ウナギ灌流肝における糖新生およびグリコーゲン分解作用

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著者	林, 征一 大城, 善太郎
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Gluconeogenesis and Glycolysis in Isolated Perfused Liver of the Eel

Seiichi HAYASHI* and Zentaro OOSHIRO*

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A method for perfusion of isolated eel (*Anguilla japonica*) liver is described. The perfusion medium employed was Krebs-Ringer bicarbonate without oxygen carriers such as red cells or fluoro carbon and albumin. The rate of gluconeogenesis in excess of lactate was almost the same as that of mammals. Insulin inhibited glycogenolysis of liver glycogen. Epinephrine and 3′, 5′,-c-AMP stimulated glycogenolysis. Activities of aspartate transaminase and alanine transaminase were maintained low during perfusion. Oxygen consumption was 1/5 to 1/7 of that in the perfused rat liver. These results indicate that our perfusion technique provides a useful method for studies of metabolic regulation of eel liver.

Perfused tissues, tissue slices and purified enzymes are often employed for the study of the metabolism in mammals. Especially, methods using isolated tissue perfusion are useful in order to examine metabolic regulations at tissue levels. In this study, isoalted liver of the eel (*Anguilla japonica*) was employed for the study of the glycogenolysis and the gluconeogenesis *in vitro*, since the eel liver is a clearly separate organ, different from some fish which have hepatopancreas. The size of the liver is also large enough for the preparation of perfusion; usually about 1.3–2.0 g in the case of adult eels. Several experiments were done using isolated perfused liver in order to know whether the perfused liver functions normally; (1) gluconeogenesis from lactate, (2) effects of insulin, epinephrine, and 3′,5′-c-AMP on glycolysis, (3) the oxygen consumption of the isolated liver during perfusion and inhibition by KCN, (4) activities of aspartate transaminase and alanine transaminase in the perfusion medium.

Experimental Procedure

Analytical methods Glucose in the perfusion medium was determined by the colorimetric method using *o*-toluidine-borate reagent described by SASAKI[1]. Lactate in the perfusion medium was determined with lactate dehydrogenase according to HOHORST[2]. For the determination of glycogen portions of liver were rapidly weighed after perfusion and digested immediately in KOH (30 g/100 ml) at 100°C according to HASID *et al.*[3] and the glycogen was determined colorimetrically as glucose. Aspartate transaminase and alanine transaminase were measured by using S.TA-Test Wako (Wako Chemicals Co., Ltd.). Oxygen content in the medium was determined by the iodometric titration based on the WINKLER's method.[4] 10 m*l* of the medium was collected into the test tube at certain intervals in which the polyethylene tubing from the hepatic vain

* Faculty of Fisheries, Kagoshima University, Kagoshima (林 征一・大城善太郎: 鹿児島大学水産学部)

was placed. At the same time the flow rate of the medium was also determined. In the collecting vessel, the freshly prepared perfusion medium was supplied at the same flow rate from a maliot flask. KCN was injected into the rubber tubing shown in Fig. 1–A by a syringe.

Perfusion medium The perfusion medium was modified KREBS-RINGER bicarbonate buffer which was freshly made each day; NaCl (120 mM), KCl (4.7 mM), $CaCl_2$ (2.4 mM), $MgSO_4$ (1.25 mM), KH_2PO_4 (1.25 mM), and $NaHCO_3$ (18 mM). This medium was gassed with humidified 95% O_2–5% CO_2 and maintained at pH 7.4±0.1 at 25°C. Usually 100 m*l* of the perfusion medium was used for each perfusion. During perfusion, samples of the medium (0.5 m*l* or 0.2 m*l*) were taken from the collecting vessel for chemical analyses and the small changes in the perfusion volume were taken into account in the calculation of metabolite content of the medium.

Perfusion apparatus The apparatus was set up based on MILLER *et al.*[5] and HEMS *et al.*[6] The whole apparatus is shown in Fig. 1–A. The cabinet (30×30×75 cm), provided with window at the front, was kept at 25±0.5°C by means of a heater, fan and thermoregulator. The perfusion medium was recirculated by microtube pump (Tokyo Rikakikai Co., Ltd. MP–2) with a sylicone tubing at a flow rate of 5.7 m*l*/min. The medium in the collecting vessel was stirred with a magnetic stirrer and gassed with humidified 95% O_2–5% CO_2. A reservoir containing oxygenated medium was set at the

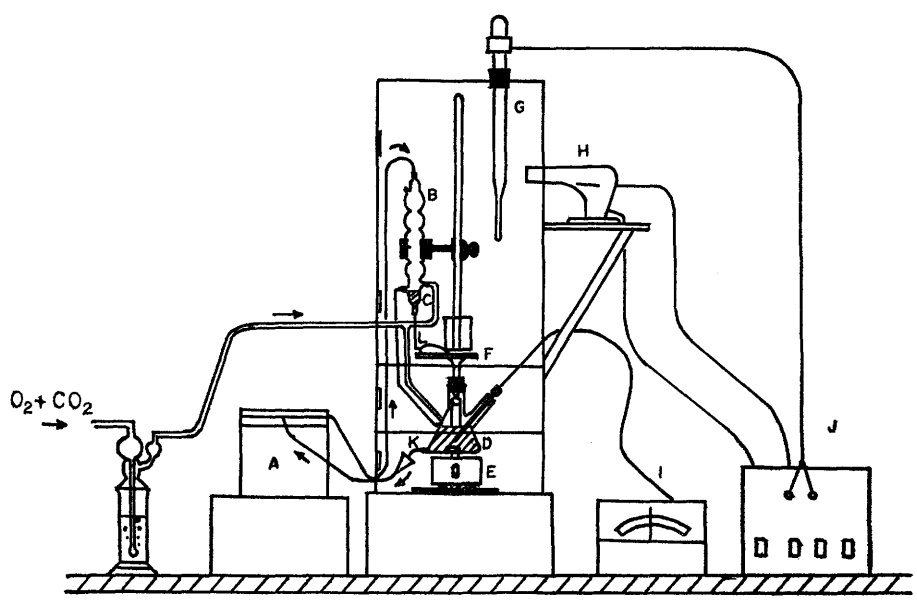

Fig. 1-A Apparatus for the perfusion of eel liver.
A. micro tube pump B. multi-bulb glass oxygenator C. reservoir D. collecting vessel E. magnetic stirrer F. platform for liver G. thermo regulator H. fan and heater I. pH meter J. relay K. rubber tubing L. silicon tubing.

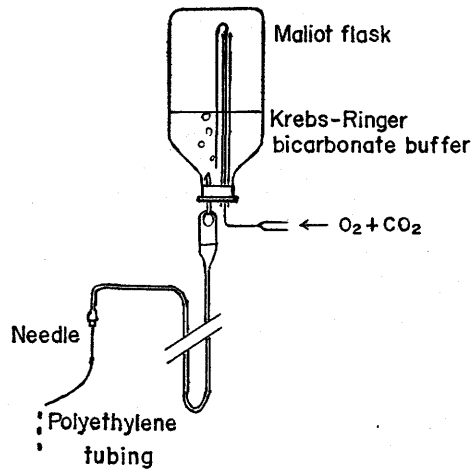

Fig. 1-B Maliot flask used for operation. For detail see the text.

bottom of the oxygenator and the distance between a reservoir and the liver was kept about 5 cm. In order to prevent progressive swelling of the liver, it is important to maintain a negative pressure in the hepatic vein. This was accomplished by placing the outlet of the tubing from the hepatic vein about 10 cm below the liver. The liver was perfused at a flow rate of 3–4 m*l*/min. The reservoir in which the oxygenated medium was collected had an outflow by which the excess medium was returned to the collecting vessel. Collecting vessel was provided with a hole to set pH electrode and with another hole to take samples of the medium or to add substrates in the medium. The window of the cabinet was used to take samples of the medium or to add substrates in the medium.

Operative technique The eel in fresh water was anaesthesized by 0.8% urethane solution. The abdomen was opened through a midline incision from the anus, and the liver, the intestine and the heart were exposed. Then heparin (100 units in 50 μl) was injected into the intestinal vein. The hepatic portal vein at about 1 cm below the liver was cut with scissors and the polyethylene tubing (outer diameter 1.1 mm, inner diameter 0.8 mm) containing the oxygenated medium as in Fig. 1–B was inserted, pushed to the liver and fixed with clip. Then the hepatic vein between the liver and the heart was cut. The color of the liver became immediately brownish yellow from brownish red. While perfusing the liver with the oxygenated medium, the liver is removed with the bile duct and the small part of the intestine. Then polyethylene tubing was inserted into the hepatic vein from the liver and tied with a ligature. The tubing inserted into the hepatic portal vein was also tied with a ligature. At this stage the needle connected to maliot flask (Fig. 1–B) was removed from the tubing inserted into the hepatic portal vein and the liver was immediately placed on platform to connect to the perfusion apparatus. The perfusion started at a flow rate of 3–4 m*l*/min.

The whole operation takes approximately 5 minutes and the liver was left without the oxygenated medium for about one minute during insertion of polyethylene tubing into the hepatic portal vein and connection of the liver to the perfusion apparatus.

Hormones and chemicals Crystalline insulin (from bovine pancreas) and LDH were obtained from Sigma Chemical Co. Crystalline epinephrine was obtained from E. Merck AG. Crystalline 3′,5′-c-AMP was generously provided by Yamasa Soybean

Souce Co., Research Institute (Choshi, Chiba).

Results

Gluconeogenesis from lactate The glucose production by the isolated perfused liver was examined in the eels subjected to starvation for various days. As shown in Fig. 2, there was no correlation between the glucose production and the duration of starvation. In order to examine the gluconeogenesis from lactate, lactate was added at 58 min. to perfused liver which showed a small glucose production for 60 min. As also shown in Fig. 3, glucose production increased immediately and the rate of glucose formation was 0.90 ± 0.07 μmole/min·g liver (n=4). On the other hand, the amount of lactate decreased at a rate of approximately 4.5 μmoles/min·g liver during the initial 30 minutes and at a rate of 2.07 ± 0.12 μmoles/min·g liver (n=4) in the following 30 minutes.

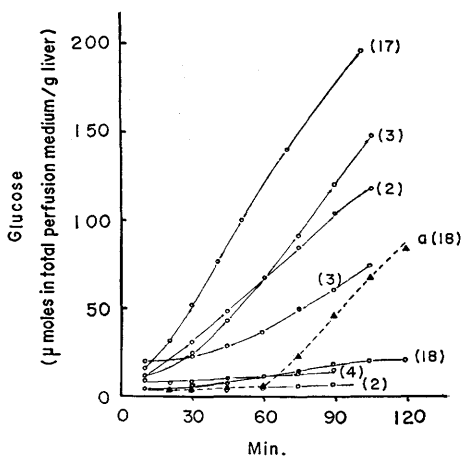

Fig. 2. Glucose production by the isolated perfused liver of the eels being subjected to starvation for various days. Starvation periods (days) in parentheses.
a – – – : Addition of lactate (10 mM) at 58 min.

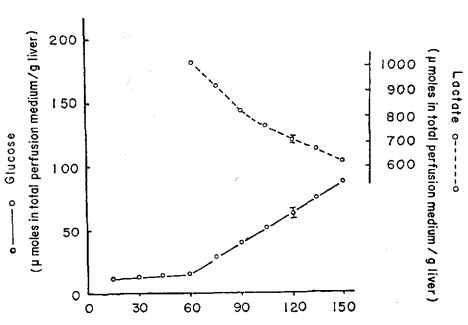

Fig. 3. Gluconeogenesis from L-lactate by the perfused eel liver. Lactate (10 mM) was added at 58 min.

Effect of insulin and epinephrine on glucose production The effect of various concentrations of insulin on glucose production is shown in Fig. 4. Insulin at a concentration of 4.4×10^{-6} mM (0.6 mμ unit/m*l*) suppressed glucose production almost completely. On the other hand, epinephrine (10^{-2} mM) caused an increase in glucose output (Fig. 5). The increase of glucose production was also observed by the addition of 3′, 5′-c-AMP (Fig.

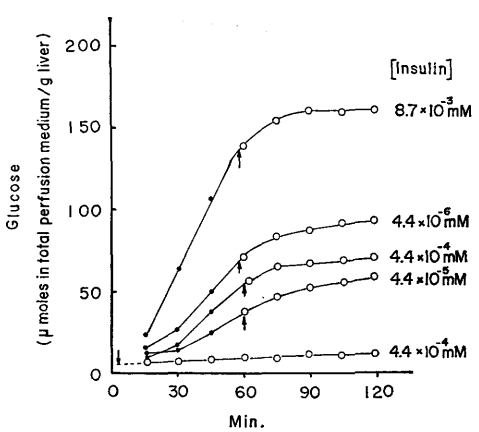

Fig. 4. Effect of insulin on glucose production. Insulin was added at zero time or 58 min. to give final concentrations indicated.

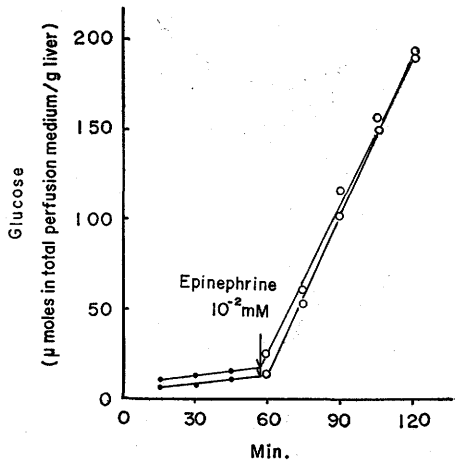

Fig. 5. Effect of epinephrine on glucose production.

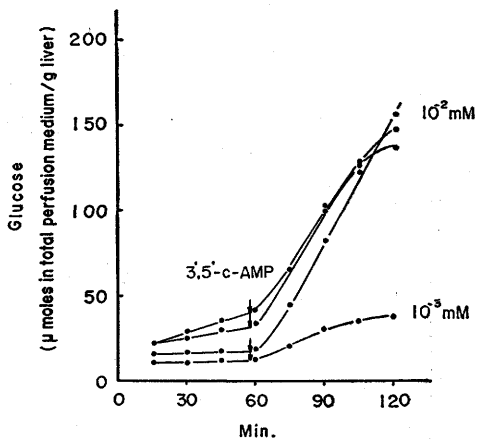

Fig. 6. Effect of 3′, 5′-c-AMP on glucose production. 3′, 5′-c-AMP was added at 58 min. to give final concentrations indicated.

Table 1. Effect of 3′, 5′-c-AMP on the amount of glycogen of the livers. 3′, 5′-c-AMP (10^{-2} mM) was added at 58 min. and liver was perfused for 60 min. after 3′, 5′-c-AMP addition. Liver glycogen was immediately determined after perfusion according the method described in experimental procedure. Livers of eels fasted for 3–6 days were used as control.

Liver glycogen (mg/g liver)	
3′, 5′-c-AMP (10^{-2}mM)	Control
0.9	15.9
4.1	22.9
1.2	20.2
3.0	33.8

6). The amount of glycogen of the livers perfused with 3′,5′-c-AMP (10^{-2} mM) was very small, as compared with that of controls (Table 1); the increase in glucose seems to be due to glycogenolysis. When epinephrine (10^{-2} mM) or 3′,5′-c-AMP (10^{-2} mM) was added to the medium containing insulin (4.4×10^{-4} mM) at 58 min, inhibition of glycogenolysis by insulin was not obserbed and glucose production increased greatly (Fig. 7). This may be due to rather high concentrations of epinephrine (10^{-2} mM) and 3′,5′-c-AMP (10^{-2} mM).

Fig. 8 and Table 2 show the effect of lactate plus 3′,5′-c-AMP on glucose production. The rate of glucose production was much greater than that of lactate removal. The increased glucose may be due to gluconeogenesis from lactate and glycogenolysis. When lactate was added to the medium containing insulin (4.4×10^{-4} mM) at 58 min, only a small increse in glucose production was observed and the rate of lactate removal was greater than that expected from glucose production (Fig. 9), suggesting an inhibitory effect of insulin on gluconeogenesis from lactate..

Oxygene consumption Table 3 shows the results of oxygen content in the medium during perfusion. The disolved oxygen content of distilled water (10 m*l*) saturated with air was 12.56±0.46 μl O_2/m*l* (n=6). As shown in Fig. 10, oxygen consumption was maintained almost constant during perfusion. KCN (0.1 mmloe) inhibited

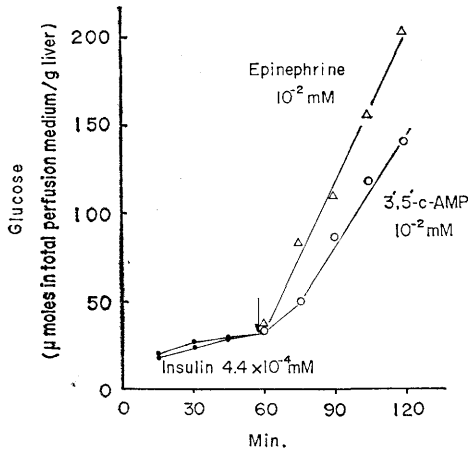

Fig. 7. Effect of insulin plus epinephrine or 3′, 5′-c-AMP on glucose production. Epinephrine (10^{-2} mM) or 3′, 5′-c-AMP (10^{-2} mM) was added to the medium containing insulin 4.4×10^{-4} mM) at 58 min.

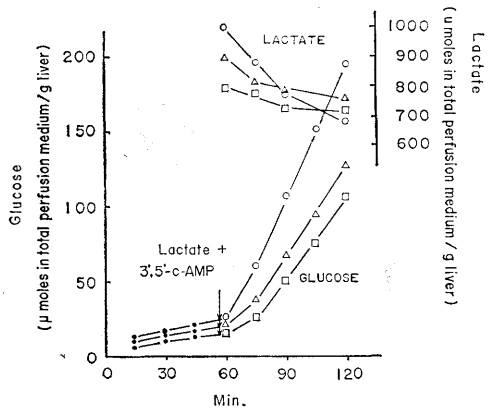

Fig. 8. Effect of lactate plus 3′, 5′-c-AMP on glucose production. 3′, 5′-c-AMP (10^{-2} mM) and L-lactate (10 mM) were added at 58 min.

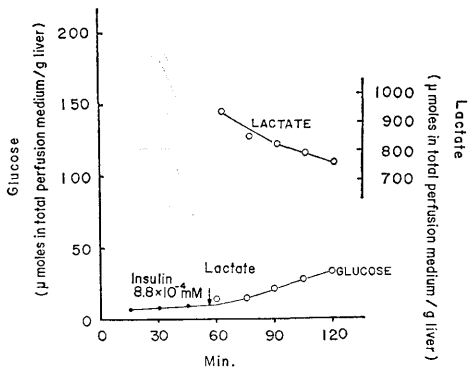

Fig. 9. Effect of insulin plus lactate on glucose production. L-Lactate (10 mM) was added to the medium containing insulin (8.8×10^{-4} mM) at 58 min.

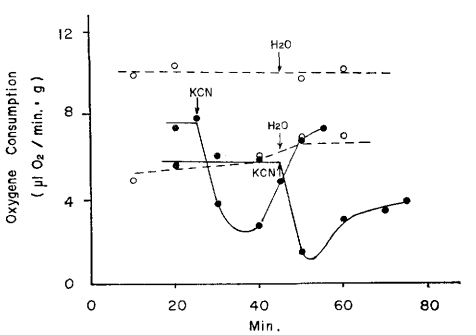

Fig. 10. Oxygene consumption by the isolated perfused eel liver and inhibition of respiration by KCN (0.1 mmole). Livers were perfused with nonrecirculating medium.

Table 2. Effect of lactate plus 3′, 5′-c-AMP on glucose production by the perfused eel liver. 3′, 5′-c-AMP (10^{-2} mM) and L-lactate (10 mM) were added at 58 min. Livers were perfused for 120 min.

Sample	Liver weight (g)	Lactate uptake (μmoles/min·g liver)	Glucose production (μmoles/min·g liver)
a	2.4	5.4	3.0
b	2.7	3.0	2.3
c	3.5	1.1	1.9

respiration immediately and recovery of respiration was observed after 30 minutes.

The oxygen consumption of the isolated perfused eel liver was 1/5 to 1/7 that of the perfused rat liver[6-8]. It is clear that these low values did not result from oxygen deficiency in the medium due to the omission of oxygen carrier such as red cells or fluoro carbon.[9]

Table 3. Oxygen content in the medium during perfusion. Livers were perfused with nonrecirculating medium

	Min								Perfusion medium
	10	20	30	40	50	60	70	75	
+H_2O (at 45 min.)	11.61	9.63	10.48	11.33	10.76	10.76			14.73
+H_2O (at 45 min.)	10.76	10.20	12.74	13.31	11.61	11.33			15.15
+KCN (at 25 min.)	10.20	9.91	12.46	13.31	11.33	11.04			15.01
+KCN (at 45 min.)		16.14	15.01	15.58	18.12	18.41	18.12	17.84	21.10

ul O_2/m*l*

Table 4. Aspartate transaminase and alanine transaminase activities in the medium during perfusion

	Min ----- 10	20	30	40	50	70	100	
Perfusate	Aspartate transaminase	5	3	5	4	5	6	6	Liver wt. 3.7 g
	Alanine transaminase	7	6	6	7	6	6	6	Karmen units
Homogenate	Aspartate transaminase				>200				Liver wt. 2.6 g
	Alanine transaminase				>200				Karmen units

Aspartate transaminase and alanine transaminase An increase in aspartate transaminase and alanine transaminase activities in the medium is recognized as a sensitive indicator of the appearance of damaged cell of the liver.[10] Since the activities of both enzymes in the medium during the perfusion were very low in the present experiment (Table 4), the damages of hepatocytes of the isolated perfused eel liver could be negligible.

Discussion

Perfusion of the eel livers with only KREBS-RINGER bicarbonate buffer did not restrict the capacity of the liver for gluconeogenesis nor its response to hormones. ARINZE *et al.*[11] and FRÖHLICH *et al.*[8] also reported that the biosynthetic capability of liver was largely unimpaired when perfused only with an electrolyte solution such as KREBS-RINGER bicarbonate buffer or KREBS-HENSELEIT buffer. The rate of glucose formation from lactate was 0.90 ± 0.07 μmole/min·g liver (n=4) in the isolated perfused eel liver. This value was almost the same as in the perfused rat liver.[6,7,11] The rate of removal of lactate during the initial 30 min. was different from the one in the following 30 min; about 4.5 μmoles/min·g liver during the initial 30 min. and 2.1 μmoles/min·g liver

in the following 30 min, suggesting that only a part of lactate was consumed to produce glucose for the initial 30 min. In the following 30 min. most of lactate seemed to be converted into glucose, since the rate of lactate removal was about 2 times of the rate of glucose formation.

The effects of epinephrine and insulin were observed in the isolated perfused eel liver as in mammals; epinephrine at a concentration of 10^{-2} mM stimulated glycogenolysis and insulin at a concentration of 10^{-6} mM (0.66 mμ unit/m*l*) suppressed glycogenolysis. Concentration of insulin (10^{-6} mM, 0.66 mμ unit/m*l*) was approximately larger by one order of magnitude than physiological concentrations of insulin in rat livers. Epinephrine and 3′,5′-c-AMP antagonized the effect of insulin on glucose output by the perfused eel liver. It has been well documented that insulin antagonizes to the effects of epinephrine and glucagon on glucose production by the perfused rat liver[12,13], or the isolated rat liver hepatocytes.[14]

Insulin seemed to inhibit gluconeogenesis from lactate and 3′,5′-c-AMP seemed to stimulate gluconeogenesis. However it was very difficult to examine the effect of 3′,5′-c-AMP or epinephrine on gluconeogenesis from lactate in the case of eel livers since it was almost impossible to make the glycogen of eel liver reduce by starvation. LARSSON *et al.*[15] reported that when the European eel (*Anguilla anguilla*) was subjected to starvation under laboratory conditions for 8 days, 95 days and 145 days, the amount of the glycogen of liver reached 32, 35 and 19 mg/g liver respectively. In the present experiment, the liver glycogen of the Japanese eel, subjected to starvation for 3–6 days, was 23 ± 8 mg/g liver (4 samples). It is likely that there is an interesting regulatory mechanism in the eel in glucose metabolism and actions of glucagon, epinephrine and insulin.

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