COMPARATIVE BIOCHEMICAL STUDIES ON AQUATIC ANIMALS II. PHOSPHORUS TURNOVER OF THE FRESHWATER FISH AND SHELLFISH

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II. PHOSPHORUS TURNOVER OF THE FRESHWATER FISH AND SHELLFISH*

By

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Introduction

In the preceding paper (1), we have reported on the calcium turnover of the freshwater fish (*Carassius auratus* L.) and shellfish (*Anodonta lauta* MARTENS), using Ca^{45} as a tracer.

Among the mineral elements essential to life, phosphorus plays a more significant rôle. To ascertain to what extent this element participates in the biological calcification of the same animals is the scope of this study. We have studied on the accumulation and disappearance of P^{32} into the various fractions of phosphorus. Furthermore, we deduced the different rôle of incorporated phosphorus in the biological calcification of both animals. The authors wish to acknowledge their thanks to Prof. Tsuchiya for his criticism.

Experimental

(1) Materials: The common goldfish (*Carassius auratus* L.) and the pond mussel (*Anodonta lauta* MARTENS).

(2) Methods: For the quantitative determination of total-P and acid soluble-P, we adopted the method of Nakamura(2) which is a modification of Allen's original method(3).

For the determination of the radioactive inorganic-P, the iso-butanol extraction method after Yoshizawa(4) was used.

These combined flow sheets are outlined in schema I.

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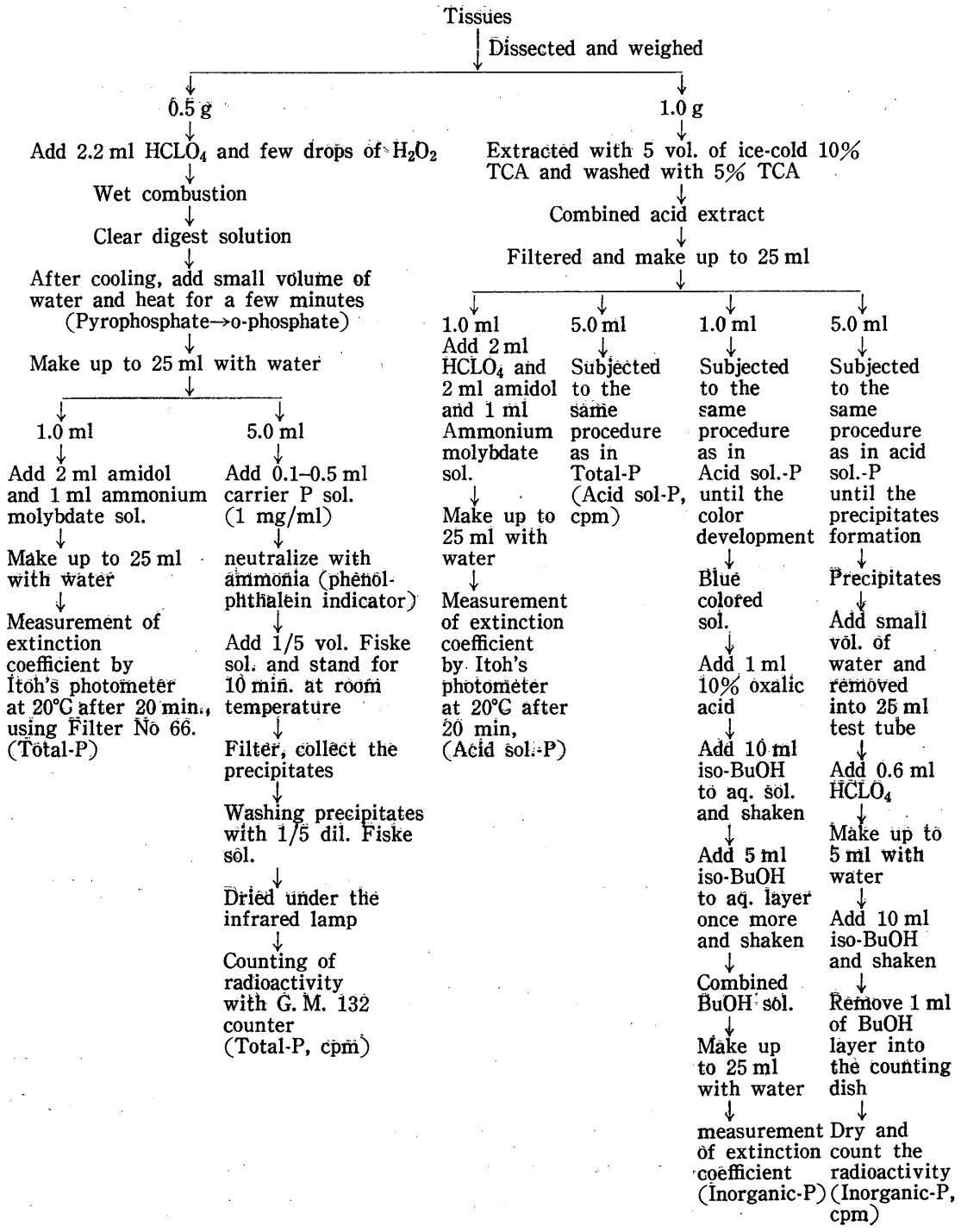
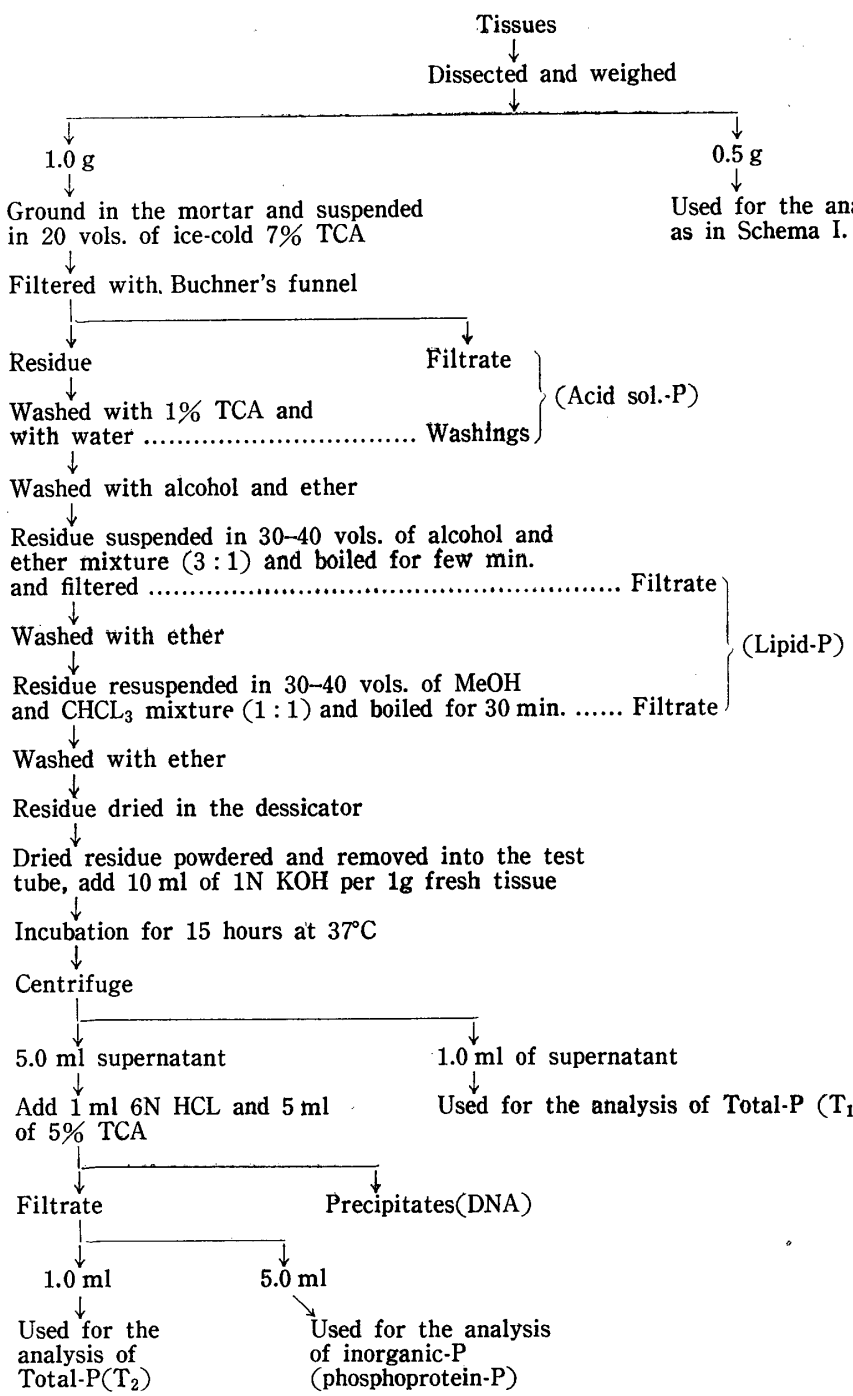
* This study was read at the annual meeting of Scientific Fisheries held on April 5, 1956 at Tokyo.

Schema 1.


Tissues
Dissected and weighed
0.5 g
Add 2.2 ml $HCLO_4$ and few drops of H_2O_2
Wet combustion
Clear digest solution
After cooling, add small volume of water and heat for a few minutes (Pyrophosphate→o-phosphate)
Make up to 25 ml with water
1.0 ml
Add 2 ml amidol and 1 ml ammonium molybdate sol.
Make up to 25 ml with water
Measurement of extinction coefficient by Itoh's photometer at 20°C after 20 min., using Filter No 66. (Total-P)
5.0 ml
Add 0.1–0.5 ml carrier P sol. (1 mg/ml)
neutralize with ammonia (phenol-phthalein indicator)
Add 1/5 vol. Fiske sol. and stand for 10 min. at room temperature
Filter, collect the precipitates
Washing precipitates with 1/5 dil. Fiske sol.
Dried under the infrared lamp
Counting of radioactivity with G. M. 132 counter (Total-P, cpm)
1.0 g
Extracted with 5 vol. of ice-cold 10% TCA and washed with 5% TCA
Combined acid extract
Filtered and make up to 25 ml
1.0 ml
Add 2 ml $HCLO_4$ and 2 ml amidol and 1 ml Ammonium molybdate sol.
Make up to 25 ml with water
Measurement of extinction coefficient by Itoh's photometer at 20°C after 20 min, (Acid sol.-P)
5.0 ml
Subjected to the same procedure as in Total-P (Acid sol-P, cpm)
1.0 ml
Subjected to the same procedure as in Acid sol.-P until the color development
Blue colored sol.
Add 1 ml 10% oxalic acid
Add 10 ml iso-BuOH to aq. sol. and shaken
Add 5 ml iso-BuOH to aq. layer once more and shaken
Combined BuOH sol.
Make up to 25 ml with water
measurement of extinction coefficient (Inorganic-P)
5.0 ml
Subjected to the same procedure as in acid sol.-P until the precipitates formation
Precipitates
Add small vol. of water and removed into 25 ml test tube
Add 0.6 ml $HCLO_4$
Make up to 5 ml with water
Add 10 ml iso-BuOH and shaken
Remove 1 ml of BuOH layer into the counting dish
Dry and count the radioactivity (Inorganic-P, cpm)

The determination of the incorporated P^{32} into the various phosphorus fractions was analysed by the method of Schmidt and Thannhauser (5)* as outlined in Schema 2.

Schema 2.



* This method was originally designed for the determination method of DNA, RNA and phosphoprotein in the animal tissue.

Calculation in Schema 2.

The calculation of DNA-P, RNA-P and phosphoprotein-P was carried out as follows;

Total-P of alkaline hydrolysate ..... T_1

Total-P of acid supernatant ..... T_2

$T_1 - T_2$ = DNA-P

T_2 – Inorganic-P of acid supernatant = RNA-P

Inorganic-P of acid supernatant = Phosphoprotein-P(residual)

Counting and calculation of the radioactivity.

Counting condition was the same as in the previous paper (1). Geiger mueller counter GM 132 was used. The distance between the sample and the window was 16 mm. We have counted more than 1000 cpm so far as possible in order to bring the error within three per cent. The correction of observed counts of P^{32} in the tissues due to the self absorption was neglected owing to its large β-energy (E_{max} = 1.71 Mev), while the decay correction ($\tau_{1/2}$ = 14.3 days) was carried out. For the illustration of radioactivity, we exclusively used the specific activity, S. A. (counts per minute per mg P).

Furthermore, total net counts of incorporated P^{32} were calculated by the following formula;

Total net counts (cpm/g wet tissue) = S. A. × phosphorus contents of a tissue (mg P/g wet tissue) .....(1)

(3) The distribution of phosphorus in the tissues of experimental animals.

As a preliminary step, the distribution of phosphorus in the tissues of experimental animals was studied as shown in Tables 1 and 2.

Table 1. The phosphorus content in the goldfish (mg P/100g wet tissue).

Tissues	Total-P	Acid sol.-P	Inorg-P	Lipid-P	Total protein-P	DNA-P	RNA-P	Phospho-protein-P
Bone	2328	1980	1730	45	173	151	12	10
Fin	1616	1044	990	48	153	74	75	4
Operculum	1556	1087	1075	43	179	112	58	9
Scale	1315	847	705	33	102	85	11	6
Gill	560	277	273	50	153	91	59	3
Viscera	262	45	34	38	153	86	61	6
Muscle	242	112	110	35	102	68	32	2

Table 2. The phosphorus content in the pond mussel (mg P/100g wet tissue).

Tissues	Total-P	Acid sol.-P	Inorg-P	Lipid-P	Total protein-P	DNA-P	RNA-P	Phospho-protein-P
Gill	1553	1147	994	40	332	30	290	12
Gonad	600	154	143	40	408	184	211	13
Digestive diverticula	585	261	223	48	306	82	214	10
Mantle interior	435	214	210	25	330	106	216	8
Mantle edge	356	108	90	25	300	76	214	10
Foot	238	46	41	48	189	22	161	6
Adductor muscle	200	80	67	25	180	21	155	4

(4) Accumulation of P^{32} in animals.

(A) Goldfish

For the accumulation of P^{32} in the goldfish, five fishes per one vat were cultured in 2 liters of the radioactive pond water. The basal diet (pupa-wheat gluten mixture) was given once a day. After varying the time intervals of culture, the animals were killed, dissected and separated into seven tissue groups. The same tissue was pooled and a part of it was taken and analysed. The results are shown in Table 3.

Table 3. The accumulation of P^{32} in the goldfish.

Tissues	Time of exposure to the radioactive media.			
	3 hours		48 hours	
	Total-P	Acid sol.-P	Total-P	Acid sol.-P
Bone	156	42	211	51
Fin	278	278	528	206
Operculum	712	254	1446	451
Scale	280	780	617	609
Gill	7868	3090	14147	7274
Viscera	2211	7048	1425	95175
Muscle	1766	494	507	210

Remarks: Figures in the Table are expressed in specific activity (cpm/mg P).
The experiment was carried out during 22th June-4th July 1955.
Water temperature. 21–23°C. Body weight of goldfish. 6.0–7.0 g.
Radioactivity of 3 hours' media. 4278 cpm/ml.
Radioactivity of 48 hours' media. 4800 cpm/ml.

(B) Pond mussel

For the accumulation of P^{32} in the pond mussel, five shellfishes per one vat were cultured in 2 liters of the pond water with an activity of 1973 cpm/ml. No diet was given. After intervals, the animals were sacrificed and treated in the same way as in the goldfish. The results are given in Table 4.

Table 4. The accumulation of P^{32} in the pond mussel.

Tissues	Time of exposure to radioactive media			
	3 hours		24 hours	
	Total-P	Acid sol.-P	Total-P	Acid sol.-P
Gill	241	270	1370	427
Gonad	179	129	—	3102
Digestive diverticula	158	79	22119	608
Mantle interior	590	263	1539	1111
Mantle edge	1677	1091	3014	11527
Foot	556	666	2911	3666
Adductor muscle	640	116	4181	2115

Remarks: Figures in the Table are expressed in specific activity.
The experiment was carried out during 16th July-2nd Aug, 1955.
Water temperature. 25–26.5°C. Shellfishes of 54–58 g body weight were used.

(5) The disappearance and decontamination of P^{32} from animals.

Five fishes and three shellfishes in the same vat were exposed to the radioactive pond water with an activity of 5050 cpm/ml. In this experiment, three different fractions were designed as indicated below;

A fraction ..... Control. After 24 hours' exposure, the animals were killed and analysed at once.

B fraction ..... After 24 hours' exposure, the animals were cultured in the non-radioactive pond water (exchanged once a day) for 6 days and then treated in the same way.

C fraction ..... After 24 hours' exposure, the animals were cultured in the non-radioactive pond water added with EDTA (0.2 g/L, exchanged once daily) for two weeks and then treated as usual.

The results are given in Tables 5 and 6.

Table 5. The disappearance and decontamination of P^{32} from the goldfish.

Tissues	Specific activities of each fractions					
	A		B		C	
	Total-P	Acid sol.-P	Total-P	Acid sol.-P	Total-P	Acid sol.-P
Bone	100	77	115	—	115	78
Fin	306	164	302	151	360	—
Operculum	339	176	415	73	35	50
Scale	912	448	630	252	203	170
Gill	9766	3500	620	946	409	331
Viscera	3889	10500	1789	2123	82	1485
Muscle	769	365	209	128	474	100

Table 6. The disappearance and decontamination of P^{32} from the pond mussel.

Tissues	Specific activities of each fractions					
	A		B		C	
	Total-P	Acid sol.-P	Total-P	Acid sol.-P	Total-P	Acid sol.-P
Gill	205	123	81	63	22	44
Gonad	560	396	75	388	12	68
Digestive diverticula	71	138	43	170	22	44
Mantle interior	864	424	103	126	46	86
Mantle edge	2656	1359	329	511	135	54
Foot	1043	1390	296	1087	12	97
Adductor muscle	2876	1500	81	593	nil	182

Remarks: The experiments in Tables 5 and 6 were carried out during 17th Aug-6th Sept 1955. Water temperature 27–28°C.
Body weight of goldfish. 6.5–6.6 g.
Body weight of pond mussel. 68–69 g.

(6) The specific activities and total net counts of P^{32} incorporated into the various phosphorus fractions.

(A) Goldfish

Five fishes per one vat were cultured in 3 liters of pond water with an activity of 18654 cpm/ml for 24 hours. After exposure, the fishes were killed and analysed by the procedure given in Schema 2. The results are indicated in Table 7.

Table 7.

Tissues	Specific activities of each fractions				
	Total-P	Acid sol.-P	Inorg-P	Lipid-P	Total protein-P
Bone	175	155	116	82	172
Fin	229	303	45	294	600
Operculum	312	391	69	329	840
Scale	920	884	102	970	5460
Gill	2500	1600	634	2890	6400
Viscera	158358	278433	92666	44106	138430
Muscle	1226	606	577	300	600
Tissues	Total net counts of incorporated P^{32} per min. per 1g wet tissue				
	Total-P	Acid sol.-P	Inorg-P	Lipid-P	Total Protein-P
Bone	3642	3069	2007	37	306
Fin	3701	3163	468	141	918
Operculum	4854	4242	740	140	1503
Scale	12100	7487	719	320	5569
Gill	14000	4432	1730	1445	9792
Viscera	414897	125295	31506	16760	211998
Muscle	2966	696	634	105	612

Remarks: The experiment was carried out during 10th Nov.-26th Nov. 1055.
Water temperature 10°C. Average body weight. 7.1 g.

(B) Pond mussel

Six shellfishes per one vat were cultured in 3 liters of the radioactive pond water with an activity of 18846 cpm/ml for 24 hours. After exposure, they were treated as indicated in Schema 2. The results are given in Table 8.

Table 8.

Tissues	Specific activities of each fractions				
	Total-P	Acid sol-P	Inorg-P	Lipid-P	Total protein-P
Gill	8708	7471	2672	3075	2449
Gonad	31041	52722	15556	42362	37775
Digestive diverticula	108500	111028	25314	65126	42000
Mantle interior	10000	9505	3021	2960	3603
Mantle edge	18700	20855	9250	12840	14984
Foot	4120	13300	2823	1252	1540
Adductor muscle	4487	5900	1277	780	550
Tissues	Total net counts of incorporated P^{32} per min. per 1 g wet tissue				
	Total-P	Acid sol.-P	Inorg-P	Lipid-P	Total Protein-P
Gill	135235	85692	26559	1230	8131
Gonad	185315	81191	22245	16944	154122
Digestive diverticula	634725	289785	56450	31260	128520
Mantle interior	43500	22337	6435	740	11890
Mantle edge	66572	22523	8325	3210	44952
Foot	9805	6118	1157	601	2787
Adductor muscle	8974	4720	856	195	1540

Remarks: The experiment was carried out during 21th Oct.-9th Nov. 1955. Water temperature 16.5°C. Average body weight 57.3 g.

Discussion

As described in the previous paper (1), for elucidating the mechanism of biological calcification, it is necessary to study the correlation between the mineral constituents (Ca, P, bicarbonate ions etc) and the relating enzymic system. Especially from the comparative biochemical point of view, it is very interesting to clarify the characteristics and differentiation of the mechanism between the fish and shellfish.

In regard to the phosphorus contents in the goldfish (Table 1), there are parallelism between the phosphorus contents (Total-P, Acid sol.-P and inorganic-P) and the degree of calcification. Calcified tissues such as bone, fin, scale and operculum have much more phosphorus than the soft tissues. In the shellfish(Table 2), the phosphorus contents are more evenly distributed in the soft tissues (except gill) than in the fish.

It is very interesting to note that the amounts of lipid-P are fairly constant throughout the tissues in both animals and the amounts of Total protein-P (including RNA-P, DNA-P and phosphoprotein-P) in the shellfish are two or four times greater than those in the fish. The amounts of DNA-P are richer than those of RNA-P in the fish, while in the shellfish the situation is reverse and, in addition, these types of phosphorus are more abundant in absolute quantity.

The accumulation of P^{32} in the animal tissue caused a rise of specific activity until the radioactive equilibrium in accordance with the time of exposure to the radioactive medium. The specific activities of the calcified tissue in the fish (Table 3) still increased within the experimental period. This increase may be attributed to the slower turnover rate, since it is time consuming for P^{32} to exchange or recrystallize with P^{31} in calcified tissues. However, the specific activities of soft tissues have already begun to decrease, indicating that the transfer of once incorporated P^{32} into another tissue took place. In the pond mussel, the specific activities of all soft tissues still increased within the experimental period, however, the increase rates are more rapid than those in the fish. (Table 4).

It is always true as in the case of Ca^{45} that a tissue containing a small quantity of total phosphorus shows higher specific activity than that of the other tissue with much inert phosphorus and the proportionality between the specific activity and incorporated net P^{32} does not always hold. (Tables 1, 2, 7 and 8)

It may be probable to postulate that there are two forms of phosphorus in tissues i. e., the stable inert phosphorus and labile dynamic one.

Next, we investigated the disappearance and decontamination of incorporated P^{32} in both animals, under the same circumstances. As is shown in Tables 5 and 6, the disappearance and decontamination of incorporated P^{32} are also more rapid in the shellfish than in the fish. The faster accumulation and disappearance (more rapid turnover rate) are the characteristics in phosphorus metabolism of the shellfish.

The distribution of incorporated P^{32} into the various phosphorus fractions has also been examined. From the comparison between Tables 7 and 8, one notices that the specific activities of the shellfish are in exceedingly higher order than those of the fish. As the cause of the discrepancy of order level, the following factors are considered to be responsible;

(1) The difference of moisture contents of both species.
(2) The difference of phosphorus contents in both animal tissues (The difference of the ratio of inert versus dynamic phosphorus).
(3) The difference of mode of phosphorus turnover.

As for the total net counts calculated by formula (1), the shellfish takes

much P^{32} for a given time interval than the fish.

According to the recent publications(6), the constitution of the bone salts in Vertebrate (human, rat, rabbit etc) is now controversial, however, its matrix is mainly composed of a hydroxyapatite structure as indicated in Figure 1.

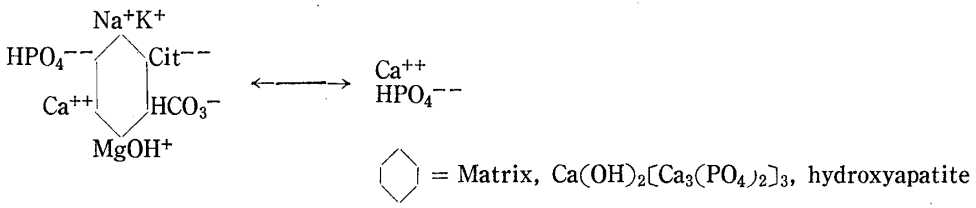


Figure 1. Bone salts in vertebrate (Hendricks 1950).

Namely, the major constituents are of hydroxyapatite and the surrounding ions such as Na^+, K^+, $MgOH^+$, HCO_3^- etc. are absorbed on the surface of bone salts. Crystalline calcium carbonate is absent from the bone salts and the most exchangeable calcium and phosphorus are surface-absorbed Ca^{++} or HPO_4^{--} ions. In the fish, one of the Vertebrate, if the constitution of bone salts are the same or similar as in the above formula, the greater parts of P^{32} are considered to incorporate gradually in bone salts through the process of exchange and recrystallization. Thus, the phosphorus which incorporated into the hydroxyapatite crystal lattice represents the inert stable phosphorus and the surface-absorbed phosphorus represents the labile dynamic phosphorus. However, in the shellfish, one of the Invertebrate, the mechanism for bone salts formation (shell formation) are different. Although not yet explained conclusively, the mechanism of shell formation may be summarized as in Figure 2 according to the reports of several workers; (7) (8) (9)

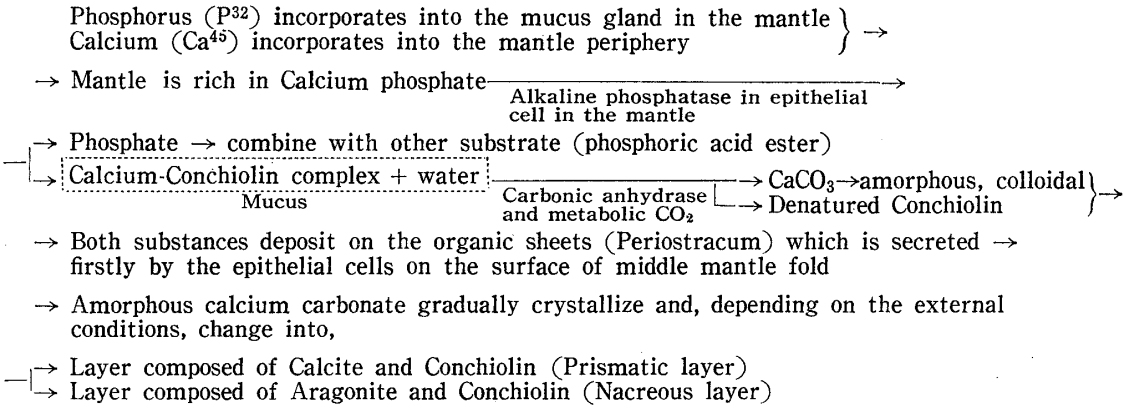


Figure 2. Shell formation in the shellfish.

In the final structure of the shell, phosphorus compounds are absent or trace if any, and the major constituents are calcium carbonate (90 per cent or more). The rest is composed of a small amount of calcium oxide and

conchiolin. Since phosphorus does not incorporate into the final shell structure, the rôle of phosphorus in the calcification of the shellfish may be considered to be its fixative function of calcium locally in the mantle as calcium phosphate which, in the next step, is dissolved and degraded into the amorphous calcium carbonate mixed with denatured conchiolin and phosphate by the aid of alkaline phosphatase. The phosphate changes into the phosphoric acid ester. Then the phosphoric acid ester enters into another metabolic pathway, therefore, the rôle of phosphorus in the calcification of the shellfish is transient in this sense although indispensable. We can interpret the less inert phosphorus and active turnover in the shellfish are attributable to this dynamic rôle of phosphorus. At any rate, quantities of P^{32} incorporate at first into the tissues which indicate more rapid turnover such as viscera, gill (goldfish) or digestive diverticula, gonad, mantle (shellfish), and then transfer gradually into the calcified tissues which indicate the slower turnover rate.

Summary

The phosphorus turnover of the freshwater fish (*Carassius auratus* L.) and the shellfish (*Anodonta lauta* MARTENS) was studied. In the goldfish, the calcified tissues have much more inert phosphorus than the soft tissues. In the shellfish, except the gill, the inert phosphorus are more evenly distributed than in the fish. It is noteworthy that the lipid-P is fairly constant throughout the tissues of both species and the amounts of total protein-P in the shellfish are far richer than those in the fish. The amounts of DNA-P are more abundant than those of RNA-P in the fish, however, the situation is reverse in the shellfish. The turnover rate of phosphorus in the shellfish is faster than in the fish. The fish has a large quantity of inert stable phosphorus and a smaller quantity of dynamic phosphorus. The former represents the phosphorus which constitutes the basal component of bone salts and the latter the phosphorus which is absorbed on the surface of matrix.

On the contrary, in the shellfish, bone salts are composed exclusively of calcium carbonate and devoid of phosphorus. Therefore, phosphorus does not incorporate into the final bone structure and its turnover is very rapid. The rôle of phoshorus in the shellfish seems to be its fixative function of calcium locally in the mantle at the initial stage of calcification. Although the uptake of P^{32} in the various phosphorus fractions are considerably rapid and widely distributed throughout the tissues of both animals, the total net incorporation of P^{32} (counts per min. per g. wet tissue) in the shellfish surpass those in the fish.

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