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PROTOCOLLAGEN PROLINE HYDROXYLASE IN ISOLATED RAT LIVER CELLS

KAZUO OHUCHI AND SUSUMU TSURUFUJI

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo (Japan)

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SUMMARY

1. Liver parenchymal cells from rats were dispersed by collagenase and hyaluronidase digestion, and by application of the bovine serum albumin flotation method. The viable cells, which were not stained with trypan blue, could be isolated with more than 95% viability. Contamination by non-parenchymal cells was less than 4%.

2. The endothelial cell fraction from rat liver was isolated by a combination of pronase digestion and bovine serum albumin flotation methods. Contamination by parenchymal cells was less than 0.4% and viability estimated with trypan blue was more than 99.8%.

3. Protocollagen proline hydroxylase activity was assayed in both the parenchymal cell and the endothelial cell fractions. The parenchymal cells showed hydroxylase activity 100 times higher than the endothelial cells in terms of the number of cells. Trypan blue-stained parenchymal cells had about a quarter of the hydroxylase activity of viable parenchymal cells.

INTRODUCTION

In biochemical studies of the liver using perfusion, slice and homogenate techniques, we often encounter difficulties in interpreting which type of cell is responsible for the changes brought about by a particular experimental treatment. Because of the difficulty in isolating any single cell species of the liver physiologically intact, there have been only a few incomplete studies[1,2] using intact and uncontaminated parenchymal cells and sinusoidal endothelial cells, the two main cell types of the liver. After reference to a number of papers reporting cell isolation techniques[1-20], we have established standard procedures for isolating parenchymal cells and endothelial cells, high both in purity and viability.

In normal liver, small amounts of collagen fibers are often found in the space of Disse, and protocollagen proline hydroxylase activity was detected in the liver homogenate[21], but it remains unknown what type of cell synthesizes these collagen fibers. In an attempt to clarify this problem, we examined the activity of proto-

collagen proline hydroxylase in parenchymal and endothelial cells which had been separated by our methods. The enzyme was found in the parenchymal cells in an amount 100 times greater than that of the endothelial cells.

MATERIALS AND METHODS

Experimental animals

Male rats of Donryu strain, 135–185 g body weight, were used.

Cell isolation

Parenchymal cells. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital. An abdominal incision was made and 200 I.U. of heparin were given through the portal vein. After 2 min the rat was killed by cutting the carotid artery. The liver was perfused *via* the portal vein first with 20 ml of Ca^{2+}-free Hanks' solution (pH 7.3)[22] and then with 15 ml of the enzyme solution containing 0.05% collagenase (Type 1, Sigma Chemical Co., U.S.A.) and 0.10% hyaluronidase (Type 1, Sigma Chemical Co., U.S.A.) in Ca^{2+}-free Hanks' solution. The liver was then quickly removed and sliced with stainless steel razor blades into 2–3-mm slices. The sliced liver was digested with 45 ml of the above-mentioned enzyme solution by constant slow shaking at 37° for 70 min in O_2–CO_2 (95:5, v/v). The resulting cell suspension was filtered first through 3 sheets of cotton gauze then through 200 mesh stainless steel gauze to remove undigested liver tissue; the filtrate was then centrifuged at 50 × *g* for 1 min. The cell pellet was washed 3 times with Ca^{2+}-free Hanks' solution and finally suspended in 4 ml of 20% bovine serum albumin solution. 4 ml of Ca^{2+}-free Hanks' solution were then overlaid on the bovine serum albumin cell suspension and centrifuged at 50 × *g* for 15 min. By the centrifugation the cells were separated into two fractions; cell pellet and cells floating at the interface of the bovine serum albumin and the Ca^{2+}-free Hanks' solutions. Together with the layer of the Ca^{2+}-free Hanks' solution the floating cells were recovered through a 1-mm diameter stainless steel tube. The recovered cells were precipitated by centrifugation at 50 × *g* for 1 min and the cell pellet was washed 2 times with Ca^{2+}-free Hanks' solution and recovered by centrifugation at 30 × *g* for 1 min to remove contaminating bovine serum albumin solution and non-parenchymal cells.

Endothelial cells. We employed ROSER's[3] method except that the enzyme solution consisted of 0.04% pronase P (Kaken Chemical Co., Japan) in EISEN's[23] solution (pH 7.3). The liver perfusion was vigorously carried out to eliminate contamination by blood cells in the endothelial cell fraction. The perfusion was carried out first with 85 ml of Eisen's solution then with 15 ml of the above enzyme solution. The liver was excised and cut into 1–2-mm cubes with scissors. The enzyme digestion was carried out first with 80 ml of the enzyme solution at 37° for 15 min in an air phase with constant shaking. Then the resulting cell suspension was removed, 70 ml of fresh enzyme solution added, and digestion continued for 15 min. The enzyme-digested solutions were combined and filtered as described in the parenchymal cell isolation method. After centrifugation at 300 × *g* for 8 min the cell pellet was washed 3 times with Eisen's solution and suspended in 4 ml of bovine serum albumin solution. 4 ml of Eisen's solution were then overlaid and centrifuged at 1000 × *g* for 15 min. The cell layer at the bovine serum albumin solution–Eisen's solution interface was re-

covered and washed 2 times with Eisen's solution by centrifugation at 300 × *g* for 8 min.

Bovine serum albumin solution. Bovine serum albumin Fraction V (Wako Pure Chemical Indst., Japan) was dissolved in distilled water with the addition of one drop of antifoam reagent, polypropyreneglycol (No. 2000, Wako Pure Chemical Indst., Japan) the pH was adjusted to 7.3 by 3 M NaOH, and the solution then made up to a concentration of 20% bovine serum albumin.

The temperature during the cell isolation procedure was maintained at 0–4° except during the enzyme digestion period.

Preparation of the substrate for the hydroxylase assay

The substrate for the hydroxylase assay was prepared according to JUVA AND PROCKOP's[24] method. From twenty 9-day-old chick embryos the tibiae and femurs were removed under sterile conditions. They were incubated first in 50 ml of KREBS'[25] saline serum substitute with 1 mM α,α'-dipyridyl at 37° for 20 min, then the incubation medium was changed, 11 ml of KREBS' saline serum substitute containing 1 mM α,α'-dipyridyl and 2.25 mC of L-[^{3}H]proline (45.0 C/mmole, Dai-ichi Kagaku, Co., Japan) being added and incubation carried out for 4 h at 37° in O_2–CO_2 (95:5, v/v). After incubation, the tibiae and femurs were homogenized in 4 ml of distilled water containing 1% of unlabeled L-proline. The homogenate was dialyzed against running tap water at 4° for 48 h and then for 24 h against 1 l of 0.05 M KCl, 0.1 M NaCl, 0.1 M glycine and 0.01 M Tris–HCl buffer adjusted to pH 7.8. The dialyzed sample was boiled for 5 min and diluted with the buffer used for dialysis to about 8 ml, and then centrifuged at 100 000 × *g* for 1 h. The supernatant solution was stored in a deep freeze (−20°). Before being used for the assay of hydroxylase, the supernatant solution was boiled for 5 min and diluted 10 times with dialysis buffer. A portion of 10 times diluted solution was used as the substrate for the hydroxylase assay.

Assay of protocollagen proline hydroxylase in liver tissues

Enzyme concentration. Liver perfused with Ca^{2+}-free Hanks' solution was homogenized in ice-cold 0.05 M KCl, 0.01 M Tris–HCl (pH 7.6) solution with a teflon homogenizer and the homogenate was centrifuged at 15 000 × *g* for 1 h at 4°. The resultant supernatant was diluted with the above solution to a protein concentration of 4.75 mg/ml. The amount of protein was measured by the method of LOWRY *et al.*[26]. 0.0, 0.1, 0.2, 0.3 and 0.4 ml of the enzyme solution was added to 0.2 ml of 10-times diluted [^{3}H]proline-labeled protocollagen and incubated at 37° for 60 min in 4.0 ml of the incubation medium, consisting of 50 mM Tris–HCl (pH 7.8), 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, 0.05 mM $FeSO_4$ and 0.05 mg/ml catalase (from beef liver, 1200–1400 Keil units/g, Tokyokasei Kogyo, Co., Japan). After the incubation, 4.0 ml of conc. HCl was added and the mixture was hydrolyzed at 105° for 16 h. Then the amount of [^{3}H]hydroxyproline formed during the incubation time was assayed according to the method described by JUVA AND PROCKOP[27].

Time course for the hydroxylation. 0.4 ml of the 15 000 × *g* supernatant solution (protein concentration, 4.60 mg/ml) was incubated with 0.4 ml of 10-times diluted [^{3}H]proline-labeled protocollagen solution in the incubation medium (final volume 4.0 ml) at 37° for 20, 40, 60, 80 and 100 min.

Effect of catalase on protocollagen hydroxylation. 1.0 ml of enzyme solution (4.72

mg/ml protein concentration) was incubated with 0.5 ml of 10-times diluted [^{3}H]-proline-labeled substrate in 4.5 ml of the incubation medium at 37° for 60 min with or without catalase (0.05 mg/ml).

Preparation of hydroxylase source in isolated liver cells

After their isolation the parenchymal and endothelial cells from 3 rats were each combined. In the case of the parenchymal cells, the nonviable cells, which were stained with trypan blue, were also collected from the pellet resulting from the centrifugation of the bovine serum albumin cell suspension solution and overlaid with Ca^{2+}-free Hanks' solution. Each cell fraction was homogenized, using a glass homogenizer, in 1.5 ml of the solution consisting of $1 \cdot 10^{-4}$ M dithiothreitol, 0.05 M KCl and 0.01 M Tris adjusted to pH 7.6. The homogenate was centrifuged at 15 000 × *g* for 1 h at 4°. The supernatant was used as a crude enzyme source.

Assay of protocollagen proline hydroxylase in isolated liver cells

A portion of the enzyme source was assayed for protein and 1.0 ml of the solution was incubated for 60 min at 37° with 0.5 ml of 10-times diluted [^{3}H]proline-labeled protocollagen solution in a final 4.5 ml of the incubation medium.

RESULTS

Cell isolation

Parenchymal cells. The cells from the layer at the bovine serum albumin solu-

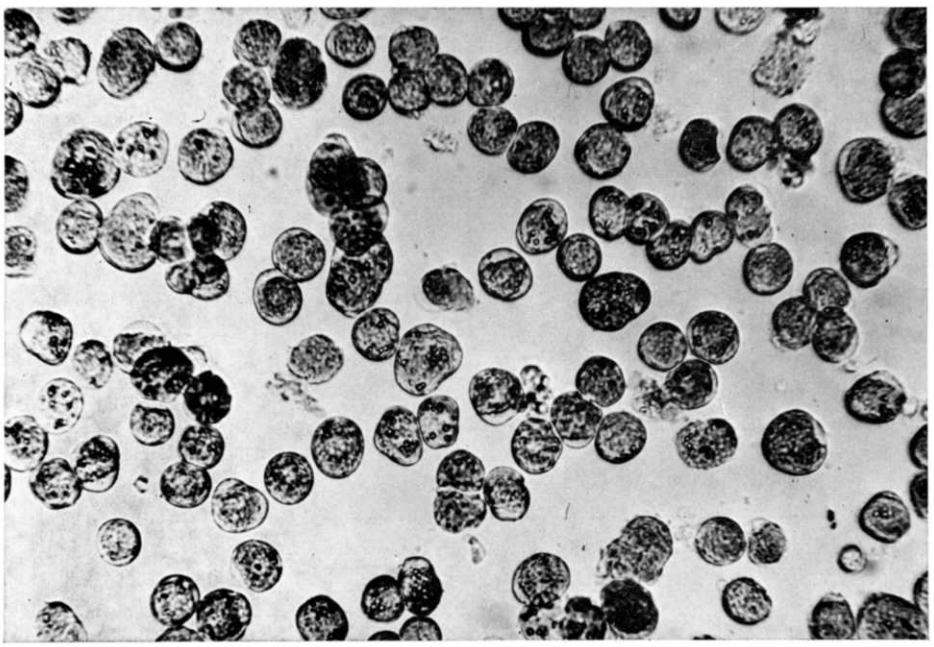


Fig. 1. Light micrograph (× 100) of isolated liver parenchymal cells suspended in Ca^{2+}-free Hanks' solution containing 0.25% trypan blue. More than 95% of the cells are not stained and the contamination by non-parenchymal cells is less than 4%.

TABLE I

NUMBERS OF CELLS, TRYPAN BLUE VIABILITY AND PURITY OF ENDOTHELIAL CELLS AND PARENCHYMAL CELLS

Viable cells are the cells which are not stained with trypan blue, nonviable cells are stained with trypan blue. Purity of endothelial cells is the % of non-parenchymal cells in total cells. Purity of viable parenchymal cells is the % of unstained parenchymal cells in total cells. The values in parentheses represent the purity of parenchymal cells including trypan bluestained cells and unstained cells in total cells.

	Number of cells	*Trypan blue viability (%)*	*Purity (%)*
Endothelial cells			
I	$1.45 \cdot 10^7$	99.8	99.7
II	$1.76 \cdot 10^7$	99.8	99.8
III	$2.60 \cdot 10^7$	99.8	99.6
Parenchymal cells			
Viable cells			
I	$1.98 \cdot 10^6$	95.2	92.3 (96.9)
II	$2.23 \cdot 10^6$	96.1	95.8 (97.6)
III	$2.23 \cdot 10^6$	96.1	95.8 (97.6)
IV	$4.71 \cdot 10^5$	95.2	93.4 (98.0)
Nonviable cells			
I	$1.94 \cdot 10^6$	3.0	95.3
II	$1.94 \cdot 10^6$	3.0	95.3
III	$3.89 \cdot 10^6$	3.0	95.3

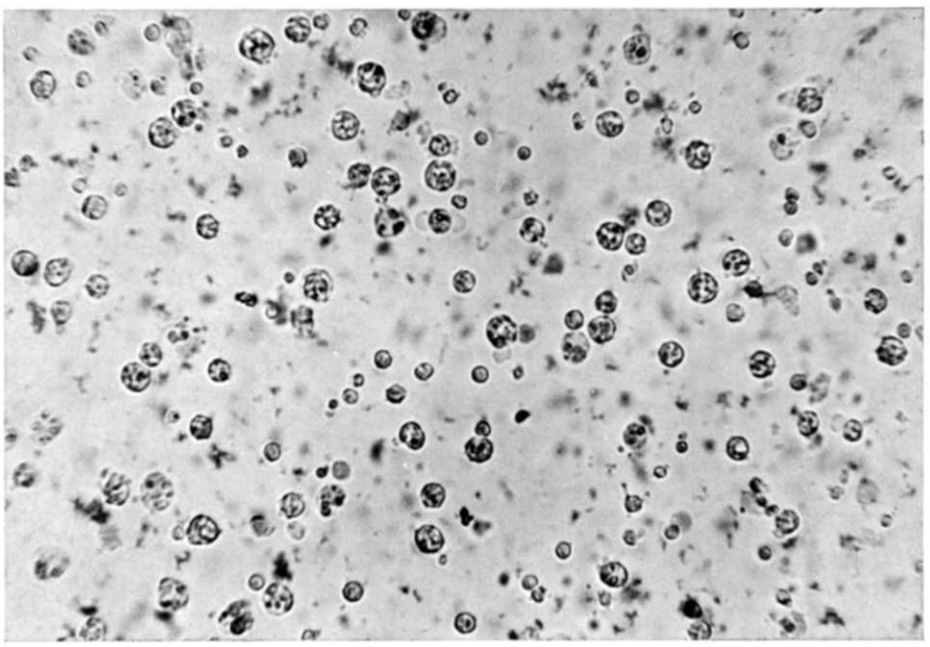
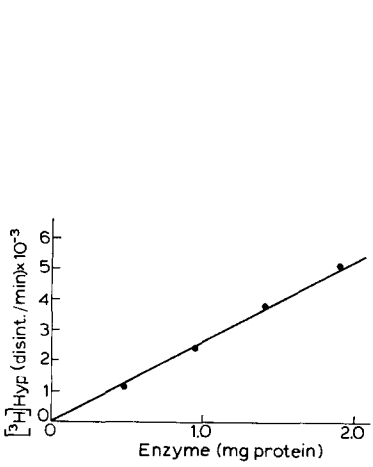


Fig. 2. Light micrograph (× 100) of isolated endothelial cells suspended in Eisen's solution containing 0.25% trypan blue. Trypan blue viability is more than 99.8% and contamination by parenchymal cells is less than 0.4%. The larger cells have a strong phagocytotic activity.



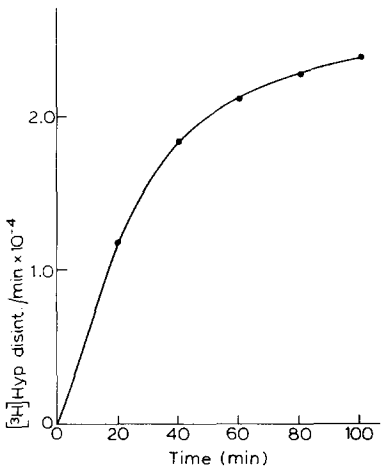


Fig. 3. The relationship between [^{3}H]hydroxyproline formed and the amount of enzyme from liver homogenate. Standard assay system is as described under MATERIALS AND METHODS. Each point represents the average of duplicate experiments.

Fig. 4. Time course for the hydroxylation of [^{3}H]proline-labeled protocollagen. Standard assay system is as described under MATERIALS AND METHODS. Each point represents the average of duplicate experiments.

tion–Ca^{2+}-free Hanks' solution interface were nearly all viable. The trypan blue exclusion test showed that more than 95% of the cells were not stained (Fig. 1). The outlines of the cells were clear and most cells were not stained with trypan blue. 5% of the cells were stained weakly, but were not completely stained as are cells which are obtained by the mechanical or the chelating agent methods. Contamination by non-parenchymal cells was less than 4%. Most of the cells from the bovine serum albumin solution pellet were stained with trypan blue; only 3% of the cells were not stained. The purity of the dead parenchymal cells was about 95% (Table I).

Endothelial cells. Fig. 2 illustrates the trypan blue viability test (×100). More than 99% of the cells were not stained with trypan blue and the contamination of the parenchymal cells was less than 0.4% (Table I). The larger cells in Fig. 2 showed strong phagocytotic activity towards carbon particles that had been given intravenously.

Protocollagen proline hydroxylase assay in liver tissue

As shown in Fig. 3, linearity was observed between the amount of [^{3}H]hydroxy-

TABLE II

EFFECT OF CATALASE ON THE HYDROXYLATION OF [^{3}H]PROLINE-LABELED PROTOCOLLAGEN

Figures represent mean ± S.E. The number of observations is given in parentheses. Standard assay system is as described under MATERIALS AND METHODS.

Catalase (mg/ml)	*[^{3}H]Hyp formed during incubation time (disint./min)*
0	5521 ± 49 (3)
0.05	7114 ± 78 (3)

TABLE III

PROTOCOLLAGEN PROLINE HYDROXYLASE IN ENDOTHELIAL CELLS AND IN PARENCHYMAL CELLS
Standard assay system is as described under MATERIALS AND METHODS.

Endothelial cells	*Enzyme (μg protein)*	*Enzyme (μg)/10⁷ cells*	*Hydroxylase activity ([³H]Hyp disint./min)*	
			per 10⁷ cells	*per μg protein*
I	340	235	53	0.23
II	380	216	34	0.21
III	440	169	58	0.34
Parenchymal cells	*Enzyme (μg protein)*	*Enzyme (μg)/10⁶ cells*	*Hydroxylase activity ([³H]Hyp disint./min)*	
			per 10⁶ cells	*per μg protein*
Viable cells				
I	460	232	497	2.14
II	740	332	697	2.10
III	740	332	637	1.92
IV	200	425	909	2.13
Nonviable cells				
I	160	83	140	1.70
II	180	93	193	1.88
III	400	103	179	1.72

proline formed and the enzyme concentration. Fig. 4 shows that hydroxylation of [³H]proline-labeled protocollagen was still occuring after 100 min incubation. Catalase increased the hydroxylation by about 29%, as shown in Table II.

Protocollagen proline hydroxylase in isolated liver cells

Table III illustrates the hydroxylase activity in the endothelial cell fraction and in parenchymal cells. In the endothelial cell fraction, the activity of protocollagen proline hydroxylase was very low, while in the parenchymal cells, especially in the cells not stained with trypan blue, the activity was more than 100 times higher in terms of the number of cells. Comparing viable parenchymal cells which were not stained with trypan blue and nonviable parenchymal cells, the hydroxylase activity per cell of the viable cells was more than 4 times higher than in the nonviable cells. When the activity was expressed per μg of cell protein, the difference between viable cells and nonviable cells was not so large.

DISCUSSION

Liver parenchymal cells have been prepared by various methods such as disruption by mechanical means[1,4–8], dissociation with chelating reagents, *e.g.* citrate[2,9–12], tetraphenylboron[11,13,14] or EDTA[9], and digestion of the intracellular matrix by enzymatic techniques with trypsin[15] or with collagenase and hyaluronidase[16,17]. When the viability of cells prepared by mechanical methods or with chelating agents, was tested with 0.25% trypan blue staining, all cells were found to

take up the vital stain and were therefore no longer viable. Mechanical methods or chelating agent methods therefore appear to be inadequate for preparing physiologically intact cells. JEZYK AND LIBERTI[18] proved that mechanically prepared cells were inferior to enzymatically prepared cells in their ability to synthesize protein, RNA and lipid, and to respire with and without added substrate. Trypsin digestion of liver slices yielded trypan blue viable cells with very low yields and most of the viable cells showed bleb formation. Using collagenase and hyaluronidase, we could disperse trypan blue viable cells which were scarcely blebbed in contrast to the cells prepared by trypsin. The viability of these cells was 20 to 30% and was not so high as reported by HOWARD AND PESCH[16]. They could isolate the cells with 75 to 95% viability but we were unable to raise the viability up to 30% in spite of following their methods closely. In order to obtain a high percentage of viable parenchymal cells, we applied the bovine serum albumin flotation method which had been used by ROSER[3] to isolate Kupffer cells from mouse liver. By the application of the bovine serum albumin flotation method, we eventually succeeded in preparing trypan blue viable cells with viability of more than 95% which was higher than that reported by HOWARD AND PESCH[16]. The trypan blue viable cells have intact cell membranes, so, under gravitation in the bovine serum albumin solution at high density, they will be able to float while nonviable cells with broken cell membranes will be unable to float and will sediment on centrifugation.

Amongst several methods for preparing endothelial cells, ROSER's[3] method was superior to any of the methods[1,19,20] we have so far examined. We modified ROSER's method such that the enzyme solution contained only 0.04% pronase P in Eisen's solution, since 0.001% deoxyribonuclease I (Sigma Chemical Co., U.S.A.) had no effect on the preparation of the rat liver endothelial cell fraction. The cells could be collected without aggregation in the absence of deoxyribonuclease I. In order to reduce the contamination of parenchymal cells in the endothelial cell fraction, the liver had to be cut into small pieces with scissors. The parenchymal cells might therefore be damaged and no longer able to float in bovine serum albumin solution and scarcely appear in the final endothelial cell fraction. Too much mincing reduced the yield of endothelial cells. In the endothelial cell fraction prepared after intravenous injection of carbon particles, about 70% of the cells phagocytized the carbon particles. ROSER[3] also showed that approx. 85% of the cells from mouse liver were phagocytotic Kupffer cells, by using radioautography after injection of radioactive colloidal gold. With the aid of electron microscopy, we could find very few fibroblasts with rough endoplasmic reticulum in this cell fraction. Most of the cells seemed to be Kupffer cells with welldeveloped microvilli and phagosomes. The remaining cells had poor cytoplasm and round nuclei and did not seem to be phagocytotic cells.

In the normal liver, small amounts of collagen are found in the space of Disse, apart from the Glisson's capsule, where there seem to be no fibroblasts. What types of cell participate in the synthesis of the collagen has not been solved. In the present investigation, we could suggest that the endothelial cells including Kupffer cells did not significantly contribute to collagen synthesis in the normal liver as they had only slight protocollagen proline hydroxylase activity. GOLDBERG AND GREEN[28] reported that in the alveolar macrophage there is no protocollagen proline hydroxylase, while in H_4II–EC_3 hepatoma cells, there is hydroxylase activity and collagen synthesis but at a small rate. In our experiment, protocollagen proline hydroxylase

was found to be about 100 times higher in the normal parenchymal cells than in the endothelial cells in terms of the number of cells. The contamination by endothelial cells of the parenchymal cell fraction would not affect the hydroxylase activity in the parenchymal cells as they had little hydroxylase activity. By rough calculation on the assumption that about 70% of the liver cells are parenchymal cells, we may say that the activity of hydroxylase found in normal liver homogenate will almost all be due to the parenchymal cells. So there arises the possibility that the liver parenchymal cells might be able to synthesize collagen in the space of Disse, though the presence of the hydroxylase does not warrant production of collagen.

In the trypan blue stained parenchymal cells there was hydroxylase activity in about one-quarter of the trypan blue viable cells. When the hydroxylase activity was expressed per μg of protein, there was less difference between the viable and the nonviable cells. This suggests that the trypan blue stained cells have damaged cell membranes and leak soluble proteins during the cell isolation procedures. The protocollagen proline hydroxylase which is in the soluble fraction of the parenchymal cells would also be lost. TAKEDA *et al.*[29] reported that the enzymes in the soluble fraction, for example glutamate–pyruvate transaminase, lactate dehydrogenase and serine dehydrogenase were released during the parenchymal cell isolation procedure with sodium citrate.

In the fibrotic liver, collagen content increases with a preceding increase in protocollagen proline hydroxylase activity in the liver homogenate[30]. Large amounts of collagen appear in the space of Disse and interparenchymal cells. POPPER[31] suggested that Kupffer cells might act as fibroblasts in cirrhotic liver and YABUMASU[32] frequently observed, using electron microscopy, that collagen was synthesized in the intraparenchymal cells. There is little knowledge of what type of cell synthesizes collagen in the fibrotic liver. In this regard, we are now investigating whether parenchymal cells and endothelial cells take part in the synthesis of collagen during carbon tetrachloride-induced liver fibrosis.

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