Isotopic Studies on the Metabolism of Protein and Ribonucleic Acid of *Mytilus* Gill

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The amount of carbons in the metabolic pool for protein synthesis and the amount of carbons in the pool consumed for the metabolism other than protein synthesis were calculated to be 7.56 $\mu\text{g}/0.5 \text{ g-gill}$ and 3.94 $\mu\text{g}/\text{h}/0.5 \text{ g-gill}$, respectively. The amount of carbons in the pool for RNA synthesis and the amount of carbons in the pool consumed for the metabolism other than RNA synthesis were calculated to be $9.85 \times 10^{-1} \mu\text{g}/\text{g-gill}$ and $1.32 \times 10^{-2} \mu\text{g}/\text{h}/\text{g-gill}$, respectively. By in vivo study, half life times were found to be 5.8 days for gill-protein, 3.96 days for midgut-protein, 2.6 days for gill-RNA and 10.6 days for midgut-RNA. In the case of gill, the proteins about mucus glands inside of a ciliated groove were very active metabolically.

Since the cilia on the gills of *Mytilus* are capable of expending energy for prolonged periods after excision from the animals it follows that the substance from which this energy is derived must be present in the cells in relatively large amounts. Gray¹⁾ suggested that glycoprotein forms the source of ciliary energy in *Mytilus*. Usuki^{2,3)} has concluded that the ciliary movement of oyster gills links closely with the oxidative metabolism of glycogen. We⁴⁾ reported that the ciliary movement of *Mytilus* gills relates with the metabolism of glycogen in the intercellular spaces abutting on the ciliated cells inside of a ciliated groove. The most obvious physiological parallels to the spontaneous rhythms so characteristic of cilia are presented by cardiac muscle cells in vertebrates and by certain locomotory muscles in the invertebrates. This similarity between muscles and gills made us study the problems of protein metabolism of gills.

1. Experimental

1.1 *In vitro* incorporation of ^{14}C -glycine into the proteins

The gills of *Mytilus edulis*, L. were excised from the ventral margin of the gill lamellae and 0.5 g of gills was taken into a Warburg flask and incubated in the artificial sea water with 5 μCi of ^{14}C -2-glycine (Daiichi Pure Chemicals Co., Ltd., 9.9 mCi/mM) at 25°C.

The composition of the artificial sea water was previously reported⁴⁾. The pH of the artificial sea water was adjusted to 7.8 with Tris-buffer and the total volume of the medium was adjusted to 2 ml. Immediately after incubation, the flask was inserted into an ice-water and 3.8 mg of ^{12}C -glycine was added to the medium to stop the reaction. Then 2 ml of 0.9% saline was added to it and the content of flask was centrifuged. The gills precipitated were washed again with 2 ml of 0.9% saline containing 3.8 mg of ^{12}C -glycine and homogenized with 0.9% saline. To the homogenate, 2 ml of cold 12% perchloric acid (PCA) was added and the mixture was allowed to stand for 10 min. The precipitated material was centrifuged down and washed with 2 ml of cold 6% PCA. Each PCA extract was combined and neutralized with 20% KOH to phenolphthalein. After standing for at least 2 hours, the resulting precipitates were centrifuged down. A suitable portion of the supernatant solution was taken as a sample of the free intracellular ^{14}C . The precipitated proteins were treated with ethanol and hot ethanol-ether (3:1) to remove lipids.

1.2 *In vitro* incorporation of ^{14}C -orotic acid into ribonucleic acid (RNA)

One g of gills was incubated with 4 μCi of ^{14}C -6-orotic acid (Daiichi Pure Chemicals Co., Ltd., 21.5 mCi/mM) as described above. The

total volume of the medium was 4 ml. After incubation the gills were taken out and homogenized with 4 ml of 0.15 M saline-0.001 M EDTA (pH 8.0). The volume of the homogenate was made to 10 ml with 0.15 M saline-0.001 M EDTA (pH 8.0), and 0.1 ml of 2% sodium dodecyl sulfate was added to the homogenate. The mixture was heated at 60°C for 10 min with occasional shaking. After cooling to room temperature, solid sodium chloride was added with vigorous stirring to it to a final concentration of 0.4 M. The turbid solution was freed of proteins by shaking it with one volume of a 9:1 mixture of chloroform-octanol for 30 min, followed by centrifugation at 4000 rpm for 15 min. To the upper water-layer, 2 volumes of ethanol were added to precipitate RNA. RNA obtained was washed with 70, 80 and 98% ethanol successively to remove any salts. The free intracellular ^{14}C sample was prepared from another incubation experiment. The preparation method was as same as in the case of ^{14}C -glycine but 3 mg of ^{12}C -orotic acid instead of ^{12}C -glycine was added to the medium after incubation.

1.3 *In vivo* incorporation of ^{14}C -leucine into the proteins

To adductor muscle of *Mytilus*, 2 μCi (0.2 ml) of ^{14}C -L-leucine (u) (Radiochemical Centre, Amersham, 100 mCi/mM) was injected. The gills and midgut were excised and proteins were isolated as described above. Some pieces of gills of the animal 2 days after the injection were fixed in diluted formaldehyde for 2 hours and then embedded in paraffin and sectioned at 8 μ in thickness. After deparaffinization, the sections were covered with a thin film of celloidine. Gill sections thus treated were taken for autoradiography by the inverting method using the emulsion of ET-2E plate for stripping method (Fuji Photo Film Co., Ltd., Japan).

1.4 *In vivo* incorporation of ^{14}C -orotic acid into RNA

To adductor muscle, 10 μCi (0.2 ml) of ^{14}C -6-orotic acid was injected. Gills and midgut were excised and RNAs were isolated as described above.

1.5 Measuring of ^{14}C -activity

Each sample prepared was dried on a metal dish under infrared rays. The dried material was weighed and its radioactivity was determined by a windowless gas-flow counter. Corrections were made for self-absorption from an empirically determined curve.

2. Results

2.1 Time-course of ^{14}C -glycine uptake by proteins *in vitro*

^{14}C -glycine uptake by proteins at various incubation periods is shown in Table 1. The time-course of the incorporation is shown in Fig. 1. We have found empirically that the

Table 1 The incorporation of ^{14}C -glycine into gill protein

Incubation period (h)	Incorporation	
	cpm/mg-protein	$\mu\text{g-}^{14}\text{C}/\text{mg-protein}$
0.5	227	4.15×10^{-5}
1.0	444	8.13×10^{-5}
1.5	556	1.02×10^{-4}
2.0	617	1.13×10^{-4}

Fig. 1 Time curve of ^{14}C -glycine uptake by gill protein.

incorporation of ^{14}C into proteins can be represented by Equation (1)

$$I_t = 1.155 \times 10^{-4} I_0 (1 - e^{-0.525t}) \quad (1)$$

where $I_t = \mu\text{g}$ of ^{14}C incorporated into one mg of proteins after t hour incubation of gills with ^{14}C -glycine and $I_0 = \mu\text{g}$ of ^{14}C added in the medium ($I_0 = 1.098 \mu\text{g-}^{14}\text{C}$).

Because one g of gills contains 72 mg of proteins as shown in Table 2 and one μCi of ^{14}C corresponds with 1.2×10^6 cpm with our counter, if glycine was taken up by proteins without decomposition, the amount of carbons consumed in the cells for the protein synthesis

Table 2 Protein content of gill

mg-protein per g of gill ¹	
86.80	
77.89	
85.85	
48.99	
50.70	
63.50	
51.80	
91.90	
90.00	
<hr/>	
Mean	72.00

Fig. 2 Time curve of free intracellular ^{14}C -content of the gill incubated with ^{14}C -glycine.

(S μg per hour per 0.5 g of gills) can be calculated from the initial velocity of the incorporation as follows.

$$S = 2.27 \times 10^3 \times 2 \times \frac{1.216 \times 10}{6 \times 10^6} \times 3.6 \times 10 = 3.31 \times 10^{-2}$$

The value of S can be regarded as the rate of protein synthesis. Assuming that the gills are in a stationary state, we may apply the method of Sprinson and Rittenberg⁵⁾ to our data as follows; Let the amount of free intracellular carbons be P μg (P can be regarded as the amount of carbons in the metabolic pool for protein synthesis), carbons from the breakdown of protein enter the pool at a constant rate C μg per hour and carbons in the pool be consumed for the metabolism other than protein synthesis at a constant rate of x μg per hour, the isotope content of

the pool, I (μg), at any instant (t) is obtained by integrating

$$\frac{dI}{I} = -\frac{x+S}{P} dt$$

$$\text{as } I = I'_0 e^{-(x+S/P)t} \quad (2)$$

where I'_0 is equal to I at $t=0$. Let I_t be the amount of isotope incorporated into one mg of proteins in microgram.

Then

$$dI_t = \frac{I}{P} S dt \quad (3)$$

is obtained.

From the Equations (2) and (3)

$$I_t = \frac{S}{x+S} I'_0 (1 - e^{-(x+S/P)t}) \quad (4)$$

is obtained.

Empirically

$$I_t = A I_0 (1 - e^{-Bt}) \quad (5)$$

where I_0 = the amount of isotope added in the medium (μg) and A and B are constants.

Comparison of Equation (4) with Equation (5) reveals

$$x = \frac{I'_0 - A I_0}{A I_0} S, \quad P = \frac{I'_0}{I_0 A B} S$$

The time course of the free intracellular ^{14}C is shown in Fig. 2. Extrapolating the time curve to $t=0$, I'_0 can be obtained as $I'_0 = 1.52 \times 10^{-2} \mu\text{g}/0.5 \text{ g-gill}$.

From Equations (1) and (5)

$$A = 1.155 \times 10^{-4} \text{ and } B = 0.525 (h^{-1})$$

is obtained.

Consequently, we can find

$$x = \frac{(1.52 \times 10^{-2} - 1.155 \times 1.098 \times 10^{-4})}{1.155 \times 1.098 \times 10^{-4}} \times 3.31 \times 10^{-2} = 3.94 \mu\text{g-C/h}/0.5 \text{ g-gill}$$

and

$$P = \frac{1.52 \times 3.31 \times 10^{-4}}{1.098 \times 1.155 \times 5.25 \times 10^{-5}} = 7.56 \mu\text{g-C}/0.5 \text{ g-gill}$$

2.2 Time course of ^{14}C -orotic acid uptake by RNA *in vitro*

From the result given in Table 3, the incorporation of ^{14}C into RNA can be represented by Equation (6) for at least 4 hour incubation.

$$I_t = -6.93 \times 10^{-6} t^2 + 8.39 \times 10^{-5} t \quad (6)$$

where I_t = μg of ^{14}C incorporated into one mg of RNA after t hour incubation of gills with ^{14}C -orotic acid.

Table 3 The incorporation of ^{14}C -orotic acid into gill RNA

Incubation period (h)	Incorporation	
	cpm/mg-RNA	$\mu\text{g}\cdot^{14}\text{C}/\text{mg}\cdot\text{RNA}$
1	374	6.84×10^{-5}
2	770	1.41×10^{-4}
3	1,080	1.97×10^{-4}
4	1,210	2.21×10^{-4}

Table 4 RNA content of gill

mg of RNA-P per g of gill	
	0.260
	0.261
	0.236
	0.258
	0.254
Mean	0.254

Since one g of gills contains 0.254 mg of RNA-P as shown in Table 4, and phosphorus content of the RNA was found to be 8.5%, one g of gills contains 2.99 mg of RNA. The amount of carbons consumed in the cells for RNA synthesis ($S \mu\text{g}$ per hour per one g of gills) can be calculated as follows.

$$S = 3.74 \times 2.33 \times 2.99 \times 10^{-4} \\ = 2.61 \times 10^{-3}$$

The amount of isotope incorporated into one mg of RNA in microgram can be described by Equation (4). In Equation (4), while value of t is small, we can write

$$e^{-(x+S/P)t} = 1 - \frac{x+S}{P}t + \frac{(x+S)^2}{2P^2}t^2$$

Then Equation (4) can be transformed as follows:

$$I_t = -\frac{S(x+S)}{2P^2}I_0 t^2 + \frac{S}{P}I_0 t \quad (7)$$

Empirically

$$I_t = At^2 + Bt \quad (8)$$

is found, where A and B are constants.

Comparison of Equation (7) with Equation (8) reveals

$$x = -S \left(1 + \frac{2AI_0}{B^2} \right), \quad P = \frac{SI_0}{B}$$

The time course of the free intracellular ^{14}C is shown in Fig. 3. Extrapolating the time curve to $t=0$, I_0' can be obtained as $I_0'=3.08$

Fig. 3 Time curve of free intracellular ^{14}C -content of the gill incubated with ^{14}C -orotic acid.

$\times 10^{-2} \mu\text{g}/\text{g}\cdot\text{gill}$.

From Equations (6) and (8)

$$A = -6.93 \times 10^{-6} (\mu\text{g}\cdot^{14}\text{C}/\text{h}^2)$$

and $B = 8.39 \times 10^{-5} (\mu\text{g}\cdot^{14}\text{C}/\text{h})$

are obtained.

Consequently we can find

$$x = 1.32 \times 10^{-2} \mu\text{g}\cdot\text{C}/\text{h}/\text{g}\cdot\text{gill}$$

and $P = 9.85 \times 10^{-1} \mu\text{g}\cdot\text{C}/\text{h}/\text{g}\cdot\text{gill}$

2.3 Time course of ^{14}C -leucine uptake by proteins *in vivo*

^{14}C -Leucine was injected to adductor muscle and plotting the logarithm of the specific activity of gill-and midgut-proteins vs. time, straight lines were obtained 2 days after injection (Fig. 4). From the slopes of the

Fig. 4 Specific activities of the gill-and midgut-proteins at various time intervals after the injection of ^{14}C -L-leucine.

Fig. 5 Autoradiogram of the gill of *Mytilus* injected with ^{14}C -L-leucine (longitudinal section).

straight lines, the half life times ($t_{1/2}$) are calculated to be 5.8 days for gill-proteins and 3.96 days for midgut-proteins. In the case of gills, a high incorporation of ^{14}C was found in the proteins about mucus glands inside of a ciliated groove as shown in Fig. 5.

2.4 Time course of ^{14}C -orotic acid uptake by RNA *in vivo*

By plotting the logarithm of the specific

Fig. 6 Specific activities of the gill- and midgut-RNAs at various time intervals after the injection of ^{14}C -orotic acid.

activity of gill- and midgut-RNAs of ^{14}C -injected *Mytilus* vs. time, the straight lines were obtained 3 days after injection (Fig. 6). From the slopes of the straight lines the half life times are calculated to be 2.6 days for gill-RNA and 10.6 days for midgut-RNA.

Discussion

In *in vivo* experiment, the results may be dependent on the rate of transportation of labeled compound to the organ concerned and the permeability to the labeled compound of the cell in which protein or RNA synthesis is situated. There are many examples that the biosynthesis of a compound from simple substances is often difficultly carried out in slices or homogenates of tissues or organs even when the compound can be easily synthesized from simple substances in an intact animal body. As the gills can be used only cutting it off from an animal, the experimental results on excised gills may be similar to those of *in vivo* experiment but normal metabolism can continue for only a short period, as nutritional substances are not supplied to gills. In *in vitro* experiments, the rate of carbon consumption for the metabolism other than protein synthesis has been obtained as $3.94 \mu\text{g/h}/0.5 \text{ g-gill}$. Previously we have found that $3.05 \mu\text{g}$ of carbons are expired per hour per 0.5 g of gills in respiration⁴⁾. Thus, $3.94 - 3.05 = 0.89 \mu\text{g}$ of carbon per hour per 0.5 g of gills is consumed for glyconeogenesis etc.

In *in vivo* experiments the half life time of gill-protein is found to be 5.8 days. The turnover rate (k) can be calculated from Equation (9).

$$k t_{1/2} = \ln 2 = 0.693 \quad (9)$$

$$k = \frac{0.693}{5.8} = 1.19 \times 10^{-1} (\text{day}^{-1})$$

As one g of gills contains 37 mg of carbons, the daily rate of protein synthesis, the replacement rate, is

$$37 \times 0.119 = 4.40 \mu\text{g-C/g-gill}.$$

It was previously reported that the active loci of glycogen metabolism of gills were intracellular spaces abutting on the ciliated cells inside of a ciliated groove⁴⁾. In the case of gill-proteins, the proteins about mucus

glands inside of a ciliated groove were active metabolically.

The half life time of gill-RNA has been found to be 2.6 days. Thus, k for gill RNA is calculated to be $2.67 \times 10^{-1} \text{ (day}^{-1}\text{)}$.

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要 旨

イ貝のエラのタンパクおよび RNA 代謝の研究

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RIを用いてイ貝のエラのタンパクおよび RNA の代謝を追及し, タンパク合成に対するプール中の炭素量はエラ 0.5 g あたり $7.56 \mu\text{g}$ であり, このうち $3.94 \mu\text{g/h}$ はタンパク合成以外に消費されること, また, RNA 合成に対するプール中の炭素量はエラ 1 g あたり $9.85 \times 10^{-1} \mu\text{g}$ であり, RNA 合成以外に消費される炭素量は $1.32 \times 10^{-2} \mu\text{g/h}$ であることが算出された。さらに *in vivo* の実験から, エラおよび中腸腺タンパクの半減期はそれぞれ 5.8 日, 3.96 日で, このときエラの場合, セン毛溝内の粘液腺近辺のタンパクがとくに代謝が活性であることがオートラジオグラフィから知られた。RNA の半減期はエラで 2.6 日, 中腸腺で 10.6 日であった。