The induction of synthesis of L-type pyruvate kinase in cultured rat hepatocytes

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Hepatocytes were isolated from preweaned neonatal and adult rats and maintained in primary monolayer culture. Cells from preweaned newborns possessed no L-type pyruvate kinase, nor did they synthesize the enzyme. Incubation for 48–72 h in culture medium supplemented with 2 mM-fructose and 0.1 μ M-insulin induced the synthesis of L-type pyruvate kinase, as judged by increased enzyme activity and the increased incorporation of [3 H]leucine into immunoprecipitable L-type pyruvate kinase. Hepatocytes isolated from 48 h-starved adult rats incorporated less [3 H]leucine into L-type pyruvate kinase than did cells isolated from high-carbohydrate-diet-fed rats. The rate of enzyme synthesis by cells from 48 h-starved rats was increased by the inclusion of fructose and insulin in the incubation medium, after a lag phase of 24–48 h. After 4 days in culture in the presence of fructose and insulin, hepatocytes from 48 h-starved rats synthesized L-type pyruvate kinase at similar rates to hepatocytes isolated from high-carbohydrate-diet-fed rats.

Weaning represents a major dietary change for the neonatal rat. From receiving a diet rich in fat, the animal changes to a diet containing a higher concentration of carbohydrate. The net result of this is a decrease in gluconeogenesis, an increase in the rates of fatty acid synthesis (Vernon & Walker, 1968*a,b*), and an increase in the plasma insulin concentration (Unger, 1971). The induced changes in enzyme concentrations that are observed on receiving a carbohydrate-rich diet may be secondary to the appearance of insulin (Katz & Ick, 1981). Pyruvate kinase occupies a position in the control of hepatic metabolism such that its regulation may influence the rate of gluconeogenesis (Feliu *et al.*, 1976, 1977), the rate of glycolysis (Scrutton & Utter, 1968; Seubert & Schoner, 1971) and the rate of fatty acid synthesis (Ochs & Harris, 1978). This enzyme has been shown to exist in several isomeric forms (Imamura *et al.*, 1972). The liver of neonatal pre-weaned rats contains the A-type isoenzyme of pyruvate kinase. Between 6 and 24 h after weaning there is a clear induction of synthesis of L-type pyruvate kinase (Weber *et al.*, 1965; Taylor *et al.*, 1967; Vernon & Walker, 1968*a,b*; Hopkirk & Bloxham, 1980), consistent with a hormone-induced synthesis *de novo* of mRNA for L-type pyruvate kinase, which is subsequently translated to form the catalytically active protein. If the animal is weaned on to a carbohydrate-rich diet

as opposed to laboratory chow, there is a significant increase in the rate at which L-type pyruvate kinase is synthesized, resulting in higher concentrations of the enzyme in the liver. Maximum rates of enzyme synthesis occur between 24 and 48 h after the commencement of weaning (Hopkirk & Bloxham, 1980). In the adult rat, the concentration of L-type pyruvate kinase is closely regulated by dietary conditions (Krebs & Eggleston, 1965). There is a decrease in activity on starvation (Van Berkel *et al.*, 1977, 1978) and a rapid increase on refeeding the animals on a carbohydrate-rich diet (Cladaras & Cottam, 1980*a*). Feeding rats on a diet rich in carbohydrate is associated with increased portal-venous concentrations of fructose, glucose, insulin and lactate. It is not possible *in vivo* to define unequivocally which stimulus is responsible for the induction of the enzyme within the complex structure of the intact animal. A less complex system which permits the study of the hormonal induction of specific liver protein synthesis is that of hepatocytes maintained in monolayer culture. Previous workers have reported the hormonal induction in such a system of glucokinase (Katz *et al.*, 1979; Schudt, 1979; Spence & Pitot, 1979, 1980), ATP citrate lyase (Spence *et al.*, 1980), acetyl-CoA carboxylase (Katz & Ick, 1981) and phosphoenolpyruvate carboxykinase (Wolfe *et al.*, 1981).

We describe below a system for the monolayer

culture of hepatocytes which permits the study of the turnover of specific hepatic enzymes *in vitro* over a period of 5–7 days. In this study the activity and synthesis of L-type pyruvate kinase were examined in hepatocytes isolated from pre-weaned neonatal rats and 48 h-starved adult rats and cultured for an extended period of time. Clear evidence is presented for the induction of enzyme synthesis.

Materials and methods

Male Wistar Albino rats were used for all experiments. Animals were either adults (125–200 g) or newborns (16–22 days). Adult animals were fed on either carbohydrate-rich diet (Bloxham *et al.*, 1977) or standard laboratory diet (chow), or were deprived of food 48 h before use. Neonatal litters (five or six animals) were kept with the mother. At day 17 food was added to the cage, and animals were given the option of eating solid food or taking maternal milk.

The experimental procedures for the preparation of rabbit anti-(L-type pyruvate kinase) and rabbit anti-(fructose 1,6-bisphosphatase) sera and the assay of hepatic pyruvate kinase and fructose 1,6-bisphosphatase have been described previously (Hopkirk & Bloxham, 1980). One unit of enzyme activity catalysed the formation of 1 μ mol of product/min at 37°C. Waymouth's tissue-culture medium was obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., foetal-calf serum was from Bio-Cult Laboratories, Glasgow, Scotland, U.K., and culture dishes were from Sterilin, Teddington, Middx., U.K. Collagenase was obtained from Boehringer Corp. (London), Lewes, Sussex, U.K. Xylene, 2,5-diphenyloxazole and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., and Synperonic NXP was from Cargo Fleet Chemicals, Eaglescliffe, Stockton, Cleveland, U.K. All radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were routinely obtained from BDH, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Poole, Dorset, U.K.

Preparation of isolated rat hepatocytes

Isolated rat hepatocytes from adult rats were prepared essentially by the method of Elliott *et al.* (1976), except that livers were cyclically perfused with a solution of collagenase (0.05%) in supplemented Waymouth's medium (Waymouth's medium containing 25 mM-NaHCO₃, 0.1 μ M-insulin, 1 mg of streptomycin/ml, 0.3 mg of penicillin/ml and 5 μ g of nystatin/ml, pH 7.4). Supplemented Waymouth's medium containing 5% foetal-calf serum was used throughout the washing procedure. Hepatocytes

from neonatal animals were prepared by a similar method, with the following modifications. The direction of perfusion was reversed: the perfusate entered the liver by the cannulated inferior vena cava; the portal vein was cut and the medium recycled by aspiration; the flow rate was decreased to 8 ml/min. Typically cells from four or five animals were pooled for culture.

Cell culture conditions

For this, 50 mm vented plastic culture dishes were pretreated at 37°C with supplemented Waymouth's medium containing 5% foetal-calf serum. After 1 h the plates were inoculated with hepatocytes (1×10^6 – 2×10^6 cells/ml final concn.). Cultures were maintained at 37°C under an atmosphere of air/CO₂ (19:1). After 4 h the medium containing unattached cells was removed and replaced with fresh Waymouth's medium containing no foetal-calf serum. The medium was then changed at intervals of 12 h.

Immunoprecipitation of L-type pyruvate kinase and fructose 1,6-bisphosphatase

A measured volume of cells was disrupted by rapid freeze-thawing three times. A sample of the homogenate was assayed for DNA (Burton, 1968). The remainder was centrifuged at 13000 g for 2 min (Eppendorf Microfuge). The pellet did not contain either L-type pyruvate kinase or fructose 1,6-bisphosphatase activities. The supernatant fraction was sequentially treated with: (a) 100 μ l of anti-(fructose 1,6-bisphosphatase) serum; (b) 10 μ g of fructose 1,6-bisphosphatase with 100 μ l of anti-(fructose 1,6-bisphosphatase) serum; (c) 150 μ l of anti-(L-type pyruvate kinase) serum; (d) 10 μ g of L-type pyruvate kinase with 150 μ l of anti-(L-type pyruvate kinase) serum. Quantitative immunotitration showed that 50 μ l of each antiserum was sufficient to neutralize 10 μ g of the corresponding enzyme. After each addition the sample was incubated at 4°C for 2 h. Inhibition of enzyme activity was confirmed by assay. The immunoprecipitate obtained after centrifugation at 13000 g for 2 min was washed in $3 \times 500 \mu$ l of phosphate-buffered saline, pH 7.2 (0.8% NaCl, 0.02% KCl, 0.115% NaHPO₄ and 0.02% KH₂PO₄) containing 0.1% Triton X-100. The pellet was dissolved in 300 μ l of formic acid and counted for radioactivity in 20 ml of Tritoscent scintillator [a solution of dimethyl-POPOP (0.05%) and 2,5-diphenyloxazole (0.4%) in xylene/Synperonic NXP (2:1, v/v)]. The secondary immunoprecipitations (b) and (d) were performed as blank control precipitations for the primary immunoprecipitations obtained from (a) and (c). The amount of radioactivity incorporated into L-type pyruvate kinase and fructose 1,6-bisphosphatase was determined by subtraction of the radioactivity in the

secondary immunoprecipitate from that in the primary immunoprecipitate (*a-b*; *c-d*). The radioactivity in the secondary immunoprecipitate was never greater than 5% of that in the primary immunoprecipitate.

Expression of results

Results are expressed as the means $\pm$ S.E.M. for *n* independent observations. Weights of cells are expressed as dry weights, and weights of liver as wet weights.

The final concentration of L-leucine in the cell culture medium was 0.38 mM. Since 10 μ Ci of L-[4,5- 3 H]leucine was added per 3 ml of culture medium this gave a specific radioactivity of 8.7 Ci/mol. An incorporation of 1000 d.p.m. represents the synthesis of protein containing 52 pmol of L-leucine.

Results

Induction of synthesis of L-type pyruvate kinase during weaning

To study the biosynthesis of L-type pyruvate kinase during weaning, rats were allowed access to either laboratory chow or carbohydrate-rich diet at day 17. Control animals were maintained solely on maternal-milk diet. These conditions are in contrast with previous experiments, which used controlled weaning at day 21 (Hopkirk & Bloxham, 1980). Total pyruvate kinase was measured at various time intervals after access to food was allowed. Animals permitted access to the carbohydrate-rich diet synthesized significantly more L-type pyruvate kinase than did animals that had been allowed access to chow (Fig. 1). Animals which received only their mother's milk did not show any increase in pyruvate kinase concentration. After 5 days access to food the livers from carbohydrate-rich diet-fed rats contained 40 ± 3 units of pyruvate kinase/g of liver, chow-fed rats 15 ± 2 units/g and milk-fed animals 6 ± 1 units/g.

Induction of synthesis of L-type pyruvate kinase in neonatal-rat hepatocytes

Isolated rat hepatocytes were prepared from preweaned 16-day-old rats and post-weaned 22-day-old rats and cultured as described in the Materials and methods section. Hepatocytes isolated from neonatal pre- and post-weaned rats appeared morphologically similar to cells isolated from adult animals. The one major difference was in the sizes of the cells. Hepatocytes from newborns appeared typically 25–30% of the size of adult hepatocytes. The number of the cells attached to the plates was determined from the amount of DNA per plate. Hepatocytes from 16- and 21-day-neonatal pre-weaned rats contained $49.81 \pm 1.60 \mu$ g of DNA/

Fig. 1. Induction of synthesis of L-type pyruvate kinase on weaning

Neonatal litters (five or six animals) were kept with the mother. At day 17 (arrow) food was introduced into the cage and animals were given the option of eating solid food or taking maternal milk. Animals were allowed access to a carbohydrate-rich diet (●), laboratory chow (○) or maternal milk only (□). Pyruvate kinase activity was measured in liver homogenates as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for at least four animals.

mg dry wt. of cells, those from 21-day carbohydrate-rich-diet-weaned rats $42.62 \pm 1.20 \mu$ g of DNA/mg of cells, and those from 21-day chow-weaned rats $45.34 \pm 1.40 \mu$ g of DNA/mg of cells.

Hepatocytes isolated from 16-day-neonatal rats were cultured on 50 mm vented plastic Petri dishes in supplemented Waymouth's tissue-culture medium with or without added 2 mM-fructose and 0.1 μ M-insulin. Pyruvate kinase activity and fructose 1,6-bisphosphatase activities were measured at 24 h intervals for up to 5 days. The rates of specific enzyme synthesis were measured by the addition of 10 μ Ci of L-[4,5- 3 H]leucine (sp. radioactivity 8.7 Ci/mol)/plate. After 3 h incubation at 37°C, fructose 1,6-bisphosphatase and L-type pyruvate kinase were sequentially precipitated and the radioactivity in the immunoprecipitates was determined as described above. Cells isolated from 16-day-neonatal rats synthesized no L-type pyruvate kinase (Fig. 2). After culture for 3 days in Waymouth's medium containing 2 mM-fructose and 0.1 μ M-insulin, hepatocytes isolated from 16-day-neonatal rats incorporated [3 H]leucine into L-type pyruvate kinase. The amount of label incorporated was increased after a further 1 day's culture and was significantly greater than ($P < 0.01$) the amount of label incorporated into L-type pyruvate kinase by cells cultured in Waymouth's medium alone. The maximum rate of synthesis by neonatal cells after 3 days in culture (485 ± 32 d.p.m./mg dry wt. of cells) was about 20% of the maximum rate of synthesis of L-type pyruvate

kinase by hepatocytes isolated from adult rats fed on a diet rich in carbohydrate (2460 ± 100 d.p.m./mg dry wt. of cells). The simultaneous presence of insulin and fructose was synergistic for the induction of synthesis of L-type pyruvate kinase, since after 3 days culture in the presence of $0.1 \mu\text{M}$ -insulin or 2 mM -fructose alone neonatal cells maximally incorporated leucine into L-type pyruvate kinase at rates of 185 ± 20 and 130 ± 10 d.p.m./mg dry wt. of cells respectively. This compares with an incorporation of 95 ± 20 d.p.m./mg dry wt. of cells after 3 days culture in the absence of any additions.

Fig. 2. Induction of synthesis of L-type pyruvate kinase in neonatal rat hepatocytes

Hepatocytes were isolated from 16-day-old rats and maintained in monolayer culture in Waymouth's medium (Δ) or Waymouth's medium supplemented with 2 mM -fructose and $0.1 \mu\text{M}$ -insulin ($\blacktriangle$). Synthesis of L-type pyruvate kinase was measured over 3 h by the incorporation of L-[4,5- ^3H]leucine into immunoprecipitable L-type pyruvate kinase. Results are the means $\pm$ S.E.M. for three independent observations. The significance of the differences in the rates of enzyme synthesis in the presence and absence of fructose and insulin was measured with Student's *t* test: * $P < 0.05$; ** $P < 0.01$.

Cells from 16-day-preweaned animals possessed 1 unit of pyruvate kinase/g dry wt. (20 units/g of DNA). The K_m of the enzyme for phosphoenolpyruvate was 0.1 mM . Addition of fructose 1,6-bisphosphate had no effect on the activity of the enzyme, which was inhibited by the addition of alanine. The pyruvate kinase activity in hepatocytes from 16-day rats was not precipitated by 50%-satd. $(\text{NH}_4)_2\text{SO}_4$. These findings are consistent with the presence of the A-type isoenzyme of pyruvate kinase within the cells (Table 1). Hepatocytes from 21-day-neonatal rats weaned on to carbohydrate-rich diet or chow had elevated activities of pyruvate kinase [63 ± 3 units/g of cells (1480 ± 70 units/g of DNA) and 35 ± 2 units/g of cells (770 ± 45 units/g of DNA) respectively]. The enzyme present in these cells had a K_m for phosphoenolpyruvate of 0.6 mM , was activated by fructose 1,6-bisphosphate and inhibited by alanine. The enzyme activity was more than 95% precipitable by 50%-satd. $(\text{NH}_4)_2\text{SO}_4$, indicative of the L-type isoenzyme. Cells, isolated from 16-day-neonatal rats, that had been cultured for 5 days in Waymouth's medium showed an increased pyruvate kinase activity (3 units/g of cells, 61 units/g of DNA). Addition of fructose and insulin in the culture medium facilitated this increase in enzyme activity (10 units/g of cells; 208 units/g of DNA). In both cases the enzyme had an increased K_m for phosphoenolpyruvate (0.6 mM and 0.7 mM respectively) compared with cells from 16-day-old rats (0.1 mM). The enzyme was activated by fructose 1,6-bisphosphate, inhibited by alanine and precipitated by 50%-satd. $(\text{NH}_4)_2\text{SO}_4$. In addition to these findings, the antibody employed to precipitate the newly synthesized pyruvate kinase was specific for the L-type isoenzyme (Hopkirk & Bloxham, 1979). These results are therefore consistent with the induction of synthesis of L-type pyruvate kinase *in vitro*.

The enzyme fructose 1,6-bisphosphatase, which is fairly constitutive under the dietary conditions employed in the present study, has been previously used as a marker of the integrity of hepatocytes

Table 1. Pyruvate kinase in neonatal rat hepatocytes

Hepatocytes were isolated from neonatal rats as described in the Materials and methods section. Pyruvate kinase activity was assayed in homogenates of freshly prepared cells or cells that had been maintained in monolayer culture for 5 days. Enzyme activities are the means $\pm$ S.E.M. for at least three independent observations.

Condition	Activity (units/g of cells)	K_m for phosphoenolpyruvate (mM)	Effect of 50%-satd. $(\text{NH}_4)_2\text{SO}_4$	Major isoenzyme
Preweaned, day 16	1 (3)	0.12 ± 0.02 (3)	Soluble	A-type
Postweaned, Chow, day 21	35 ± 2 (4)	0.63 ± 0.05 (3)	Precipitable	L-type
Postweaned, high-carbohydrate, day 21	63 ± 3 (6)	0.62 ± 0.05 (3)	Precipitable	L-type
Preweaned, 5 day culture	3 (3)	0.64 ± 0.05 (3)	Precipitable	L-type
Preweaned, 5 day culture + 2 mM -fructose + $0.1 \mu\text{M}$ -insulin	10 ± 1 (3)	0.70 ± 0.05 (3)	Precipitable	L-type

maintained in monolayer culture (Poole & Bloxham, 1982). The concentration of fructose 1,6-bisphosphatase did not significantly alter in hepatocytes from 16-day-old rats (24.3 ± 0.8 units/g of cells, 488 ± 20 units/g of DNA) cultured in the presence (25.3 ± 1.2 units/g of cells; 504 ± 25 units/g of DNA) or absence of fructose and insulin (23.7 ± 3.2 units/g of cells; 476 ± 64 units/g of DNA). The rates of incorporation of [^3H]leucine into immunoprecipitable fructose 1,6-bisphosphatase were also unaffected by culture. Freshly prepared cells from 16-day-old animals incorporated 4280 ± 540 d.p.m./mg of cells (86 ± 11 d.p.m./ μg of DNA) into fructose 1,6-bisphosphatase. Hepatocytes cultured for 4 days in the presence of insulin and fructose incorporated 3970 ± 960 d.p.m./mg of cells (80 ± 20 d.p.m./ μg of DNA), and cells cultured in the absence of fructose and insulin incorporated 3630 ± 870 d.p.m./mg of cells (73 ± 17 d.p.m./ μg of DNA).

Induction of the synthesis of L-type pyruvate kinase in cultured adult rat hepatocytes

Starvation decreases both the rate of synthesis and the total liver concentration of L-type pyruvate kinase (Van Berkel *et al.*, 1977, 1978). Refeeding, particularly with a diet rich in carbohydrate, causes an increase in the rate of enzyme synthesis (Hopkirk & Bloxham, 1979; Cladaras & Cottom, 1980a). To investigate the factors that possibly cause this increase in the rate of enzyme synthesis, the rates of enzyme synthesis were measured in monolayer cultures of hepatocytes isolated from 48 h-starved rats. Enzyme synthesis *de novo* was measured as the rate of incorporation of [^3H]leucine into immunoprecipitable L-type pyruvate kinase. Incorporation of [^3H]leucine into L-type pyruvate kinase, fructose 1,6-bisphosphatase and total cell protein was linear with time for at least 3 h. Freshly prepared hepatocytes from 48 h-starved rats incorporated 98 ± 12 d.p.m. of [^3H]leucine/3 h per mg of cells into L-type pyruvate kinase. After 24 h culture the amount of label incorporated into L-type pyruvate kinase in 3 h did not increase (Fig. 3). Inclusion of 2 mM-fructose and 0.1 μM -insulin in the culture medium had no effect on the rate of synthesis of L-type pyruvate kinase in freshly prepared cells. After 48 h culture in the presence of fructose and insulin, there was a significant increase in the amount of label incorporated into L-type pyruvate kinase in cells cultured with fructose and insulin compared with cells cultured in Waymouth's medium alone. After 72 h culture in medium containing fructose and insulin, cells synthesized L-type pyruvate kinase at similar rates to cells isolated from carbohydrate-rich-diet-fed rats. Cells cultured in Waymouth's medium alone synthesized L-type pyruvate kinase at rates 20–25% of that in cells

Fig. 3. Induction of synthesis of L-type pyruvate kinase in hepatocytes isolated from 48 h-starved adult rats. Hepatocytes were isolated from 48 h-starved (Δ , $\blacktriangle$, $\square$, $\blacksquare$) or carbohydrate-rich-diet-fed ($\circ$, $\bullet$) rats and cultured in either Waymouth's medium (Δ , $\square$, $\circ$) or Waymouth's medium containing 2 mM-fructose and 0.1 μM -insulin ($\blacktriangle$, $\blacksquare$, $\bullet$). Synthesis of L-type pyruvate kinase and fructose 1,6-bisphosphatase was measured over 3 h by the incorporation of L-[4,5- ^3H]leucine into immunoprecipitable L-type pyruvate kinase ($\circ$, $\bullet$, $\square$, $\blacksquare$) and fructose 1,6-bisphosphatase (Δ , $\blacktriangle$). Results are the means $\pm$ S.E.M. for three independent observations. The significance of the differences between the rates of synthesis of L-type pyruvate kinase by hepatocytes from 48 h-starved rats cultured in the presence and absence of fructose and insulin was determined by using Student's *t* test. * $P < 0.05$; ** $P < 0.01$.

cultured with insulin and fructose. Neither insulin nor fructose when added alone for 3 days in culture were capable of reproducing the same magnitude of induction of synthesis of L-type pyruvate kinase by hepatocytes as when both were added together. Fructose alone was virtually without effect, and insulin alone gave only 25% of the induction observed when fructose was also included. These values are similar to the observations on culture of hepatocytes from pre-weaned rats (see above).

Hepatocytes isolated from carbohydrate-rich-diet-fed rats incorporated similar amounts of [^3H]leucine into L-type pyruvate kinase after 3–4 days in culture as did freshly prepared cells. Addition of insulin and fructose to the culture medium had no effect on the rate of enzyme synthesis.

As an internal control, the amount of label incorporated into the constitutive enzyme fructose 1,6-bisphosphatase was determined. The rates of incorporation of [^3H]leucine into fructose 1,6-bisphosphatase did not significantly alter during the experiment and were not affected by the addition of fructose and insulin to the culture medium (Fig. 3). The increased incorporation of [^3H]leucine into L-type pyruvate kinase appears therefore to be due to a specific induction of synthesis of L-type pyruvate kinase, rather than a general increase in protein synthesis.

Discussion

The ability to maintain primary cultures of hepatocytes for extended periods of time provides a valuable tool with which to study the regulation of a variety of metabolic processes in a strictly defined medium in the absence of complicating factors which apply to experiments performed *in vivo* or to perfusion.

We have demonstrated here, for the first time, that with such a culture system it is possible to induce synthesis of L-type pyruvate kinase in hepatocytes isolated from both preweaned neonatal rats and 48 h-starved adult rats. These two cases are typified either by very low concentrations of L-type pyruvate kinase and low rates of synthesis of L-type pyruvate kinase in the starved adult animal or by the presence of only the foetal A-type isoenzyme in the newborn. Induction by fructose and insulin in cells from adult animals occurred after a lag period of between 24 and 48 h. Maximum rates of synthesis of L-type pyruvate kinase were reached after 72 h in culture. Animals that were weaned at day 17 on to chow or carbohydrate-rich diet showed a significant increase in L-type pyruvate kinase after 48–72 h. A similar lag in the induction of synthesis of L-type pyruvate kinase *de novo* was observed in cultured hepatocytes isolated from 16-day-old rats. After a further 24 h in culture the rate of synthesis had reached 25% of the maximum rate obtained with cells from adult rats fed on a carbohydrate-rich diet. After 5 days in culture the total enzyme concentration had reached 20% of that of animals weaned on to carbohydrate-rich diet. Previous experiments have shown that the greatest increases in L-type pyruvate kinase activity *in vivo* may be obtained by feeding rats on a diet rich in sucrose (Hopkirk & Bloxham, 1979; Cladaras & Cottam, 1980a). The net effect of this is to increase the portal-venous concentrations of insulin, glucose, lactate and fructose. The induction of synthesis of L-type pyruvate kinase in cultured cells was maximal when both insulin and fructose were present together; generally the insulin effect was greatly decreased when fructose was omitted from the culture medium. The effective insulin concentration (0.1 μM) is considerably higher than the physiological concentration of insulin (0.1 nM). However, it should be remembered that hepatocytes possess a considerable ability to degrade insulin (Poole *et al.*, 1980). Therefore high initial concentrations of hormone are required to maintain an effective concentration of insulin throughout the culture.

The expression of insulin action is clearly involved with an increase in hepatic protein synthesis. In cultured rat hepatocytes it has been shown to be at least in part responsible for the induction of glucokinase (Spence & Pitot, 1979, 1980; Schudt, 1979; Katz *et al.*, 1979), ATP citrate lyase (Spence

et al., 1980), phosphoenolpyruvate carboxykinase (Wolfe *et al.*, 1981) and acetyl-CoA carboxylase (Katz & Ick, 1981). The mechanism whereby this control is achieved is not understood, although generally it is appreciated that these effects are probably related to a dual effect involving the control of the rate of translation of mRNA on the ribosome or of the increased transcription of specific DNA regions to generate mRNA (Ochoa & De Haro, 1979). Increased synthesis of L-type pyruvate kinase *in vivo* is associated with increased concentrations of mRNA for L-type pyruvate kinase (Cladaras & Cottam, 1980b). The induced enzyme synthesis observed *in vivo* proceeds after a lag phase of up to 24 h, and maximum rates of enzyme synthesis occur 48 h after weaning (Hopkirk & Bloxham, 1980). The accumulation of mRNA precedes this synthesis. The results reported here show there to be a longer lag phase (48–72 h) for enzyme synthesis in newborn than in 48 h-starved adult rats (24–48 h). This may reflect the current status of the animal; neonatal-rat hepatocytes synthesized no L-type pyruvate kinase, whereas hepatocytes from adult rats were already synthesizing the enzyme, but at much decreased rates, and therefore must have contained mRNA for L-type pyruvate kinase. The comparison is therefore one of increased enzyme synthesis and the induction of synthesis of a novel enzyme.

Translation of RNA is also facilitated by phosphorylated sugars, including glucose 6-phosphate, fructose 6-phosphate and sedoheptulose 7-phosphate (Giloh & Mager, 1975; Ernst *et al.*, 1978); it has been postulated that this may be as a result of increased flux through the hexose monophosphate pathway (Ochoa & De Haro, 1979), as intermediates of glycolysis have no effect. Animals fed on a diet rich in fructose display high hepatic and serum triacylglycerol concentrations (Zakim *et al.*, 1967). High rates of lipogenesis demand the production of large quantities of substrate in the form of acetyl-CoA and NADPH. Correspondingly L-type pyruvate kinase and glucose 6-phosphate dehydrogenase both display marked increases in total activity in animals fed on high-sucrose diets (Cohen *et al.*, 1972). The large increase in total glucose 6-phosphate dehydrogenase activity is reflected in significant increases in NADPH (Fitch & Chaikoff, 1960) and 6-phosphogluconate (Smith & Freedland, 1979) in animals fed on high-carbohydrate diets. In turn, 6-phosphogluconate has been shown to be an allosteric activator of L-type pyruvate kinase (Smith & Freedland, 1979, 1981). Whether or not this also serves to initiate protein synthesis *de novo* and to induce the expression of latent genes is as yet unknown, but further developments in hepatocyte culture techniques should provide a system with which to understand at a molecular level

the regulation of the expression of a number of genes.

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