2.タップミノウの尾鰭の再生に及ぼすインシュリンの効果について

Effect of Insulin on the Tail Fin Regeneration in the Topminnow, *Gambusia affinis*

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It is widely known that insulin is a material which promotes the carbohydrate metabolism. Of late several investigators have reported that insulin accelerates even the cell division. OSANAI showed that insulin pretreatment has an effect on promoting the growth of chick heart fibroblasts by using the tissue culture method (1956). KATAYAMA has also shown that the pretreatment with insulin for 10 minutes produces a promoting effect on the number of mitosis in the mouse ear epidermis *in vitro* (1957). KANATANI observed that insulin is effective in promoting in the growth involved in head regeneration in the planarian, invertebrate.

This study is an attempt to investigate whether or not insulin does produce an effect on the tail fin regeneration in the topminnow, *Gambusia affinis.*

Material and Method

Gambusia affinis were collected in the pond Ryūtan, Shuri, Naha City, Okinawa Island. Only female fish were used for the experiments, since the length and size of the tail fin of the male fish is different from those of the tail of the female. Care was also taken that just fish of equal body length, tail fin length, tail fin thickness were used as the experimental fish and control fish. The two experiments were made as follows.

I. Tests were made in order to decide the proper spot in the tail fin for amputation. Tail fins were cut both at the middle of the tail fin and at a point, one third from the tail end. Then the differences in regeneration of the tail fin

Table 1 Comparison between the regenerated parts in the tail fin tip amputated at the middle of the tail and at a point, one third from the tail end.

Size of fish (mm)		Length of regenerating tail fins (mm)			
Standard length	Tail fin length	Cut at the middle of the tail		Cut at one third from the end	
		7th day	14th day	7th day	14th day
20.4	4.00	0.76	1.96	0.72	1.60
21.6	3.00	0.70	1.96	0.64	1.40
20.0	4.80	0.80	2.00	0.80	1.80
21.0	4.00	0.76	1.96	0.60	1.60
21.6	3.80	0.60	1.96	0.60	1.80
22.0	3.80	0.92	2.00	0.64	1.60
Average		0.76	1.97	0.66	1.63

in the previous two cases were closely examined. As shown in Table I, in the tail fin regeneration the former cutting (in the middle of the tail fin) is faster than the latter cutting (at a point, a third from the tail end.) In the latter case, if the temperature in the solution is low, the regeneration is so slow that one feels difficulty in observing the process of the regeneration. In the case of medaka, *Oryzyas*, the regeneration is fastest when the tail fin is amputated at a point, two fifths from the end, in the tail fin. While, if the tail fin of topminnow is cut in the same place as in the case of medaka, the topminnow tends to bleed to death. In consequence, I have decided to amputate the tail fin of topminnow at the middle point. Since it was found in the series of tests that the differences were found in the growth of the regeneration, if the amputated point shifts front or back from the middle of the tail fin; the special care was taken that the tail fin was correctly cut at the middle point of the tail.

II. The following tests were made in order to examine the effects of the temperature in the experimental place on the growth of the regeneration. Some fish which were amputated in the tail fins were kept in the place 9°–15° C, while other fish also cut in the tails were stored in the place 26°–32° C. The results were as shown in Table 2. First of all, the fish of the first group presented a

Table 2 Effect of the temperature in the experimental place on the growth of the regeneration. The fish were kept in water both at the temperature 9°–15° C and at 26°–32° C.

	Size of fish (mm)		Length of regenerated tail fins (mm)			
	Standard length	Tail fin length	9°–15° C			26°–32° C
			7th day	14th day	21st day	28th day
Lower temperature	22.8	5.20	0.12	0.52	0.81	2.80
	22.8	5.20	0.12	0.52	0.81	2.80
	22.8	5.20	0.08	0.40	0.80	2.52
	22.8	5.20	0.08	0.40	0.80	2.52
	22.8	5.20	0.08	0.40	0.80	2.52
			26°–32° C			
Higher temperature	22.8	5.20	1.40	2.60	2.88	2.88
	22.8	5.20	1.40	2.60	2.88	2.88
	22.8	5.20	1.40	2.60	2.88	2.88
	22.8	5.20	1.40	2.60	2.88	2.88
	22.8	5.20	1.20	2.20	2.40	2.40

slight growth during the period of 21 days after amputation; but during the period of 7 days after the fish were transfered to the place 26°–32° C from the previous condition, the tail fin tip showed a remarkable progress in regeneration. In the case of the second group, over the period of the first 7 days after amputation, the regeneration was active, however it slowed down a little in the period of the second 7 days, and during this period the regeneration reached almost the point of the former length (pre-amputated condition) in the tail fins. After 14 days

since the tail fins were cut, no regression or a very slight regression were observed. As the above experiments were shown, regeneration is influenced much by the temperature. Therefore care was taken that no differences between the temperature in the place where experimental fish were stored and that in the place the controlled fish kept would take place while the experiments were going on.

As to the fish amputated in the tail fins, one was kept in the control, a glass vessel which held water in it; and another fish was stored in the experimental, a glass vessel which held dilute insulin medium in it. These vessels were put in the dark place. It is known that the movement of the tail fin of medaka, *Oryzyas* produces an effect on the regeneration (Mori, 1942). All the fish kept in the place, which is dark and the temperature of which is low, are found to have kept motionless. To keep the fish under experiment healthy, they were taken out into the light, warm place for two to three hours every day (while the tests were going on). When the fish were moved out in this way, they were given food and the culture mediums were removed. In this method fish kept healthy throughout the experiments.

Insulin used for experiments was Fiselin (trade name) manufactured by Torii Co. Ltd. When a strong insulin needed, Insulin Novo (trade name) was put to use. The former solution (20 unit/ml.), contains 0.1 w/v % Cresol, as a preservative, 1.4 w/v % Glycerin as a stabilizer, and it smells of cresol, but the latter doesn't. In the Fiselin, 1 unit/ml., fish die immediately. Consequently for the tests the Fiselin, which concentration was lower than that of 1 unit/ml, and when a strong insulin needed, Insulin Novo was used. Insulin was treated to the fish under experiment for three days, seven days, and fourteen days respectively after amputation. For a week after that the fish were kept at the laboratory.

The regenerated tail tip was observed and measured every 7 days, using a lower binocular microscope. The regenerated parts of these fish were colorless and could be clearly distinguished from the other parts. (Fig.)

Results

First of all fish amputated in the tail fin were kept in several different dilute insulin solution (0.025, 0.05, 0.1, 0.2, 0.3 unit/ml.), for 7 days or 14 days after cutting and the regeneration was observed. The results of the observation are shown in Table 3. It is clear that, the growth of tail tips is accelerated when the fish were treated with 0.05, 0.1 unit/ml. insulin solution, and especially the growth is remarkable in the 0.1 unit/ml. insulin solution. On the contrary, the retarded growth of regeneration was noticed both in the thicker solution 0.3 unit/ml. and in the more dilute solution 0.025 unit/ml. than the immediately previous one. Seeing that, it is thought that this retarding function, observed in the 0.3 unit/ml. insulin solution, in the growth of the tail fin regeneration is caused by cresol involved in insulin solution, the following test was made. In this experiment were used a little thicker insulin 0.3 unit/ml. and 0.5 unit/ml. Insulin Novo, and the results of the tests are given in Table 4. As shown in Table 4, regeneration was

Table 3 Effect of insulin on the growth of tail fin regeneration after treating with 0.025, 0.05, 0.1, 0.2, 0.3 unit/ml. insulin solution for 7 days or 14 days at 9°–15° C.

	Size of fish (mm)		Length of regenerating tail fins (mm)							
			7th day		14th day		21st day		28th day	
	Standard length	Tail fin length	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
0.025 unit/ml.	21.6	4.6	0.40	0.60	0.80	0.72	1.04	1.00	2.40	2.80
	22.0	5.2	0.60	0.40	1.00	0.88	1.60	1.08	2.80	2.40
	22.4	5.0	0.60	0.48	0.88	0.92	1.08	1.06	2.80	3.00
	22.8	5.2	0.60	0.60	0.90	1.00	1.60	1.20	2.90	2.90
	23.6	5.3	0.80	0.80	1.20	1.00	2.00	2.00	3.20	2.80
	Average		0.60	0.58	0.96	0.90	1.46	1.27	2.82	2.78
0.05 unit/ml.	23.6	5.0	0.56	0.56	0.96	0.96	1.20	1.40	2.80	2.80
	19.6	4.2	0.40	0.72	1.26	1.00	1.40	1.40	3.20	3.00
	23.6	5.3	0.64	0.64	1.60	0.96	2.00	1.68	3.28	2.80
	20.4	4.8	0.48	0.48	0.80	1.20	1.32	1.42	2.40	2.80
	19.4	4.8	0.52	0.52	0.80	0.90	1.40	1.40	2.40	2.90
	Average		0.52	0.58	1.08	1.00	1.46	1.46	2.82	2.86
0.1 unit/ml.	24.8	5.6	0.40	0.72	0.64	1.20	1.00	1.60	2.80	3.40
	23.2	5.4	0.40	0.48	0.88	1.20	1.08	1.40	3.00	3.00
	22.4	5.0	0.48	0.80	0.80	1.00	1.00	1.20	2.88	2.88
	23.2	5.4	0.60	0.60	0.92	0.92	1.60	1.60	2.80	2.80
	24.0	5.4	0.40	0.48	0.80	1.00	1.20	1.20	3.00	3.00
	Average		0.46	0.62	0.81	1.06	1.18	1.40	2.90	3.02
0.2 unit/ml.	22.4	4.8	0.48	0.48	0.84	0.90	2.44	2.28	2.68	2.44
	21.6	4.6	0.48	0.48	0.84	0.84	2.52	2.04	2.74	2.28
	22.4	4.6	0.48	0.48	0.80	0.68	2.28	2.28	2.48	2.48
	21.6	4.6	0.40	0.40	0.84	0.80	2.48	2.40	2.64	2.64
	22.8	4.8	0.48	0.48	0.68	0.60	2.40	2.40	2.68	2.76
	Average		0.46	0.46	0.80	0.76	2.42	2.28	2.64	2.52
0.3 unit/ml.	22.4	4.8	0.40	0.48	1.00	1.08	2.00	2.00		
	21.6	4.6	0.44	0.44	1.04	1.04	3.00	2.80		
	22.4	4.6	0.60	0.44	1.04	1.04	2.40	2.48		
	21.6	4.6	0.80	0.52	1.12	1.00	2.40	2.40		
	22.8	4.8	0.48	0.40	0.88	1.20	2.60	2.60		
	Average		0.54	0.46	1.02	1.07	2.48	2.46		

also promoted in these two given solutions. So far, in the experiments, fish were treated with insulin solution for 7 days or 14 days, but this time insulin was given in a shorter period. The fish amputated in the tail fins were put in thicker insulin solution (0.5, 0.1 unit/ml.) for 3 days, and then they were transfered into

Table 4 Effect of insulin on the growth of tail fin regeneration after treating with 0.5, 1 unit/ml. insulin for 3 days, at 9°-15° C.

	Size of fish (mm)		Length of regenerating tail fins (mm)					
	Standard length	Tail fin length	7th day		14th day		21st day	
			Control	Experimental	Control	Experimental	Control	Experimental
1 unit/ml. insulin	20.4	4.80	0.40	0.40	1.04	1.00	2.40	2.40
	21.2	4.80	0.44	0.44	0.80	0.80	2.00	2.40
	22.4	4.60	0.44	0.72	1.00	1.00	2.60	2.40
	22.4	5.00	0.60	0.80	0.88	1.04	2.40	3.00
	22.4	4.80	0.48	0.48	0.80	0.80	3.00	2.80
	Average		0.47	0.57	0.90	0.93	2.48	2.60
0.5 unit/ml. insulin	24.0	5.20	0.40	0.48	0.80	0.80	2.40	2.40
	24.0	5.40	0.60	0.60	1.20	1.20	2.80	2.80
	24.4	5.60	0.48	0.48	1.00	1.00	2.40	2.40
	21.6	4.60	0.60	0.60	1.20	1.00	2.80	2.80
	26.8	5.60	0.44	0.60	1.04	1.04	3.00	3.00
	Average		0.50	0.55	1.05	1.01	2.68	2.68

water. In the 7th and 14th day tail tips were measured. The results are recorded in Table 5. As seen in the Table, regeneration is also accelerated in the two solutions.

Table 5 Effect of insulin on the growth of tail fin regenerating with 0.3, 0.5 unit/ml. insulin Novo, for 14 days, at 9°-15° C.

	Size of fish (mm)		Length of regenerating tail fins (mm)							
	Standard length	Tail fin length	7th day		14th day		21st day		28th day	
			Control	Experi-mental	Control	Experi-mental	Control	Experi-mental	Control	Experi-mental
0.3 unit/ml.	24.0	5.20	0.40	0.48	0.80	0.84	1.04	1.08	2.90	2.88
	24.0	5.20	0.40	0.40	0.84	0.80	1.08	0.92	2.80	2.40
	26.0	5.60	0.48	0.48	0.92	0.80	1.20	1.60	2.80	3.00
	26.0	5.20	0.48	0.60	0.80	1.00	0.92	1.20	2.40	2.80
	27.0	5.80	0.60	0.60	0.92	0.92	1.40	1.20	3.00	2.80
	Average		0.47	0.51	0.86	0.87	1.13	1.20	2.78	2.78
0.5 unit/ml.	23.6	5.20	0.48	0.48	0.80	0.88	1.00	1.08	2.80	3.00
	24.0	5.40	0.48	0.48	0.88	0.84	1.08	1.08	3.00	3.00
	25.6	5.60	0.40	0.48	0.90	0.92	1.20	1.20	3.00	2.80
	25.6	5.60	0.40	0.60	0.72	1.00	0.92	1.60	2.80	3.20
	27.6	5.80	0.60	0.52	1.00	1.08	1.60	1.20	3.20	3.00
	Average		0.47	0.51	0.86	0.94	1.16	1.23	2.96	3.00

Table 6 Effect of insulin on the tail fin regeneration of Topminnow, at 9°–15° C, insulin solution sith 0.02 and 0.03 M glucose was applied for 7 days after amputation.

	Size of fish (mm)		Length of regenerated tail fins (mm)															
			7th day				14th day				21st day				28th day			
	Standard length	Tail fin length	Control	Experimental			Control	Experimental			Control	Experimental			Control	Experimental		
				Insulin	Glucose	Insulin+Glucose		Insulin	Glucose	Insulin+Glucose		Insulin	Glucose	Insulin+Glucose		Insulin	Glucose	Insulin+Glucose
0.005 unit/ml. Insulin and 0.02 M, 0.03 M Glucose	24.0	5.60	0.12	0.12	0.12	0.20	0.40	0.40	0.20	0.28	0.84	0.80	0.52	0.84	1.92	2.20	2.20	2.20
	25.6	5.60	0.20	0.20	0.20	0.20	0.44	0.44	0.48	0.44	0.80	0.88	0.72	0.76	2.00	2.00	2.00	2.00
	24.4	5.60	0.12	0.20	0.24	0.28	0.40	0.44	0.40	0.44	0.80	0.80	0.60	0.76	2.00	2.08	2.00	2.20
	23.2	5.20	0.24	0.24	0.24	0.28	0.40	0.40	0.48	0.48	0.60	0.80	0.60	0.60	1.60	2.00	1.60	2.40
	22.8	4.48	0.40	0.24	0.40	0.40	0.48	0.40	0.40	0.48	0.96	0.96	0.80	0.84	2.16	2.16	1.92	2.16
	Average		0.22	0.20	0.24	0.27	0.42	0.42	0.39	0.42	0.80	0.85	0.65	0.76	1.94	2.09	1.94	2.19
0.01 unit/ml. Insulin and 0.02 M, 0.03 M Glucose	23.2	6.00	0.12	0.12	0.12	0.12	0.40	0.40	0.40	0.48	0.80	0.80	0.80	0.80	2.20	2.20	2.20	2.20
	24.0	5.60	0.12	0.12	0.16	0.24	0.32	0.48	0.24	0.52	0.60	0.92	0.92	0.92	2.08	2.08	2.16	2.08
	28.4	5.80	0.12	0.12	0.16	0.20	0.35	0.40	0.36	0.48	0.72	0.80	0.88	0.80	2.08	2.08	2.16	2.08
	22.8	4.64	0.08	0.08	0.08	0.08	0.40	0.40	0.44	0.60	0.80	0.80	0.92	0.92	2.24	2.24	2.24	2.24
	24.0	5.20	0.20	0.20	0.20	0.32	0.40	0.40	0.40	0.60	0.72	0.72	0.80	0.84	1.88	1.88	1.88	1.88
	Average		0.13	0.13	0.14	0.19	0.37	0.42	0.37	0.54	0.73	0.81	0.86	0.86	2.10	2.10	2.13	2.10
0.1 unit/ml. Insulin and 0.02 M, 0.03 M Glucose	23.6	5.20	0.20	0.24	0.24	0.68	0.68	0.60	0.40	0.72	1.04	1.40	1.04	1.04	2.48	2.08	2.08	2.48
	22.4	4.48	0.16	0.36	0.32	0.32	0.52	0.52	0.81	0.60	1.24	1.24	1.24	0.92	2.40	2.40	2.48	2.08
	26.4	5.20	0.20	0.24	0.35	0.48	0.48	0.48	0.60	0.60	1.08	1.08	1.08	0.92	2.40	2.40	2.40	2.40
	23.2	5.20	0.28	0.40	0.52	0.48	0.48	0.52	0.60	0.60	1.20	1.20	0.92	1.20	2.40	2.40	2.40	2.24
	22.8	5.20	0.40	0.40	0.40	0.40	0.72	0.72	0.72	0.72	0.81	0.81	0.80	0.80	2.52	2.52	2.08	2.08
	Average		0.25	0.33	0.37	0.47	0.58	0.57	0.63	0.65	1.07	1.15	1.02	0.98	2.44	2.36	2.29	2.26

Insulin is closely connected with carbohydrate metabolism by promoting the uptake of carbohydrate in various tissues. Then for the next experiment was performed in order to see whether insulin plus glucose is effective in promoting the regeneration. As experimental solution, three kinds of the dilute insulin solution (0.005, 0.01, and 0.1 unit/ml.) were used. And 0.02 M glucose and 0.03 M glucose were put to use. The 0.02 M glucose was added to each solution for the first week and 0.03 M glucose for the second week. The results are given in Table 6. From the data given in this Table, it is seen that the promoting function of regeneration takes place in the fish kept in the solution treated with only 0.1 unit/ml. insulin, while the regeneration is remarkable composed with insulin plus glucose is applied. In the solution treated with more dilute insulin (0.005, 0.01 unit/ml.) the regeneration is not actively made, but it is greatly promoted if 0.2 M glucose is added.

After the fish were kept in water for 14 days after amputation, they were transfered into insulin solution of 0.05 and 0.5 unit/ml. and these were kept for 24 hours, the tail tips were fixed with Bouin's fluid (containing urea and acetic acid) and stained by Harris Acid hematoxilin. (Roberts Rugh: Experimental Embryology, p. 28). The number of epidermal cells in which chromosomes were observed distinctly were counted for the tail tip areas of five fishes in each treatment under a microscope. The results showed that the number of epidermal cells with distinct chromosomes were largest in the fishes kept in the 0.5 unit/ml. solution and smallest in the fishes kept in water. In the fishes treated by the 0.05 unit/ml. solution, the number was a little larger than in the case of the fishes kept in water.

Discussion

In the experiments made for this study, the promoting effect of the regeneration was observed in the solution treated with the dilute 0.05, 0.1 unit/ml. insulin solution. This effect was not identified in the 0.3 unit/ml. However, in the 0.3 unit/ml. and in the thicker solution (0.5 unit/ml.), if Insulin Novo is used the regeneration is accelerated. It seems possible that the retarding function of the previous 0.3 unit/ml. insulin is influenced by cresol contained in the test solutions. It is because the appetite of the fish falls off and this may cause the poor regeneration. The decrease of the fish appetite was also seen to some degree when they were kept in the insulin solution. As seen in Table 3, the regeneration is actively made in the 0.2 unit/ml. insulin solution at the initial stage of the experiment, however if the insulin solution of the same concentration is kept on treating for a certain period of time, the regeneration will be retarded.

So far investigators have reported that insulin stimulates various tissue cells. Many investigators conducted their experiments by treating the solution with insulin for a short time. However, KANATANI reported that it produces a promoting effect on the planarian regeneration to treat the experimental solution with insulin for 7 days. In the present study, to treat the solution with insulin for 7 days was

effective in the promoting effect on the tail tip regeneration. Since it was regarded as also effective to treat insulin for the period of shorter than 7 days, the insulin was treated for 3 days and it proved to be effective after all.

Thus, insulin given at the begining stage of regeneration was effective, while, as shown in various tables, if insulin was given continuously for a certain period of time, less or no effect of insulin was observed.

KATAYAMA has shown that insulin plus 0.02 M glucose increases the number of mitosis in the mouse ear epidermis. OSANAI has also shown that the growth of chick heart fibroblasts was augumented by the presence of insulin added 0.01 M glucose, but the growth in the medium containing 0.03 M glucose was depressed. In the present study, insulin plus 0.02 M glucose evidently augumented the growth of regeneration, but insulin added 0.03 M glucose did not take effect in that of regeneration. The results of the present study are in agreement with OSANAI's view that the growth of chick heart fibroblasts is augumented by the tissue culture method. The results observed in these experiments also were essentially similar to those described by KATAYAMA that the number of mitosis in the mouse ear epidermis is increased by *in vitro* as described before.

According to the results of the observation, made by using a microscope, of regenerating tail tips treated with insulin for 24 hours, many chromosomes were found in the experimental, but not so many in the control. This fact shows that the cells in regenerating tail tips were directly stimulated by the presence of insulin, and further that the growth takes place in the regenerated parts by the active process of the cell division.

Summary

1. The effect of insulin on the tail fin of the topminnow, *Gambusia affinis* regeneration was studied. After amputating the tail fin, the fish were put in the dilute insulin solution at the temperature 9°–15° C. The growth of tail tips was then measured every 7 days during the experiment. It was observed that insulin produces a promoting effect on the growth of tail tips.

2. When, the fish cut in the tail fin were put into the solution containing insulin plus glucose, the promoting effect of insulin on the regeneration was found to be noticeably augumented.

3. It is known that there is some insulin in the body of the fish. The results of the present study shows that cell division in the tail tip seems to be promoted by treating with insulin.

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Fig. 1 Tail fin regeneration in the Topminnow, *Gambusia affinis*, for 14 days after amputation.

A: Original tail fin.
B: Amputated at a point, in the middle of the tail fin.
C: 7 days after amputation.
D: 14 days after amputation.

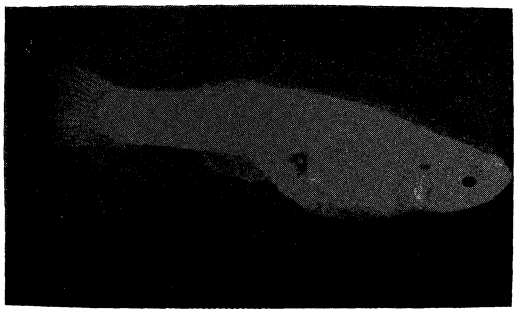

Fig. 1, A

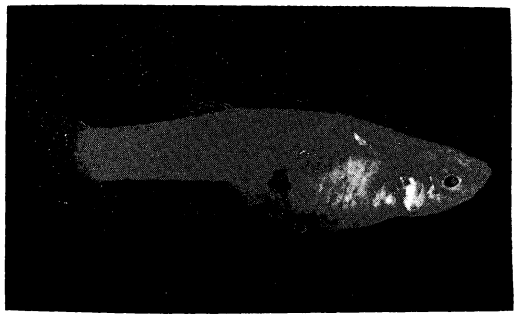

Fig. 1, B

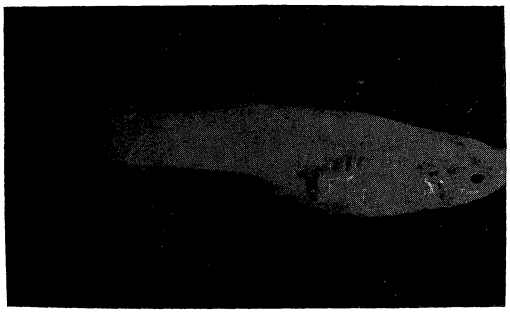

Fig. 1, C

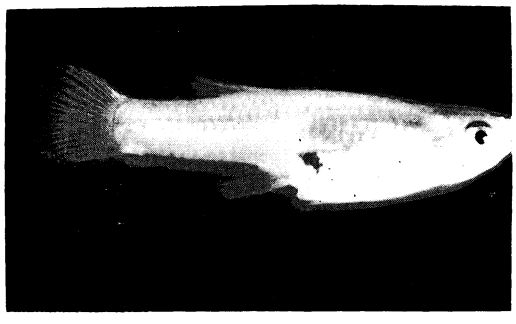

Fig. 1, D

Fig. 2 Chromosomes observed in the regenerating tail tip. ×1500.

A: Prophase, B: Metaphase, C: Anaphase.

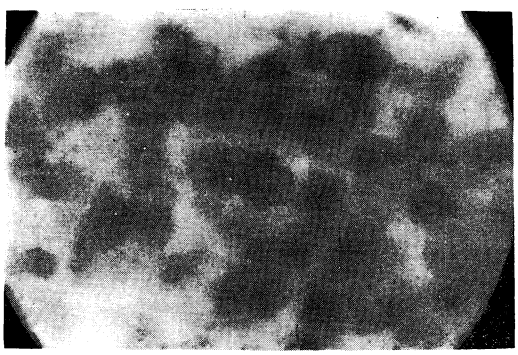

Fig. 2, A

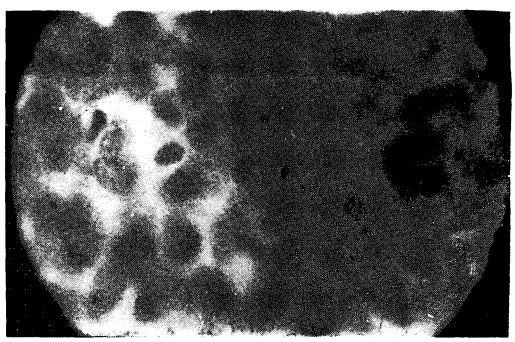

Fig. 2, B

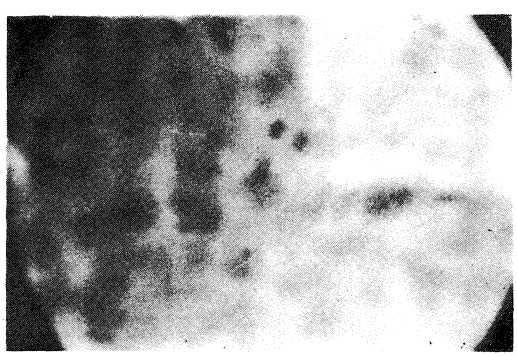

Fig. 2, C