supported by a project grant from the Medical Research Council (U.K.) to D. G. W. and Dr. A. Cornish-Bowden and Dr. I. P. Trayer. M. J. O. W. holds a Training Studentship in Research Methods from the same Council.

References

- Adelman, R. C. & Freeman, C. (1972) *Endocrinology* **90**, 1551–1560
- Allen, M. B., Aragon, C., Gimenez, C. & Walker, D. G. (1978) *Biochem. Soc. Trans.* **6**, 176–177
- Beaudry, M.-A., Chiasson, J.-L. & Exton, J. H. (1977) *Am. J. Physiol.* **233**, E175–E180
- Blazquez, E., Montova, E. & Lopez, C. (1970) *J. Endocrinol.* **48**, 553–561
- Blazquez, E., Sugase, T., Blazquez, M. & Foa, P. P. (1972) *Acta Diabetol. Latinoam.* **9**, Suppl. 1, 13–35
- Brown, J. C. & Otte, S. C. (1978) *Diabetes* **27**, 782–787
- Carrick, D. T. (1979) Ph.D. Thesis, University of Birmingham
- Christofferson, T., Morland, J., Osnes, J. B. & Oye, I. (1973) *Biochim. Biophys. Acta* **313**, 338–349
- Dahlqvist, A. (1978) *Int. Rev. Biochem.* **16**, 179–207
- DiMarco, P. N. & Oliver, I. T. (1978) *Eur. J. Biochem.* **87**, 235–241
- Dymsza, H. A., Czajka, D. M. & Miller, S. A. (1964) *J. Nutr.* **84**, 100–106
- Girard, J. R., Kervran, A. & Assan, R. (1975) in *Early Diabetes in Early Life* (Camerini-Davalose, R. A. & Cole, H. S., eds.), pp. 57–70, Academic Press, New York
- Greengard, O. (1971) *Essays Biochem.* **7**, 159–205
- Greengard, O. (1975) *J. Steroid Biochem.* **6**, 639–642
- Grossbard, L. & Schimke, R. T. (1966) *J. Biol. Chem.* **241**, 3546–3560
- Höhn, P., Schäfer, A. & Gabbert, H. (1977) *Mech. Ageing Dev.* **6**, 35–51
- Holroyde, M. J., Allen, M. B., Storer, A. C., Warsy, A. S., Chesher, J. M. E., Trayer, I. P., Cornish-Bowden, A. & Walker, D. G. (1976) *Biochem. J.* **153**, 363–373
- Jamdar, S. C. & Greengard, O. (1970) *J. Biol. Chem.* **245**, 2779–2783
- Katz, N. R., Nauck, M. A. & Wilson, P. T. (1979) *Biochem. Biophys. Res. Commun.* **88**, 23–29
- Keppens, S. & DeWulf, H. (1976) *FEBS Lett.* **68**, 279–282
- Kirk, C. J., Rodrigues, L. M. & Hems, D. A. (1979) *Biochem. J.* **178**, 493–496
- Marks, V. & Turner, D. S. (1977) *Essays Med. Biochem.* **3**, 109–152
- Macy, I. G., Kelly, H. J. & Sloan, R. E. (1953) *N.A.S.N.R.C. Publ.* 254
- McMichael, H. B. (1975) *Biochem. Soc. Trans.* **3**, 223–227
- Miller, S. A. & Dymaza, H. A. (1963) *Science* **141**, 517–518
- Morgan, L. M., Wright, J. W. & Marks, V. (1979) *Diabetologia* **16**, 235–239
- Niemeyer, H., Perez, N. & Rabajille, E. (1966) *J. Biol. Chem.* **241**, 4055–4059
- Niemeyer, H., Ureta, T. & Clark-Turri, L. (1975) *Mol. Cell. Biochem.* **6**, 109–126
- Novak, E., Drummond, G. I., Skala, J. & Hahn, P. (1972) *Arch. Biochem. Biophys.* **150**, 511–518
- Oliver, I. T. (1979) *Proc. Aust. Biochem. Soc.* **12**, Q26
- Partridge, N. C., Hoh, C. H., Weaver, P. K. & Oliver, I. T. (1975) *Eur. J. Biochem.* **51**, 49–54
- Pederson, R. A. & Brown, J. C. (1978) *Endocrinology* **103**, 610–615
- Pilkis, S. J. (1970) *Biochim. Biophys. Acta* **215**, 461–476
- Ross, S. A. & Dupre, J. (1978) *Diabetes* **27**, 327–333
- Schudt, C. (1979) *Eur. J. Biochem.* **98**, 77–82
- Seitz, H. J., Lüth, W. & Tarnowski, W. (1979) *Arch. Biochem. Biophys.* **195**, 385–391
- Simon, E., Scow, R. O. & Chernick, S. S. (1961) *Am. J. Physiol.* **201**, 1073–1077
- Storer, A. C. & Cornish-Bowden, A. (1974) *Biochem. J.* **141**, 205–209
- Ureta, T., Radojkovic, J. & Niemeyer, H. (1970) *J. Biol. Chem.* **245**, 4819–4824
- Vernon, R. G. & Walker, D. G. (1968) *Biochem. J.* **106**, 331–338
- Wakelam, M. J. O., Aragon, C., Gimenez, C., Allen, M. B., Walker, D. G. (1979) *Eur. J. Biochem.* **100**, 467–475
- Walker, D. G. & Eaton, S. W. (1967) *Biochem. J.* **105**, 771–777
- Walker, D. G. & Holland, G. (1965) *Biochem. J.* **97**, 845–854
- Walker, D. G. & Rao, S. (1964) *Biochem. J.* **90**, 360–368
- Walker, D. G., Khan, H. H. & Eaton, S. W. (1965/66) *Biol. Neonat.* **9**, 224–239
- Walker, D. G., Allen, M. B., Wakelam, M. J. O., Gimenez, C. & Aragon, C. (1978) *Arch. Biol. Med. Exp.* **11**, 27
- Walker, P. R., Bonney, R. J. & Potter, V. R. (1974) *Biochem. J.* **140**, 523–529
- Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50
- Wright, B. E. (1979) *Trends Biochem. Sci.* **4**, N110–N111