# Glyconeogenesis in Chum Salmon Alevin

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# Glyconeogenesis in Chum Salmon Alevin

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## Summary

1. Using egg and alevin of the chum salmon, *Oncorhynchus keta*, changes of general chemical components during its development, the relation between total free amino acids (FAA) and sugar level in exercised alevin, and the incorporation of $^{14}$C-labeled alanine into glycogen in alevin were studied in order to consider energy metabolism and to get and evidence of glyconeogenesis.

2. Dry weight, protein and lipid conspicuously decreased after hatching. The consumption ratio of protein was larger than it of lipid at early stage after hatching. Glycogen enormously increased after hatching and also glucose gradually increased.

3. Glycogen and total FAA in alevin decreased after an hour of exercise and glucose obviously increased. Glycogen and glucose returned to initial amount during 5 hours of recovery under the static condition after an hour of exercise, but total FAA continued to keep low level.

4. By the injection with 2 $\mu$l of a solution containing $^{14}$C-labeled alanine 0.1 $\mu$Ci per 2 $\mu$mol of alanine, it was revealed that the alanine rapidly incorporated into glycogen after 5 hours of the injection under the static condition. The ratios incorporated from alanine into glycogen were 1.2, 3.2 and 4.8 percent at 2, 5 and 24 hours after the injection respectively.

5. From these results, it was concluded that glycogen and glucose played an important role in energy metabolism, and that FAA was not only consumed during exercise but also contributed to glyconeogenesis as precursor.

Protein is known to be a major source of energy during yolk sac stage of fishes (1). However in the previous paper, we suggested that glycogen would contribute to energy metabolism during larval stage, based on the fact that much glycogen continuously accumulated in liver cells from the egg stage untill the end of the yolk sac stage (2).

Recently, the hepatic glyconeogenesis have been studied in mammal and it is clarified that precursors of glyconeogenesis are mainly amino acids such as alanine and serine (3). Then several studies on glyconeogenesis of fish are carried out (4–10), but no evidence of glyconeogenesis during the larval stage is reported. There are activation of nitrogen metabolism and glycometabolism such as elevation of the rate of protein consumption (11), the rate of ammonia excretion (12) and the

level of total free amino acid (13) during larval stage after hatching and the accumulation of glycogen in liver cells of chum salmon alevin (2), therefor it may be possible to be the mechanism of glyconeogenesis from free amino acids derived from yolk protein during larval stage.

It is the purpose of this study to get the evidence of glyconeogenesis of chum salmon alevin and to elucidate the mechanism of energy metabolism. The present paper describes about the changes of general components during development of egg and alevin of fish, the relation between total free amino acid (FAA) and sugar level in the exercised alevin, and glycogen synthesis from $^{14}C$-labeled alanine in the alvein.

## Materials and Methods

*Experiment I. Changes of General Components during Development of Egg and Alevin*

The experiments were carried out with the same group of eggs and alevins as in the previous paper (2). Samples of eggs or alevins were used for various chemical analyses at selected intervals during development.

*Wet and Dry Weight*: Ten eggs or 10 alevins were used for measurement of wet weights and dry weights, which were dried in a incubator maintained at 105°C, and water contents of each groups were obtained by calculating from their results.

*Protein-N*: Samples of 10 eggs or 10 alevins were homogenized in 10 percent trichloroacetic acid (TCA) and after precipitation, the precipitates were dried at 80°C for 24 hours. A part of dried precipitates were used for the determination of protein-N by semimicro-Kjeldahl procedure.

*Lipids*: Lipids were extracted from 20 eggs or 20 alevins with chloroform – methanol (2:1) mixture solvent by the method of Folch et al. (14). Aliquots of the lipid extracts were spotted onto thin layer chromatography (TLC) plates (Silica Gel, Wakogel B–5) and developed in the solvent mixture (n-hexane/diethylether/acetic acid, 75:25:1). The chromatograms were dried briefly in an incubator maintained at 110–115°C. Charring of the spots was carried out by heating the plates in an oven for 25 minutes at 180°C after spraying them lightly with a saturated solution of $K_2Cr_2O_7$ in 70 percent by volume of aqueous sulfuric acid, and the proportion of lipid components of the TLC spots were measured by a densitometer (15).

*Glycogen and Glucose*: Samples of 5 eggs or 5 alevins were immediately put in 80 percent cold methanol solution and homogenated. The suspensions were centrifuged two times for 5 minutes at 3,000 rpm. They were separated to the supernatant liquids containing the glucose and the precipitate sediment containing the glycogen. The glucose and the glycogen were purified by the method of Kemp and Heijningen (16) and the method of Roy and Deiley (17), respectively. Both of the glucose and glycogen were determined by the phenol-sulfate method (18).

*Experiment II. Changes in Total FAA and Sugar Contents according to Exercise of Alevins*

*Procedure of Exercising and Sampling*: The fish used in this experiment were alevins of 30 days after hatching and weighing 370–380 mg. The alevins were reared by the same method as described in previous paper (2). These experiments were carried out in the room maintained at 10°C. About 30 alevins were put in one liter beaker with 800 ml of water and it was stirred for 1–3 hours at the constant speed by the magnetic stirrer (Auto mixer, M21, Yamato Scientific Co., Ltd.). Alevins swam against water current to keep the same place. After exercising of designated periods of time, they were separated to several groups and each group composed of 5 alevins, brought in several big petri dishes with 300 ml of water and gravels. They immediately took refuge in a space among gravels and recovered under the static condition. At the selected time alevins were picked up and immediately frozen in acetone dry-ice and 80 percent cold methanol solution for analyses of the content of total FAA and sugars, respectively.

*Determination of Total FAA*: Frozen alevins were homogenated in 6 percent TCA and after two times of precipitation for 10 minutes at 300 rpm, the supernatant liquid were used to determine by the method of Lee and Takahashi (19).

*Histological procedure*: A part of samples were fixed with Bouin a fluid and the glycogen in liver was detected according to previous procedure (2).

*Experiment III. Synthesis of Glycogen from $^{14}C$-Labeled Alanine in Alevins*

*$^{14}C$-Labeled Alanine*: Uniformly labeled L-alanine-$^{14}C$, obtained from The Radiochemical Centre Amersham, had a specific activity of 10 mCi per mmol. It was diluted with L-alanine to a solution containing 50 $\mu$Ci per mmol in 1 ml.

*Procedure of Administration of $^{14}C$-Labled Alanine*: Alevins of 35 days after hatching were exercised for one hour by the same method as described in the experiment II. After exercising, alevins were briefly anesthetized in urethane solution of 2 percent, and 2 $\mu$l of a solution containing $^{14}C$-labeled alanine, 0.1 $\mu$Ci per 2 $\mu$mol alanine, was injected into the abdomen of embryo of alevin with a finely-honed syringe (10 $\mu$l capacity; Hamilton Co., Ltd.). Administrated alevins were put in several big petri dishes and were recoveried under the static condition described in the experiment II.

*Procedure of Glycogen Purification and Determination of Radioactivity*: After various recovery times, 0.5, 1, 2 and 24 hours, alevins were picked up and put in a test tube with 30 percent KOH solution. The glycogen was purified according to method of Hassid and Abraham (20). The purified glycogen was disolved with 5 ml of distilled water and 1 ml of glycogen solution was added to 10ml of toluene containing 4 g of 2,5-diphenyloxazole (PPO), 0.1 g of 1, 4-bis(5-phenyloxazole-2-yl) benzen (POPOP) and 500 ml of Triton X–100 per liter as a scintillator and the

radioactivity was determined by a liquid scintillation spectrometer (Pakard Co., Ltd.).

All experiments were repeated twice. When two results did not correspond, experiments were carried out all over again. When two results almostly corresponded, results were relied and averaged.

## Results

*Experiment I. Changes in General Components during Development of Egg and Alevin*

As shown in Fig. 1, wet weights, dry weights and water contents are nearly constant during the egg stage. After hatching, wet weight was gradually increased in the contrast with decrease in dry weight according to development of alevin. This conflicting phenomenon was obviously caused by the conspicuous increase in water contents.

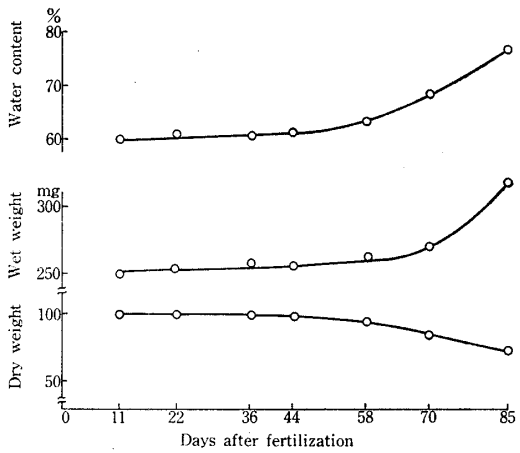


FIG. 1. Changes in wet weight, dry weight and water content of an egg and alevin during development.

Protein-N and total lipid also maintained constant level before hatching, but decreased after hatching (Table 1). The Protein-N decreased rapidly and conspicuously during development of alevin after hatching (Fig. 2). At 85 days after fertilization (37 days after hatching), the ratio of protein-N to initial amount of it was 63 percent. While the conspicuous decrease in the total lipid content started at later stage, i.e. at 20 days after hatching (Fig. 2 and Table 1). At 85 days after fertilization, the ratio of total lipid to initial amount of it was 75 percent. Namely, the consumption of protein was evidently larger than it of total lipid.

The proportion of lipid components don't change during development of egg before hatching (Table 2). Namely, the eggs contained 60–62 percent triglyceride (TG), 16–18 percent phospholipid (PL), 11.5–12.5 percent sterol (S), and 9–10 percent added total with diglyceride (DG), free fatty acid (FFA), hydrocarbon (HC)

TABLE 1. *Actual Amounts of General Components in the Egg and Alevin of Chum Salmon*

| Components \ Days after fertilization (Days after hatching) | 11 | 22 | 36 | 44 (−4) | 58 (10) | 70 (22) | 85 (37) |
|---|---|---|---|---|---|---|---|
| Wet weight (mg) | 250 | 255 | 258 | 256 | 262 | 271 | 314 |
| Dry weight (mg) | 100 | 100 | 102 | 99 | 95 | 86 | 73 |
| Water content (%) | 60.1 | 61.0 | 60.6 | 61.3 | 63.8 | 68.4 | 76.7 |
| Kjeldahl-N in TCA precipitate (mg) | 10.0 | 10.1 | 10.2 | 10.0 | 9.4 | 7.9 | 6.3 |
| Total lipid (mg) | 29.0 | 29.5 | 29.4 | 27.4 | 27.8 | 27.4 | 21.6 |

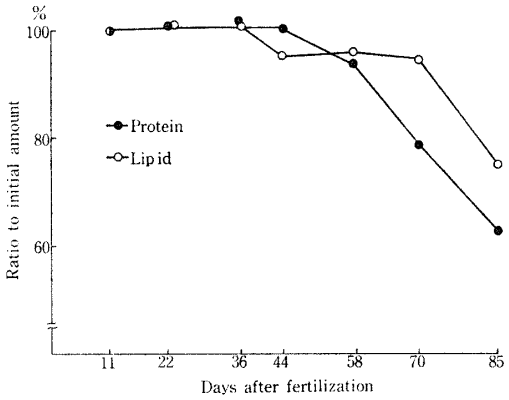


FIG. 2. Changes in relative amounts of protein and lipid of an egg and alevin during development.
Relative amounts are represented as ratio to initial amount of egg at 11 days after fertilization.

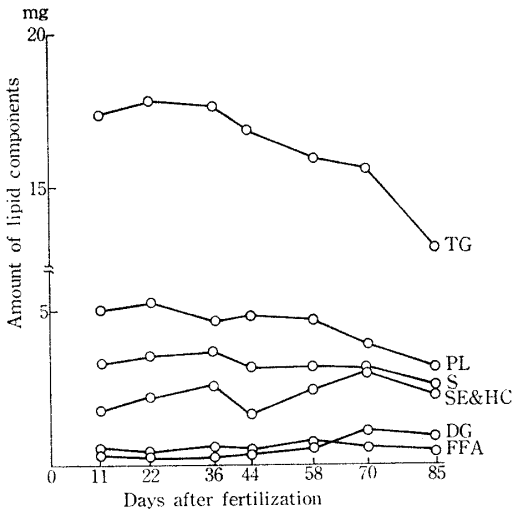


FIG. 3. Changes in amount of lipid components on an egg and alevin during development.

and sterol ester (SE). After hatching the proportion of TG and PL decreased, and there were slightly the changes in other components. Fig. 3 shows the changes in actual amount of lipid components per an individual. After hatching, the amount of TG, PL and SE decreased during the development after hatching. In particular, TG strongly decreased during the period 70 days to 85 days after fertilization and the decreasing of TG extensively influenced upon the change of total lipid. While, it was difficult to be found the conspicuous changes in other components, DG, FFA and HC.

The glycogen and glucose showed characteristic changes respectively during development of egg and alevin (Fig. 4). The glycogen increased gradually during development of egg before hatching and conspicuously during development of

TABLE 2. *Proportions of Lipid Components in the Egg and Alevin of Chum Salmon*

| Components (%) \ Days after fertilization (Days after hatching) | 11 | 22 | 36 | 44 (−4) | 58 (10) | 70 (22) | 85 (37) |
|---|---|---|---|---|---|---|---|
| Phospholipid | 17.8 | 17.9 | 16.1 | 17.8 | 16.8 | 14.2 | 14.8 |
| Diglyceride | 1.2 | 0.8 | 0.8 | 1.2 | 2.0 | 4.1 | 4.4 |
| Sterol | 11.5 | 11.9 | 12.4 | 11.5 | 11.1 | 11.1 | 12.3 |
| Free fatty acid | 1.8 | 1.2 | 2.0 | 1.8 | 2.9 | 2.4 | 2.2 |
| Triglyceride | 61.7 | 60.9 | 60.0 | 61.7 | 58.3 | 57.2 | 55.8 |
| Hydrocarbon + Sterol ester | 6.1 | 7.3 | 8.8 | 6.1 | 8.7 | 11.0 | 10.5 |

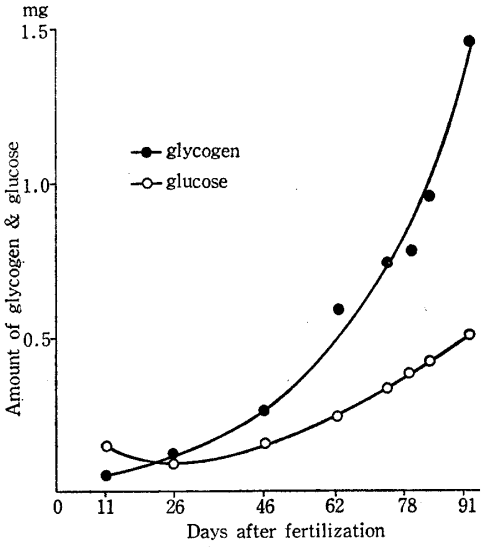


Fig. 4. Changes in amount of glycogen and glucose of an egg and alevin during development.

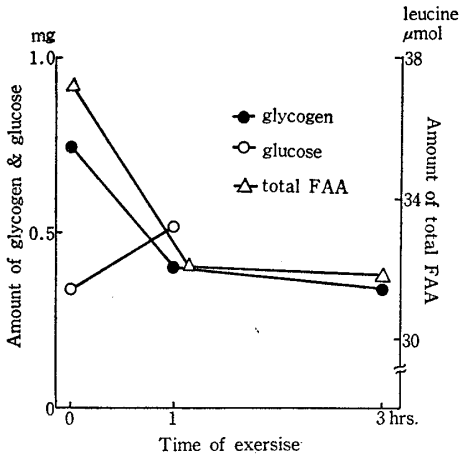


FIG. 5. Changes in amount of glycogen, glucose and total FAA according to exercise in alevin at 30 days after hatching.

alevin after it. Actual amount of glycogen was about 0.05 mg per an egg of 11 days after fertilization, while it was about 1.5 mg per an alevin of 43 days after hatching. Namely, the latter increased by about 30 folds on comparing with the former. On the other hand, the glucose temporally decreased at 26 days after fertilization and thereafter gradually increased throughout development of egg and alevin.

*Experiment II. Changes in Total FAA and Sugar Content according to Exercise of Alevins*

As shown in Fig. 5, during first an hour of exercise by magnetic stirrer, the conspicuous decrease occur in contents of total FAA and glycogen, and furthermore

obvious increase in content of glucose. However, during next two hours of exercise, total FAA and glycogen almostly kept constant levels. On this basis, followed experiments were carried out after an hour of exercise.

In the next experiment, the behavior of glycogen, glucose and total FAA according to recovery after exercise were investigated. As a natural results, amount of glycogen, glucose and total FAA showes similar pattern to the experiment of Fig. 5 during first an hour of exercise. Namely glycogen and total FAA decreased about 0.3 mg and 15 mol respectively. While glucose increased about 0.1 mg during the period of exercise. According to the time elapsed of recovery under the static condition after exercise, the glycogen contents gradually increased, and nearly returned to the initial contents before exercise for 5 hours of recovery. However total FAA did not still increased for 5 hours of recovery. On the other hand, glucose content gradually decreased during recovery and also returned to the initial content during 5 hours of recovery.

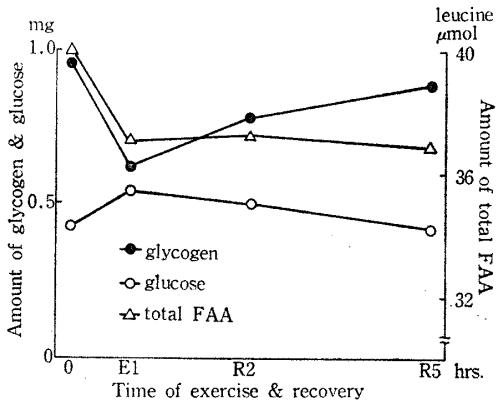


FIG. 6. Changes in amount of glycogen, glucose and total FAA according to recovery after an hour of exercise in alevin at 3 days after hatching. E: exercise, R: recovery

The similar change of glycogen according to exercise and recovery was also accepted by the histological observation of liver cells in the experimental alevins. As shown in Fig. 7-a much PAS-positive material accumulated in liver cells before exercise. As the PAS-positive material disappeared by the salivary digestion (Fig. 7-b), it was decided to be glycogen. The glycogen in liver cells was decreased after an hour of exercise (Fig. 7-c). Then the glycogen considerably accumulated again in liver cells after 5 hours of recovery (Fig. 7-d).

*Experiment III. Glycogen Synthesis from $^{14}C$-Labeled Alanine in Alevins*

Alevins, which was administered with $^{14}C$-labeled alanine after an hour of exercise, did not die at all for 24 hours of recovery. To ascertain the incorporation of the alanine into glycogen, the glycogen was precipitated several times with ethyl alcohol. During the consecutive precipitations, the radioactivity of the

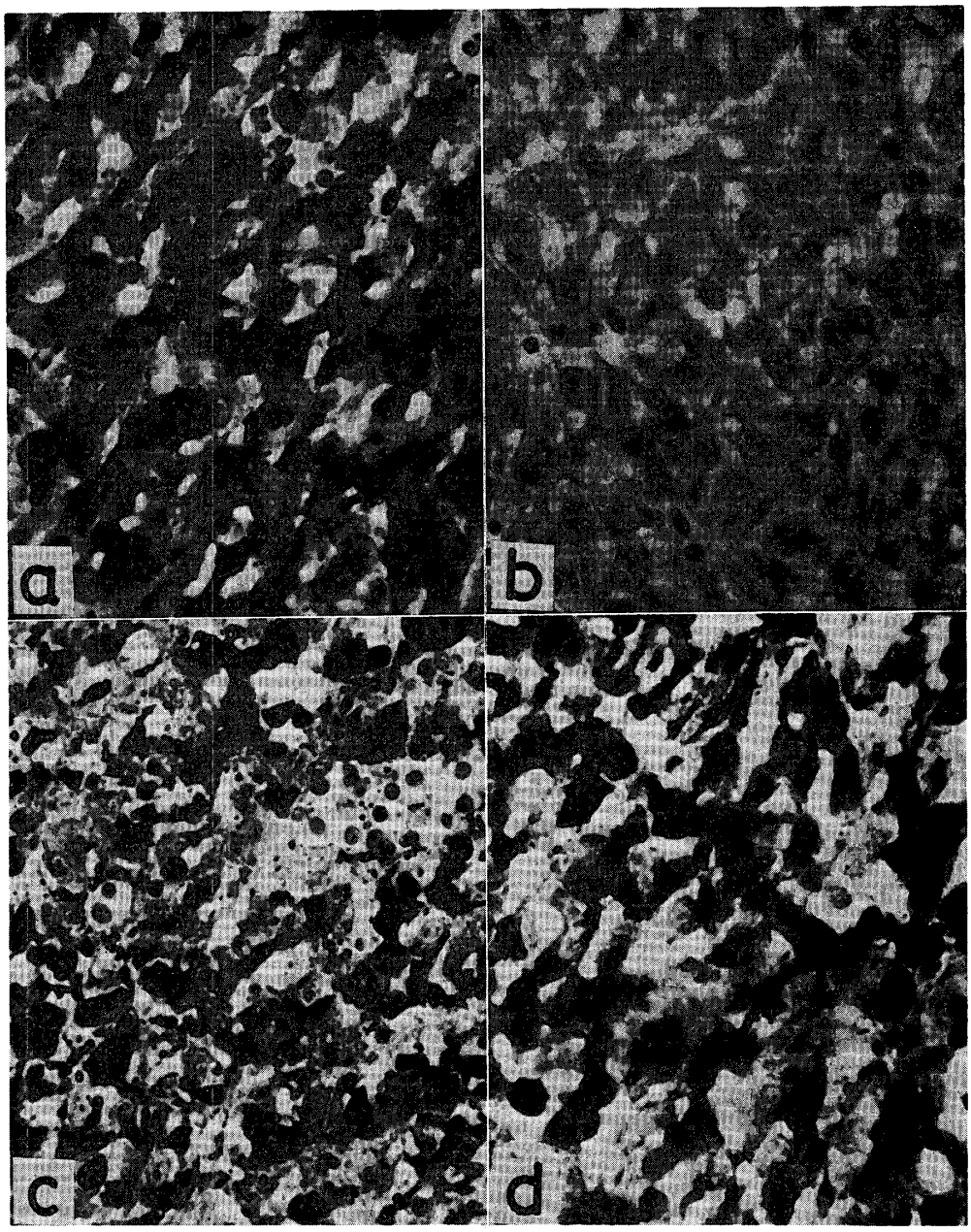


FIG. 7. Histological changes of liver cells according to exercise and thereafter recovery in alevins at 33 days after hatching, × 930, PAS reaction-hematoxylin.

a) Liver cells of alvein before exercise, PAS-positive material, represented as black, is remarkable in cytoplasm.
b) Liver cells, treated with saliva, of alevin before exercise, PAS-positive material is washed out and empty space is observed in cytoplasm of liver cells.
c) Liver cells of alevin after an hour of exercise, PAS-positive material is obviously decreased as compared with liver cells of alevin before exercise.
d) Liver cells of alevin after 5 hours of recovery, PAS-positive material is increased as compared with exercising alevin.

glycogen did not almost decreased. For example, the activity in the three time precipitation of samples of 5 hours of recovery was 7182.2 dpm and it of 4 and 5 times precipitations was 6975.0 dpm and 6932.8 dpm respectively. Hence, three

TABLE 3. *Incorporation into Glycogen after Injection with* 0.1 *μCi and* 0.2 *μmol of L-[U-$^{14}$C] Alanine into Chum Salmon Alevin*

| Item culculated \ Time after injection (hours) | 0.0 | 0.5 | 1.0 | 2.0 | 5.0 | 24.0 |
|---|---|---|---|---|---|---|
| A. Radioactivity of glycogen (dpm) | 8.0 | 426.9 | 1623.2 | 2692.0 | 7182.2 | 10736.9 |
| B. Ratio of $^{14}$C-alanine incorporated into glycogen (%) | 0.00 | 0.19 | 0.73 | 1.21 | 3.24 | 4.84 |
| C. Specific activity (dpm/mg) | 10 | 505 | 1796 | 2733 | 6386 | 9513 |
| D. Amount of glycogen synthesized from 0.2 mol of administered alanine ($\gamma$) | 0.00 | 0.34 | 1.31 | 2.16 | 5.83 | 8.71 |
| E. Amount of total glycogen ($\gamma$) | 831.2 | 845.4 | 904.0 | 984.9 | 1124.7 | 1128.7 |

Each value is average of 10 individuals. B: A(dpm)/0.1 $\mu$Ci ($2.22\times10^5$ dpm) $\times$ 100, C: 1 $\mu$mol of glucose (180$\gamma$) $\times$ B/100, D: A/B $\times$ 1000

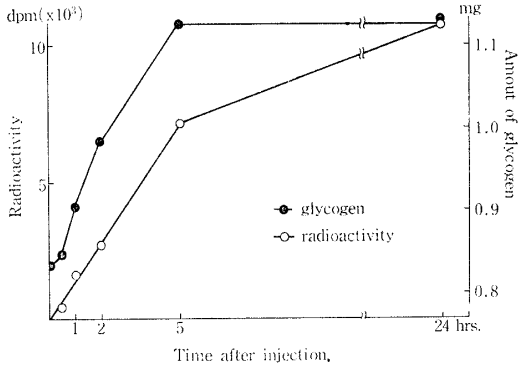


FIG. 8. Changes in amount of total glycogen and radioactivity incorporated from administered U-$^{14}$C-[L-alaine], with 2 $\mu$mol of alanine containing 0.1 $\mu$Ci of $^{14}$C-alanine.

times precipitations is enough to purification of glycogen.

As shown in Fig. 8, the radioactivities incorporated into glycogen conspicuously increased for 5 hours after administered and thereafter increased somewhat further at 24 hours. The incorporated activity was about 7,200 dpm per an individual at 5 hours of recovery, and furthermore it reached to about 10,000 dpm at 24 hours of recovery (Table 3). The ratio of the alanine incorporated into glycogen, incorporated activity/0.1 $\mu$Ci ($2.22\times10^5$ dpm) $\times$ 100, was from 0.2 percent to 4.8 percent after administered. The amount of $^{14}$C-labeled glycogen synthesized from administered alanine, 1 $\mu$mol of glucose (180$\gamma$) $\times$ incorporated ratio/100, was from 0.3 to 8.7 $\gamma$.

The contents of total glycogen in experimental alevins obviously increased during 5 hours of recovery after exercise and administered. This result was similar to it of the experiment II. Thereafter the contents of glycogen did not increased and showed the same value at 5 hours and 24 hours of recovery (Fig. 8 and Table 3).

## Discussion

The results of experiment I shows that each chemical components of chum salmon egg and alevin conspicuously change after hatching. Firstly, dry weight obviously decreased after hatching, in the contrast with the increase of wet weight. The decrease of dry weight can be accounted to be caused by the decrease of protein and lipid after hatching. Furthermore, consumption ratio of protein is larger than it of lipid during development of alevin. Hence, protein will be important as energy sourse for chum salmon alevin, being simultaneous with that protein is major component for embryogenesis. The similar notion about protein consumption is suggested in aquatic embryo by Hayes (21). If protein is consumed as energy, it will be thought that protein is digested to FAA. This inference is supported by the fact that FAA extensively increase after hatching in rainbow trout (13).

On the other hand, TG and PL in the lipid components, in particular TG, are thought to be mostly used as energy sourse during development. The TG especially decreased during the later period of development. The TG is known to be major components of oily droplet and to be used as energy at later period of development in teleost larvae (22). In chum salmon, it is clarified that protein in yolk matter is used during earlier stage of alevin and thereafter TG is consumed at later stage.

The glycogen and glucose of chum salmon egg and alevin almostly increased during development. In particular the glycogen enormously increased after hatching. The increase of glycogen is well correspond to previous histological study which glycogen accumulation in liver cells started just before hatching and continued to the end of yolk sac stage (2). On this basis, glycogen and glucose is thought to play an important role in energy metabolism during development of egg and alevin. Simultaneously, it is also an interest question what glycogen is derived from. The changes during development show that it is most possible for the protein to be original precursor of glycogen and glucose. In fact, FAA derived from body protein is well known to be precursor, especially when liver glycogen is decreased in mammal (3) and adult eel (5).

The experiment II was carried out in order to examine the relation between sugar, such as glycogen and glucose, and total FAA under the condition of exercise. By an hour of exercise, glycogen decreased chemically and histologically, in the contrast with increase of glucose. The behaviors of glycogen and glucose are well correspond with decrease of glycogen in liver cells and increase of blood sugar in mammal. On this basis, it is judged that alevins have an ability of glycolysis mechanism with exercise, although they are generally static. Furthermore, FAA will be also consumed, since total FAA decrease during exercise.

The alevins can not continue to exercise physiologically for long time, because amount of glycogen and total FAA did not almost change from one hour to three

hours of exercise as shown in Fig. 5. Actually, alevins could not sufficiently swim against water current and tended to be driven out after three hours of exercise.

On the other hand, during three hours of recovery under the static condition after an hour of exercise, glycogen and glucose returned to initial amount of them before exercise. Increase of glycogen was also ascertained by histological observation with liver cells. However, total FAA did not return to the amount to initial level. This phenomenon may be able to be understood that FAA is consumed as precursors of glycogen synthesis.

The experiment III was undertaken in order to conform the hypothesis that FAA is one of precursors of glycogen synthesis in alevin. This hypothesis was actually conformed by the result that the radioactivity incorporated into glycogen rapidly increased during 5 hours after injection with $^{14}C$-labeled alanine. While incorporated ratio increased from 0.2 to 3.2 percent during the time from 0.5 to 5 hours after injection. These results is not very different from results with fasted rat that labeled alanine is rapidly incorporated into liver glycogen during 0.5 to 4 hours after injection and that incorporated ratio increased from 0.1 to 1.6 percent during the time from 0.5 to 4 hours after injection (23). Some variation of incorporated ratio in two studies may be due to different materials, namely whole body is used in chum salmon alevin, while liver is only used in fasted rat, for purification of glycogen. Since increase of incorporated radioactivity is slower during first 5 hours after injection, glycogen is almostly synthesized during earlier period after exercise and injection. However amount of glycogen which was synthesized from the alanine administered is only 5.8 $\gamma$ and 8.7 $\gamma$ at 5 and 24 hours after injection respectively. This value is very small, on comparing with about 300 $\gamma$ of increase in total glycogen during 5 hours and 24 hours after exercise and injection with alanine. The fact, that only small amount of alanine administered contribute to glycogen synthesis, is thought to occur from existense of other precursors. For example, glucose, glycolytic materiales and many amino acids can be listed as precursors of glycogen synthesis in mammal (3). The alanine have been known to be most influential precursors of glyconeogenesis in mammal (24). Therefor alanine was used as administered material in this study because of strong possibility of alanine as precursor of glyconeogenesis. However it is also said that all amino acids almostly have potential ability as precursors of glyconeogensis in mammal (25). Especially, the serine have been known to be secondly useful precursor of glyconeogenesis in amino acids in rat (25). Since the serine is contained about 6 percent of total FAA in yolk protein of salmon egg (13, 26), it can be precursor of glyconeogenesis in alevin.

In this study, it was clarified that glycogen and glucose played an important role in energy metabolism, and that FAA was not only consumed directly as an energy source but also contributed to glyconeogenesis as a precursor. Most of FAA in alevin is understood to be used for embryogenesis as component of body protein

under the static condition, but FAA will be abundantly consumed as an energy source under the condition of exercise by stressing factor. In fact, it is mentioned by Micheil and Bailey (27) that growth of alevin is inhibited by stressing factors such as light stimulates and water currents. Accordingly, the influence of these to the growth of alevin must be studied in detail in order to develop the incubation system of alevin. On the other hand, it is also necessary to examine biochemically (in particular emzymechemically) the glyconeogenesis in order to clarify energy metabolism of egg and alevin.

## Acknowledgement

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## References

1) Smith, S., "*The Physiology of Fishes I.*" ed by Brown, M.E., Academic Press Inc., New York, 323 (1957)
2) Takahashi, K., Hatta, N., Sugawara, Y. and Sato, R., *Tohoku J. Agr. Res.* (in press)
3) Felig, P., *Ann. Rev. Biochem.*, **44**, 933, (1975)
4) Inui, Y. and Yokote, M., *Bull. Fresh Water Fish. Res. Lab.*, **24**, 33 (1974)
5) Inui, Y. and Yokote, M., *Bull. Jap. Soc. Sci. Fish.*, **41**, 291 (1975)
6) Inui, Y. and Yokote, M., *Bull. Jap. Soc. Sci. Fish.*, **41**, 965 (1975)
7) Inui, Y. and Yokote, M., *Bull. Jap. Soc. Sci. Fish.*, **41**, 973 (1975)
8) Inui, Y. and Yokote, M., *Bull. Jap. Soc. Sci. Fish.*, **41**, 1101 (1975)
9) Inui, Y., Arai, S. and Yokote, M., *Bull. Jap. Soc. Sci. Fish.*, **41**, 1105 (1975)
10) Hayashi, S. and Ooshiro, Z., *Bull. Jap. Soc. Sci. Fish.*, **41**, 201 (1975)
11) Ogino, C. and Suyama, M., *Bull. Jap. Soc. Sci. Fish.*, **23**, 227, (1957) (in Japanese, with English summary)
12) Rice, S.D. and Stokes, R.M., "*The Early Life Histology of Fish*", ed by Blaxter, J.H.S., Springer-Verlag, Berlin Heidelberg New York, p 325 (1974)
13) Suyama, M., *Bull. Jap. Soc. Sci. Fish.*, **23**, 789 (1958) (in Japanese, English summary)
14) Folch, J., Lees, M. and Stanley, G.H.S., *J. Biol. Chem.*, **226**, 497 (1957)
15) Blank. M.L., Schmidt, J.A. and Privett, O.S., *J. Am. Chem. Soc.*, **41**, 371 (1964)
16) Kemp, A. and Kits Van Heijningen, A.J.H., *Biochem*, **56**, 646, (1954)
17) Roy, J.H. and Deiley, R.E., *Anal. Biochem.*, **15**, 245 (1966)
18) Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., *Anal. Chem.*, **28**, 350 (1956)
19) Lee, Y.P. and Takahashi, T., *Anal. Biochem.*, **14**, 71 (1966)
20) Hassid, W.Z. and Abraham, S., *Methods Enzymol.*, **3**, 34 (1957)
21) Hayes, F.R., *Quart. Rev. Biol.*, **24**, 281 (1941)

22) Blaxter, J.H.S., "*Fish Physiology III.*", ed by Hoar, E.S. and Randall, D.J., Academic Press, New York, p 177 (1969)
23) Friedman, B., Goodman, E.H., and Wennhouse, S., *J. Biol. Chem.*, **240**, 3729, (1965)
24) Aikawa, T., Matsutaka, H., Takezawa, K. and Ishikawa, E., *Biochem. Biophys. Acta.*, **279**, 234 (1972)
25) Sugimoto, E., *Protein, Nucleic acid and Enzyme*, **22**, 1841 (1977) (in Japanese)
26) Yoneta, T., *Bull. Fac. Fish. Hokkaido Univ.*, **28**, 29 (1977) (in Japanese, with English summary)
27) Micheil, W.J. and Bailey, J.E., "*Salmon Rancher's Manual*", Northwest Fisheries Center Processed Report, National Marine Fisheries Service, Seattle, Washington (1975)