Studies on Eel Liver Functions Using Perfused Liver and Primary Cultured Hepatocytes

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Abstract
Perfused eel livers, isolated eel hepatocytes, and cultured eel hepatocytes are used to investigate eel liver functions such as gluconeogenesis, glycogen synthesis, and lipoprotein synthesis and methods for preparation are described. A novel phosphoenolpyruvate (PEP) synthesis pathway from pyruvate in gluconeogenesis in eel liver was elucidated and PEP synthesis pathways in eel, rat, and pigeon livers were compared. Glycogen synthesis from pyruvate, lactate, and glucose was investigated by using cultured eel hepatocytes. It was found that $10^{-6}$ or $10^{-7}$ M glucagon didn’t stimulate glycogen degradation in the presence of pyruvate but stimulated glycogen degradation in the presence of lactate. Glycogen synthesis from pyruvate was observed even when $10^{-6}$ or $10^{-7}$ M glucagon was present. The characteristics of the lipoprotein synthesized and secreted by cultured eel hepatocytes are clarified. Thyroxine and eel serum high-density lipoprotein (HDL) stimulated the lipoprotein synthesis by cultured eel hepatocytes. In the presence of estradiol-17β, eel serum HDL stimulated vitellogenin synthesis in eel hepatocytes. HDL specifically bound to eel hepatocytes and the ligand of HDL receptor in the plasma membrane of eel hepatocytes was identified to be ganglioside GM4 of eel serum HDL.

1. Introduction

A liver has many functions such as gluconeogenesis, synthesis of serum proteins, lipid synthesis, and detoxification. Hormones, cytokines, and many metabolites in serum regulate liver functions in vivo. These materials are produced by various extra-hepatic tissues as well as by liver. Therefore, isolation of a liver from a living animal is necessary for investigating liver functions. A perfused liver, isolated hepatocytes, and primary culture of hepatocytes have been developed for investigating such functions.

A perfused liver was used for the first time when Claude Bernard (1957) investigated the conversion of glycogen to glucose in a liver. However, this technique was not widely accepted until the early 1960s when Miller et al. (Miller et al. 1951; Miller 1973) developed an apparatus and an operative procedure for the perfusion of isolated rat liver. A perfused liver is able to keep liver functions active for 2 to 3 h and so is usually available for experiments lasting 2 to 3 h.

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The current methods of isolated hepatocytes are based on the procedure of Berry and Friend (1969). Berry (1976) has pointed out that any successful method for liver cell separation must involve three critical steps, namely exposure of the liver to a calcium-free medium, digestion with collagenase, and gentle mechanical treatment. Liver cell suspension consists of several kinds of cells, which are hepatic parenchymal cells (hepatocytes), endothelial and epithelial cells, Kupfer cells, and Ito cells. The hepatocytes constitute the major and most representative cellular subpopulation in terms of liver functions and are easily separated from other liver cells by low-speed centrifugation of about 60 × g for 90 s. Isolated hepatocytes, as well as a perfused liver are available for around 2 to 3 h for experiments, but many experiments with different conditions can be conducted by using isolated hepatocytes prepared from one liver.

Primary culture of hepatocytes has been used for investigating liver functions since Bissell et al. (1973) cultured adult rat hepatocytes as a monolayer. Cultured hepatocytes maintain liver functions for several days in a culture medium (Gómez-Lechón and Castell 1994).

A perfused liver, isolated hepatocytes, and cultured hepatocytes are used to investigate fish liver functions as well as mammalian liver. Particularly, all these three methods are available for studying eel liver functions. Eel livers also have many important functions as have mammalian livers. Their functions are gluconeogenesis (Hayashi and Ooshiro 1975a, 1979), lipogenesis (Ndiaye and Hayashi 1996), and synthesis and secretion of serum proteins (Yu et al. 1991; Hayashi and Yu 1993).

Here, gluconeogenesis, gluconeogenic pathway, glycogen synthesis, lipoprotein synthesis, high-density lipoprotein (HDL) metabolism, and vitellogenin synthesis in eel liver are described. In addition, integrity of a perfused eel liver, isolated and cultured eel hepatocytes is also discussed.

2. Eel as an experimental fish for studying liver functions

2-1. Availability of eel (Anguilla japonica) as an experimental fish

A perfused liver (Hayashi and Ooshiro 1975a, 1975b), isolated liver cells (Hayashi and Ooshiro 1978), and cultured hepatocytes (Hayashi and Ooshiro 1985c, 1986; Hayashi 1988, 1991; Hayashi and Komatsu 1998) have been used for investigation of eel liver functions. Eel liver should be made available for these experimental methods. There are five reasons why eel liver should be available. (1) Compared to those of other fishes, eel portal and hepatic veins are thicker and stronger, making it possible to insert polyethylene tubing into the veins. Furthermore, the portal vein of eel is very easy to recognize because of the short length of the intestine. (2) Eels of 200 to 250 g body weight have livers (2 to 3 g) large enough for perfusion. (3) Eels can be kept in fresh water in a laboratory for several months without feeding. (4) Eel liver is a separate organ from the pancreas, but in other fishes such as carp the liver and pancreas coexist as a hepatopancreas and perfusing such a liver is impossible. (5) Eels weighing 200 to 250 g can be obtained at any season throughout the year, because eels are actively aquacultured in freshwater at 25 to 31°C at Kagoshima, Japan.

2-2. Perfusion of eel liver

Perfusion apparatus is shown in Fig. 1 and is set up in a box kept at 28°C with a thermoregulator and a hair-dryer. The perfusion medium is Krebs–Ringer bicarbonate buffer (pH 7.4 ± 0.1) oxygenated continuously with 5% CO₂/95% O₂. Eel liver, portal vein, intestine, and heart are shown in Fig. 2A. The pancreas is found around the portal vein like an adipose tissue on the intestine and the gall bladder is contacted and connected to the liver and is hidden at the back of the liver in Fig. 2A. Figure 2B shows how to connect polyethylene tubings into a liver. Detailed procedures of perfusion of eel liver have previously been described in the papers by Hayashi and Ooshiro (1975a), Hayashi (1991), and Hayashi and Komatsu (1998).
2.3. Primary culture of eel hepatocytes

2.3A. Isolated eel hepatocytes

Isolated eel hepatocytes are prepared by collagenase digestion of a perfused liver (Hayashi and Ooshiro 1978). The average number of isolated eel hepatocytes prepared from one eel liver

Fig. 1. Perfusion apparatus. (I) A, flask (100 mL); B, silicon tubing (100 cm, 2-mm inner diameter, 4-mm outer diameter); C, reservoir; D, three-way cock; E, silicon tubing (15 cm, 4-mm inner diameter, 6-mm outer diameter); F, platform; G, polyethylene tubing for O₂ and CO₂; a, about 6 cm; b, about 7 cm; I, 2, silicon tubing (1-mm inner diameter, 3-mm outer diameter). (II) A, peristaltic pump; B, reservoir; C, tube for O₂ and CO₂; D, flask; E, stirrer; F, platform; G, J, thermoregulator; H, hair dryer; I, pH meter; L, liver. Reproduced with permission from Primary culture of eel hepatocytes—Synthesis and secretion of lipoprotein, Hayashi and Komatsu. In: Cell & Tissue Culture: Laboratory Procedures, Doyle, Griffiths, and Newell, eds. 1998 © John Wiley & Sons Limited.
(2 to 2.5 g) is $5 \times 10^8$ hepatocytes. If $5 \times 10^8$ of hepatocytes are used in one experiment, up to 100 experiments are possible using one liver. Though a perfused liver is good experimental material for investigating liver functions, one experiment requires one eel. Since eel as an experimental fish is not homogeneous in age, sex, nutritional conditions, heredity, and weighing, we do sometimes get results with relatively large deviations by using perfused eel livers. Isolated eel hepatocytes can remove such obstacles because many experiments are possible by using isolated hepatocytes prepared from one liver.

A stringent test of the metabolic integrity of hepatocytes is the ability to synthesize glucose from pyruvate or lactate, because gluconeogenesis involves both the mitochondrial and cytosolic fractions of hepatocytes working in union. Furthermore, four moles of ATP and two moles of GTP (or ITP) are required for the synthesis of one mole of glucose from two moles of lactate or pyruvate. The ability to synthesize glucose from lactate, pyruvate, or alanine is stable at least for 60 min in isolated eel hepatocytes (Hayashi and Ooshiro 1978, 1979). However, as described by Söling and Kleineke (1976), the functional state of isolated hepatocytes are sometimes impaired in comparison to intact livers. The results obtained with isolated hepatocytes must be interpreted with great caution.

Fig. 2. (A) Eel liver, portal vein, intestine and heart. (B) Polyethylene tubing inserted into portal and hepatic veins. I, Insert polyethylene tubing into the portal vein and fix by ligation. II, Remove a liver with a gall bladder, a part of the intestine and the heart from the body. III, Separate the heart from the liver. Insert another polyethylene tube into the hepatic vein between the heart and the liver, and fix by ligation. Reproduced with permission from Primary culture of eel hepatocytes—Synthesis and secretion of lipoprotein, Hayashi and Komatsu. In: Cell & Tissue Culture: Laboratory Procedures, Doyle, Griffiths, and Newell, eds. 1998 © John Wiley & Sons Limited.
As shown in Fig. 3, isolated eel hepatocytes could be cultured in Williams’ E medium (WE medium) with 0.16 μM insulin and 5 to 10% fetal bovine serum (FBS) (Table 1A) at 28°C in a CO₂ incubator (Yu et al. 1991; Ndiaye and Hayashi 1996; Hayashi and Komatsu 1998). WE medium contains 11 mM glucose, 20 kinds of amino acids, some kinds of vitamins and inorganic salts. Isolated hepatocytes are necessary to attach the surface of culture dishes for culture. However, eel hepatocytes are difficult to attach and the dishes precoated with fibronectin or poly L-lysine must be prepared to make the eel hepatocytes attach. Poly-L-lysine precoated on culture dishes could make eel hepatocytes attach to the dishes electrostatically. Fibronectin, purified from horse serum by a gelatin-Sepharose column, is an extracellular matrix and allows eel hepatocytes to attach by its biological activities (March et al. 1974; Dessau et al. 1978).

Kondo and Watabe (2006) have reported the effects of carp serum on goldfish culture cells and carp serum has a more extensive growth promoting effect compared with FBS. In general, fish serum contains high concentrations of lipoproteins, particularly high-density lipoprotein (HDL). Concentration of fish serum HDL is several to 10 times higher than that of human (Babin and Vernier 1989), but in contrast to HDL, albumin is a very minor component in fish serum.

Fig. 3. Eel hepatocytes cultured 0 and 5 days at 30°C. Bar represents 40 μm.
Although about half the amount of serum proteins is albumin in human serum. Therefore, in fish cell culture, the use of FBS is not the best choice. Kondo and Watabe (2006) have examined the components of carp serum to promote growth of goldfish cells and they have found lipoprotein fraction containing HDL has a promoting effect. However, the mechanism is still uncertain but fish cells seem to require more lipid than mammalian cells.

Eel hepatocytes were cultured in a serum-free medium, as shown in Table 1B, for 2 to 3 weeks (Hayashi and Ooshiro 1986). Insulin, glucagon, prolactin, growth hormone, epidermal growth factor (EGF), and H$_2$SeO$_3$ were used for culture instead of FBS. The serum-free medium is the modified medium reported by Enat et al. (1984) and all these hormones and EGF originated from mammals. Asami et al. (1984) have reported that rat hepatocytes do not survive in a serum-free medium over 2 days without a trypsin inhibitor because a trypsin-like enzyme is present in the plasma membrane of rat hepatocytes and this enzyme damages cultured rat hepatocytes. As FBS contains a trypsin inhibitor, rat hepatocytes can be cultured in Williams’ medium E with 10% FBS. Eel hepatocytes could be cultured in a serum-free medium without a trypsin inhibitor.

To investigate the effect of hormones or EGF in a serum-free medium on cultured eel hepatocytes, the eel hepatocytes were cultured in a serum-free medium deprived of one hormone or EGF (Hayashi and Ooshiro 1986). The hepatocytes cultured for 11 days in a complete serum-free medium are shown in Fig. 4(1). An insulin-deficient medium had the most remarkable effect on the morphology and cell survival of the hepatocytes (Fig. 4(2)). Their morphology was of a round shape like isolated hepatocytes, even after 11 days and the cell numbers were about half compared with those of the control in a complete serum-free medium. This result shows that insulin is essential for the attachment and spreading of eel hepatocytes.

The morphology of the hepatocytes cultured in a glucagon or prolactin-deficient medium was normal during the first 7 days but oil-like droplets in the cultured hepatocyte were observed at 11 days (Figs. 4(3) and (5)). The cell numbers of both hepatocytes were about 60% of the control cells. The hepatocytes cultured in an EGF or growth hormone-deficient medium had a normal morphology but the EGF deficient medium made the cell numbers decrease to about 60% of the control cells (Figs. 4(4) and (6)).

3. Glucose metabolisms in eel liver

3-1. Gluconeogenesis in rat liver

Generally, gluconeogenesis means glucose synthesis from pyruvate or lactate. Four moles of ATP and two moles of GTP (or ITP) are required for the synthesis of one mole of glucose from two moles of pyruvate or lactate. The pathway of gluconeogenesis is principally the reverse of the glycolysis pathway except for three steps, specifically between glucose and

<table>
<thead>
<tr>
<th>A (100 mL)</th>
<th>B (100 mL)</th>
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<tbody>
<tr>
<td>William’s E medium</td>
<td>1.1 g</td>
</tr>
<tr>
<td>Penicillin</td>
<td>7 mg</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>23 mM</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.16 μM</td>
</tr>
<tr>
<td>Others</td>
<td>FBS 5 or 10 mL</td>
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Table 1. Culture medium: A, Williams’ E medium containing fetal bovine serum; B, Williams’ E medium without serum.
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When lactate is used as a substrate for gluconeogenesis, lactate is converted to pyruvate by lactate dehydrogenase (LDH) in the cytosol. Then the pyruvate passes through the mitochondrial membrane and is converted to oxaloacetate by pyruvate carboxylase. Because the liver pyruvate carboxylase of all animals investigated so far is localized in the mitochondria (Gumbmann and Tappel 1962; Böttger et al. 1969; Garber and Hanson 1971) and oxaloacetate can not pass through the mitochondrial membrane (Haslam and Griffiths 1968), the reaction of PEP-synthesis from oxaloacetate by PEP-carboxykinase should be carried out in the mitochondria. However, subcellular distribution of PEP-carboxykinase is different among animals. Three subcellular distributions of PEP-carboxykinase are known. PEP-carboxykinases of pigeon (Gevers...
1967), rat (Böttger et al. 1969), and eel (Hayashi and Ooshiro 1979) liver distribute in the mitochondria, in the cytosol, and in both the mitochondria and the cytosole, respectively. Therefore, the step from pyruvate to PEP boxed in Fig. 5 is rather complex. In rat liver PEP-carboxykinase distribute in the cytosol, therefore oxaloacetate, which cannot pass through the mitochondrial membrane, must be transported to the cytosol from the mitochondria. In rat liver, PEP is synthesized from oxaloacetate and GTP by cytosolic PEP-carboxykinase in the cytosol (Böttger et al. 1969). This transportation of oxaloacetate to the cytosol is done by an aspartate-oxaloacetate or a malate-oxaloacetate pathway.

When lactate is used as a gluconeogenic substrate, lactate is converted to pyruvate by a cytosolic enzyme of LDH and, at the same time, an equivalent amount of NADH is formed. But when pyruvate is used, about half of the pyruvate is converted to lactate by LDH, as shown in Fig. 6B (Krebs 1968) and so an equivalent amount of NADH in the cytosol is consumed. Therefore, cytosolic NADH is increased when lactate is used as a gluconeogenic substrate, but decreased when pyruvate is used. NADH in the cytosol is necessary for glyceraldehyde-3-phosphate dehydrogenase reaction in gluconeogenesis, as shown in Fig. 5. The reason why the quantity of the cytosolic NADH is changed by lactate or pyruvate seems to be due to the muscle-type LDH of rat liver (Fine et al. 1963; Ogihara 1975).

There are two types of LDH-isozymes. One is muscle-type and the other is heart-type. Catalytic properties of heart- and muscle-type LDHs are different from each other. It is known that substrate inhibition of LDH is due to the formation of a ternary complex such as LDH-NAD*-pyruvate (Everse et al. 1971). The formation of a ternary complex proceeds more readily.

Fig. 5. Pathway of gluconeogenesis. * and ** represent glucose-6-P phosphatase and fructose-1,6-P2 phosphatase for gluconeogenesis, respectively. The PEP synthesis pathway is boxed.
with heart-type LDH than with muscle-type LDH. As shown in Fig. 6B, about half of the pyruvate is converted to lactate and this is due to little inhibition of rat liver LDH by the pyruvate. If rat liver LDH is of the heart-type, little lactate is produced, as shown in Fig. 6A which also shows gluconeogenesis from pyruvate by a perfused eel liver. Eel liver LDH is heart-type, as described in Section 3-2A.

In rat liver when pyruvate is used as a gluconeogenic substrate, the malate–oxaloacetate pathway is used to supplement the decreased cytosolic NADH, as shown in Fig. 7 (Lardy et al.)

Fig. 6. Comparison of gluconeogenesis from pyruvate by the (A) perfused eel and (B) rat liver. (A) The initial concentration and radioactivity of 14C-pyruvate were 5 mM and 4.63 kBq mL⁻¹, respectively; (B) The initial concentration of pyruvate was 5 mM (data from Table 3 in Krebs 1968). Reproduced from Hayashi, Gluconeogenesis. In: Metabolism in Fish, Nagayama, ed., 1983. Kouseisha Kouseikaku Co., Ltd. with permission of the Japanese Society of Fisheries Science and Kouseisha Kouseikaku Co., Ltd.

1965). The pathways within the box are the mitochondrial pathways. NADH formed by malate dehydrogenase in the cytosol is used for glyceraldehyde-3-phosphate dehydrogenase in the gluconeogenetic pathway, but when lactate is used, the aspartate–oxaloacetate pathway is used for gluconeogenesis. These metabolic pathways were also confirmed by inhibitors of enzymes. Amino-oxyacetate (AOA), D-malate, and quinolinic acid inhibit glutamate-oxaloacetate transaminase, malate dehydrogenase and PEP carboxykinase, respectively. PEP-carboxykinase in the cytosol is also inhibited by tryptophan. In rat liver, AOA inhibits gluconeogenesis from lactate, but not from pyruvate, while D-malate inhibits gluconeogenesis from pyruvate, but not from lactate. Thus, the aspartate–oxaloacetate pathway functions for gluconeogenesis from lactate and the malate–oxaloacetate pathway functions for that from pyruvate (Krebs et al. 1967; Rognstad and Katz 1970; Anderson et al. 1971; Arinze et al. 1973; Arinze and Rowley 1975; Meijer et al. 1978).

3-2. Gluconeogenesis in eel liver

3-2A. Gluconeogenesis by perfused eel liver

Gluconeogenesis from lactate (10 mM) by a perfused eel liver is shown in Fig. 8. Glucose was determined by the colorimetric method using a o-toluidine-borate reagent (Sasaki 1969) and lactate was determined by the enzymatic method (Noll 1984). The rate of glucose synthesis from lactate was $0.90 \pm 0.07 \mu mol \text{ (g-liver)}^{-1} \text{ min}^{-1}$. The rate of removal of lactate during the initial 30 min was $4.5 \mu mol \text{ (g-liver)}^{-1} \text{ min}^{-1}$ and that during the following 30 min was $2.07 \mu mol \text{ (g-liver)}^{-1} \text{ min}^{-1}$ (Hayashi and Ooshiro 1975a). During the initial 30 min, lactate was used in another metabolic pathway as well as gluconeogenesis. However, in the following 30 min, gluconeogenesis was the main fate of lactate and one mole of glucose was synthesized from 2 moles of lactate. The ability of gluconeogenesis by a perfused eel liver was almost the same as that by a perfused rat liver (Hems et al. 1966), although the experimental temperature at 28°C for eel was lower than that at 37°C for rat.

Eel livers contain glycogen in spite of a long starvation period such as several months (Larsson and Lewander 1973; Dave et al. 1975). Therefore, glucose produced by gluconeogenesis in the perfusion medium can not be distinguished from glucose produced by glycolysis of glycogen. To avoid this confusion, a radio-isotope labeled precursor was used for determining the gluconeogenesis. Gluconeogenesis from 5 mM $^{14}$C-pyruvate by a perfused eel liver is shown in Fig. 6A (Hayashi et al. 1982b; Hayashi 1983). The incorporation of $^{14}$C-pyruvate into glucose was determined as follows. A part of the perfusion medium (0.5 mL) was taken at the indicated time and applied to pencil-type columns of Dowex 1 × 8 (formate type) and Dowex 50 × 8 (H+ type). $^{14}$C-Glucose was eluted by 4 mL of water and $^{14}$C-glucose in the eluate was measured...
by a liquid scintillation counter (Beckman LS-230). Lactate and pyruvate were determined by enzymatic methods (Noll 1984; Lamprecht and Heinz 1984).

Gluconeogenesis from pyruvate by a perfused rat liver prepared from a starved rat is also shown in Fig. 6B (Krebs 1968). As there is almost no glycogen in the liver of rat starved for 48 h, the glucose in the perfusion medium is postulated to be derived from gluconeogenesis. A remarkable difference between eel and rat is the production of lactate. As shown in Figs. 6A and B, lactate production at 20 min perfusion in eel and rat perfused liver was 1.6 and 43 μmol (g-liver)^{-1}, respectively. Pyruvate consumption at 20 min perfusion in eel and rat perfused liver was 40 and 85 μmol (g-liver)^{-1}, respectively. Whereas glucose production at 20 min perfusion in eel and rat perfused liver was 15 and 16 μmol (g-liver)^{-1}, respectively.

The difference of lactate production between eel and rat liver is due to the difference of the type of LDH. The LDH of rat liver is of the muscle type (Fine et al. 1963; Ogihara 1975), whereas that of eel liver is of the heart type, as shown in Fig. 9 (Hayashi et al. 1985). Furthermore, LDH types of cod (Sensabaugh and Kaplan 1972), rainbow trout (Bailey and Wilson 1968), and other fish livers (Whitt et al. 1975) have been reported to be of the heart type. Catalytic properties of heart- and muscle-type LDHs are different as described in Section 3-1. The substrate inhibition proceeds more readily with heart-type enzyme than with muscle-type enzyme. Actually, the activity of the eel liver LDH is inhibited by 70% in the presence of 1 mM pyruvate and NAD+, whereas that of the eel muscle LDH is inhibited only by 9% (Hayashi et al. 1985). As shown in Fig. 6A, little lactate formation from pyruvate seems to be due to the substrate inhibition of the eel liver LDH. The heart type of the eel liver LDH seems to induce a different PEP synthesis pathway in gluconeogenesis from pyruvate from that of rat, as described in Section 3-1. The PEP synthesis pathway in the eel liver will be described in Section 3-3.

Gluconeogenesis in eel liver from 14C-lactate was investigated by a non-circulated perfusion system to measure the mean time for conversion of lactate to glucose. As shown in Fig. 10, the mean time for gluconeogenesis from lactate was 60 ± 15 sec (Hayashi and Ooshiro 1975b). This mean time is the same as that of a perfused rat liver, about 75 sec (Exton and Park 1967).
3-2B. Gluconeogenesis by isolated eel hepatocytes and cultured eel hepatocytes

The average gluconeogenesis from 5 mM 14C-pyruvate, 14C-lactate, and 14C-alanine by isolated eel hepatocytes were 19 ± 7 (n = 5), 24 ± 3 (n = 4), and 11 ± 2 (n = 4) μmol (g-wet weight)−1 h−1, respectively (Hayashi and Ooshiro 1979). These were determined by the incorporation of 14C-substrate into glucose. The reaction mixture with 2 mL of final volume contained 1 mL of isolated eel hepatocytes, 20 μL of 14C-substrate and a Krebs–Ringer bicarbonate buffer gassed with 95% O2/5% CO2. The mixture was incubated at 30°C for 60 min. The reaction was initiated by the addition of isolated eel hepatocytes and stopped by the addition of 0.2 mL of 60% HClO4. After centrifugation, the supernatant was neutralized with 2 M K2CO3 and 1 mL of neutralized supernatant was applied to pencil-type columns of Dowex 1 × 8 and Dowex 50 × 8. 14C-Glucose was recovered in the eluate. The wet weight of the cells was calculated from the dry weight and the water content of the cells (73.2 ± 2.8%, n = 10). All these procedures are described in the paper by Hayashi and Ooshiro (1979).

Isolated eel hepatocytes were used within 2 h after the preparation of isolated eel hepatocytes. They retain gluconeogenetic ability within 2 h. The average number of isolated eel hepatocytes prepared from one eel liver is 5 × 10⁸ cells and 50 mL of hepatocytes suspension is possible for 45 experiments on gluconeogenesis.

After eel hepatocytes were cultured in WE-5% FBS (Table 1A) or a WE-serum-free defiened medium (Table 1B) on dishes precoated with fibronectin for 7 to 10 days, the hepatocytes were washed with glucose-free MEM medium containing 0.5% bovine serum albumin (BSA) and incubated in the same medium with or without 10 mM pyruvate or lactate. The medium was removed at the indicated time and the glucose in the medium was determined by the colorimetric method with glucose oxidase and peroxidase (Kunst et al. 1984).

Gluconeogenesis from 10 mM pyruvate and lactate in the cultured eel hepatocytes were determined as 0.104 ± 0.029 (n = 3) (Fig. 11) and 0.032 (n = 2) μmol (mg-cell protein)−1 h−1, respectively. If the protein content of the cultured eel hepatocytes is hypothesized to be 20%, the rates of glucose synthesis from 10 mM pyruvate and lactate are calculated as 21 and 6.4 μmol-glucose (g-cell)−1 h−1, respectively. Gluconeogenesis from 5 mM pyruvate was determined as...
45 μmol (g-liver)⁻¹ h⁻¹ by perfused eel livers and as 19 μmol-glucose (g-cell)⁻¹ h⁻¹ by isolated eel hepatocytes as described above. The value of 21 μmol-glucose (g-cell)⁻¹ h⁻¹ is about 47% and 111% of gluconeogenesis from 5 mM pyruvate obtained by perfused eel livers and isolated eel hepatocytes, respectively. However, as the concentration of 10 mM pyruvate used for the cultured eel hepatocytes was two times higher than those used for the perfused eel livers and for the isolated eel hepatocytes, gluconeogenesis from 5 mM pyruvate and lactate by cultured eel hepatocytes seem to be lower than 21 and 6.4 μmol-glucose (g-cell)⁻¹ h⁻¹, respectively.

3-3. Phosphoenolpyruvate synthesis pathway in eel liver

As described in Section 3-1, PEP synthesis from oxaloacetate in gluconeogenesis is different among animals. These differences are mainly due to the difference of LDH type (heart- or muscle-type) and to the difference of subcellular distribution of PEP-carboxykinase, which catalyze the synthesis of PEP from oxaloacetate and GTP (or ITP).

In rat liver, LDH is muscle-type and PEP-carboxykinase distributes in the cytosole. These factors induce two pathways to transport mitochondrial oxaloacetate to the cytosole, namely aspartate–oxaloacetate pathway and malate–oxaloacetate pathway (Fig. 7). These pathways were confirmed by the investigations of the type of LDH, subcellular distributions of some enzymes, and effects of inhibitors, amino acids, and fatty acids. Effects of amino acids on gluconeogenesis are investigated relating to the aspartate–oxaloacetate pathway containing glutamate-oxaloacetate transaminase reaction. Effects of fatty acids are also investigated relating to the NADH/NAD⁺ ratio by fatty acid oxidation or to glutamate dehydrogenase reaction, which concern the glutamate concentration in the mitochondria.

In the following sections, effects of inhibitors, amino acids, and oleic acid on gluconeogenesis, are described to clarify the PEP synthesis pathway in the eel liver gluconeogenesis and subcellular distribution of enzymes related to gluconeogenesis are also described.

3-3A. Effects of inhibitors

The effects of AOA, α-malate, quinolinic acid, and tryptophan on gluconeogenesis were investigated by isolated eel hepatocytes (Hayashi and Ooshiro 1979). AOA, α-malate, and quinolinic acid inhibit glutamate-oxaloacetate transaminase, malate dehydrogenase, and PEP carboxykinase, respectively. Tryptophan also inhibits PEP carboxykinase in the cytosole as well as quinolinic acid. Because the PEP carboxykinase of pigeon liver distributes in the mitochondria, quinolinic acid has no effect on gluconeogenesis by perfused pigeon livers (Söling et al. 1971).
As shown in Table 2, AOA inhibited gluconeogenesis from lactate and pyruvate by about 60%. However, D-malate, quinolinic acid, and tryptophan had no effect. These results agreed with the report on isolated hepatocytes of rainbow trout (Walton and Cowey 1979). It has been reported that the permeability of quinolinic acid to the plasma membrane is different among animals (Elliot et al. 1977). No effect of quinolinic acid on gluconeogenesis in eel and rainbow trout hepatocytes seemed to be due to the impermeability of the cell membrane to quinolinic acid or to the properties of the enzyme itself.

In eel and rainbow trout liver, D-malate did not inhibit gluconeogenesis from pyruvate. It seems that the oxaloacetate–malate pathway with malate dehydrogenase is not included in the pathway of PEP synthesis in gluconeogenesis from pyruvate. In contrast to the effect of D-malate, AOA, an inhibitor of transaminase, inhibits gluconeogenesis from both pyruvate and lactate. That AOA, but not D-malate, showed an inhibitory effect on gluconeogenesis from pyruvate in eel and rainbow trout liver is remarkably different from gluconeogenesis in rat liver. As described in Section 3-1, when pyruvate is used as a substrate for gluconeogenesis in rat liver, about half of the pyruvate is converted into lactate with the consumption of an equimolar NADH in the cytosole (Fig. 6B) because rat liver LDH is of the muscle type. Therefore, supplying NADH in the cytosole is necessary for glyceraldehyde-3-phosphate dehydrogenase reaction in gluconeogenesis from pyruvate in rat liver and the oxaloacetate–malate pathway supplies NADH through malate dehydrogenase in the cytosole (Krebs et al. 1967; Anderson et al. 1971). As shown in Fig. 6A, pyruvate is used to synthesize glucose, and little lactate is formed in perfused eel livers. This means that even if pyruvate is used as a gluconeogenic substrate, there is no decrease in the cytosolic NADH in eel liver. This seems mainly due to the heart type of LDH in eel liver, as shown in Fig. 9 (Hayashi et al. 1985). Therefore, the oxaloacetate–malate pathway for gluconeogenesis is not essential in eel liver, as supported by the fact that D-malate did not inhibit gluconeogenesis from pyruvate.

Table 2. Effects of amino-oxyacetate (AOA), D-malate, quinolinic acid (QA) and tryptophan on gluconeogenesis in isolated eel liver cells.

<table>
<thead>
<tr>
<th>Addition</th>
<th>(mm)</th>
<th>Gluconeogenesis (μmoles glucose/g wet weight·h)</th>
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<tr>
<td></td>
<td></td>
<td>Substrate</td>
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<tr>
<td>A. None</td>
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</tr>
<tr>
<td>AOA</td>
<td>(0.2)</td>
<td>23.8 ± 1.0 (100)</td>
</tr>
<tr>
<td>D-Malate</td>
<td>(5)</td>
<td>9.3 ± 0.2 (39)*</td>
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<td></td>
<td>(10)</td>
<td>25.5 ± 3.7 (107)</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>B. None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QA</td>
<td>(2.5)</td>
<td>18.7 ± 0.7 (100)</td>
</tr>
<tr>
<td></td>
<td>(5.0)</td>
<td>18.9 ± 0.7 (101)</td>
</tr>
<tr>
<td>C. None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>(2.5)</td>
<td>28.2 ± 1.7 (100)</td>
</tr>
<tr>
<td></td>
<td>(5.0)</td>
<td>26.0 ± 1.9 (92)</td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of substrates were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. for three observations, *p < 0.01. Reprinted from J. Comp. Physiol. B, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, Anguilla japonica, Hayashi and Ooshiro, Table 1. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.
3-3B. Subcellular distribution of enzymes

Subcellular distribution of pyruvate carboxylase, glutamate oxaloacetate transaminase, and PEP carboxykinase, as well as marker enzymes of succinate dehydrogenase of mitochondria and lactate dehydrogenase of cytosole in eel hepatocytes, is shown in Table 3. Pyruvate carboxylase of eel liver is a mitochondrial enzyme as well as that of carp (Gumbmann and Tappe 1962), rat (Böttger et al. 1969), and guinea pig liver (Garber and Hanson 1971). Glutamate-oxaloacetate transaminase and PEP carboxykinase of eel liver was distributed in both the mitochondria and the cytosole. Approximately 30% of PEP-carboxykinase activity was localized in the mitochondria, therefore a part of PEP seemed to be synthesized in the mitochondria by mitochondrial PEP-carboxykinase (oxaloacetate–PEP pathway). That the degree of inhibition by AOA on gluconeogenesis from lactate or pyruvate was 60% (Table 2) seems due to the inhibition of cytosolic glutamate-oxaloacetate transaminase by AOA. The AOA insensitive portion is likely due to the oxaloacetate–PEP pathway in the mitochondria.

Effects of quinolinic acid on the PEP carboxykinase activity in the cytosolic and the mitochondrial fractions were investigated. The activities of the cytosolic and the mitochondrial

Table 3. Subcellular distribution of enzymes in eel liver.

| Fraction       | Protein mg/g liver | SDH                    | LDH         |               |                  |
|----------------|--------------------|------------------------|-------------|----------------|--|----------------|
|                |                    | U/g liver              | U/mg protein| U/g liver     | U/mg protein    |
| Homogenate     | 41.8 ± 6.7         | 0.59 ± 0.20            | 0.014 ± 0.004 | 1.34 ± 0.30  | 0.032 ± 0.005       |
| Nuclei         | 2.9 ± 1.2          | 100%                   | 0.07 ± 0.06  | 0.025 ± 0.019 | 0.11 ± 0.07   | 0.039 ± 0.018  |
| Mitochondria   | 8.1 ± 2.5          | 10.3%                  | 0.45 ± 0.11  | 0.050 ± 0.011 | 0.03 ± 0.01  | 0.003 ± 0.004 |
| Microsomes     | 5.3 ± 1.5          | 12.7%                  | 0.03 ± 0.02  | 0.007 ± 0.005 | 0.01 ± 0.01  | 0.003 ± 0.003 |
| Cytosol        | 19.7 ± 5.3         | 47.2%                  | 0%           | 0.000 ± 0.000 | 0.01 ± 0.01 | 0.003 ± 0.003 |
|                | Recovery           | 86.0%                  | 96.3%        | 89.2%         | 82.6%         | 0.056 ± 0.028 |

(A) Marker enzymes of succinate dehydrogenase (SCD) and lactate dehydrogenase (LDH). (B) Pyruvate carboxylase (PC), aspartate transaminase (GOT), and PEP-carboxykinase (PEPCK). Activities were measured at 37°C. The data are means ± S.D. of three observations. Reproduced from J. Comp. Physiol. B, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, Anguilla japonica, Hayashi and Ooshiro, Tables 6 and 7. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.
fraction were inhibited by 30 and 25%, respectively (Hayashi and Ooshiro 1979). No effect of quinolinic acid on the gluconeogenesis in isolated eel hepatocytes shown in Table 2 seems due to the impermeability of the plasma membrane of eel hepatocytes to quinolinic acid. PEP carboxykinase of rat liver is found only in the cytosole (Nordie and Lardy 1963; Böttger et al. 1969), but that of guinea pig liver is found in both the cytosole and the mitochondria (Nordie and Lardy 1963). In pigeon liver PEP carboxykinase is found only in the mitochondria (Gevers 1967). Thus, there are three types of subcellular distribution of PEP carboxykinase among animals. Comparison of the PEP synthesis pathway between eel, rat, and pigeon liver will be described in Section 3-4.

The PEP synthesis pathway in eel liver is shown in Fig. 12. The pathways within the square show the mitochondrial pathways. These are assumed from the results of the effects of inhibitors such as AOA, α-malate, and quinolinic acid and of the subcellular distributions of pyruvate carboxylase, glutamate-oxaloacetate transaminase, and PEP carboxykinase.

3-3C. Effects of leucine and other amino acids

As shown in Fig. 12, oxaloacetate in the mitochondria is synthesized by mitochondrial pyruvate carboxylase and then converted to aspartate by the mitochondrial glutamate-oxaloacetate transaminase. Aspartate formation is affected by the glutamate concentration in the mitochondria and the glutamate concentration is affected through glutamate dehydrogenase or some transaminases. Glutamate dehydrogenase is stimulated by leucine and some amino acids stimulate transaminases. Then the effects of leucine and other amino acids on gluconeogenesis were investigated.

The stimulatory effect of leucine was observed in gluconeogenesis from 5 mM 14C-lactate, 14C-pyruvate, and 14C-alanine (Table 4). Gluconeogenesis from 5 mM 14C-lactate, 14C-pyruvate, and 14C-alanine was stimulated by 5 mM leucine by 8, 76, and 45% compared with the control, respectively. The stimulation of gluconeogenesis by leucine depended on the concentrations of leucine. For example, 10 mM leucine stimulated gluconeogenesis from 14C-lactate but 2.5 mM leucine did not affect it, as shown in Table 4.
Incorporation of $^{14}$C-pyruvate into glucose and amino acids in the presence or absence of leucine (5 mM) was investigated (Table 5). The incorporation into glucose was increased 1.8 fold by leucine and the incorporation into neutral, acidic, and basic amino acids was increased 2.6, 3.7, and 1.6 fold by leucine, respectively. Furthermore, effects of leucine on aspartate and glutamate concentrations and on the incorporation of $^{14}$C-pyruvate into these amino acids were investigated. As shown in Table 6, the glutamate content increased 3.6 fold in the

**Table 4.** Effect of leucine on gluconeogenesis.

<table>
<thead>
<tr>
<th>Leucine (mM)</th>
<th>Gluconeogenesis (μmoles glucose/g wet weight·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate (%)</td>
</tr>
<tr>
<td>None</td>
<td>24.7 ± 0.7 (100)</td>
</tr>
<tr>
<td>2.5</td>
<td>25.3 ± 0.7 (102)</td>
</tr>
<tr>
<td>5.0</td>
<td>26.6 ± 1.2 (108)</td>
</tr>
<tr>
<td>10.0</td>
<td>31.8 ± 1.6 (129)*</td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of substrates were as described in Table 2. The data are the means ± S.D. of three observations. $^p < 0.01$. Reprinted from *J. Comp. Physiol. B*, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, *Anguilla japonica*, Hayashi and Ooshiro, Table 2. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.

**Table 5.** Incorporation of $^{14}$C-pyruvate into glucose and amino acids in the presence or absence of leucine (Leu), tryptophan (Trp) or amino-oxyacetate (AOA).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control</th>
<th>Leu (5 mM)</th>
<th>Leu Control</th>
<th>Trp (2 mM)</th>
<th>Trp Control</th>
<th>AOA (0.2 mM)</th>
<th>AOA Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (Amino acids)</td>
<td>3,341 ± 88</td>
<td>6,075 ± 360</td>
<td>1.8</td>
<td>3,799 ± 136</td>
<td>1.1</td>
<td>2,094 ± 57</td>
<td>0.6</td>
</tr>
<tr>
<td>Neutral</td>
<td>773</td>
<td>1,994</td>
<td>2.6</td>
<td>1,047</td>
<td>1.4</td>
<td>535</td>
<td>0.7</td>
</tr>
<tr>
<td>Acidic</td>
<td>627</td>
<td>2,337</td>
<td>3.7</td>
<td>696</td>
<td>1.1</td>
<td>424</td>
<td>0.7</td>
</tr>
<tr>
<td>Basic</td>
<td>76</td>
<td>120</td>
<td>1.6</td>
<td>107</td>
<td>1.4</td>
<td>68</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of $^{14}$C-pyruvate were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. of three observations. Reprinted from *J. Comp. Physiol. B*, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, *Anguilla japonica*, Hayashi and Ooshiro, Table 4. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.

**Table 6.** Effects of leucine on aspartate and glutamate concentrations and on incorporation of $^{14}$C-pyruvate into these amino acids.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Gluconeogenesis (μmoles/h·g wet wt)</th>
<th>Glutamate (μmoles/g wet wt)</th>
<th>Aspartate (μmoles/g wet wt)</th>
<th>$^{14}$C-Glutamate (cpm)</th>
<th>$^{14}$C-Aspartate (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33.18 ± 4.26</td>
<td>1.49 ± 0.26</td>
<td>1.23 ± 0.09</td>
<td>479 ± 315</td>
<td>108 ± 88</td>
</tr>
<tr>
<td>Leucine (5 mM)</td>
<td>67.55 ± 10.77*</td>
<td>5.48 ± 0.25</td>
<td>1.51 ± 0.06</td>
<td>2,858 ± 332</td>
<td>581 ± 385</td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of $^{14}$C-pyruvate were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. of three observations. $^p < 0.001$, $^p < 0.05$. Reprinted from *J. Comp. Physiol. B*, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, *Anguilla japonica*, Hayashi and Ooshiro, Table 5. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.
presence of 5 mM leucine, but no change was observed in the aspartate content, while the incorporation of \(^{14}C\)-pyruvate into both glutamate and aspartate was increased 5 to 6 fold by leucine supplement. As a result, the specific radioactivity (cpm \(\mu\)mol\(^{-1}\)) of \(^{14}C\)-aspartate was 4.3 times higher than that of the control. These results indicate that the increase of the turnover of aspartate was due to an increase of the glutamate content in the mitochondria.

Two enzyme reactions affecting the glutamate content in the mitochondria are assumed; one is a glutamate dehydrogenase (GDH) reaction and the other is a transaminase reaction, as shown in Fig. 12. Glutamate dehydrogenase in eel liver was purified from the mitochondria and the physico-chemical properties were investigated (Hayashi et al. 1982a; Tang et al. 1992). Eel liver GDH activity is stimulated 2.5 fold to the direction of glutamate synthesis and 1.4 fold to the deamination direction in the presence of 5 mM leucine. The stimulatory effect of leucine on gluconeogenesis seems due to activation of the GDH by leucine followed by an increase in the glutamate content in the mitochondria.

Another enzyme affecting the glutamate content by leucine in the mitochondria is leucine transaminase. However, there is no data on leucine transaminase in eel liver mitochondria. Investigations into leucine transaminase need to carried out to clarify the situation.

Effects of some amino acids on gluconeogenesis were investigated by isolated eel hepatocytes. There are many transaminase reactions in the mitochondria and the glutamate content is influenced by these reactions. Table 7 shows the effects of amino acids on gluconeogenesis from 5 mM \(^{14}C\)-lactate, \(^{14}C\)-pyruvate, and \(^{14}C\)-alanine by eel isolated hepatocytes and the comparison with that from 10 mM lactate by rat isolated hepatocytes (Cornell et al. 1974).

### Table 7. Effect of amino acids on gluconeogenesis in isolated cells of eel and rat liver.

<table>
<thead>
<tr>
<th>A.A.</th>
<th>Eel (5mM)</th>
<th>Rat (10mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Gly</td>
<td>83 (-)</td>
<td>104 (+)</td>
</tr>
<tr>
<td>L-Val</td>
<td>90 (-)</td>
<td>81 (-)</td>
</tr>
<tr>
<td>L-Leu</td>
<td>108 (+)</td>
<td>145 (+)</td>
</tr>
<tr>
<td>L-Ile</td>
<td>93 (+)</td>
<td>92 (+)</td>
</tr>
<tr>
<td>L-Phe</td>
<td>152 (+)</td>
<td>106 (+)</td>
</tr>
<tr>
<td>L-Pro</td>
<td>84 (-)</td>
<td>96 (+)</td>
</tr>
<tr>
<td>L-Trp</td>
<td>85 (-)</td>
<td>89 (-)</td>
</tr>
<tr>
<td>L-Ser</td>
<td>51 (-)</td>
<td>144 (+)</td>
</tr>
<tr>
<td>L-Met</td>
<td>101 (+)</td>
<td>115 (+)</td>
</tr>
<tr>
<td>L-Thr</td>
<td>93 (+)</td>
<td>110 (+)</td>
</tr>
<tr>
<td>L-Asn</td>
<td>97 (+)</td>
<td>71 (-)</td>
</tr>
<tr>
<td>L-Gln</td>
<td>159 (+)</td>
<td>101 (+)</td>
</tr>
<tr>
<td>L-Asp</td>
<td>90 (+)</td>
<td>92 (+)</td>
</tr>
<tr>
<td>L-Glu</td>
<td>117 (+)</td>
<td>105 (+)</td>
</tr>
<tr>
<td>L-Tyr</td>
<td>69 (-)</td>
<td>88 (-)</td>
</tr>
<tr>
<td>L-His</td>
<td>131 (+)</td>
<td>97 (+)</td>
</tr>
<tr>
<td>L-Lys</td>
<td>99 (+)</td>
<td>102 (+)</td>
</tr>
<tr>
<td>L-Arg</td>
<td>108 (+)</td>
<td>83 (+)</td>
</tr>
</tbody>
</table>

Eel and rat hepatocytes were incubated at 30°C for 60 min and at 37°C for 20 min, respectively.

In isolated eel liver the gluconeogenesis from \(^{14}\text{C}\)-lactate was stimulated by glutamine, phenylalanine, and histidine, but that from \(^{14}\text{C}\)-pyruvate was stimulated by leucine, glutamine, histidine, serine, arginine, valine, methionine, tyrosine, and asparagine. Gluconeogenesis from \(^{14}\text{C}\)-alanine was stimulated only by serine. On the contrary, serine and tyrosine inhibited gluconeogenesis from \(^{14}\text{C}\)-lactate and only glycine inhibited that from \(^{14}\text{C}\)-pyruvate. However, the number of amino acids showing an inhibitory effect on the gluconeogenesis was less than that showing a stimulatory effect.

The stimulatory effects of valine, leucine, isoleucine, phenylalanine, and histidine on gluconeogenesis from 5 mM \(^{14}\text{C}\)-pyruvate disappeared after the addition of 1 mM AOA in the incubation medium of isolated eel hepatocytes (Table 8). The addition of AOA in the presence of these amino acids decreased the gluconeogenesis from 5 mM \(^{14}\text{C}\)-pyruvate to 46 to 55% compared to the control value obtained in the absence of both the amino acids and AOA. These results strongly suggest that the amino acids stimulate the site of aspartate–oxaloacetate pathway in gluconeogenesis in the eel hepatocytes.

In isolated rat hepatocytes, lysine, tyrosine, arginine, and asparagine stimulate gluconeogenesis from lactate, though the incubation time of 20 min for isolated rat hepatocytes is shorter than the incubation time of 60 min for isolated eel hepatocytes (Cornell et al. 1974) and histidine, threonine, serine, and tryptophan inhibited the gluconeogenesis in rat hepatocytes. Amino acids giving stimulatory or inhibitory effects are different between eel and rat but the exact reason of this difference is still uncertain.

3-3D. Effect of oleic acid

\(\beta\)-Oxidation of oleic acid produces acetyl-CoA which induces NADH production through tricarboxylic acid cycle and an increase in the NADH/NAD\(^+\) ratio in the mitochondria (Söling et al. 1970). Acetyl-CoA is an activator of pyruvate carboxylase and the increased NADH/NAD\(^+\) ratio in the mitochondria leads to an increased NADH/NAD\(^+\) ratio in the cytosol, which affects LDH reaction.

Gluconeogenesis from lactate (20 mM) in perfused rat livers is stimulated by 2 mM oleic acid (Söling et al. 1968, 1970; Williamson et al. 1966). Söling et al. (1968, 1970) have explained that the stimulatory effect of oleic acid in rat liver occurs between pyruvate and PEP, namely oxaloacetate production is increased by the activation of pyruvate carboxylase by acetyl-CoA.

Whereas gluconeogenesis from lactate (20 mM) in perfused guinea pig livers is inhibited by 2 mM oleic acid by about 20% compared with the control (Jomain-Baum and Hanson 1975; Tutwiler and Brentzel 1982), Söling et al. (1970) have explained why oleic acid inhibits gluconeogenesis from lactate in guinea pig liver, where the redox state of the lactate/pyruvate system

Table 8. Gluconeogenesis from pyruvate in the presence of amino acids and AOA.

<table>
<thead>
<tr>
<th>Amino acid (5 mM)</th>
<th>Gluconeogenesis (relative rates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-\text{AOA})</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Val</td>
<td>139 ± 13*</td>
</tr>
<tr>
<td>Leu</td>
<td>167 ± 18*</td>
</tr>
<tr>
<td>Ile</td>
<td>127 ± 13b</td>
</tr>
<tr>
<td>Phe</td>
<td>120 ± 11</td>
</tr>
<tr>
<td>His</td>
<td>155 ± 14b</td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of \(^{14}\text{C}\)-pyruvate were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. of three observations. \(^*p < 0.01, ^bp < 0.05. Reprinted from J. Comp. Physiol. B, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, Anguilla japonica, Hayashi and Ooshiro, Table 3. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media.
is already more negative than that in rat liver and the addition of oleic acids leads to a further increase of the lactate/pyruvate ratio resulting in a considerable drop in the concentration of pyruvate. This is the most probable reason for the decreased gluconeogenesis from lactate in guinea pig livers in the presence of oleic acid, since under these conditions the rate of pyruvate carboxylation in guinea pig liver seems to depend much more on the concentration of pyruvate than on the level of acetyl-CoA.

In isolated eel hepatocytes, oleic acid strongly inhibited gluconeogenesis from lactate and pyruvate (Hayashi and Ooshiro 1985b). Table 9 shows the effects of oleic acid on gluconeogenesis, protein synthesis, and the incorporation into amino acids fraction from 5 mM 14C-lactate or 14C-pyruvate by isolated eel hepatocytes. Oleic acid at 0.25 and 2.50 mM inhibited gluconeogenesis from 14C-lactate by 36 and 99%, respectively. The incorporation of 14C-lactate into amino acids in the presence of 2.50 mM of oleic acid decreased by 40% compared with that of the control, but protein synthesis from 14C-lactate was not affected by the oleic acid. Similarly, oleic acid at 0.25 and 2.50 mM inhibited gluconeogenesis from 14C-pyruvate by 37 and 94%, respectively. These inhibitory effects on gluconeogenesis in eel liver are quite different from the stimulatory effect of oleic acid on gluconeogenesis in rat liver. The degree of inhibitory effects of oleic acid in eel liver is stronger than that in guinea pig liver.

Table 9 shows the effects of oleic acid, α-ketoglutarate, and NH₄Cl on the glutamate content as well as on gluconeogenesis from 5 mM 14C-lactate or 14C-pyruvate by the isolated eel hepatocytes. When 14C-lactate was used as a precursor, the glutamate content decreased by 29% in the presence of 2.50 mM of oleic acid. Similarly, when 14C-pyruvate was used, the glutamate content decreased by 24% in the presence of 2.50 mM of oleic acid (Hayashi and Ooshiro 1985b).

In the presence of 5 mM α-ketoglutarate and NH₄Cl, gluconeogenesis from 5 mM 14C-lactate and 14C-pyruvate increased by 23 and 50%, respectively. But 2.5 mM oleic acid completely inhibited the gluconeogenesis from both precursors in the presence of 5 mM α-ketoglutarate and NH₄Cl (Hayashi and Ooshiro 1985b).

The effect of oleic acid on O2 consumption and 14CO2 production in isolated eel hepatocytes in the presence of 5 mM 14C-lactate was investigated using a Warburg manometer. As shown in Fig. 13, 2.5 mM oleic acid slightly increased the O2 consumption and 14CO2 production (Hayashi and Ooshiro 1985b). These results show that the utilization of 14C-lactate slightly increased as an energy source. Because gluconeogenesis from 5 mM 14C-lactate was inhibited almost completely by 2.5 mM oleic acid, the metabolic flow of lactate to the tricarboxylic acid cycle and electron transport chain seems to increase.

**Table 9.** Effects of oleic acid on gluconeogenesis, protein synthesis, and the incorporation into amino acids fraction from 14C-lactate and 14C-pyruvate by isolated eel liver cells.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Addition mM</th>
<th>Glucose (μmol/g wet wt-h) (%)</th>
<th>Protein (cpm/g wet wt-h) (%)</th>
<th>Amino Acids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14C-Lactate</td>
<td>None</td>
<td>8.47±1.02 (100)</td>
<td>43,010±2,970 (100)</td>
<td>58,020±6,850 (100)</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>5.39±0.34**(64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5.05±0.04*(1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>8.68±0.03 (100)</td>
<td>40,610±6,400 (94)</td>
<td>34,590±7,950**(60)</td>
</tr>
<tr>
<td>14C-Pyruvate</td>
<td>None</td>
<td>8.47±1.02 (100)</td>
<td>40,610±6,400 (94)</td>
<td>34,590±7,950**(60)</td>
</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td>5.48±0.03*(63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5.56±0.09* (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>5.56±0.09* (6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of substrates were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. of three observations. *p < 0.01, **p < 0.05. Reprinted with permission from *Nippon Suisan Gakkaishi*, 51, Hayashi and Ooshiro, Effect of oleic acid on gluconeogenesis in isolated liver cells of eel, 447–452, 1985, the Japanese Society of Fisheries Science.
Fig. 13. Effects of oleic acid on O₂ consumption and ¹⁴CO₂ production. The initial concentration and radioactivity of ¹⁴C-lactate were 5 mM and 9.25 kBq, respectively. The wet weight of cells used was 136.81 ± 0.01 mg. The data are the means ± S.D. of three observations. Reprinted with permission from Nippon Suisan Gakkaishi, 51, Hayashi and Ooshiro, Effect of oleic acid on gluconeogenesis in isolated liver cells of eel, 447–452, 1985, the Japanese Society of Fisheries Science.

Table 10. Effects of oleic acid, α-ketoglutarate (α-KG), and NH₄Cl on glutamate content and gluconeogenesis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Addition</th>
<th>Glutamate (μmol/g wet wt)</th>
<th>Gluconeogenesis (μmol glucose/g wet wt·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹⁴C-Lactate</td>
<td>None</td>
<td>1.19±0.23 (100)</td>
<td>4.52±0.52 (100)</td>
</tr>
<tr>
<td></td>
<td>Oleic Acid (2.5)</td>
<td>0.86±0.17*** (71)</td>
<td>0.06±0.02* (1)</td>
</tr>
<tr>
<td></td>
<td>α-KG (5.0)</td>
<td>1.36±0.29 (114)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oleic Acid (2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-KG (5.0)</td>
<td>0.01±0.01* (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH₄Cl (5.0)</td>
<td>5.57±0.25 (123)</td>
<td></td>
</tr>
<tr>
<td>¹⁴C-Pyruvate</td>
<td>None</td>
<td>0.83±0.15 (100)</td>
<td>5.03±0.51 (100)</td>
</tr>
<tr>
<td></td>
<td>Oleic Acid (2.5)</td>
<td>0.62±0.11*** (76)</td>
<td>0.52±0.09* (10)</td>
</tr>
<tr>
<td></td>
<td>α-KG (5.0)</td>
<td>1.70±0.15* (205)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oleic Acid (2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-KG (5.0)</td>
<td>0.29±0.03* (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH₄Cl (5.0)</td>
<td>7.51±0.59** (150)</td>
<td></td>
</tr>
</tbody>
</table>

The initial concentration and radioactivity of substrates were 5 mM and 9.25 kBq, respectively. The data are the means ± S.D. of three observations. *p < 0.01, **p < 0.05, ***p < 0.2. Reprinted with permission from Nippon Suisan Gakkaishi, 51, Hayashi and Ooshiro, Effect of oleic acid on gluconeogenesis in isolated liver cells of eel, 447–452, 1985, the Japanese Society of Fisheries Science.
The site of the inhibitory effect of oleic acid in isolated eel hepatocytes is not a pyruvate carboxylase reaction. Because acetyl-CoA derived from the oxidation of oleic acid in the mitochondria is an activator of pyruvate carboxylase, oxaloacetate production by pyruvate carboxylase increases and then gluconeogenesis is stimulated. However, oleic acid strongly inhibited gluconeogenesis in eel liver. The reaction site of the inhibitory effect of oleic acid seems a more later reaction than the pyruvate carboxylase reaction. As shown in Fig. 12, the aspartate–oxaloacetate pathway is the main pathway of gluconeogenesis in eel liver and the glutamate concentration affects the glutamate-oxaloacetate transaminase reaction. Glutamate is formed by glutamate dehydrogenase (GDH), and GDH and glutamate-oxaloacetate transaminase are intimately connected. The activity of GDH purified from eel liver mitochondria was completely inhibited by 10 μM of oleic acid or oleoyl-CoA (Hayashi and Ooshiro 1985b). Palmitic acid and caproic acid at 10 μM also inhibited eel GDH by 85 and 20%, respectively.

The effect of leucine on gluconeogenesis was described in the previous section and it is known that it plays an important role as the activator of eel liver GDH. In contrast to leucine, oleic acid plays the role of the inhibitor of GDH. Both leucine and oleic acid affect GDH activity which in turn affects the glutamate concentration in the mitochondria followed by the regulation of gluconeogenesis.

3-4. Comparison of PEP synthesis pathways between eel, rat, and pigeon liver

Figures 14A, B and C show PEP synthesis pathways of eel, rat, and pigeon liver, respectively. The pathways within the boxes show the mitochondrial pathways which have been characterized by the subcellular distribution of enzymes such as pyruvate carboxylase, glutamate-oxaloacetate transaminase, and PEP carboxykinase, by the effects of enzyme inhibitors on gluconeogenesis, and by the effects of leucine and oleic acid on gluconeogenesis.

Table 11 compares the subcellular distribution of pyruvate carboxylase and PEP carboxykinase in rat, eel and pigeon liver. Pyruvate carboxylase from all three animals was localized in the mitochondria (Gevers 1967; Böttger et al. 1969; Hayashi and Ooshiro 1979). However, PEP carboxykinase of rat, eel, and pigeon liver is localized in the cytosole (Nordie and Lardy 1963), in both the cytosole and the mitochondria (Hayashi and Ooshiro 1979), and in the mitochondria (Gevers 1967), respectively.

![Fig. 14. Comparison of PEP synthesis pathways between eel (A), rat (B), and pigeon (C) livers. The pathways within squares show the mitochondrial pathways. (A) is reprinted from J. Comp. Physiol. B, 132, 1979, 343–350, Gluconeogenesis in isolated liver cells of the eel, Anguilla japonica, Hayashi and Ooshiro, Figure 3. © 1979, Springer-Verlag. With kind permission of Springer Science+Business Media. (B) is reproduced from Lardy et al., Paths of carbon in gluconeogenesis and lipogenesis: The role of mitochondria in supplying precursors of phosphoenolpyruvate. Proc. Natl. Acad. Sci. USA, 53, 1965; 1410–1415. © 1965, The National Academy of Sciences with kind permission of Prof. Henry Lardy and Prof. Verner Paetkau.](image-url)
In rat liver, oxaloacetate formed by pyruvate carboxylase in the mitochondria must be transported to the cytosole to synthesize PEP by cytosolic PEP carboxykinase. Oxaloacetate is impermeable to the mitochondrial membrane and then the malate–oxaloacetate pathway or aspartate–oxaloacetate pathway is used to transport the mitochondrial oxaloacetate to the cytosole in rat liver. Another characteristic point in the PEP synthesis pathway in rat liver is that there are two PEP synthesis pathways. When pyruvate is used for the gluconeogenesis in rat liver, the malate–oxaloacetate pathway is used for the PEP synthesis pathway. While lactate is used for the gluconeogenesis, the aspartate–oxaloacetate pathway is used for the PEP synthesis pathway, as described in Section 3-1.

In the eel liver, the remarkable characteristic for the PEP synthesis pathway is absence from the malate–oxaloacetate pathway in gluconeogenesis from pyruvate. This is mainly due to the heart type of LDH. As shown in Fig. 6A, most pyruvate was used to synthesize glucose, and lactate is not formed by LDH. Contrasting with eel perfused liver, rat perfused liver converts about half the amount of consumed pyruvate to lactate (Fig. 6B) in association with the consumption of equivalent of cytosolic NADH. Therefore, if the cytosolic NADH/NAD⁺ ratio is similar in rat and eel liver, the supply of cytosolic NADH is not required for gluconeogenesis from pyruvate in eel liver. However, the redox state in eel hepatocytes still remains to be investigated.

In pigeon liver, PEP carboxykinase is localized in the mitochondria. Oxaloacetate is converted to PEP in the mitochondrial PEP carboxykinase and PEP is transported to the cytosole for gluconeogenesis. This PEP synthesis pathway is the simplest of the three species (Fig. 14C).

### 3-5. Glycogen metabolisms in eel liver

The reason why eel liver glycogen is retained in spite of a long period of starvation (Larsson and Lewander 1973; Dave et al. 1975) is uncertain. The ability of glycogen synthesis from 10 mM pyruvate or lactate was determined by using cultured eel hepatocytes. After eel hepatocytes were cultured in WE–5% FBS–0.16 μM insulin on dishes precoated with fibronectin for 7 to 10 days, the hepatocytes were washed with glucose-free MEM medium containing 0.5% BSA and cultured in the same medium at least for 4 h. Then the hepatocytes were incubated in glucose-free MEM medium–0.5% BSA with or without 10 mM pyruvate or lactate. After incubation the medium was removed and the cells were homogenized in HClO₄. The supernatant of the homogenate after centrifugation was neutralized with KHCO₃ and after centrifugation the neutralized supernatant was used for the determination of glycogen which was digested by glucoamylase (Keppler and Decker 1984) and the glucose formed was determined by an enzymatic method (Kunst et al. 1984).

The glycogen synthesis from 5 mM ¹⁴C-pyruvate or ¹⁴C-glucose was also determined by using cultured eel hepatocytes which were cultured in WE–5% FBS–0.16 μM insulin for 7 to 10 days. Then the hepatocytes were washed with serum- and insulin-free WE medium and incubated in the same medium in the presence of 5 mM ¹⁴C-pyruvate or ¹⁴C-glucose. The cells were then homogenized in HClO₄. After centrifugation glycogen in the supernatant of the homogenate was precipitated with ethanol and then washed with ethanol. After washing, the glycogen was dissolved in water and the radioactivity was determined.

### Table 11. Comparison of subcellular distribution of pyruvate carboxylase and PEP carboxykinase among eel, rat, and pigeon livers.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Pyruvate Carboxylase</th>
<th>PEP Carboxykinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eel</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Rat</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>Pigeon</td>
<td>–</td>
<td>+++</td>
</tr>
</tbody>
</table>

In rat liver, oxaloacetate formed by pyruvate carboxylase in the mitochondria must be transported to the cytosole to synthesize PEP by cytosolic PEP carboxykinase. Oxaloacetate is impermeable to the mitochondrial membrane and then the malate–oxaloacetate pathway or aspartate–oxaloacetate pathway is used to transport the mitochondrial oxaloacetate to the cytosole in rat liver. Another characteristic point in the PEP synthesis pathway in rat liver is that there are two PEP synthesis pathways. When pyruvate is used for the gluconeogenesis in rat liver, the malate–oxaloacetate pathway is used for the PEP synthesis pathway. While lactate is used for the gluconeogenesis, the aspartate–oxaloacetate pathway is used for the PEP synthesis pathway, as described in Section 3-1.

In the eel liver, the remarkable characteristic for the PEP synthesis pathway is absence from the malate–oxaloacetate pathway in gluconeogenesis from pyruvate. This is mainly due to the heart type of LDH. As shown in Fig. 6A, most pyruvate was used to synthesize glucose, and lactate is not formed by LDH. Contrasting with eel perfused liver, rat perfused liver converts about half the amount of consumed pyruvate to lactate (Fig. 6B) in association with the consumption of equivalent of cytosolic NADH. Therefore, if the cytosolic NADH/NAD⁺ ratio is similar in rat and eel liver, the supply of cytosolic NADH is not required for gluconeogenesis from pyruvate in eel liver. However, the redox state in eel hepatocytes still remains to be investigated.

In pigeon liver, PEP carboxykinase is localized in the mitochondria. Oxaloacetate is converted to PEP in the mitochondrial PEP carboxykinase and PEP is transported to the cytosole for gluconeogenesis. This PEP synthesis pathway is the simplest of the three species (Fig. 14C).
As shown in Fig. 11, glycongen synthesis from 10 mM pyruvate was observed in eel hepatocytes cultured in glucose-free MEM medium. The rates of glycongen synthesis from 10 mM pyruvate and lactate were 0.407 ± 0.127 (n = 4) and 0.163 (n = 2) μmol-glucose (mg-cell protein)−1 h−1 in the absence of glucose, respectively. If the protein content of the cultured eel hepatocytes is hypothesized to be 20%, the rates of glycongen synthesis from 10 mM pyruvate and lactate are calculated as 81 and 33 μmol-glucose (g-cell)−1 h−1, respectively. It was found that the rates of glycongen synthesis from pyruvate and lactate were 3.9 and 5 times higher than the rates of glucose synthesis from pyruvate and lactate because gluconeogenesis from 10 mM pyruvate and lactate by cultured eel hepatocytes was 21 and 6.4 μmol-glucose (g-cell)−1 h−1, respectively.

The effects of glucagon and insulin on balancing blood glucose are well known in mammalian livers (Darnell et al. 1990). Glucagon stimulates glycogen degradation and gluconeogenesis, whereas insulin stimulates glycogen synthesis and inhibits gluconeogenesis. We investigated the effect of glucagon or insulin on glycogen metabolism and gluconeogenesis by using cultured eel hepatocytes.

The eel hepatocytes were cultured in the defined serum-free WE medium, as shown in Table 1B, for 7 to 9 days and then washed with glucose-free MEM–0.5% BSA. The cells were incubated in glucose-free MEM–0.5% BSA for 4 h. After that the cells were again washed with glucose-free MEM–0.5% BSA and incubated for 1 h in the same medium in the presence of 10 mM pyruvate or lactate with or without different concentrations of glucagon.

In the presence of 10 mM pyruvate without glucagon, glycogen was synthesized at the rate of 0.560 μmol-glucose (mg-cell protein)−1 h−1. However, glycogen was still synthesized from 10 mM pyruvate in the presence of 10−6 and 10−7 M glucagon at the rates of 0.313 and 0.286 μmol-glucose (mg-cell protein)−1 h−1, respectively (Fig. 15), although glucagon at 10−6 and 10−7 M repressed glycogen synthesis compared with glycogen synthesis without glucagon. Glucagon at 10−6 and 10−7 M did not stimulate glycogen degradation to glucose in the presence of 10 mM pyruvate without glucagon.

![Fig. 15. Effect of glucagon on the glycogen metabolism in the presence of 10 mM pyruvate or 10 mM lactate.](image-url)
pyruvate. When 10 mM pyruvate was used, the glucose appeared in the medium which was possibly due to gluconeogenesis from 10 mM pyruvate, because the glycogen content in the eel hepatocytes was always higher than that in the eel hepatocytes at 0 min incubation, as shown in Fig. 15.

Whereas the glycogen content in the eel hepatocytes incubated with 10 mM lactate was decreased by 10⁻⁴ or 10⁻⁷ M glucagons, below the glycogen content in the eel hepatocytes before incubation (Fig. 15). These results show that glucagon at 10⁻⁴ and 10⁻⁷ M stimulated glycogen degradation. Therefore, the glucose appeared in the medium in the presence of 10⁻⁴ and 10⁻⁷ M glucagon seemed due to glycogen degradation to glucose.

When eel hepatocytes were cultured in a serum- and insulin-free WE medium, which contains 11 mM glucose, glucagon at 2.5 × 10⁻⁷ M stimulated not only gluconeogenesis from 5 mM ¹⁴C-pyruvate but also glycogen synthesis from 5 mM ¹⁴C-pyruvate (Fig. 16). These results were obtained from the incorporation of 5 mM ¹⁴C-pyruvate into glycogen or glucose. In the presence of 11 mM glucose in a WE medium, glycogen synthesis and gluconeogenesis from 5 mM ¹⁴C-pyruvate were stimulated by 2.5 × 10⁻⁷ M glucagon 2.5- and 37-fold compared with those in the absence of glucagon, respectively (Hayashi and Ooshiro 1985d). Ui and Tokumitsu (1979) reported that glucose inhibits glycogen phosphorylase and activates glycogen synthetase.

Fig. 16. Effects of insulin (I), 0.25 µM, and glucagon (G), 0.25 µM, on the incorporation of ¹⁴C-precursor into glucose, glycogen, and proteins in cultured eel hepatocytes. Control cells were cultured in Williams’ E medium with 10% NU-serum (Flow Laboratory) for 6 days. After 6 days, the hepatocytes were washed with a serum-free medium and the cells were incubated in the serum-free medium with a ¹⁴C-precursor at 30°C for 4 h. The incorporation of 5 mM ¹⁴C-pyruvate (9.25 kBq) into the intracellular protein, extracellular protein, glucose, and glycogen in the control cells were 36,200, 4,600, 735, and 563 dpm (mg-protein)⁻¹, respectively. The incorporation of 5 mM ¹⁴C-glucose (9.25 kBq) into the intracellular protein, extracellular protein, and glycogen in the control cells were 4,930, 690, and 2,250 dpm (mg-protein)⁻¹, respectively. The incorporation of 5 mM ¹⁴C-leucine (9.25 kBq) into the intra- and extra-cellular proteins in the control cells were 23,160 and 4,740 dpm (mg-protein)⁻¹, respectively. Percentages show the ratio of the dpm (mg-protein)⁻¹ obtained in the presence of insulin or glucagon or both to the dpm (mg-protein)⁻¹ obtained in the absence of insulin and glucagons. Reprinted with permission from Nippon Suisan Gakkaishi, 51, Hayashi and Ooshiro, Effects of glucagon, insulin, and the eel serum in the eel liver cells in primary culture, 1123–1127, 1985, the Japanese Society of Fisheries Science.
Glucose in WE medium seems to inhibit glycogen phosphorylase and to activate glycogen synthetase in the cultured eel hepatocytes.

In contrast to glucagon, insulin at $2.5 \times 10^{-7}$ M inhibited gluconeogenesis from 5 mM $^{14}$C-pyruvate by 33% and slightly stimulated glycogen synthesis by 18% compared with those of the control. However, in the presence of both glucagon and insulin at $2.5 \times 10^{-7}$ M, gluconeogenesis and glycogen synthesis from 5 mM $^{14}$C-pyruvate were stimulated 35- and 10-fold compared with those of the control, respectively (Fig. 16) (Hayashi and Ooshiro 1985d). In the presence of insulin alone, glycogen synthesis from 5 mM $^{14}$C-pyruvate was slightly stimulated by 18% compared with the control. In the presence of glucagon alone, glycogen synthesis was stimulated 2.5-fold, as described above.

Glycogen synthesis from 5 mM $^{14}$C-glucose was stimulated by $2.5 \times 10^{-7}$ M insulin 2.3-fold and inhibited by $2.5 \times 10^{-7}$ M glucagon by 78% as shown in Fig. 16. In the presence of both glucagon and insulin at $2.5 \times 10^{-7}$ M, glycogen synthesis from 5 mM $^{14}$C-glucose was inhibited by 66%.

Intracellular- and extracellular-protein synthesis from 5 mM $^{14}$C-leucine was stimulated by $2.5 \times 10^{-7}$ M insulin by 73 and 52% compared with those of the control, respectively, and also stimulated by $2.5 \times 10^{-7}$ M glucagon by 63 and 31% compared with those of the control, respectively.

The effects of eel serum on glycogen synthesis and gluconeogenesis were investigated by using cultured eel hepatocytes. After the eel hepatocytes were cultured in WE medium containing 5% eel serum (final protein concentration of 1.35 mg mL$^{-1}$) instead of NU serum (final protein concentration of 1.36 mg mL$^{-1}$, artificial serum equivalent to FBS) for 4 days, the hepatocytes were washed with a serum- and insulin-free WE medium and incubated in the same medium for 4 h in the presence of 5 mM $^{14}$C-pyruvate. As shown in Fig. 17, gluconeogenesis and glycogen synthesis by eel hepatocytes cultured in eel serum increased 39-fold and 8-fold higher than those by the control hepatocytes cultured in NU serum, respectively (Hayashi and Ooshiro 1985d).

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**Fig. 17.** Effects of eel serum and eel serum with insulin (1.6 μM) on the incorporation of 5 mM $^{14}$C-pyruvate (9.25 kBq) into the glucose, glycogen, and proteins in cultured eel hepatocytes. Reprinted with permission from *Nippon Suisan Gakkaishi*, 51, Hayashi and Ooshiro, Effects of glucagon, insulin, and the eel serum in the eel liver cells in primary culture, 1123–1127, 1985, the Japanese Society of Fisheries Science.
The stimulatory effects of eel serum on glycogen synthesis and gluconeogenesis from 5 mM $^{14}$C-pyruvate, resembled the stimulatory effects of glucagon, as shown in Fig. 16, but the substance giving stimulatory effects in eel serum is uncertain. During 4 days’ culture in a WE medium containing 5% eel serum, the substance seemed to affect glycogen synthesis and gluconeogenesis in the cultured eel hepatocytes and to maintain the stimulatory effects during 4 h incubation in a serum-free WE medium. Glucose concentrations of eel serum and NU serum in the culture medium are almost the same, about 0.61 mM, and so stimulatory effects by eel serum are not due to glucose. The stimulatory effects of eel serum on glycogen synthesis and gluconeogenesis from 5 mM $^{14}$C-pyruvate disappeared after the addition of $1.6 \times 10^{-6}$ M insulin (Fig. 17).

4. Lipoprotein metabolisms in eel liver

4-1. Characteristics of fish serum lipoproteins

Babin and Vernier (1989) reviewed fish serum lipoproteins that, except for Chondrichthyes such as skates and shark, Agnatha such as lamprey and Teleostei such as carp and rainbow trout are classified as hyperlipidemic and hypercholesterolemic compared with human serum. For example, concentrations of cholesterol and triglyceride of eel serum are 476 and 749 mg dL$^{-1}$, respectively, whereas concentrations are 180 and 100 mg dL$^{-1}$ in human serum, respectively (Hayashi 1999). Total lipid concentrations of eel and human serum are 2,100 and 480 mg dL$^{-1}$, respectively. On the other hand, protein concentrations of eel and human serum are 4,000 to 5,000 and 8,000 mg dL$^{-1}$, respectively. In fish, most serum lipids are associated with lipoproteins. Therefore, lipoproteins are the main component of eel serum proteins, whereas albumin is the main component in human serum protein. In general, fish serum contains more lipoproteins than human serum. This leads to a hyperapolipoproteinemia in fish and the plasma concentration of apolipoproteins accounts for about 36% of plasma proteins in rainbow trout (Babin 1987) and 30% in channel catfish (McKay et al. 1985). However, in human serum it is lower than 10%.

In fish lipoproteins, high-density lipoprotein (HDL) is the main lipoprotein although very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) are also present. In human serum LDL is a main lipoprotein. Protein concentrations of HDL in eel and human serum are 770 mg dL$^{-1}$ and 170 to 190 mg dL$^{-1}$, respectively (Hayashi 1999). Fish VLDL and LDL contain apoB48-like proteins as human VLDL and LDL and fish HDL contains apoA-like proteins. However, fish VLDL and LDL lack apoB100-like proteins and contain apoA-like proteins as well as in HDL. Contrary to fish VLDL and LDL, human VLDL and LDL contain apoB100 but do not contain apoA.

Why the amounts of fish lipoproteins are so high is still unclear and the role of the lipoproteins is also unclear, particularly the role of HDL is an interesting problem, but why such a high concentration of HDL is necessary is unresolved.

On the other hand, the role and function of vitellogenin is clear. Female fish serum during maturation for spawning contains vitellogenin as do other oviparous animals. Vitellogenin is a precursor of egg yolk and classified as very high-density lipoprotein (VHDL). It is induced and synthesized in liver by 17$\beta$-estradiol, circulated through blood, and incorporated into oocyte by a specific receptor in the oocyte plasma membrane. The lipid content of VHDL is about 20% and the main lipid component is phospholipids.

4-2. Lipoproteins secreted by primary cultured eel hepatocytes

We determined the lipoprotein secreted by cultured eel hepatocytes, isolated the lipoprotein, and the secreted lipoprotein was defined as VLDL-like lipoprotein. The secreted lipoprotein was determined as described below.

Hepatocytes of $1.5 \times 10^7$ cells were cultured on a 10-cm plastic dish in WE medium containing 5% FBS and 0.16 $\mu$M insulin for 6 days. After the hepatocytes were incubated with $^{14}$C-leucine in WE medium without serum and insulin for the indicated period, a medium (7 mL)
and PBS (3 mL), used to wash cells, were combined and the combined medium was applied to a Sephadex G-25 column (1.6 × 15 cm). The protein fraction was pooled as extracellular protein and lyophilized. The lyophilized protein was dissolved in 50 μL of sample buffer for SDS-PAGE and used for the measurement of radioactivity and autoradiography.

The intracellular protein was recovered as follows. Three milliliters of 0.1 N NaOH was added to the cells on a dish, and the dissolved cells were transferred to a test tube. Then proteins in the test tube were precipitated with 5% trichloroacetic acid. The resulting precipitate was dissolved in 2 mL of 0.1 N NaOH, and the solution was used for the measurement of radioactivity and to assay the proteins as an intracellular protein.

The time course of the syntheses of intra- and extra-cellular proteins is shown in Fig. 18. Syntheses of intra- and extra-cellular proteins from 2 to 32 h were increased over 32 h, and the synthesis of intracellular protein was higher at any time than that of the extracellular protein (Fig. 18A). A fluorogram of extracellular protein shows that after 16 h incubation almost all proteins corresponding to eel serum proteins were observed (Fig. 18B).

The lipoprotein secreted by cultured eel hepatocytes was also isolated by density gradient ultracentrifugation, as described below.

After hepatocytes of 1.5 × 10^7 cells on a 10 cm plastic dish were cultured in WE medium containing 5% FBS and 0.16 μM insulin for 5 to 7 days. The hepatocytes were washed three times with a serum- and insulin-free WE medium and 7 mL of the serum- and insulin-free medium and 14C-leucine was added to the cells. Then hepatocytes were incubated at 28°C for 24 h. After incubation, the medium was recovered as described above. The medium and the PBS used to wash the cells were combined. The medium pooled from 6 to 10 dishes was centrifuged at 3,000 rpm for 5 min to remove cell and cell debris, and the supernatant was applied to a Sephadex
G-25 column (5 × 18.6 cm) equilibrated with PBS. The protein fraction was pooled and concentrated with a membrane of YM10 (Amicon).

The lipoprotein was fractionated by density gradient ultracentrifugation (Chung et al. 1980). An equal amount of 0.75% NaCl was gently laid over 19 mL of the sample containing 7.6 g of KBr, and centrifuged at 35,000 rpm (150,000 × g) for 14 h at 15°C in a 410-rotor using an International B/60 model ultracentrifuge (DAMON/IBC). After centrifugation, 40 fractions were collected from the bottom of the tube by a peristaltic pump and a fraction collector (Pharmacia).

Proteins secreted by cultured hepatocytes were fractionated by density gradient ultracentrifugation, as shown in Fig. 19A. The radioactivity of proteins labeled with 14C-leucine and the proteins determined by the colorimetric method were observed at densities above 1.21 g mL⁻¹ and below 1.063 g mL⁻¹. The fractions with densities below 1.063 g mL⁻¹ appeared to have triglycerides, which were determined by the enzymatic method (Determiner TG-S555, Kyowa Medix), as the main lipid component (Fig. 19A(2)).

**Fig. 19.** Fractionation of secreted and serum lipoproteins by density gradient ultracentrifugation. After hepatocytes cultured on a 10-cm dish were incubated with 14C-leucine (37 kBq/dish) for 24 h, the medium pooled from 10 dishes was centrifuged and applied to a Sephadex G-25 column (5 × 18.6 cm). Then the protein was fractionated by density gradient ultracentrifugation. Eel serum was fractionated in the same way. (A) Fractionation of secreted lipoprotein: 1, Distribution of protein (○) and radioactivity (×); 2, Distribution of lipid. (B) Fractionation of serum lipoproteins: 1, Distribution of protein (○); 2, Distribution of lipid (■, triglyceride; □, phospholipids; △, cholesteryl ester; ▲, free cholesterol). Reprinted from Cell Struct. Funct., 16, Yu et al. Characterization of lipoprotein secreted by cultured eel hepatocytes and its comparison with serum lipoproteins, 347–355, 1991, with permission from the Japan Society for Cell Biology.
On the other hand, eel serum proteins fractionated in the same manner as secreted proteins were observed at densities above 1.21 g mL\(^{-1}\), between 1.08 and 1.17 g mL\(^{-1}\), and below 1.063 g mL\(^{-1}\) (Fig. 19B(1)). Fractions with densities between 1.08 and 1.17 g mL\(^{-1}\) had phospholipids and cholesterol as the main lipid components (Fig. 19B(2)). Phospholipid, total cholesterol, and free cholesterol were determined by enzymatic methods of Determiner PL, Determiner TC555, and Determiner FC555 (Kyowa Medix), respectively. As shown in Table 12, the fraction (1.08 < \(d\) < 1.17 g mL\(^{-1}\)) was found to resemble a typical HDL found in human and rat serum in respect of protein and lipid compositions. Furthermore, the fraction contained two main apolipoproteins corresponding to apoAI and AII of mammalian HDL. This fraction was defined as HDL. The lipoprotein with densities below 1.063 g mL\(^{-1}\) had triglycerides as the main lipid component. The protein content was 6.2% and this lipoprotein had apolipoproteins corresponding to apoB48. These results show that this lipoprotein is VLDL.

However, secreted proteins contained no lipoprotein corresponding to serum HDL, as shown in Fig. 19A. Recent research on HDL formation suggests that HDL is formed from serum free apoAI and AII and an intracellular free cholesterol mediated ATP-binding cassette transporter I (ABCAI) in plasma membranes (Tsujita et al. 2005; Brunham et al. 2006). Actually, free apoAI and AII in the secreted proteins were detected in the fraction with a density above 1.21 g mL\(^{-1}\) by antibodies against the apoAI and AII purified from eel serum HDL (Katoh et al. 2000). Furthermore, although the secreted lipoprotein is a VLDL-like lipoprotein, apoAI and AII of the secreted lipoprotein are easily transferred to liposome particles by incubation with the secreted lipoprotein and liposomes (Yu et al. 1992).

When the secreted lipoprotein with densities below 1.063 g mL\(^{-1}\) was compared with eel serum VLDL, 12% of protein and 69% of triglycerides contents of the secreted lipoprotein were higher than those of eel serum VLDL (Table 12). The total cholesterol (4%) and phospholipid (15%) content of the secreted lipoprotein were lower. Apolipoproteins of the secreted lipoprotein consisted of apoA and B48 as the main components (Fig. 20A). Molecular weights of the secreted apolipoproteins are as follows: proteins corresponding to apoB48 are 290,000, 265,000, 245,000, and 220,000, and proteins corresponding to apoAI and AII are 28,000 and 14,000, respectively. These apolipoproteins were found in eel serum VLDL (Fig. 20B).

VLDL-like lipoprotein was secreted at the rate of 10.9 ± 4.34 μg protein (mg-cell protein\(^{-1}\)) (24 h\(^{-1}\)) (\(n = 6\)). It has been reported that in rat hepatocytes VLDL is secreted at the rate of 0.28 μg-protein (mg-cell protein\(^{-1}\)) (24 h\(^{-1}\)) (Bell-Quint and Forte 1981). This value was much lower than that of eel hepatocytes.

### Table 12. Composition of VLDL-like lipoprotein secreted by eel hepatocytes, serum VLDL, and HDL. VLDL-like lipoprotein was pooled from fractions 37 to 40 shown in Fig. 19A.

<table>
<thead>
<tr>
<th>VLDL-like lipoprotein (%)</th>
<th>Eel serum VLDL (%)</th>
<th>HDL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>3.8 ± 0.3</td>
<td>9.6 ± 0.8</td>
</tr>
<tr>
<td>CE</td>
<td>0.36 ± 0.50</td>
<td>4.5 ± 1.1</td>
</tr>
<tr>
<td>TG</td>
<td>69.0 ± 5.1</td>
<td>53.3 ± 7.0</td>
</tr>
<tr>
<td>PL</td>
<td>14.7 ± 2.9</td>
<td>26.4 ± 4.7</td>
</tr>
<tr>
<td>Protein</td>
<td>12.0 ± 4.8</td>
<td>6.2 ± 1.1</td>
</tr>
</tbody>
</table>

The values of VLDL-like lipoprotein, VLDL, and HDL are the means ± S.D. for 5, 4, and 4 experiments, respectively. FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipids. Reprinted with permission from *Cell Struct. Funct.*, 16, Yu et al. Characterization of lipoprotein secreted by cultural eel hepatocytes and its comparison with serum lipoproteins, 347–355, 1991, the Japan Society for Cell Biology.
4-3. Effects of maturation on eel lipoprotein metabolism

4-3A. Comparison of body length, body weight, gonad-somatic index, and plasma thyroxine between silver and yellow eels

Yellow eels are immature and grow in rivers. Silver eels migrate to the sea from the river for spawning. We captured silver and yellow eels in the Sendai River in Kagoshima, Japan and determined the body length, body weight, GSI [(gonad weight/body weight) × 100], and concentrations of the plasma thyroxine. As shown in Table 13, body lengths and weights of silver eels were significantly higher than those of yellow eels. The GSI of silver eels was also significantly higher than that of yellow eels. We determined total thyroxine concentrations by enzyme immunoassay (ICN Pharmaceutical) in the plasma of silver and yellow eels. The former was $10.9 \pm 8.2 \mu g \text{dL}^{-1} (1.40 \times 10^{-7} \text{M}, n = 20)$ and the latter was $3.0 \pm 4.3 \mu g \text{dL}^{-1} (3.86 \times 10^{-8} \text{M}, n = 20)$. The thyroxine concentration of silver eels was significantly higher than that of yellow eels. The high concentration of plasma thyroxine of silver eels strongly suggests a correlation between the migration of eels for spawning and thyroxine. Namely, when eels migrate to the sea for spawning as silver eels, the thyroxine concentration in their plasma increases.

4-3B. Comparison of plasma lipoproteins between silver and yellow eels

Plasma lipoproteins of silver and yellow eels were separated by density gradient ultracentrifugation. The VLDL of silver eels has two remarkable characteristics. The first is the high ratio of protein (apolipoproteins). The protein composition of silver eel VLDL was 17% and the value of yellow eel VLDL was 6%. The second is the low composition of triglycerides, and the value in silver eels was 40%, whereas the value of yellow eels was 53%. However, phospholipids compotion of silver eel (30%) was slightly higher than that of yellow (26%).
The HDL of silver eels also had the same tendency as the VLDL of silver eels. The high compositon of protein and the low composition of triglycerides were recognized in silver HDL. Particularly, the total cholesterol composition was remarkably low, 8%, and that of yellow eel was 18%, but the phospholipid composition of silver eel (35%) was slightly higher than that of yellow eel (32%) (Ndiaye and Hayashi 1997a).

4-3C. Comparison of lipoprotein synthesis by cultured hepatocytes of silver and yellow eels

The incorporation of $^3$H-leucine and $^{14}$C-acetate into the lipoprotein synthesized by cultured hepatocytes of silver eels was higher than those of yellow eels (Ndiaye and Hayashi 1997a). Particularly, the incorporation of $^3$H-leucine into the lipoprotein by silver eel hepatocytes was significantly higher than that by yellow eel hepatocytes.

The secretion rate $\mu$g (mg-cellular protein)$^{-1}$ (24 h)$^{-1}$ of lipoprotein by cultured hepatocytes of silver eels was $291 \pm 49$ ($n = 4$), whereas that of yellow eels was $130 \pm 19$ ($n = 3$). The secretion rate of silver eels is significantly higher than that of yellow eels. The chemical compositions of protein and triglycerides of the secreted lipoprotein by cultured silver eel hepatocytes were 9 and 67.2%, respectively, whereas those by cultured yellow eel hepatocytes were 18 and 60%, respectively. These characteristics of the compositions were the reverse with plasma VLDL of silver eels.

Serum VLDL of silver eels has a high composition of protein and low composition of triglycerides. The synthesized lipoprotein by cultured hepatocytes of silver eels has a low composition of protein and high composition of triglycerides, though the incorporation of $^3$H-leucine into lipoprotein by silver eel hepatocytes was 2.4 times higher than that by yellow eel hepatocytes. These results allow us to speculate that the lipoprotein synthesized by silver eel hepatocytes transports the triglycerides to other organs such as muscle and then serum VLDL of silver eels with a high protein composition is formed.

Stimulation of lipoprotein synthesis by silver eel hepatocytes seems to be due to hormonal effects such as by thyroxine or estradiol. In avian liver, estradiol stimulates VLDL synthesis and

---

### Table 13. Comparison of body length, body weight, and gonad-somatic index (GSI) between yellow and silver eels.

<table>
<thead>
<tr>
<th>Eels</th>
<th>Silver Eel</th>
<th>Yellow Eel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length (mm)</td>
<td>585</td>
<td>458</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>253.3</td>
<td>96.4</td>
</tr>
<tr>
<td>GSI</td>
<td>0.76</td>
<td>0.26</td>
</tr>
<tr>
<td>Sex</td>
<td>♂</td>
<td>♀</td>
</tr>
<tr>
<td>Body length (mm)</td>
<td>757</td>
<td>467</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>699.8</td>
<td>84.8</td>
</tr>
<tr>
<td>GSI</td>
<td>1.62</td>
<td>0.33</td>
</tr>
<tr>
<td>Sex</td>
<td>♂</td>
<td>♀</td>
</tr>
</tbody>
</table>

GSI = (gonad weight/body weight) × 100.

*Significantly different from yellow eels. $p < 0.01$.

$x \pm S.D.\ 669.2 \pm 118.2^a \quad 521.3 \pm 341.3^a \quad 1.02 \pm 0.53^a \quad 549.1 \pm 77.2 \quad 230.8 \pm 134.7 \quad 0.49 \pm 0.24$

4-3D. Effect of thyroxine on lipoprotein synthesis by cultured eel hepatocytes

Hepatocytes were prepared from aquacultured immature eels and cultured in a WE medium containing 5% FBS and 0.16 μM insulin. Addition of 10⁻⁸ M thyroxine (T4) in the WE medium stimulated lipid synthesis by hepatocytes. Thyroxine (10⁻⁴ M) increased the amount of intracellular free cholesterol, phospholipid, and triglyceride, and the sum of these lipids was 510 μg (mg-cell protein)⁻¹ corresponding to 143% of the control (Table 14) (Ndiaye and Hayashi 1997b). Thyroxine also increased the amount of extracellular free cholesterol, phospholipids, and triglycerides, and the sum of these lipids corresponded to 143% of the control.

The incorporation of ¹⁴C-acetate into cholesterol, phospholipid, and triglyceride in cultured hepatocytes was stimulated by thyroxine. The incorporation into the sum of these lipids corresponded to 157% of the control. The incorporation of ¹⁴C-acetate into extracellular lipids of thyroxine treated hepatocytes corresponded to 133% of the control (Table 15).

These results suggest that thyroxine stimulates lipid synthesis in hepatocytes and lipid efflux into the culture medium. Most of extracellular lipids associate with lipoproteins. Then we investigated the effect of thyroxine on lipoprotein synthesis and secretion.

Table 14. Lipid contents of intra- and extra-cellular fraction of eel hepatocytes cultured with or without thyroxine.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intracellular Lipid</th>
<th>Extraacellular Lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg cell prot.</td>
<td>µg/mg cell prot.</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>PL</td>
</tr>
<tr>
<td>Control</td>
<td>29.4 ± 2.7</td>
<td>233.2 ± 56.5</td>
</tr>
<tr>
<td>+ T4(10⁻⁸ M)</td>
<td>44.0* ± 4.2</td>
<td>326.8** ± 60.6</td>
</tr>
</tbody>
</table>

Hepatocytes (2 × 10⁷/dish) were cultured with or without 10⁻⁸ M thyroxine (T4) in 7 mL of Williams’ E medium (WE medium) containing 5% FBS and 0.16 μM insulin for 5 days. Then the medium was changed to 7 mL of FBS- and insulin-free WE medium with or without 10⁻⁸ M T4. After 24 h, intra- and extra-cellular lipids were extracted. FC, PL, and TG represent free cholesterol, phospholipids, and triglyceride, respectively. The values are the means ± S.D. for three experiments. *p < 0.01, **p < 0.05. Reprinted from Comp. Biochem. Physiol. B, 116, Ndiaye and Hayashi, A lipoprotein secreted by cultured hepatocytes of silver or yellow eel: Comparison with their plasma lipoproteins, 209–216, © 1997, Elsevier Inc. with permission from Elsevier.

Table 15. Effect of thyroxine on lipid synthesis and efflux in cultured eel hepatocytes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intracellular</th>
<th>Extraacellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>× 10⁻⁵ dpm/mg cell prot.</td>
<td>× 10⁻¹ dpm/mg cell prot.</td>
</tr>
<tr>
<td></td>
<td>FC</td>
<td>PL</td>
</tr>
<tr>
<td>Control</td>
<td>0.88 ± 0.04</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>+ T4(10⁻⁸ M)</td>
<td>1.69* ± 0.05</td>
<td>0.11* ± 0.05</td>
</tr>
</tbody>
</table>

Hepatocytes (2 × 10⁷/dish) were cultured with or without 10⁻⁸ M thyroxine (T4) in 7 mL of Williams’ E medium (WE medium) containing 5% FBS and 0.16 μM insulin for 5 days. Then the medium was changed to 7 mL of FBS- and insulin-free WE medium containing 74 kBq ¹⁴C-acetate with or without 10⁻⁸ M T4. After 24 h, phospholipids (PL), triglyceride (TG), free cholesterol (FC), and cholesteryl ester (CE) of the extracted lipid were separated by TLC. The each lipid after TLC was cut into pieces and the radioactivity of each piece in 5 mL of scintillation-cocktail was measured by a scintillation counter. The values are the means ± S.D. for three experiments. *p < 0.01, **p < 0.05. Reprinted from Comp. Biochem. Physiol. B, 116, Ndiaye and Hayashi, A lipoprotein secreted by cultured hepatocytes of silver or yellow eel: Comparison with their plasma lipoproteins, 209–216, © 1997, Elsevier Inc. with permission from Elsevier.
Hepatocytes prepared from immature and aquacultured eel were cultured with or without thyroxine (10⁻⁸ M) for 5 days. After the 5 days, the medium was changed to an FBS- and insulin-free WE medium containing ³H-leucine and ¹⁴C-acetate, and hepatocytes were incubated for 24 h. After incubation, the medium was recovered, centrifuged, and filtered with a membrane of 3.0 μm pore size. 100 μL of the filtrate was applied to a Superose 12 HR 10/30 column. Lipoprotein and other proteins were eluted by PBS, as shown in Fig. 21.

As shown in Table 16, the incorporation of ³H-leucine and ¹⁴C-acetate into lipoprotein synthesized by thyroxine-treated hepatocytes were 206% and 132% of the control, respectively (Ndiaye and Hayashi 1997b). On the other hand, the incorporation of ¹⁴C-acetate into the proteins, except lipoprotein synthesized by both thyroxine-treated and control hepatocytes, was not observed, but the incorporation of ³H-leucine into the proteins, except lipoprotein synthesized by both hepatocytes, was almost the same. These results show that thyroxine specifically stimulates lipoprotein synthesis.

Silver eels migrate to the sea from the river for spawning. The concentration of the thyroxine in their plasma is significantly higher than that of yellow eels. Thyroxine seems to stimulate lipogenesis and lipoprotein synthesis in the liver of silver eels. Cultured hepatocytes from silver eels show high activity of synthesis of protein as well as lipoprotein. However, the effect of thyroxine on lipoprotein and protein synthesis in the cultured hepatocytes prepared from immature eel showed that thyroxine specifically stimulated lipoprotein synthesis but not proteins excluding lipoprotein. These disagreements seem to be due to the effect of another factor excluding thyroxine on the liver of silver eels. Actually, isolated hepatocytes prepared from silver eels showed higher gluconeogenetic activity than hepatocytes prepared from yellow eels (Hayashi and Ooshiro 1985a).

Fig. 21. Gel-filtration of the medium from cultured hepatocytes treated with or without thyroxine by a Superose 12 HR 10/30 column. Hepatocytes (2 × 10⁷/dish) were cultured with or without 10⁻⁸ M thyroxine (T4) in 7 mL of Williams’ E medium (WE medium) containing 5% FBS and 0.16 μM insulin for 5 days. Then the medium was changed to 7 mL of FBS- and an insulin-free WE medium containing 185 kBq ³H-leucine and 74 kBq ¹⁴C-acetate with or without 10⁻⁸ M T4 for 24 h. After incubation, the medium was centrifuged at 3,000 rpm for 5 min and the supernatant was filtered with a membrane of pore size 3.0 μm (Millipore). One hundred microliters of the filtrate was applied to a Superose 12 HR 10/30 column equilibrated with PBS and secreted lipoprotein and other proteins were eluted with PBS at a flow rate of 18 mL h⁻¹. A fraction was collected every 0.5 mL and the radioactivity of each fraction was measured with a scintillation counter (Aloka LS-3500). Reprinted from Comp. Biochem. Physiol. B, 116, Ndiaye and Hayashi, A lipoprotein secreted by cultured hepatocytes of silver or yellow eel: Comparison with their plasma lipoproteins, 209–216, © 1997, Elsevier Inc. with permission from Elsevier.
HDL binding to primary cultured eel hepatocytes

4-4. HDL binding to primary cultured eel hepatocytes

4-4.1. Stimulatory effect of HDL on VLDL-like lipoprotein synthesis and secretion

The primary function of HDL is known as a reverse transport of cholesterol from peripheral tissues to liver. Liver is the only tissue that metabolizes excess cholesterol to bile acid to exclude or to use as a detergent for lipid absorption in intestine. The main apolipoproteins of HDL are apoAI and AII. Free apoAI and AII are synthesized in liver and secreted into circulation. Excess cholesterol of peripheral tissues are transported to free apoAI and AII through ABCAI of peripheral tissues and thereafter a mature HDL is formed (Yokoyama 2004). Information on the mechanism of HDL formation and HDL function seem to be available for eels, but there are few reports on HDL formation and the reverse transport of cholesterol in fish.

When HDL transports cholesterol to liver, HDL binds to an HDL receptor in the plasma membrane of hepatocytes. Scavenger receptor BI is most well known as an HDL receptor of liver in mammals (Acton et al. 1996). In fish serum, including eel serum, HDL is a main component and its concentration is about several to ten times higher than that of human (Babin and Vernier 1989). We investigated whether eel hepatocytes have an HDL receptor.

HDL isolated from eel serum by density gradient ultracentrifugation was labeled by lipophilic dye of \( \text{N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP)} \), FITC, or \( \text{125I} \). By using di-15-ASP-HDL or FITC-HDL, binding of HDL to cultured eel hepatocytes was investigated. Incorporation of HDL into hepatocytes was also investigated by using \( \text{125I-HDL} \).

Freshly isolated eel hepatocytes were incubated with di-15-ASP-HDL at different concentrations in a WE medium at \( 0^\circ \text{C} \) for 2 h in the dark. After the incubation, the cells were washed with cold PBS and then suspended with 0.5 mL of cold PBS. The cell suspension was analyzed by a flow cytometer (Epics Elite, Coulter Electronics). As shown in Fig. 22, specific binding of di-15-ASP to hepatocytes was saturated at a concentration of over 100 \( \mu \text{g-HDL-protein mL}^{-1} \) and \( K_\text{d} \) was about 20 \( \mu \text{g-HDL-protein mL}^{-1} \) (Ndiaye et al. 1995).

Eel hepatocytes were cultured in a 96-well microplate, and different concentrations of FITC-HDL/mL-PBS were added to each well and incubated for 30 min at \( 28^\circ \text{C} \) in a CO\(_2\) incubator. After incubation, each well was washed with cold PBS and 100 \( \mu \text{L} \) of cold PBS was added. The binding of FITC-HDL to cultured eel hepatocytes was determined by measuring the fluorescence intensity using a Cyto Fluor 4000 (Applied Biosystems). The binding of FITC-HDL to the hepatocytes was inhibited in the presence of 50 times excess of non-labeled HDL. These results show that FITC-HDL bound specifically to cultured hepatocytes (Kumagai et al. 2007).

\( \text{125I-HDL} \) was linearly incorporated into the acid-insoluble and acid-soluble fractions of the cultured hepatocytes (Fig. 23). Though the amount of incorporation of \( \text{125I-HDL} \) into the acid-soluble fractions was about 7% of the total incorporation amount, which was relatively low
compared with that of the acid-insoluble fractions, the appearance of radioactivity in the acid-soluble fraction shows that $^{125}$I-HDL incorporated into the hepatocytes was decomposed within the cells during 1 h incubation. In the presence of 100 times excess of cold HDL, the incorporation of $^{125}$I-HDL was extremely inhibited, as shown in Fig. 23 (Ndiaye et al. 1995). These results show that eel hepatocytes have an HDL receptor in the plasma membrane of eel hepatocytes. The $K_d$ value between the HDL receptor and HDL was determined to be about $20 \mu g$-HDL $mL^{-1}$, which corresponds to $40 \mu g$-HDL $mL^{-1}$ because HDL consists of about 50% protein. This value is almost the same as those of rat liver, the $K_d$ values are 10 to $50 \mu g$-HDL $mL^{-1}$ (Leitersdorf et al. 1984; Glass et al. 1985; Mitchel et al. 1987; Morrison et al. 1992).

When cultured hepatocytes prepared from immature eels were incubated with HDL, intracellular phospholipids and cholesterol ester contents increased, but not significantly, compared with the control. The levels of intracellular free fatty acids, triglycerides, and free cholesterol showed a tendency to decrease (Ndiaye et al. 1995). However, the rate of secretion of lipoprotein by the cultured eel hepatocytes incubated with HDL increased 2-fold higher than that by the control hepatocytes (Table 17). The composition of the secreted lipoprotein was 53.9% triglycerides, 5.0% free cholesterol, 6.2% cholesterol ester, 21.6% phospholipids, and 13.3% protein. The compositions of free cholesterol, cholesterol ester, and phospholipids of the secreted lipoprotein increased significantly. These results show that the addition of HDL to
cultured hepatocytes stimulated the efflux of lipids from hepatocytes through secreted lipoprotein. Furthermore, the synthesis of lipoprotein was specifically stimulated by HDL because only the incorporation of \(^{14}\)C-leucine into the secreted lipoprotein increased by HDL and the incorporation of intracellular proteins or other secreted proteins, except that the lipoprotein, showed almost no change compared with that of the control (Table 18) (Ndiaye et al. 1995).

The primary effect of HDL on the cultured eel hepatocytes seems to be an increase of intracellular cholesterol ester and phospholipids and this increases stimulates the efflux of intracellular lipids through the secreted lipoprotein. The effect of thyroxine on the cultured eel hepatocytes described above was an increase of lipogenesis in the cells and the increase of lipogenesis stimulated the efflux of intracellular lipids through the secreted lipoprotein. Therefore, both HDL and thyroxine seem to induce an increase of intracellular lipids and the efflux of intracellular lipids through the secreted lipoprotein.

4-4B. ApoAI and apoaII of HDL do not function as a ligand for eel HDL receptor

As described in Section 4-4A, eel hepatocytes have HDL receptors, but the ligand of HDL receptor is still unknown. Scavenger receptor BI (SR-BI) is most well known as an HDL receptor of mammals (Acton et al. 1996) and the apoAI of HDL is identified as the ligand of SR-BI (Acton et al. 1996). ApoAI and AII are the main proteins of both HDL and mammal HDL.

Two methods are applied to investigate whether apoAI or AII functions as the ligand of eel HDL receptor. One is the method for investigating the effect of anti-apoAI and AII antibodies on binding of FITC-HDL to the cultured eel hepatocytes and the other is the method for investigating the effect of apoAI and AII on ligand blotting by FITC-HDL. The results from the former

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**Table 17.** Effect of HDL on the lipid composition and secretion rate of lipoprotein (A) and on the lipid contents in cultured hepatocytes (B).

<table>
<thead>
<tr>
<th>Condition</th>
<th>TG (mg/mL)</th>
<th>FC (mg/mL)</th>
<th>CE (mg/mL)</th>
<th>PL (mg/mL)</th>
<th>Protein (mg/mL)</th>
<th>Total (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>101.7±29.8</td>
<td>4.32±1.58</td>
<td>3.77±1.62</td>
<td>21.12±6.32</td>
<td>29.7±6.8</td>
<td>160.61±31.29</td>
</tr>
<tr>
<td>HDL</td>
<td>170.9±33.3</td>
<td>15.92±3.67</td>
<td>19.64±7.81</td>
<td>68.56±24.54</td>
<td>42.2±4.5</td>
<td>317.22±42.49</td>
</tr>
</tbody>
</table>

TG, FC, CE, PL, and FFA represent triglyceride, free cholesterol, cholesterol ester, phospholipids, and free fatty acids, respectively. Addition of HDL to the cultured hepatocytes was 2.6 mg-protein mL\(^{-1}\). The values are the means ± S.D. for three experiments. *p < 0.01, **p < 0.05. Reprinted from Comp. Biochem. Physiol. B, 140, Kumagai and Hayashi, Participation of high-density lipoprotein in vitellogenesis in Japanese eel hepatocytes, 543–550, © 2005, Elsevier Inc. with permission from Elsevier.

**Table 18.** Effect of HDL on the incorporation of \(^{14}\)C-leucine into the secreted protein including secreted lipoprotein and the intracellular proteins.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Secreted Protein (dpm/mg cell prot.)</th>
<th>Secreted Lipoprotein (dpm/mg cell prot.)</th>
<th>Secreted Protein (mg/dish)</th>
<th>Intracellular Protein (mg/dish)</th>
<th>Cellular Protein (mg/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(2.38±0.57) \times 10^6</td>
<td>(3.18±0.95) \times 10^6</td>
<td>(1.33±0.30) \times 10^6</td>
<td>(1.31±0.32) \times 10^6</td>
<td>3.71±0.75</td>
</tr>
<tr>
<td>HDL</td>
<td>(2.61±0.21) \times 10^6</td>
<td>(4.55±0.78) \times 10^6</td>
<td>(1.33±0.09) \times 10^6</td>
<td>(1.47±0.14) \times 10^6</td>
<td>3.89±0.10</td>
</tr>
</tbody>
</table>

Addition of HDL to the cultured hepatocytes was 2.6 mg-protein mL\(^{-1}\). The values are the means ± S.D. for three experiments. Reprinted from Comp. Biochem. Physiol. B, 140, Kumagai and Hayashi, Participation of high-density lipoprotein in vitellogenesis in Japanese eel hepatocytes, 543–550, © 2005, Elsevier Inc. with permission from Elsevier.

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method were that FITC-HDL treated with anti-apoAI or AII antibody bound to hepatocytes with the same affinity as FITC-HDL without treatment with the antibody (Table 19). The results from the latter method were that FITC-HDL binding to 67 kDa plasma membrane protein of eel hepatocytes was not affected in the presence of an excess of apoAI or AII (Fig. 24). These results suggest that neither apoAI nor AII functions as the ligand of eel HDL receptor (Kumagai et al. 2007).

4-4C. Ganglioside of HDL functions as a ligand for an HDL receptor of eel hepatocytes

Hakomori (1981) have reviewed that oligosaccharide side chains of intrinsic membranes of glyco-sphingo lipids, particularly gangliosides, specifically bind to receptors. For example, ganglioside GM1 of the plasma membrane of intestinal epithelial cells is known as the ligand of cholera toxin protein (Fishman 1982). Chisada et al. (2005) reported that Vibrio trachuri, which causes vibriosis in fish, adheres to ganglioside GM4 of the intestine of red sea bream (Pagras

Table 19. Effects of HDL, anti-apoAI antibody, anti-apoAII antibody, and the purified ganglioside from the eel HDL on binding of FITC-HDL to hepatocytes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>FITC-HDL (30 μg-protein mL⁻¹) bound to hepatocytes</th>
<th>Relative fluorescence intensity</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (1.2 × 10⁵ cells)</td>
<td>24.8 ± 1.20</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>Non-labeled HDL (1.5 mg/mL)</td>
<td>24.7 ± 2.50</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>Anti-apoAI antibody</td>
<td>24.7 ± 1.20</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>Anti-apoAII antibody</td>
<td>24.7 ± 1.20</td>
<td>76%</td>
<td></td>
</tr>
<tr>
<td>Ganglioside from eel HDL (6 μg/mL)</td>
<td>36.8 ± 3.20*</td>
<td>100%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Non-labeled HDL incubated with ganglioside from eel HDL</td>
<td>7.68 ± 0.700*</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Eel hepatocytes (1.2 × 10⁵ cells) were cultured into a 96-well microplate. One hundred microliters of FITC-HDL (30 μg-protein mL⁻¹) with or without non-labeled HDL, antibody against apoAI or AII, and the purified ganglioside was added to each well, followed by incubation on ice for 30 min. After incubation, each well was washed with twice cold PBS, and then added to 100 μL of cold PBS. FITC-HDL (30 μg-protein mL⁻¹) was incubated with 1.5 mg of non-labeled HDL, 6.0 μg of the purified ganglioside or 1.5 mg of non-labeled HDL incubated with 6.0 μg of the purified ganglioside. FITC-HDL (30 μg-protein mL⁻¹) was treated with 15 μg of the antibodies. The binding of FITC-HDL was determined by measuring the fluorescence intensity using Cyto Fluor 4000. Each number ± S.D. represents the relative fluorescence intensity of FITC-HDL binding to hepatocytes. *Significantly different from the value of FITC-HDL, p < 0.01. **Significantly different from the value of FITC-HDL, p < 0.02. Reprinted from Comp. Biochem. Physiol. B, 147, Kumagai et al., Ganglioside from eel serum high density lipoprotein (HDL) and its role as a ligand for HDL binding protein, 635–644, © 2007, Elsevier Inc. with permission from Elsevier.

Fig. 24. Effects of apoAI and apoAII on binding of FITC-HDL by ligand blotting. Plasma membrane proteins of hepatocytes were applied to a gradient gel from 5 to 17.5% polyacrylamide, followed by transblotting to a PVDF membrane. Lane 1 was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol–7% glacial acetic acid. Lane 2 was incubated with 2 μg protein of FITC-HDL. Lanes 3, 4, and 5 were incubated with 2 μg protein of FITC-HDL in the presence of 50 times excess of non-labeled HDL, apoAI, and apoAII, respectively. Reprinted from Comp. Biochem. Physiol. B, 147, Kumagai et al., Ganglioside from eel serum high density lipoprotein (HDL) and its role as a ligand for HDL binding protein, 635–644, © 2007, Elsevier Inc. with permission from Elsevier.
major). Whether the ganglioside of eel serum HDL functions as the ligand of eel HDL receptor was investigated.

4.4C.1. Ganglioside GM4 isolated from eel serum HDL

Ganglioside GM4 was purified from lyophilized eel serum HDL, as reported by Kumagai et al. (2007). The mobilities of the purified GM4 and its lyso-form were the same as those of authentic GM4 from human brain and its lyso-form on HPTLC analysis. As shown in Fig. 25, the lyso-form of GM4 was positive against a ninhydrin reaction, because the amino-group appeared in lyso-form of ganglioside after ganglioside is treated by sphingolipid ceramide N-deacylase.

The mass of purified GM4 from eel serum HDL was 1088.7 and authentic GM4 from human brain was 1118.8. The masses of ceramide of the purified and authentic GM4s were 618.6 and 648.6, respectively (Fig. 26). From these values, masses of oligosaccaride of both GM4s were calculated as 470.1 and 470.2, respectively. The oligosaccaride of GM4 consists of N-acetyleneuraminic acid and galactose, the mass of which corresponds to 470. Recombinant endoglycoceramidase II (TAKARA Bioscience) did not hydrolyze GM4, but hydrolyzed gangliosides such as GM3, the ceramide of which binds to the glucose of the oligosaccaride. This means that purified ganglioside has no glucose in its oligosaccaride. The difference of 30 mass between GM4 of eel serum HDL (1088.7) and that of human brain (1118.8) is due to the difference of fatty acids of the GM4s. The fatty acid of GM4 of eel serum HDL seems to be 22:1 (C22H41O, 321) and the fatty acid of GM4 of human brain seems to be 24:0 (C24H47O, 351).

Fig. 25. HPTLC of the purified ganglioside and its lyso-form. (A) HPTLC of the purified ganglioside from eel serum HDL and authentic GM4 with a solvent system of chloroform/methanol/0.2% CaCl$_2$H$_2$O (65/25/4, v/v). Lane 1, authentic GM4 from human brain (1 μg); Lane 2, the purified ganglioside from eel serum HDL (0.5 μg); (B) HPTLC of lyso-form of the purified ganglioside, authentic GM4 and GM3. The purified ganglioside from eel serum HDL and authentic GM4 were treated with sphingolipid ceramide N-deacetylase, and the lyso-form of the purified ganglioside was further purified using a Sep-Pak Plus Silica column. The lyso-form of authentic GM4 (lane 1, 5 μg), the purified ganglioside (lane 2, 1.5 μg), and authentic GM3 from bovine milk (lane 3, 5 μg) were developed on HPTLC with a solvent system of chloroform/methanol/0.2% CaCl$_2$H$_2$O (65/25/4, v/v) and visualized with resorcinol-HCl reagent and 0.5% (w/v) ninhydrin in 100% acetone. Reprinted from Comp. Biochem. Physiol. B, 147, Kumagai et al., Ganglioside from eel serum high density lipoprotein (HDL) and its role as a ligand for HDL binding protein, 635–644, © 2007, Elsevier Inc. with permission from Elsevier.
Fig. 26. TOF mass spectrometry and assumed structures of the purified ganglioside from eel HDL. Authentic GM4 from human brain and the purified ganglioside from eel HDL were dissolved in chloroform/methanol (2/1, v/v) at a final concentration of 0.17 and 0.12 μg μL⁻¹, respectively. Each sample of 1 μL was applied to an Agilent 1100 TOF mass spectrometer (California, USA) with an HPLC of Agilent 1100 system equipped with a column of ZORBAX SB-08. The column equilibrated with 10 mM ammonium acetate/acetonitrile (80:20) was eluted at a flow rate of 0.3 mL min⁻¹ at 40°C. (A) Authentic GM4 from human brain. (B) Purified ganglioside from eel HDL. Reprinted from Comp. Biochem. Physiol. B, 147, Kumagai et al., Ganglioside from eel serum high density lipoprotein (HDL) and its role as a ligand for HDL binding protein, 635–644, © 2007, Elsevier Inc. with permission from Elsevier.
Serum HDL of immature eels contained 47.8 ± 17.8 nmol (n = 6) GM4 (MW 1089) per 100 mg dry weight of HDL calculated from the amount of N-acetylneuraminic acid. The HDL contained 52.6 ± 16.4 mg protein per 100 mg dry weight of HDL, and about the half of the HDL proteins consist of apoAI (MW 28,000), namely apoAI of 26.3 mg protein, which corresponds to 939.2 nmol of apoAI, containing per 100 mg dry weight of HDL. The molecular ratio of apoAI to GM4 in 100 mg dry weight of HDL is roughly 20:1. If the molecular weight of eel HDL is about 400,000 as well as mammalian HDL, 5 molecules of apoAI and AII (MW 14,000) are contained in each HDL particle. Therefore, taking an average that one in four molecule of HDL contains one molecule of GM4. If 1 molecule of HDL contains 20 molecules of GM4, the molar ratio of the HDL with GM4 to that without GM4 is 1/80, although how many molecules of GM4 are contained in 1 molecule of HDL has not yet been determined.

Although there are reports on gangliosides of serum lipoproteins in human serum (Chatterjee and Kwiterovich 1976; Clark 1980), the report on ganglioside GM4 of eel HDL is the first one in Teleostei (Kumagai et al. 2007). In human serum the major gangliosides of serum HDL are gangliosides GM3, GD3, and GM2, but GM4 is not detected (Senn et al. 1989). On the other hand, other gangliosides, excluding GM4, were not detected in eel serum HDL.

4.4C.2. GM4 as the ligand for eel HDL receptor

Two methods were applied to investigate whether GM4 functions as the ligand of eel HDL receptor. One is the method for investigating the effect of GM4 on binding of FITC-HDL to cultured eel hepatocytes. The other is the method for investigating the effect of GM4 and GM1 on ligand blotting by FITC-HDL. The results from the former method were that FITC-HDL incubated preliminarily with GM4 bound to the eel hepatocytes with a higher affinity than FITC-HDL without incubation with GM4. However, in the presence of non-labeled HDL incubated preliminarily with GM4, binding of FITC-HDL to the eel hepatocytes was strongly inhibited (Table 19).

The results from the latter method were that FITC-HDL incubated preliminarily with GM4 bound to 67 and 80 kDa plasma membrane proteins of eel hepatocytes with a higher affinity than FITC-HDL without incubation with GM4 (Fig. 27). Particularly, 80 kDa plasma membrane protein could be detected only by FITC-HDL incubated preliminarily with GM4. However, the binding of FITC-HDL incubated preliminarily with GM1 was strongly inhibited (Fig. 27). These results suggest that GM4 of eel HDL is the ligand of the eel HDL receptor.
As described in Section 4-4A, the $K_d$ value for binding eel HDL to eel HDL receptor is about 20 μg-HDL-protein mL$^{-1}$, which corresponds to 40 μg-HDL mL$^{-1}$ because HDL consists of about 50% protein. If the molecular weight of eel HDL is about 400,000, the $K_d$ value is calculated as $1 \times 10^2$ mol L$^{-1}$. The concentration of HDL containing GM4 is calculated as follows. As the concentration of eel serum HDL is about 500 mg dL$^{-1}$, the HDL concentration is calculated as 1.25 $\times$ 10$^{-5}$ mol L$^{-1}$. As described in Section 4-4C, if 20 molecules of GM4 are contained in one molecule of HDL, the molar ratio of the HDL with GM4 to the HDL without GM4 is 1/80. Then the concentration of the HDL containing GM4 is calculated as 1.56 $\times$ 10$^{-7}$ mol L$^{-1}$, which is comparable to the $K_d$ value. However, this concentration is calculated on the hypothesis that one molecule of HDL among 80 molecules of HDL contains 20 molecules of GM4.

It is thought that the ganglioside of serum lipoprotein is synthesized in a liver (Senn et al. 1989). Fish livers such as horse mackerel, Pacific mackerel, bonito, red sea bream, and shark contain GM4 as a main ganglioside, but mammalian liver contains GM3 as the main ganglioside (Saito et al. 2001; Li et al. 2002). Ganglioside is secreted into blood as a component of secreted lipoprotein (Kivatinitz et al. 1992), probably VLDL. The concentration of GM4 in serum HDL probably varies depending on the degree of GM4 synthesis, synthesis and secretion of lipoprotein, and transportation of GM4 to HDL from secreted lipoprotein. These variations of GM4 concentration in HDL may affect the interaction between eel serum HDL and the eel HDL receptor.

4-5. Vitellogenin induction by cultured eel hepatocytes

4-5A. Vitellogenin induction by estradiol-17β

Vitellogenin, a calcium-binding glycosylphospholipoprotein, is a precursor of primary egg yolk proteins and is synthesized and induced in the liver by estradiol-17β. Vitellogenin secreted from the liver is circulated through the blood and is incorporated into the oocyte by receptor-mediated endocytosis.

When aquacultured immature eels were intraperitoneally injected with 2 mg estradiol-17β per kg weight three times every 4 days, vitellogenin appeared in the plasma. Vitellogenin is a very-high density lipoprotein with a density from 1.27 to 1.30 g mL$^{-1}$ and was isolated by density gradient ultracentrifugation. Vitellogenin was further purified by a DEAE-Toyopearl 650S column. During the purification procedures, aprotinin (1,000 KIE mL$^{-1}$) and 20 mM EDTA were necessary to protect against protease digestion (Komatsu and Hayashi 1997). The purified vitellogenin had a single protein with 196 kDa under both reduced and non-reduced conditions (Fig. 28).

4-5B. Vitellogenin induction by cultured eel hepatocytes

Cultured hepatocytes prepared from aquacultured immature eels induced a synthesis of 196 kDa protein by $10^{-4}$ M estradiol-17β, which was the same protein as that appearing in the plasma of estradiol-17β injected eels. The synthesized protein with 196 kDa by cultured hepatocytes seems a component of vitellogenin (Fig. 29). However, a pharmacological dose of estradiol-17β was necessary for induction of vitellogenin (Komatsu and Hayashi 1997). The hepatocytes cultured with $10^{-4}$ M estradiol-17β contained many oily droplets in the cytoplasm, but the control cells had only a few droplets.

The effect of coating with poly-l-lysine and fibronectin on culture dishes used for attachment of hepatocytes were investigated for vitellogenin synthesis by $10^{-4}$ M estradiol-17β. At the same time, the effects of cell densities of $3.5 \times 10^{5}$ and $7 \times 10^{5}$ cells cm$^{-2}$ were investigated. The hepatocytes cultured on fibronectin-coated dishes and at low cell density synthesized vitellogenin 14 μg-protein (mg-cell protein)$^{-1}$ (Figs. 30 and 31) (Komatsu and Hayashi 1998). These conditions were optimal for vitellogenin induction under tested conditions.

Hepatocytes prepared from preliminarily estradiol-17β injected eels induced a synthesis of 196 kDa protein by $10^{-6}$ M estradiol-17β, a physiological concentration. After 3 days’ culture, the vitellogenin was observed in the medium supplemented with $10^{-6}$ M estradiol-17β and its amount was 0.66 and 1.32 μg-protein (mg-cell protein)$^{-1}$ at 9 and 11 days, respectively (Fig. 32), but control cells without estradiol-17β did not synthesize vitellogenin (Komatsu and Hayashi 1997).
Fig. 28. SDS-PAGE of the purified vitellogenin (Vg) from eel plasma. The purified Vgs from estradiol-17β-treated eels (lanes F and G) and female silver eel (lanes H and I) were prepared by a DEAE-Toyopearl 650S following density gradient ultracentrifugation. The whole plasma of control eels (lane B), estradiol-17β-treated eels (lane C), wild eels (lane D) and a female silver eel (lane E) were electrophoresed. Each sample of 15 μg (lanes B, C, D and E) or 3 μg (lanes F, G, H and I) was loaded on an SDS polyacrylamide gradient (4.5–18%) slab gel. Lanes B, C, D, E, F and H were performed under reduced conditions, and lanes G and I were performed under non-reduced conditions. Lane A, low molecular weight marker; Lane J, high molecular marker. Reprinted with permission from *Fish. Sci.*, 63, Komatsu and Hayashi, Pharmacological dose of estradiol-17β induces vitellogenin synthesis in cultured hepatocytes of immature eel Anguilla japonica, 989–994, 1997, the Japanese Society of Fisheries Science.

Fig. 29. SDS-PAGE of the plasma protein (lanes B, C, and D) prepared from control eels (lane B), estradiol-17β-treated eels (lane C), and a female silver eel (lane D) and that of the secreted protein (lanes E, F, G, H, I, and J) by non-treated eel hepatocytes cultured with 10–4 M estradiol-17β (lanes H, I, and J) and without estradiol-17β (lanes E, F, and G). Aliquots (15 μg) of several samples were loaded on an SDS polyacrylamide gradient (4.5–18%) slab gel. Lane A, molecular weight marker; VTG, apolipoprotein of Vg. Reprinted with permission from *Fish. Sci.*, 63, Komatsu and Hayashi, Pharmacological dose of estradiol-17β induces vitellogenin synthesis in cultured hepatocytes of immature eel Anguilla japonica, 989–994, 1997, the Japanese Society of Fisheries Science.
Fig. 30. SDS-PAGE of plasma proteins and secreted proteins by cultured eel hepatocytes. The hepatocytes were cultured on plastic dishes precoated with poly-L-lysine (E and C) or fibronectin (F and H). Aliquots (15 μg) of samples were loaded on an SDS polyacrylamide gradient (4.5–18%) slab gel. Lane A, molecular weight marker; lanes B, C, and D, the plasma protein prepared from control, estradiol-17β-treated, and a female silver eel, respectively; lanes E, F, G, and H, the secreted protein by the eel hepatocytes cultured with 10⁻⁷ M estradiol-17β (G and H) and without estradiol-17β (E and F). Reprinted with permission from Fish. Sci., 63, Komatsu and Hayashi, Pharmacological dose of estradiol-17β induces vitellogenin synthesis in cultured hepatocytes of immature eel Anguilla japonica, 989–994, 1997, the Japanese Society of Fisheries Science.

Fig. 31. Effects of materials for cell adhesion and cell density on the levels of apolipoprotein of vitellogenin (VTG) secretion by cultured eel hepatocytes. Hepatocytes were cultured under low cell density (3.5 × 10⁵ cells cm⁻²) or high cell density (7 × 10⁵ cells cm⁻²) condition on plastic dishes which were precoated with poly-L-lysine or fibronectin in the presence or absence of 10⁻⁴ M estradiol-17β. After 4 days’ culture, the intracellular (A) and extracellular (B) proteins were recovered. Several secreted proteins were loaded on SDS polyacrylamide gradient (4.5–18%) gel. After electrophoresis, the VTG content (C) in extracellular proteins was determined by a densitometer. Percentage of the VTG to total secreted proteins is given in parentheses in (C). Numbers of experiments are given in parentheses in (A). Reprinted with permission from Fish. Sci., 64, Komatsu and Hayashi, Optimal conditions for estradiol-17β induced vitellogenin synthesis by cultured hepatocytes of eel Anguilla japonica, 658–659, 1998, the Japanese Society of Fisheries Science.
Why hepatocytes prepared from preliminarily estradiol-17β-injected eels can induce vitellogenin at a physiological concentration of estradiol-17β (10^{-6} M) seems due to the increased numbers of estrogen receptor in the hepatocytes. The $K_d$ value of hepatic estrogen receptor of Japanese eel is known to be in the nM range (Todo et al. 1995, 1996). High concentrations of estradiol-17β, such as at 10^{-4} M, are not necessary for estradiol-17β to bind estrogen receptors.

Hepatocytes prepared from preliminarily estradiol-17β-injected eels seem to contain higher numbers of the cytosolic estrogen receptor than that in the hepatocytes from eels without estradiol-17β treatment.

4-5C. Stimulatory effect of HDL on vitellogenin synthesis and secretion

Hepatocytes prepared from immature eels were cultured in a WE medium containing 10% FBS, and 0.16 μM insulin on 60 mm dishes precoated with 0.02% poly-l-lysine. HDL was added at 400 μg protein per dish and estradiol-17β at 10^{-5}, 10^{-4}, or 10^{-3} M was also added. In the presence of 10^{-5} M estradiol-17β, HDL stimulated the induction of vitellogenin synthesis which in the presence of 10^{-3} M estradiol-17β and HDL was three times higher than that in the presence of 10^{-5} M estradiol-17β alone (Fig. 33) (Kumagai and Hayashi 2005). At concentrations of 10^{-4} and 10^{-3} M estradiol-17β, vitellogenin induction in the absence of HDL was not detected by the ELISA method. However, for the same concentrations of estradiol-17β vitellogenin induction was detected if HDL was present, although the amount of vitellogenin was about 1/4 compared with that of vitellogenin induced in the presence of 10^{-5} M estradiol-17β and HDL.

5. Discussion

5-1. Integrity of a perfused eel liver, isolated and cultured hepatocytes

The integrity of a perfused liver, isolated and cultured hepatocytes are checked by morphological, biochemical, and physiological methods. Biochemical integrities are mainly checked.
by synthetic abilities such as gluconeogenesis, protein synthesis, urea synthesis, bile acid synthesis, and lipogenesis. Oxygen consumption and CO₂ production are used as indicators of physiological integrity. In this section we focus on biochemical integrities.

Gluconeogenesis is a good indicator for biochemical integrity because gluconeogenesis from pyruvate or lactate needs 4 ATP and 2 GTP per mole of glucose synthesis. Furthermore enzymes necessary for gluconeogenesis are distributed in both cytosol and mitochondria. Namely, having a gluconeogenetic ability reflects normal energy production and integrity of intracellular compartments.

Perfused eel livers, isolated and cultured eel hepatocytes have the ability of gluconeogenesis. Table 20 shows a comparison of gluconeogenesis by perfused eel livers (Section 3-2A), isolated eel hepatocytes (Section 3-2B) and cultured eel hepatocytes (Section 3-2B). The highest rate of gluconeogenesis was obtained by the perfused eel livers and the lowest was obtained by the cultured eel hepatocytes. The values of 21 and 6.4 μmol-glucose (g-cell) h⁻¹ for cultured eel hepatocytes in Table 20 were the rates of gluconeogenesis from 10 mM pyruvate and
lactate, respectively. Therefore, when 5 mM pyruvate or lactate was used, gluconeogenesis by cultured eel hepatocytes seems lower than 21 or 6.4 μmol-glucose (g-cell)⁻¹ h⁻¹. If only gluconeogenesis is used as an indicator of the integrity of an eel liver, perfused eel livers retain the best liver functions. Certainly, perfused eel livers maintain tissues that consist of several kinds of cells and to maintain liver tissue seems to be the best way of keeping the liver functions. But perfused eel livers can retain liver functions only for 2 to 3 h and it is impossible to retain liver functions of perfused eel livers for over 3 h at present. Whereas gluconeogenesis by isolated eel hepatocytes was higher than that by cultured eel hepatocytes, as shown in Table 20. From this result, postulating that the integrity of isolated eel hepatocytes is higher than that of cultured eel hepatocytes is not reasonable. We then compared glycogen synthesis and intracellular protein synthesis between isolated eel hepatocytes and cultured eel hepatocytes.

Isolated eel hepatocytes were suspended in Ringer solution or serum- and insulin-free WE medium immediately after preparation and suspension of the isolated eel hepatocytes was used for experiments. Eel hepatocytes were cultured in WE–5% FBS–0.16 μM insulin for 4 to 5 days and then washed with Ringer solution or serum- and insulin-free WE medium. After washing, the cultured eel hepatocytes were used for experiments. Incubation time for experiments by isolated eel hepatocytes and by cultured eel hepatocytes were 1 and 2 h, respectively.

As shown in Table 21, glycogen synthesis was the highest in the cultured eel hepatocytes incubated in a serum- and insulin-free WE medium and then in the cultured eel hepatocytes incubated in Ringer solution. Among the three substrates, glycogen synthesis from 5 mM ¹⁴C-glucose was the highest and that from 5 mM ¹⁴C-lactate was the lowest. Both isolated eel hepatocytes suspended in Ringer solution and in a serum- and insulin-free WE medium synthesized little glycogen.

Intracellular protein synthesis was the highest in the cultured eel hepatocytes incubated in a serum- and insulin-free WE medium as glycogen synthesis. Among the three substrates, intracellular protein synthesis from 5 mM ¹⁴C-pyruvate was the highest and that from 5 mM ¹⁴C-glucose was the lowest. Isolated eel hepatocytes synthesized about 1/30 to 1/230 of the intracellular protein obtained by cultured eel hepatocytes.

These results show that judging the integrity of perfused eel livers or eel hepatocytes from only the ability of gluconeogenesis is not reasonable. Putting all these results together, cultured eel hepatocytes seem to retain the highest integrity. However, which method of the three is used is due to the purpose of the experiment.

5-2. Gluconeogenesis and glycogen metabolisms in eel liver

5-2A. Gluconeogenesis

The main focus of the pathway for gluconeogenesis from pyruvate or lactate is the PEP synthesis pathway. PEP synthesis is different among animals such as rat, eel, and pigeon, as
shown in Fig. 14. The reason why the PEP synthesis pathway is different is due to a different type of LDH and to the difference of the subcellular distribution of PEP carboxykinase. The PEP synthesis pathway was also clarified from the effects of some enzyme inhibitors and some amino acids on gluconeogenesis. The stoichiometric analysis of gluconeogenesis from 5 mM pyruvate by perfused eel livers suggests that the LDH type in eel liver is different from that in rat liver. Actually, the LDH of the eel liver was found to be of the heart type by starch gel electrophoresis and not of the muscle type as in rat liver (Fig. 9). An important characteristic of heart-type LDH was elucidated by intense substrate inhibition. Namely, the ternary complex of LDH-pyruvate-NAD\(^+\) inhibits LDH reaction. While the muscle type of LDH hardly forms the ternary complex. Because rat liver LDH is of the muscle type, the LDH reaction is little inhibited by pyruvate. Therefore, in rat liver about half of the amount of consumed pyruvate is converted to lactate with consumption of NADH in the cytosol, whereas when pyruvate is used for gluconeogenesis in eel liver no lactate is produced, therefore NADH in the cytosol is not consumed.

The difference of LDH type between rat and eel liver brings about a different consumption of cytosolic NADH. This seems to be the reason why a malate–oxaloacetate pathway for PEP synthesis is required in rat liver and not in eel liver (Figs. 14A, B). The malate–oxaloacetate

Table 21. Comparison of glycosgen synthesis, gluconeogenesis, and protein synthesis between isolated eel hepatocytes and cultured eel hepatocytes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glycogen cpm/(mg-protein·h)</th>
<th>Glucose cpm/(mg-protein·h)</th>
<th>Protein cpm/(mg-protein·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Isolated eel hepatocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM (^{14})C-Lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>0</td>
<td>5.79 (\times) 10(^3)</td>
<td>89</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>0</td>
<td>2.70 (\times) 10(^3)</td>
<td>34</td>
</tr>
<tr>
<td>5 mM (^{14})C-Pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>4</td>
<td>4.77 (\times) 10(^3)</td>
<td>134</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>6</td>
<td>2.33 (\times) 10(^3)</td>
<td>46</td>
</tr>
<tr>
<td>5 mM (^{14})C-Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>10</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>8</td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

<p>| <strong>B) Cultured eel hepatocytes</strong> |</p>
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glycogen cpm/(mg-protein·2 h)</th>
<th>Glucose cpm/(mg-protein·2 h)</th>
<th>Protein cpm/(mg-protein·2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM (^{14})C-Lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>96</td>
<td>3.45 (\times) 10(^3)</td>
<td>2.44 (\times) 10(^3)</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>508</td>
<td>711</td>
<td>4.93 (\times) 10(^3)</td>
</tr>
<tr>
<td>5 mM (^{14})C-Pyruvate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>120</td>
<td>5.02 (\times) 10(^3)</td>
<td>4.39 (\times) 10(^3)</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>1.47 (\times) 10(^3)</td>
<td>1.76 (\times) 10(^3)</td>
<td>10.6 (\times) 10(^3)</td>
</tr>
<tr>
<td>5 mM (^{14})C-Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ringer</td>
<td>615</td>
<td></td>
<td>1.06 (\times) 10(^3)</td>
</tr>
<tr>
<td>Serum-free WE medium</td>
<td>6.02 (\times) 10(^3)</td>
<td></td>
<td>1.06 (\times) 10(^3)</td>
</tr>
</tbody>
</table>

The values of isolated eel hepatocytes and cultured eel hepatocytes are the means for three experiments and for two experiments, respectively.
pathway is available to supply the cytosolic NADH through cytosolic malate dehydrogenase. Cytosolic NADH is necessary for glyceraldehydes-3-phosphate dehydrogenase in gluconeogenesis.

PEP carboxykinase of rat, eel, and pigeon distributes in the cytosol, in both the mitochondria and the cytosol, and in the mitochondria, respectively. Pyruvate carboxylase, which catalyzes the conversion of pyruvate to oxaloacetate, localizes in the mitochondria of rat, eel, and pigeon liver. Therefore, oxaloacetate is produced in the mitochondria and then the oxaloacetate is converted to PEP by PEP carboxykinase. In pigeon liver PEP carboxykinase localizes in the mitochondria and PEP is synthesized in the mitochondria (Fig. 14C).

PEP can pass through the mitochondrial membrane, but oxaloacetate cannot (Haslam and Griffiths 1968). Therefore, in rat and eel liver, oxaloacetate must be transported from the mitochondria to the cytosol. The effects of some enzyme inhibitors on gluconeogenesis, such as amino-oxyacetate (transaminase inhibitor), d-malate (malate dehydrogenase inhibitor), quinolinic acid (PEP carboxykinase inhibitor), and tryptophan (PEP carboxykinase inhibitor), suggest two PEP synthesis pathways in rat and eel liver, malate–oxaloacetate pathway and aspartate–oxaloacetate pathway (Fig. 7). As eel liver LDH is of the heart type, when pyruvate is used for gluconeogenesis, the malate–oxaloacetate pathway is not required because cytosolic NADH is not consumed by LDH. In eel liver, only the aspartate–oxaloacetate pathway is necessary for PEP synthesis, as shown in Fig. 14B, although about 30% of PEP is synthesized in the mitochondria by mitochondrial PEP-carboxykinase.

That the glutamate oxaloacetate transaminase reaction plays an important role in gluconeogenesis in eel liver, as shown in Fig. 12, is supported by the results of the effects of leucine and oleic acid on gluconeogenesis. Gluconeogenesis in eel liver is controlled by the amount of mitochondrial glutamate which is affected by glutamate dehydrogenase. When leucine was added to the reaction mixture, gluconeogenesis from pyruvate increased 1.5 to 2-fold and the amount of glutamate increased 3.7-fold. Increased glutamate seems to accelerate the transaminase reaction from oxaloacetate to aspartate in the mitochondria. The glutamate dehydrogenase purified from the mitochondrial fraction of the eel liver was stimulated 2.5-fold to the direction of glutamate formation by leucine, but 1.4-fold to the deamination direction (Hayashi et al. 1982a).

Furthermore, eel liver glutamate dehydrogenase was completely inhibited by 10 μM oleic acid or oleoyl-CoA. Addition of 2.5 mM oleic acid in the reaction mixture inhibited gluconeogenesis from lactate and pyruvate by over 95%. At the same time, the amount of glutamate in the eel liver decreased significantly compared to that of the control. This decrease seems due to the inhibitory effect of oleic acid on glutamate dehydrogenase. However, 2.5 mM oleic acid did not inhibit O2 consumption and CO2 production in the eel hepatocytes.

Inui and Yokote (1975b) have reported that the hydrocortisone-administered eel stimulates gluconeogenesis and the activity of glutamate oxaloacetate transaminase of the hydrocortisone-administered eel increases. They suggest some significance of aspartate in the amino acid metabolism of the eel. This seems to correspond to the important role of the aspartate–oxaloacetate pathway in gluconeogenesis of the eel liver.

5-2B Glycogen metabolisms

Glycogen stocke in eel liver is an important material as an energy source as well as lipid. There are few quantitative investigations on glycogen synthesis in fish liver. Investigations have become possible by using cultured eel hepatocytes incubated with a glucose-free MEM medium–0.5% BSA and the hepatocytes synthesized glycogen from 10 mM pyruvate and lactate at the rates of 81 (n = 4) and 33 (n = 2) μmol-glucose (g-cell)–1 h–1, respectively. Glycogen synthesis from pyruvate and lactate by cultured eel hepatocytes is higher than gluconeogenesis from pyruvate and lactate.

A remarkable characteristic on glycogen metabolism in eel liver is that the effect of glucagon on glycogen metabolism in the presence of 10 mM pyruvate was different from that in the presence of 10 mM lactate. In mammalian liver, it is well known that glucagon stimulates glycogen degradation and gluconeogenesis, whereas insulin stimulates glycogen synthesis and inhibits gluconeogenesis (Darnell et al. 1990). However, glucagon at 10–4 and 10–5 M in the presence
of 10 mM pyruvate did not stimulate glycogen degradation, but the same concentrations of glucagon in the presence of 10 mM lactate stimulated glycogen degradation. Glycogen synthesis from 10 mM pyruvate was observed even if 10−6 and 10−7 M glucagon was present. Furthermore, glycogen synthesis from 5 mM 14C-pyruvate by eel hepatocytes incubated with a serum- and insulin-free WE medium, which contains 11 mM glucose, was stimulated by 2.5 × 10−7 M glucagon 2.5-fold compared with glycogen synthesis without glucagon. As stimulatory effects of glucose on glycogen synthesis have been reported by Ui and Tokumitsu (1979), glucose in a WE medium also seems to stimulate glycogen synthesis in the eel hepatocytes.

In the presence of both glucagon and insulin at 2.5 × 10−7 M, glycogen synthesis from 5 mM 14C-pyruvate was further stimulated 10 times higher than glycogen synthesis in the absence of glucagon and insulin. In the presence of insulin alone, glycogen synthesis from 5 mM 14C-pyruvate was slightly stimulated by 18% compared with the control. These results show that glycogen synthesis from pyruvate was enhanced by the addition of glucose and insulin. However, the reason why glycogen synthesis from pyruvate, not from lactate, was stimulated by glucagon remains to be clarified in future.

Inui and Yokote (1975a, b) reported that liver glycogen content increased in the alloxanized eel and hydrocortisone-administered eel. Alloxan is well known for destroying the islet B cells and the alloxan-treated eel induces insulin deficiency. The alloxanized eel increased blood glucose and liver glycogen. They suggest that gluconeogenesis from amino acids was stimulated in the alloxanized eel. Similarly, the administration of hydrocortisone to the eel stimulated gluconeogenesis. Both the alloxan-treated eel and the hydrocortisone-administered eel stimulated gluconeogenesis and glucagon also stimulated gluconeogenesis in the cultured eel hepatocytes. Therefore, the stimulation of gluconeogenesis may induce glycogen synthesis. The mechanism of glucagon action on glycogen synthesis from pyruvate seems to be an interesting problem.

5-3. Lipoprotein metabolisms in eel liver

5-3A. Lipoprotein synthesized by cultured eel hepatocytes

There are three characteristics of lipoprotein synthesized by cultured eel hepatocytes. The first one is that the synthesized and secreted lipoprotein is only one kind of lipoprotein and it is a VLDL-like lipoprotein. As shown in Table 12, chemical compositions of the lipoprotein were 69.0% triglycerides, 14.7% phospholipid, 3.8% free cholesterol, 0.36% cholesterol ester, and 12.0% protein. The density of the lipoprotein is 1.06 g mL−1. These properties represent VLDL-like lipoprotein and the rate of secretion of the lipoprotein was very high at 10.9 ± 4.34 μg protein per mg cell protein per 24 h, which was 39 times higher than that of rat (Bell-Quint and Forte 1981). The high synthesis and secretion of the lipoprotein by eel hepatocytes seem to reflect the high concentration of eel serum lipoproteins. Although HDL is a main lipoprotein in eel serum, cultured eel hepatocytes do not synthesize and secrete HDL. The lipoprotein synthesized by the hepatocytes may be converted to LDL and HDL after secretion. Experiments on the conversion from secreted VLDL-like lipoprotein to HDL have not yet been performed, but apoAI and AII proteins of the secreted lipoprotein were easily transferred to artificial liposomes (Yu et al. 1992). Furthermore, free apoAI and AII in the secreted proteins were detected by anti apoAI or AII antibody (Katoh et al. 2000). These free apoAI and AII seem to be converted to HDL mediated through ABCA1 protein in plasma membranes of peripheral tissues (Tsujita et al. 2005; Brunham et al. 2006).

The second characteristic is the components of apolipoproteins of the lipoprotein. In spite of VLDL-like lipoprotein, it contains apoAI and AII as the main components. Another main component of the lipoprotein is apoB48, but apoB100 is not contained. These characteristics of apolipoprotein components are the same in eel serum VLDL and LDL. Main apolipoprotein of mammalian VLDL is apoB100, and apoAI and AII are not contained in mammalian VLDL. ApoAI and AII are the main components of mammalian HDL. Eel serum HDL also consists of apoAI and AII but both consist of two isomers. The two apoAI have 28 kDa and different N-terminal amino acid sequences and the two apoAII have 14 kDa and identical N-terminal amino acid sequences (Kondo et al. 2001). Because protein in mammalian apoB48 is a component
of chylomicron, the lipoprotein synthesized and secreted by cultured eel hepatocytes is also characterized as chylomicron-like lipoprotein.

The third characteristic is that the lipoprotein synthesis and secretion are controlled by the amounts of intracellular lipids of the hepatocytes in cooperation with external factors. Thyroxine (10^{-8} M) and HDL (2.6 mg mL^{-1}) stimulated the lipoprotein synthesis and secretion. Thyroxine also stimulated lipogenesis in the hepatocytes and thereby the amounts of intracellular lipids such as cholesterol, phospholipid, and triglycerides increased. At the same time, thyroxine stimulated the efflux of lipids to extracellular medium. Almost all extracellular lipids are associated with the lipoprotein in the medium. The effect of thyroxine on the incorporation of ^3H-leucine and ^14C-acetate into the secreted proteins including lipoprotein revealed that thyroxine specifically stimulated the incorporation of ^3H-leucine and ^14C-acetate into the lipoprotein.

Addition of HDL to the hepatocytes induced an increase of intracellular phospholipid and cholesterol ester, which are the main lipids of HDL. The amount of secreted lipoprotein increased by 2-fold by the addition of HDL and the incorporation of ^14C-leucine into the secreted lipoprotein increased by the addition of HDL, but there was no change in the incorporation into other secreted proteins. From the results of the effects of thyroxine and HDL on the synthesis and secretion of lipoprotein, it is concluded that stimulation of the synthesis and secretion of lipoprotein is induced when the amount of intracellular lipids of hepatocytes increases. Eel liver, different from cod liver, does not store lipids and excess liver lipids seem to transport immediately from a liver to serum through the secreted VLDL-like lipoprotein. Because eel muscle contains high amounts of lipid, about 20% of wet weight, the lipid of eel muscle seems to be transported through the secreted lipoprotein.

5.3B. HDL metabolism

We found that eel serum HDL functioned as stimulators of lipoprotein and vitellogenin synthesis and secretion. Eel HDL is also believed to play a key role in the process of reverse cholesterol transport. Excess cholesterol is transported to the liver by HDL and metabolized to bile acids in the liver to eliminate into the intestine. In any way, HDL binds specifically to eel hepatocytes and probably binds to the HDL receptor of the hepatocytes. However, the ligand for the HDL receptor have not been identified in fish. In mammals, the ligand for HDL receptors is known to be apoAI (Acton et al. 1996). However, apoAI or AII of eel serum HDL did not function as the ligand for the HDL receptor, as shown in Fig. 24 and Table 19. This seems to be due to a high concentration of HDL in eel serum, actually the HDL concentration was calculated as 1.25 × 10^{-5} mol L^{-1}. Whereas the K_d value for the HDL receptor of eel hepatocytes was determined to be 40 μg protein HDL mL^{-1}, which corresponds to 1 × 10^{-7} mol L^{-1}. The HDL concentration was calculated on the assumption that the concentration of eel serum HDL is about 500 mg dL\(^{-1}\) and the molecular weight of HDL is 400,000. Each HDL molecule contains five apoAI and AII molecules as described in Section 4-4C.

Ganglioside GM4 was purified from eel serum HDL and stimulated the binding of FITC-HDL to hepatocytes and to plasma membrane proteins (Fig. 27, Table 19). GM1 inhibited binding of FITC-HDL to plasma membrane proteins (Fig. 27). Furthermore, in the presence of non-labeled HDL incubated preliminarily with GM4, the binding of FITC-HDL to the eel hepatocytes was inhibited (Table 19). These results show that GM4 in eel HDL is the ligand for the HDL receptor. The molar ratio of apoAI to GM4 was calculated as 20. As 1 molecule of HDL contains 5 molecules of apoAI and AII, 1 molecule of HDL among 4 molecules of HDL contains 1 molecule of GM4. If the hypothesis that 1 molecule of HDL contains 20 molecules of GM4 is acceptable, the concentration of the HDL with GM4 is 1/80 of the HDL without GM4. Namely, the concentration of HDL with GM4 is calculated as 1.56 × 10^{-7} mol L^{-1}, which is comparable with K_d value of 1 × 10^{-7} mol L^{-1}.

This is the first report that GM4 is the ligand for the HDL receptor. Further investigations on the HDL receptor of fish including eel are necessary. Scavenger receptor BI (SR-BI) is the most well investigated as the HDL receptor in mammals. Recently, SR-BI of Atlantic salmon has been cloned (Kleveland et al. 2006). This has revealed that expressed salmon SR-BI has approximately 80 kDa on reducing SDS-PAGE. Among eel plasma membrane proteins, 80 kDa
protein was also detected by ligand blotting with FITC-HDL, but this 80 kDa protein has not yet been cloned.

5-3C. Induction of vitellogenin synthesis by cultured eel hepatocytes

Characteristic of vitellogenin induction in cultured eel hepatocytes by estradiol-17β (E2) is the necessity of a pharmacological dose of E2. If cultured hepatocytes were prepared from an immature eel, 10⁻⁴ M E2 would be necessary for vitellogenin induction. However, if cultured hepatocytes were prepared from the immature eel injected preliminarily with E2, vitellogenin would be induced by 10⁻⁶ M E2, which is a physiological concentration, but in the absence of 10⁻⁶ M E2 vitellogenin would not be induced in the same hepatocytes. Todo et al. (1995) have reported that the Kᵣ value of E2 receptor is known to be of nM order. An E2 concentration such as 10⁻⁴ M is not necessary to bind to an E2 receptor, perhaps the amount of E2 receptor in immature eel hepatocytes seems to be too small to bind to E2. Cultured hepatocytes prepared from an immature eel preliminarily injected E2 seems to be present in a large amount of E2 receptor. E2 probably not only induces vitellogenin but also the E2 receptor in vivo.

5-4. General Discussion

Glucose, glycogen, and lipids are important energy materials. These materials seem to play important roles in the spawning migration of eel. Gluconeogenesis, glycogen synthesis, and lipoprotein synthesis, therefore, also seem to play important roles.

Gluconeogenesis, glycogen synthesis, and lipoprotein synthesis also are all important liver functions. Perfused eel liver, isolated eel hepatocytes, and cultured eel hepatocytes have made it possible to quantitatively investigate all these important liver functions.

Gluconeogenesis was investigated by using perfused eel livers and isolated eel hepatocytes, and a novel pathway for gluconeogenesis in eel liver has been clarified which is different from those in rat and pigeon livers.

Glycogen synthesis and degradation was investigated by using cultured eel hepatocytes and unique results were as follows: Glucagon at 10⁻⁶ and 10⁻⁷ M in the presence of 10 mM pyruvate did not stimulate glycogen degradation but the same concentrations of glucagon in the presence of 10 mM lactate stimulated glycogen degradation. Glycogen synthesis from 10 mM pyruvate was observed, even if 10⁻⁶ and 10⁻⁷ M glucagon was present. These results suggest that the effect of glucagon on glycogen metabolism in eel liver is different from that in mammalian liver.

Lipoprotein synthesis and secretion was investigated by using cultured eel hepatocytes. In general, fish serums including eel serum contain high concentrations of lipoproteins. The primary function of lipoproteins is to transport lipids between tissues although the reason why eel serum contains high concentrations of lipoproteins is uncertain. The lipoprotein secreted by cultured eel hepatocytes was found to be VLDL-like lipoprotein. The rate of synthesis and secretion of VLDL-like lipoprotein by cultured eel hepatocytes was over 30 times higher than that of VLDL by cultured rat hepatocytes. This seems to reflect the high concentrations of eel serum lipoproteins.

Perfused eel liver, isolated eel hepatocytes, and cultured eel hepatocytes are all useful methods for investigating the metabolism of eel. However, some improvements in these methods are necessary. For example, the usefulness of eel serum instead of FBS for cultured eel hepatocytes must be examined in future.

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