The Development of Hepatic Glucokinase in the Neonatal Rat

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1. Glucokinase and hexokinase activities have been determined in the livers of newborn rats and attempts made to influence *in vivo* the development of the glucokinase. 2. Glucokinase first appears in rat liver about 16 days after birth and adult activities are reached 10–12 days later. Evidence is presented which indicates that this represents synthesis of new protein. Hexokinase activities remain constant throughout the period of glucokinase development. 3. Both exogenous glucose and insulin are necessary for the natural development of glucokinase, for this is retarded in starved and alloxan-diabetic neonatal rats. 4. The absence of glucokinase during the first 2 weeks of extrauterine life in the rat is not due to lack of insulin. 5. Attempts to advance the time at which glucokinase first appears by infusions of glucose, insulin and chlorpropamide alone and in various combinations have resulted in marginal effects only. 6. When rats are starved for 3 days during the period of glucokinase development and then re-fed, glucokinase is more rapidly synthesized, indicating that the potential ability to synthesize glucokinase continues to develop throughout the period of starvation. 7. Some possible reasons for the comparatively late development of glucokinase are discussed.

The first report (Walker, 1962) that adult liver contains two enzymes capable of catalysing the phosphorylation of glucose by ATP depended on a comparison of the effects of glucose concentration on the rate of phosphorylation of glucose by dialysed preparations of the supernatant fractions of homogenates of adult and foetal liver. Whereas foetal liver appears to contain only a single hexokinase (EC 2.7.1.1) having a low K_m value for glucose, adult liver contains both the hexokinase and an enzyme having a high K_m (about 10mM) that has been designated glucokinase (EC 2.7.1.2). These observations have subsequently been extended and confirmed (e.g. DiPietro, Sharma & Weinhouse, 1962; Walker, 1963*b*; Viñuela, Salas & Sols, 1963), and the adaptive nature of the glucokinase has been demonstrated (Salas, Viñuela & Sols, 1963; Sharma, Manjeshwar & Weinhouse, 1963; Walker & Rao, 1964). It is changes in the glucokinase activity that account for changes in the total glucose-phosphorylating capacity of the liver (e.g. DiPietro & Weinhouse, 1960; Niemeyer, Clark-Turri & Rabajille, 1963; Blumenthal, Abraham & Chaikoff, 1964; Borrebaek, Abraham & Chaikoff, 1964).

The glucokinase must first appear at some stage during the growth of the animal. Preliminary observations with dialysed liver-supernatant preparations indicated that glucokinase develops

immediately after birth in the guinea pig, whereas in the rat the enzyme is not detectable until some days after birth (Walker, 1963*b*). The present paper reports in detail on the progress of the development of glucokinase in the neonatal rat, and examines some of the factors responsible for the control of this development. Evidence is presented which suggests that glucokinase cannot be synthesized until a certain stage of maturation of the liver cell is reached. Brief accounts of some of these results have appeared (Walker, 1964, 1965).

MATERIALS AND METHODS

Animals. The rats were of the Wistar albino strain maintained as described by Walker & Rao (1964). Very young animals were selected at random from a large breeding colony for the normal development study. Birth dates of all litters were carefully recorded after daily inspection. For the study of effects of various treatments on glucokinase development, litters of rats were selected having ten to 12 animals/litter. Any obviously weakling animals were rejected.

Special care was taken when starving neonatal rats owing to their being poikilothermic. Such animals were kept in small boxes close to a radiator so that the ambient temperature was about 28°. The younger animals, which could not drink water from the normal bottles, were offered water by teat pipette three times daily.

The production of alloxan-diabetes in neonatal rats

proved extremely difficult owing to a wide range of tolerance to the drug. Use of the procedure employed for adult rats by Walker & Rao (1964) resulted in less than 20% of the young animals becoming diabetic (assessed on the basis of lack of weight increase and glucosuria). The most successful procedure employed was intraperitoneal injection of animals previously starved overnight with alloxan monohydrate in the form of a 2.5% (w/v) solution in 0.1M-sodium citrate buffer, pH 5, at the rate of 250 mg. of alloxan/kg. body wt. The young alloxan-diabetic rats treated with insulin were given 2 units of soluble insulin (Burroughs Wellcome and Co., London)/100 g. body wt. on the first day and 4 units of protamine-zinc-insulin B.P. Novo (Evans Medical Ltd., Speke, Liverpool)/100 g. body wt. on each of 3 days (see below).

Litters of young rats were divided into groups by ear-marking but left with their mothers. One group remained untreated as normal control animals. Insulin was administered intramuscularly and all other agents by intraperitoneal injection of solutions or suspensions in 0.9% NaCl in the following dosages (all expressed/100 g. body wt.): glucose, 2 m-moles (18%, w/v); chlorpropamide, 5 mg. (1%, w/v); phenylalanine, 100 mg. (2%, w/v); *p*-fluorophenylalanine, 50 mg. (1%, w/v); methionine, 100 mg. (2%, w/v); ethionine, 50 mg. (1%, w/v); actinomycin D, 0.07 mg. (1.75 mg./100 ml.; 1 ml./25 g. body wt.). Details of courses of injections are given in the Results section alongside the relevant results. In general, the young animals tolerated these treatments surprisingly well and the few animals that did appear to be extremely sick were omitted from the experiments.

Chemicals and enzyme. ATP (disodium salt), NADP⁺ (sodium salt), L-methionine and L-ethionine were obtained from Sigma (London) Chemical Co. Ltd.; 2-mercaptoethanol was from L. Light and Co. Ltd., Colnbrook, Bucks.; glucose-determination kits and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were from C. F. Boehringer und Soehne G.m.b.H (obtained through Courtin and Warner Ltd., Lewes, Sussex); L-β-phenyl-α-alanine and alloxan were from Hopkin and Williams Ltd., Chadwell Heath, Essex; DL-*p*-fluorophenylalanine (A grade) was from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; chlorpropamide (*N*-chlorobenzene-*p*-sulphonyl-*N'*-propylurea) was from Pfizer Ltd., Sandwich, Kent. Actinomycin D was a generous gift from Merck, Sharp and Dohme International, New York, N.Y., U.S.A., through the courtesy of Dr Horace D. Brown. All other reagents were as described by Walker & Rao (1964).

Determination of glucokinase and hexokinase. The procedures used followed the general pattern described by Walker & Rao (1964) but with certain modifications described below. The livers from more than one young animal were pooled when necessary as indicated in the Tables. Homogenates (33.3%, w/v) were prepared in a medium containing: KCl (0.15M); EDTA (sodium salt) (5mM); MgCl₂ (5mM); mercaptoethanol (10mM); final pH 7.3. Glucose phosphorylation was estimated by coupling the glucose 6-phosphate formed to NADP⁺ in the presence of exogenous glucose 6-phosphate dehydrogenase in incubation mixtures (total vol. 1.5 ml.) containing (final concentrations): glycylglycine buffer (50mM), adjusted to pH 7.5 with NaOH; MgCl₂ (7.5mM); ATP (5mM); NADP⁺ (0.5mM); 0.2 international unit of glucose 6-phosphate dehydrogenase; 0.02 ml. of liver-supernatant preparation

(prepared at 100000g for 60 min. in an MSE Super-Speed 40 refrigerated centrifuge, 8 × 10 ml. angle rotor); glucose (100mM or 0.5mM). The temperature used was 28° and NADPH production was recorded on a Beckman recorder coupled to a Beckman model DB spectrophotometer. Glucokinase is calculated from the difference in rates with 100mM- and 0.5mM-glucose, and hexokinase from the difference in rates with 0.5mM-glucose in the presence and absence of ATP (Walker & Rao, 1964; Salas *et al.* 1963). Frequent checks were also made with no ATP but 100mM-glucose to ensure that none of the recorded activity was due to the presence of glucose dehydrogenase [β-D-glucose-NAD(P) oxidoreductase, EC 1.1.1.47], which also has a high *K_m* for glucose and will also reduce NADP⁺ (Metzger, Wilcox & Wick, 1964), either in the rat-liver preparation or in the added glucose 6-phosphate dehydrogenase.

Two additional points about methods must be made at this stage. (a) Duplicate assays with crude supernatant preparations showed that the rates of NADPH production were reproducible to within ± 0.002 extinction unit/min. However, when the glucokinase activity is low its measurement depends on the small difference of rates of NADPH production with 100mM- and 0.5mM-glucose and the percentage error of reproducibility is correspondingly greater. Thus glucokinase activities lower than about 0.05 μmole/min./g. wet wt. of liver are subject to considerable error. (b) NADPH formation is due to oxidation of the glucose 6-phosphate formed being oxidized in the presence of exogenous glucose 6-phosphate dehydrogenase and also endogenous 6-phosphoglucanate dehydrogenase (EC 1.1.1.44). Some workers (e.g. Blumenthal *et al.* 1964) express results as μmoles of NADPH formed and others (e.g. Viñuela *et al.* 1963; Salas *et al.* 1963) divide this by a factor between 1.5 and 2 to correct for the NADPH production by the second dehydrogenase and then express results as μmoles of glucose phosphorylated. We have preferred to continue to divide the rate of NADP⁺ reduction by 2 (DiPietro & Weinhouse, 1960; Sharma *et al.* 1963; Walker, 1963b; Walker & Rao, 1964) for reasons previously stated, because the activities of 6-phosphoglucanate dehydrogenase do not change significantly during the period of development under study (Lea & Walker, 1964), and particularly because rates of glucose phosphorylation *in vivo* are unlikely in any event to reach those recorded *in vitro* with 100mM-glucose (Walker & Rao, 1964). Rates of glucose phosphorylation are expressed per g. wet wt. of liver, per whole liver and per 100 g. body wt. Statistical significance of means and differences were calculated by a *t* test.

Blood glucose and glucose tolerance. Blood glucose was determined as described by Walker & Rao (1964). Glucose tolerance was estimated in animals previously starved overnight by giving glucose intragastrically [1g./kg. body wt. as a 20% (w/v) solution in water] by stomach tubes (made of fine nylon tubing) and killing the animals at specified times later. Litters of young rats were used in a procedure essentially the same as that used for fructose tolerance in neonatal rabbits by Walker (1963a).

RESULTS

Preliminary evidence about the post-natal appearance of glucokinase in the rat was obtained with dialysed liver-supernatant preparations (Walker,

Table 1. *Activities of the glucose-phosphorylating enzymes in the livers of neonatal rats during the period of development of hepatic glucokinase*
 Each result represents a single animal taken at random from the breeding colony. Details of the assay procedure are given in the text. The results are given as means $\pm$ S.D.

Age (days)	No. of animals	Body wt. (g.)	Liver wt. (g.)	100 $\times$ Mean liver wt./mean body wt.	Glucose-phosphorylating activity (μ moles of glucose phosphorylated/min.)					
					Glucokinase			Hexokinase		
					(per g.)	(per whole liver)	(per 100g. body wt.)	(per g.)	(per whole liver)	(per 100g. body wt.)
1-14	34	—	—	—	0.02 $\pm$ 0.02	—	—	0.33 $\pm$ 0.17	—	—
15	10	27.3 $\pm$ 3.1	0.92 $\pm$ 0.14	3.4	0.03 $\pm$ 0.02	0.02 $\pm$ 0.02	0.08 $\pm$ 0.08	0.33 $\pm$ 0.11	0.30 $\pm$ 0.09	1.1 $\pm$ 0.4
16	5	30.5 $\pm$ 5.6	1.09 $\pm$ 0.24	3.6	0.05 $\pm$ 0.04	0.06 $\pm$ 0.05	0.17 $\pm$ 0.15	0.36 $\pm$ 0.08	0.39 $\pm$ 0.09	1.3 $\pm$ 0.1
17	5	34.2 $\pm$ 8.5	1.21 $\pm$ 0.37	3.5	0.06 $\pm$ 0.05	0.08 $\pm$ 0.08	0.23 $\pm$ 0.05	0.53 $\pm$ 0.22	0.38 $\pm$ 0.19	1.0 $\pm$ 0.7
18	4	31.5 $\pm$ 3.2	1.25 $\pm$ 0.21	4.0	0.06 $\pm$ 0.06	0.09 $\pm$ 0.06	0.24 $\pm$ 0.15	0.27 $\pm$ 0.08	0.33 $\pm$ 0.06	1.1 $\pm$ 0.2
19	4	35.7 $\pm$ 5.2	1.47 $\pm$ 0.04	4.1	0.19 $\pm$ 0.10	0.31 $\pm$ 0.21	0.81 $\pm$ 0.41	0.26 $\pm$ 0.05	0.39 $\pm$ 0.04	1.1 $\pm$ 0.1
20	5	42.5 $\pm$ 7.2	1.77 $\pm$ 0.35	4.2	0.23 $\pm$ 0.07	0.39 $\pm$ 0.06	0.97 $\pm$ 0.09	0.25 $\pm$ 0.10	0.44 $\pm$ 0.16	1.0 $\pm$ 0.4
21	21	36.0 $\pm$ 7.2	1.47 $\pm$ 0.31	4.1	0.37 $\pm$ 0.26	0.58 $\pm$ 0.40	1.48 $\pm$ 1.09	0.28 $\pm$ 0.15	0.43 $\pm$ 0.29	1.1 $\pm$ 0.6
22	10	41.0 $\pm$ 7.5	1.71 $\pm$ 0.37	4.2	0.34 $\pm$ 0.09	0.59 $\pm$ 0.21	1.44 $\pm$ 0.35	0.21 $\pm$ 0.09	0.36 $\pm$ 0.14	0.9 $\pm$ 0.3
23	17	45.0 $\pm$ 6.6	2.07 $\pm$ 0.38	4.6	0.63 $\pm$ 0.42	1.32 $\pm$ 0.92	2.84 $\pm$ 1.84	0.24 $\pm$ 0.11	0.50 $\pm$ 0.23	1.1 $\pm$ 0.5
24	21	47.0 $\pm$ 5.7	2.00 $\pm$ 0.23	4.3	0.65 $\pm$ 0.27	1.31 $\pm$ 0.42	2.84 $\pm$ 1.18	0.30 $\pm$ 0.15	0.61 $\pm$ 0.28	1.3 $\pm$ 0.7
25	20	53.1 $\pm$ 8.8	2.23 $\pm$ 0.44	4.2	0.73 $\pm$ 0.24	1.72 $\pm$ 0.81	3.14 $\pm$ 1.22	0.27 $\pm$ 0.14	0.59 $\pm$ 0.29	1.2 $\pm$ 0.2
26	3	53.6 $\pm$ 11.9	2.27 $\pm$ 0.54	4.2	1.02 $\pm$ 0.33	2.34 $\pm$ 0.95	4.36 $\pm$ 0.70	0.29 $\pm$ 0.03	0.65 $\pm$ 0.16	1.2 $\pm$ 0.1
27	8	61.3 $\pm$ 7.8	2.85 $\pm$ 0.66	4.7	1.11 $\pm$ 0.37	3.09 $\pm$ 1.10	5.11 $\pm$ 1.76	0.28 $\pm$ 0.03	0.81 $\pm$ 0.40	1.3 $\pm$ 0.5
28	4	67.1 $\pm$ 4.4	3.13 $\pm$ 0.23	4.7	1.37 $\pm$ 0.08	4.27 $\pm$ 0.33	6.36 $\pm$ 0.35	0.22 $\pm$ 0.10	0.71 $\pm$ 0.35	1.0 $\pm$ 0.5
29	6	61.2 $\pm$ 5.2	2.87 $\pm$ 0.28	4.7	1.20 $\pm$ 0.53	3.52 $\pm$ 1.76	5.57 $\pm$ 2.30	0.26 $\pm$ 0.04	0.75 $\pm$ 0.20	1.2 $\pm$ 0.2
30	5	59.6 $\pm$ 14.0	2.63 $\pm$ 0.87	4.4	1.23 $\pm$ 0.35	3.23 $\pm$ 1.21	5.50 $\pm$ 1.73	0.28 $\pm$ 0.14	0.78 $\pm$ 0.44	1.3 $\pm$ 0.7
Adult males*	20	191 $\pm$ 29	8.43 $\pm$ 1.65	4.4	1.24 $\pm$ 0.35	10.6 $\pm$ 3.9	5.5 $\pm$ 1.8	0.43 $\pm$ 0.09	3.5 $\pm$ 1.2	1.4 $\pm$ 0.5

* Results reported previously (Walker & Rao, 1964) and confirmed by control assays on adult animals during the present study.

1963b). Dialysis invariably leads to at least some loss of glucokinase activity and it is clearly advantageous to use fresh supernatant preparations. The accuracy of the determination is then subject only to the limitations mentioned in the Materials and Methods section with particular reference to the comments on low glucokinase activities.

Table 1 shows that glucokinase is first detectable in rat liver about 15 or 16 days after birth. The rate of appearance of the enzyme is slow at first but then increases, the maximum rate of increase occurring during the period 21–25 days, with adult activities being recorded from about 27 days of age onwards. The range of activities at any one age were considerable, as indicated by s.d. values. These biological variations were nearly as great between siblings as between animals from various litters. No differences were noted between the sexes with respect to the development of the glucokinase. There was no correlation between either the age at which hepatic glucokinase activity could be detected or the age at which half adult activities were attained and the body weight of the neonatal animal at that time.

Throughout the whole of this period and also during the first 2 weeks of post-natal life the hexokinase activity remains essentially constant. A group of six determinations of hexokinase activity in foetal livers from rats of gestational age 19–21 days have activities of 0.31–0.61 μ mole of glucose phosphorylated/min./g. of liver (cf. Burch *et al.* 1963).

The slow development and the wide variation in glucokinase activities, especially when the variation is considered as a percentage of the mean, has imposed a severe restriction on the design of further experiments on factors affecting the development of the glucokinase. First, the most suitable control animals would be littermates. This limits any one experiment to a maximum of about ten animals

including both experimental and control animals. Secondly, a period of 3 days appears to be the minimum time over which a treatment must be given to permit a significant natural increase in the glucokinase activities in the control animals against which the effect of treatment may be assessed.

Evidence that glucokinase development represents synthesis of new protein. The low rate of appearance of glucokinase suggests that synthesis of new protein is involved, for an enzymic activation process is unlikely to take 10–12 days to complete. Tests were performed designed to detect the presence of an activator in adult liver or an inhibitor in foetal and immediately post-natal liver that would affect the glucokinase activity of liver preparations measured *in vitro*. These included the mixing of liver-supernatant preparations made from animals of various stages of development, the mixing of homogenates similarly before preparing mixed supernatant samples and the effects of short periods of incubation of mixed preparations before assay. Hexokinase and glucokinase activities of mixed samples were always the calculated arithmetic sum of the activities of the component liver-supernatant preparations within the limits of experimental error.

Agents known to inhibit synthesis of protein seriously retarded the natural development of glucokinase. Litters of rats were treated with an amino acid antimetabolite alone or together with the natural analogue (Table 2). Simultaneous treatment of animals with methionine overcame an inhibitory effect of ethionine, and phenylalanine prevented inhibition of glucokinase development by *p*-fluorophenylalanine. All animals remained with their mothers during the experimental period so that individual food intakes could not be measured. Food (both milk and solids) was present in the stomachs of all animals when killed and the liver wt./body wt. ratio remained normal. The

Table 2. *Effect of inhibitors of protein synthesis on the development of glucokinase in the neonatal rat*

Each experiment was performed on one litter of young rats. They were treated with the antimetabolite with or without the natural analogue on each of 3 days as described in the Materials and Methods section when 21–23 days old. On day 24 they were killed and enzyme assays performed as described in the text. The animals remained with the mothers throughout the period of treatment and the stomachs of all the animals (including those treated with either ethionine or *p*-fluorophenylalanine only) contained both curdled milk and solid food when the animals were killed. The other animals of the litters were used to establish normal values for the litter. Results are given as means $\pm$ S.E.M. Similar patterns of results were obtained in duplicate experiments on other litters.

Expt. no.	Total no. of animals in litter	Treatment	No. of animals	Body wt. (g.)	Liver wt. (g.)	100 $\times$ Mean liver wt./mean body wt.	Activity (μ mole of glucose phosphorylated/min./g. of liver)		
							Total	Glucokinase	Hexokinase
1	9	Ethionine	3	36 $\pm$ 2	1.55 $\pm$ 0.02	4.3	0.44 $\pm$ 0.02	0.02 $\pm$ 0.02	0.42 $\pm$ 0.06
		Ethionine + methionine	4	43 $\pm$ 4	1.85 $\pm$ 0.19	4.3	0.59 $\pm$ 0.07	0.33 $\pm$ 0.04	0.26 $\pm$ 0.10
2	10	<i>p</i> -Fluorophenylalanine	3	38 $\pm$ 1	1.73 $\pm$ 0.04	4.5	0.79 $\pm$ 0.07	0.40 $\pm$ 0.07	0.39 $\pm$ 0.03
		<i>p</i> -Fluorophenylalanine + phenylalanine	3	42 $\pm$ 1	1.87 $\pm$ 0.08	4.5	1.10 $\pm$ 0.12	0.71 $\pm$ 0.08	0.39 $\pm$ 0.06

effects of the inhibitors were not therefore due to starvation. Neonatal animals from other litters were treated with either puromycin or actinomycin D (for doses see the Materials and Methods section) during the period 21–24 days old. These antibiotics proved extremely toxic to the neonatal animals and only a few animals survived the 3-day period of treatment; these all had low glucokinase activities, suggesting that marked inhibition of glucokinase had occurred.

Glucose tolerance and insulin activity in the newborn rat. The maintenance of normal glucokinase in the adult rat depends on adequate dietary carbohydrate and insulin (Salas *et al.* 1963; Sharma *et al.* 1963; Walker & Rao, 1964; Blumenthal *et al.* 1964). The stomach of the newborn rat left with its mother is never empty, so that dietary carbohydrate is never lacking from the time of the first feed of milk within a few hours of birth. Insulin is present in the β -cells of the islets of Langerhans of rat pancreas from about the seventeenth day of gestation (Dixit, Lowe, Heggested & Lazarow, 1964). Blood glucose concentrations in the neonatal rat (Dawkins, 1963) are essentially normal compared with adult animals, and the young rat cannot be considered as being diabetic from the point of view of 'fasting' blood glucose concentrations, glucosuria, ketonuria etc.

Oral glucose tolerance tests were performed on several litters of rats at ages both before and after the development of glucokinase. Animals up to 16 days old started with slightly higher blood glucose concentrations, probably because of the small amount of food remaining in the stomach at the end of the 16 hr. period of starvation. Glucose tolerance in litters 1L, 1G and 1I (Table 3) was abnormal, but in litters 1K and 1J the oral dose of 1 mg. of glucose/g. body wt. was very efficiently tolerated. This difference correlates with the development of hepatic glucokinase but could also be due to a lack of an insulinogenic response to the glucose in the younger litters of animals. Insulin (soluble)

was injected intramuscularly into young rats 8–13 days old and the blood glucose concentrations were determined 4 hr. later. The blood glucose concentration was lowered by insulin (doses of 1–2 units/100 g. body wt.) from the control value of 111 ± 6 mg./100 ml. of blood to 41 ± 3 (means $\pm$ S.E.M.) ($P < 0.01$). Little extra effect was seen with larger amounts of insulin (4–10 units). Chlorpropamide given intraperitoneally had little or no effect on the blood glucose concentration after 4 hr., but given by stomach tube it caused a significant fall in the blood glucose concentration in both starved and fed animals (47 ± 5 and 69 ± 10 mg./100 ml. of blood respectively; $P < 0.01$). Animals examined between 8 and 24 hr. after the intraperitoneal injection of chlorpropamide had lower-than-normal blood glucose concentrations, suggesting that administration by this route resulted in a slower but longer-lasting effect. It is concluded that chlorpropamide exerted an insulinogenic response in the young rats. There is no reason to suppose that glucose could not also cause insulin release, although direct blood insulin assays have not been performed.

Attempts to advance the age at which glucokinase first appears. If endogenous insulin can be released by normal insulinogenic stimuli then there is no apparent reason why glucokinase does not appear earlier. Attempts to bring forward the age at which glucokinase develops by treating neonatal rats with glucose, which is both the substrate of glucokinase and also an insulinogenic agent, with the insulinogenic drug chlorpropamide and with exogenous insulin were essentially negative. Half of three litters of rats were injected intraperitoneally with 2 m-moles of glucose/100 g. body wt. each day from the age of 8 days until they were killed at 14, 16 or 18 days. Glucose treatment raised the glucokinase activities slightly but the difference was only statistically significant at 14 days of age (controls, 0.05 ± 0.02 ; glucose-treated, 0.13 ± 0.01 ; $P < 0.01$). Glucose treatment of other animals commencing

Table 3. *Glucose tolerance in neonatal rats before and after the development of hepatic glucokinase*

All animals were previously starved overnight (16 hr.) and then given glucose (1 mg./g. body wt.) in the form of 4% (w/v) solution by stomach tube. The results presented statistically represent means $\pm$ S.D. with the numbers of separate determinations on individual animals in parentheses.

Litter code	No. in litter	Age (days)	Blood glucose (mg./100 ml. of blood) in individual animals at the given times after glucose administration orally				
			0	30 min.	1 hr.	2 hr.	3 hr.
1L	12	12	73, 75	126, 155	180, 166	176 ± 11 (3)	130 ± 14 (3)
1G	11	14	85, 86	181 ± 18 (3)	217 ± 18 (3)	214 ± 31 (3)	—
1I	13	16	98, 118	185, 210	237 ± 21 (3)	211 ± 65 (3)	171 ± 18 (3)
1K	9	28	50, 48	—	83, 89	79, 84	77 ± 2 (3)
1J	10	29	55, 56	—	86, 88	78 ± 4 (3)	78 ± 7 (3)

at various ages from 3 days to 12 days old resulted in no other significant advance in glucokinase development over that in the control littermates.

Neonatal rats were treated with insulin, without and with glucose to prevent the hypoglycaemic effect of the insulin, and also with chlorpropamide. Over 120 animals from 14 litters were used for this series of experiments and in no instance was the glucokinase activity in an experimental group significantly higher than the corresponding control group of littermates. The non-appearance of glucokinase before a certain age and the subsequent low rate of development cannot therefore be due to a lack of the substrate, glucose, or of insulin.

Exogenous glucose and insulin are both necessary for maximum glucokinase activities in the adult rat. Because of this lack of positive effects of glucose and insulin before the age of about 14 days it was desirable to show that they are necessary for normal glucokinase development to proceed.

Effect of starvation and alloxan-diabetes on glucokinase development in the neonatal rat. Young rats starved at 15 days of age had negligible (less than $0.02 \mu\text{mole}$ of glucose phosphorylated/min./g. of liver) glucokinase activities when killed at 18 days of age. Similarly, animals starved for 3 days when 18–21 days old still had low activities at 21 days of age. Animals were starved over the period 21–24 days old and compared with normal fed littermates (Table 4). The starved animals showed significantly lower glucokinase activities after 48 hr. of starvation and thereafter. Glucokinase does not completely disappear, however, in starvation, as observed for adult animals by Walker & Rao (1964).

The production of alloxan-diabetes in neonatal rats proved very difficult. The dose response was extremely varied and the number of animals that survived the treatment and became grossly glycosuric was only about 20% of those injected. Table 5 presents results which indicate that alloxan-diabetic animals at 24 days old had negligible glucokinase activities, whereas insulin-treated alloxan-diabetic animals of the same age possessed normal glucokinase activities for that age. Insulin is therefore necessary for normal glucokinase development in the neonatal animal.

Effect of re-feeding on glucokinase activities in starved neonatal rats. Inspection of the results of different groups of workers (e.g. Salas *et al.* 1963; Sharma *et al.* 1963) reveals some variations in the time taken for normal glucokinase activities to be reached when starved adult rats are re-fed. This in part depends on the magnitude of the decrease in activity during the starvation period. However, a period of 16 hr. (i.e. overnight) is always adequate and this has been confirmed for adult rats by us.

Neonatal rats were starved for 3 days during the period when the rate of increase of glucokinase

Table 4. *Effect of starvation on the development of glucokinase in the neonatal rat*

Animals	Age of animals when killed (days)	No. of animals	Body wt. (g.)	Liver wt. (g.)	100 × Mean liver wt./mean body wt.	Activity (μmoles of glucose phosphorylated/min.) (per g. of liver)				Significance of difference from normal animals of the same age for the activities of glucokinase expressed		
						Total		Glucokinase		Per g. of liver	Per 100 g. body wt.	
						Glucokinase	Hexokinase	Glucokinase	Hexokinase			
Normal fed rats	21	3	35 ± 1	1.64 ± 0.12	4.7	0.52 ± 0.12	0.36 ± 0.10	0.16 ± 0.12	2.42 ± 0.54	1.89 ± 0.44	0.73 ± 0.49	
	22	3	39 ± 3	1.75 ± 0.13	4.5	0.52 ± 0.09	0.46 ± 0.06	0.15 ± 0.03	2.40 ± 0.26	1.81 ± 0.19	0.58 ± 0.14	
	23	3	42 ± 1	1.97 ± 0.15	4.7	0.64 ± 0.05	0.46 ± 0.09	0.16 ± 0.05	3.16 ± 0.43	2.11 ± 0.45	1.01 ± 0.34	
	24	3	48 ± 1	2.30 ± 0.06	4.6	0.71 ± 0.19	0.59 ± 0.09	0.12 ± 0.10	3.75 ± 0.77	2.81 ± 0.44	0.94 ± 0.37	
Rats starved from when 21 days old	22	3	31 ± 2	1.03 ± 0.08	3.3	0.39 ± 0.18	0.27 ± 0.14	0.12 ± 0.02	1.30 ± 0.75	0.89 ± 0.77	0.41 ± 0.02	0.2 < P < 0.3
	23	6	27 ± 2	0.90 ± 0.17	3.3	0.31 ± 0.15	0.15 ± 0.13	0.15 ± 0.04	1.06 ± 0.69	0.55 ± 0.52	0.51 ± 0.18	0.02 < P < 0.05
	24	6	26 ± 1	0.90 ± 0.16	3.5	0.34 ± 0.02	0.22 ± 0.03	0.12 ± 0.03	1.18 ± 0.08	0.78 ± 0.17	0.40 ± 0.11	0.01 < P < 0.02 P < 0.01

The 27 animals used in this experiment were from three litters each containing nine neonatal rats. Each group of three animals in the Table comprised one animal from each litter. Each result is for the liver of a single animal except for animals starved for 2 and 3 days where the livers from two animals were pooled for assay. Details of the assay procedure are given in the text. The results are given as means ± s.d.

Table 5. *Role of insulin in the development of hepatic glucokinase in the neonatal rat*

The animals used originated from three litters. The normal values quoted were for two animals from each litter. Alloxan-diabetes was induced at 17 days old by intraperitoneal injection, and insulin (2 units/100 mg. body wt. daily) was injected intramuscularly when animals were 21, 22 and 23 days old. All animals were killed when 24 days old for enzyme and blood glucose determinations; further details of these treatments and of the assay procedures are given in the text. Results are expressed as means $\pm$ S.E.M.

Description	No. of animals	Body wt. (g.)	Liver wt. (g.)	Weight increase over period 21-24 days old (g.)	Blood glucose (mg./100 ml. of blood)	Activity (μ moles of glucose phosphorylated/min./g. of liver)		
Normal	6	53 $\pm$ 3	2.20 $\pm$ 0.05	12 $\pm$ 1	112 $\pm$ 6	1.00 $\pm$ 0.18	0.75 $\pm$ 0.14	0.25 $\pm$ 0.05
Alloxan-diabetic	5	39 $\pm$ 2	2.03 $\pm$ 0.03	7 $\pm$ 2	381 $\pm$ 41	0.25 $\pm$ 0.04	0.04 $\pm$ 0.02	0.21 $\pm$ 0.04
Alloxan-diabetic insulin-treated	5	47 $\pm$ 4	2.11 $\pm$ 0.07	13 $\pm$ 2	153 $\pm$ 29	1.30 $\pm$ 0.20	1.08 $\pm$ 0.19	0.22 $\pm$ 0.02

activities during natural development proceeds at the maximum rate, i.e. in the age region 21-25 days. They were then re-fed with glucose by stomach tube and permitted access to normal diet, and killed 16 hr. later. The glucokinase activities (Table 6) in these animals were normal. Thus, although normal glucokinase development proceeds comparatively slowly and starvation results in lowered glucokinase activity, a rapid return to what are normal values for the given age of animal is achieved during the comparatively brief re-feeding period.

Indirect evidence that this rapid recovery was due to net synthesis of protein came from experiments in which neonatal animals were starved for the same 3-day period and then re-fed in the presence of known inhibitors of protein synthesis. The recovery of glucokinase activity on re-feeding was inhibited by *p*-fluorophenylalanine and actinomycin D, and the inhibitory effect of the former was essentially prevented by simultaneous injections of phenylalanine (Table 6).

DISCUSSION

The high apparent K_m value for glucose possessed by glucokinase provides an enzymic explanation for the dependence on glucose concentration of various aspects of glucose utilization by the liver. The development of the enzyme at a comparatively late stage of development of the animal represents one of the final steps of liver maturation and coincides with the time at which rats first consume solid foods (Murphy & Dunn, 1949; and our own observations). Until the enzyme has developed the immature animal lacks the means of homeostatic control that glucokinase is believed to provide.

The absence of glucokinase in foetal liver is probably physiologically unimportant. Control of the foetal blood glucose concentration can be maintained by the maternal liver, for glucose readily traverses the placenta (Widdas, 1961). The newborn rat during the first 2 weeks of extrauterine life can exert considerable control of its own carbo-

hydrate metabolism without possessing hepatic glucokinase. Marked hyperglycaemia is not observed and the only known significant difference is a lowered tolerance to glucose (Table 3). One implication is that hepatic glucokinase may be of limited importance in the normal utilization of glucose by the animal and exists primarily to act as an overflow mechanism, i.e. to phosphorylate glucose when the blood glucose concentration increases above normal and thence initiate net hepatic glucose utilization (glycogen formation and oxidation). As emphasized by Walker & Rao (1964), the glucokinase activities measured *in vitro* with 100 mM-glucose as substrate are never likely to be achieved *in vivo*, so that at all normal blood glucose concentrations the actual glucokinase activities may be only 25-50% of the maximum possible. This suggested confined role of glucokinase is perhaps additional support for the physiological concept (see, e.g., Weber, 1963, for enzymic substantiation of this) that, under conditions other than those of high blood glucose concentrations, the primary function of the liver is to make glucose rather than to utilize it (Pesch & Topper, 1963). The formation of respiratory carbon dioxide from glucose in the liver is low (Majchrowicz & Quastel, 1963), and fatty acids are a major energy source of the liver (Fritz, 1961).

Assuming that it is an essential first step, a fact that has been questioned (Figuerola, Pfeifer & Niemeyer, 1962), the phosphorylation of glucose by the liver in the absence of glucokinase presents some problems. The non-specific low- K_m hexokinase is available in the liver before glucokinase development (Burch *et al.* 1963; this paper), but its activity is low compared with that in other animal tissues (Long, 1952; Sols, 1964) and it may be subject to inhibition by glucose 6-phosphate (Crane & Sols, 1955). The finding of high hexokinase activities in carcinomas arising from non-parenchymal (e.g. bile duct) liver cells (Sharma, Manjeshwar & Weinhouse, 1964) suggested that hexokinase was not present in the parenchymal cells (Sols, Salas & Viñuela, 1964). However, the recent report (Sharma, Sharma, Donnelly, Morris & Weinhouse, 1965) of high

Table 6. *Reappearance of hepatic glucokinase in neonatal rats re-fed for 16 hr. after starvation for 3 days*Further details of the treatment of animals and of the assay procedure are given in the text. Results represent the means $\pm$ S.E.M.

Expt. no.	No. of litters involved	Age of animals when killed for assay (days)	Previous treatment of animals including age period of treatment	No. of individual results	Body wt. (g.)	Liver wt. (g.)	100 x Liver wt./body wt.	Activity (μ moles of glucose phosphorylated/min./g. of liver)		
								Total	Glucokinase	Hexokinase
1	2	22	Starved for 3 days (19-22 days)	4	23 $\pm$ 2	0.69 $\pm$ 0.07	3.0 $\pm$ 0.1	0.19 $\pm$ 0.01	0.08 $\pm$ 0.01	0.11 $\pm$ 0.01
		23	Normal, fed	4	47 $\pm$ 4	2.21 $\pm$ 0.18	4.7 $\pm$ 0.1	1.27 $\pm$ 0.13	0.91 $\pm$ 0.20	0.36 $\pm$ 0.07
		23	Starved for 3 days (19-22 days), then re-fed for 16 hr.	6	30 $\pm$ 3	1.62 $\pm$ 0.08	5.4 $\pm$ 0.1	1.20 $\pm$ 0.11	1.10 $\pm$ 0.06	0.10 $\pm$ 0.01
2	2	24	Starved for 3 days (21-24 days)	4	30 $\pm$ 3	1.07 $\pm$ 0.05	3.6 $\pm$ 0.1	0.80 $\pm$ 0.05	0.59 $\pm$ 0.01	0.22 $\pm$ 0.05
		25	Normal, fed	4	60 $\pm$ 4	2.71 $\pm$ 0.15	4.6 $\pm$ 0.1	1.42 $\pm$ 0.08	1.07 $\pm$ 0.01	0.36 $\pm$ 0.07
		25	Starved for 3 days (21-24 days), then re-fed for 16 hr.	6	41 $\pm$ 3	2.41 $\pm$ 0.37	5.8 $\pm$ 0.1	1.49 $\pm$ 0.08	1.35 $\pm$ 0.10	0.14 $\pm$ 0.01
3	3*	24	Starved for 3 days (20-23 days), then re-fed for 16 hr.	3	31 $\pm$ 4	1.26 $\pm$ 0.24	5.2 $\pm$ 0.4	0.98 $\pm$ 0.16	0.72 $\pm$ 0.14	0.26 $\pm$ 0.02
		24	Starved for 3 days (20-23 days), then re-fed for 16 hr., plus <i>p</i> -fluorophenylalanine on 23rd day	5	24 $\pm$ 1	0.87 $\pm$ 0.08	3.5 $\pm$ 0.3	0.44 $\pm$ 0.08	0.32 $\pm$ 0.11	0.12 $\pm$ 0.02
		24	Starved for 3 days (20-23 days), then re-fed for 16 hr., plus <i>p</i> -fluorophenylalanine and phenylalanine on 23rd day	4	26 $\pm$ 2	0.98 $\pm$ 0.17	3.7 $\pm$ 0.5	0.72 $\pm$ 0.04	0.57 $\pm$ 0.02	0.15 $\pm$ 0.01
4	3*	25	Starved for 3 days (21-24 days), then re-fed for 16 hr.	5	34 $\pm$ 3	1.89 $\pm$ 0.18	5.5 $\pm$ 0.1	1.25 $\pm$ 0.18	1.13 $\pm$ 0.18	0.11 $\pm$ 0.02
		25	Starved for 3 days (21-24 days), then re-fed for 16 hr., plus actinomycin D on 24th day	6	31 $\pm$ 3	1.27 $\pm$ 0.14	4.0 $\pm$ 0.2	0.48 $\pm$ 0.07	0.09 $\pm$ 0.02	0.39 $\pm$ 0.07

* Other animals in these litters were used to confirm that normal values were obtained in untreated animals.

hexokinase activities in certain parenchymal-cell hepatomas supports the suggestion (Walker, 1963b; Walker & Rao, 1964) that hexokinase and glucokinase are both present in the parenchymal cell. Other evidence obtained *in vitro* (Walker & Lea, 1964; Lea & Walker, 1965) implies that hexokinase can participate in glucose utilization by liver tissue from both foetal and neonatal rats under some conditions.

The low rate of development of glucokinase, the steady hexokinase activities during this period of glucokinase development, the absence of any indication of enzymic activators or inhibitors and the effects of known inhibitors of protein synthesis all suggest, but do not prove, that the development of the enzyme represents an initiation of and increasing ability to synthesize new protein. Dietary glucose and insulin are clearly necessary for normal development, but it is apparently not a lack of these agents that accounts for the absence of the enzyme before a certain stage of maturity. The suppression of glucokinase synthesis may be affected by physiological conditions existing in the neonatal animal the nature of which are at present unknown.

The most significant results (Table 6) indicate that, during a period of starvation imposed at a time when glucokinase activity would normally be increasing at a maximum rate, the potential ability to synthesize glucokinase increases even though the observed activity actually decreases. This increase reveals itself on re-feeding the starved animal, when a rapid increase in glucokinase activity ensues, and is prevented by *p*-fluorophenylalanine and actinomycin, as is the natural increase in activity during the normal period of development. Ethionine and *p*-fluorophenylalanine have been used previously to prevent glucokinase synthesis on re-feeding starved adult rats (Salas *et al.* 1963; Sharma *et al.* 1963). *p*-Fluorophenylalanine competes with its natural analogue in protein synthesis (Kit, Dubbs & Piekarski, 1962) at the stage of activation and attachment to transfer RNA (Arnstein & Richmond, 1964). The inhibitory effect of actinomycin D (Table 10), which inhibits protein synthesis by preventing the formation of messenger RNA (Reich, 1963), implies that the rapid increase in glucokinase depends on the formation of messenger RNA.

The interpretation of experiments that employ inhibitors of protein synthesis as evidence that an increase in measurable enzymic activity represents new synthesis of protein always assumes that the rate of breakdown of the enzyme protein remains constant, i.e. dependent only on the steady-state concentration of the enzyme. Recent work on hepatic tryptophan pyrrolase (Schimke, Sweeney & Berlin, 1964) and arginase (Schimke, 1964) clearly indicates that such an assumption is not always valid and that, in some mammalian metabolic

adaptations, marked changes in the rate of enzyme degradation can take place. Although rates of glucokinase breakdown have not been measured, it seems unlikely that the induction of enzyme synthesis, where the initial enzyme activity is virtually zero, could be due to a marked decrease in rate of degradation. Such a mechanism of enzyme induction would, in any case, be a biologically wasteful process.

An explanation is required as to how glucose and insulin affect glucokinase synthesis and the reason why they cannot exert their effect before a certain time. Considering the problem against the background of current models for the induction and regulation of protein synthesis (Jacob & Monod, 1961), either transcription or translation or both sites of the process might be affected. The dependence of the control of glucokinase synthesis on messenger-RNA formation is implied by the inhibitory action of actinomycin D. If insulin itself is necessary for glucokinase synthesis, and all the evidence from studies *in vivo* suggests that it is (Sharma *et al.* 1963; Salas *et al.* 1963), then we must ask whether it acts directly or indirectly (Steiner & King, 1964) as an inducer. Some of the evidence for the role of insulin as a protein anabolic agent has been mentioned by Walker & Rao (1964) (for a recent review see Lukens, 1964), and an effect of insulin on transcription is attractive in explaining how insulin can have a marked effect, not only on the synthesis of certain enzymes such as hepatic glucokinase and UDP-glucose-glycogen glucosyl-transferase (EC 2.4.1.11) (Steiner & King, 1964), but also in apparently repressing the stimulating effect of glucocorticosteroids on several of the key enzymes involved in hepatic gluconeogenesis (Weber, Singhal, Stamm, Fisher & Mentendiek, 1964).

From studies on the effect of insulin on messenger-RNA synthesis in muscle tissue, Wool & Moyer (1964) proposed that an effect of insulin on transcription cannot explain all the observed facts and they have suggested that insulin may also have an effect at the ribosomal level. Walker (1965) collated some of the changes in the physical nature of the protein-synthetic apparatus of the cell that are known to occur during the post-natal development of the liver. In view of the dependence of protein synthesis on morphological structure (Campbell & Sargent, 1964; Villa-Trevino, Farber, Staehelin, Wettstein & Noll, 1964) in the liver, the synthesis of glucokinase may have to await a rather late stage in the maturation of the liver cell. Insulin may be involved in or necessary for the maturation process. The very low glucokinase activities in the livers of alloxan-diabetic rats could also be due to insulin lack at the sites of protein synthesis, an effect which may involve stabilizing in some way

the messenger-RNA templates (Pitot, 1964) necessary for protein synthesis by the liver ergosomes.

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