Isoenzyme Patterns in Parenchymal and Non-Parenchymal Cells Isolated from Regenerating and Regenerated Rat Liver

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Parenchymal and non-parenchymal cells were isolated from adult rat liver that had been fully regenerated after a 70% partial hepatectomy. The characteristics of the parenchymal cell preparations from regenerated rat liver indicated that they were a homogeneous population and comparable with parenchymal cells isolated from intact liver. The parenchymal cells from regenerated adult rat liver contain glucokinase, hexokinase, pyruvate kinase type I and aldolase B. The non-parenchymal cells contain hexokinase, pyruvate kinase type III and aldolase B. When cells were isolated at different times of the day from rats on controlled feeding schedules, variation of tyrosine aminotransferase activity and liver glycogen content were observed in the parenchymal cells in keeping with the reported diurnal oscillations found in whole liver extracts. When parenchymal cells were isolated from rats 48 and 72 h after partial hepatectomy, different isoenzyme patterns were observed. These cells appeared to synthesize pyruvate kinase type III, a function that was assigned previously to non-parenchymal cells or to foetal rat liver hepatocytes.

The isolation and separation of the various cell types from adult liver represents a further degree of sophistication in studying liver biochemistry. Since the report of Berry & Friend (1969) describing the dissociation of liver cells by enzymic methods, many modified and improved methods for the isolation of intact and viable parenchymal cells from adult rat liver have been described (Hommes *et al.*, 1970; Cantrell & Bresnick, 1972; Crisp & Pogson, 1972; Capuzzi *et al.*, 1971; Lentz & Di Luzio, 1971; Ingebretsen & Wagle, 1972; Van Wyk *et al.*, 1971; Müller *et al.*, 1972; Seglen, 1972). Biochemical studies on the parenchymal cells, separated from the remaining cell types, indicate that certain enzymes in the liver are present either exclusively in the parenchymal cells or in the non-parenchymal cells, whereas other enzymes are present in both parenchymal and non-parenchymal cells. For example, Cantrell & Bresnick (1972) reported that benzpyrene hydroxylase activity is present and inducible in both cell types. Crisp & Pogson (1972) demonstrated that certain glycolytic enzymes in mouse liver such as glucokinase and L-type pyruvate kinase (pyruvate kinase type I) were found only in the parenchymal cells. Hexokinase and M-type pyruvate kinase (pyruvate kinase type III) were found to be present in the non-parenchymal fractions. Crisp & Pogson (1972) also concluded that

non-parenchymal cells from the liver contained no gluconeogenic capacity because they lacked glucose 6-phosphatase.

The assignment of pyruvate kinase type I and pyruvate kinase type III activities to the two cell types of the liver was confirmed by Van Berkel *et al.* (1972). These workers used a differential assay on the basis of the assumption that only pyruvate kinase type I shows an allosteric response to one of its substrates, phosphoenolpyruvate, whereas pyruvate kinase type III is a non-allosteric enzyme. However, because pyruvate kinase type III can be readily converted into an enzyme form exhibiting allosteric kinetics in both cell culture and in cell-free systems (Walker *et al.*, 1973; Walker & Potter, 1973) the above type of differential assay is unreliable.

Several isoenzyme patterns have been found to vary according to the differentiated state of the liver. The developmental pattern for glucokinase, hexokinase, pyruvate kinase type I, pyruvate kinase type II, pyruvate kinase type III and aldolase A and B have been studied by several workers (Walker & Holland, 1965; Jamdar & Greengard, 1970; Hommes & Draisma, 1970; Middleton & Walker, 1972; Walker & Potter, 1972a). It appears from these reports that in foetal life, the liver is characterized by the absence of glucokinase and low activities of pyruvate kinase type I and aldolase B. The developing liver contains hexokinase as the major glucose phosphorylating enzyme, pyruvate kinase type II as the major pyruvate kinase activity and aldolase A. During the first 3 days of liver regeneration after a 70% partial hepatectomy

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the liver is characterized by a change in the relative activities of pyruvate kinase type I and pyruvate kinase type III (Bonney *et al.*, 1973) as a result of a decrease in pyruvate kinase type I activity and an increase in pyruvate kinase type III activity. The liver isoenzyme pattern returned to the normal state 2 weeks after the operation.

Pyruvate kinase type III may be synthesized by the parenchymal cells under the conditions of induced hyperplasia, but this could only be ascertained by the isolation of liver parenchymal cells during the early phase of regeneration. The present paper also describes the isolation of liver parenchymal cells from adult rats 4–8 weeks after they have been subjected to partial hepatectomy. The results show that these cells are comparable with liver parenchymal cells isolated from unoperated rats and that they are suitable controls for the isolation of parenchymal cells at several shorter time-intervals after partial hepatectomy. In addition, the isolated parenchymal cells may be used to study diurnal rhythms of enzyme activities in the parenchymal cells (Bonney *et al.*, 1973; Hopkins *et al.*, 1973).

Materials and Methods

Animals

White male rats were purchased from Sprague-Dawley, Madison, Wis., U.S.A. and maintained on the '8+16' feeding schedule described previously (Bonney *et al.*, 1973; Potter *et al.*, 1968) on a diet which consisted of 30% protein and 61% carbohydrate (Watanabe *et al.*, 1968). Partial hepatectomies were performed under ether anaesthesia at 12:00h by the method of Higgins & Anderson (1931).

Liver parenchymal cell isolation

Liver parenchymal cells and non-parenchymal cells were isolated by a variation of the original technique of Berry & Friend (1969) discussed by Crisp & Pogson (1972).

While developing the procedure for perfusion of the rat liver it was discovered that, in many instances, the median and left lateral lobes blanched slowly compared with the rest of the liver. It was therefore decided to remove surgically these large lobes by performing a 70% partial hepatectomy. The rats were allowed to recover from the operation for 6 to 8 weeks at which time the residual lobes had regenerated to approximately 88% of the normal liver weight as a percent of body weight. Both parenchymal and non-parenchymal cells were isolated from regenerated rat liver.

The perfusion medium consisted of Hanks Ca^{2+} -free medium (Grand Island Biological Co., Oakland, Calif., U.S.A.), containing 10munits of insulin/ml

(Eli Lilly and Co., Indianapolis, Ind., U.S.A.), 0.5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo., U.S.A.); 1.0mM-MgSO₄ and 2 units of heparin/ml (Riker Laboratories Inc., Northridge, Calif., U.S.A.), and was maintained at 37°C and gassed with O₂+CO₂ (95:5%). Perfusion medium (100–150ml) was pumped into the liver at 8–10ml/min via the inferior vena cava until the liver was completely blanched. At this point the enzyme perfusion medium, also at 37°C, was perfused through the liver. This solution was similar to the perfusion medium except that heparin was omitted, 0.05% collagenase 1 (Sigma Chemical Co.) and 0.1% hyaluronidase (Sigma Chemical Co.) were added. After approximately 15min the liver was transferred to a plastic beaker containing 20ml of the enzyme perfusion medium. The Glisson capsule was cut with microdissecting scissors and the cells were dispersed by gently drawing them up and down in a wide-bore Pasteur pipette. The suspension was filtered first through nylon gauze of 253 μm mesh (Tabler, Ernst and Traber, Inc., New York, N.Y., U.S.A.) and then through a smaller nylon mesh (64 μm) into a plastic centrifuge tube. The cells passing through were mainly parenchymal cells. The cells remaining on the first filter were scraped off the nylon gauze and reincubated with the enzyme perfusion solution at 37°C for 15min with intermittent gentle shaking and then refiltered through the larger-size nylon gauze. The cells remaining on the nylon gauze after the last filtration were scraped into a plastic centrifuge tube and designated non-parenchymal cells. The parenchymal cells were centrifuged twice at 50g for 2min at 4°C and the resulting pellets washed with ice-cold perfusion medium. The cells were next centrifuged at 100g for 10min at 4°C, washed with the same medium twice and the final pellet was weighed. The parenchymal cells were then suspended in the perfusion medium and nuclear counts and viable-cell determinations were performed. Nuclear counts were determined with a haemocytometer by using a citric acid–Crystal Violet stain (Morse & Potter, 1965). Viable cells were determined by their ability to exclude Trypan Blue (0.1%). This final suspension may be used for the primary cell cultures or biochemical analysis. The non-parenchymal cells were centrifuged at 100g for 10min at 4°C and washed three times with ice-cold perfusion medium.

Analytical methods

The purified cell preparations were suspended in an equal volume of homogenizing buffer (0.25M-sucrose, 0.5mM-dithiothreitol, 0.1M-KCl and 0.075M-Tris-HCl, pH7.5). The cells were broken by a Potter-Elvehjem tissue homogenizer. Samples were taken for determination of DNA and protein. The remainder of the homogenate was centrifuged at

105000g for 1 h and the resulting supernatant was used for enzyme and protein determinations.

The DNA of the parenchymal cells may be labelled before isolation of the cells as follows: injections of 40 μ Ci of [3 H]thymidine in each of three intramuscular injections were given at 1 and $\frac{1}{2}$ h intervals beginning approximately 18 h after partial hepatectomy and the rats were allowed to recover from the surgery for 4–6 weeks. This technique for labelling DNA has been called 'the integrated pulse technique' (J. D. Yager & V. R. Potter, personal communication).

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V, Sigma Chemical Co.) as standard and DNA extracted and estimated as described by Bonney *et al.* (1973). The enzymes were assayed by the indicated standard methods: tyrosine aminotransferase (Diamondstone, 1966); glucokinase and hexokinase (Walker & Parry, 1966); pyruvate kinase (Bücher & Pfeiderer, 1955); aldolase A and B (Blostein & Rutter, 1963). The contributions of aldolases A and B to the total aldolase activity were calculated by the fructose diphosphate/fructose monophosphate activity ratio as described by Endo *et al.* (1970). The isoenzymes of pyruvate kinase were separated by starch-block zonal electrophoresis before being eluted and assayed as described by Tanaka *et al.* (1967) and modified by Walker & Potter (1972a). For all enzymes, 1 unit of activity is the amount of enzyme required to metabolize 1 μ mol of substrate/min at 25°C (37°C in the case of tyrosine aminotransferase).

In some experiments cells were isolated at different times of the day to see if the diurnal rhythm in glycogen content seen in intact liver could be observed in the isolated parenchymal cells. Portions of the purified cell preparations were fixed in buffered formalin (10%, v/v) and stained for glycogen by Best's Carmine stain (Mallory, 1942). The percentage of cells staining pink for glycogen was scored.

The volume and diameter of the isolated parenchymal cells were determined by the use of a Coulter counter (model no. 2B1 with a Coulter Channelyzer) with ragweed pollen (diam. 19.5 μ m) as a standard.

Results

Characteristics of isolated cells

The emphasis of the present work was on the isolation of parenchymal cells rather than non-parenchymal cells. Parenchymal cells from fully regenerated liver are comparable with those of cells isolated from whole (unoperated) livers by other workers using similar techniques. The protein yield of 1.0 mg/10⁶ nuclei is comparable with the 1.15 mg/10⁶ nuclei found by Van Wyk *et al.* (1971) and to values of 1.58 mg/10⁶ cells and 1.93 mg/10⁶ cells obtained by

Crisp & Pogson (1972) and Lentz & Di Luzio (1971) respectively. When the results are expressed as protein per cell, the values appear higher, probably because of the presence of binucleated parenchymal cells (Wheatley, 1972).

The average diameter of the isolated parenchymal cells was 27.7 μ m (range 19.5–33.2 μ m) which is in good agreement with the literature values for hepatic cord cells (Jacob & Bhargava, 1962). There were not a significant number of cells observed with diameter of 1–20 μ m in any of the preparations examined, indicating little or no contamination with erythrocytes (5–7 μ m diam.) or other cells smaller than parenchymal cells such as bile duct and Kupffer cells. The suspension also did not appear to contain many clumps of cells, as no particles with a diameter greater than 33 μ m were detected by the Coulter counter. The DNA content of the parenchymal cells was found to be 17.1 pg/nucleus (or 21.0 μ g/mg of protein) and is similar to the values reported by Crisp & Pogson (1972) and Lentz & Di Luzio (1971).

The cells also appeared to be of uniform size as observed by phase-contrast microscopy. In addition, electron micrographs (not shown) of the parenchymal cells showed that they contained intact cellular and nuclear membranes, rough and smooth endoplasmic reticulum and numerous mitochondria characteristic of liver cells.

In two experiments the regenerated parenchymal cells were isolated containing radioactive DNA labelled by the integrated pulse technique described in the Materials and Methods section. In two preparations the isolated cells contained approximately 500 d.p.m./ μ g of DNA.

Enzyme activities

The specific activities of several enzymes of glycolysis and of tyrosine aminotransferase in high-speed supernatants prepared from regenerated liver compare closely with those of normal liver (Table 1). All animals were killed at 08:00 h and these activities are in agreement with those reported previously (Bonney *et al.*, 1973; Walker & Potter, 1972a,b). In normal liver and regenerated liver, pyruvate kinase type III represents approximately 8–10% of the total pyruvate kinase activity, hexokinase represents 12–14% of the ATP-linked glucose-phosphorylating activity and all the aldolase activity is due to aldolase B.

The next three columns in Table 1 show enzyme specific activities found in parenchymal and non-parenchymal cells isolated from regenerated liver of rats killed at 08:00 h. Glucokinase activity in the preparations was as high or higher than that found in intact liver indicating that the isolated cells do not leak this enzyme into the medium. Hexokinase activity in the parenchymal cells represented only 3–5% of the total ATP-linked glucose phosphorylating

(1972). This procedure digests the contaminating parenchymal cells whereas the non-parenchymal cells remain intact and can be isolated by employing several centrifugations and washings in Hanks' medium. The purity of these cells was indicated by the lack of detectable glucokinase activity and pyruvate kinase type I; the cells did contain hexokinase and pyruvate kinase type III activity. In addition, tyrosine aminotransferase activity was detectable but did not appear to be increased at 17:00h.

Glycogen content

Portions of the parenchymal and non-parenchymal cells were isolated at three different times on the controlled feeding schedule (no. 13 at 08:00h, no. 16 at 14:00h and no. 21 at 17:00h) and stained for glycogen. The percentage of parenchymal cells containing glycogen (pink granules) at the three times are: 08:00h, 18%; 14:00h, 61%; 17:00h, 71%. In the non-parenchymal cell preparations, the only cells that stained positively for glycogen were the low number of contaminating parenchymal cells seen in non-parenchymal cell preparation no. 25.

Pyruvate kinase isoenzymes during regeneration

Bonney *et al.* (1973) demonstrated that the isoenzyme pattern of pyruvate kinase in adult rat liver shifted toward that of foetal liver during regeneration. The greatest change in the pattern was observed 48 and 72h after partial hepatectomy. The observed increase in pyruvate kinase type III activity represented a net increase in units/liver per 100g body weight. Crisp & Pogson (1972) demonstrated that pyruvate kinase type III activity was not found in parenchymal cells from normal liver. Thus it was not certain whether the increase in pyruvate kinase type III activity after partial hepatectomy (Bonney *et al.*, 1973) was due to synthesis of the enzyme by the parenchymal cells under conditions of liver growth, or to an increased synthesis of the enzymes by non-parenchymal cells. Another possibility is that there is an increased amount of non-parenchymal cells containing this enzyme. Therefore, after establishing that regenerated liver was suitable for the isolation of parenchymal cells, an attempt was made to isolate parenchymal cells 48 and 72h after a 70% partial hepatectomy. The isolated parenchymal cells 48h after partial hepatectomy exhibited only one peak of cells on the Coulter counter corresponding to an average diameter of $26.7\mu\text{m}$ (range $18.4\text{--}33.4\mu\text{m}$), which is comparable with that seen with fully regenerated liver parenchymal cells. The yield of parenchymal cells/g of regenerating liver was comparable with that of control (regenerated) liver. The percent of parenchymal cells excluding Trypan Blue was 80–88%. The isolated parenchymal cells were homo-

Fig. 1. Pyruvate kinase starch-block zonal electrophoresis isoenzyme patterns in isolated cells and in intact rat liver

Portions from 105000g supernatants prepared from intact liver (a) or isolated non-parenchymal (b) and parenchymal cells (c) obtained from regenerated liver were subjected to starch-block zonal electrophoresis. Pyruvate kinase activity was determined after elution from the starch.

genized as described in the Materials and Methods section and the enzyme activities were determined on the 105000g supernatants. In addition, a portion of this supernatant was subjected to starch-block zonal electrophoresis to separate the two pyruvate kinase activities. In adult regenerated liver homogenates (Fig. 1a) pyruvate kinase type I represents 91% of the total pyruvate kinase activity and pyruvate

Fig. 2. Pyruvate kinase starch-block zonal electrophoresis isoenzyme patterns in isolated parenchymal cells and in whole liver extracts after partial hepatectomy (regenerating livers)

Portions from 105000g supernatant preparations from liver remnants and from isolated parenchymal cells from liver 48 and 72h after partial hepatectomy were subjected to starch-block zonal electrophoresis. Pyruvate kinase activity was determined after elution from the starch. Parenchymal cells, 48h (a), 72h (b); whole liver extracts, 48h (c), 72h (d).

Table 2. Isoenzyme patterns in liver parenchymal cells after partial hepatectomy

The parenchymal cells were isolated from the liver at zero time (regenerated), or 48 and 72h after partial hepatectomy. The specific activities for the zero-time points are the average of five determinations. Three experiments (1, 2 and 3) were performed 48 and 72h after the operation.

Time after operation (h)	Enzyme	Specific activity (munits/mg of protein)							
		Liver			Parenchymal cells				
0	Glucokinase		7.29				8.28		
	Hexokinase		1.05				0.42		
	Pyruvate kinase type I		443				586		
	Pyruvate kinase type III		39				19		
	Aldolase B		71				71		
	Aldolase A		<0.1				<0.1		
48		Expts.	...	1	2	3	1	2	3
	Glucokinase		—		1.00	3.10	—	2.33	5.65
	Hexokinase		—		1.83	1.90	—	0.48	0.65
	Pyruvate kinase type I		196		68	158	128	268	248
	Pyruvate kinase type III		293		155	282	355	132	192
	Aldolase B		—		31	39	—	30.7	35.0
	Aldolase A		—		<0.1	<0.1	—	<0.1	<0.1
72	Glucokinase		—		1.61	3.10	—	2.80	2.35
	Hexokinase		—		1.90	2.10	—	0.49	0.87
	Pyruvate kinase type I		168		137	108	144	92	183
	Pyruvate kinase type III		223		102	292	336	85	154
	Aldolase B		—		26.1	—	—	24.6	—
	Aldolase A		—		<0.1	—	—	<0.1	—

kinase type III approximately 8–9%. However, in the cell preparations isolated from regenerated liver the activities appeared to be separated with pyruvate kinase type I being almost exclusively in the parenchymal cells and pyruvate kinase type III in the non-parenchymal cells in agreement with other workers (Crisp & Pogson, 1972; Van Berkel *et al.*, 1972).

The pyruvate kinase isoenzyme pattern in whole regenerating liver extracts 48 and 72 h after partial hepatectomy are shown in Figs. 2(c) and 2(d). When parenchymal cells were isolated from regenerating livers 48 and 72 h after partial hepatectomy, nearly identical patterns were seen (Figs. 2a and b).

Along with the calculated enzyme activities of pyruvate kinase types II and III, the activities of glucokinase, hexokinase and aldolase B in parenchymal cells during regeneration are of interest. Glucokinase activity decreased in the whole liver (Table 2) in agreement with our previous report (Bonney *et al.*, 1973) and this enzyme activity was also decreased in the isolated parenchymal cells. Although hexokinase activity appeared to increase in the liver, it is not certain whether the small increases in hexokinase in parenchymal cells are significant. As seen in Fig. 2, the activity of pyruvate kinase type I decreased and that of pyruvate kinase type III increased in the whole liver and in the parenchymal cells at both times. However, the pattern of aldolase isoenzymes was not altered at these two selected times after the operation but aldolase B activity was decreased. The change in pyruvate kinase isoenzyme patterns observed in the present study is greater than in the previous report (Bonney *et al.*, 1973) possibly because the operations were performed at a different time of the day (12:00 h rather than 20:00 h).

Discussion

The use of regenerated liver as a source of parenchymal and non-parenchymal cells has several advantages. In comparison with intact liver the remnant liver lobes perfuse rapidly and completely and they are more easily wrapped in sterile bags, which greatly aids in the prevention of contamination from rat hair and other external sources. More importantly, the liver DNA can be labelled with radioactive thymidine during regeneration and since the DNA in liver turns over slowly, parenchymal cells can be isolated containing radioactive DNA (Bonney *et al.*, 1973; Hopkins *et al.*, 1973). The characteristics of parenchymal cells from the above source appear to be identical with those of cells isolated from normal liver by other workers.

The activities of the several enzymes examined in the parenchymal cells were as high or higher than in extracts from normal or regenerated intact liver homogenates. This indicates that the isolated cells do

not leak these cytoplasmic enzymes and are representative of liver parenchymal cells *in vivo* in that sense. The small amount of hexokinase activity observed in most of the preparations of parenchymal cells was not found in some of the preparations of Crisp & Pogson (1972) but their preparations were from mice. However, since there are at least three 'low K_m ' hexokinases found in rat liver (Grossbard & Schimke, 1966), it is not unreasonable that one of these activities might reside in the parenchymal cell. In some preparations (not shown), where pyruvate kinase III activity was undetectable there was still detectable hexokinase activity. Our results with parenchymal cells from regenerated liver agree with those of Werner *et al.* (1972), who detected hexokinase type II in isolated rat liver parenchymal cells.

The isolation of liver cells from animals maintained on controlled feeding and lighting schedules allows one to establish whether or not the observed diurnal oscillation in whole liver is restricted to one cell type (parenchymal cell) or is a product of changes in activity of the particular enzyme in all cell types of the liver (Bonney *et al.*, 1973; Hopkins *et al.*, 1973). It appears that the oscillation of tyrosine aminotransferase and glucokinase activities observed in intact liver is indeed restricted to the parenchymal cells but further studies are needed on this point. It is not certain if tyrosine aminotransferase activity is present in non-parenchymal cells but the activity found in this fraction does not appear to increase after feeding on the '8+16' schedule.

The major pyruvate kinase activity found in the liver when the cells are dividing, such as during foetal life (Walker & Potter, 1972a) or in hepatomas (Walker & Potter, 1972a) is pyruvate kinase type III. In addition, several reports have shown the pyruvate kinase type III activity is increased during liver regeneration (Walker & Potter, 1972a; Tanaka *et al.*, 1967). Bonney *et al.* (1973) have shown this increase is greatest 48 and 72 h after a 70% partial hepatectomy. Because the cells of the 'adult' liver are dividing under controlled conditions during liver regeneration when the parenchymal cells are dividing to replace those lost during surgery, they may acquire a function they presumably had in foetal life, that of synthesizing pyruvate kinase type III. In Fig. 2, the starch-block electrophoretic patterns of pyruvate kinase activity, when compared with fully regenerated liver parenchymal cells (Fig. 1) indicate that pyruvate kinase type III is being synthesized by the parenchymal cells. The procedure used in the present study for parenchymal and non-parenchymal cell separation might be different when the source of the cells is fully regenerated liver, or liver with dividing cells, such as regenerating liver 48 and 72 h after partial hepatectomy. This seems unlikely because the yield of cells/g of regenerating liver is the same as that from normal (regenerated) liver. Also the average size of the cells

is 26.7 μm diam. compared with 27.0 μm for fully regenerated parenchymal cells, and no other populations of cells were detected by Coulter-counter scanning or by phase-contrast microscopy. The enzyme activities of glucokinase, pyruvate kinase types I and III and aldolase B in parenchymal cells are very similar to that of whole liver extracts prepared at 48 and 72 h after partial hepatectomy (Table 2).

The distribution of the isoenzymes of pyruvate kinase between the two cell types has been previously suggested (Crisp & Pogson, 1972; Van Berkel *et al.*, 1972) although the study of Crisp & Pogson (1972) was with mouse liver. However, the experiments reported here suggest that when the adult liver parenchymal cell is caused to divide or prepares for division it may be capable of acquiring a function it once had during development, that of synthesizing pyruvate kinase type III. This may also be true for transformed parenchymal cells. The existence of an enzyme, normally associated with foetal liver, in the adult parenchymal cells may indicate that all dividing liver cells share some common properties.

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