The separate roles of glucose and insulin in the induction of glucokinase in hepatocytes isolated from neonatal rats

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1. The specificity of the effect of glucose on the induction of glucokinase activity that occurs when hepatocytes freshly isolated from 13-day-old rats are incubated in Medium 199 together with insulin [Wakelam & Walker (1980) *FEBS Lett.* **111**, 115–119] was examined. A pattern that is different from other known effects of glucose is found, and metabolism of this compound is not necessary to account for this particular effect. 2. The effects of a raised glucose concentration and of insulin on the induction can be separated. The hexose initiates the process in the absence of insulin in a manner that is sensitive to actinomycin D but not to cycloheximide. The subsequent effect of insulin is dependent on the prior effect of glucose or other positive analogue, does not require the presence of glucose and is inhibited by cycloheximide but not by actinomycin D. 3. Induction of glucokinase *in vitro* in hepatocytes from neonatal animals is inhibited by adrenaline, glucagon and dibutyryl cyclic AMP, but not by vasopressin or angiotensin II. The inhibition by cyclic AMP is on the stage requiring insulin and is comparatively specific, because total protein synthesis is not apparently diminished. 4. The implications of these results are discussed with reference to possible mechanisms of induction and to the situation *in vivo*.

Many lines of evidence (for reviews see Niemeier *et al.*, 1975; Weinhouse, 1976) from experiments *in vivo* have indicated that insulin is in some way required for the synthesis of hepatic glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2). Because the substrate glucose is also insulinogenic, it has not been possible to ascertain whether the hexose is also essential. Studies on the development of glucokinase activity in the neonatal rat, which apparently involves synthesis of the enzyme *de novo* (Wakelam *et al.*, 1979, 1980), have served to illustrate the complexities of the *in vivo* regulation. Other hormones affect enzyme induction. Those, such as glucagon and adrenaline, that act via cyclic AMP inhibit the development of glucokinase activity both in the adult (Ureta *et al.*, 1970; Pilkis, 1970) and in the neonatal (Wakelam *et al.*, 1980) rat. The hepatic cyclic AMP concentration shows an inverse correlation with glucokinase activity (Seitz *et al.*, 1979).

It would clearly be of advantage to be able to study the induction of glucokinase in a liver preparation isolated from extrahepatic influences. Wakelam & Walker (1980a) reported that freshly prepared hepatocytes from 13-day-old rats (which

contain no glucokinase) can be used to demonstrate comparatively rapid appearance of glucokinase activity *in vitro* over a period of a few hours. Synthesis of new enzyme protein was inferred on the basis of inhibition of induction by actinomycin D and cycloheximide, electrophoretic evidence and immunotitration. Induction was dependent on the glucose concentration over the range 5.5–20.5 mM and required insulin (Wakelam & Walker, 1980a). In the present paper the roles of glucose, insulin and other factors in the induction of glucokinase in these cells are investigated in more detail and discussed. A preliminary account of some of these results has been given (Wakelam & Walker, 1980b).

Materials and methods

Materials and animals

The sources of most materials have been given previously (Wakelam *et al.*, 1979, 1980; Wakelam & Walker, 1980a). L-[4,5-³H]Leucine (190 Ci/mmol) and [6-¹⁴C]orotic acid (59 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Mono-component pig insulin (25.6 i.u./mg)

was from Novo Research Institute, Bagsvaerd, Denmark.

Rats were from our departmental colony of specific-pathogen-free Wistar rats. They were maintained as described previously (Wakelam *et al.*, 1979, 1980).

Methods

Hepatocytes were isolated from the livers of 13-day-old animals of either sex by preperfusion with Ca^{2+} -free Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932), and then re-circulating perfusion (Kirk *et al.*, 1977), in via the portal vein and out from the inferior vena cava, with the same buffer containing 2.54 mM- Ca^{2+} ions, 0.025% (w/v) collagenase, 5 mM-glucose and the amino acid mixture described previously (Wakelam & Walker, 1980a). Two animals were perfused in parallel to provide sufficient cells for the experiments on any one day. The yield and quality of the isolated cells was checked frequently by light-microscopy, exclusion of Trypan Blue and leakage of lactate dehydrogenase activity (Wakelam & Walker, 1980a). The isolated hepatocytes (1.5×10^8 – 2×10^8) were washed twice in well-gassed (O_2/CO_2 , 19:1) Krebs–Ringer bicarbonate buffer, pH 7.4 (containing Ca^{2+} ions), and resuspended in the medium to be used in the particular experiment, which was commenced immediately.

Medium 199 (Morgan *et al.*, 1950), a fully defined Earle's balanced salt solution containing amino acids, lipids, vitamins, nucleic acid derivatives and 5.5 mM-glucose, was supplemented with 0.4 mM-ornithine, which has been shown (Leffert & Koch, 1978) to suppress growth of non-parenchymal cells and maintain specific protein synthesis and urea-cycle function in isolated parenchymal cells. In later experiments (see the Results section) a glucose-free Medium 199 was employed and supplemented as given in the Tables.

Additions to the incubation medium were made in a very small volume (usually 10 μl). About 3×10^6 cells were suspended in 3 ml of medium at 37°C previously gassed with O_2/CO_2 (19:1) contained in 25 ml silicone-treated glass conical flasks, which were shaken (75 oscillations/min) in an incubator (Mickle) with a gassing hood through which was passed a steady stream of the same gas mixture. Incubations were usually performed in quadruplicate. In experiments during which the incubation medium was changed, the contents of a flask were transferred to a plastic conical tube, and the cells were collected by centrifugation (80 g for 2 min), resuspended in well-gassed (same gas mixture) Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°C by 30 inversions of the tubes, collected again by centrifugation and then resuspended similarly in the new incubation medium. At the end of the

incubation cells were collected and washed similarly (the washing being necessary to lower the glucose concentration in readiness for subsequent determination of enzyme activities), and then homogenized in a small hand-held glass Dounce homogenizer in 2 ml of 0.154 M-KCl/10 mM- NaHCO_3 /1 mM-EDTA, pH 7.0. Homogenates were centrifuged, and glucokinase and hexokinase activities were determined (normally with 200 μl of supernatant in a final volume of 1 ml of assay medium) as described and evaluated previously (Wakelam *et al.*, 1979). Because no change in the hexokinase activity was seen in any of the experiments reported (for typical results see Wakelam & Walker, 1980a), the values are not given in the present paper. Glucokinase activities are expressed as munits/mg of soluble protein, where 1 unit is the activity catalysing the phosphorylation of 1 μmol of glucose/min. Protein determinations were performed on the supernatants by the method of Lowry *et al.* (1951), as modified by Peterson (1977), with dried bovine serum albumin as standard.

As shown in the Results section, a routine check on the suitability of each preparation of cells was made by determining the amount of glucokinase activity present after 2 h under 'standard' conditions, i.e. when cells were incubated in the presence of 20.5 mM-glucose and 0.1 μM -insulin. Small variations between experiments can be due to individual animal differences and to modifications of hormone-sensitivity of the hepatocytes resulting from the collagenase treatment.

The rate of protein synthesis in hepatocytes was determined by adding in 10 μl approx. 10^6 d.p.m. of L-[4,5- ^3H]leucine to the 'standard' 3 ml incubation mixture (see above) and allowing synthesis to proceed for 2 h. The cells were then collected, washed and homogenized as before. Protein in a 1 ml sample of the supernatant was precipitated with 10% (w/v) trichloroacetic acid, and the precipitate was washed five times in 5% (w/v) trichloroacetic acid before dissolution in 0.5 ml of 0.5 M-NaOH. This solution was neutralized, and the radioactivity was determined by liquid-scintillation counting. Incorporation was calculated as described by Seglen (1978) on the basis that Medium 199 contains 120 mg of DL-leucine/litre and assuming a fractional leucine content of 760 μmol per g of liver protein (Schreiber *et al.*, 1971).

The rate of RNA synthesis in hepatocytes was determined by adding in 10 μl approx. 10^6 d.p.m. of [^{14}C]orotic acid to the 'standard' 3 ml incubation system. At the end of the 2 h incubation the cells were collected, washed and homogenized as before. Samples (50 μl) of homogenates were spotted on to glass-fibre discs and the amount of radioactivity incorporated into RNA was determined by the method of Yu & Feigelson (1971).

Results

Specificity of the glucose effect

No appearance of glucokinase activity occurs when hepatocytes from 13-day-old rats are incubated with insulin in Medium 199, which contains 5.5 mM-glucose, but induction is seen when the glucose concentration is increased, with a maximum effect at about 20 mM-glucose (Wakelam & Walker, 1980a). The specificity of this effect of increased glucose concentration was explored by adding various analogues at 15 mM and incubating with insulin for 2 h. Table 1 (column *a*) shows that fructose is as effective as glucose in inducing glucokinase activity. Galactose and mannose have smaller effects. Other analogues are also effective, but 6-deoxy-D-glucose, 3-O-methyl-D-glucose and L-glucose did not induce. At a later stage in the present work (see below) the question arose as to whether the basal 5.5 mM-glucose was necessary for, or influenced the effects of, the other sugars. This does not appear to be so (Table 1, column *b*), and it was further established that several of the analogues effective at 20.5 mM had no effect at 5 mM. The form of the concentration-dependent induction of glucokinase activity by the effective glucose analogues was found, in other experiments, to be essentially similar to that previously found for glucose (Wakelam & Walker, 1980a). The sugar alcohol D-mannitol and the readily utilizable gluconeogenic precursors lactate/pyruvate and dihydroxyacetone did not induce.

The role of mannoheptulose, which had been previously used *in vivo* (Wakelam *et al.*, 1980) as an inhibitor of insulin secretion and hence of glucokinase induction, was explored; it was found, not only to be a weak inducer (Table 1, column *a*), but also to inhibit the effectiveness of 20.5 mM-glucose in a concentration-dependent manner, such that 10 mM-mannoheptulose inhibited induction by about 90%.

Separation of effects of glucose and insulin

The effect of raising the glucose concentration can occur in the absence of insulin because the concentration can be returned to the non-inducing one of 5.5 mM before insulin is added and induction is still observed. This is seen, e.g. in Table 2, Expt. (*a*), line 3, and Expt. (*c*), line 2. The glucose effect must precede that of insulin, because incubation with insulin and then glucose separately is ineffective (Expt. *b*, line 3). Together the three experiments in Table 2 demonstrate clearly the sequential roles of glucose and insulin; an interval of 1 h between the treatments with glucose and insulin (Expt. *c*, line 3) does not lower the amount of glucokinase activity at the end of the incubation period.

The initiating effect of a raised glucose concentration is inhibited by the presence of actinomycin D but not by cycloheximide, whereas the subsequent effect of insulin is inhibited by cycloheximide but not by actinomycin D (Table 3, Expts. *a* and *b*). In another experiment it was shown that the concentration of actinomycin D used, which

Table 1. *Specificity of the glucose effect*

Hepatocytes prepared from 13-day-old rats were incubated under the standard conditions described in the text for 2 h. Insulin was added at zero time and after 1 h to give a final concentration of 0.1 μ M. Two sets of detailed conditions were used as follows. The results in column (*a*) were obtained when the incubation medium contained normal Medium 199 (which contains 5.5 mM-D-glucose) and the additional sugar or derivative was added to give a final concentration of 15 mM (i.e. 20.5 mM in the case of D-glucose). The results in column (*b*) were obtained with the glucose-free Medium 199 and the sugar was added to give a final concentration of 20.5 mM. Glucokinase activities and protein contents were determined on supernatant fractions from homogenates of cells as described in the Materials and methods section. Results are given as means $\pm$ S.E.M. for the numbers of results given in parentheses. In parallel incubations (4), indicated by asterisk (*), the addition of this sugar at a final concentration of 5 mM resulted in no glucokinase activity being induced.

	Glucokinase activity (munits/mg of protein)	
	(a)	(b)
None	0	0
D-Glucose	2.61 $\pm$ 0.13 (24)	2.22 $\pm$ 0.33 (12)
D-Fructose	2.31 $\pm$ 0.30 (4)	2.07 $\pm$ 0.36 (4)*
α -Methyl D-glucoside	1.98 $\pm$ 0.30 (4)	—
N-Acetyl-D-glucosamine	1.70 $\pm$ 0.49 (4)	—
D-Glucosamine	1.46 $\pm$ 0.58 (4)	1.17 $\pm$ 0.24 (4)*
2-Deoxy-D-glucose	1.18 $\pm$ 0.16 (4)	1.38 $\pm$ 0.15 (4)
D-Galactose	1.09 $\pm$ 0.30 (4)	0.94 $\pm$ 0.23 (4)*
D-Mannose	0.50 $\pm$ 0.31 (4)	1.35 $\pm$ 0.25 (4)*
β -Methyl D-glucoside	0.42 $\pm$ 0.17 (4)	—
D-Mannoheptulose	0.32 $\pm$ 0.18 (4)	—

Table 2. *Separation of the effects of glucose and insulin*

Hepatocytes prepared from 13-day-old rats were incubated for 2 or 3 h in Medium 199 containing the basal 5.5 mM-glucose. In the protocols given, the presence of glucose means that the glucose concentration was increased to 20.5 mM. Insulin was added hourly as indicated to give a final concentration of 0.1 μ M. Other details are as given in the legend to Table 1. Results are means $\pm$ S.E.M. for four observations.

Expt.	Protocol	Glucokinase activity (munits/mg of protein)
(a)	Glucose + insulin for 2 h	3.15 $\pm$ 0.73
	Glucose (only) for 2 h	0
	Glucose for 1 h, wash, then insulin for 1 h	1.03 $\pm$ 0.34
	Glucose for 2 h + insulin for 2nd 1 h only	3.17 $\pm$ 0.88
(b)	Glucose + insulin for 2 h	2.77 $\pm$ 0.58
	Insulin (only) for 2 h	0
	Insulin for 1 h, wash, then glucose for 1 h	0
	Insulin for 2 h + glucose for 2nd 1 h only	1.72 $\pm$ 0.42
(c)	Glucose + insulin for 2 h	2.27 $\pm$ 0.41
	Glucose for 1 h, wash, then insulin for 1 h	2.40 $\pm$ 0.24
	Glucose for 1 h, wash, leave for 1 h, then insulin for 1 h	2.91 $\pm$ 0.28

Table 3. *Inhibition of the effect of glucose and insulin by actinomycin D and cycloheximide respectively*

The experimental details were similar to those in Table 2. When added the concentrations of inhibitors were: actinomycin D, 0.4 μ g/ml; cycloheximide, 10 μ g/ml. Results are means $\pm$ S.E.M. for the numbers of observations given in parentheses.

Expt.	Protocol	Glucokinase activity (munits/mg of protein)
(a)	Glucose + insulin for 2 h	2.26 $\pm$ 0.17 (8)
	Glucose for 1 h, wash, then insulin for 1 h	2.42 $\pm$ 0.42 (8)
	Glucose + actinomycin D for 1 h, wash, then insulin for 1 h	0 (4)
	Glucose for 1 h, wash, then insulin + actinomycin D for 1 h	2.47 $\pm$ 0.51 (8)
	Glucose + actinomycin D for 1 h, wash, then insulin + actinomycin D for 1 h	0 (4)
(b)	Glucose + insulin for 2 h	2.30 $\pm$ 0.19 (8)
	Glucose for 1 h, wash, then insulin for 1 h	1.98 $\pm$ 0.07 (8)
	Glucose + cycloheximide for 1 h, wash, then insulin for 1 h	1.49 $\pm$ 0.28 (8)
	Glucose for 1 h, wash, then insulin + cycloheximide for 1 h	0 (4)
	Glucose + cycloheximide for 1 h, wash, then insulin + cycloheximide for 1 h	0 (4)

inhibited the glucose effect, did not inhibit the incorporation of [14 C]orotic acid into total RNA during the second phase of incubation with insulin after the inhibitor had been washed out [control, 3.54 $\pm$ 0.46 (4) % incorporation/h; plus actinomycin D, 2.70 $\pm$ 0.43 (4); $P > 0.1$], but did inhibit this incorporation when present during the first phase with the raised glucose concentration [0.85 $\pm$ 0.46 (4); $P < 0.01$]. In a further experiment, in which incorporation of [3 H]leucine into total cytosolic protein was measured, the concentration of cycloheximide employed significantly ($P < 0.01$) lowered the incorporation [control, 1.08 $\pm$ 0.14 (4)

% incorporation/h; plus cycloheximide, 0.20 $\pm$ 0.08 (4)] during the incubation with insulin but had no effect [0.99 $\pm$ 0.13 (4)] if present during the incubation with glucose and then removed by wash-out before the addition of insulin.

The separate and sequential roles of glucose and insulin are demonstrated further in experiments (Table 4) in which the period of incubation with the raised glucose concentration (Expt. a) or with insulin (Expt. b) were varied. Although the effects observed were not strictly proportional to time, the trend is apparent and supports the above observations.

The basal (5.5 mM) concentration of glucose

present in Medium 199 does not induce glucokinase activity, but was present during the second phase incubation with insulin in the above experiments. To establish whether the low concentration of glucose was necessary for insulin to be effective, the glucose-free Medium 199 was used in the experiments reported in Table 5. Expt. (a) shows that 5.5 mM-glucose can be replaced by the same concentration of lactate/pyruvate, and the absence of any added metabolizable substrate did not significantly lower glucokinase induction on subsequent incubation with insulin ($P > 0.05$). Expt. (b) (Table 5) shows that the overall induction initiated by 2-deoxyglucose, but not apparent as glucokinase activity until subsequent incubation with insulin, does not require glucose at any stage.

Cyclic AMP and glucokinase induction

The inhibition of glucokinase induction *in vivo* by certain other hormones can also be observed in the system studied *in vitro*. Vasopressin and angiotensin II do not prevent glucokinase induction in the basic test system (Expt. a, Table 6), but adrenaline and glucagon are inhibitory (Expts. b and c). This inhibition is mimicked by dibutyryl cyclic AMP (Expt. c). The results in Table 7 indicate that the effect of glucagon and cyclic AMP is not on the first phase initiated by the raised glucose concentration, but is on the subsequent insulin phase. This inhibition by cyclic AMP is not, however, a generalized effect on protein synthesis, because the incorporation of [^3H]leucine into total cytosolic protein is not lowered under conditions in which

Table 4. *Effect of time on the roles of glucose and insulin in glucokinase induction*

Hepatocytes from 13-day-old rats were incubated in Medium 199 containing the basal 5.5 mM-glucose to which was added either more glucose to a final concentration of 20.5 mM or insulin for the time periods stated. Other details are as given in the legend to Table 1. Results are means $\pm$ S.E.M. for four observations.

Expt.	Protocol	Glucokinase activity (munits/mg of protein)
(a)	Glucose for 15 min, wash, then insulin for 1 h	0.83 ± 0.20
	Glucose + actinomycin D for 15 min, wash, then insulin for 1 h	0
	Glucose for 30 min, wash, then insulin for 1 h	1.04 ± 0.08
	Glucose + actinomycin D for 30 min, wash, then insulin for 1 h	0
	Glucose for 1 h, wash, then insulin for 1 h	1.60 ± 0.20
(b)	Glucose + insulin for 2 h	1.57 ± 0.36
	Glucose for 1 h, wash, then insulin for 30 min	1.19 ± 0.11
	Glucose for 1 h, wash, then insulin for 1 h	1.27 ± 0.14
	Glucose for 1 h, wash, then insulin for 2 h	2.06 ± 0.46
	Glucose for 1 h, wash, then insulin + cycloheximide for 2 h	0

Table 5. *Lack of a requirement for added glucose during the action of insulin in glucokinase induction*

Hepatocytes prepared from 13-day-old rats were incubated in glucose-free Medium 199 as indicated; thus, although sometimes glucose was added as part of the protocol (final concn. 20.5 mM), exogenous glucose was absent during the action of insulin, except for the control results in line 1 of each experiment and in line 3 of experiment (a). When present, the 2-deoxyglucose concentration was 20.5 mM. Results are means $\pm$ S.E.M. for four observations.

Expt.	Protocol	Glucokinase activity (munits/mg of protein)
(a)	Glucose + insulin for 2 h	2.25 ± 0.61
	Glucose for 1 h only	0
	Glucose for 1 h, wash, then insulin + 5.5 mM-glucose for 1 h	1.54 ± 0.19
	Glucose for 1 h, wash, then insulin + 5.5 mM-lactate/pyruvate (9:1) for 1 h	1.36 ± 0.35
	Glucose for 1 h, wash, then insulin only for 1 h	1.04 ± 0.15
(b)	Glucose + insulin for 2 h	2.02 ± 0.18
	Glucose for 1 h, wash, then insulin only for 1 h	1.95 ± 0.24
	2-Deoxyglucose for 1 h only	0
	2-Deoxyglucose + insulin for 2 h	1.41 ± 0.20
	2-Deoxyglucose for 1 h, wash, then insulin only for 1 h	1.20 ± 0.32

Table 6. *Inhibition of glucokinase induction by certain other hormones*

Hepatocytes from 13-day-old rats were incubated for 2 h in Medium 199 with 20.5 mM-glucose, 0.1 μ M-insulin and other hormones as indicated. Other details are as given in the legend to Table 1. Results are means $\pm$ S.E.M. for four observations in each case.

Expt.	Addition	Glucokinase activity (munits/mg of protein)
(a)	None	3.47 $\pm$ 0.93
	Vasopressin (0.23 μ M)	2.53 $\pm$ 0.72
	Angiotensin II (1 μ M)	3.50 $\pm$ 0.75
(b)	None	2.53 $\pm$ 0.55
	Adrenaline (10 μ M)	0.70 $\pm$ 0.19
(c)	None	2.42 $\pm$ 0.75
	Glucagon (0.001 μ M)	0.28 $\pm$ 0.10
	Glucagon (0.1 μ M)	0
	Dibutyl cyclic AMP (10 μ M)	0

Table 7. *Inhibition of the insulin effect by glucagon and dibutyl cyclic AMP*

Hepatocytes from 13-day-old rats were incubated in Medium 199 (containing 5.5 mM-glucose) plus additions as indicated. Other details are as given in the legend to Tables 3 and 6.

Expt.	Protocol	Glucokinase activity (munits/mg of protein)
(a)	Glucose + insulin for 2 h	2.56 $\pm$ 0.09
	Glucose for 1 h, wash, then insulin for 1 h	1.59 $\pm$ 0.31
	Glucose + 0.1 μ M-glucagon for 1 h, wash, then insulin for 1 h	1.42 $\pm$ 0.35
	Glucose for 1 h, wash, then insulin + 0.1 μ M-glucagon for 1 h	0.44 $\pm$ 0.15
(b)	Glucose + insulin for 2 h	2.04 $\pm$ 0.10
	Glucose for 1 h, wash, then insulin for 1 h	1.60 $\pm$ 0.05
	Glucose + 10 μ M-dibutyl cyclic AMP for 1 h, wash, then insulin for 1 h	1.08 $\pm$ 0.24
	Glucose for 1 h, wash, then insulin + 10 μ M-dibutyl cyclic AMP for 1 h	0.04 $\pm$ 0.04

glucokinase induction is prevented. Cycloheximide, however, inhibited both the induction of glucokinase activity and the synthesis of total cytosolic protein (results not shown).

Discussion

The system studied *in vitro* that permits induction of glucokinase in hepatocytes isolated from neonatal rats in response to glucose plus insulin (Wakelam & Walker, 1980a) has been found suitable for further exploration of the phenomenon. We have previously presented substantial evidence that this appearance of glucokinase activity is due to synthesis *de novo* (Wakelam & Walker, 1980a). Nevertheless, in the absence of direct evidence for this, such as incorporation of labelled amino acids into immunoprecipitable protein, we have referred to the phenomenon so far as induction, with no implication for the mechanisms involved.

The requirement for a glucose concentration greater than 5.5 mM applies to a number of glucose

analogues (Table 1). Physiological monosaccharides (e.g. fructose, mannose, galactose) and certain other derivatives at higher concentrations result, in the presence of insulin, in the appearance of glucokinase activity. The list of positive effectors includes 2-deoxy-D-glucose, which will be taken up and phosphorylated but not then further metabolized. Metabolites of glucose (dihydroxyacetone, lactate/pyruvate) are ineffective. Structural features of the specificity includes the fact that a ring formation appears to be necessary, because D-mannitol is ineffective; an osmotic effect is also excluded by this result. A hydroxy group on C-2 is not necessary, because 2-deoxyglucose, glucosamine and N-acetyl-D-glucosamine are positive effectors. A hydroxy group on C-3 may be required, because 3-O-methylglucose is ineffective. The utility of fructose (Table 1) may be related to its rapid conversion into glucose. The positive and inhibitory effects exerted by mannoheptulose are not readily explainable, but need to be considered in any interpretation of experiments performed *in vivo*,

such as those previously reported (Wakelam *et al.*, 1979, 1980).

This specificity of the glucose effect (Table 1) is different from that of known mammalian hexokinases (Crane, 1962; Walker, 1966; Colowick, 1973) or of hepatic glucokinase itself (Parry & Walker, 1966). The relative $V_{\max}$ and K_m values also do not match, and, in any case, metabolism of the effectors does not appear to be necessary. The specificity of the carrier-mediated diffusion of monosaccharides across the plasma membrane of hepatocytes (Baur & Heldt, 1977) is also different (Craik & Elliott, 1979). Whitton & Hems (1977) proposed the existence in liver of a hexose receptor concerned in the regulation of glycogen synthesis in addition to the better-documented effect of glucose on phosphorylase phosphatase (Stalmans *et al.*, 1974). The specificities of both of these hepatic glucose effects are again different.

The presence in the hepatocytes from 13-day-old rats of a distinct glucose receptor for glucokinase induction is therefore postulated. This would be different from that concerned, as an example, with the recognition of glucose as a signal for insulin biosynthesis and release by the B-cells of the islets of Langerhans of the pancreas (Ashcroft, 1980). In that cell type the glucose almost certainly has to be metabolized, and certain gluconeogenic substrates such as dihydroxyacetone elicit insulin release. A hydroxy group on the C-2 position is also important for insulin release in the B-cell (Weaver *et al.*, 1979).

Our results permit no further comments on the location of the hypothetical receptor. The glucose-concentration-dependence suggests that its interaction with glucose must be complex.

Irrespective of the nature of the glucose receptor, the separation of the roles of glucose and insulin has been demonstrated (Tables 2–5). The effect of glucose and certain analogues looks remarkably like a case of substrate induction of enzyme synthesis, but this remains to be proved. One of the major interests aroused about glucokinase after its discovery in the early 1960's (for review see Weinhouse, 1976) was the apparent involvement of insulin in its synthesis. No evidence for the presence of preformed inactive glucokinase in the untreated neonatal rat has been found. Because the primary effect of a raised glucose concentration in our system is sensitive to actinomycin D and the subsequent effect of insulin is sensitive to cycloheximide, but not vice versa (Table 4), our observations support the previous indirect evidence (Wakelam & Walker, 1980*a,b*), but do not prove, that synthesis of enzyme protein is involved in the process. The possibility arises that the two phases represent the formation and subsequent translation into protein of specific mRNA for glucokinase. It would need the quantification of translatable mRNA

by using a cell-free protein-synthesizing system coupled with incorporation of radioactive amino acids into immunoprecipitable glucokinase in order for such a hypothesis to be proved. An effect of insulin on protein degradation cannot be excluded by our results, but insulin has been shown to stimulate what has been interpreted as a translational stage in the synthesis of both lactate dehydrogenase (Suleiman & Vestling, 1979) and tyrosine aminotransferase (Kletzien *et al.*, 1976) in cultures of adult rat hepatocytes.

The inhibition of the overall synthesis of glucokinase in hepatocytes from 13-day-old rats by the same hormones (Table 6) that also prevent precocious induction by glucose administration *in vivo* at that age (Wakelam *et al.*, 1980) suggests that the hepatocyte system is suitable for the further exploration of the inhibitory effect of cyclic AMP, the likely mediator of those hormonal effects *in vivo* and that is itself also inhibitory *in vitro* (Table 6). The addition of dibutyl cyclic AMP does not significantly alter the effect of glucose on the system studied *in vitro*, but clearly inhibits the subsequent effect of insulin (Table 7, Expt. b). This inhibitory effect of cyclic AMP must nevertheless be fairly specific; the mechanism remains to be established, but is not the general inhibition of hepatic protein synthesis by cyclic AMP first seen about 10–12 days after birth and that apparently increases over the next 10 days (Klaipongpan *et al.*, 1977; Bloxham & Klaipongpan, 1979).

Katz *et al.* (1979), Schudt (1979) and Spence & Pitot (1979) have independently described systems of adult hepatocytes studied *in vitro* in primary culture in which increases of glucokinase activity occurred in response to incubation for 2–3 days in the presence of insulin and other factors. Similar results were obtained with cultured hepatocytes from 14-day-old rats (Nakamura *et al.*, 1979). An essential or enhancing role of glucocorticoids in this apparent synthesis was described in all those reports. The systems used were different from ours, and we have not yet explored possible glucocorticoid effects on our system.

If the induction of glucokinase activity by a raised glucose concentration demonstrated *in vitro* is a mechanism that operates around the commencement of weaning to initiate the appearance of glucokinase activity *in vivo*, then the question arises as to how such a raised glucose concentration could arise. The change of diet during weaning on to a high-carbohydrate solid chow is gradual, and glucokinase activity appears even if the rat is weaned on to a carbohydrate-free diet (Walker & Holland, 1965) or weaning is prevented (Walker *et al.*, 1974). In spite of the evidence discussed above of the need for a glucose receptor to explain the observations made *in vitro*, the existence of other inducing factors

that operate around the 16th postnatal day must be contemplated. Perhaps glucose is one of several agents that together, maybe synergistically, regulate initiation of glucokinase induction.

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