Developmental changes in hepatic fructose 2,6-bisphosphate content and phosphofructokinase-1 activity in the transition of chicks from embryonic to neonatal nutritional environment

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Within 2 days of hatching in chicks, there are parallel increases in hepatic fructose 2,6-bisphosphate content and phosphofructokinase-1 activity. The changes observed are a consequence of feeding on the carbohydrate-rich diet of neonatal life: lack of access to food after hatching prevents changes for either parameter. The results are discussed in relation to changes in the activities of hepatic lipogenic enzymes during the embryonic/neonatal transition of chicks and the role of insulin in co-ordination of developmental processes.

INTRODUCTION

Fructose 2,6-bisphosphate (F26BP) is a powerful allosteric regulator which exerts reciprocal control over phosphofructokinase-1 (PFK-1) and fructose-1,6-bisphosphatase (F16BPase). Within the liver this metabolite plays a critical role in the determination of the balance between glycolysis and gluconeogenesis (Hers & Hue, 1983; Pilkis *et al.*, 1981). Hepatic F26BP content alters in response to dietary and hormonal conditions. Starvation of rats decreases F26BP content, and re-feeding after prolonged starvation produces F26BP contents which are supranormal by comparison with normal fed animals (Masamichi & Uyeda, 1982; Neely *et al.*, 1981). Genetically obese mice exhibit elevated hepatic concentrations of F26BP (Claus & Pilkis, 1982; Hue & van de Werve, 1982). Changes have been observed in hepatic F26BP content throughout different stages of foetal and neonatal development of rats, and insulin/glucagon ratios have been implicated in the control of F26BP concentration (Schubert *et al.*, 1986).

Changes in hepatic F26BP content may be a consequence of modification in the supply of fructose 6-phosphate or of changes in the activity of the bifunctional enzyme (PFK-2/F26BPase) which synthesizes and degrades F26BP (Hue *et al.*, 1981*b*; Bartrons *et al.*, 1983). The activity of liver PFK-2 can be regulated by changes in enzyme phosphorylation state (a cyclic AMP-dependent process) and by changes in the concentrations of allosteric effectors (Van Schaftingen *et al.*, 1982*a*; Claus *et al.*, 1984; Sakakibara *et al.*, 1984).

The effects of F26BP, and the regulation of its concentration, are similar in mammalian and avian species, despite several unique features in the hepatic metabolism in birds (Chaekal *et al.*, 1983; Van Schaftingen & Hers, 1986). The chick embryo develops in an environment which lacks carbohydrate *in ovo*. Hepatic gluconeogenesis is fully activated in the embryo to maintain blood glucose concentration (Dickson, 1983). After hatching, the available food is rich in carbohydrate; this environmental change is associated with dramatic changes in key regulatory enzymes of

glucose and lipid metabolism (Pearce, 1977; Goodridge, 1986). For fatty acid synthase and malic enzyme a regulation of specific gene transcription has been proposed (Morris *et al.*, 1984). The co-ordinated changes after hatching produce a hepatic metabolic profile which would (i) favour glycolysis and lipogenesis and (ii) inhibit gluconeogenesis. As the liver is the major site of lipogenesis in chickens, co-ordinate regulation of glycolysis and lipogenesis may be crucial, given the importance of glucose as a lipogenic precursor in this species (Bickerstaffe *et al.*, 1970; Goodridge, 1968*a,b*; Goodridge *et al.*, 1974).

In the present study we describe the changes in hepatic F26BP content during the developmental transition from the embryonic (net gluconeogenic) to neonatal (net glycolytic) state in chicks. Given the interrelationship between F26BP and PFK-1 activity, in terms of both allosteric regulation and possibly control of enzyme degradation (Van Schaftingen & Hers, 1983), we have measured the correlation between activity of hepatic PFK-1 and F26BP content throughout development. Our studies indicate that both F26BP content and PFK-1 activity increase in parallel after hatching, provided that food ingestion occurs. Lack of access to food after hatching prevents changes in either parameter. The events observed at hatching are discussed in relation to hormonal changes known to occur during the chick embryo/neonatal transition. In addition, the findings are extended to generalized consideration of hormonal regulation of F26BP content and to the linkage between F26BP content and turnover of PFK-1.

MATERIALS AND METHODS

Animals

Fertile chick (*Gallus domesticus*) eggs were obtained from Northern Biologicals, St. Annes on Sea, Lancs., U.K. Eggs were incubated until hatching (20–21 days from onset of incubation) in a conventional forced-draught incubator at 40 °C and at a constant humidity of 60%. After hatching, chicks were maintained in a

Abbreviations used: F16BPase, fructose-1,6-bisphosphatase (EC 3.1.3.11); F26BPase, fructose-2,6-bisphosphatase (EC 3.1.3.46); PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); F26BP, fructose 2,6-bisphosphate.

warm environment and allowed free access to water and food *ad libitum* (Labsure CRM diet). Where specified, animals were deprived of food alone for the stated interval.

Reagents

Aldolase, AMP, ATP, fructose 6-phosphate, glucose 6-phosphate, glycerol-3-phosphate dehydrogenase, NADH, peroxidase and triosephosphate isomerase were purchased from B.C.L., Lewes, Sussex, U.K. F26BP and glucose oxidase were obtained from Sigma, Poole, Dorset, U.K. All other chemicals were of the purest grade available from standard suppliers.

Enzyme assays

Embryonic and neonatal chicks were killed by cervical dislocation, and livers were removed for extraction and analysis of enzyme activities. Tissues were either processed immediately or frozen in liquid N₂ and stored at -80 °C for later extraction; the latter treatment did not impair analysis. Enzyme activities are expressed as units (μ mol of product formed/min at 30 °C) per g wet wt. of liver, and for comparison as units/mg of tissue DNA.

PFK-1. The assay is based on the methods of Castano *et al.* (1979) and Van Schaftingen *et al.* (1980a,b). Livers (50–300 mg wet wt.) were homogenized by ten passes of a hand-powered Teflon/glass homogenizer in 9 vol. of 50 mM-triethanolamine, pH 7.35 (20 °C), containing 1 mM-EDTA, 15 mM-KF, 2 mM-MgCl₂ and 3 mM-EGTA. Homogenates were centrifuged in a micro-centrifuge (12000 g, 2 min, 4 °C), and samples of the resultant supernatants were centrifuged in a Beckman Airfuge (100000 g, 10 min, 4 °C) to produce a clear extract for analysis of PFK-1 activity. Enzyme activity was measured at pH 8.14 (total enzyme activity) in an assay system (1 ml total volume) which contained (final concns.) 50 mM-Hepes, pH 8.14, 5 mM-KH₂PO₄, 1 mM-NH₄Cl, 0.15 mM-NADH, 0.1 mM-AMP, 5 mM-fructose 6-phosphate, 1.5 mM-ATP, 0.5 unit of aldolase, 5 units of triosephosphate isomerase and 0.5 unit of glycerol-3-phosphate dehydrogenase. Assays were performed at 30 °C with 0.01 ml portions of extracts and, after measurement of the low blank rate, ATP was added to initiate the reaction.

PFK-2. The assay was based on the stopped assay described by Bartrons *et al.* (1983). Livers (50–300 mg wet wt.) were homogenized by ten passes of a hand-powered Teflon/glass homogenizer in 5 vol. of 20 mM-KH₂PO₄, pH 7.0, containing 10 mM-EDTA and 100 mM-KCl. Homogenates were centrifuged (27000 g, 30 min, 2 °C), and portions (0.015 ml) of supernatants were used for analysis of the total (pH 8.5) and active (pH 6.6) forms of PFK-2 at 30 °C, by using the exact assay constituents described by Bartrons *et al.* (1983) in a total assay volume of 0.25 ml. Reactions were started by addition of liver extracts and stopped by addition of 0.1 M-NaOH (0.25 ml). After neutralization with 0.1 M-acetic acid, samples (0.01 ml) from reaction mixtures were analysed for F26BP content as described below. Production of F26BP was constant for at least 20 min; a reaction time of 10 min was used routinely for

measurement of enzyme activity. In all cases a zero-time blank was included to determine F26BP content in liver extracts.

Metabolite assays

F26BP was measured in freeze-clamped livers and from PFK-2 assays by the method of Van Schaftingen *et al.* (1982b). The metabolite was extracted from livers by homogenization in 0.05 M-NaOH (20–50 vol.). After heat treatment (80 °C, 5 min), extracts were cooled, and 1 M-Hepes, pH 7.5 (0.05 ml/ml), was added. Samples were neutralized with 0.5 M-acetic acid, then centrifuged (12000 g, 4 min, 2 °C) to provide a clear supernatant suitable for analysis. The F26BP content of liver extracts (2.5–20 μ l) and of PFK-2 assays (10 μ l) was determined relative to standards (0–4 pmol of F26BP) analysed on the same day. Coupling enzymes were desalted before use by centrifugation (Beckman Airfuge: 100000 g, 10 min, 4 °C) and resuspended in glycerol/20 mM-ammonium acetate (1:1, v/v). Possible contamination of commercial fructose 6-phosphate with F26BP was removed by acid treatment as described by Van Schaftingen & Hers (1983). Tissue (50–100 mg wet wt.) DNA content of homogenized extracts was analysed by the method of Burton (1956). Glycogen content of intact liver (50–100 mg wet wt.) was determined by methods reported previously (Picardo & Dickson, 1982).

Statistical analysis

Results are presented as means $\pm$ S.E.M. for the numbers of animals specified. Significance of differences between groups was determined by Student's *t* test.

RESULTS AND DISCUSSION

The content of F26BP in freeze-clamped liver samples is relatively low during the embryonic life of chicks until the last 3–4 days of embryonic existence (Fig. 1). A peak F26BP content is observed on the day immediately before hatching. Hatching produces a rapid decrease in F26BP (to early-embryonic values), but, given normal access to food, the metabolite content increases in the first 1–2 days of neonatal life, to the maximal values observed just before hatching. Throughout a subsequent 2-week period hepatic F26BP content decreases to values similar to those reported in the livers of mature fed chickens (Chaekal *et al.*, 1983; Bannister, 1984).

As with rodent liver, the hepatic concentration of F26BP in chickens is controlled by the relative activities of PFK-2 and F26BPase. This bifunctional enzyme from chicken liver has been purified and characterized, and the activity is regulated in response to cyclic AMP-dependent phosphorylation (Chaekal *et al.*, 1983; Van Schaftingen & Hers, 1986). Determination of PFK-2 activity in livers from embryos 2 days before hatching showed that the enzyme was present in significant amounts (6.3 ± 0.4 units/g wet wt.; three experiments), and 80–90% of the enzyme is in the active form (Bartrons *et al.*, 1983). The transient increase in hepatic F26BP before hatching follows the mobilization of hepatic glycogen stores (Fig. 2). Although glucose 6-phosphate produced by hepatic glycogenolysis is predominantly converted into glucose (to support muscular exertion at hatching; Campbell & Langslow, 1978; Freeman, 1969), increased glucose 6-phosphate, and hence fructose 6-phosphate, will favour synthesis of F26BP (Hue *et al.*, 1981a,b).

Fig. 1. Changes in liver F26BP content throughout development of embryonic and neonatal chicks

The F26BP content of embryonic and neonatal liver samples was determined as described in the Materials and methods section. The values shown are means $\pm$ S.E.M. for either 10–18 (for embryos) or 5–12 (for hatched chicks) determinations ($\blacktriangle$). In certain experiments, chicks (five or six) were deprived of food for up to 3 days after hatching (∇). Values for hatched chicks ($\blacktriangle$) were obtained from livers removed within 3 h after the actual act of hatching (H). Significance of difference compared with fed animals: * $P < 0.05$.

Fig. 2. Changes in liver glycogen content throughout development of embryonic and neonatal chicks

The glycogen content of embryonic and neonatal liver samples was determined as described in the Materials and methods section. The values shown are means $\pm$ S.E.M. for 7–13 animals ($\blacksquare$). In certain experiments, chicks (five or six) were deprived of food for up to 2 days after hatching ($\square$). Values for hatched chicks ($\blacksquare$) were obtained from livers removed within 3 h after the actual act of hatching (H). Significance of difference compared with fed animals: * $P < 0.05$.

Increased hepatic F26BP before hatching parallels an increased deposition of triacylglycerols within liver (in correlation with mobilization of non-esterified fatty acids from the yolk stores). A transient acceleration of hepatic glycolysis at this time may be essential for provision of glycerol 3-phosphate for triacylglycerol synthesis.

In contrast with the report by previous workers (Wallace & Newsholme, 1967), we have found PFK-1 activity in embryonic-chick liver to be appreciable (Fig. 3). Significant glycolysis should be possible for embryonic liver if the concentrations of allosteric effectors (e.g. F26BP) are favourable. Thus the transient increase in F26BP content before hatching may be of physiological value in regulation of glycolytic flux. Hatching and subsequent food intake provoke a modest (2.0–2.5-fold) increase in enzyme activity (Fig. 3). By 2 weeks after hatching, hepatic PFK-1 activity has decreased to values similar to those determined for mature fed chickens (Bannister, 1984; Kono & Uyeda, 1973). The overall relative pattern of change was identical when results were expressed on the basis of cellular DNA content. Low activities of PFK-1 in embryonic life reported by Wallace & Newsholme (1967) may be an artefact, owing to the extreme instability of avian PFK-1. Rapid and irreversible loss of PFK-1 activity occurs on storage of dilute homogenates unless F26BP is added to buffers (M. J. Hamer, A. J. Bate & A. J. Dickson, unpublished work). Similar observations have been made for PFK-1 extracted from isolated rat liver cells (Pilkis *et al.*, 1983).

Lack of access to food after hatching prevents increases in both PFK-1 activity and F26BP content (Figs. 1 and 3). Although no food is available from outside sources, neonatal chicks can survive on the contents of their residual yolk sac for 2–3 days. The nutrient supply under those conditions will, however, be identical with the situation *in ovo*. Provided that food is

Fig. 3. Changes in PFK-1 activity throughout development of embryonic and neonatal chicks

PFK-1 activity was measured in homogenates prepared from embryonic and neonatal livers as described in the Materials and methods section. Values are means $\pm$ S.E.M. for either five to ten (for embryos) or five to six (for hatched chicks) determinations (●). In certain experiments, chicks (four to six) were deprived of food for up to 2 days after hatching (○). Significance of difference compared with fed animals: * $P < 0.05$. H, hatching.

available, hatching is normally associated with increased plasma insulin concentrations; lack of food, however, maintains insulin concentrations at the lower embryonic values (Raheja *et al.*, 1972). Other workers have reported changes in key enzymes of lipid metabolism during the embryonic/neonatal transition in chicks (Morris *et al.*, 1984). Changes in these enzyme activities follow a time course similar to that which we describe here for PFK-1 and F26BP. Increased plasma insulin concentration has been implicated in the production of a co-ordinated switch in the hepatic metabolic profile of neonatal chicks (Wilson *et al.*, 1986). Indeed, insulin activates the synthesis of fatty acid synthase, malic enzyme and acetyl-CoA carboxylase in cultures of chick-embryo liver cells (Goodridge *et al.*, 1979; Morris *et al.*, 1984). The increase in hepatic F26BP content after hatching may be related to changes in expression of glucokinase activity and, hence, glucose utilization. Although avian liver had been stated to lack glucokinase (Ureta *et al.*, 1972), a membrane-bound form of the enzyme has been identified in chicken liver (Wals & Katz, 1981), and this enzyme has been shown to be inducible (Klandorf *et al.*, 1986).

Increased activity of PFK-1 after hatching could be dependent on, or be independent of, the changes in hepatic F26BP content. F26BP has been implicated in protection of PFK-1 from proteolysis (Van Schaftingen & Hers, 1983). However, there is no direct relationship between F26BP content and PFK-1 activity before hatching (Figs. 1 and 3). In addition, we have shown a direct effect of insulin on synthesis of PFK-1 in primary cultures of chick-embryo hepatocytes (M. J. Hamer & A. J. Dickson, unpublished work). Thus changes in PFK-1 activity at hatching may be divorced from modulation of F26BP content and may represent a direct effect on enzyme synthesis in response to changes in plasma insulin.

It has been suggested that hepatic PFK-1 has a crucial function in the control of lipogenesis (Rognstad & Katz, 1980). In the transition from embryonic to neonatal life in chicks, there appears to be co-ordinate regulation between expression of a key glycolytic enzyme (PFK-1) and lipogenic enzymes (e.g. fatty acid synthase). Co-ordination by insulin of synthesis of a set of key regulatory enzymes is of crucial importance to elucidation of metabolic defects in several stress situations and hormonal imbalances. Investigation of integration of synthesis of specific proteins by chick-embryo hepatocytes in culture offers a valuable model system for elucidation of the molecular events in the mechanism of insulin action.

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REFERENCES

- Bannister, D. W. (1984) *Int. J. Biochem.* **16**, 895–899
- Bartrons, R., Hue, L., Van Schaftingen, E. & Hers, H. G. (1983) *Biochem. J.* **214**, 829–837
- Bickerstaffe, R., Clive, E. & Annison, E. F. (1970) *Biochem. J.* **118**, 427–431
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Campbell, R. S. & Langslow, D. R. (1978) *Biochem. Soc. Trans.* **6**, 149–152
- Castano, J. G., Nieto, A. & Feliu, J. E. (1979) *J. Biol. Chem.* **254**, 5576–5579
- Chaekal, O., Boaz, J. C., Sugano, T. & Harris, R. A. (1983) *Arch. Biochem. Biophys.* **225**, 771–778
- Claus, T. H. & Pilkis, S. J. (1982) *Biochem. Biophys. Res. Commun.* **109**, 664–668
- Claus, T. H., El-Maghrabi, M. R., Regen, D. M., Stewart, H. B., McGrane, M., Kountz, P. D., Nyferer, F., Pilkis, J. & Pilkis, S. J. (1984) *Curr. Top. Cell. Regul.* **23**, 57–86

- Dickson, A. J. (1983) *Int. J. Biochem.* **15**, 861–865
- Freeman, B. M. (1969) *Comp. Biochem. Physiol.* **28**, 1169–1176
- Goodridge, A. G. (1968a) *Biochem. J.* **108**, 655–661
- Goodridge, A. G. (1968b) *Biochem. J.* **108**, 663–666
- Goodridge, A. G. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2399–2405
- Goodridge, A. G., Garay, A. & Silpananta, P. (1974) *J. Biol. Chem.* **249**, 1469–1475
- Goodridge, A. G., Civelli, O., Yip, C. & Scherrer, K. (1979) *Eur. J. Biochem.* **96**, 1–8
- Hers, H. G. & Hue, L. (1983) *Annu. Rev. Biochem.* **52**, 617–653
- Hue, L. & van de Werve, G. (1982) *FEBS Lett.* **145**, 263–266
- Hue, L., Van Schaftingen, E. & Blackmore, P. F. (1981a) *Biochem. J.* **194**, 1023–1026
- Hue, K., Blackmore, P. F. & Exton, J. H. (1981b) *J. Biol. Chem.* **256**, 8900–8903
- Klandorf, H., Clark, B. L., Scheck, A. C. & Brown, J. (1986) *Biochem. Biophys. Res. Commun.* **139**, 1086–1093
- Kono, N. & Uyeda, K. (1973) *J. Biol. Chem.* **248**, 8603–8609
- Masamichi, K. & Uyeda, K. (1982) *Biochem. Biophys. Res. Commun.* **104**, 84–88
- Morris, S. M., Winberry, L. K., Fisch, J. E., Back, D. W. & Goodridge, A. G. (1984) *Mol. Cell. Biochem.* **64**, 63–68
- Neely, P., El-Maghrabi, M. R., Pilkis, S. J. & Claus, T. H. (1981) *Diabetes* **30**, 1062–1064
- Pearce, J. (1977) *Int. J. Biochem.* **8**, 267–275
- Picardo, M. & Dickson, A. J. (1982) *Comp. Biochem. Physiol. B* **71**, 689–693
- Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J. & Claus, T. (1981) *J. Biol. Chem.* **256**, 3619–3622
- Pilkis, S. J., Chrisman, T. D., El-Maghrabi, M. R., Colosia, A., Fox, A., Pilkis, J. & Claus, T. H. (1983) *J. Biol. Chem.* **258**, 1495–1503
- Raheja, K. L., Tepperman, H. & Tepperman, J. (1972) *Horm. Metab. Res.* **4**, 69–73
- Rognstad, R. & Katz, J. (1980) *Arch. Biochem. Biophys.* **203**, 642–646
- Sakakibara, R., Kitajima, S. & Uyeda, K. (1984) *J. Biol. Chem.* **259**, 41–46
- Schubert, C., Boehme, H. J. & Hofmann, E. (1986) *Biosci. Rep.* **6**, 513–518
- Ureta, T., Radojkovic, J., Slebe, J. C. & Reichberg, S. B. (1972) *Int. J. Biochem.* **3**, 103–110
- Van Schaftingen, E. & Hers, H. G. (1983) *Biochem. Biophys. Res. Commun.* **113**, 548–554
- Van Schaftingen, E. & Hers, H. G. (1986) *Eur. J. Biochem.* **159**, 359–365
- Van Schaftingen, E., Hue, L. & Hers, H. G. (1980a) *Biochem. J.* **192**, 263–271
- Van Schaftingen, E., Hue, L. & Hers, H. G. (1980b) *Biochem. J.* **192**, 887–895
- Van Schaftingen, E., Davies, D. R. & Hers, H. G. (1982a) *Eur. J. Biochem.* **124**, 143–149
- Van Schaftingen, E., Lederer, B., Bartrons, R. & Hers, H. G. (1982b) *Eur. J. Biochem.* **129**, 191–195
- Wallace, J. C. & Newsholme, E. A. (1967) *Biochem. J.* **104**, 378–384
- Wals, R. A. & Katz, J. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1543–1548
- Wilson, S. B., Back, D. W., Morris, S. M., Swierczynski, J. & Goodridge, A. G. (1986) *J. Biol. Chem.* **261**, 15179–15182

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