Studies on the Biosynthesis of Hepatic Pyruvate Kinase and its Correlation with Enhanced Hepatic Lipogenesis in Meal-Trained Rats

By Timothy J. HOPKIRK and David P. BLOXHAM

Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Southampton SO9 3TU, U.K.

(Received 4 January 1979)

Metabolic and enzymic changes were measured in meal-trained rats fed on high-carbohydrate diet. Rates of hepatic fatty acid synthesis are probably greater than rates of gluconeogenesis throughout the 24 h day provided that animals are fed. The daily enhancement of fatty acid synthesis on meal feeding coincided with the maximum activation of hepatic pyruvate kinase. Maximum activation of this enzyme was reflected in increased total catalytic activity ($V_{\max}$), increased activity at 0.5 mM-phosphoenolpyruvate ($v_{0.5}$), decreased $V_{\max}/v_{0.5}$ ratio and a decrease in co-operativity of phosphoenolpyruvate binding as measured by the Hill coefficient (h). The latter changes are consistent with a decrease in enzyme phosphorylation during activation of the enzyme. To estimate changes in enzyme protein, quantitative enzyme precipitation with rabbit antisera was used. Giving a high-carbohydrate diet to meal-trained animals induced enzyme synthesis within a few hours. Adaptations in diet that enhanced fatty acid synthesis (chow to high carbohydrate; starved to high carbohydrate) led to an increased steady-state concentration of pyruvate kinase protein. An approximate estimate of the half-life of hepatic pyruvate kinase was 56 h. Whenever pyruvate kinase specific activity was measured in liver tissue extracts it was always considerably less (20–100 $\mu\text{mol}/\text{min}$ per mg of protein, depending on dietary status) than the specific activity of pure pyruvate kinase (200 $\mu\text{mol}/\text{min}$ per mg of protein). Antigenically active, catalytically inactive protein was removed during enzyme purification from cytosol at the stage of $(\text{NH}_4)_2\text{SO}_4$ fractionation. The fraction precipitated by 30–45% satd. $(\text{NH}_4)_2\text{SO}_4$ was enzymically active, antigenically active and had a specific activity of 200 $\mu\text{mol}/\text{min}$ per mg of protein. Antigenically reacting protein was identified in the remaining $(\text{NH}_4)_2\text{SO}_4$ fractions (0–30% and 45–85% satd.) and this contained no enzyme activity. These may correspond to inactive proteolytic fragments of pyruvate kinase. The rate-determining step in adjusting enzyme concentration seems to be proteolysis.

Considerable interest has been focused on the role of pyruvate kinase in the control of hepatic metabolism. Originally it was recognized that the maximal activity of hepatic pyruvate kinase was considerably higher than that of many key gluconeogenic enzymes (Scrutton & Utter, 1968). Since liver has been regarded as primarily gluconeogenic rather than glycolytic in function (Clark *et al.*, 1974; Bloxham, 1974), the high concentration of pyruvate kinase presented an apparent anomaly. This was partially resolved by the demonstration that hepatic pyruvate kinase was an allosteric enzyme showing positive co-operativity in substrate binding and subject to regulation by ligands such as fructose 1,6-bisphosphate (Taylor & Bailey, 1967; Engstrom, 1978). More recently it has been shown that the enzyme is phosphorylated by cyclic AMP-dependent kinase and this phosphorylation can be inhibitory, especially at low phosphoenolpyruvate concentrations (Engstrom, 1978; Foster & Blair, 1978). The observation that

this phosphorylation is stimulated by glucagon has focused attention on the possibility that pyruvate kinase phosphorylation represents the control point for glucagon stimulation of gluconeogenesis by the enhanced conversion of pyruvate into phosphoenolpyruvate (Van Berkel *et al.*, 1977; Feliu *et al.*, 1977). This control envisages the function of a substrate cycle constituted by pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Clark *et al.*, 1975; Rognstad & Katz, 1977), and indeed there is evidence for the operation of this cycle (Rognstad & Katz, 1977). In the presence of glucagon, inhibition of pyruvate kinase occurs and the cycle is broken. This would allow the net outflow of phosphoenolpyruvate and enhanced gluconeogenesis would result.

The scheme as presented is perfectly plausible, but suggests that pyruvate kinase operates in a purely negative manner. If this was the case there seems little reason to have concentrations of pyruvate kinase

which are far in excess of those of the other enzymes in the substrate cycle. To account for the high concentration of pyruvate kinase it seems to us that perhaps its importance to gluconeogenesis has been over-emphasized, and as an alternative we would suggest that this enzyme may play an important role in the maintenance of fatty acid synthesis in the liver.

The liver is a major organ of fatty acid synthesis in the rat (Brunengraber *et al.*, 1973; Godbole & York, 1978), and rates of fatty acid synthesis may be greater than those of gluconeogenesis (Clark *et al.*, 1974). In liver, glucose is an extremely poor precursor for fatty acid synthesis even when fatty acid synthesis is dramatically increased in obese animals (Salmon *et al.*, 1974; Hems *et al.*, 1975; Bloxham *et al.*, 1977). However, both lactate and acetate are excellent precursors (Salmon *et al.*, 1974). This would seem to argue in favour of those experiments that have shown that glycolysis from glucose in liver is negligible (D. G. Clark *et al.*, 1973; M. G. Clark *et al.*, 1974). Alternative experiments showed that fatty acid synthesis could be correlated with hepatic glycogen depletion (Woods & Krebs, 1971; Salmon *et al.*, 1974). These experiments suggest that, whereas glucose entering the hepatocyte is a poor precursor for fatty acid synthesis, glycogen is an excellent precursor. Clearly there are still fundamental problems in explaining these observations. It does mean, however, that there would be a major role for pyruvate kinase in the conversion of glycosyl units from glycogen into fatty acids.

In order to study this possibility we have attempted to correlate changes in the activity and the rate of synthesis of pyruvate kinase with changes in fatty acid synthesis. For these experiments we have chosen to use meal-trained animals in which metabolic changes can be closely regulated. All our evidence indicates that the activation of pyruvate kinase coincides with the activation of fatty acid synthesis. In addition, our experiments with the antibody to hepatic pyruvate kinase point towards another control point for pyruvate kinase which regulates the fraction of hepatic pyruvate kinase present in the fully active form at any time. More active enzyme is generated as fatty acid synthesis increases. This control may be distinguished from effects caused by phosphorylation by cyclic AMP-dependent protein kinase. These experiments are complementary to similar observations made by Kohl & Cottam (1976).

Materials and Methods

Maintenance of rats

Male Wister Albino rats (250 g) were used for all experiments. Meal-trained animals were fed between 09:00 and 13:00 h each day. It requires 7 to 10 days to train animals to this feeding regime. Animals were in light from 07:00 to 19:00 h each day. Thus our

conditions of feeding change rats from nocturnal feeders to daytime feeders. Animals were fed on either standard laboratory diet (chow) or high-carbohydrate diet (Bloxham *et al.*, 1977).

Measurement of lipogenesis

In vivo, the incorporation of radioactivity in hepatic fatty acid synthesis was measured 1 h after the intravenous injection of 2 mCi of $^3\text{H}_2\text{O}$ (Windmueller & Spaeth, 1966). Results were converted from μg -atoms of ^3H incorporated into fatty acids/min per g wet wt. of liver into μmol of acetyl units/min per g wet wt. of liver. Assuming a C_{16} fatty acid, this conversion is given by the following equation (Windmueller & Spaeth, 1966):

$$\mu\text{mol of acetyl unit} = \frac{\mu\text{g-atoms of H} \times 8}{13.3}$$

Preparation and incubation of hepatocytes

Hepatocytes were prepared by using the recirculating-digestion procedure described by Zahltén & Stratman (1974). Isolated hepatocytes were suspended in Krebs bicarbonate Ringer medium [0.9% (w/v) NaCl, 1.15% (w/v) KCl, 0.11 M-CaCl₂, 3.82% (w/v) MgCl₂, 2.11% (w/v) KH₂PO₄ and 1.3% (w/v) NaHCO₃, in the proportions 200:8:3:2:2:40, by vol.] containing 20 mM-glucose, 3 mM-[U- ^{14}C]lactate (0.01 $\mu\text{Ci}/\mu\text{mol}$) and 0.1 mCi of $^3\text{H}_2\text{O}/\text{ml}$. The hepatocyte protein concentration was usually 20 mg/ml. The concentration of lactate is the maximum concentration that we have observed in hepatic portal-venous blood (Hopkirk & Bloxham, 1977). The incorporation of radioactivity into fatty acids has been described previously (Bloxham & Akhtar, 1972). Routinely a 2 ml suspension of hepatocytes was gassed with O₂/CO₂ (19:1) and incubated for 1 h in a 37°C water bath with shaking at 60 strokes/min.

Enzyme activity in tissue extracts

A 10% (w/v) homogenate was prepared in Krebs bicarbonate Ringer medium by using a motor-driven homogenizer. The preparation was centrifuged at 20000 g for 30 min at 4°C and the supernatant was decanted for assay. The total protein concentration was determined with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951). Enzyme activity is usually expressed as μmol of product/min per 100 mg of soluble protein. We find that usually 1 g of liver gives 100–120 mg of soluble protein.

To assay the maximum activity of pyruvate kinase a reaction mixture containing 25 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 1 mM-MgCl₂, 0.5 mM-ADP, 0.15 mM-NADH, 4 μg of pig heart lactate dehydrogenase, 0.5 mM-fructose 1,6-bisphosphate and up to 0.1 unit of pyruvate kinase/ml was set up and incubated at

37°C for 3 min. The reaction was started by the addition of 0.5 mM-phosphoenolpyruvate and was followed by the decrease in A_{340} . For assays in crude homogenates 2 mM-KCN was included to inhibit NADH dehydrogenase. This has no effect on the activity of purified pyruvate kinase. To measure the Hill coefficient for phosphoenolpyruvate binding, fructose 1,6-bisphosphate was omitted from the assay and phosphoenolpyruvate concentration was varied between 0.5 and 10 mM. The reaction rates were analysed for co-operativity in binding by the method of Atkins (1972). The maximum velocity at saturating phosphoenolpyruvate concentration in the absence of fructose 1,6-bisphosphate was identical with the reaction velocity at 0.5 mM-phosphoenolpyruvate in the presence of saturating fructose 1,6-bisphosphate. A unit of pyruvate kinase produces 1 μ mol of pyruvate/min at 37°C. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glucokinase (EC 2.7.1.2), lactate dehydrogenase (EC 1.1.1.27), fructose 1,6-bisphosphate aldolase (EC 4.1.2.13), enolase (EC 4.2.1.11), phosphoglycerate mutase (EC 5.4.2.1) and fructose 1,6-bisphosphatase (EC 3.1.3.11) were all assayed by standard spectrophotometric assays (Bergmeyer, 1974).

Purification of hepatic pyruvate kinase

All stages in the purification were performed at 2–4°C. All phosphate buffers contained either 30% (v/v) glycerol or 30% (v/v) ethylene glycol. The initial stages in purification involved the preparation of a post-mitochondrial supernatant (stage 1), the preparation of a cytosol fraction (stage 2), pH 5 treatment (stage 3) and $(\text{NH}_4)_2\text{SO}_4$ fractionation (stage 4). The details of these steps are identical with the procedure of Ljungstrom *et al.* (1974).

DEAE-cellulose chromatography (stages 5 and 6). The $(\text{NH}_4)_2\text{SO}_4$ precipitate from stage 4 was dissolved in 50 ml of 20 mM-potassium phosphate, pH 7.2, and dialysed for 18 h against 5 litres of the same buffer.

The enzyme fraction was diluted with an equal volume of buffer and mixed with 0.5 ml packed volume of DEAE-cellulose/mg of protein. This was equilibrated for 2 h until all the enzyme activity was bound to the resin. Unbound protein was eluted from the column with starting buffer until the eluate gave $A_{280} < 0.05$. A linear gradient of 20 mM-potassium phosphate, pH 7.2, to 100 mM-potassium phosphate, pH 6.0, was applied to the column in a volume approx. 6 times the total column volume. Fractions containing enzyme activity were pooled and dialysed for 18 h against 5 litres of 20 mM-potassium phosphate, pH 7.2, containing 0.1 mM-fructose 1,6-bisphosphate. In all subsequent stages fructose 1,6-bisphosphate was present in the buffers used at a concentration of 0.1 mM. The enzyme fraction was adjusted to pH 7.0 with 1 M-potassium phosphate, pH 7.2, and bound to

DEAE-cellulose as described above. Unbound protein was eluted with starting buffer. The enzyme was eluted by using a linear gradient (3 times column volume) of 0–300 mM-KCl in 20 mM-potassium phosphate, pH 7.2. Fractions containing enzyme activity were pooled and dialysed successively for 12 h each against 5 litres of 20 mM-potassium phosphate, pH 7.2, 5 litres of 1 mM-potassium phosphate, pH 7.2, and 5 litres of 1 mM-potassium phosphate, pH 5.5.

CM-Sephadex chromatography (stage 7)

The enzyme fraction was applied to a column of CM-Sephadex (C-50) equilibrated with 1 mM-potassium phosphate, pH 5.5 (2 ml packed volume of CM-Sephadex/mg of protein). Unbound protein was eluted by the starting buffer. The enzyme was eluted by 5 mM-potassium phosphate, pH 7.2. Fractions containing enzyme activity were pooled and dialysed for 18 h against 5 litres of 20 mM-potassium phosphate, pH 7.2.

Concentration on DEAE-cellulose (stage 8)

The enzyme fraction was applied to a small DEAE-cellulose column (0.5 ml packed volume/mg of protein applied). The enzyme was eluted with 20 mM-potassium phosphate buffer, pH 7.2, containing 300 mM-KCl. Fractions (1 ml) were collected and those containing enzyme activity were pooled. The enzyme was stored at -10°C .

As an alternative procedure the enzyme may be collected by precipitation with 70%-satd. $(\text{NH}_4)_2\text{SO}_4$ after diluting the glycerol to 10% (v/v).

A summary of the enzyme purification is shown in Table 1. The enzyme was obtained in 10% overall yield and was homogeneous by gel electrophoresis (Fig. 1). The enzyme specific activity was measured at 30°C for direct comparison with the results of other purification procedures (Kohl & Cottam, 1976; Ljungstrom *et al.*, 1974). A value of 200 μ mol of product/min per mg of protein for the pure enzyme compares favourably with the other preparations quoted in these references. The concentration of pure protein was estimated by assuming that 1 mg/ml has $A_{280}^{1\text{cm}} = 1.0$. This value is an assumption and the correct value awaits the determination of the molar absorption coefficient of rat liver pyruvate kinase. The concentration of protein was also confirmed by determinations with Folin reagent (Lowry *et al.*, 1951) and Coomassie Brilliant Blue (Fazekas *et al.*, 1963). The three methods of assaying protein concentration agreed within 10%.

Purification of rabbit muscle pyruvate kinase

Rabbit muscle pyruvate kinase was purified as described by Tietz & Ochoa (1962). The stages in the purification referred to in Table 3 are exactly as described in the original reference. For raising

Table 1. *Purification of rat liver pyruvate kinase*

The Table shows a typical purification from 300g of liver. Results are averaged from five successive preparations. The details are described in the Materials and Methods section. Rats were starved for 48h and re-fed on high-carbohydrate diet for 72h before death. All protein concentrations were determined by the method of Lowry *et al.* (1951). Activity was assayed at 30°C.

Purification step	Volume (ml)	Protein (mg/ml)	Total enzyme activity ($\mu\text{mol/min}$)	Enzyme specific activity ($\mu\text{mol/min per mg}$)	Purification (fold)
1. Post-mitochondrial supernatant	900	100	24700	0.27	1
2. Cytosol	650	50	22100	0.68	2.5
3. pH 5 treatment	690	20	22100	1.60	5.9
4. 30–45%-satd. $(\text{NH}_4)_2\text{SO}_4$	70	26	17000	9.34	34.6
5. DEAE-cellulose (1)	350	0.7	13900	56.7	210
6. DEAE-cellulose (2)	130	1.0	12200	93.8	348
7. CM-Sephadex	175	0.06	2500	238	881
8. Concentration on DEAE-cellulose	16	0.80	2550	200	737

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of liver pyruvate kinase*

Liver pyruvate kinase was purified as described in the Materials and Methods section from rats fed on a high-carbohydrate, low-protein diet. Portions (10, 20 and 40 μg) of the enzyme were applied to 7.5% acrylamide gels and electrophoresed in the presence of 0.2% sodium dodecyl sulphate. Protein was stained with Coomassie Brilliant Blue. The protein had a mobility close to that of muscle-type pyruvate kinase.

antibodies the enzyme was purified by chromatography on CM-Sephadex (Stammers & Muirhead, 1975). All procedures for use of the enzyme were

described in previous papers (Bloxham & Chalkley, 1976; Bloxham *et al.*, 1978).

Production of antibodies to rat liver and rabbit muscle pyruvate kinase

Antisera to rat liver pyruvate kinase were raised in 2.5 kg male white New Zealand rabbits. Antisera to rabbit muscle pyruvate kinase were raised in 20-week-old pre-laying chickens. Animals were injected each time with 1 mg of protein emulsified in Freund's complete adjuvant (Difco, West Molesey, Surrey, U.K.). Three injections were given subcutaneously at 1-week intervals. After a 2-week gap a fourth injection was given, and 14 days later the antibody titre was maximum and the animals were killed by exsanguination. Alternatively, for rabbits, the animals can be maintained alive and 40 ml of blood withdrawn periodically from the ear vein. A booster injection of 1 mg of liver pyruvate kinase was given every 2 months. The work described in this project used four separate preparations of antisera raised with three batches of pyruvate kinase with constant results.

Blood was allowed to clot at 18°C for 18 h and centrifuged at 25000g for 30 min at 4°C. The serum was decanted and could be used directly for enzyme-antibody titrations. Alternatively the antibody was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Hudson & Hay, 1976).

Pyruvate kinase antisera titrations

The amount of liver pyruvate kinase present in a cytosol fraction was quantified by standardizing each batch of antiserum against purified liver pyruvate kinase. Different amounts of liver pyruvate kinase (0–100 μg routinely) were incubated with a constant volume of antiserum [50 μl of antiserum; 60 mg of protein/ml; 25 μl of $(\text{NH}_4)_2\text{SO}_4$ -purified antibody/ml, 12 mg/ml] in 1 ml of 10 mM-potassium phosphate, pH 7.4, at 4°C for 2 h. The antibody-enzyme complex

retained 15% catalytic activity, but was completely removed from solution by centrifugation at 20000g for 30 min. The supernatants were assayed for pyruvate kinase activity at 20°C. The protein pellets were washed twice with 0.9% (w/v) NaCl and dissolved in 500 μ l of 0.1 M-NaOH. Protein content was assayed with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951). Enzyme-activity measurements were plotted as units/ml of supernatant versus the amount of liver pyruvate kinase protein added. Back-projection of the line to zero enzyme activity in the supernatant gave the enzyme equivalence point. This always corresponded closely to the point of maximum protein precipitation. At equivalence, liver pyruvate kinase antibody forms a 1:12 complex. The amount of protein precipitated at equivalence was always constant $\pm 20\%$ (S.E.M.) irrespective of the source of liver pyruvate kinase.

Experimentally, pyruvate kinase in liver extracts was assayed by adding different volumes (0–800 μ l) to a constant volume of antiserum. The samples were then analysed as described above for unbound enzyme activity and protein precipitate. From the calibration of the antiserum the equivalence point can be used to estimate the amount of liver pyruvate kinase protein (Q ; μ g/mg of soluble protein). If the total enzyme activity was also measured as μ mol of product formed at 37°C/min per mg of soluble protein (A), then the specific activity of pyruvate kinase in liver extracts (μ mol of product at 37°C/min per mg of pyruvate kinase) was calculated as follows:

$$\text{Specific activity} = \frac{A}{Q} \times 1000$$

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified enzyme and antigen-antisera complexes in 7.5% polyacrylamide gels was performed as described by Weber & Osborn (1969). Ouchterlony double-diffusion plates were analysed by standard procedures by using 1% (w/v) agarose in 0.1 M-barbitone acetate buffer, pH 8.5.

Sources of chemicals and reagents

All the materials in this work were obtained from BDH, Poole, Dorset, U.K., or Boehringer Corp. (London), Lewes, Sussex, U.K. DEAE-cellulose (DE52) was obtained from Whatman, Maidstone, Kent, U.K. CM-Sephadex (C-50) was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Results

Fatty acid synthesis in meal-trained rats in vivo

The initial part of this study examined the changes seen *in vivo* in hepatic lipogenesis under controlled feeding conditions. The use of the '4+20' feeding regime allowed study of the changes in hepatic metabolism in response to a controlled feeding period

(09:00–13:00h each day) without recourse to the extreme comparisons of feeding and starvation. The high-sucrose diet induced high rates of synthesis of hepatic fatty acids and allowed study of fluctuations in this pathway and its related enzymes that are less clear on an ordinary chow diet.

The diurnal fluctuations in the rats of hepatic fatty acid synthesis *in vivo* were measured after the injection of $^3\text{H}_2\text{O}$. A preliminary report of these measurements has been presented (Hopkirk & Bloxham, 1977). In both chow-fed and sucrose-fed rats an increase in fatty acid synthesis was observed within 1 h of feeding. The maximum rate of synthesis occurred 5–16 h after the start of giving high-carbohydrate diet and 5–13 h after giving chow diet. The maximum rates of synthesis were 2.2 ± 0.15 (S.E.M., $n = 20$) and 1.30 ± 0.08 (S.E.M., $n = 12$) μ mol of acetyl units incorporated/min per g wet wt. of liver for sucrose- and chow-fed animals respectively. Fatty acid synthesis summed over 24 h was 1270 ± 80 (S.E.M., $n = 20$) and 610 ± 50.5 (S.E.M., $n = 12$) μ mol of acetyl units incorporated/24 h per g wet wt. of liver for sucrose- and chow-fed animals respectively. Thus both maximum synthetic rates and total fatty acid synthesis were approx. twice as great in the sucrose-fed rats as in the chow-fed rats. In both groups of animals the increased synthetic rate was associated with feeding. If the feeding period was omitted from the daily routine there was no increase in hepatic fatty acid synthesis (Hopkirk & Bloxham, 1977). Thus changes that occur to increase hepatic fatty acid synthesis occur each day and are initiated by the animal feeding.

The maximum rates of fatty acid synthesis can be compared with estimates of gluconeogenesis *in vivo* (Clark *et al.*, 1974). On the high-carbohydrate or normal chow diet the maximum rates of fatty acid synthesis were 2.2 and 1.30 μ mol of acetyl unit incorporated/min per g wet wt. of liver. This compares with maximum estimates of gluconeogenesis *in vivo* of 1.6 μ mol of lactate consumed/min per g wet wt. of liver. Assuming that the acetyl unit is derived from lactate, then these two sets of values are comparable. However, the maximum rate of gluconeogenesis was estimated in starved animals given a body load of 20 μ mol of lactate/g body wt. In fed and starved animals, at physiological lactate concentration (2 μ mol/g) the rate of gluconeogenesis varied between 0.06 and 0.2 μ mol of lactate consumed/min per g wet wt. It is clear that in the fed animal the rate of fatty acid synthesis exceeds the estimate of gluconeogenesis throughout the day. Gluconeogenesis only exceeds fatty acid synthesis when the feeding period is omitted.

Fatty acid synthesis in hepatocytes isolated from meal-trained rats

The changes in fatty acid synthesis represent stable modifications of hepatic metabolism which are not

solely dependent on short-term hormonal control. To show this, hepatocytes were isolated from rats at various times during feeding and post-prandially, and their capacity for fatty acid synthesis was measured (Fig. 2). We used 3 mM-lactate for these incubations, since this concentration is approximately physiological. We observed a maximum rate of fatty acid synthesis of $0.1 \mu\text{mol}$ of acetyl unit incorporated/min per g wet wt. of hepatocytes. This increased to $0.5 \mu\text{mol}$ of acetyl unit incorporated/min per g wet wt. of hepatocytes if incubations were repeated with 10 mM-lactate and 1 mM-pyruvate. The latter rate corresponds to rates observed for fatty acid synthesis *in vitro* (Harris, 1975). Although the absolute rates of fatty acid synthesis were lower *in vitro*, the synthetic rate varied during the day much as it did *in vivo*. There was a clear enhancement of hepatocyte fatty acid synthesis coincidental with feeding.

The rates of fatty acid synthesis from $[\text{U-}^{14}\text{C}]$ -lactate and $^3\text{H}_2\text{O}$ can be compared by converting the incorporation data for $^3\text{H}_2\text{O}$ into μmol of acetyl unit/min per g wet wt. of hepatocytes as described in the Materials and Methods section. When this is done, the estimated fatty acid synthesis from $^3\text{H}_2\text{O}$ is at least twice that from lactate. This demonstrates that lactate cannot provide all of the acetyl units for fatty acid synthesis. As expected from previous experiments

(Salmon *et al.*, 1974), we observed glycogen depletion in hepatocyte incubations at each of the time points. Presumably glycogen may account for the extra carbon atoms for fatty acid synthesis, and this conversion will require active pyruvate kinase. Therefore we investigated the activation status and the rate of synthesis of pyruvate kinase to see how this correlated with enhanced fatty acid synthesis. Pyruvate kinase was chosen for investigation, since the activity of the liver enzyme is altered by dietary conditions (Krebs & Eggleston, 1965; Tanaka *et al.*, 1967; Seubert & Schoner, 1971).

Diurnal variations in the catalytic activity of pyruvate kinase

Preparation of liver extracts. A large number of different extraction media have been chosen for the preparation of liver extracts (Feliu *et al.*, 1977; Van Berkel *et al.*, 1977; Foster & Blair, 1978). We compared the activity of pyruvate kinase extracted by a variety of buffers (Table 2). The maximum activity was obtained with either Krebs bicarbonate Ringer medium or 0.25 M-sucrose containing 1 mM-EDTA and 0.1 mM-dithiothreitol, pH 7.0. These two buffers were chosen for preparation of enzyme extracts and enzyme purification respectively. Krebs bicarbonate

Fig. 2. Fatty acid synthesis in hepatocytes from high-carbohydrate-trained rats

Hepatocytes were isolated from rats meal-trained on the high-carbohydrate diet for 10 days both during the feeding period and at various times post-prandially. Their capacity for fatty acid synthesis was measured by the incorporation of ^3H from $^3\text{H}_2\text{O}$ (●) and the incorporation of $[\text{U-}^{14}\text{C}]$ lactate (○). All incubations were carried out as described in the Materials and Methods section in the presence of 3 mM-lactate and 20 mM-glucose. The black area below the abscissa represents the period of darkness. The hatched area above the abscissa represents the feeding period. Results are the means of three observations; error bars indicate the S.E.M.

Table 2. Influence of extraction buffer on hepatic pyruvate kinase activity

The liver from a rat meal-trained on the high-carbohydrate diet for 20 days was homogenized in different buffers and the content of pyruvate kinase in the cytosol was measured. Buffers 3–6 all contained 1 mM-EDTA and 0.1 mM-dithiothreitol. Enzyme assays were all carried out at 30°C.

Extraction buffer	Enzyme activity in cytosol fraction ($\mu\text{mol/min per 100 mg of cytosolic protein}$)
1. Krebs bicarbonate Ringer	103 ± 7.5
2. Krebs bicarbonate Ringer + 2-mercaptoethanol	90.5 ± 4.5
3. 0.25 M-Sucrose, pH 7.0	109.3 ± 8.0
4. 25 mM-Tris/HCl, pH 7.4	67.2 ± 5.0
5. 20 mM-Potassium phosphate, pH 7.0	70.9 ± 6.5
6. 20 mM-Imidazole, pH 7.0	62.5 ± 3.0

Ringer medium has been used in previous experiments (Van Berkel *et al.*, 1977), which means that our results will be directly comparable. This extraction buffer appears to preserve the phosphorylation/dephosphorylation status of hepatic pyruvate kinase (Van Berkel *et al.*, 1978)

Diurnal variation of maximal catalytic activity. Pyruvate kinase activity was measured throughout the day in meal-trained animals fed on either high-carbohydrate or chow diets. Maximum catalytic activity was measured in the presence of 0.5 mM-fructose 1,6-bisphosphate. This concentration of fructose 1,6-bisphosphate overcomes any inhibitory effect caused by protein phosphorylation (Kohl & Cottam, 1976, 1977), and phosphoenolpyruvate binding is no longer co-operative (K_m for phosphoenolpyruvate = 0.15 mM). Fig. 3(a) shows that for both groups of animals the total activity of enzyme increases during feeding and is maximum 1–5 h post-prandially. Thus feeding should give a rapid induction of synthesis (see below). Also the period of maximum enzyme activity coincides with the period of maximum fatty acid synthesis quite closely. The total activity of pyruvate kinase was about 3 times as great in homogenates from high-carbohydrate-fed animals compared with chow-fed animals. This increase parallels the enhanced fatty acid synthesis in high-carbohydrate-fed rats.

Diurnal variation in the co-operativity of phosphoenolpyruvate binding to pyruvate kinase. Hepatic pyruvate kinase may be phosphorylated to produce an enzyme with decreased activity (Van Berkel *et al.*, 1977; Ljungstrom *et al.*, 1974; Titanji *et al.*, 1976). The decrease in activity is best observed by assaying enzyme activity at low phosphoenolpyruvate concentrations in the absence of fructose 1,6-bisphos-

Fig. 3. Variations in the catalytic activity of hepatic pyruvate kinase

Liver extracts were prepared from meal-trained rats during the day and kinetic parameters of liver pyruvate kinase were measured. Details are given in the text. Closed symbols represent data from chow-trained animals (●, ■, ▲). Open symbols represent data from high-carbohydrate-trained animals (○, □, △). (a) The maximum enzyme activity in the presence of fructose 1,6-bisphosphate ($V_{\max}$) is shown in each group (●, ○). (b) The activity of the enzyme at 0.5 mM-phosphoenolpyruvate, $v_{0.5}$ (●, ○), is shown with the ratio of $V_{\max}/v_{0.5}$ (▲, △). (c) The change in the Hill coefficient (h) during the day (■, □,) is shown. By analysis of variance with unweighted means in both groups the fall in the value of h during the day is significant ($P < 0.001$). The change in the value of h during the day is significantly different between the two dietary groups ($P < 0.01$). Results are the means of five observations; bars where shown are the S.E.M. Other details are as in Fig. 2.

phate. Pyruvate kinase activity was measured throughout the day at 0.5 mM-phosphoenolpyruvate ($v_{0.5}$; Fig. 3b), and there was a substantial increase after feeding, which subsequently declined. This

increase in activity potentially reflects both induction (as in Fig. 3a) and a decrease in enzyme phosphorylation. The first of these two effects can be eliminated by plotting the ratio $V_{max}/v_{0.5}$ (Fig. 3b). This ratio clearly declines on feeding and is consistent with a decrease in enzyme phosphorylation which will result in a relative enhancement of enzyme activity at low phosphoenolpyruvate concentration. This result can be confirmed by measuring the co-operativity of phosphoenolpyruvate binding to enzyme throughout the day and estimating the Hill coefficient (h). Co-operativity (h) decreases from 2.3 ± 0.2 before feeding to 1.8 ± 0.1 2 h after feeding (Fig. 3c). The decrease in h is greater for animals fed on high-carbohydrate diet. Furthermore, h stays decreased for a longer period of time for pyruvate kinase from animals fed on a high-carbohydrate diet. Since phosphorylation results in an increase in co-operativity of phosphoenolpyruvate binding (Van Berkel *et al.*, 1977; Feliu *et al.*, 1977), the decreased h value could represent a decrease in phosphorylation after feeding.

Adaptation from chow to high-carbohydrate diet. The changes in pyruvate kinase are clearly more marked for animals fed on a high-carbohydrate diet. We were interested in following the exact changes as the rats were adapted from meal-fed on chow to meal-fed on high-carbohydrate diet. For this purpose animals were trained to meal-feed on chow and then the diet was changed to high carbohydrate. Each day after the change rats were analysed before feeding and at 15:00h (the time of maximum enzyme activation) for the total hepatic pyruvate kinase (Fig. 4) and for the co-operativity of phosphoenolpyruvate binding. The adaptation to the diet does not lead to a marked transitional alteration in h values (results not shown). Thus the h value after feeding is always lower than the prefeeding value ($P < 0.001$). When h values for pyruvate kinase from fed animals were compared with each other there was no statistically significant difference. A similar result was obtained when all the h values from prefed animals were compared. Therefore if the decrease in h values on feeding represents a decrease in phosphorylation of hepatic pyruvate kinase then it appears that adaptation to the diet results in only small changes in the daily phosphorylation pattern of the enzyme. Rather, there is a much more profound alteration in the total activity of the enzyme (Fig. 4). Complete adaptation of total hepatic pyruvate kinase activity takes 8 days. During this period, adaptation is characterized by a rapid daily increase in the activity of the enzyme on feeding, especially at days 2 and 3. Most interestingly, the feeding schedule does not enable the activity to return to its prefeeding value during the adaptation period. This demonstrates that the degradation of pyruvate kinase cannot keep pace with the increased formation of pyruvate kinase protein during the adaptation phase.

Fig. 4. Changes in the activity of hepatic pyruvate kinase in adaptation from chow to the high-carbohydrate diet. Rats were meal-trained on chow for 10 days and were transferred to meal-feeding on high-carbohydrate diet on day 1. The total liver pyruvate kinase activity was measured in a cytosol fraction each day before feeding (●) and 1.5 h after the end of feeding (○). Results are expressed as the means $\pm$ S.E.M. of seven observations at each point.

Use of rabbit anti-(liver pyruvate kinase) sera to estimate hepatic pyruvate kinase concentration

Rabbit anti-(liver pyruvate kinase). Changes in total pyruvate kinase activity during a single day of meal-feeding appear to be associated with changes in total enzyme protein. In order to measure these changes quantitatively we chose to use antibody-titration procedures. For this purpose hepatic pyruvate kinase was purified to homogeneity from the livers of rats fed on high-carbohydrate diet. The procedure is described in detail in the Materials and Methods section. Antisera were then raised by repeated injection of hepatic pyruvate kinase into rabbits. For most of the work described here, antisera were used without further processing; however, an immunoglobulin-enriched fraction has been obtained by $(\text{NH}_4)_2\text{SO}_4$ fractionation and gave qualitatively identical results. Partially purified anti-(liver pyruvate kinase) quantitatively precipitates more enzyme. An example of a quantitative precipitation experiment between anti-(liver pyruvate kinase) sera and purified liver pyruvate kinase is shown in Fig. 5. The equivalence point for neutralization of enzyme activity and protein precipitation coincide closely. At the equivalence point shown $20 \mu\text{g}$ of liver pyruvate kinase (mol.wt. 224000) forms $200 \mu\text{g}$ of protein precipitate. If rabbit immunoglobulin G has a mol. wt. of 150000 (see below), this means that 0.09 nmol (0.36 nmol in subunits) of pyruvate kinase complexes

Fig. 5. Quantitative precipitation of liver pyruvate kinase by anti-(liver pyruvate kinase) sera

Increasing amounts of enzyme were incubated, as detailed in the Materials and Methods section, with a constant amount (50 µl) of antiserum (open symbols). Parallel incubations with pre-immune serum gave control values (closed symbols). The supernatants of the incubations were assayed for residual enzyme activity (●, ○) and the pellets were assayed for protein content (■, □). Back-projection of the residual activities of incubations plus antiserum to the abscissa gives the equivalence point of enzyme neutralization. This point closely coincides with the point of maximal protein precipitation.

1.2 nmol of immunoglobulin G. This corresponds to three immunoglobulin G molecules bound per subunit of pyruvate kinase.

Precipitation of liver pyruvate kinase from liver extracts. To demonstrate that anti-(liver pyruvate kinase) serum would precipitate pyruvate kinase from liver extracts, protein precipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Liver extracts were prepared from starved and fed rats and made to react with anti-(liver pyruvate kinase) serum. The precipitate formed was analysed by gel electrophoresis (Fig. 6). Gel scans show that both precipitates contain heavy chain (mol.wt. 50000) and light chain (mol.wt. 25000). Clearly both these proteins can mask other polypeptides on the gel. In addition there is a protein of mol.wt. 56000 which corresponds exactly to standard pyruvate kinase and to the protein precipitated by the reaction of purified pyruvate kinase with antisera. There are additional proteins (particularly at mol.wt. 40000) found in the protein precipitates from liver extracts. The exact significance of these proteins is unclear (see below). The precipitates produced with both extracts were formed under conditions of antibody excess and the pyruvate kinase was quantitatively precipitated. Comparison of the scans in Fig. 6 shows that there is

Fig. 6. Scans of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of immune complexes

Pure liver pyruvate kinase was made to react with anti-(liver pyruvate kinase) serum and the precipitate dissolved in 1% sodium dodecyl sulphate to give an approximate protein concentration of 2 mg/ml. A portion (100 µg) of protein was applied to a 7.5% acrylamide gel and electrophoresed. Protein was stained with Coomassie Brilliant Blue and the gel scanned (a). The reactions between anti-(liver pyruvate kinase) and liver cytosol extracts from fed (b) and 48 h-starved (c) rats were analysed in a similar manner. Three of the proteins were identified as indicated.

more pyruvate kinase protein in livers from fed animals than in those of starved animals. This is quantified in work described below.

Ouchterlony double-diffusion analysis. When the reaction of purified hepatic pyruvate kinase with antisera was measured by double-diffusion analysis, only a single precipitin line was formed. A single precipitin line was also obtained when diluted antiserum (1:10; 6 mg of protein/ml) was diffused against extracts. However, when undiluted antiserum (60 mg of protein/ml) was diffused against extracts a minor additional precipitin band was also observed. A similar observation has been reported by Kohl & Cottam (1976).

Interactions of anti-(liver pyruvate kinase) sera with liver enzymes. To estimate the specificity of the antiserum its effect on a number of liver enzymes was

tested. Of the enzymes tested, glucose 6-phosphate dehydrogenase, glucokinase, lactate dehydrogenase, fructose 1,6-bisphosphate aldolase, enolase, phosphoglycerate mutase and phosphoglucose isomerase, no inhibition or precipitation of enzyme activity was observed under conditions where liver pyruvate kinase was inhibited (Fig. 7). Of the enzymes tested, several use substrates which also participate in the pyruvate kinase reaction, i.e. MgATP (glucokinase), pyruvate (lactate dehydrogenase), phosphoenolpyruvate (enolase) and fructose 1,6-bisphosphate (fructose 1,6-bisphosphate aldolase). These enzymes might be expected to have similar binding sites that may be antigenically cross-reactive with the anti-(liver pyruvate kinase) sera.

Of the spectrum of cytosolic enzymes tested, only one gave any cross-reactivity with the anti-(liver pyruvate kinase) sera, and this was hepatic fructose 1,6-bisphosphatase, the inhibition of which was similar to that of liver pyruvate kinase in the liver cytosol. This suggests that hepatic pyruvate kinase and fructose 1,6-bisphosphatase have immuno-

logically cross-reacting sites. All our purified pyruvate kinase preparations are completely free of fructose 1,6-bisphosphatase. Muscle-type pyruvate kinase activity showed no inhibition by incubation with the anti-(liver pyruvate kinase) sera, and gave no reaction on double-diffusion plates.

Quantitative changes in the amount and specific activity of liver pyruvate kinase

Induction of pyruvate kinase on feeding. Results given above have shown that feeding results in an increase in total enzyme activity within a few hours. This increase in activity is accompanied by the induction of formation of pyruvate kinase protein (Fig. 8). The induction was shown by comparing pyruvate kinase protein reacting with antisera in liver extracts from prefed and fed animals (1.5h post-prandially). Induction is demonstrated on feeding by the small but statistically significant ($P < 0.01$, $n = 14$) decrease in enzyme equivalence point. This is consistent with the increased slope of the plot of enzyme activity versus soluble protein in titrations using liver extracts from fed rats.

Pyruvate kinase synthesis during dietary adaptation. By using the quantitative precipitation approach, the amount of enzyme present during adaptation from chow to high-carbohydrate diets was estimated (Fig. 9a). A high-carbohydrate diet results in the enhanced formation of pyruvate kinase protein. This was measured as a decrease in the amount of cytosolic protein

Fig. 7. Titrations of liver cytosolic enzymes against anti-(liver pyruvate kinase) serum

A constant volume of liver cytosol (1 ml) extract was incubated with increasing amounts of antisera. The activities of various enzymes were assayed after a 2h incubation and expressed as a percentage of their activity in a parallel incubation without antiserum. The titrations of lactate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphoglucose isomerase, glucokinase, fructose 1,6-bisphosphate aldolase, enolase and phosphoglycerate mutase are shown (■), together with the titrations of fructose 1,6-bisphosphatase (▲) and liver pyruvate kinase (●).

Fig. 8. Change in titratable liver pyruvate kinase in a single day

Liver extracts were prepared from rats meal-trained on the high-carbohydrate diet for 8 days, before feeding (closed symbols) and 1.5h after the end of feeding (open symbols). A constant amount of antiserum was incubated with increasing volumes of cytosolic extract and the enzyme activity remained in the supernatant assayed (●, ○). The back-projection of the lines generated to the abscissa gave the point of equivalence. Assays of the protein precipitated (■, □) are also shown.

added to reach equivalence. The amount of pyruvate kinase rose from 0.53 to 1.4 mg/100 mg of cytosolic protein. The first induction of enzyme activity was easily observed within the first day of adaptation.

We now know the total liver pyruvate kinase protein concentration (i.e. Fig. 9a) and the total enzyme activity (Fig. 9b). It is clear that the ratio of these two values gives the specific activity of the enzyme as it exists in the cytosol. This is shown in Fig. 9(b) and reveals a fascinating aspect of the current work, namely that the adaptation to high-carbohydrate diet results in an increase in enzyme specific activity (50 ± 10 to $90 \pm 10 \mu\text{mol/min}$ per mg of liver pyruvate kinase). However, even the maximum enzyme specific activity is only a fraction of the specific activity of the purified enzyme ($200 \mu\text{mol/min}$ per mg of liver pyruvate kinase). The change of enzyme activity is independent of phosphorylation, since all assays are performed at saturating fructose 1,6-bisphosphate concentration. A similar change in specific activity has been observed by Kohl & Cottam

(1976, 1977), using different antisera and titration techniques.

The current results indicate that the change in enzyme specific activity is a slow process, since the major alteration occurs 2–4 days after alteration of the diet: it should be remembered that induction of enzyme protein and changes in enzyme co-operativity occurred within a few hours.

We investigated whether a change in enzyme specific activity was a general phenomenon by adapting rats to two different nutritional states. Firstly starved rats (72 h) were fed *ad lib.* on high-carbohydrate diet (Fig. 10). Secondly animals meal-trained on high-carbohydrate diet were starved (Fig. 11). It is clear that the first dietary adaptation, which enhances fatty acid synthesis, produces a 6-fold increase in enzyme protein over 50 h. The enzyme specific activity is also enhanced (22.5 to $50 \mu\text{mol/min}$ per mg of liver pyruvate kinase) within 24 h. Both of these changes are more rapid than when rats were adapted from chow to high-carbohydrate diet.

Fig. 9. Changes in the quantity and activity of pyruvate kinase in the liver during adaptation to the high-carbohydrate diet

Liver extracts were prepared from rats trained on chow diet and transferred to the high-carbohydrate diet on day 1. On each day sampled, rats were killed before feeding (closed symbols) and 1.5 h after feeding was finished (open symbols). Liver extracts were prepared, assayed for total enzyme activity and titrated for enzyme amount. (a) shows the change in the equivalence point ($\blacktriangle$, $\triangle$) and the accompanying increase in the amount of liver pyruvate kinase in the liver ($\blacksquare$, $\square$); (b) shows total liver pyruvate kinase activity ($\blacksquare$, $\square$) as adaptation to the diet occurs. The ratio of the total enzyme activity to the total enzyme amount gives the specific activity of the enzyme protein ($\bullet$, $\circ$).

Fig. 10. Changes in the amount and activity of liver pyruvate kinase during the adaptation from starvation to re-feeding on the high-carbohydrate diet

Rats meal-trained on chow were starved for 72 h. They were re-fed with high-carbohydrate diet *ad libitum*, and changes in the quantity and activity of liver pyruvate kinase were measured, as described in Fig. 9 and the text, in liver extracts prepared at various times during the re-feeding period. The changes in equivalence point ($\blacktriangle$) and enzyme amount ($\blacksquare$) are shown in (a). The changes in the total enzyme activity ($\blacksquare$) and the resultant enzyme specific activity ($\bullet$) are shown in (b).

Fig. 11. Adaptation of activity and amount of liver pyruvate kinase during starvation

Rats were meal-trained for 10 days on the high-carbohydrate diet. They were starved for up to 96h and liver extracts were prepared at various times during this period. As described in the text, total enzyme activity was assayed and the extracts were titrated for the amount of enzyme present. The change in the equivalence point ($\blacktriangle$) and the change in total enzyme amount ($\blacksquare$) are shown in (a). The change in total enzyme activity in the liver ($\blacksquare$) and the specific activity of the enzyme protein ($\bullet$) are shown in (b).

In the reverse situation, when animals were starved from high-carbohydrate diet, changes in enzyme protein and enzyme activity were very slow (Fig. 11). Thus there was a lag of almost 30h before there was a statistically significant decrease in pyruvate kinase protein. From the time that enzyme protein starts to decline (24h), it takes a further 56h for the enzyme to decline to half its initial concentration. Presumably 56h is similar to the expected half-life for the protein if one assumes that starvation completely suppresses enzyme synthesis. A half-life of 56h confirms our previous suggestion that the rate of degradation of the protein is slow in relation to the rate of synthesis. In addition to changes in enzyme protein, the specific activity of the enzyme also falls slowly on starvation. After 96h starvation, the total enzyme protein and specific activity correspond approximately to the situation existing in the liver of meal-trained rats on chow diet.

Investigation of hepatic pyruvate kinase specific activity during enzyme purification. Purified hepatic

pyruvate kinase assayed under standard conditions in our laboratory has a specific activity of $200 \pm 20 \mu\text{mol}$ of product/min per mg at 30°C . This pure enzyme is used as the standard for all antisera titrations. When assayed by titration with antisera, the specific activity is considerably lower than $200 \pm 20 \mu\text{mol}$ of product/min per mg in rat liver cytosol. It follows that during the enzyme purification from cytosol there should occur a stage(s) when this discrepancy is removed. To examine this, pyruvate kinase was purified from the livers of rats fed on a high-carbohydrate diet. At each stage in the purification the enzyme was assayed to determine catalytic activity and the equivalence point was estimated to measure the concentration of immunologically active protein. The use of these two techniques enables the determination of enzyme specific activity in crude preparations. Table 3 shows that during the purification the apparent enzyme specific activity increased sharply at the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step (stage 4) and after this the specific activity was unaltered by further purification. This is the expected result if the $(\text{NH}_4)_2\text{SO}_4$ fractionation removed an antigenically active protein(s) which contains negligible enzyme activity. Certainly we can account for all the enzyme activity at the $(\text{NH}_4)_2\text{SO}_4$ -fractionation stage. Of the total activity (units) recovered, 1, 92 and 7% were present in the 0–30%, 35–45% and 45–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitates respectively. The enzyme activity in the 45–80%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction is almost certainly muscle-type pyruvate kinase (Tanaka *et al.*, 1965). Addition of antisera to this fraction does not inhibit enzyme activity, as expected for an L-type-specific antiserum.

The result on the specific-activity increase was confirmed by purification from the livers of chow-fed rats. The total antigenically active protein and enzyme activity were lower in the livers of these animals, as expected from Fig. 9. Exactly the same qualitative result was obtained at the $(\text{NH}_4)_2\text{SO}_4$ -fractionation stage 4. Interestingly, there was a larger increase at this step for chow-fed animals. It can be estimated that only 30% of the antigenically active protein contains full catalytic activity. This contrasts with 50% for the high-carbohydrate-fed animals.

As a control experiment we have compared the antiserum-titration pattern for a purification of hepatic pyruvate kinase with a purification of rabbit muscle pyruvate kinase. The specific activity determined by antiserum titration of the muscle enzyme was clearly constant during purification (Table 3). This shows that in sarcoplasm all the antigenically active pyruvate kinase has full catalytic activity.

The fractionation experiment has accounted for the bulk (92%) of enzyme activity and demonstrated the main fraction containing antigenically active pyruvate kinase protein. To account for the low specific activity in liver extracts, there must still

Table 3. Comparison of the titratable specific activity of liver and muscle pyruvate kinase during enzyme purification. Liver pyruvate kinase was purified from rats meal-trained on both chow and high-carbohydrate diets, as described in the Materials and Methods section. At each step the pooled fractions were titrated with anti-(liver pyruvate kinase) serum and the enzyme specific activity was calculated for the particular stage in the purification. As a comparison a similar procedure was carried out during the purification of rabbit muscle pyruvate kinase. The asterisks indicate that these enzymes were used to standardize the antiserum titration.

Enzyme purified	Stage of purification	Enzyme activity neutralized by antiserum (units)	Sp. activity ($\mu\text{mol/min per mg of}$ pyruvate kinase)
Liver pyruvate kinase from high-carbohydrate-fed rats	2	0.85 ± 0.08	80.95
	3	1.10 ± 0.09	105.0
	4	2.20 ± 0.05	209.5
	5	2.00 ± 0.07	190.5
	6	2.00 ± 0.07	190.5
	7	1.96 ± 0.07	187
	8	2.10 ± 0.04	200*
Liver pyruvate kinase from chow-fed rats	2	0.65 ± 0.05	62.0
	3	0.66 ± 0.04	62.9
	4	2.10 ± 0.10	200
	5	2.05 ± 0.09	195
Rabbit muscle pyruvate kinase	Initial extract	0.66 ± 0.06	155.0
	$(\text{NH}_4)_2\text{SO}_4$ fractionation	0.67 ± 0.06	157.0
	Ethanol precipitate	0.81 ± 0.07	189.8
	Second heat treatment	0.77 ± 0.11	180.5
	Crystallization	0.64 ± 0.08	150*

remain a protein fraction present which is antigenically active but catalytically inactive. Therefore the three fractions obtained in the $(\text{NH}_4)_2\text{SO}_4$ fractionation were each tested by double diffusion against anti-(liver-pyruvate kinase) serum. The 30–45%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction gave a strong precipitin band, and only a single band was observed when the ratio of antiserum/enzyme was varied over a wide range (cf. results on unfractionated cytosol, when a second minor band was observed). The 0–30%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction, which contained the least protein, gave a small reaction on double diffusion. However, the 45–85%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction formed a single precipitin line. Thus this fraction must contain antigenically active but catalytically inactive protein. It should be remembered that, although the 45–85%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction contains a small amount of muscle-type pyruvate kinase, this does not form a complex with anti-(liver pyruvate kinase) serum. Although the 0–30%-satd.- $(\text{NH}_4)_2\text{SO}_4$ fraction gave a weak precipitin line, we did confirm the presence of antigenically active protein by the quantitative precipitation of protein measured by the Folin reagent.

Discussion

Importance of fatty acid synthesis in hepatic metabolism and the relation to pyruvate kinase activity

Although lactate is an excellent precursor for fatty acid synthesis (Hems *et al.*, 1975; Bloxham *et al.*,

1977), alternative precursors may be important, and of these glycogen (Salmon *et al.*, 1974) is particularly relevant to the present discussion, since its conversion into fatty acids will depend on the participation of pyruvate kinase. Thus regulation of hepatic pyruvate kinase could be primarily related to control of fatty acid synthesis in the fed rat. As shown in the present study, activation of pyruvate kinase both by enzyme synthesis and by changes in catalytic activity responds rapidly to dietary changes which enhance fatty acid synthesis. Furthermore, the period of enhanced fatty acid synthesis during meal training coincides reasonably with the activation of pyruvate kinase. Finally, removal of pyruvate kinase either by decrease in enzyme protein or by enzyme inactivation is a slow process. This is particularly well shown in the adaptation from chow to high-carbohydrate diet.

Short-term regulation of pyruvate kinase by phosphorylation is also consistent with the control of fatty acid synthesis. Thus glucagon inhibits hepatic fatty acid synthesis (see Ma *et al.*, 1978, for references and discussion) and also stimulates the phosphorylation of pyruvate kinase (Ekman *et al.*, 1976; Van Berkel *et al.*, 1977; Ochs & Harris, 1978), which will inhibit the enzyme (Ljungstrom *et al.*, 1974).

Although for the purposes of the current work we have focused special interest on the role of hepatic pyruvate kinase in regulation of fatty acid synthesis, it must be appreciated that hormonal control of fatty acid synthesis is a multifactorial event. A complete discussion would require analysis of the contribution of several additional enzymes.

Pyruvate kinase in liver cytosol

The present work using meal-trained animals provides reasonable evidence for the rapid induction of enzyme synthesis within 4h, or at least for the formation of antigenically active protein. Furthermore, the evidence is clear that proteolysis of pyruvate kinase must be a slow step. This is indicated by our estimate of the half-life of enzyme protein as 56h. Thus the overall picture of biosynthesis may be represented as follows:

If this scheme is correct, inactive pyruvate kinase may be present in the cytosol. If the inactive pyruvate kinase fragments still react with antisera, then it would be expected that measurements of enzyme activity in cytosol will give a lower result than the specific activity for pure pyruvate kinase. This result was observed experimentally in the present work using meal-trained rats, and was also observed by Kohl & Cottam (1976, 1977) comparing starved and fed rats. We have now shown that active antigenically active enzyme can be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation from catalytically inactive antigenically active protein.

The low specific activity of pyruvate kinase in tissue extracts may be caused by the antisera to pyruvate kinase being too non-specific, and competing titration reactions may be responsible for variations in specific activity. Certainly our purified pyruvate kinase is substantially homogeneous (>95%); however, we have observed a cross-reaction with fructose 1,6-bisphosphatase. Fructose 1,6-bisphosphatase was completely absent from our enzyme preparations. Also, during purification pyruvate kinase was separated from fructose 1,6-bisphosphatase at the first DEAE-cellulose-chromatography stage. This is a later step than the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, which separates active antigenic pyruvate kinase. Although it seems that cross-reaction with fructose 1,6-bisphosphatase cannot explain the low specific activity of pyruvate kinase in tissue extracts, the result does provide a cautionary note against overinterpretation of our results. Thus both fructose 1,6-bisphosphatase and hepatic pyruvate kinase have binding sites for fructose 1,6-bisphosphate, and possibly these sites are structurally so similar in both proteins (potentially an important result for understanding protein structure and function) that they give a cross-reaction with antisera. The possibility of common antigenic sites on apparently unrelated proteins cannot be overlooked.

In conclusion, the current work has shown that the biosynthesis and activation of hepatic pyruvate kinase are especially well adapted to respond rapidly to dietary changes that enhance fatty acid synthesis. However, dietary changes that will enhance gluconeogenesis only slowly change enzyme activity and concentration. We consider that this pattern of response may indicate that the control of pyruvate kinase may be more closely related to the regulation of fatty acid synthesis rather than to the control of gluconeogenesis.

T. J. H. is grateful to the Science Research Council for the award of a research studentship.

References

- Atkins, G. L. (1972) *Eur. J. Biochem.* **33**, 175–180
- Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, 2nd edn., Academic Press, London
- Bloxham, D. P. (1974) *Int. J. Biochem.* **5**, 429–435
- Bloxham, D. P. & Akhtar, M. (1972) *Int. J. Biochem.* **3**, 294–308
- Bloxham, D. P. & Chalkley, R. A. (1976) *Biochem. J.* **159**, 201–211
- Bloxham, D. P., Fitzsimons, J. T. R. & York, D. A. (1977) *Horm. Metab. Res.* **9**, 304–309
- Bloxham, D. P., Coghill, S. J. & Sharma, R. P. (1978) *Biochim. Biophys. Acta* **525**, 61–73
- Brunengraber, H., Boutry, M. & Lowenstein, J. M. (1973) *J. Biol. Chem.* **248**, 2656–2669
- Clark, D. G., Rognstad, R. & Katz, J. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1141–1148
- Clark, D. G., Lee, D., Rognstad, R. & Katz, J. (1975) *Biochem. Biophys. Res. Commun.* **67**, 212–219
- Clark, M. G., Bloxham, D. P., Holland, P. C. & Lardy, H. A. (1974) *J. Biol. Chem.* **249**, 279–290
- Ekman, P., Dahlqvist, U., Humble, E. & Engstrom, L. (1976) *Biochim. Biophys. Acta* **429**, 374–382
- Engstrom, L. (1978) *Curr. Top. Cell. Regul.* **13**, 29–51
- Fazekas, S., Webster, R. G. & Datyner, A. (1963) *Biochim. Biophys. Acta* **71**, 377
- Feliu, J. E., Hue, L. & Hers, H. G. (1977) *Eur. J. Biochem.* **81**, 609–617
- Foster, J. L. & Blair, J. B. (1978) *Arch. Biochem. Biophys.* **189**, 263–276
- Godbole, V. & York, D. A. (1978) *Diabetologia* **14**, 191–197
- Harris, R. A. (1975) *Arch. Biochem. Biophys.* **169**, 168–180
- Hems, D. A., Rath, E. A. & Verinder, T. R. (1975) *Biochem. J.* **150**, 161–173
- Hopkirk, T. J. & Bloxham, D. P. (1977) *Biochem. Soc. Trans.* **5**, 1294–1297
- Hudson, L. & Hay, F. C. (1976) *Practical Immunology*, 1st edn., pp. 1–2, Blackwell Scientific Publications, Oxford

- Kohl, E. A. & Cottam, G. L. (1976) *Arch. Biochem. Biophys.* **176**, 671–682
- Kohl, E. A. & Cottam, G. L. (1977) *Biochim. Biophys. Acta* **484**, 49–58
- Krebs, H. A. & Eggleston, L. V. (1965) *Biochem. J.* **94**, 3c–4c
- Ljungstrom, O., Helmqvist, G. & Engstrom, L. (1974) *Biochim. Biophys. Acta* **358**, 289–298
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ma, G. Y., Gove, C. D. & Hems, D. A. (1978) *Biochem. J.* **174**, 761–768
- Ochs, R. S. & Harris, R. A. (1978) *Arch. Biochem. Biophys.* **190**, 193–201
- Rognstad, R. & Katz, J. (1977) *J. Biol. Chem.* **252**, 1831–1833
- Salmon, D. M. W., Bowen, N. L. & Hems, D. A. (1974) *Biochem. J.* **142**, 611–618
- Scrutton, M. C. & Utter, M. F. (1968) *Annu. Rev. Biochem.* **37**, 249–302
- Seubert, W. & Schoner, W. (1971) *Curr. Top. Cell. Regul.* **3**, 237–267
- Stammers, D. K. & Muirhead, H. (1975) *J. Mol. Biol.* **95**, 213–225
- Tanaka, T., Harano, Y., Morimura, H. & Mori, R. (1965) *Biochem. Biophys. Res. Commun.* **21**, 55–60
- Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91
- Taylor, C. B. & Bailey, E. (1967) *Biochem. J.* **102**, 32c–33c
- Tietz, A. & Ochoa, S. (1962) *Methods Enzymol.* **5**, 365–369
- Titanji, V. P. K., Zetterqvist, O. & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun.* **422**, 98–108
- Van Berkel, T. J. C., Kruijt, J. K. & Koster, J. F. (1977) *Eur. J. Biochem.* **81**, 423–432
- Van Berkel, T. J. C., Kruijt, J. K., Van den Berg, G. B. & Koster, J. F. (1978) *Eur. J. Biochem.* **92**, 553–561
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Windmueller, H. G. & Spaeth, A. E. (1966) *J. Biol. Chem.* **241**, 2891–2899
- Woods, H. F. & Krebs, H. A. (1971) *Biochem. J.* **125**, 129–139
- Zahlten, R. N. & Stratman, F. W. (1974) *Arch. Biochem. Biophys.* **163**, 600–608