

## The Regulation of Gluconeogenesis in Pouch Young of the Tammar Wallaby, *Macropus eugenii* (Desmarest)

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### Abstract

Parenchymal cells have been prepared from wallaby pouch-young liver by enzymic means. Such cells exclude Trypan blue, lose little lactate dehydrogenase into the incubation medium, have an adenylate energy charge of about 0.75 and increase production of glucose from endogenous sources in the presence of  $10^{-9}$ M glucagon. We conclude that the cells are metabolically viable. Hepatocytes isolated from fed pouch young released glucose in the absence of added substrates. The rate of glucose release was increased when L-lactate, L-lactate + pyruvate, propionate or fructose were added. After 24 h without food, glucose release in the absence of substrate was greatly reduced but the effect of substrate addition was greater with all substrates other than fructose.

The concentration of some intermediates of the gluconeogenic pathway was measured in livers of fed and fasted pouch young. After fasting, the concentrations of lactate, pyruvate, malate, fructose-6-phosphate, glucose-6-phosphate and glucose fell whilst the concentrations of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate rose.

The activities of glucose-6-phosphatase, hexosediphosphatase, mitochondrial phosphoenolpyruvate carboxykinase (GTP) and pyruvate carboxylase were similar in livers from fed and fasted pouch young. Cytosolic phosphoenolpyruvate carboxykinase (GTP) activity increased several-fold after fasting.

We conclude that hepatic gluconeogenesis is regulated in wallaby pouch young and that cytosolic phosphoenolpyruvate carboxykinase (GTP) is involved in this regulation.

The regulation of both glucose synthesis and phosphoenolpyruvate carboxykinase (GTP) activity occurred at all stages of pouch life from 75 to 220 days after birth. Homeothermy is developed at about 150 days of age and the development of regulation of glucose synthesis clearly precedes this.

### Introduction

Regulation of blood glucose concentration has been studied very little in marsupials; indeed, little is known about the intermediary metabolism of these animals. Yet they are of interest to the student of metabolism, because of their unusual development. Although the adults are often large, the gestation period is always short and the young are small at birth. For example, the tammar wallaby, *Macropus eugenii*, weighs 5-10 kg when adult but only 300 mg when born after a gestation period of 27 days. The young then suckles for more than 200 days in the pouch. At about 200 days the young begins to supplement its diet with grass and it leaves the pouch after about 250 days (Murphy and Smith 1970).

Little is known of metabolic regulation at any point in its development. It is likely that the foetus derives glucose and amino acids from the blood of the mother (Renfree 1970, 1973) but nothing is known of the subsequent metabolism of these compounds. For the greater part of pouch life the young relies wholly on milk as a source of fluid, energy and substrates for growth. Both lipid and protein components

of the milk change as pouch life proceeds in the red kangaroo (Bailey and Lemon 1966; Griffiths *et al.* 1972), the quokka (Jordan and Morgan 1968) and the tammar (Ezzy 1975). Adult kangaroos are deficient in some enzymes of galactose metabolism (Stephens *et al.* 1974), and intestinal lactase content in kangaroo pouch young is low (Kerry 1969) but nothing is known of the way in which the milk components are metabolized by pouch young.

The adult has a compartmented fore-stomach with a large microbial population. The blood glucose level is low at about 65 mg/100 ml. Other macropods utilize volatile fatty acids as an energy source (Barker 1961) and it is likely that this is the case in the tammar.

At about 150 days after birth the pouch young begins to regulate its body temperature (Setchell 1974). We have measured rates of glucose production by hepatocytes isolated from tammar pouch young both before and after development of homeothermy. Points at which regulation of gluconeogenesis might occur were investigated by assaying the concentrations of intermediates of the gluconeogenic pathway in liver tissue and by determining the activities of relevant enzymes.

## Materials and Methods

### *Animals*

Male pouch young of the tammar wallaby, *Macropus eugenii* (Desmarest) were taken from a colony maintained at the Division of Wildlife Research, CSIRO, Gungahlin, A.C.T. The original stock for this colony were wild tammars captured on Kangaroo Island, South Australia. The adults were held in open paddocks and their diet was supplemented with lucerne. They had free access to water.

The head and pes length of each animal was measured and the age determined from the age regression curves of Murphy and Smith (1970). The body weight of each animal was also recorded.

After removal from the pouch, the young were treated either as fed or fasted animals. Fed young were used within 1 h of removal from the teat. Fasted young were used 24 h after removal. During this time they were kept in an incubator at 35°C and high humidity. Throughout this paper the terms 'fed' and 'fasted' are used as defined here.

### *Preparation of Isolated Hepatocytes*

Isolated hepatocytes were prepared essentially by the method of Berry and Friend (1969) as modified by Johnson *et al.* (1972). Pouch young were anaesthetized by intraperitoneal injection of Nembutal (60 mg/kg body weight) and heparin (1 i.u./g body weight) in saline was injected into the saphenous vein. The bile duct was cannulated and the hepatic portal vein fitted with a cannula connected to a Braun perfusor (B. Braun, Melsungen, Germany). The liver was removed from the body cavity and pre-perfused with Ca<sup>2+</sup>-free Hanks saline (Johnson *et al.* 1972) for 10 min at 5 ml/min to remove blood components. It was then connected to a recirculating perfusion apparatus containing 100 ml of Ca<sup>2+</sup>-free Hanks saline, and perfused at a rate of 3–5 ml (g liver)<sup>-1</sup> min<sup>-1</sup>. 5 min after the start of perfusion, 35 mg collagenase (Collagenase Type I, Sigma, St Louis, Missouri, U.S.A.) dissolved in 2 ml of perfusion medium was added. The pH remained at 7.1 throughout the perfusion. When the liver began to lose its turgidity, usually after 45–60 min, it was chopped into fine pieces in a small volume of perfusate and shaken at 37°C for a further 15 min. The cell suspension was then sieved through nylon gauze and centrifuged at 40 *g* for 2 min after which the supernatant was discarded. The cells were washed three times and resuspended in Krebs bicarbonate Ringer, pH 7.1 (Krebs and Henseleit 1932) containing 2% (w/v) bovine serum albumen (Fraction V, poor in fatty acids, Calbiochem, San Diego, U.S.A.) and gassed with 5% CO<sub>2</sub> in oxygen.

This final cell suspension was examined under the light microscope, the cells were tested for their ability to exclude Trypan blue, and cell numbers were determined in a Neubauer improved haemocytometer (American Optical Co., Buffalo, U.S.A.). Only those preparations in which the cells were well dispersed and well rounded and in which the nuclei were clearly visible, and where more than 90% of cells excluded Trypan blue, were used in metabolic experiments.

### *Incubation of Isolated Hepatocytes*

Incubations were carried out in stoppered 50-ml Erlenmeyer flasks in a shaking water bath at 37°C under an atmosphere of 5% CO<sub>2</sub> in oxygen. Each incubation contained about  $1 \times 10^6$  cells in 2.0 ml of medium. This gave a large surface area and a shallow depth of medium which ensured efficient gassing. Samples, usually 200  $\mu$ l, of the cell suspension were taken at specific time intervals and quenched in 20  $\mu$ l 50% (v/v) HClO<sub>4</sub>. After each sampling the incubation flask was regassed with 5% CO<sub>2</sub> in oxygen and restoppered. Samples were centrifuged to remove the precipitated proteins, and the supernatants were assayed for glucose by the GOD-Period Method using a Biochemica Test Combination (Böhringer GMBH, Mannheim, Germany). This method of assay was compared with that using hexokinase and glucose-6-phosphate dehydrogenase (Bergmeyer *et al.* 1974). These two assays always gave similar results and so the GOD-Period Method, being more convenient, was used routinely.

### *Determination of Lactate Dehydrogenase (EC 1.1.1.27) Activity in Incubation Media*

In order to determine the amount of leakage of cytosolic enzymes into the incubation medium, some cell samples were separated from the medium by centrifugation at 40 *g* either at the start of incubation or after 30 min. The clear supernatants were stored on ice and the cells were resuspended in a small volume of fresh incubation medium. They were sonicated for four periods of 8 s in an MSE sonicator (MSE, London, U.K.) with cooling during and between each period of sonication. The temperature of the sample did not exceed 15°C at any time. The suspension was then centrifuged at 14 000 *g* for 2 min in a Quickfit Microcentrifuge (James A. Jobling, Stone, U.K.). Lactate dehydrogenase activity in the stored media and in the supernatants after sonication was determined by the method of Bergmeyer and Bernt (1974).

### *Determination of Adenine Nucleotide Concentration in Hepatocytes*

In some preparations, after 30 min, the entire incubation was quenched by addition of one-tenth volume of 50% (v/v) HClO<sub>4</sub>. The preparation was centrifuged to remove precipitated proteins, the supernatant neutralized by addition of 3 M KHCO<sub>3</sub>, and the precipitated potassium perchlorate removed by centrifugation after standing on ice for 30 min. The concentrations of ATP, ADP and AMP in the final supernatant were determined by the methods of Lamprecht and Trautschold (1974) and Jaworek *et al.* (1974).

### *Measurement of Intermediary Metabolites in Whole Liver*

Pouch young were decapitated using scissors, or a guillotine (Harvard Instrument Co., Millis, Mass., U.S.A.) for larger animals, the body cavity was opened and the liver removed and clamp-frozen in liquid nitrogen using tongs described by Wollenberger *et al.* (1960). The total time elapsed between decapitation and freezing was between 20 and 30 s. The thin slice of frozen liver was powdered in a metal mortar in liquid nitrogen and added to a preweighed beaker containing a known volume of 8% (v/v) HClO<sub>4</sub> in an ethanol-ice water bath. The liver powder was stirred into the ice-cold perchloric acid slurry until the powder was fully dispersed. After reweighing the beaker to determine the liver weight, the liver-perchloric acid suspension was homogenized in a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 15 000 *g* for 20 min at 0°C, the supernatant neutralized with 3 M KHCO<sub>3</sub>, and, after 30 min on ice, the precipitated potassium perchlorate was removed by centrifugation. Throughout this procedure the sample was kept on ice.

Assay of metabolic intermediates was performed on the final supernatant on the day of preparation. Specific enzymic techniques were used as follows: pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate (Czok and Eckert 1965), lactate and malate (Gutman and Wahlefeld 1974), glucose-6-phosphate and fructose-6-phosphate (Lang and Michal 1974); fructose-1,6-diphosphate (Michal and Beutler 1974), and glucose (Bergmeyer *et al.* 1974).

### *Enzyme Assays*

Pouch young were decapitated and the liver removed and placed on ice. A sample of liver was homogenized in three volumes of buffered sucrose containing 200 mM sucrose, 1 mM reduced glutathione, 1 mM dithiothreitol, 1 mM EDTA, triethanolamine-HCl 40 mM, pH 7.4. One volume

of 2% (w/v) sodium deoxycholate in sucrose buffer was added to four volumes of this homogenate and a second sample of homogenate was diluted equivalently with sucrose buffer. Both samples were centrifuged for 1 h at 100 000 *g* at 0°C. The supernatants were used for assay of pyruvate carboxylase (EC 6.4.1.1) (Henning and Seubert 1964) phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (Nordlie and Lardy 1963; Henning *et al.* 1966) and hexosediphosphatase (EC 3.1.3.11) (Pontremoli *et al.* 1965).

A further sample of liver was homogenized in 0.1 M citrate buffer pH 6.5 and glucose-6-phosphatase (EC 3.1.3.9) activity was assayed using the whole homogenate by the method of Harper (1965).

Pyruvate carboxylase, phosphoenolpyruvate carboxykinase (GTP) and hexosediphosphatase activities were always measured in fresh liver. Glucose-6-phosphatase activity was sometimes measured after the liver had been frozen for 2 days at -20°C. There was no change in enzyme activity during this time although longer periods of storage resulted in loss of activity. Enzyme activities are expressed in units per gram wet weight. One unit produces one  $\mu$ mole of product in one minute.

### Chemicals

All enzymes, coenzymes, adenine nucleotides, inosine triphosphate, L-lactate, pyruvate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, 1,3-diphosphoglycerate, fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, acetyl phosphate, L-alanine, coenzyme A, dithiothreitol, glutathione and triethanolamine-HCl were from Boehringer GMBH, Mannheim, Germany; EDTA and sodium deoxycholate came from Merck, Darmstadt, Germany; [ $^{14}$ C]-pyruvate used in the pyruvate carboxylase assay was from the Radiochemical Centre, Amersham, U.K.; glucagon was from Calbiochem, San Diego, U.S.A.; all other chemicals were analytical grade and glass distilled water was used throughout.

## Results

### *Viability of Isolated Hepatocytes*

It is more difficult to prepare hepatocytes from wallaby pouch young than from the rat. Whereas we are able to prepare excellent hepatocytes from rat liver after only 15 min perfusion with collagenase, wallaby pouch-young livers had to be perfused for much longer and the cell yield ( $10^7$  cells/g) was lower than from rat liver. Dispersion of wallaby hepatocytes was not improved by increasing the concentration of collagenase in the perfusion medium or by adding hyaluronoglucosidase. The use of gelatine rather than bovine serum albumen in incubation media (Zahlten and Stratman 1974) caused the cells to clump badly and decreased the rate of glucose synthesis.

The viability of wallaby liver cells was established by several criteria. At least 90% of the cells excluded Trypan blue and appeared well rounded with the nuclei clearly visible. A typical preparation had 8.7 nmol ATP, 3.7 nmol ADP and 2.2 nmol AMP per  $10^6$  cells and an adenylate energy charge of 0.724. Freeze-clamped livers from four pouch young 190–220 days old contained  $1.425 \pm 0.195$   $\mu$ mol ATP,  $0.689 \pm 0.034$   $\mu$ mol ADP and  $0.207 \pm 0.028$   $\mu$ mol AMP per g wet weight and the adenylate energy charge was 0.76. These values were not significantly changed in either freeze-clamped preparations or in hepatocytes after 24 h fasting.

Lactate dehydrogenase activity in the livers of two 200-day-old pouch young was 170 and 182 units/g liver whilst in two cell preparations from animals of similar age the activity of this enzyme was 1.56 and 1.47 units/ $10^6$  cells. After 30 min incubation the lactate dehydrogenase concentration in the incubation medium had increased by only about 3% of the total activity in the cells. It is not clear whether this leakage represents cell breakage or some leakiness of cell membranes.

### Endogenous Glucose Production

Isolated hepatocytes prepared from fed pouch young released glucose at an average rate of  $1.15 \pm 0.25 \text{ mg } (10^6 \text{ cells})^{-1} \text{ min}^{-1}$  ( $n = 11$ ) and a typical time course of glucose release is shown in Fig. 1. The amount of glycogen in the livers of fed pouch young is  $45.3 \pm 7.9 \text{ mg/g}$  ( $n = 8$ ) and so the glucose released by fed pouch young can probably be attributed to glycogen breakdown.

Perfused livers from four fed pouch young released glucose at an average rate of  $0.98 \pm 0.14 \text{ } \mu\text{mol g}^{-1} \text{ min}^{-1}$  which is about 150 times the rate of release from  $1 \times 10^6$  cells.

After 24 h without food, the glycogen concentration in the liver had fallen to  $0.72 \pm 0.14 \text{ mg/g}$  ( $n = 8$ ) and very little glucose was released by hepatocytes from fasted animals incubated in the absence of added substrate (Fig. 1).

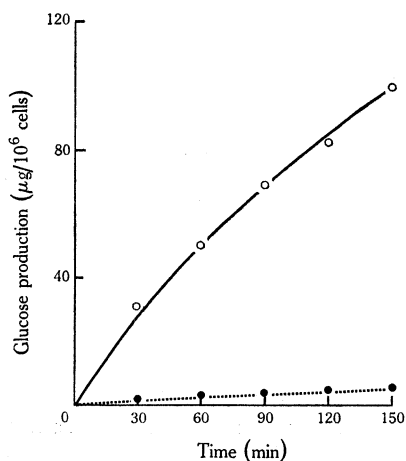


Fig. 1. Endogenous glucose production by hepatocytes isolated from the livers of one fed (○) and one fasted (●) pouch young. Hepatocytes were incubated in Krebs bicarbonate Ringer + 2% (w/v) bovine serum albumen with no added substrate.

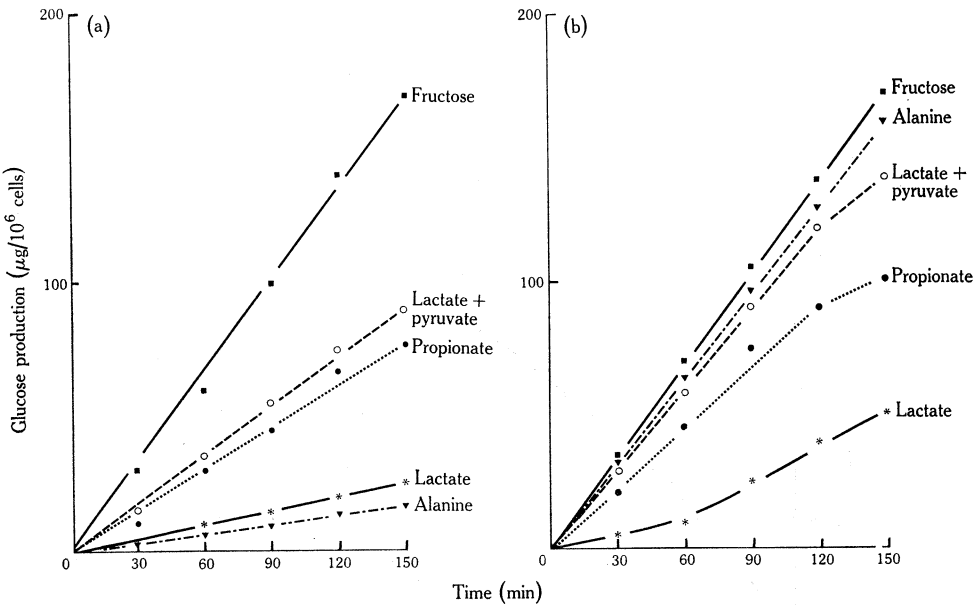
### Glucose Production from Added Substrates

When hepatocytes from fed pouch young were incubated in the presence of added substrate, the rate of production of glucose increased (Fig. 2a). Addition of 10 mM L-lactate increased the rate of glucose production by about  $0.15 \text{ } \mu\text{g } (10^6 \text{ cells})^{-1} \text{ min}^{-1}$ . When 10 mM L-alanine, 10 mM L-lactate + 1 mM pyruvate, 10 mM propionate or 10 mM fructose was added, glucose was produced at higher rates. Thus, cells from fed pouch young of all ages studied were capable of gluconeogenesis, albeit at low rates. These rates did not vary with the age of the animal (Table 1).

When cells from fasted animals were incubated with the same substrates at the same concentrations, rates of glucose production were increased with all except fructose (Fig. 2b). This increase occurred at all ages of development studied (Table 1) although it was less pronounced with L-lactate or propionate than with the other substrates.

When differing concentrations of each substrate were incubated with hepatocytes from fed or fasted animals, rates of synthesis of glucose were maximal at substrate concentrations less than 10 mM. Maximum rates of glucose synthesis were supported by 0.5 mM L-alanine and propionate, by 5.0 mM L-lactate and by 5.0 mM L-lactate + 0.5 mM pyruvate. When the substrate concentration was 10 mM there was no

inhibition of glucose synthesis, synthetic rates were maintained linear for longer periods and the substrate concentration was never reduced by more than 1 mM or 10% during the incubation. Thus 10 mM substrate was used in all experiments except where substrate concentration is specifically mentioned.



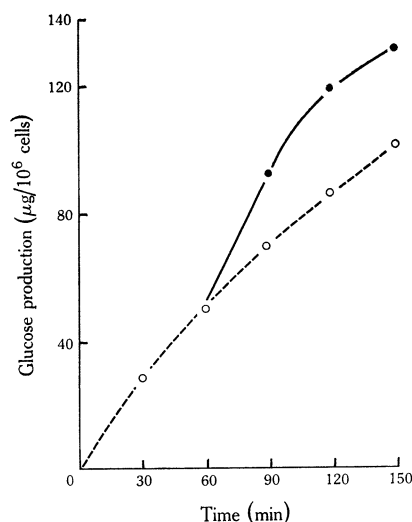
**Fig. 2.** Gluconeogenesis from added substrates by hepatocytes isolated from (a) a fed pouch young aged 170 days and (b) a fasted pouch young aged 165 days. Hepatocytes were incubated in Krebs bicarbonate Ringer + 2% (w/v) bovine serum albumen; various substrates were added at the start of the incubation to give a final concentration of 10 mM. Duplicate runs with each substrate gave similar results and the endogenous glucose production in the absence of substrate was subtracted from that when substrate was present to give the actual rate of glucose synthesis from added substrate.

**Table 1.** Rates of gluconeogenesis in cells from fed and fasted pouch young of *M. eugenii*. Results are expressed as μg glucose per 10<sup>6</sup> cells per minute. Each value is the mean ± s.e. for the number of pouch young in each group (shown in parentheses). A one-tailed Student's *t* test was used

Substrate	Young aged 30–100 days			Young aged 100–200 days		
	Fed	Fasted	<i>P</i>	Fed	Fasted	<i>P</i>
10 mM Lactate	0.284 ± 0.071 (5)	0.393 ± 0.112 (7)	> 0.2	0.228 ± 0.070 (8)	0.515 ± 0.097 (8)	< 0.05
10 mM Propionate	0.475 ± 0.179 (5)	—		0.744 ± 0.176 (9)	1.353 ± 0.214 (8)	< 0.05
10 mM Alanine	0.167 ± 0.092 (5)	0.556 ± 0.052 (6)	< 0.005	0.066 ± 0.027 (8)	1.452 ± 0.163 (8)	< 0.001
10 mM Lactate + 1 mM pyruvate	—	—		0.502 ± 0.196 (7)	1.713 ± 0.172 (6)	< 0.001
10 mM Fructose	—	—		1.38 ± 0.326 (9)	1.540 ± 0.351 (10)	> 0.1

### Effects of Added Glucagon

Glucagon at concentrations of  $10^{-9}$  M and higher stimulated glucose production by cells from fed animals (Fig. 3). At lower concentrations glucagon had no effect. When added to cells from fasted animals in the presence of lactate, propionate or fructose, glucagon at concentrations as high as  $10^{-6}$  M had no effect upon the rate of glucose production.



**Fig. 3.** Effect of glucagon on endogenous glucose production by hepatocytes isolated from a fed pouch young aged 125 days.  $10^{-8}$  M glucagon (●) was added to the incubation medium at 60 min after the start of incubation. ○ Control.

**Table 2.** Concentration of various gluconeogenic intermediates in the livers of fed and fasted pouch young of *M. eugenii*

Results are expressed as nmoles per gram. Each value is the mean  $\pm$  s.e. for four animals. A one-tailed Student's *t* test was used

Gluconeogenic intermediate	Young aged 75–120 days			Young aged 190–220 days		
	Fed	Fasted	<i>P</i>	Fed	Fasted	<i>P</i>
Lactate	3300 $\pm$ 558	781 $\pm$ 293	< 0.01	3768 $\pm$ 970	2088 $\pm$ 207	n.s.
Pyruvate	109 $\pm$ 11	63 $\pm$ 14	< 0.05	188 $\pm$ 65	47 $\pm$ 5	n.s.
Malate	2254 $\pm$ 100	477 $\pm$ 166	< 0.001	2797 $\pm$ 89	1098 $\pm$ 60	< 0.001
Phosphoenolpyruvate	60 $\pm$ 16	159 $\pm$ 14	< 0.01	71 $\pm$ 20	222 $\pm$ 13	< 0.001
2-Phosphoglycerate	19 $\pm$ 25	40 $\pm$ 5	< 0.02	20 $\pm$ 1.7	43 $\pm$ 1.6	< 0.001
3-Phosphoglycerate	143 $\pm$ 26	377 $\pm$ 54	< 0.02	170 $\pm$ 44	490 $\pm$ 35	< 0.001
Fructose-6-phosphate	48 $\pm$ 15	14 $\pm$ 3	n.s.	53 $\pm$ 5	37 $\pm$ 8	n.s.
Glucose-6-phosphate	156 $\pm$ 37	44 $\pm$ 9	< 0.05	223 $\pm$ 17	135 $\pm$ 34	n.s.
Glucose	7260 $\pm$ 222	2953 $\pm$ 223	< 0.001	11439 $\pm$ 1116	5417 $\pm$ 544	< 0.005

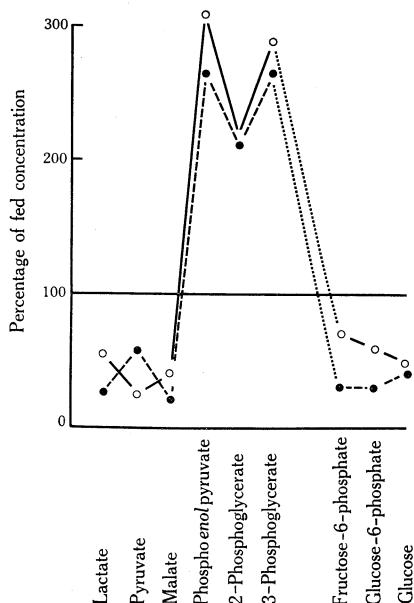
### Levels of Gluconeogenic Intermediates in Pouch-young Liver

When the concentrations of several intermediates of the gluconeogenic pathway were compared in livers from fed and fasted pouch young, clear differences could be seen at both early and late stages of pouch life (Table 2). At both ages there were significant decreases in the concentrations of both malate and glucose, and significant increases in the concentrations of phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate. When these results are expressed as cross-over plots (Fig. 4)

there is a positive cross over between malate and phosphoenolpyruvate in both age groups.

### Enzyme Activities

Four major enzymes have been implicated in the regulation of gluconeogenesis in eutherian mammals: pyruvate carboxylase, phosphoenolpyruvate carboxykinase (GTP), hexosediphosphatase and glucose-6-phosphatase. When assayed in fed and fasted pouch young of different ages, the activities of pyruvate carboxylase, hexosediphosphatase and glucose-6-phosphatase were unchanged (Table 3). Membrane-bound phosphoenolpyruvate carboxykinase (GTP) activity was also unaltered whilst in contrast, cytosolic activity of this enzyme was increased several-fold after fasting at all ages studied (Table 3).



**Fig. 4.** Effects of fasting on the metabolic pattern (cross-over plot) in freeze-clamped livers of pouch young aged 75–120 days (○) and 190–220 days (●). Fed values are taken as 100%. The points are mean values for four animals in each group.

### Discussion

Several criteria, including the ability to maintain a high adenylate energy charge and the integrity of the plasma membrane, have been used to establish the viability of hepatocytes isolated from wallaby pouch young. The fact that such hepatocytes can synthesize glucose at rates similar to both eutherian hepatocytes and the isolated perfused eutherian liver provides further evidence for their metabolic viability.

In the isolated perfused rat liver, the rate of gluconeogenesis from L-lactate is usually about  $1 \mu\text{mol (g liver)}^{-1} \text{ min}^{-1}$  (Söling *et al.* 1970); similar rates have been reported in the mouse (Assimakopoulos-Jeannet *et al.* 1973) and the guinea-pig (Söling *et al.* 1970). Somewhat lower rates have been found in hepatocytes isolated from the rat (Berry and Kun 1972; Johnson *et al.* 1972; Zahlten and Stratman 1974) although comparisons are complicated by the different methods used for calculating the number of hepatocytes per gram of liver. Zahlten and Stratman (1974) have estimated that there are about  $100 \times 10^6$  hepatocytes/g liver in the rat.

Wallaby hepatocytes are about the same size as those in the rat (17–20  $\mu\text{m}$  mean diameter), and the DNA content of kangaroo and rat diploid cells are similar at about 8.5 pg DNA/cell (Martin and Hayman 1967; Bachmann 1972). Since the amount of DNA per gram of liver is similar in rat and wallaby (Janssens *et al.*, unpublished data) it seems likely that the number of hepatocytes per gram of liver is similar in the two species.

**Table 3. Enzyme activities in the livers of adult and fed and fasted pouch young of *M. eugenii* at various stages of development**

Enzyme activities are expressed as units/g tissue. One unit produces 1  $\mu\text{mol}$  product/min. Each value is the mean  $\pm$  s.e. for the number of pouch young in each group (shown in parentheses)

Age (days)	State	Phosphoenolpyruvate carboxykinase (GTP) (Cytosolic)	(Membrane-bound)	Hexosediphosphatase (Total)	Glucose-6-phosphatase (Total)	Pyruvate carboxylase (Membrane-bound)
50–75	Fed	0.685 $\pm$ 0.096 (7)	5.66 $\pm$ 0.27 (7)	4.66 $\pm$ 0.51 (5)	22.57 $\pm$ 2.70 (4)	—
	Fasted	6.34 $\pm$ 1.56 (5)	5.84 $\pm$ 0.63 (5)	6.00 $\pm$ 0.71 (5)	20.00 $\pm$ 1.37 (3)	—
80–100	Fed	0.610 $\pm$ 0.108 (3)	5.43 $\pm$ 0.50 (3)	4.77 $\pm$ 1.09 (3)	21.15 $\pm$ 3.51 (3)	—
	Fasted	8.02 $\pm$ 1.09 (4)	5.76 $\pm$ 0.59 (4)	5.76 $\pm$ 1.32 (4)	28.47 $\pm$ 2.46 (4)	—
190–230	Fed	0.486 $\pm$ 0.105 (4)	5.64 $\pm$ 0.97 (4)	4.46 $\pm$ 0.74 (5)	18.98 (2)	1.489 $\pm$ 0.053 (6)
	Fasted	4.63 $\pm$ 0.77 (5)	5.41 $\pm$ 0.73 (5)	5.07 $\pm$ 0.25 (4)	22.26 $\pm$ 1.77 (3)	1.193 $\pm$ 0.149 (4)
Adult	Fed	2.66 $\pm$ 0.54 (5)	4.05 $\pm$ 0.43 (5)	3.54 $\pm$ 0.36 (5)	9.40 $\pm$ 1.61 (4)	—

This contention is supported by estimates of both lactate dehydrogenase activity and total adenylate concentration in fresh liver and isolated cells from fed and fasted pouch young. Lactate dehydrogenase activity in 1 g of liver was approximately 124 times that in  $1 \times 10^6$  isolated cells, whilst the total adenylate concentration was about 150 times that in  $1 \times 10^6$  cells. The adenylate energy charges and ATP/ADP ratios were very similar in liver and cells of both fed and fasted pouch young. These results suggest that the isolated cells are in a similar condition to those in the whole liver and further support the suggestion that there are approximately  $100 \times 10^6$  cells/g liver. As emphasized by many workers in this field (for example Zahlten and Stratman 1974) all such calculations are only approximate and we prefer to express our results as product per  $10^6$  cells per minute. This can lead to problems when animals of different ages are compared because the size of hepatocytes may change as development progresses in some species (Clark *et al.* 1976). We do not yet have good estimates of the number of cells per gram of liver in small pouch young, largely because of the small amount of tissue available, and so the relative rates of activity at the different ages studied may not be a true reflection of the capacity for hepatic glucose synthesis by the whole animal. We believe, however, that the effects of fasting in each age group are accurately portrayed.

L-Alanine, propionate, L-lactate and L-lactate+pyruvate stimulated a greater increase in the rate of glucose production by hepatocytes from fasted pouch young than by cells from fed animals. There was no change in the rate of glucose release in the presence of fructose. Although the rate of glycogen breakdown may be affected by the presence of added substrates (Howard and Widder 1976) the results also suggest that gluconeogenesis may be regulated in pouch young and that such regulation may occur early in the pathway.

It is interesting that L-lactate is a rather poor substrate in this species when it is such a good one in most eutherians. This may reflect a lack of ATP for gluconeogenesis because when pyruvate and L-lactate are added together gluconeogenesis proceeds rapidly. Pyruvate can act as a substrate for the tricarboxylic acid cycle and ATP synthesis, whereas L-lactate, entering a relatively reduced cytoplasm, may well not supply sufficient pyruvate for ATP generation. Exton and Park (1967) suggested that fatty acid oxidation could supply such an ATP requirement in the rat but this may not be so in the wallaby.

Propionate is a good substrate for gluconeogenesis—this is not unexpected in an animal which has a ruminant-like digestion. L-Alanine is also a good substrate, but only in fasted animals. Indeed hepatocytes from fasted pouch young synthesize glucose from L-alanine several times more rapidly than hepatocytes from fed animals, particularly when they are older (Table 1). This is not surprising because an unfed animal will rely upon amino acids from protein breakdown rather than volatile fatty acids from the diet as substrates for gluconeogenesis. L-Alanine is a much poorer gluconeogenic substrate than L-lactate plus pyruvate in cells from fed animals, whilst the two substrates are about equally good in cells from fasted animals. This suggests that the conversion of L-alanine to pyruvate may be limiting in fed pouch young. Preliminary experiments (Janssens *et al.*, unpublished data) show that alanine aminotransferase activity is increased several-fold after starvation and the entry of alanine to the gluconeogenic pathway may well be controlled here.

When the concentrations of several intermediates of the gluconeogenic pathway in fed and fasted animals are compared (Fig. 4), there is a clear positive cross over between malate and phosphoenolpyruvate. If we assume that malate and oxaloacetate are at equilibrium in both conditions, then this suggests that phosphoenolpyruvate carboxykinase (GTP) is a likely enzyme of regulation. The substrates for pyruvate carboxylase and glucose-6-phosphatase were also reduced in amount in the unfed animals and so these enzymes could also be involved in regulation. Fructose-diphosphate concentrations are very low in wallaby pouch-young liver and were not accurately measured by the assay used. Hence no conclusions pertaining to the role of hexosediphosphatase can be drawn from these measurements.

When these four enzymes were assayed in livers from fed and fasted pouch young, the only enzyme to have increased in activity was phosphoenolpyruvate carboxykinase (GTP), but this change was limited to the fraction in the cytosol. This result fits in well with the conclusions drawn from the cross-over plots and it seems clear that an increased activity of cytosolic phosphoenolpyruvate carboxykinase (GTP) is a major factor in enhancing gluconeogenesis in fasted wallaby pouch young. Fasting is similarly followed by large increases in cytosolic phosphoenolpyruvate carboxykinase (GTP) activity in the rat, rabbit (Usatenko 1970), guinea-pig (Söling *et al.* 1970) and pig (Swiatek *et al.* 1970), whilst mitochondrial phosphoenolpyruvate carboxykinase (GTP) activity is increased only slightly, if at all. In contrast, Taylor *et al.* (1971)

reported no change in the activity of this enzyme in either cytosol or mitochondria in the sheep after 6–7 days without food; this difference between the sheep and the other eutherians has been attributed to its ruminant digestion. Ruminants must synthesize all the glucose they need even when fully fed and consequently gluconeogenic rates are probably not increased and may be reduced on fasting. Certainly the glucose turnover rate is reduced in fasting sheep (Annison *et al.* 1967) which may imply a decreased rate of gluconeogenesis.

Thus, the wallaby pouch young is apparently more like a eutherian non-ruminant than a eutherian ruminant in its response to fasting, despite the fact that the adult wallaby has a ruminant-like digestion. Of course, the pouch young studied were all milk-feeders and even the oldest were not eating large quantities of grass. It could well be that their gluconeogenic pathway would increase in activity when the bacterial flora in the gut became fully established. Indeed the activity of phosphoenolpyruvate carboxykinase in the cytosol of the fed adult liver is much higher than in the fed pouch young (Table 3). It is worth noting here that rates of gluconeogenesis in lambs increase with increasing age (Jarrett *et al.* 1975). The metabolic effects of the change from a milk to a grass diet in the wallaby are currently under investigation.

The observation that the rate of gluconeogenesis is unaffected by glucagon is also of interest. Although glucagon stimulates gluconeogenesis in the starved rat (Johnson *et al.* 1972; Garrison and Haynes 1973; Zahlten *et al.* 1973) it has only a slight effect at the high concentration of  $10^{-7}$  M in the mouse (Müller *et al.* 1976) and no effect at even higher concentrations in the guinea-pig (Söling *et al.* 1970). This variation in response to glucagon in different species may reflect a genuine difference between species or a difference in response of the species to starvation or some other pretreatment. For example, in wallaby pouch young, gluconeogenic rates are increased markedly after 24 h without food and perhaps no further adaptation is possible. This is not to say that glucagon has had no effect during the period without food or that glucagon plays no role in regulation of gluconeogenic rates. We are fairly sure that the lack of response to glucagon is not a consequence of membrane damage. Glucagon stimulates glycogenolysis in wallaby hepatocytes as in all other species so far studied (Söling and Kleineke 1976).

Finally, there seems to be little change in the response of the gluconeogenic pathway to fasting as the animal develops the ability to thermoregulate. Perhaps in older animals fasting increases gluconeogenic rates more than in younger animals (Table 1) but pouch young of less than 100 days regulate both gluconeogenesis and phosphoenolpyruvate carboxykinase (GTP) activity. Clearly the development of the ability to regulate gluconeogenesis precedes the development of the ability to thermoregulate in this species.

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### References

- Annison, E. F., Brown, R. E., Leng, R. A., Lindsay, D. B., and West, C. E. (1967). Rates of entry and oxidation of acetate, glucose, D(–)- $\beta$ -hydroxybutyrate, palmitate, oleate and stearate, and rates of production and oxidation of propionate and butyrate in fed and starved sheep. *Biochem. J.* **104**, 135–47.

- Assimakopoulos-Jeannet, F., Exton, J. H., and Jeanrenaud, B. (1973). Control of gluconeogenesis and glycogenolysis in perfused livers of normal mice. *Am. J. Physiol.* **225**, 25–32.
- Bachmann, K. (1972). Genome size in mammals. *Chromosoma* **37**, 85–93.
- Bailey, L. F., and Lemon, M. (1966). Specific milk proteins associated with resumption of development by the quiescent blastocyst of the lactating red kangaroo. *J. Reprod. Fertil.* **11**, 473–5.
- Barker, J. M. (1961). The metabolism of carbohydrate and volatile fatty acids in the marsupial, *Setonix brachyurus*. *Q. J. Exp. Physiol.* **46**, 54–68.
- Bergmeyer, H. U., and Bernt, E. (1974). Lactate dehydrogenase. UV-assay with pyruvate and NADH. In 'Methods of Enzymatic Analysis', 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 574–9. (Verlag Chemie: Weinheim.)
- Bergmeyer, H. U., Bernt, E., Schmidt, F., and Stork, H. (1974). D-glucose. Determination with hexokinase and glucose-6-phosphate dehydrogenase. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 1196–201. (Verlag Chemie: Weinheim.)
- Berry, M. N., and Friend, D. S. (1969). High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* **43**, 506–20.
- Berry, M. N., and Kun, E. (1972). Rate-limiting steps of gluconeogenesis in liver cells as determined with the aid of fluoro-dicarboxylic acids. *Eur. J. Biochem.* **27**, 395–400.
- Clark, M. G., Filsell, O. H., and Jarrett, I. G. (1976). Gluconeogenesis in isolated intact lamb liver cells. Effects of glucagon and butyrate. *Biochem. J.* **156**, 671–80.
- Czok, R., and Eckert, L. (1965). D-3-phosphoglycerate, D-2-phosphoglycerate, phosphoenolpyruvate. Determination with phosphoglycerate mutase, enolase, pyruvic kinase and lactic dehydrogenase. In 'Methods of Enzymatic Analysis'. 1st English Edn. (Ed. H. U. Bergmeyer) pp. 229–33. (Verlag Chemie: Weinheim.)
- Exton, J. H., and Park, C. R. (1967). Control of gluconeogenesis in liver. I. General features of gluconeogenesis in the perfused livers of rats. *J. Biol. Chem.* **242**, 2622–36.
- Ezzy, D. M. (1975). Changes in the production rate and composition of milk during lactation in the tammar wallaby *Macropus eugenii* (Desmarest). Unpublished Honours Thesis, Australian National University, Canberra.
- Garrison, J. C., and Haynes, R. C. Jr. (1973). Hormonal control of glycogenolysis and gluconeogenesis in isolated rat liver cells. *J. Biol. Chem.* **248**, 5333–43.
- Griffiths, M., McIntosh, D. L., and Leckie, R. M. C. (1972). The effects of cortisone on nitrogen balance and glucose metabolism in diabetic and normal kangaroos, sheep and rabbits. *J. Zool.* **166**, 265–75.
- Gutman, I., and Wahlefeld, A. W. (1974). L-(+)-lactate and L-(–)-malate. Determination with lactate dehydrogenase, malate dehydrogenase and NAD. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 1464–8 and 1585–9. (Verlag Chemie: Weinheim.)
- Harper, A. E. (1965). Glucose-6-phosphatase. In 'Methods of Enzymatic Analysis'. 1st English Edn. (Ed. H. U. Bergmeyer.) pp. 788–92. (Verlag Chemie: Weinheim.)
- Henning, H. V., and Seubert, W. (1964). Zum Mechanismus der Gluconeogenese und ihrer Steuerung. I. Quantitative Bestimmung der Pyruvatcarboxylase in Rattenleber. *Biochem. Z.* **340**, 160–70.
- Henning, H. V., Stumpf, B., Ohly, B., and Seubert, W. (1966). On the mechanism of gluconeogenesis and its regulation. III. The gluconeogenic capacity and the activities of pyruvate carboxylase and PEP-carboxylase of rat kidney and rat liver after cortisol treatment and starvation. *Biochem. Z.* **344**, 274–88.
- Howard, R. B., and Widder, D. J. (1976). Substrate control of glycogen levels in isolated hepatocytes from fed rats. *Biochem. Biophys. Res. Commun.* **68**, 262–9.
- Jarrett, I. G., Filsell, O. H., and Clark, M. G. (1975). Preparation and use of isolated lamb liver cells for studies on gluconeogenesis. *Proc. Aust. Biochem. Soc.* **8**, 54.
- Jaworek, D., Gruber, W., and Bergmeyer, H. U. (1974). Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 2127–9. (Verlag Chemie: Weinheim.)
- Johnson, M. E. M., Das, N. M., Butcher, F. R., and Fain, J. N. (1972). The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyl cyclic adenosine monophosphate and fatty acids. *J. Biol. Chem.* **247**, 3229–35.
- Jordan, S. M., and Morgan, E. H. (1968). The changes in the serum and milk whey proteins during lactation and suckling in the quokka (*Setonix brachyurus*). *Comp. Biochem. Physiol.* **25**, 271–83.

- Kerry, K. R. (1969). Intestinal disaccharidase activity in a monotreme and eight species of marsupials (with an added note on the disaccharidases of five species of sea birds). *Comp. Biochem. Physiol.* **29**, 1015–22.
- Krebs, H. A., and Henseleit, K. (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyles's Z. Physiol. Chem.* **210**, 33–66.
- Lamprecht, W., and Trautschold, I. (1974). Adenosine-5'-triphosphate. Determination with hexokinase and glucose-6-phosphate dehydrogenase. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 2101–10. (Verlag Chemie: Weinheim.)
- Lang, G., and Michal, G. (1974). D-glucose-6-phosphate and D-fructose-6-phosphate. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 1238–42. (Verlag Chemie: Weinheim.)
- Martin, P. G., and Hayman, D. L. (1967). Quantitative comparisons between the karyotypes of Australian marsupials from three different superfamilies. *Chromosoma* **37**, 290–310.
- Michal, G., and Beutler, H. O. (1974). D-fructose-1,6-diphosphate, dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. In 'Methods of Enzymatic Analysis'. 2nd English Edn. (Ed. H. U. Bergmeyer.) pp. 1314–19. (Verlag Chemie: Weinheim.)
- Müller, P., Singh, A., Orci, L., and Jeanrenaud, B. (1976). Secretory processes, carbohydrate and lipid metabolism in isolated mouse hepatocytes. Aspects of regulation by glucagon and insulin. *Biochim. Biophys. Acta* **428**, 480–94.
- Murphy, C. R., and Smith, J. R. (1970). Age determination of pouch young and juvenile Kangaroo Island wallabies. *Trans. R. Soc. South Aust.* **94**, 15–20.
- Nordlie, R. C., and Lardy, H. A. (1963). Mammalian liver phosphoenolpyruvate carboxykinase activities. *J. Biol. Chem.* **238**, 2259–63.
- Pontremoli, S., Traniello, S., Luppis, B., and Wood, W. A. (1965). Fructose diphosphatase from rabbit liver. *J. Biol. Chem.* **240**, 3459–63.
- Renfree, M. B. (1970). Protein, amino acids and glucose in the yolk sac fluids and the maternal blood sera of the tammar wallaby. *Macropus eugenii* (Desmarest). *J. Reprod. Fertil.* **22**, 483–92.
- Renfree, M. B. (1973). The composition of fetal fluids of the marsupial *Macropus eugenii*. *Dev. Biol.* **33**, 62–79.
- Setchell, P. J. (1974). The development of thermoregulation and thyroid function in the marsupial *Macropus eugenii* (Desmarest). *Comp. Biochem. Physiol.* **47A**, 1115–21.
- Söling, H.-D., and Kleineke, J. (1976). Species dependent regulation of hepatic gluconeogenesis in higher animals. In 'Gluconeogenesis: Its Regulation in Mammalian Species'. (Eds R. W. Hanson and M. A. Mehlmán.) pp. 369–462. (John Wiley & Sons: New York.)
- Söling, H.-D., Willms, B., Kleineke, J., and Gehlhoff, M. (1970). Regulation of gluconeogenesis in the guinea-pig liver. *Eur. J. Biochem.* **16**, 289–302.
- Stephens, T., Irvine, S., Mutton, P., Gupta, J. D., and Harley, J. D. (1974). Deficiency of two enzymes of galactose metabolism in kangaroos. *Nature (London)* **248**, 524–5.
- Swiatek, K. R., Chao, K. L., Chao, H. L., Cornblath, M., and Tildon, T. (1970). Distribution of phosphoenolpyruvate carboxylase in pig liver. *Biochim. Biophys. Acta* **206**, 229–35.
- Taylor, P. H., Wallace, J. C., and Keech, D. B. (1971). Gluconeogenic enzymes in sheep liver. Intracellular localization of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in normal, fasted and diabetic sheep. *Biochim. Biophys. Acta* **237**, 179–91.
- Usatenko, M. S. (1970). Hormonal regulation of phosphoenolpyruvate carboxykinase activity in liver and kidney of adult animals and formation of this enzyme in developing rabbit liver. *Biochem. Med.* **3**, 298–310.
- Wollenberger, A., Ristau, O., and Schoffa, G. (1960). Eine einfache Technik der extrem schnellen Abkühlung grösserer Gewebe stücke. *Pflügers Arch. Gesamte Physiol. Menschen Tiere* **270**, 339–412.
- Zahlten, R. N., and Stratman, F. W. (1974). The isolation of hormone-sensitive rat hepatocytes by a modified enzymatic technique. *Arch. Biochem. Biophys.* **163**, 600–8.
- Zahlten, R. N., Stratman, F. W., and Lardy, H. A. (1973). Regulation of glucose synthesis in hormone-sensitive isolated rat hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3213–18.

