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Abstract

Isolated hepatic cells from adult rats were prepared by perfusing the livers with trypsin. The highest yield of viable cells was obtained by perfusing the liver with 0.1% trypsin, pH 7.0, at 37 degrees C for 30 min. Following this treatment about 70% of cells excluded trypan blue. The isolated cells contained many binucleate cells. Between 60 and 70% of DNA present originally in the liver was recovered from the isolated hepatic cells, which had higher glucose 6-phosphatase activity than the liver. Thus the resulting cell population seems to be rich in hepatocytes. The isolated hepatic cells, however, lost some of their cellular proteins such as alanine and tyrosine aminotransferases. It was suggested that the membranes of isolated hepatic cells might be damaged by both enzymatic digestion and mechanical destruction.

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PRIMARY CULTURE OF ADULT RAT LIVER CELLS. I. PREPARATION OF ISOLATED CELLS FROM TRYPSIN-PERFUSED LIVER OF ADULT RAT

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Abstract. Isolated hepatic cells from adult rats were prepared by perfusing the livers with trypsin. The highest yield of viable cells was obtained by perfusing the liver with 0.1% trypsin, pH 7.0, at 37°C for 30 min. Following this treatment about 70% of cells excluded trypan blue. The isolated cells contained many binucleate cells. Between 60 and 70% of DNA present originally in the liver was recovered from the isolated hepatic cells, which had higher glucose 6-phosphatase activity than the liver. Thus the resulting cell population seems to be rich in hepatocytes. The isolated hepatic cells, however, lost some of their cellular proteins such as alanine and tyrosine aminotransferases. It was suggested that the membranes of isolated hepatic cells might be damaged by both enzymatic digestion and mechanical destruction.

The isolation of hepatocytes allows a direct approach to study the hepatic metabolisms and the carcinogenesis of the liver *in vivo* by short or long-term culture. Early methods depend upon the use of mechanical force to separate cells from liver (1). Various chelating agents such as citrate (2), tetraphenylboron (3) and ethylenediaminetetraacetate (EDTA) (4) were thereafter used in the conjunction with mechanical destruction.

A method for preparing liver cells by incubating liver slices with collagenase and hyaluronidase has been described by Howard *et al.* (5, 6). The enzymatically prepared cells were morphologically and metabolically more like untreated hepatocytes than cells obtained by other methods (5-9). Unfortunately, the cell yields obtained by this enzymatic technique are low, typically 5×10^6 cells per g of liver (6). Berry and Friend (10) have reported that the enzymatic procedure could be modified by perfusing the liver with collagenase and hyaluronidase using, *in situ*, a recirculating perfusion apparatus prior to the incubation. This modification greatly increased the cell yields and improved the subcellular integrity of the isolated liver cells. Trypsin was used for preparing the isolated liver cells since various proteolytic enzymes have been employed for similar procedures (11, 12). When liver cells were obtained in this laboratory

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using the former procedure (*i.e.* incubating minced liver tissue with 0.1 to 0.2% trypsin in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline) (13–15), the cell yields obtained were less than 10% of those by the recirculating perfusion method described above, and biochemical functions of the growing cells in culture were not similar to those of the mature hepatocytes (16, 17).

In this report, a detailed method is described for obtaining much larger numbers of isolated hepatic cells by perfusing the liver with trypsin. Biochemical properties of the cells thus obtained are described and also compared with those of the liver cells obtained by perfusing with collagenase and hyaluronidase.

MATERIALS AND METHODS

Animals and chemicals. Adult Donryu rats, weighing 200 to 300 g, inbred in this institute were used in the present experiment. Animals were fed *ad libitum* on Oriental Laboratory Chow MF and water. Trypsin (1 : 250) was obtained from Difco. Collagenase (type I) and hyaluronidase (type I) were obtained from Sigma Chemical Co.

Preparation of isolated hepatic cells. The hepatic cells were prepared by the methods developed by Berry and Friend (10) and modified by Crisp and Pogson (18). The recirculating perfusion system used in the present experiment is illustrated in Fig. 1. The liver was perfused through its inferior vena cava with

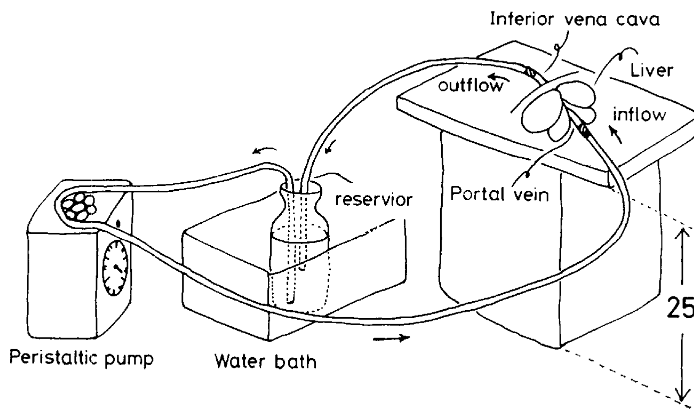

Fig. 1. Recirculating-perfusion system of the liver. The diagrams illustrates the perfusion system used in this experiment. The liver was perfused *in situ*. Arrows show the flow direction of the perfusate.

a flow rate of 10 ml per min using a Perista-Mini Pump (Mitsumi Business in Fluid Technology). A rat was positioned about 25cm above a reservior, so that drainage of the liver could be assisted by a siphoning effect. The first hundred mls of perfusate (Ca^{2+} and Mg^{2} -free Hanks' balanced salt solution (CMF-HBSS) (19), pH 7.0) was discarded, thereby flushing all of the circulating blood

from the organ. The inflow tube was then connected with a reservoir containing proteolytic enzyme dissolved in CMF-HBSS, which was then recirculated through the liver for 20 to 50 min. The perfused and minced liver tissue was transferred to a spitz containing 30 ml of CMF-HBSS at 4°C and dissociated by gentle pipetting. Crude cell suspensions were filtered through a mesh (150-mesh) into a glass centrifuge tube and centrifuged at 500 rpm for 5 min at room temperature. The supernatant was decanted and replaced by 30 ml of fresh CMF-HBSS, gently pipetted and recentrifuged. This procedure was repeated 3 times. Cells were separated from debris by centrifugation. The final cell suspensions were used for light microscopic and biochemical studies.

Determination of cell viability and yield. The cell viability was judged from exclusion of the vital stain, trypan blue (20). The cell yields were expressed as cells obtained, culculated from liver per 100 g body weight.

Light microscopic observation of dispersed cells. The cell suspensions were transferred to Falcon plastic dishes and observed under a phase contrast microscope. For light microscopy the cells were fixed for 5 min in methanol and stained for 30 min with 5% Giemsa in phosphate-buffer.

Assay of enzyme activity. The cells obtained from 1 g of perfused livers were washed twice with a cold solution of 0.154 M KCl containing 0.32 mM $KHCO_3$ and 4 mM EDTA (pH 7.5) and resuspended in approximately 4 volumes of the same solution and homogenized for 1 min in a glass homogenizer with a Teflon pestle. The supernatants were separated by centrifuging the homogenates at 25000 g and 4°C for 60 min. Perfused and non-perfused livers with CMF-HBSS were also homogenized, and the supernatants similarly separated. The homogenates were used for measuring the activity of glucose 6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9, G6Pase) (21, 22) and the supernatants for tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.6.1.5, TAT) (23) and alanine aminotransferase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2, GPT) (24). Specific enzyme activities are expressed in milliunits (nmoles of product formed per min) per mg of supernatant protein for TAT and of homogenate for G6Pase and in Karmen units (K.U.) (24) per mg of supernatant protein for GPT. Protein contents were determined by the method of Lowry *et al.* (25).

DNA determination. Liver DNA fractions were separated by the method reported by Schmidt and Thannhauser (26). The DNA content of the final extracts were determined spectrophotometrically with diphenylamine (27).

RESULTS

Effect of various conditions of liver perfusion on cell yields. The numbers of single cells were shown at the various concentrations of trypsin in the perfusate, pH 7 0 (Fig. 2-A). The liver perfusion was carried out at 37°C for 30 min. The total numbers of isolated cells were nearly constant up to 0.1% concentration of trypsin used, but decreased at a concentration of 0.2%. The cell viabilities diminished at lower concentrations of trypsin. The maximal yield of viable cells

was obtained with 0.1% trypsin. The perfused and minced liver tissues were stirred by a magnetic stirrer in the same solution at 37°C in order to isolate cells gently from the perfused livers. The viability of cells isolated by stirring was higher than that by pipetting, although the total number of viable cells isolated by stirring was lower than by pipetting. It seems likely that epithelial cells are sensitive to physical force (28).

When isolated cells were prepared from the liver perfused with 0.1% trypsin at 37°C for 30 min, at pHs of 6.5, 7.0, 7.5 and 8.0, respectively, the total cell numbers were fairly constant under the various conditions studied but the number of viable cells at pH 7.0 was the highest (Fig. 2-B).

When livers were perfused with 0.1% trypsin, pH 7.0, kept at 20, 30, 37 and 45°C respectively, in a water bath, the total cell numbers were nearly constant at the temperatures used, but the highest yield of viable isolated cells was obtained at 37°C (Fig. 2-C).

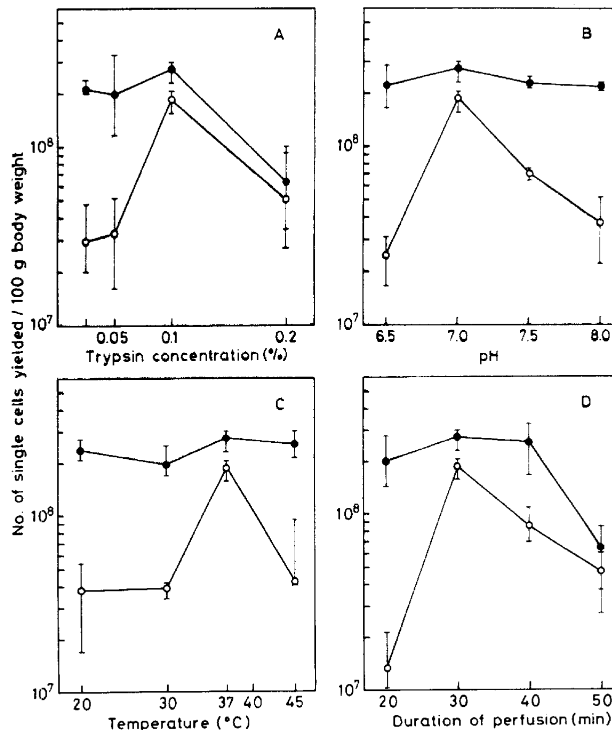

Fig. 2. Effect of various conditions of liver perfusion on the cell yields. The isolated hepatic cells were prepared by gently pipetting the perfused and minced tissue in CMF-HBSS until the dispersion of cells were almost complete. Almost all the cells in the resulting suspension were single. Average results of 3 experiments are indicated. ●, Total numbers of isolated cells and ○, numbers of viable cells. Vertical bars indicate the upper and lower experimental values, respectively.

The liver perfusion was tried with 0.1% trypsin at pH 7.0 and 37°C for various times as indicated (Fig. 2-D). The continuance of the recirculation of the perfusate was impossible for longer than 50 min, since loss of the circulating medium from the liver surface became too great to maintain perfusion. The liver became enough soft to obtain the maximal cell yield when it was perfused for 30 min.

From the above results it can be concluded that the highest yield of viable cells is obtained from the liver perfused with 0.1% trypsin at pH 7.0 and 37°C for 30 min. These conditions were used throughout the following experiments.

Light microscopic study of isolated cells. About seventy percent of the isolated cells excluded trypan blue (Table 1). Many binucleate cells were present in the isolated cells which were of different sizes (Fig. 3-A). In contrast to damaged cells, which were irregularly shaped, the intact parenchymal cell appeared as a spherical shape with a well defined and sharply refractile plasma membrane (Fig. 3-B). Silver-impregnation of isolated liver cells showed that cells dispersed with collagenase were rounded with argyrophylic fibers, while cells dispersed with trypsin rarely retained them on cell surfaces.

Effect of various perfusion media on biological parameters of perfused and non-perfused livers. The total number of cells obtained from the liver perfused with trypsin was nearly equal to that obtained with 0.05% collagenase and 0.1% hyaluronidase. Sixty to 70% DNA content of the liver was recovered by the trypsin-perfusion method (Table 1). The viability of cells prepared by the trypsin-perfusion method was lower than that by the collagenase-perfusion method (10, 18). Protein and DNA content of the liver perfused with CMF-HBSS alone was less than in the non-perfused liver while collagenase and hyaluronidase or trypsin perfusion resulted in further decreases.

Enzyme activities in perfused and non-perfused liver. The G6Pase activity in the liver perfused with CMF-HBSS alone was higher than that in the non-perfused liver (Table 2). The specific activity of G6Pase in the isolated cells prepared with either trypsin or collagenase and hyaluronidase was also about twice as high. Increases in this enzyme activity in the liver perfused with CMF-HBSS with or without proteolytic enzymes seemed to be due to leakage of cellular proteins. It seems clear from these results that G6Pase itself was well retained in the hepatocytes (29) and that hepatocyte-rich fractions were obtained. The TAT activity in the liver perfused with CMF-HBSS alone was also higher than that in the non-perfused liver, however the activity in the isolated cells prepared enzymatically was lower. This seems likely to result from leakage of TAT itself (29, 30). Leakage of GPT from the liver perfused with CMF-HBSS with or without proteolytic enzymes was observed (31). The activities of both transaminases were higher in the cells isolated with collagenase and hyaluronidase than with trypsin (18).

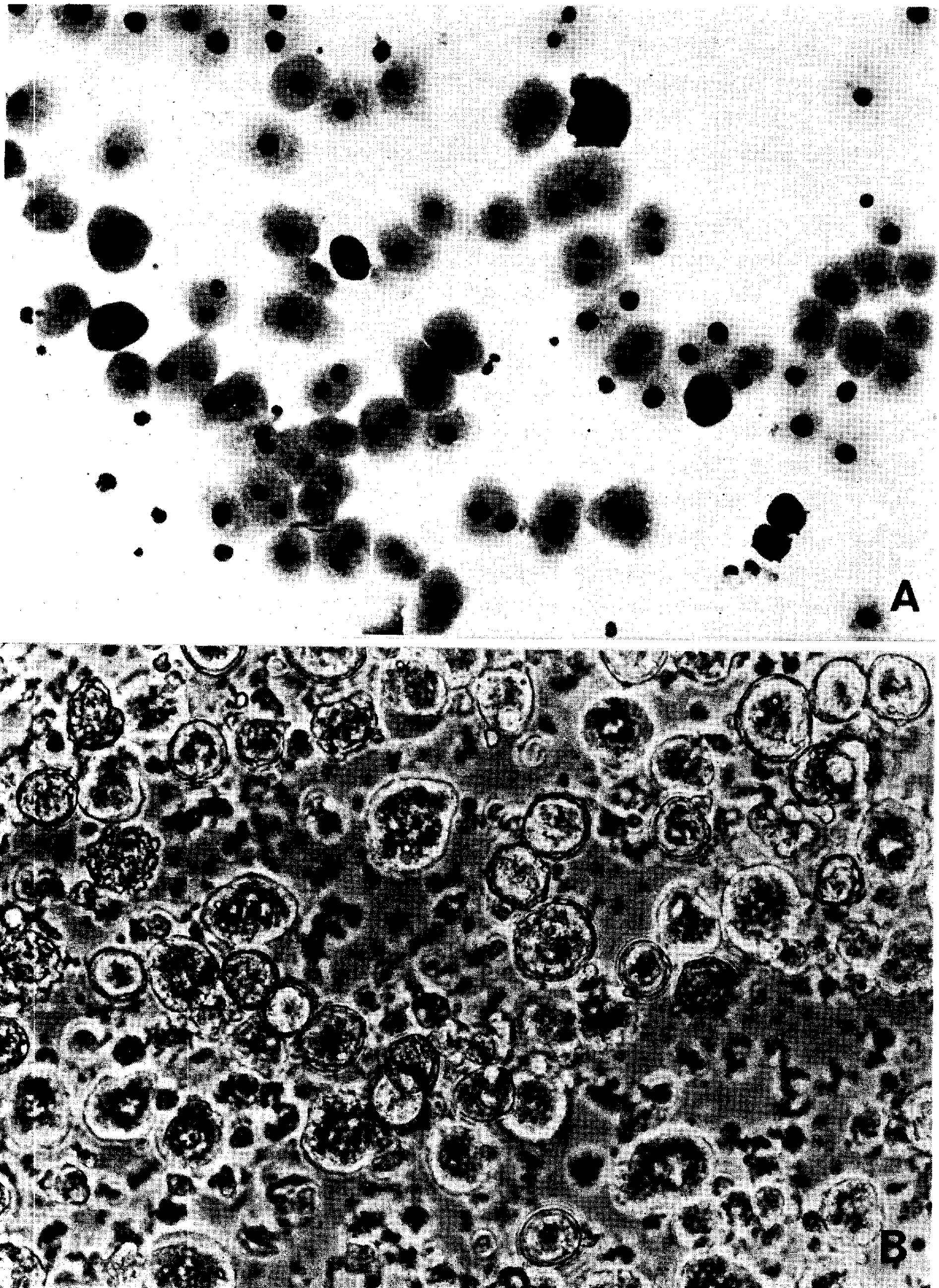

Fig. 3. Isolated cells from adult rat livers. The cells were prepared by perfusing the liver with 0.1% trypsin at pH 7.0 and 37°C for 30 min. A, Giemas stain; ×200 and B, phase contrast; ×200.

TABLE 1. EFFECT OF VARIOUS PERFUSION MEDIA ON BIOLOGICAL PARAMETERS OF CELL SUSPENSIONS FROM PERFUSED OR NON-PERFUSED LIVER

Perfusion medium	Cell number ($\times 10^6$/liver/ 100 g body weight)	Cell Viability (%)	Number of Viable cells ($\times 10^6$/liver/ 100 g body weight)	Protein (mg/liver /100 g body weight)	DNA (mg/liver /100 g body weight)
I. Liver tissue					
None	–	–	–	732±49	7.8±0.1
CMF-HBSS	–	–	–	562±89	6.3±0.2
II. Isolated cells					
0.1% trypsin	275±39	68±10	188±27	324± 8	4.4±0.1
0.05% collagenase +0.1% hyaluronidase	288±24	81± 4	232±10	436± 9	5.6±0.5

Results from 3 experiments are expressed as mean ± standard deviation.

TABLE 2. ENZYME ACTIVITIES IN PERFUSED OR NON-PERFUSED LIVER

Perfusion medium	GPT (K.U./mg protein)	TAT (mU/mg protein)	G6Pase (mU/mg protein)
I. Liver tissue			
None	482±35	44± 7	53± 5
CMF-HBSS	388±18	72± 7	84±15
II. Isolated cells			
0.1% trypsin	104±32	24± 3	110±13
0.05% collagenase +0.1% hyaluronidase	201±11	35± 4	103± 6

Results from 3 experiments are expressed as mean ± standard deviation.

DISCUSSION

The liver was perfused with 0.1% trypsin at pH 7.0 and 37°C for 30 min in order to obtain the highest yield of viable cells. Although trypsin is most active at pH 8 to 9 (28), the perfusion at pH 7.0 is rather suitable for the purpose mentioned above. The yield of viable cells obtained by this method did not increase with vigorous bubbling of a gas-mixture of 95% O_2 and 5% CO_2 through the enzyme medium, although Berry and Friend (10) recommended to gas the enzyme solution with the carbogen. The isolation of the hepatic cells by using the recirculating liver-perfusion method can be carried out in a short time with 10 times higher cell yields than when minced-liver tissues were incubated with trypsin (13–15). The perfusion method thus offers sufficient numbers of isolated hepatic cells for biochemical experiments. Most of the cells isolated by the incubation method were mononucleate and relatively small in cell size (unpublished data). The results of the present experiment indicate that the cells obtained by

the perfusion method with trypsin reflect more exactly the organization of cells *in vivo* than those isolated by the incubation method.

The perfusion of the liver with CMF-HBSS with or without proteolytic enzymes resulted in leakage of enzymes such as GPT and TAT (29-31), an effect also observed in rats treated with various hepatotoxins (32, 33). Enzyme leakage probably reflects the membrane damages of the isolated cells. Grisham *et al.* (12) reported that suspensions of single liver cells prepared with trypsin were enriched in non-parenchymal cells as compared to the cellular populations in the liver *in vivo*, resulting from more marked lysis of hepatocytes by trypsin rather than of other liver cells. As the specific activity of G6Pase in the cells dispersed by the trypsin-perfusion method is higher than that of the non-perfused liver, most of the cells obtained by this method seem to be hepatic parenchymal cells. The hepatic cells prepared by this method produced albumin and maintained a relatively higher activity of TAT up to 4 days after intiating primary culture (34). An increased activity of TAT and enhancement of albumin production by addition of dexamethasone or hydrocortisone were observed in the primary cultured cells (35). We can conclude from the findings described above that the hepatic cells prepared by this method will facilitate a more direct approach to study the hepatic metabolisms *in vivo*.

It is well known that any successful procedure for isolating liver cells must involve three critical steps, *i. e.*, exposure of tissue to a calcium-free medium, digestion by proteolytic enzymes and gentle mechanical treatment. Berry and Friend (10) recognized that portions of cell-membranes containing desmosomes invaginated during the perfusion procedure. Of considerable interest were the findings that the addition of calcium ions to the medium after the pre-perfusion did not lessen the yield of isolated cells but no intact isolated-cells were obtained when calcium ions were present in the pre-perfusion solution (36). It can be suggested therefore, that the pre-perfusion with calcium-free medium brings about an irreversible cleavage of desmosomes (36). Splitting of desmosomes is a key element in the procedure of cell separation, since no success can be achieved if calcium ions are present throughout the preparations (10, 36). The differences between the biochemical and cytological properties of the cells isolated by the perfusion with trypsin and those with collagenase seem to be derived from the different proteolytic properties of these enzymes. It seems probably that while collagenase digests only the substances supporting hepatic cell cords in the liver lobules, trypsin also digests the substances between adjacent cells in the cell cords.

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