Long-term modulation of type L pyruvate kinase activity in young and mature rats

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The regulation of type L pyruvate kinase concentrations in liver of young (35–45 days old) and adult (60–85 days old) rats starved and re-fed a 71% sucrose diet was investigated. Re-feeding is accompanied by an increase in the enzyme level in liver determined kinetically and immunologically. A constant ratio of kinetic activity to immunological activity was observed under all conditions examined, indicating that activity changes are the result of a regulation of synthesis or degradation and not an interconversion between kinetically active and inactive forms of the enzyme. Synthesis of pyruvate kinase was directly examined by using hepatocytes isolated from starved and re-fed rats. A stimulation of pyruvate kinase synthesis is observed on re-feeding. This increase in synthesis of pyruvate kinase is retained by the isolated hepatocyte for up to 7 h in the absence of hormonal stimuli. Administration of glucagon ($1\mu\text{M}$) to the isolated hepatocytes had no influence on synthesis of pyruvate kinase and no evidence for a glucagon-directed degradation of the enzyme was found. Re-feeding the rat was followed by a transient increase in the synthesis of pyruvate kinase. The peak rate of synthesis was observed before a detectable increase in the enzyme concentration. After a rapid synthesis period, a new steady-state level of the enzyme was achieved and synthesis rates declined. The time course and magnitude for the response to the sucrose diet was dependent on the age of the rat. In young rats, an increase in pyruvate kinase synthesis is observed within 6 h and peak synthesis occurs at 11 h after re-feeding sucrose. The peak synthesis rate for pyruvate kinase for young rats represents approx. 1% of total protein synthesis. With adult rats, increased pyruvate kinase synthesis is not observed for 11 h, with peak synthesis occurring at 24 h after re-feeding. In the older rats, peak pyruvate kinase synthesis constitutes greater than 4% of total protein synthesis. Continued re-feeding of the adult rat beyond 24 h is accompanied by a decline of pyruvate kinase synthesis to approx. 1.5% of total protein synthesis. The concentration of the enzyme, however, does not decline during this period, suggesting that control of pyruvate kinase degradation as well as synthesis occurs.

Liver pyruvate kinase (type L; EC 2.7.1.40) is regulated by a variety of mechanisms that serve to minimize recycling of phosphoenolpyruvate to pyruvate during active gluconeogenic conditions and to enhance glycolytic formation of pyruvate for lipogenesis when carbohydrate supplies are abundant. Rapid regulation of the enzyme activity in response to changes in liver metabolism can be accomplished through allosteric modulation by a variety of liver metabolites as well as through phosphorylation by cyclic AMP-dependent protein kinases (for reviews, see Blair, 1980; Denton & Halestrap, 1979). Long-range regulation of the enzyme activity in response to dietary and hormonal factors, involving changes in the enzyme concentration, has also been

reported. The biochemical basis for this long-range regulation of the enzyme level in liver, however, is not well understood. Cladaras & Cottam (1980a) demonstrated that re-feeding a high-carbohydrate diet to a starved rat is accompanied by a marked increase in the synthesis of type L pyruvate kinase in the liver. More recently these investigators (Cladaras & Cottam, 1980b) reported that the concentration of translatable mRNA for the enzyme also increases with the high-carbohydrate diet. On the basis of studies with the purified enzyme, Bergström *et al.* (1978) postulated that phosphorylation of pyruvate kinase may serve as a signal for degradation. Direct evidence for regulation of degradation, however, has not been available. In fact, measurements of the

half-life for the enzyme during starvation and feeding would not support the concept of regulation of degradation, since the half-life is long (47–75 h) and is not greatly altered with diet (Hopkirk & Bloxham, 1979; Cladaras & Cottam, 1980a). Immunological studies have also indicated that the fall in rat liver pyruvate kinase activity during starvation is not accompanied by an equivalent decrease in the enzyme protein concentration (Kohl & Cottam, 1976; Hopkirk & Bloxham, 1979). These latter observations open the possibility that conversion between active and inactive forms of the enzyme also occurs. Whether the kinetically inactive forms of the enzyme observed in those investigations represent proteolysis products from the active enzyme is not known.

We have been interested in the regulation of liver pyruvate kinase and have studied long-range control of the enzyme activity in rat liver. The results of our investigation do not support the concept of interconversion between active and inactive forms of the enzyme. We find, in agreement with Cladaras & Cottam (1980a), that re-feeding a high-carbohydrate diet to the rat is accompanied by increased synthesis of the enzyme. Our studies also extend previous reports to demonstrate that the increased synthesis of the enzyme in response to re-feeding is transient and that it is dependent on the age of the rat. These and other findings are presented in this report.

Materials and methods

Tissue preparation

All studies were carried out on male Wistar-strain rats obtained from Hilltop Laboratories, Scottdale, PA, U.S.A. Two groups of rats representing different ages were used. Immature rats were 35–45 days old and weighed 150–220 g. Mature rats were 60–85 days old and weighed 275–350 g. For re-feeding studies, the rats were starved for 48 h, then re-fed a 71% sucrose, 18% casein, 11% vegetable oil diet supplemented with USP Salt Mix XIV (Nutritional Biochemicals) and vitamins as described by Sorof & Young (1967). Normal chow-fed rats received Wayne Blox (67% carbohydrate, 24% protein, 8% fat) *ad libitum*. All animals were provided with free access to water and were maintained on a 12 h-light/12 h-dark schedule.

Homogenates and $(\text{NH}_4)_2\text{SO}_4$ -precipitated fractions of whole liver used for immunotitration of pyruvate kinase activity were prepared as previously described (Blair *et al.*, 1976). The livers were homogenized at 4°C in 3 vol. of buffer, pH 7.5, consisting of 20 mM-Tris/HCl, 100 mM-KCl, 5 mM-MgSO₄, 1 mM-EDTA, 0.2 mM-fructose 1,6-bisphosphate, 10 mM-2-mercaptoethanol and 1 mM-phenyl-

methanesulphonyl fluoride. Isolated hepatocytes were prepared by collagenase digestion of the liver as previously described (Foster & Blair, 1978).

Antibody preparation

Crystalline rat liver pyruvate kinase was prepared by a modification of the procedure of Harada *et al.* (1978). The modifications were: (1), fresh liver was used rather than frozen material; (2), 0.1 mM-phenylmethanesulphonyl fluoride was added to all buffers as a proteolysis inhibitor; (3), dialysis of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated material was completed within 8 to 10 h to minimize proteolysis. Harada *et al.* (1978) reported the electrophoretic properties of the enzyme were altered during purification. With the modifications employed in the present investigation we found the enzyme to be kinetically indistinguishable from that found in fresh extracts from rat hepatocytes (Foster & Blair, 1978). The enzyme prepared by this method was also found to be kinetically and physically comparable with that prepared by the procedure of Titanji *et al.* (1976). The purified enzyme has a specific activity of 200 units/mg of protein under the standard assay conditions employed in the present investigation (see below) and exhibited a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Goats that had been prestimulated by injection with Freund's complete adjuvant were injected with 200 µg of purified type L pyruvate kinase mixed with 2 ml of 20 mM-KH₂PO₄ (pH 7.0) and 2.0 ml of Freund's incomplete adjuvant at biweekly intervals. Antigen injections were given intramuscularly at four sites. Antibody titres in three separate goats were found to be at a maximum after four injection periods. The antiserum preparations used in this investigation on Ouchterlony double-diffusion against liver extracts gave a single precipitin band showing identity with purified pyruvate kinase and had a titre of approx. 72 units of pyruvate kinase-neutralizing activity/ml of serum. Other criteria used to assess the suitability of the serum used in the present studies are discussed in the Results section.

For immunotitration studies, the antiserum preparations were heated at 56°C for 60 min in the presence of 1 mM-phenylmethanesulphonyl fluoride and 1 mM-EDTA; then, a globulin-enriched fraction was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation (0–50%). The globulin-enriched fraction was then exhaustively dialysed against 0.15 M-NaCl at 4°C to remove $(\text{NH}_4)_2\text{SO}_4$ and other contaminating materials and stored frozen. For rapid isolation of radiolabelled pyruvate kinase from tissue extracts, the enriched globulin fraction was coupled to Sepharose 6B by using CNBr-activated Sepharose obtained commercially (Sigma) or prepared by the

procedure of Axén *et al.* (1967). The resin-coupled antiserum neutralized 40 units of pyruvate kinase activity/ml of packed resin. Pre-immune serum or resin-bound pre-immune serum used as a control was prepared from non-stimulated goats.

Incubation of hepatocytes

To investigate the synthesis of pyruvate kinase, rat hepatocytes were preincubated at a density of approx. 7×10^6 cells/ml for 30 min at 37°C in a buffer at pH 7.4 containing 120 mM-NaCl, 5 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 25 mM-NaHCO₃, 1.25 mM-CaCl₂, 10 mM-glucose, 4% (w/v) dialysed bovine serum albumin (Miles Laboratories) and an amino acid mixture, modified from that described by John & Miller (1969) except that leucine was omitted for isotopic labelling studies (see the legend to Fig. 4 for complete amino acid composition). The incubation vials were gassed with CO₂/O₂ (1:19). The purpose of the pre-incubation was to obtain a low level of endogenous leucine before adding the radiolabel. After pre-incubation, the hepatocytes were washed into fresh buffer at 0°C and incubation at 37°C was initiated after addition of 5 μ Ci of [³H]leucine/ml. (sp. radioactivity 50 Ci/mmol; New England Nuclear). Unless otherwise noted, the cells were incubated with the isotope for 1 h at 37°C. The reaction was terminated by adding (NH₄)₂SO₄ to a final concentration of 2.6 M (Foster & Blair, 1978) and the precipitated cell pellet was washed twice and stored at 4°C in 2.6 M-(NH₄)₂SO₄ containing 30 mM-2-mercaptoethanol. The (NH₄)₂SO₄ cell pellet was extracted at 4°C with 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol for 1 h at an enzyme/buffer ratio calculated to give approx. 10 units of enzyme activity/ml. The supernatant from this extract obtained by centrifuging at 10000 g for 10 min was used for determining total radioactivity in protein by the filter-disc technique of Bollum (1968) and for determination of radio-labelled pyruvate kinase. With this procedure, incorporation of radiolabelled leucine into total protein and into pyruvate kinase was generally linear for 1 h incubation of hepatocytes with greater than 70% of the added isotope being incorporated into cellular protein. Similar results were obtained when hepatocytes were incubated with a complete amino acid-containing medium (0.38 mM-leucine); however, longer incubation periods (6 h) were necessary to attain a high degree of labelling of the enzyme due to the low specific radioactivity of the leucine pool.

Quantitative isolation of radiolabelled pyruvate kinase

Radiolabelled pyruvate kinase in extracts from hepatocytes was rapidly isolated by using Sepharose-coupled goat anti-(rat type L pyruvate kinase).

Pyruvate kinase (4 units) was incubated with 0.1 ml (packed volume) antiserum-resin for 1.5 h at 4°C in a buffer, pH 7.0, containing 10 mM-potassium phosphate, 100 mM-KF and 1 mM-dithiothreitol. Quantitative binding of the enzyme was assessed by measuring loss of enzyme activity. No loss of pyruvate kinase activity was observed with control resins prepared with pre-immune serum. After binding the resin was collected by centrifugation (200 g for 2 min) and washed twice at 4°C with fresh buffer containing 500 mM-NaCl. The resin was then washed three times at room temperature with 4 ml of 500 mM-NaCl containing 1% Triton X-100 and three times with 1% Triton X-100 in water to remove excess salt, which interferes with electrophoresis. Radiolabelled pyruvate kinase was dissociated by incubating the immunoresins with 1% (w/v) sodium dodecyl sulphate for 15 h at room temperature. Dissociation of the antibody-antigen complex is not complete in shorter periods of time. Quantitative estimates of the specific radiolabelling of pyruvate kinase were made by subtracting the non-specific radioactivity bound to control resins incubated with a duplicate sample from that bound to anti-(pyruvate kinase) resins. This procedure gives quantitative recovery of purified pyruvate kinase, which was labelled by phosphorylation and has several advantages over direct immunoprecipitation procedures. In addition to quantitative recovery, the immunoresin procedure is rapid and minimizes proteolysis or denaturation of the enzyme that was observed with direct immunoprecipitation techniques. Also, availability of a direct control consisting of sample incubated with pre-immune serum coupled to Sepharose, we feel, is superior to second-precipitation procedures applied with immunoprecipitation techniques (Hopgood *et al.*, 1973).

Other procedures

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done by the procedure of Laemmli (1970) with the exception that 1.4% NN'-diallyltartardiamide was substituted as the cross-linking agent. After electrophoresis, the gels were sliced in 2 mm sections, dissolved in 0.2 ml of 2% (w/v) periodic acid and counted for radioactivity. Scintillation counting was done using ACS (Amersham) counting fluid in a Packard Tri-Carb counter. All counts were corrected for efficiency by use of an external standard.

Protein analysis was done by the procedure of Lowry *et al.* (1951) with dialysed bovine serum albumin as standard. Bovine serum albumin standards were calibrated spectrophotometrically using $A_{279}^{1\%} = 6.67$ (Foster & Sterman, 1956). Pyruvate kinase activity was determined at pH 7.5 and 25°C in the presence of 1.8 mM-phosphoenolpyruvate,

2.5 mM-ADP and 75 μ M-fructose 1,6-bisphosphate as described previously (Blair *et al.*, 1976; Foster & Blair, 1978). One unit of activity is defined as that catalysing the formation of 1 μ mol of pyruvate/min at 25°C. Fructose 1,6-bisphosphatase activity was assayed by the procedure of Pontremoli (1966). Enolase was assayed by the procedure of Westhead (1966).

Results

Response of liver pyruvate kinase activity to high-sucrose diet in rats of different ages

Several investigators have reported that re-feeding a high-sucrose diet to the rat is accompanied by a marked increase in the activity of pyruvate kinase in liver (Gunn & Taylor, 1973; Kohl & Cottam, 1976; Blair, 1980). We find that the time course of this response as well as the overall magnitude of the increase in the enzyme activity is dependent on the age of the rats studied. The results of studies measuring pyruvate kinase activity in livers from young, immature rats and livers from adult rats starved and re-fed a high-sucrose diet are summarized in Fig. 1.

With adult rats, only a small increase in pyruvate kinase activity is observed during the first 24 h of re-feeding. Between 24 and 48 h, however, a sharp increase in the enzyme activity is observed and a plateau is reached between 48 and 120 h. With younger rats, the response of pyruvate kinase activity to re-feeding is more prompt, with a significant increase in activity per g of liver being noted within 12 h and a plateau being reached within 48 h.

Pyruvate kinase levels are approx. 40 units/g of liver in rats fed standard chow *ad libitum* and this declines to 20 units/g of liver on starvation for 48 h in both young and adult rats. The differences between the two age groups of rats is apparent only on re-feeding the sucrose diet. The difference in the plateau level of pyruvate kinase activity between the young and older rats is particularly apparent when the increase in liver weight accompanying the sucrose diet is taken into consideration. When the results are expressed in terms of total pyruvate kinase activity per liver, re-feeding the young rat for 48 h gives an approx. 4-fold increase in the enzyme activity above that seen in the starving state. With the older, mature rats the increase is approx. 8-fold over the starved state (see the legend to Fig. 1).

Validity of the immunological technique

Because of the controversy in the literature concerning the relationship between kinetically measured and immunologically determined levels of pyruvate kinase in the liver after the starving and re-feeding schedules, an assessment of the immuno-

Fig. 1. *Pyruvate kinase activity in liver of rats re-fed a high sucrose diet*

Young (●) and adult (■) rats were fed on standard laboratory chow *ad libitum* or starved for 48 h and re-fed a high-sucrose diet. At various times the livers were removed and homogenized in 3 vol. of homogenization buffer, pH 7.5. Pyruvate kinase activity was precipitated with 2.6 M-(NH₄)₂SO₄ and the enzyme activity was measured at optimal conditions in the presence of 75 μ M-fructose 1,6-bisphosphate. The results are expressed as means $\pm$ S.E.M. and are units/g of liver for multiple determinations from three to ten animals per group. * $P < 0.005$ versus levels in starved rats. Statistical analysis was by Student's *t* test. In young rats starved for 48 h, mean liver weight was $4.6 \pm 0.4\%$ of total body weight and this value increased to $6.5 \pm 0.3\%$ of total body weight after re-feeding for 48 h. In starved adult rats, liver weight was $4.3 \pm 0.4\%$ of total body weight and this value increased to $5.8 \pm 0.1\%$ of total body weight after re-feeding.

logical levels of the enzyme was undertaken in the present investigation. The success and reliability of such immunological studies is highly dependent on the monospecificity of the antibody preparations applied. The suitability of the goat anti-(rat type L pyruvate kinase) raised for the present investigation has been assessed by several criteria.

The antiserum appears to have specificity for type L pyruvate kinase in that it inhibited pyruvate kinase activity only in those tissues known to contain significant amounts of the type L isoenzyme (liver, red cell and kidney). No inhibition of pyruvate kinase activity from skeletal muscle and spleen, which contain the M₁ and M₂ isoenzymes respectively, was observed. Furthermore, the antibody

preparation exhibited no significant inhibition of fructose 1,6-bisphosphatase activity nor enolase activity from rat liver, indicating that the antibody does not cross-react with other enzymes that bind metabolites interacting with type L pyruvate kinase (results not shown).

A single precipitin band showing identity with purified pyruvate kinase was observed in Ouchterlony double-diffusion patterns for the antibody diffused against extracts from rat liver (results not shown). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the liver proteins bound to the Sepharose-coupled antibody gives one band that stains with Coomassie Brilliant Blue (result not shown) and that is radiolabelled when hepatocytes are incubated with radioactive amino acids (Fig. 2). This radiolabelled protein band electrophoreses with purified pyruvate kinase. Hopkirk & Bloxham (1979) reported two molecular-weight bands for immunoreactive pyruvate kinase from rat liver if the liver extracts are examined before $(\text{NH}_4)_2\text{SO}_4$ fractionation. Our results do not confirm their observation. We find only one band which co-migrates on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with purified rat liver pyruvate kinase both before and after $(\text{NH}_4)_2\text{SO}_4$ fractionation (Fig. 2). These various lines of evidence have been taken to collectively indicate that the goat anti-(rat pyruvate kinase) serum used in the present investigation is monospecific for type L pyruvate kinase.

Immunotitration of rat liver extracts

To immunologically assess the concentration of type L pyruvate kinase in extracts of livers from starved and re-fed rats, various amounts of liver extract were incubated with a fixed level of goat anti-(type L pyruvate kinase) serum and the amount of enzyme activity remaining after neutralization was plotted against the enzyme activity originally added as shown in Fig. 3. The titre was then determined by extrapolating the linear portion of the titration curve to the abscissa. Control serum had no influence on the enzyme activity (Fig. 3).

In similar studies to those described above, Hopkirk & Bloxham (1979) reported that the immunologically determined level of pyruvate kinase differs between unpurified liver extracts and extracts partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, suggesting an immunoreactive yet catalytically inactive protein is removed by the salt treatment. We also examined the influence of $(\text{NH}_4)_2\text{SO}_4$ fractionation on the immunoreactivity of pyruvate kinase and found no difference from that obtained for non-fractionated liver extracts using fed rats (Fig. 3) and 48h-starved rats (results not shown). For further studies, we routinely included $(\text{NH}_4)_2\text{SO}_4$ precipitation before immunotitration

Fig. 2. Electrophoresis of radiolabelled proteins bound to control and anti-(type L pyruvate kinase) serum covalently bound to Sepharose 6B resins

Hepatocytes from a rat starved for 48 h and re-fed a high-sucrose diet for 24 h were incubated with $[^3\text{H}]$ leucine for 1 h. A portion of the cells was extracted immediately and the extract was incubated with resin-bound anti-(type L pyruvate kinase) serum (●). A second portion of the extract was precipitated with 2.6 M $(\text{NH}_4)_2\text{SO}_4$ and the resulting pellet was dissolved in 10 mM-potassium phosphate buffer, pH 7.0, with 1 mM-dithiothreitol before incubation with the resin-bound antiserum (□), or control (pre-immune) serum (○). After washing and eluting the antibody resins, the radiolabelled proteins were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The developed gels were then sliced in 2 mm slices, dissolved in 2% H_2IO_6 and counted for radioactivity. The observed radioactivity corrected for counting efficiency in each slice is presented as a function of the migration distance relative to the Bromophenol Blue dye front. The relative position of purified type L pyruvate kinase electrophoresed simultaneously in a paired gel is indicated by the arrow.

because of the stabilizing effect $(\text{NH}_4)_2\text{SO}_4$ had on the enzyme activity, particularly in studies with the isolated hepatocytes (Foster & Blair, 1978).

The results of immunotitration studies of pyruvate kinase activity in extracts of livers from rats starved and re-fed a high-sucrose diet show a constant relationship between enzyme activity and immunoreactivity (Table 1), suggesting that the

Fig. 3. Immunotitration curves for type L pyruvate kinase in rat liver extracts

A high-speed supernatant from rat liver was prepared by centrifuging homogenates in 50 mM-Tris/HCl, pH 7.5, for 30 min at 12000g. A portion of the supernatants was then brought to 2.6 M- $(\text{NH}_4)_2\text{SO}_4$ by addition of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. The precipitated protein was collected and dissolved in 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol. Various amounts of the fresh supernatant (○ and ●) or $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction (□ and ■) were then incubated for 2 h at 4°C with a fixed amount (0.02 ml) of anti-(type L pyruvate kinase) serum (● and ■) or control serum (○ and □) in a final volume of 1.0 ml. Pyruvate kinase activity was determined at the beginning of the incubation period (initial activity) and after 2 h (final activity). The immunotitre was then estimated by extrapolation of the antiserum curves to the ordinate as shown by the broken line. As demonstrated by the slope and intercept of the line obtained for incubations with control serum, no inactivation of pyruvate kinase was observed in this situation. Inactivation of the enzyme activity was caused specifically by anti-serum raised against purified type L pyruvate kinase.

increase in the enzyme activity accompanying re-feeding is a consequence of an increase in the amount of enzyme and not an activation of pre-existing enzyme.

The relationship between pyruvate kinase activity and immunoreactivity was also found to remain constant in liver from adult rats that had been re-fed the high-sucrose diet for 48 h to induce the enzyme level, and then starved. The activity of type L pyruvate kinase in the liver of re-fed rats, however, promptly decreases after initiation of starvation, whereas those animals maintained on the sucrose

diet retain the high level of enzyme activity (Table 1).

Pyruvate kinase synthesis

To investigate the synthesis of pyruvate kinase, hepatocytes isolated from rats maintained on various diets were incubated with radiolabelled amino acids and radiolabelled pyruvate kinase that was formed was isolated immunologically. The pool sizes of leucine in the hepatocytes were not determined in these studies; thus absolute rates of protein synthesis cannot be assessed. Pyruvate kinase synthesis relative to total incorporation of isotope into soluble proteins was determined. The incorporation of isotope into cellular protein was similar among hepatocytes obtained from rats of various ages and those starved and re-fed a high-sucrose diet (Table 2). The specific incorporation of radiolabel into pyruvate kinase, however, was markedly influenced by age as well as diet. Fig. 4 summarizes the results obtained concerning the relative rate of synthesis of pyruvate kinase in hepatocytes from young and adult rats re-fed a high-sucrose diet. With both young and adult rats, re-feeding this diet after starving is followed by a marked increase in the relative rate of pyruvate kinase synthesis. The synthesis rate peaks then declines as re-feeding is continued, although the enzyme activity remains constant with prolonged re-feeding (Fig. 1). The time course and magnitude of this response to re-feeding differs between young and adult rats. In immature rats that have been starved for 48 h, pyruvate kinase synthesis peaks at approx. 1.3% total soluble protein synthesis within 12 h after re-feeding. With adult rats, peak enzyme synthesis rates occur 24 h after re-feeding and is nearly 4% of total soluble protein synthesis. The relative rate of pyruvate kinase synthesis in hepatocytes from both young and adult rats fed a standard laboratory chow is approx. 0.35% of total protein synthesis. Cycloheximide ($10 \mu\text{M}$) inhibited incorporation of $[^3\text{H}]$ leucine into soluble protein and into pyruvate kinase by greater than 99% (Table 2), suggesting that isotope incorporation into protein is dependent on protein synthesis.

Lack of influence of glucagon on pyruvate kinase synthesis

Glucagon has been reported to inhibit the synthesis of several enzymes induced by re-feeding high-carbohydrate diets including: glucose 6-phosphatase dehydrogenase (Garcia & Holten, 1975) and glucokinase (Spence & Pitot, 1979). The influence of glucagon on pyruvate kinase was examined in this investigation using hepatocytes from rats that were starved then re-fed a high-sucrose diet for 24 h. High rates of pyruvate kinase synthesis were retained in these hepatocytes for up

Table 1. *Immunotitration of rat liver extracts*

Young (150–220 g) and adult (275–350 g) rats were fed standard laboratory chow *ad libitum* or starved for 48 h and re-fed a high-sucrose diet. One group of 48 h re-fed adult rats was starved for various times to examine degradation of newly synthesized enzyme. The livers were extracted and immunotitration of type L pyruvate kinase activity was carried out as described in the legend to Fig. 3. The equivalence point from each titration was determined from extrapolation of the titration curve and is presented as the mean $\pm$ S.E.M. of results obtained from the number of determinations given in parentheses. The antiserum was calibrated against three preparations of purified rat type L pyruvate kinase (specific activity = 185 ± 18 units/mg of protein). The 'apparent specific activity' of the pyruvate kinase in liver extracts was calculated from the immunotitration data and is presented as units of pyruvate kinase activity/mg of immunotitratable protein.

Rats	Diet	Pyruvate kinase activity (units/liver)	Equivalence point (units/ml of serum)	'Apparent immunospecific activity' (units/mg)
Young	Standard chow (3)	378 ± 24	61.5 ± 0.5	176 ± 1
	Starved for 48 h (3)	159 ± 12	58.5 ± 3.0	168 ± 9
	Re-fed for 48 h (3)	649 ± 17	66.0 ± 7.0	189 ± 20
Adult	Standard chow (8)	506 ± 32	60.7 ± 7.0	174 ± 7
	Starved for 48 h (4)	221 ± 14	63.0 ± 3.6	180 ± 10
	Re-fed for 24 h (4)	609 ± 47	64.1 ± 2.1	184 ± 6
	Re-fed for 48 h (5)	1680 ± 63	66.9 ± 4.1	192 ± 12
	Re-fed for 96 h (3)	1498 ± 122	72.7 ± 3.8	198 ± 10
	Re-fed for 48 h/starved for 48 h (3)	605 ± 19	75.5 ± 4.2	205 ± 11
	Re-fed for 48 h/starved for 96 h (3)	367 ± 41	68.7 ± 5.6	187 ± 15
Purified type L pyruvate kinase (3)			64.6 ± 2.6	(185 ± 18)

Table 2. *Incorporation of radiolabelled leucine into soluble protein of isolated rat hepatocytes*

Hepatocytes from rats starved for 48 h or starved and re-fed a high-sucrose diet were incubated with [3 H]leucine for 1 h as described in the Materials and methods section. Total radioactivity incorporated into soluble protein was determined by a filter-disc procedure (Bollum, 1968) and is presented as the mean $\pm$ S.E.M. (d.p.m./ 10^8 cells) for three to ten determinations for each condition. Cycloheximide ($10 \mu\text{M}$) was added at initiation of the incubation period where indicated.

Diet	$10^{-6} \times$ Radioactivity in soluble protein (d.p.m./ 10^8 cells)
Starved for 48 h	8.0 ± 2.6
Re-fed 6–8 h	8.7 ± 1.7
Re-fed for 12 h	6.5 ± 1.5
Re-fed for 24 h	7.5 ± 1.6
Re-fed for 72 h	4.2 ± 1.1
Re-fed for 24 h + cycloheximide	0.12 ± 0.04

glucagon had no influence on the total level of the enzyme activity with prolonged incubation (7 h). The hormone was effective in decreasing the activity ratio of pyruvate kinase at 1.8 mM-phosphoenolpyruvate, indicating phosphorylation did occur (Foster & Blair, 1978).

The long-term influence of glucagon on the physical properties of pyruvate kinase was also examined by using hepatocytes from re-fed rats that were pulse-labelled with [3 H]leucine for 1 h and then washed and incubated for an additional 5 h in the presence of a complete amino acid mixture containing an unlabelled leucine chase (0.38 mM). Glucagon had no influence on the amount of radiolabelled enzyme measured after 5 h and a single radiolabelled protein peak that co-migrated with purified pyruvate kinase was observed after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the labelled enzyme from both control and glucagon-treated hepatocytes (results not shown).

Discussion

The long-term regulation of pyruvate kinase activity in the liver in response to dietary and hormonal stimuli such as that presented in Fig. 1 has been recognized for many years (Krebs & Eggleston, 1965; Tanaka *et al.*, 1967; Weber, 1969; Gunn

to 7 h in the absence of hormonal stimuli (results not shown). Glucagon ($1 \mu\text{M}$) had no influence on the synthesis of the enzyme during this period. Also,

Fig. 4. Synthesis of type L pyruvate kinase in hepatocytes from starved and re-fed rats

Hepatocytes were prepared from adult (○) or young (□) rats maintained on standard laboratory chow *ad libitum* or starved for 48 h and re-fed a high-sucrose diet for the time period indicated. The cells were pre-incubated for 30 min in leucine-free incubation buffer supplemented with 340 μ M-alanine, 536 μ M-arginine, 955 μ M-asparagine, 812 μ M-glycine, 167 μ M-histidine, 396 μ M-isoleucine, 640 μ M-lysine, 74 μ M-methionine, 242 μ M-phenylalanine, 278 μ M-proline, 590 μ M-serine, 436 μ M-threonine, 122 μ M-tryptophan, 99 μ M-tyrosine, 341 μ M-valine, 11 μ M-cysteine and 415 μ M-glutamine. After pre-incubation, the cells were washed once and placed in fresh incubation medium containing [3 H]leucine (5 μ Ci/ml). Incubation at 37°C was then continued for 1 h. The cells were harvested in 2.6 M-(NH₄)₂SO₄ and extracted in 10 mM-potassium phosphate, pH 7.0, containing 1 mM-dithiothreitol. Total soluble radioactive protein and radioactive pyruvate kinase were determined for each extract. The contribution of radioactivity in pyruvate kinase as a percentage of total soluble protein is presented as the mean $\pm$ S.E.M. for the number of experiments shown in parentheses. Duplicate and single determinations at particular time points are presented as individual points. * P < 0.01 versus 48 h-starved rats; ** P < 0.01 versus 24 h-re-fed rats (mature); *** P < 0.01 versus 12 h-re-fed rats (young). Statistical analysis was conducted by Student's t test.

strated that re-feeding the starved rat a high-carbohydrate diet is followed by an increase in the levels of translatable mRNA for type L pyruvate kinase and markedly increased synthesis of the enzyme. The present investigation demonstrates that the high rates of synthesis of the enzyme brought about by the high-carbohydrate diet are retained in the isolated hepatocyte in the absence of hormonal stimuli for several hours. This demonstration opens the possibility for further investigation of those factors that act directly on the liver to modulate synthesis of the enzyme. The present investigation further expands to studies of Cladaras & Cottam (1980a) by showing that the time course and the magnitude of the response of pyruvate kinase synthesis to the high-carbohydrate diet is dependent on the age of the rat. We do not know the factors that determine the magnitude and time course for the response in pyruvate kinase synthesis or the changes that occur during maturation. Adelman (1970) demonstrated that the initiation of glucokinase synthesis in response to a high-carbohydrate diet is dependent on the age of the rat and suggested that the age factors represent overall changes in the hormonal response of the animal to dietary carbohydrate (Sartin *et al.*, 1980). Although a complete understanding of the age-dependent factors influencing the synthesis of pyruvate kinase must await further investigation, recognition that pyruvate kinase may constitute up to 4% of soluble protein synthesis in hepatocytes from adult rats compared with 1% in young rats will prove valuable in further isolation of mRNA for this important liver enzyme.

The development of an isolated hepatocyte system for studying synthesis of pyruvate kinase allows a direct examination of agents that may have an impact on this enzyme. Other investigators have proposed that phosphorylation of type L pyruvate kinase such as that elicited by glucagon may serve as a signal for degradation of the enzyme (Bergström *et al.*, 1978; Hall *et al.*, 1979). Interestingly, the synthesis of a number of hepatic enzymes, which, like pyruvate kinase, are induced by a high-carbohydrate diet, is inhibited by glucagon. These enzymes include glucokinase (Spence & Pitot, 1979; Schudt, 1979), glucose 6-phosphate dehydrogenase (Garcia & Holten, 1975; Winberry *et al.*, 1980) and fatty acid synthase (Volpe & Marasa, 1975) among others. In the case of glucose 6-phosphate dehydrogenase it would appear that glucagon does not act directly on the liver (Winberry *et al.*, 1980), although it does inhibit the enzyme synthesis when administered to the rat. Spence & Pitot (1979), however, demonstrated that glucagon rapidly inhibits glucokinase synthesis in rat hepatocytes in primary culture. Their studies also demonstrated that glucagon enhances degradation of the enzyme.

& Taylor, 1973); however, little is known of those factors that act directly on the liver to mediate this regulation, or the mechanisms by which these factors act. Several proposals concerning the long-term regulation of pyruvate kinase have been made, but very few of these proposals have been directly tested. Cladaras & Cottam (1980a,b) first demon-

The results of the present investigation indicate that glucagon does not rapidly modulate either the synthesis or degradation of hepatic pyruvate kinase. It will be necessary to expand these studies to assess whether the hormone has longer-range consequences. One of the unexpected findings of the present investigation is that the synthesis of pyruvate kinase is maintained for several hours by the isolated hepatocyte in the absence of hormonal stimuli. Either the mRNA for the enzyme is quite stable or it is continually being synthesized, thus maintaining a fixed level of the message. If the mRNA is stable, actions of glucagon on synthesis of the mRNA may not be apparent within the time frame employed in the present investigation. Similar arguments can be made with regard to glucagon actions on the turnover of the enzyme, particularly in the light of reports that the enzyme has a half-life ranging from 47 to 75 h (Cladaras & Cottam, 1980a; Hopkirk & Bloxham, 1980). Expansion of the present studies to rat hepatocytes maintained in primary culture is required to address those problems.

Immunological studies reported by other investigators have indicated that the changes in pyruvate kinase activity in the liver during starving and re-feeding are not accompanied by equivalent changes in the enzyme concentration measured immunologically (Kohl & Cottam, 1976; Hopkirk & Bloxham, 1979; Johnson & Veneziale, 1980). One implication of that finding would be that changes in the enzyme activity could represent conversion between active and inactive forms of the enzyme. It was also suggested that inactive forms detected represent proteolytic products of the native enzyme (Hall *et al.*, 1979; Hopkirk & Bloxham, 1979). No evidence for immunologically active yet kinetically inactive forms of the enzyme was found in the present investigation. The 'apparent specific activity' of the enzyme determined by immunotitration was constant under all of the conditions examined and agreed well with the specific activity determined for the purified enzyme (Table 1). The basis for this difference in our observations from those of others is not clear. One possibility is that the antibody used in the present investigation does not react with low-molecular-weight inactive forms of the enzyme that were observed by others. It is also possible that inactive forms of the enzyme observed by others were generated after extraction of the liver. The rapid immunoresin technique used in the present investigation was specifically developed to minimize proteolysis occurring during isolation of the radio-labelled enzyme. Hopkirk & Bloxham (1979) also reported that the anti-(pyruvate kinase) serum used in their investigation neutralized fructose 1,6-bisphosphatase. The antiserum used in the present investigation did not cross-react with fructose

1,6-bisphosphatase or enzymes other than type L pyruvate kinase that were examined. Thus differences in the nature of the immunological reagents employed in various laboratories could also account for the different observations. One conclusion of the present investigation is that the rapid increase in pyruvate kinase activity observed in liver of rats fed on a high-carbohydrate diet is primarily the consequence of the marked increase in synthesis of the enzyme occurring during this time period (Figs. 1 and 4). The transient nature of time course for pyruvate kinase synthesis after re-feeding, however, was not an anticipated observation (Fig. 4). The fact that pyruvate kinase synthesis peaks during the time when the enzyme concentration rises within the liver but declines once a new steady-state level of the enzyme is achieved might suggest that the high-carbohydrate diet is also accompanied by decreased degradation of the enzyme. No direct evidence for decreased turnover of the enzyme was found in the present investigation. Estimates of the turnover of the enzyme in liver of starving and re-fed rats also would not support a control of degradation of the enzyme during re-feeding (Cladaras & Cottam, 1980a). Thus, those factors that modulate the synthesis of the enzyme and the basis for decreased synthesis of the enzyme once the steady-state level of the enzyme is achieved remain to be elucidated.

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