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Glycolytic and Gluconeogenic Enzyme Activities in Parenchymal and Non-parenchymal Cells from Mouse Liver

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1. Parenchymal cells have been prepared from mouse liver by enzymic and mechanical means. 2. The dry weights, protein and DNA contents of these cells have been determined. 3. Mouse liver 'M-' and 'L-type' pyruvate kinases have been prepared free of contamination with each other; their kinetic properties have been examined and a method has been developed for their assay in total liver homogenates. 4. Recoveries of phosphoglycerate kinase, lactate dehydrogenase and phosphofructokinase in enzymically prepared cells indicate that little, if any, cytoplasmic protein is lost during preparation. 5. Parenchymal cells exhibit a very substantial increase in the activity ratio of glucokinase to hexokinase over that in total liver homogenate; in three out of eight experiments, hexokinase activity was undetectable. 6. 'L-type' pyruvate kinase alone occurs in the parenchymal cell. Non-parenchymal cells are characterized by the presence of 'M-type' activity only. 7. Parenchymal cells contain both glucose 6-phosphatase and fructose 1,6-diphosphatase. The non-parenchymal fraction appears to contain fructose 1,6-diphosphatase, but is devoid of glucose 6-phosphatase. 8. No aldolase A was detectable in the whole liver. Aldolase B occurs in both parenchymal and non-parenchymal tissue. 9. Parenchymal cells prepared by mechanical disruption of mouse liver with 20% polyvinyl alcohol exhibit a similar enzyme profile to those prepared enzymically. 10. The methodology involved in the preparation of isolated liver cells is discussed. The importance of the measurement of several parameters as criteria for establishing the viability of parenchymal cells is stressed. 11. The metabolic implications of the results in the present study are discussed.

It has been recognized for a number of years that the livers of rats and other species contain several enzymes capable of catalysing the phosphorylation of glucose by ATP (Gonzalez *et al.*, 1964). These have been resolved by electrophoresis (Katzen *et al.*, 1965) into three 'low K_m' hexokinases (EC 2.7.1.1) and one 'high-K_m' glucokinase (EC 2.7.1.2). The presence of this high-K_m, insulin-inducible, glucokinase has been correlated with the responsiveness of the liver to glucose at concentrations in the range normally observed for this sugar in the blood (Cahill *et al.*, 1959; Di Pietro *et al.*, 1962). This apparent duplication of glucose-phosphorylating capacities, considered together with the low total hexokinase activities (in the rat), has stimulated the suggestion that glucokinase alone is associated with the parenchymal cells and that the hexokinases occur in the mesenchymatous tissue (Kupffer cells etc.) (Sols *et al.*, 1964). This view has, however, been contested (Walker, 1966) on the basis of developmental (Ballard & Oliver, 1964; Walker & Lea, 1964) and hepatic tumour studies (Sharma *et al.*, 1965).

A similar situation arises with the enzyme pyruvate kinase (EC 2.7.1.40). Several workers (Tanaka *et al.*, 1965; Passeron *et al.*, 1967; Tanaka *et al.*, 1967*a*; Taylor & Bailey, 1967; Susor & Rutter, 1968) have shown the existence in liver of at least two distinct activities, one apparently similar to, although not identical with (Jiménez de Asuá *et al.*, 1971), muscle 'M-type' pyruvate kinase, the other exhibiting both a sigmoid rate response towards the substrate phosphoenolpyruvate and a sensitivity to activation by fructose 1,6-diphosphate (Tanaka *et al.*, 1967*b*; Taylor & Bailey, 1967; Carminatti *et al.*, 1968). The presence of the latter type of pyruvate kinase in tissues has been correlated with the presence of an active dicarboxylate shuttle and gluconeogenic or glyceroneogenic potential (Pogson, 1968*a*; Llorente *et al.*, 1970). In view of the high affinity for phosphoenolpyruvate of the 'M-type' pyruvate kinase, and of the physiological amounts of phosphoenolpyruvate in the liver under various dietary and hormonal conditions, it is difficult to explain the presence in liver of the two species of pyruvate kinase unless one invokes the presence of as yet unknown but very potent effector molecules or compartmentation either within cells or between the various hepatic cell types.

The problem of liver cell heterogeneity and enzyme

distribution may be theoretically most easily solved by the isolation of one or more pure cell types. Many attempts to prepare parenchymal cells from a number of species have been made (for references, see Bhargava, 1968); most of the techniques used, however, result in the preparation of leaky or otherwise 'non-viable' cells (Berry & Friend, 1969; Suzangar & Dickson, 1970). A recent attempt to determine the distribution of hexose-phosphorylating activities in isolated rat parenchyma has indicated that glucokinase may predominate in these cells and that hexokinase is more associated with non-parenchymal elements (Sapag-Hagar *et al.*, 1969); considerable leakage of protein and cell damage were encountered, however, so that the authors were unable to reach categorical conclusions on the distribution of the two enzymes. The use of the enzyme perfusion technique of Berry & Friend (1969), a procedure that yields intact and metabolically active parenchymal cells, has permitted a further and more clearcut investigation of this problem. In this paper we report the results of such a study.

Materials and Methods

Materials

Chemicals. 3-Phosphoglycerate (sodium salt), fructose 1-phosphate (sodium salt), fructose 1,6-diphosphate (tetracyclohexylammonium and potassium salts), glucose 6-phosphate (sodium salt), sodium pyruvate, ATP, ADP, AMP, $NADP^+$, NADH and NADPH and triethanolamine hydrochloride were from Boehringer Corp. (London) Ltd. Phosphoenolpyruvate (monocyclohexylammonium salt) was prepared as described by Clark & Kirby (1966). Calf thymus DNA, 2-mercaptoethanol and tris, as Trizma base, were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Bovine serum albumin, fraction V, was from Armour Pharmaceutical Co. (Eastbourne, Sussex). Crystalline insulin was given by Boots Pure Drug Co. Ltd., Nottingham, U.K., and was dissolved in 3.3mM-HCl. Nembutal (sodium pentobarbitone) was obtained as a solution (60mg/ml) in aq. 10% ethanol–20% propylene glycol from Abbot Laboratories, Ltd., Queenborough, Kent, U.K. Heparin (140–170 units/mg) was from Evans Medical Ltd., Speke, Liverpool, U.K.

Enzymes. Aldolase (EC 4.1.2.7), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), glycerol 1-phosphate dehydrogenase (EC 1.1.1.8), lactate dehydrogenase (pig heart; EC 1.1.1.27), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), phosphoglucose isomerase (EC 5.3.1.9), pyruvate kinase (EC 2.7.1.40) and triose phosphate isomerase (EC 5.3.1.1) were from the Boehringer Corp. (London) Ltd. Collagenase (EC 3.4.4.19), glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7) and bovine testicular hyaluronidase (EC 4.2.99.1) were from Sigma (London) Chemical Co. Ltd.

Animals. Male white mice, 3–4 weeks old, 20–30g, were used throughout. The animals were allowed diet M1B (Oxo Ltd., London S.E.1, U.K.) and water *ad libitum.*

Preparation of parenchymal cells

Cells were prepared by a modification of the technique developed by Berry & Friend (1969) for the isolation of rat liver cells. Since a different animal was used in these studies, we reproduce the method used in detail.

Animals were anaesthetized by intraperitoneal injection of Nembutal (12mg) in 0.9% NaCl containing 200i.u. of heparin as an anticoagulant (total volume 0.2ml). After 10–15min the abdomen was opened and the portal vein and inferior vena cava were cannulated with 21-gauge needles. Blood vessels above the liver were clamped, care being taken not to impede flow through the liver itself. The liver was perfused through the vena cava with 100ml of Ca^{2+}-free Hanks solution (Hanks & Wallace, 1949) containing 1–5m-units of insulin/ml at 37°C and gassed with O_2+CO_2 (95:5). The flow rate was maintained at 8–10ml/min by means of a peristaltic pump (American Instrument Co., Silver Spring, Md., U.S.A.); distension of the liver was minimized by maintaining the animal 20cm above the reservoir. The first 50ml of medium was used to flush the liver of blood and was allowed to go to waste. Collagenase (0.05%) and hyaluronidase (0.1%; Howard & Pesch, 1968) were then added to the remaining medium, which was recirculated through the liver for a further 20–40min. After approx. 15min the consistency of the liver was such that a proportion of the circulating medium leaked out of the organ into the body cavity; this was returned to the reservoir until the rate of fluid loss became too great for continuance of perfusion. The liver was removed with a spoon spatula, washed gently with 0.9% NaCl and was then transferred to a plastic beaker containing 20ml of perfusion medium at 4°C. Lumps were dispersed by gentle pressure with a spatula; undue force at this point resulted in the production of progressively leakier cells, as judged by the lack of retention of cytoplasmic enzymes. The resulting suspension was then transferred to a plastic flask and was shaken at 100 strokes/min for 15min at 37°C under air. This procedure breaks up cell clumps and aids the digestion of cell débris. In most cases, however, the suspension resulting from the perfusion alone was sufficient for the preparation of viable cells. In addition the 15min incubation required rapid shaking to avoid reaggregation of cells; this frequently led to decreased yields and cell damage (as

observed by 'blistering' of the cell membrane seen under the microscope and by leakage of cytoplasmic enzymes).

The final cell suspension was filtered through two layers of nylon mesh (pore size 250 and 61 μm; Henry Simon Ltd., Cheadle Heath, Stockport, Cheshire, U.K.) into a plastic centrifuge tube. Cells were separated from débris by centrifugation at 50*g* for 2 min at room temperature. The cells were washed several times by gentle redispersion in Ca^{2+}-free Hanks solution at 37°C, followed by centrifugation as above.

Microscopic observation of the cells revealed a preparation of parenchymal cells very little contaminated by red blood cells, non-parenchymal hepatic cells and cell débris (<1%). The absence of cytoplasmic staining by Trypan Blue (Hoskins *et al.*, 1956) indicated that more than 99% of the cells were 'intact'.

Cells were finally resuspended in approx. 4 vol. of 50 mM-tris chloride, pH 7.5, containing 100 mM-KCl, 20 mM-$MgSO_4$ and 10 mM-2-mercaptoethanol, and were extracted by homogenization with a motor-driven all-glass tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) for 1 min at 0°C.

'Non-parenchymal' tissue retained by the nylon mesh was carefully washed three times with Hanks solution and homogenized in 4 vol. of the tris chloride extraction medium.

Supernatants from the 50*g* centrifugation steps were buffered by the addition of 1 ml of tenfold concentrated tris chloride extraction medium/10 ml sample.

Samples for enzyme assays were prepared as follows. (A) Portions of the homogenized parenchymal cells, non-parenchymal cells and the 50*g* supernatants were kept at 0–4°C. (B) Further samples were centrifuged at 12000*g* for 4 min (Eppendorf Zentrifuge 3200). The supernatants from (B) were split into two portions: (i) kept at room temperature, and (ii) kept at 0–4°C.

Hexokinase, glucokinase and glucose 6-phosphatase were assayed in samples A. This was found necessary since variable but significant proportions of the activities of both hexokinase and glucose 6-phosphatase were found in the precipitates after centrifugation; for glucose 6-phosphatase this is attributed to the inclusion, or co-precipitation, of a portion of the microsomal fraction with the cell débris. Although most glucokinase is soluble, the activity is normally assayed with hexokinase; it was therefore convenient to perform glucokinase assays with samples (A). Glucokinase activities reported thus include both the soluble and the particulate enzymes (Berthillier *et al.*, 1970; Berthillier & Got, 1970). For reasons outlined below, pyruvate kinase activity was assayed in samples (Bi). The activities of all other enzymes were assayed in samples (Bii).

Cell preparation by other methods. In preliminary experiments, a number of other techniques were applied.

(1) Cells were prepared by mechanical disruption after pre-perfusion of the liver with Ca^{2+}-free Locke's solution (Dawson *et al.*, 1959), as described by Jacob & Bhargava (1962). The extraction medium comprised 0.25 M-sucrose, 20 mM-D-glucose and 2 mM-ATP, pH 7.0 (Sapag-Hagar *et al.*, 1969). Parenchymal cells were finally separated by filtration through nylon stocking (Jacob & Bhargava, 1962).

(2) Cells were prepared by the method of Branster & Morton (1957), involving pre-perfusion and extraction of the liver with Locke's solution, pH 7.3, followed by filtration as before.

(3) In a few experiments, a modification of the method of Howard *et al.* (1967) was used, in which the perfusion medium reported by these authors was replaced by one comprising Krebs & Henseleit (1932) buffer with half the stated concentration of calcium, together with 1% collagenase and 1.5% bovine serum albumin, pH 7.3.

(4) The method recommended by Suzangar & Dickson (1970) for the preparation of non-leaky cells by extraction in polyvinyl alcohol was also used. The liver was pre-perfused with 10 ml of Ca^{2+}-free Locke's solution, pH 7.4, at 37°C. Perfusion was through the inferior vena cava, the portal vein also being cut to allow free flow. The liver was then rapidly removed and washed in Ca^{2+}-free Locke's solution at 37°C. After gentle blotting, the liver was dispersed in 20% polyvinyl alcohol in 0.25 M-sucrose (adjusted to pH 7.4 by addition of solid $NaHCO_3$) in a homogenizer constructed as described by Jacob & Bhargava (1962). The dispersate was filtered through nylon mesh of 250 μm pore size; filtration was aided by the addition of a further 10 ml of dispersion medium. The filtrate was then centrifuged at 1000*g* for 5 min to precipitate isolated cells. These were subsequently resuspended in either Ca^{2+}-free Locke's solution or the dispersion medium, as discussed in the Results section. Cells were reprecipitated by centrifugation at either 100*g* for 2 min (Ca^{2+}-free Locke's solution) or 1000*g* for 5 min (polyvinyl alcohol medium).

In all cases cells were finally homogenized in tris chloride medium and were treated as described previously.

Determination of dry weight. The moisture content of whole liver was determined by drying a known weight of freshly perfused (Ca^{2+}-free Locke's solution) tissue at 110°C to constant weight.

To determine the dry weight in cell suspensions, suitable portions were pipetted into preweighed glass vials; after centrifugation at 50*g* for 2 min the supernatant medium was carefully removed, firstly by aspiration and finally by absorption with filter paper. The remaining pellet was then dried at 110°C to

constant weight. Duplicate determinations were within 2% of the mean value.

Cell counting. Cell counts were made with an improved Neubauer haemocytometer with a chamber depth of 0.1mm. For determination of the total number of cells per liver, a nuclear count was also made in supernatant fractions. This value was adjusted to give the number of cells on the basis that 16% of the total number of cells were binucleate (Bhargava, 1968).

Determination of protein. Protein in cell fractions was determined by the biuret method as described by Layne (1957). Soluble protein refers to that fraction remaining in the supernatant after centrifugation at 12000rev./min for 4min.

Determination of DNA. DNA was determined in cold trichloroacetic acid-insoluble precipitates of cell fractions by the method of Burton (1956) with calf thymus DNA as standard.

Electrophoresis. Pyruvate kinase activities were separated electrophoretically on cellulose acetate strips (Sepraphore III; Gelman-Hawksley, Lancing, Sussex, U.K.) by using a modification of an earlier procedure (Pogson, 1968*b*). The buffer consisted of 15mM-imidazole, 5mM-EDTA and 10mM-2-mercaptoethanol, pH7.4. Runs were for 1h at 700V (0.8–0.9mA/cm) at 1–5°C. Cooling was effected by circulation of ice–water through the platen of the apparatus (Shandon Scientific Co. Ltd., Willesden, London, U.K.).

Activity was developed by the application of strips of filter paper presoaked in a medium containing 0.1M-tris chloride, 0.1M-KCl, 10mM-$MgCl_2$, 4mM-phosphoenolpyruvate, 4mM-ADP, 2mM-NADH and lactate dehydrogenase (5μl/ml), pH7.5. The appearance of spots (dark on a fluorescent blue ground), indicating the presence of pyruvate kinase activity, was observed under ultraviolet illumination. The time for development was 0–5min. Appropriate blanks for NADH oxidase activity were carried out simultaneously.

Enzyme assays

All enzymes were assayed at optimum substrate concentrations at 30°C through a 1cm light-path in a Hilger–Gilford spectrophotometer with recording attachment. Activities are expressed as μmol of substrate utilized/min.

Hexokinase and glucokinase were assayed by the method of Parry & Walker (1966). The medium consisted of 50mM-tris chloride, 100mM-KCl, 7.5mM-$MgSO_4$, 1.6mM-ATP, 0.5mM-$NADP^+$, 2.5μg of glucose 6-phosphate dehydrogenase, 10μg of 6-phosphogluconate dehydrogenase and either 0.5mM-D-glucose (hexokinase) or 100mM-D-glucose (hexokinase plus glucokinase), in a final volume of 3.0ml, pH7.5. Blanks (without ATP) were carried out simultaneously. 'NADPH oxidase' activities were too low to be detectable.

Phosphofructokinase (EC 2.7.1.11) was assayed in a medium containing 50mM-tris chloride, 200mM-KCl, 5mM-$MgCl_2$, 2mM-AMP, 3.3mM-ATP, 1.0mM-fructose 6-phosphate, 0.01% bovine serum albumin, 0.1mM-NADH, 100μg of aldolase, 7μg of glycerol 1-phosphate dehydrogenase and 0.4μg of triose phosphate isomerase in a final volume of 3.0ml, pH8.2 (Underwood & Newsholme, 1965*b*). The extent of the blank rate of NADH oxidation (fructose 6-phosphate omitted) was decreased by addition of 50μM-rotenone.

Aldolases A and B (Sols, 1968) were assayed as described by Sapag-Hagar *et al.* (1969) with 2mM-fructose 1,6-diphosphate and 10mM-fructose 1-phosphate respectively. The medium otherwise contained 50mM-tris chloride, 0.1mM-NADH, 7μg of glycerol 1-phosphate dehydrogenase and 0.4μg of triose phosphate isomerase in a final volume of 1.0ml, pH7.5. In simultaneous blanks, sugar phosphate was omitted.

Phosphoglycerate kinase (EC 2.7.2.3) was assayed in a medium containing 100mM-tris chloride, 50mM-KCl, 10mM-magnesium acetate, 3.3mM-ATP, 1.6mM-3-phosphoglycerate, 0.2mM-NADH and 30μg of glyceraldehyde 3-phosphate dehydrogenase in a final volume of 3.0ml, pH7.4. 3-Phosphoglycerate was omitted in blank determinations.

Lactate dehydrogenase was assayed in a medium comprising 100mM-tris chloride, 50mM-KCl, 10mM-magnesium acetate, 0.33mM-pyruvate and 0.2mM-NADH; the final volume was 3.0ml, pH7.4.

Fructose 1,6-diphosphatase (EC 3.1.3.11) was assayed as recommended by Underwood & Newsholme (1965*a*). The medium consisted of 50mM-tris chloride, 5mM-$MgSO_4$, 20mM-2-mercaptoethanol, 50μM-fructose 1,6-diphosphate, 0.2mM-$NADP^+$ and 30μg each of phosphoglucose isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in a final volume of 3.0ml, pH7.5. After determination of the appropriate blank rates, reactions were initiated by addition of fructose 1,6-diphosphate.

Glucose 6-phosphatase (EC 3.1.3.9) activity was determined in a 'stopped-assay' system as described by Nordlie & Arion (1966). After deproteinization with perchloric acid, formation of D-glucose was determined by the method of Huggett & Nixon (1957).

Total pyruvate kinase activity was measured in a medium containing 100mM-tris chloride, 100mM-KCl, 10mM-$MgCl_2$, 2mM-ADP, 4mM-phosphoenolpyruvate, 0.2mM-NADH and 30μg of lactate dehydrogenase in a final volume of 3.0ml, pH7.5. Reaction was initiated by addition of either ADP or phosphoenolpyruvate after measurement of blank rates. The relative proportions of 'L'- and 'M-type' pyruvate kinases in whole liver homogenates and

cellular fractions were determined from measurements of rates at two different phosphoenolpyruvate concentrations, as discussed in the Results section.

Preparation of 'M-type' pyruvate kinase

Two mice were injected with Nembutal and heparin as described above. The livers were perfused through the inferior vena cava with 10ml of 50mM-tris chloride, 1mM-EDTA and 1mM-2-mercaptoethanol, pH7.5; the hepatic artery was clamped and the portal vein sectioned to allow free flow. It is important at this stage to ensure removal of as much blood as possible, since red blood cells contain a third form of pyruvate kinase (Koler *et al.*, 1964; Ibsen *et al.*, 1971) that may otherwise appear as a contaminant in the final product.

The livers were finally removed and homogenized in 5vol. of the same buffer at 0°C. After centrifugation at 18000rev./min for 30min at 4°C (MSE Highspeed 18), the supernatant was applied to a 10cm×2cm column of Whatman DE-52 DEAE-cellulose (W. & R. Balston Ltd., Springfield Mill, Maidstone, Kent, U.K.) previously equilibrated with the same buffer. The fraction was washed in with a further 20ml of buffer and the total eluate containing 'M-type' pyruvate kinase was collected. 'L-type' pyruvate kinase is adsorbed on to DE-52 under these conditions. The eluate was then brought successively to 45, 55 and 70% saturation by careful addition of powdered $(NH_4)_2SO_4$ at 0°C. After centrifugation at 18000rev./min for 30min at 0°C, the final precipitate was dissolved in 2ml of the tris buffer; the resulting solution was kept at 0–4°C during subsequent assay procedures. All measurements with this preparation were carried out within 24h. The absence of 'L-type' activity was confirmed by demonstration of the insensitivity of the preparation towards activation by fructose 1,6-diphosphate at low phosphoenolpyruvate concentrations.

Preparation of 'L-type' pyruvate kinase

Several attempts to purify the 'L-type' activity from whole mouse liver met with only partial success. This was attributable to the ease with which allosteric desensitization towards fructose 1,6-diphosphate occurred at low temperature (0–4°C) (D. M. Crisp, unpublished work; Tanaka *et al.*, 1967*b*) and under the conditions necessary for purification.

Fully sensitive 'L-type' pyruvate kinase was prepared from pure parenchymal cells, isolated as described above. The cells, washed several times, were homogenized in 50mM-tris chloride, 100mM-KCl, 20mM-$MgSO_4$ and 10mM-2-mercaptoethanol, pH7.5, at room temperature. The resulting homogenate was centrifuged at 12000*g* for 2min and the supernatant was maintained at room temperature during subsequent assay procedures.

Kinetic investigations were performed as soon as possible after preparation of the enzyme. The absence of 'M-type' activity was ascertained from the absence of detectable rates at phosphoenolpyruvate concentrations below 50μM.

Results

Properties of pyruvate kinase

The relative proportions of 'M-' and 'L-type' pyruvate kinases in a mixture of the two may be theoretically determined from measurements of rates

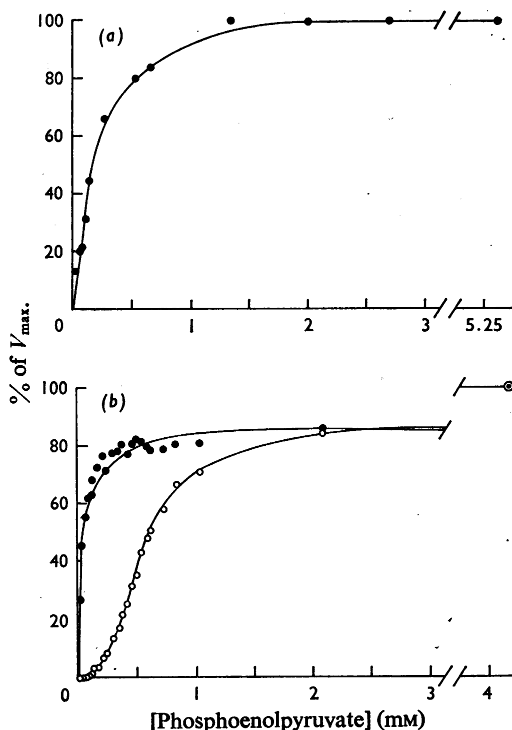

Fig. 1. *Plots of reaction velocity against phosphoenolpyruvate concentration for 'M-type' (a) and 'L-type' (b) pyruvate kinases from mouse liver*

Enzymes were prepared as described in the Materials and Methods section. $V_{max.}$ for the 'M-type' pyruvate kinase was 0.2μmol·min⁻¹·ml⁻¹; for the 'L-type' pyruvate kinase it was 0.46μmol·min⁻¹·ml⁻¹. In (*b*): ○, rates in the absence of fructose 1,6-diphosphate; ●, rates with added fructose 1,6-diphosphate (0.5mM final concentration).

at two substrate concentrations, in a manner analogous to that employed for hexokinase and glucokinase. To this end, the properties of both types of pyruvate kinase, free of each other, were investigated. Fig. 1 shows plots of reaction velocity at varying phosphoenolpyruvate concentrations. Under these conditions 'M-type' pyruvate kinase exhibits a K_m for phosphoenolpyruvate of 0.17mM [virtually identical with that of 0.18mM reported by Jiménez de Asuá *et al.* (1971) for the same enzyme from rat liver]. The addition of fructose 1,6-diphosphate had no effect on measured reaction rates.

The K_m ($S_{0.5}$, substrate concentration at half-maximal velocity) for phosphoenolpyruvate for 'L-type' pyruvate kinase was found to be 0.63 mM, with a value of 2.4 for n, the Hill coefficient. This compares with values of 0.64mM and n 1.8 for the rat liver enzyme at pH 7.25 (Rozengurt *et al.*, 1969). In the presence of fructose 1,6-diphosphate (0.5mM) the rate response curve becomes hyperbolic with a K_m for phosphoenolpyruvate of 46μM; this is significantly lower than that given by Rozengurt *et al.* (1969) (0.18–0.20mM).

At phosphoenolpyruvate concentrations below 50μM the rates obtained with 'L-type' pyruvate kinase are too low to be measured in our system (recorder full-scale deflexion = 0.2 extinction unit). The 'M-type', on the contrary, has appreciable activity at these phosphoenolpyruvate concentrations. The relative proportions of the two forms may thus be evaluated from rate measurements at both 50μM- and 4mM-phosphoenolpyruvate, namely:

$$[\text{'L-type'}] = v_2 - v_1\,[(K+S)/S]$$

and

$$[\text{'M-type'}] = v_1\,[(K+S)/S] \text{ units/ml of extract}$$

where v_1 = rate at low phosphoenolpyruvate concentration (expressed as μmol/min per ml of extract), v_2 = rate at high phosphoenolpyruvate concentration (4mM; expressed as μmol/min per ml of extract), $K = K_m$ for phosphoenolpyruvate of 'M-type' pyruvate kinase, i.e. 0.17mM, S = low phosphoenolpyruvate concentration. The validity of this method is dependent upon accurate determination of the phosphoenolpyruvate concentration used; in all experiments reported in this paper, the molarity of phosphoenolpyruvate solutions was always determined immediately after pyruvate kinase assays.

As reported previously (Pogson, 1968*b*), pyruvate kinase activities may be separated electrophoretically on cellulose acetate. This method has been used in these studies to check the purity of enzyme and cell preparations. The results are shown schematically in Fig. 2. The pyruvate kinase mobilities observed are in agreement with previous observations (Tanaka *et al.*, 1967*a*; Pogson, 1968*b*).

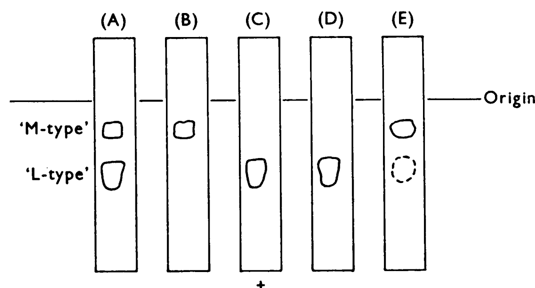

Fig. 2. *Schematic representation of cellulose acetate electrophoretograms of pyruvate kinase activities from mouse liver*

Runs were for 1h at 700V at 1–5°C; the buffer contained 15mM-imidazole, 5mM-EDTA and 10mM-2-mercaptoethanol, pH 7.4. Spots were visualized as described in the Materials and Methods section. (A) Whole liver homogenate; (B) purified 'M-type' pyruvate kinase; (C) purified 'L-type' pyruvate kinase; (D) extract of isolated parenchymal cell fraction, Expt. C17; (E) extract of non-parenchymal cell fraction, Expt. C17.

Preparations of parenchymal cells

Parenchymal cells have been isolated by a number of different methods in attempts to obtain metabolically viable 'non-leaky' preparations. Results with cells prepared as described by Jacob & Bhargava (1962) or Branster & Morton (1957) bear out the conclusions of Suzangar & Dickson (1970) in that very little, if any, cytoplasmic enzyme activity is retained. Leakage of cell material occurred during mechanical disruption and continued through several washing procedures. Enzymic activity remaining in the cells was similar to that in the suspending media, and was thus unlikely to be due to specific retention of protein.

The collagenase-perfusion method developed by Howard *et al.* (1967) gave somewhat better, although variable, results. Although cell yields were satisfactory, more contamination with other cell types was found with this method than with others. Since in our hands this method was less good and more tedious than that of Berry & Friend (1969), further detailed studies were discontinued.

Good yields of cells with high retention of cytoplasmic enzymes have been obtained only with the technique of Berry & Friend (1969). The basic properties of these cells are summarized in Table 1. Similar results for cells prepared as described by Jacob and Bhargava (1962) are included for comparison. The DNA content of both types of cells is higher than that of the whole liver on a dry-weight basis. This is a reflection of the number of binucleate

Table 1. *Basic parameters of isolated parenchymal cells from mouse liver*

Whole liver refers to livers freed of blood by pre-perfusion with Hanks solution. Mechanically prepared cells were isolated by the method of Jacob & Bhargava (1962); enzymically prepared cells were isolated as described by Berry & Friend (1969). Numbers in parentheses indicate the number of determinations.

	Dry wt. (% wet wt.)	Soluble protein (mg/g wet wt.)	Total protein (mg/g wet wt.)	100×Ratio soluble protein / total protein	DNA (mg/g dry wt.)	DNA (μg/mg of total protein)	10^{-6}×Total number of cells/g wet wt.	DNA (pg/cell)	Total protein (ng/cell)	Dry wt. per cell (ng)
Whole liver (6)	28.8±0.5	95.6±1.7	175 ±5	54.9±1.5	4.10±0.38	6.38±0.56				
Mechanically prepared cells (6)	9.6±0.1	26.6±1.1	52.6±2.0	50.7±1.5	11.4 ±0.7	20.6 ±1.3	47.5±6.2	36.8±2.1	1.80±0.11	3.23±0.10
Enzymatically prepared cells (4)	10.6±0.5	35.4±3.4	67.9±2.7	51.7±3.1	10.8 ±1.2	16.7 ±1.2		26.0±1.1	1.58±0.13	2.48±0.28

tetraploid cells amongst the parenchyma and may also be due in part to surface binding of DNA released by breakage of other cells. Despite the possession of a higher content of cytoplasmic enzymes, the enzymically prepared cells have a lower mean dry weight than those prepared mechanically. This may be attributable to the larger amount of extracellular and non-cellular material adhering to the latter cells. This extracellular material constitutes up to 50% of the total liver dry weight (Bhargava, 1968), and is presumably degraded and removed from enzymically prepared cells by the mixture of collagenase and hyaluronidase.

Activities of cytoplasmic enzymes

The recovery of cytoplasmic enzymes is shown in Table 2. Three glycolytic enzymes of widely differing molecular weight were chosen to test the leakiness of parenchymal cells: phosphoglycerate kinase is a monomer, mol.wt. approx. 40000 (Krietsch & Bücher, 1970); lactate dehydrogenase is a tetramer, mol.wt. approx. 150000 (Klotz & Darnell, 1969); phosphofructokinase is a multiple subunit-associating system, minimum mol.wt. approx. 360000 (Ling *et al.*, 1965). Fraction S1 represents the activity released by breakage of cells during either perfusion or filtration. The low activity of phosphofructokinase in this fraction may be correlated with the known instability of this enzyme in dilute solution (Underwood & Newsholme, 1965*b*); this is borne out from a comparison of the figures for specific activities in the three fractions shown.

Two washing steps were performed, S2 and S3, but no activity of any of the three enzymes was detectable in either fraction. The values for percentage total recoveries of phosphoglycerate kinase and lactate dehydrogenase, taken together with the observation that 88% of the volume of mouse liver consists of parenchymal cells (Wilson *et al.*, 1953), indicate that the recovery of intact parenchymal cells in this experiment was 20–25%. The recovery of parenchymal cells varies somewhat from experiment to experiment, and is dependent on the efficacy of the perfusion process; a substantial proportion of the cells remain in the non-filtrable 'non-parenchymal' fraction. Evidence of this is given in Table 3, where the appearance of glucokinase in the non-parenchymal fraction is a direct indication of residual parenchymal cells (see Expt. C6 and below). In Expt. D1, where the non-parenchymal fraction was further incubated with collagenase and hyaluronidase before refiltration, all parenchymal cells were removed and hexokinase was the only glucose-phosphorylating activity detectable. In all cases (eight experiments) parenchymal cells showed a very substantial increase in the ratio of glucokinase to hexokinase activities (Table 3) over that seen in total

Table 2. *Distribution of lactate dehydrogenase, phosphoglycerate kinase and phosphofructokinase in various fractions during the preparation of parenchymal cells from mouse liver*

The basic procedure was as described in the Materials and Methods section. The filtrate after perfusion (containing parenchymal cells, débris and soluble components) was centrifuged (supernatant = S1), and the precipitated cells were washed twice with Ca^{2+}-free Hanks solution. The resulting supernatants, S2 and S3, contained no measurable enzyme activity and have been excluded. Figures in parentheses indicate the number of determinations; for total liver homogenate, values are given ± S.E.M. n.d., Not determined.

Enzyme	Expt.	Parameter	Fractions			Total liver homogenate
			S1	Parenchymal	'Non-parenchymal'	
Lactate dehydrogenase	C8	m-units/μg of DNA	456	70.3	77.1	117 ± 26 (4)
		units/mg of protein	2.44	3.99	2.44	1.90 ± 0.20 (4)
		% total activity	47.1	17.5	35.4	—
Phosphoglycerate kinase	C8	m-units/μg of DNA	194	21.3	24.6	66.1 ± 9.9 (4)
		units/mg of protein	1.04	1.21	0.78	1.09 ± 0.02 (18)
		% total activity	42.5	15.0	42.5	—
Phosphofructo-kinase	C8	m-units/μg of DNA	0.02	1.13	1.21	n.d.
		m-units/mg of protein	1.26	64.0	38.3	78.4 ± 4.0 (35)
		% total activity	1.7	27.0	71.3	—

liver homogenate (where the ratio is 2.1:1). In five experiments, including C6 and C10, the hexokinase activities were between 1 and 10% of the total glucose-phosphorylating capacity; these values are close to the limit of detection in the assay system used. In three experiments, including C16 and D1, no hexokinase activity could be detected in the parenchymal fraction. These results confirm and extend the tentative conclusions of Sapag-Hagar *et al.* (1969).

In preliminary experiments, perfusion was carried out without added insulin. In these no glucokinase activity was detectable in any fraction. Addition of insulin alone was sufficient to ensure retention of glucokinase activity.

Similar results to the above were obtained in pyruvate kinase assays (Table 4). Parenchymal cells contain 'L-type' pyruvate kinase alone; the non-parenchymal fraction contains all 'M-type' activity, and, even when 'contaminated' with residual parenchymal tissue, exhibits a higher ratio of 'M-' to 'L-type' pyruvate kinase than observed in homogenates of the whole liver (mean value for ratio = 0.15:1). In several experiments (e.g. Expt. D2), no 'L-type' pyruvate kinase activity was detectable in the non-parenchymal fraction. These results were further confirmed by cellulose acetate electrophoresis (Fig. 2).

The activities of the two gluconeogenic enzymes, fructose 1,6-diphosphatase and glucose 6-phosphatase, were also measured in several experiments (Table 5). In all cases it was found that glucose 6-phosphatase is restricted to the parenchymal cells, as expected. This finding agrees with that of Lentz & Di Luzio (1971) with different techniques for preparation of isolated cells, but contrasts with a report of the histochemical localization of glucose 6-phosphatase activity within the Kupffer cells (Rosen, 1970). This difference may be explicable either by postulating complete digestion of Kupffer cells during perfusion, or on the basis of the lack of specificity and quantitative evaluation implicit in the histochemical procedure.

The situation with fructose 1,6-diphosphatase is more complex. Although the specific activity of the enzyme is reproducibly higher in the parenchymal cells, some activity was always present in the non-parenchymal tissue. This was true even in experiments where 'L-type' pyruvate kinase was shown to be absent in this fraction. Although this activity may be due in part to non-specific phosphatase activity, it is probable that some true fructose 1,6-diphosphatase is indeed present, as has been shown, for example, in skeletal muscle (Opie & Newsholme, 1967). This is borne out by demonstration of the sensitivity of the enzyme to 5′-AMP added directly to the cuvette.

Attempts were made to determine the distribution of aldolases A and B (Rutter *et al.*, 1963) in mouse liver, by using the kinetic method of Sillero *et al.* (1969) with fructose 1,6-diphosphate and fructose 1-phosphate as the respective substrates. Whole liver homogenate, however, exhibits a ratio of fructose 1,6-diphosphate aldolase to fructose 1-phosphate aldolase activities of only 0.94 (Table 6). This indicates either that no significant concentration of aldolase A is present or that the kinetic properties

Table 3. *Typical experiments showing the distribution of glucokinase and hexokinase between parenchymal and 'non-parenchymal' cells from mouse liver*

Parenchymal cells were washed three times with Ca^{2+}-free Hanks solution; activities in the resulting supernatants (S2, S3, S4) are not shown. S2 contained small amounts of hexokinase and, on occasion, low activity of glucokinase. S3 and S4 were devoid of enzyme activity. In Expt. D1, the 'non-parenchymal' fraction was freed of parenchymal tissue by further incubation at 37°C for 15min with Ca^{2+}-free Hanks solution containing collagenase and hyaluronidase. Enzyme activities in this fraction were determined in the non-filtrable material. Figures for protein concentration refer to the total protein content of each fraction. n.d., Not determined. The hexokinase and glucokinase contents of whole mouse liver were found to be 1.50 ± 0.12 and 3.16 ± 0.24 m-units/mg of protein respectively (means of 36 determinations ± S.E.M.). Other experimental details were as detailed in the Materials and Methods section. n.d., Not determined.

		Parenchymal cells		S1		'Non-parenchymal' cells	
Expt.	Parameter	Hexokinase	Glucokinase	Hexokinase	Glucokinase	Hexokinase	Glucokinase
C6	m-units/ml of extract	7.2	83.2	13.3	0	57.9	118
	m-units/mg of protein	0.4	4.5	4.7	—	2.4	4.9
	% of glucose-phosphorylating capacity in fraction	8	92	100	0	33	67
C10	m-units/ml of extract	14.5	154	10.9	4.8	60.3	49.4
	m-units/μg of DNA	0.03	0.34	0.44	0.19	0.07	0.06
	m-units/mg of protein	n.d.	n.d.	2.55	1.13	3.94	3.23
	% of total enzyme	7.2	61.2	54.1	19.2	29.9	19.6
	% of glucose-phosphorylating capacity in fraction	9	91	69	31	55	45
C16	m-units/ml of extract	0	73.3	2.9	8.7	42.4	40.0
	m-units/μg of DNA	—	0.23	n.d.	n.d.	0.15	0.14
	m-units/mg of protein	—	2.44	9.7	29.0	2.8	2.7
	% of glucose-phosphorylating capacity in fraction	0	100	25	75	51	49
D1	m-units/ml of extract	0	32.3	3.1	8.4	38.6	0
	m-units/mg of protein	—	1.12	3.3	8.8	8.6	—
	% of total enzyme	0	30.6	48.7	60.0	30.1	0
	% of glucose-phosphorylating capacity in fraction	0	100	27	63	100	0

of the two enzymes may be markedly different from those in other species. This observation would agree, however, with the appearance of one aldolase band only during electrophoresis of rabbit liver extract (Penhoet *et al.*, 1966). The ratio of fructose 1,6-diphosphate aldolase to fructose 1-phosphate aldolase activities in parenchymal cells is furthermore generally similar to that in the whole liver homogenate and the non-parenchymal tissue (Table 6).

The specific activities of parenchymal cell enzymes (as units or m-units/mg of protein) are similar to or greater than those found in whole liver homogenate. Furthermore the specific activities in terms of DNA content are similar to those found in whole homogenates, allowing for the increased cell DNA content (Table 1). These results thus indicate that the parenchymal cells isolated have lost little or none of their intracellular protein; this is in agreement with the retention of complex metabolic activities in these cells, namely gluconeogenesis (Berry & Friend, 1969) and albumin synthesis (Weigand *et al.*, 1971).

Studies with mechanically prepared cells

Cells prepared by dispersion in polyvinyl alcohol retain a high proportion of their cytoplasmic enzymes (Suzangar & Dickson, 1970). The filtered parenchymal cells may be washed with several changes of the extraction medium (containing 20% polyvinyl alcohol) without significant leakage. Attempts to

Table 4. *Typical experiments showing the distribution of 'M-' and 'L-type' pyruvate kinases between parenchymal and 'non-parenchymal' cells from mouse liver*

Parenchymal cells were washed two or three times with Ca^{2+}-free Hanks solution; values for the resulting supernatants (S2, S3, S4) are not shown. Of these, S2 alone contained measurable pyruvate kinase activity. In Expt. D2, non-parenchymal tissue was reincubated with lytic enzymes as detailed in the Materials and Methods section and the legend to Table 3. Values for protein concentration are derived from measurements of soluble protein alone. The results in Expt. C12 are means of four determinations ± S.E.M. The total liver content of 'M-' and 'L-type' pyruvate kinases was found to be 44.8±3.7 and 349±21 m-units/mg of protein respectively (means of 29 determinations±S.E.M.). Other experimental details were as detailed in the Materials and Methods section.

		Parenchymal cells		S1		'Non-parenchymal' cells	
Expt.	Parameter	'M-type'	'L-type'	'M-type'	'L-type'	'M-type'	'L-type'
B20	units/ml of extract	0	2.01	0	0.46	0.53	2.02
	m-units/mg of protein	—	293	—	244	61	232
	% of total 'M-type' or 'L-type'	0	20.1	0	35.9	100	40.4
	% of pyruvate kinase in fraction	0	100	0	100	21	79
C10	units/ml of extract	0	3.33	0	0.76	1.08	1.30
	m-units/μg of DNA	—	7.3	—	30.7	1.23	1.49
	m-units/mg of protein	—	320	—	312	132	158
	% of total 'M-type' or 'L-type'	0	25.6	0	58.6	100	10.0
	% of pyruvate kinase in fraction	0	100	0	100	46	54
D2	units/ml of extract	0	2.46	0	0.64	1.52	0
	m-units/mg of protein	—	393	—	602	780	—
	% of total 'M-type' or 'L-type'	0	28.3	0	57.2	100	0
	% of pyruvate kinase in fraction	0	100	0	100	100	0
C12	units/ml of extract	0	1.86±0.28			1.09±0.11	1.42±0.50
	m-units/μg of DNA	—	4.7 ±0.7			4.3 ±0.6	5.3 ±1.4
	m-units/mg of protein	—	391 ±39			294 ±101	304 ±19
	% of pyruvate kinase in fraction	0	100			47 ±10	53 ±10

remove the polyvinyl alcohol by washing in more aqueous media (e.g. Ca^{2+}-free Locke's solution) are accompanied by very rapid and total loss of glycolytic enzymes into the medium.

Because of the difficulty in working with so viscous a medium, the cell yield is contaminated with other cell lines and débris. This is shown in Table 7; the parenchymal cell fraction contains a higher ratio of 'L-type' pyruvate kinase to 'M-type' pyruvate kinase than does the total homogenate, but the presence of 'M-type' activity indicates incomplete separation of cell types. In this experiment, recovery of 'M-type' pyruvate kinase activity was quantitative. Of the 'L-type' activity 55% was lost, presumably owing to inactivation by the polyvinyl alcohol suspending medium in the S1 fraction. Although this method is more rapid than that involving perfusion, it is technically more tedious and less effective; the results, however, are consistent with those obtained with enzymically prepared cells.

Discussion

Preparation of isolated cells

A large and growing number of techniques are now available for the isolation of parenchymal cells. This fact is in itself an indication of the generally unsatisfactory nature of most of the methods, and contrasts, for example, with the situation of fat cell isolation from adipose tissue, where a single technique has become predominant (Rodbell, 1964). All procedures used hitherto are open to criticism on the

Table 5. *Distribution of glucose 6-phosphatase and fructose 1,6-diphosphatase between parenchymal and 'non-parenchymal' cells from mouse liver*

Details of experimental procedures are as given in the Materials and Methods section and in the legend to Table 3. Values for protein content refer to total protein for glucose 6-phosphatase and to soluble protein for fructose 1,6-diphosphatase. Activities in total liver homogenates are expressed as means±S.E.M.; the number of determinations is given in parentheses.

Enzyme activity	Parameter	Fraction: Parenchymal	Fraction: 'Non-parenchymal'	Total liver homogenate
Glucose 6-phosphatase	m-units/μg of DNA	1.17	0	2.06± 0.55 (4)
	m-units/mg of protein	15.9	0	17.8 ± 3.5 (4)
Fructose 1,6-diphosphatase	m-units/μg of DNA	3.37	1.71	11.0 ± 2.3 (4)
	m-units/mg of protein	249	151	182 ±17 (4)

Table 6. Distribution of aldolase between parenchymal and 'non-parenchymal' cells from mouse liver

Details of experimental procedures are as given in the Materials and Methods section and in the legend to Table 3. Values for protein content refer to soluble protein alone. Results in parenchymal and 'non-parenchymal' fractions are the averages of two determinations; those for total liver homogenate are expressed as means±S.E.M. with the number of determinations in parentheses.

Enzyme activity	Parameter	Fraction: Parenchymal	Fraction: 'Non-parenchymal'	Total liver homogenate
Fructose 1,6-diphosphate aldolase (*A*)	m-units/μg of DNA	4.74	4.30	15.6± 2.5 (4)
	m-units/mg of protein	371	311	261 ±14 (4)
Fructose 1-phosphate aldolase (*B*)	m-units/μg of DNA	5.26	4.25	16.4± 2.4 (4)
	m-units/mg of protein	413	301	276 ± 7 (4)
Ratio (*A*)/(*B*)		0.90	1.01	0.94± 0.04 (4)

grounds of possible membrane and general cell damage by lytic enzymes or from the use of non-physiological chelating agents coupled with a degree of comparatively violent mechanical disruption, or both. For any given series of experiments therefore it is necessary to decide in advance which parameter or parameters of the whole organ should be conserved during the isolation procedures, and to select a method appropriate to that decision.

A number of criteria have been variously applied to test the 'viability' of isolated parenchymal cells: (1) microscopic appearance of cells; (2) oxygen uptake as a measure of respiratory activity, both endogenous and with added substrate (2-oxoglutarate or other tricarboxylic acid-cycle intermediate) (Iype & Bhargava, 1965; Ontko, 1967; Howard & Pesch, 1968; Berry & Friend, 1969; Jezyk & Liberti, 1969; Hommes *et al.*, 1970); (3) membrane permeability, both in terms of leakage of cell contents and in specific and non-specific uptake of various solutes; (4) complex metabolic activities, e.g. protein synthesis (Bhargava & Bhargava, 1962), urea-cycle activity (Berry, 1962), nucleic acid synthesis (Jacob & Bhargava, 1965*a,b*; Jezyk & Liberti, 1969), gluconeogenesis (Berry & Friend, 1969) and ethanol oxidation (Berry, 1971); (5) retention of tissue organizational specificity (Bhargava & Bhargava, 1968).

Of these parameters, some are of more value than others. (1) Microscopic appearance is a poor guide to membrane integrity. Electron micrographs of preparations obtained by various techniques purport to demonstrate intactness of cells, although leakage of cell components and lack of metabolic integrity can be simultaneously demonstrated (Jezyk & Liberti, 1969; Suzangar & Dickson, 1970). (2) Oxygen uptake in the presence of tricarboxylic acid-cycle intermediates is a criterion merely of mitochondrial integrity. Any 'cell' consisting of a permeable membrane and intact mitochondria will show respiratory activity under appropriate conditions. (3) Membrane permeability is a specific phenomenon. As an example, cells prepared mechanically by the method of Jacob & Bhargava (1962) lose cytoplasmic protein, are permeable to RNA, DNA and ribonuclease, but are less permeable than whole liver to free purine and pyrimidine bases and are impermeable to orotic acid,

Table 7. *A typical experiment showing the distribution of 'M-' and 'L-type' pyruvate kinases between mechanically prepared parenchymal and 'non-parenchymal' cells of mouse liver*

Cells were prepared by mechanical disruption of the liver in 20% polyvinyl alcohol as described in the Materials and Methods section. Values for protein content refer to soluble protein determinations.

Fractions ..	S1		S2		Parenchymal		'Non-parenchymal'		Total liver homogenate	
Parameter	'M-type'	'L-type'	'M-type'	'L-type'	'M-type'	'L-type'	'M-type'	'L-type'	'M-type'	'L-type'
m-units/mg of protein	43.2	167	133	212	40.6	537	147	153	31.4	185
Total units	1.39	5.38	0.26	0.42	0.48	6.3	3.91	4.06	6.03	35.5
% of total 'M-type' or 'L-type'	23	15	4	1	8	18	65	11	100	100
% of pyruvate kinase in fraction	21	79	39	61	7	93	49	51	15	85

commonly used experimentally as a convenient permeant RNA precursor *in vivo* (Bhargava, 1968). Again, cells prepared by incubation of liver with lysozyme (Hommes *et al.*, 1970) retain all their nucleotide despite losing a large proportion of cytoplasmic glycolytic activities (D. M. Crisp & C. I. Pogson, unpublished work). Furthermore it has been reported that leakage of enzymes may be restricted by the presence of corticosteroids (Takeda *et al.*, 1964); this provides additional evidence that such protein loss is not due to general breakdown of membrane structure. (4) The ability to carry out processes as complex as that of protein and nucleic acid synthesis is not necessarily related to capacity for other metabolic processes or to general short-term biochemical viability. Thus, although several preparations possess activity in the former processes, few show ability to respond metabolically to the presence of, for example, simple sugars (see e.g. Iype & Bhargava, 1965).

Despite their altered permeability properties, mechanically prepared cells still retain the capacity to recognize the original tissue, and form reaggregated cell colonies when injected intraperitoneally into a second animal (Laws & Stickland, 1961; Bhargava, 1970). Again cells isolated by the method of Rappaport & Howze (1966) with tetraphenylboron are reported to be metabolically inert (Friedman & Epstein, 1967; Murthy & Petering, 1969; Suzangar & Dickson, 1970), but have been used successfully as starting material for cell culture over periods of more than 6 months (Gerschenson & Casanello, 1968); these cultured cells synthesize glycogen and retain sensitivity to insulin (Gerschenson & Andersson, 1971).

The volume of literature devoted to the properties of liver cell suspensions indicates that even the establishment of several criteria of cell 'viability' may give only a partial answer to the question of whether total liver cell function is retained in any given case. The ease and rapidity of isolation, coupled with the high yield, of the mechanically prepared cells suggests that this may be the method of choice for investigations of cell differentiation and interaction. From the results reported in this paper, however, it is our contention that, for biochemical studies involving cytoplasmic systems, the more tedious and time-consuming enzymic method of Berry & Friend (1969) provides the most satisfactory cell preparations.

Biochemical properties of parenchymal cells

From the studies reported here it is clear that parenchymal cells possess both glycolytic and gluconeogenic capacities, and that the non-parenchymal cells (reticuloendothelial cells including littoral, Kupffer and sinusoidal lining cells; Daoust, 1958) are solely glycolytic. In correlation with the total distribution studies, both hexokinase and 'M-

type' pyruvate kinase are identified with the non-parenchymal fraction; these enzymes are identical with or similar to the hexokinase and pyruvate kinase respectively of skeletal muscle, a tissue that is wholly glycolytic.

In the parenchymal cell, the problem of the 'energetically wasteful cycles', at the levels of glucose phosphorylation and pyruvate formation, is now somewhat simplified. The observations that hexokinase activity is absent or, at best, present at very low concentrations only in parenchymal cells, implies that the rate of glucose phosphorylation is largely independent of the concentration of glucose 6-phosphate, and may be markedly sensitive to the blood glucose concentration, which approximates to the K_m for glucokinase (Salas *et al.*, 1965) and which may at the same time suffice to act as a modulator of glucose 6-phosphatase activity (Arion & Nordlie, 1964). The balance between glucokinase and glucose 6-phosphatase activities *in vivo* is, in addition, known to be subject to hormonal influences (see e.g. Ashmore & Weber, 1968), which may thus further influence the rate of non-productive 'cycling', a process that itself has been considered to be of some regulatory import (Newsholme & Gevers, 1967). The ultimate understanding of the physiological control mechanisms at this point must, however, await the results of investigations into the true properties of glucose 6-phosphatase *in vivo*. In this context, it is noteworthy that hexokinase and glucose 6-phosphatase are reported to be present together in both kidney cortex (Grossbard & Schimke, 1966; Szepesi *et al.*, 1970) and, together with glucokinase, in pancreatic islets (Ashcroft & Randle, 1970).

The physiological concentrations of phosphoenolpyruvate, alanine and ATP in liver are sufficient, in the absence of fructose 1,6-diphosphate, to effect a substantially total inhibition of 'L-type' pyruvate kinase in rat liver *in vivo* (Sols & Marco, 1970). The measured concentrations of fructose 1,6-diphosphate in the whole liver, however, even under gluconeogenic conditions, are in fact apparently sufficient to cause nearly full activation of the enzyme; if this were true *in vivo* the combination of active pyruvate carboxylase (EC 6.4.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and pyruvate kinase would merely serve as an ATPase system with no overall synthesis of phosphoenolpyruvate. Sols & Marco (1970) overcome this difficulty by postulating that a significant proportion of the fructose 1,6-diphosphate apparently present may be bound to other enzymes, notably aldolase. In addition to this, however, we point out the possibility that a significant part of the total fructose 1,6-diphosphate may be in cells other than the parenchyma; although such cells constitute a relatively small part only (10–20%) of the total liver cell volume, the amounts of fructose 1,6-diphosphate in glycolysing cells are reported to be much higher than those in gluconeogenic tissues (Newsholme & Randle, 1961, 1964).

The question of compartmentation of metabolites is a vexed one. With liver, as indeed with many other tissues, the question of inter-, as well as intra-, cellular compartmentation must be considered. The use of isolated pure parenchymal cell preparations may help in reducing the total number of variables that are present, for example, with the perfused liver system. As an example, Veneziale *et al.* (1970) and Veneziale (1971) have postulated the presence of a novel gluconeogenic pathway on the basis of specific radioactivity measurements of the expected metabolic intermediates, despite direct evidence in favour of total gluconeogenic carbon flow via the 'orthodox' pathway involving phosphoenolpyruvate carboxykinase (Rognstad & Katz, 1970; Williamson *et al.*, 1971). It is possible that such specific radioactivity measurements may be misleading in the whole organ, in view of the known 'pools' in the different cell types. Computer simulation studies may be similarly upset by the provision of whole tissue data (e.g. Heath & Threlfall, 1968). Indeed Achs *et al.* (1971) have restated the need for good data from single cell lines in future simulation attempts.

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