CHAPTER IV

RESULTS

1. Freshwater Fish Cell Cultures.

1.1 Morphology :

a) Morphology of Anabas testudineus cells in culture.

Cell cultures were made from the caudal trunks of A. testudineus (6 pieces) which were 10-11 cm in size. During the first 10^{th} passage, the cells were predominantly fibroblast-like. After that, the cells began to change from fibroblast to epithelial-like. In the 15^{th}-16^{th} passage, they began to degenerate.

But, culture from the caudal trunks of the fishes (10 pieces) which were 2-3 cm in size produced a cultivation composed of fibroblast cells after culturing 3-5 days(Figure 8) and these formed a monolayer or confluent sheet. Thereafter, the splitting ratio was 1:3 to 1:4. They had a fibroblast-like morphology (Figure 9).

b) Morphology of Cyprinus carpio cells in culture.

Cell cultures from the caudal trunks of C. carpio (6 cm, 10 pieces and 18-20 cm, one piece) could be grown in the same way as A. testudineus. They could be

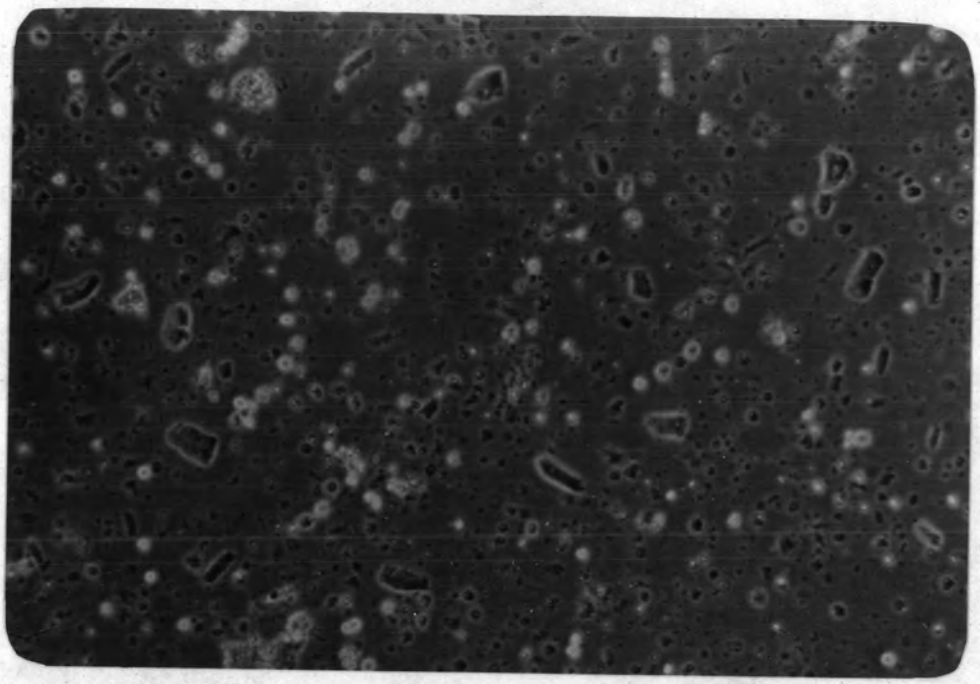

Figure 7. Cell and tissue fragment suspension obtained by trypsinization of Anabas testudineus caudal trunks. This degree of dispersion was ideal for successful planting of a primary monolayer culture.

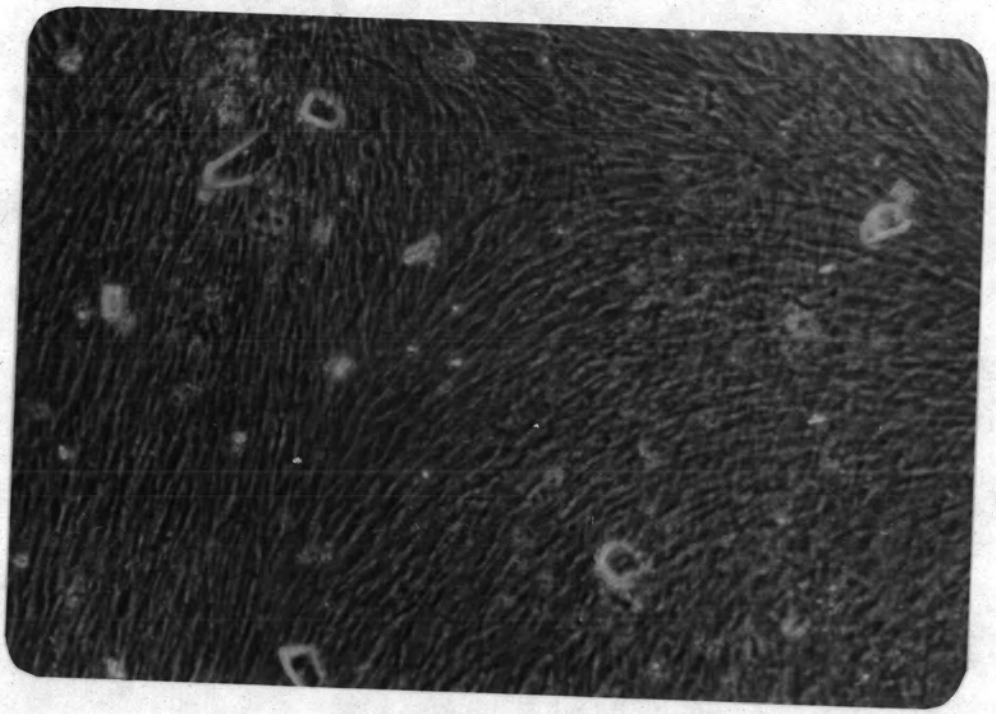

Figure 8. Photograph of Anabas teatudineus cells in the first passage after 3-5 days, showing characteristic of fibroblast-like morphology (phase contrast 10x).

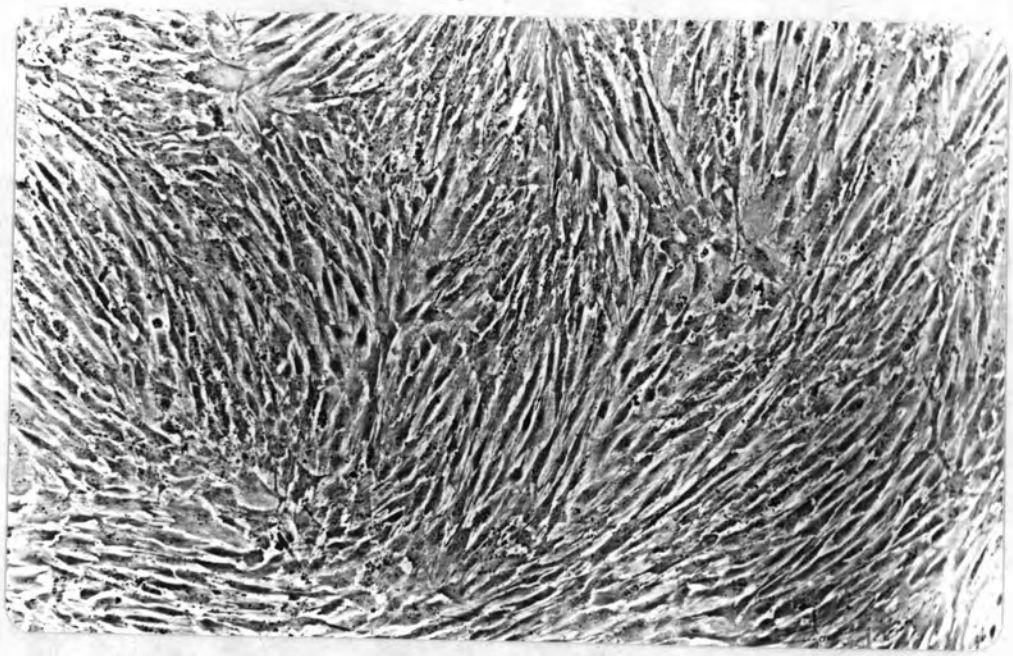

Figure 9. Photograph of Anabas testudineus cells at 12th passage, 2 days, showing characteristic of fibroblast-like morphology (phase contrast 10x).

splitted at a ratio of 1:2 to 1:3 at 28°C. The morphology was fibroblast-like (Figure 10). After fifty passages, they began to degenerate.

c) Morphology of Lebeo rohita cells in culture.

Fifty caudal trunk samples of L. rohita, 1-2 cm in size were used for cell cultures. They could be cultured in the same way as samples from other fishes (Figure 11). But these cultures were very sensitive to a change of media or temperature. They tended to clump, some cells died and some of them grew. Thus, they were unsuitable for studying viral multiplication. After the 15^{th}-16^{th} passage, they began to degenerate.

d) Morphology of Ophicephalus lucius cells in culture.

The cells were cultured from the caudal trunks of O. lucius (6 pieces) which were 6-9 cm in size. Cells were predominantly fibroblast-like. They were able to form a confluent monolayer sheet within 5-7 days after the first incubation. The first four splitting cells of the monolayer were performed at ratio of 1:2, at 28°C. Thereafter, the splitting ratio was 1:3-1:4 (Figure 12).

1.2 Growth curve and optimal growth temperature.

The results of the experiments on cell growth as a function of the incubation temperature were

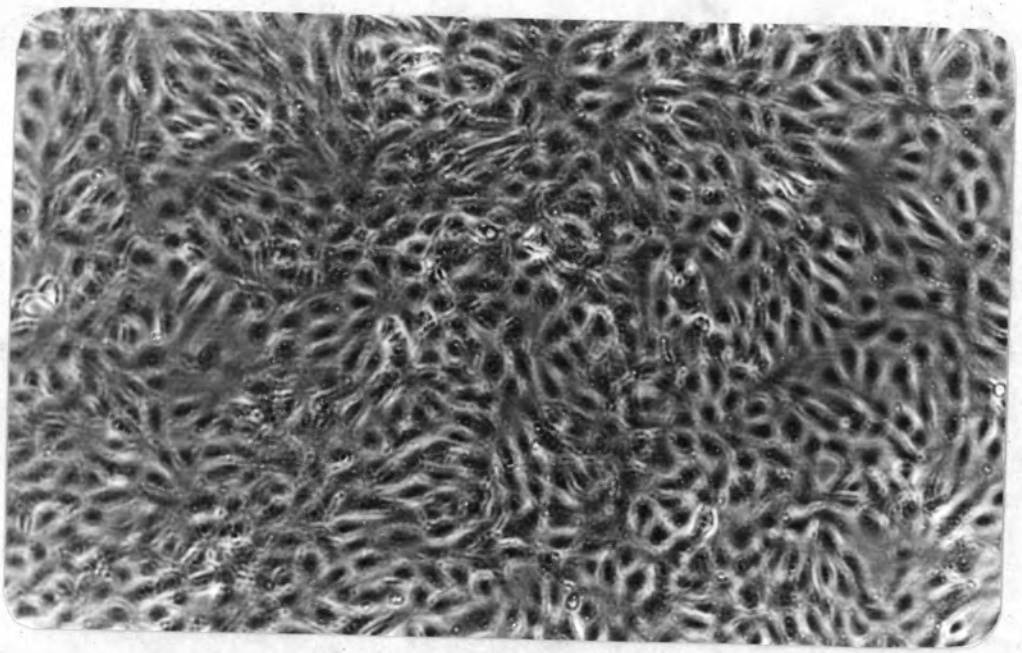

Figure 10. Photograph of Cyprinus carpio cells at the 10th passage, 2 days, showing characteristics of fibroblast-like morphology (phase contrast 10x).

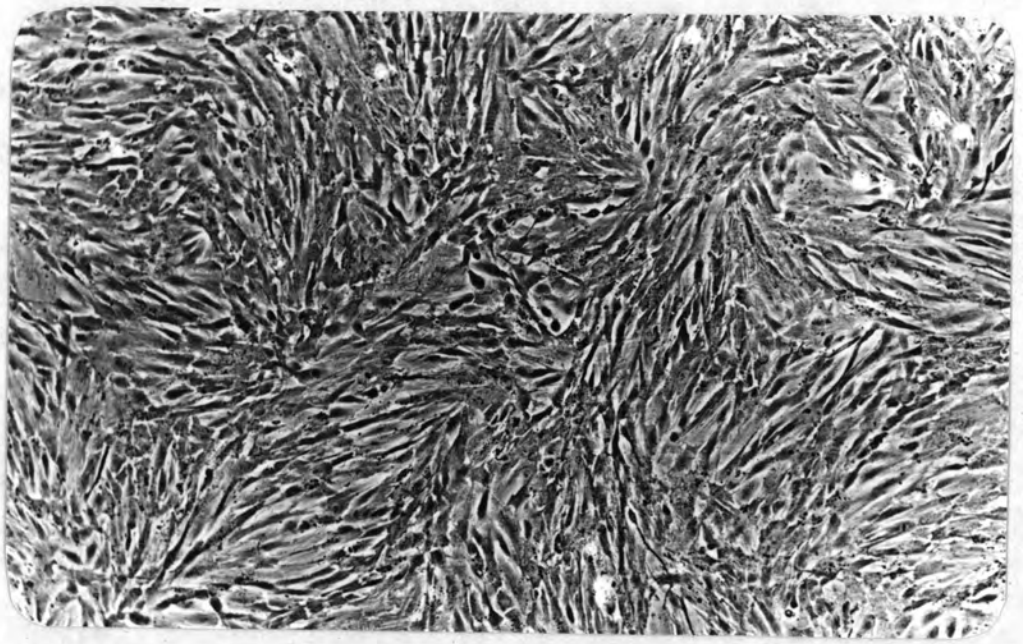

Figure 11. Photograph of Lebeo rohita cells at the 10th passage, 2 days, showing characteristics of fibroblast-like morphology (phase contrast 10x).

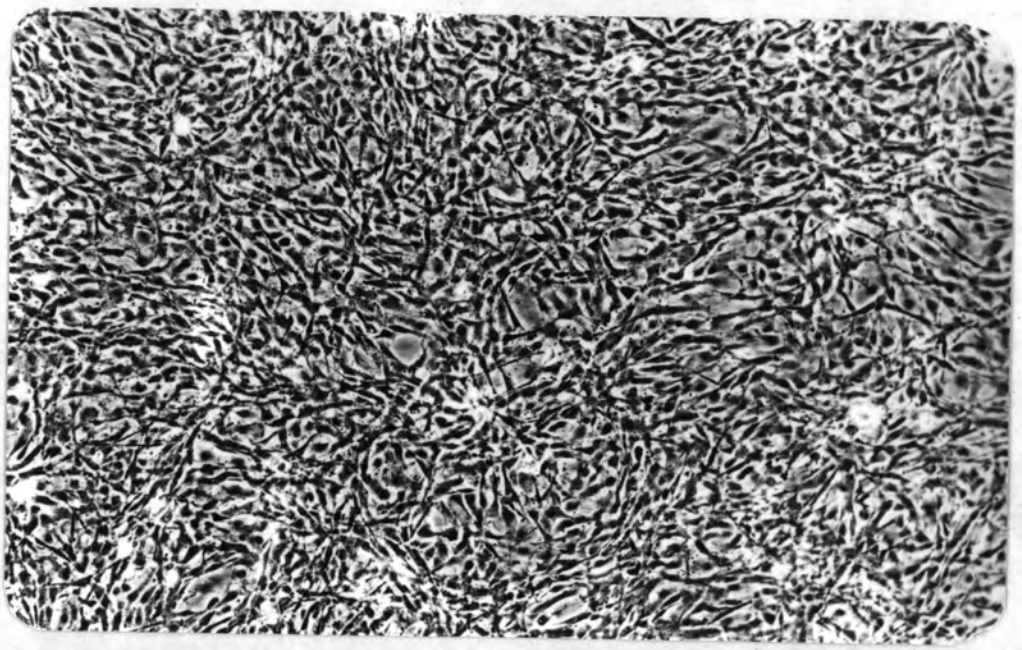

Figure 12. Photograph of Ophicephalus lucius cells at the 18th passage showing characteristics of fibroblast-like morphology (phase contrast 10x).

as follows :

a) A. testudineus cell cultures.

Cell cultures from A. testudineus (27th subculture) were viable from 16 to 37 °C or higher and were able to grow between 28 °to 37 °C or higher. The optimal growth temperature was 28 °C (Figure 13). When 4 ° 16 ° 22 °, 28 ° and 37 °C were used, at 28 °C, they achieved maximum division within 14 days. Afterthat, they began to degenerate. At 16 and 22 °C, they could be survived but did not divide and remained viable for 14 to 28 days. At 4 °C they could not grow (Figure 14).

b) C. carpio cells cultures.

The cell cultures from C. carpio (29th subculture) were viable from 4 °up to 37 °C or higher and were able to grow between 16 ° to 37 °or higher. The optimal growth temperature was 37 °C (Figure 15) or higher. When the cells were seeded with 1 x 10^6 cell/ml and cultured at 4 °, 16 °, 22 °, 28 ° and 37 °C. They achieved maxinum division within 14 days at 28 °C. After that, they began to degenerate. At 16 ° to 22 °C, C. carpio cells survived but did not divide or did so very slowly and remained viable for a month. At 4 °C, they could be survived for only a short time and at 37 °C, they divided very well but died within 7 days (Figure 16).

c) O. lucius cell cultures.

Cells from O. lucius (29th subculture) were viable

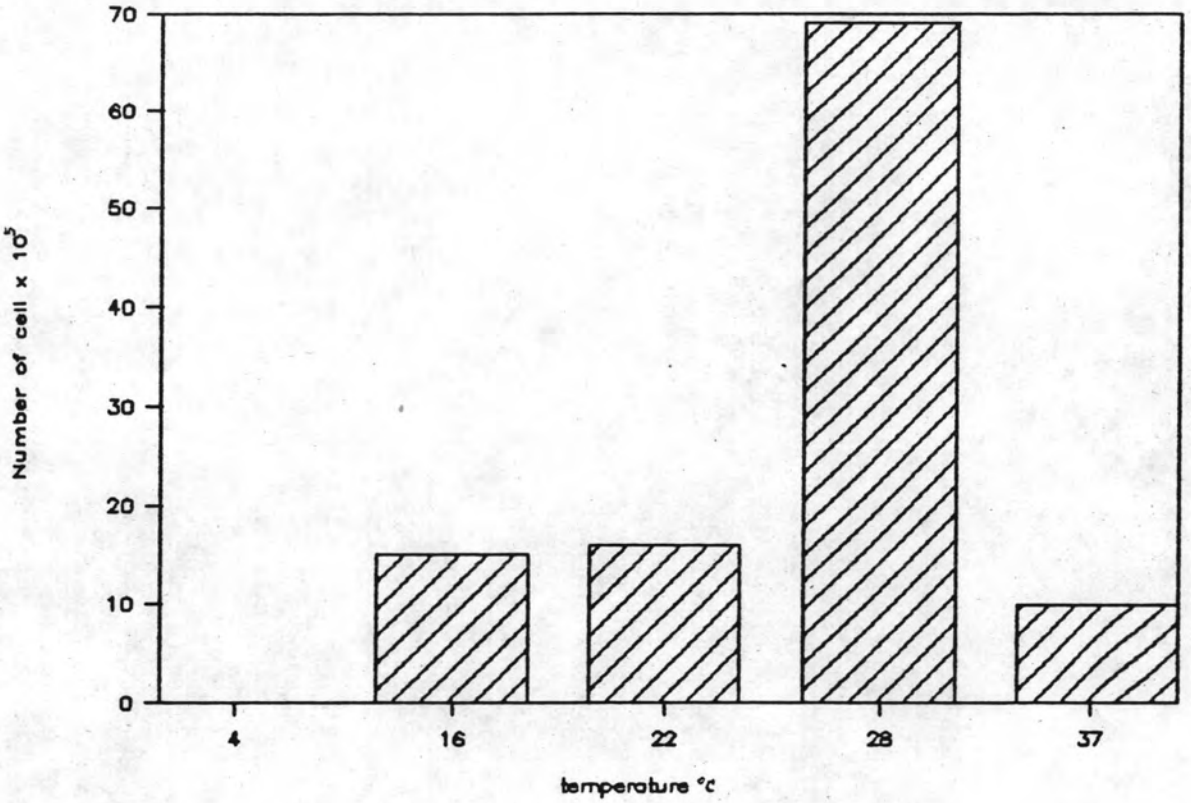

Figure 13. Mean number of cells from Anabas testudineus (27^{th} passage) per 25 cm^2 flask after incubation for 7 days at selected temperatures.

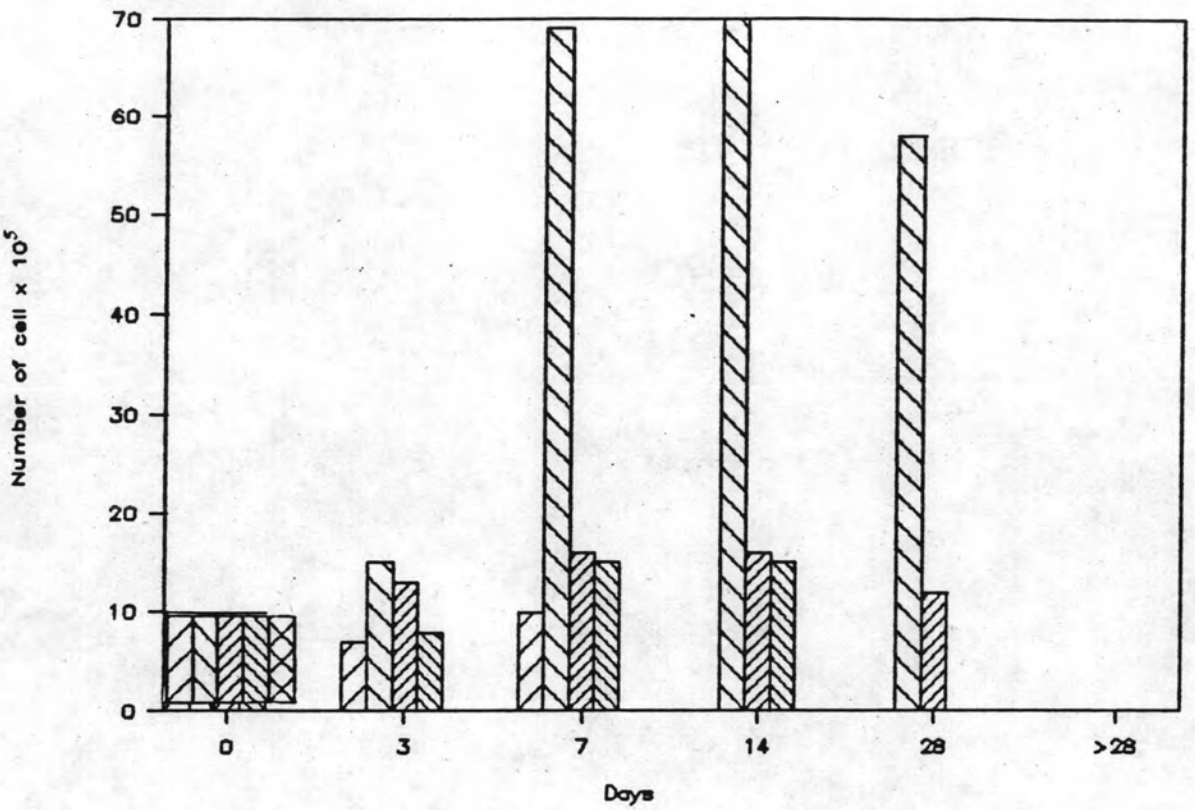

Figure 14. Growth of A. testudineus cells at different incubation temperatures. Culture flasks (25 cm^2) were seeded with $1x10^5$ cell/ml and divided into 5 groups, each incubated at one of the following temperature:

37 °C 28 °C
22 °C 16 °C 4 °C

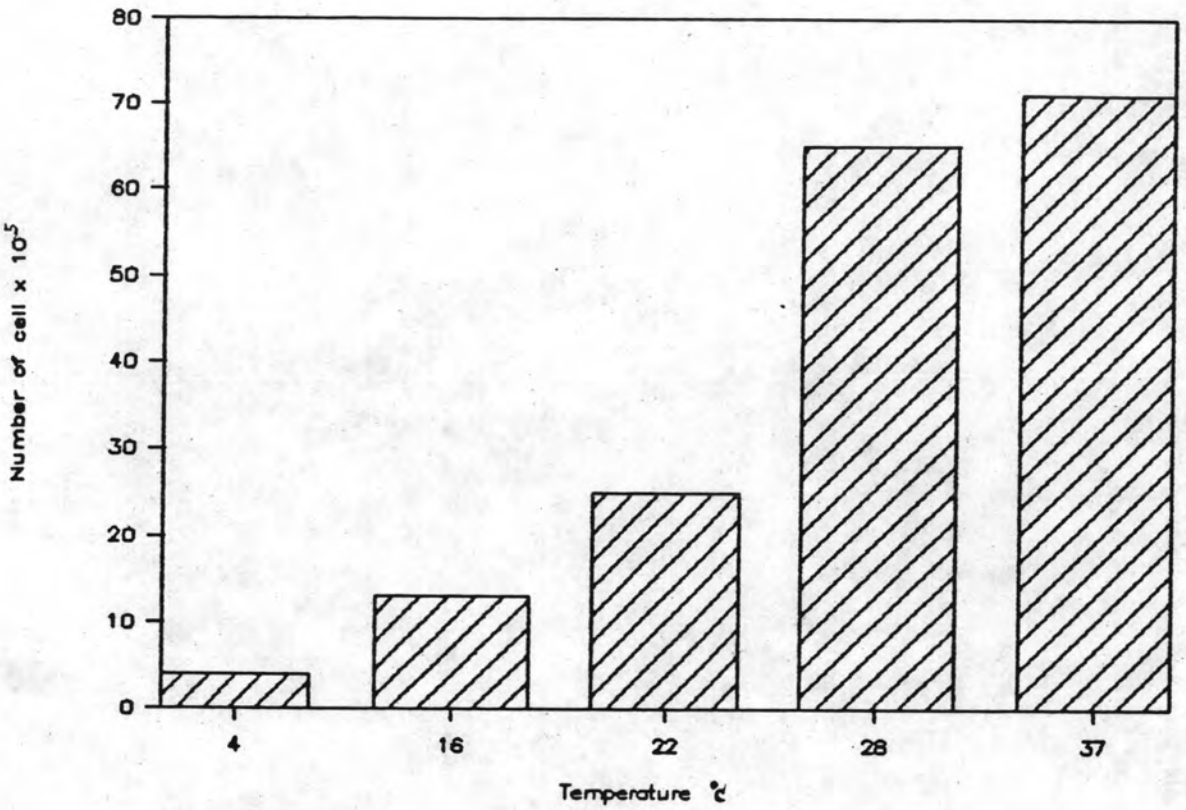

Figure 15. Mean number of cells from Cyprinus carpio (29th passage) per 25 cm^2 flask after incubation for 7 days at selected temperatures.

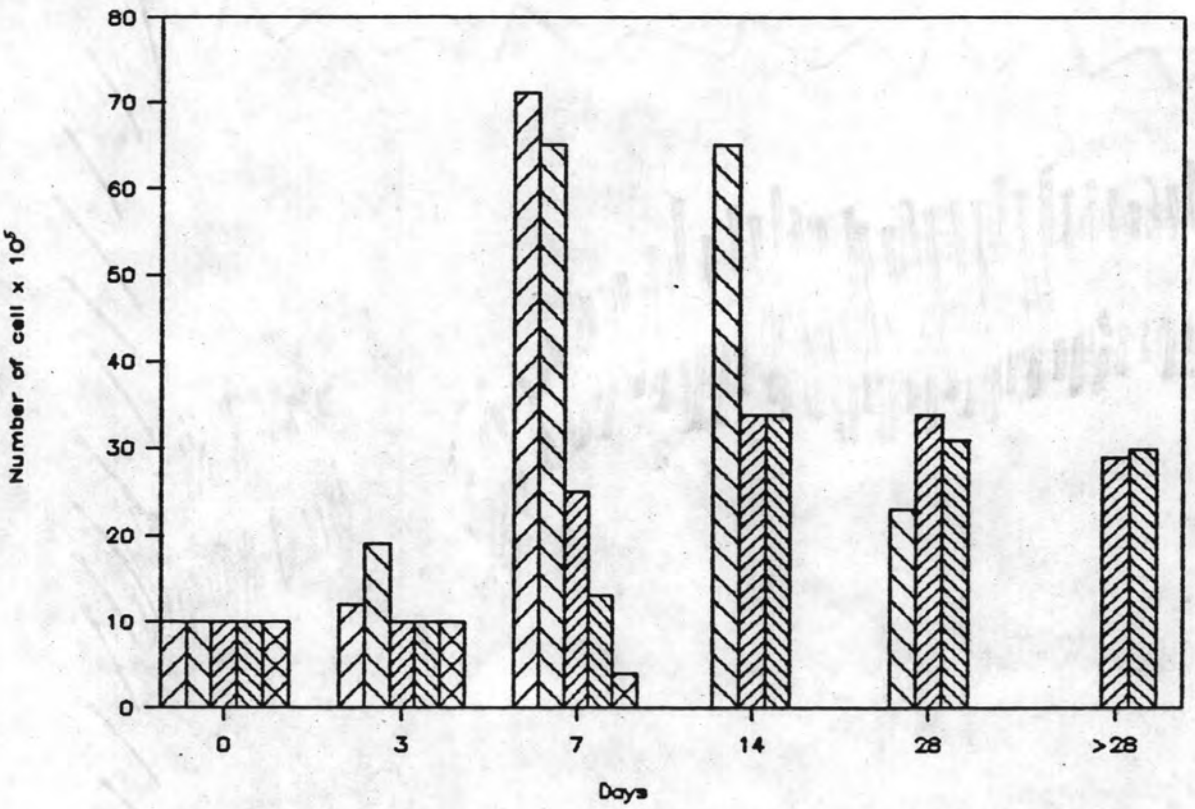

Figure 16. Growth of C. carpio cells at differnt incubation temperatures. Culture flasks (25 cm^2) were seeded with $1x10^6$ cell/ml and divided into 5 groups, each incubated at one of the following temperatures :

37 °C 28 °C
22 °C 16 °C 4 °C

from 16°C up to 37°C or higher and were able to grow between 22° and 37°C or higher. The optimal growth temperature was 28°C (Figure 17). When the cells were seeded with 1 x 10^6 cell/ml and incubated at 4°, 16°, 22°, 28°and 37°C. At 4°C, they could not grow or survived. O. lucius cells could be survived within 28 days at 16° and 22°C. At 37 °C, the cells could be grown and degenerated within 7 days (Figure 18).

1.3 Sterility of the cell cultures.

By using thioglycollate broth for testing bacterial contamination were not found in cell cultures from O. lucius, A. testudineus and C. carpio.

Fungus contamination was not present in these cell cultures when used Sabouraud's agar plate test.

1.4 Cell preservation.

After preservation of the cells in 10 % DMSO and 20 % fetal bovine serum more than three months, the recovery of viable cells could be done by thawing running tap water, seeding into L-15 (growth medium) and incubating at 28°C. The quantity of cells contained in a frozen tube, when seeded into 25 cm^2 flasks, led to a harvest of 2-3 x 10^6 cell/ml within 7 days from O. lucius, 5-6 x 10^6 cell/ml of C. carpio and A. testudineus.

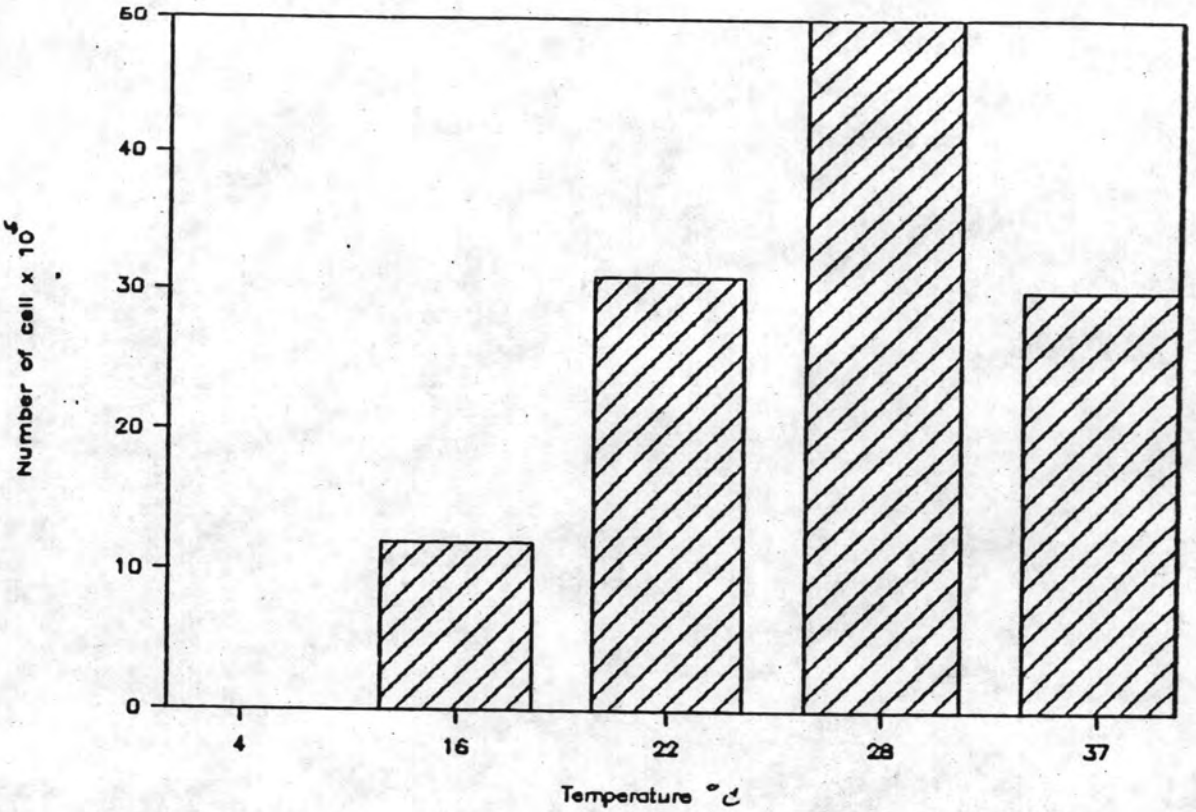

Figure 17. Mean number of cells from Ophicephalus lucius (29th passage) per 25 cm² flask after incubation for 7 days at selected temperatures.

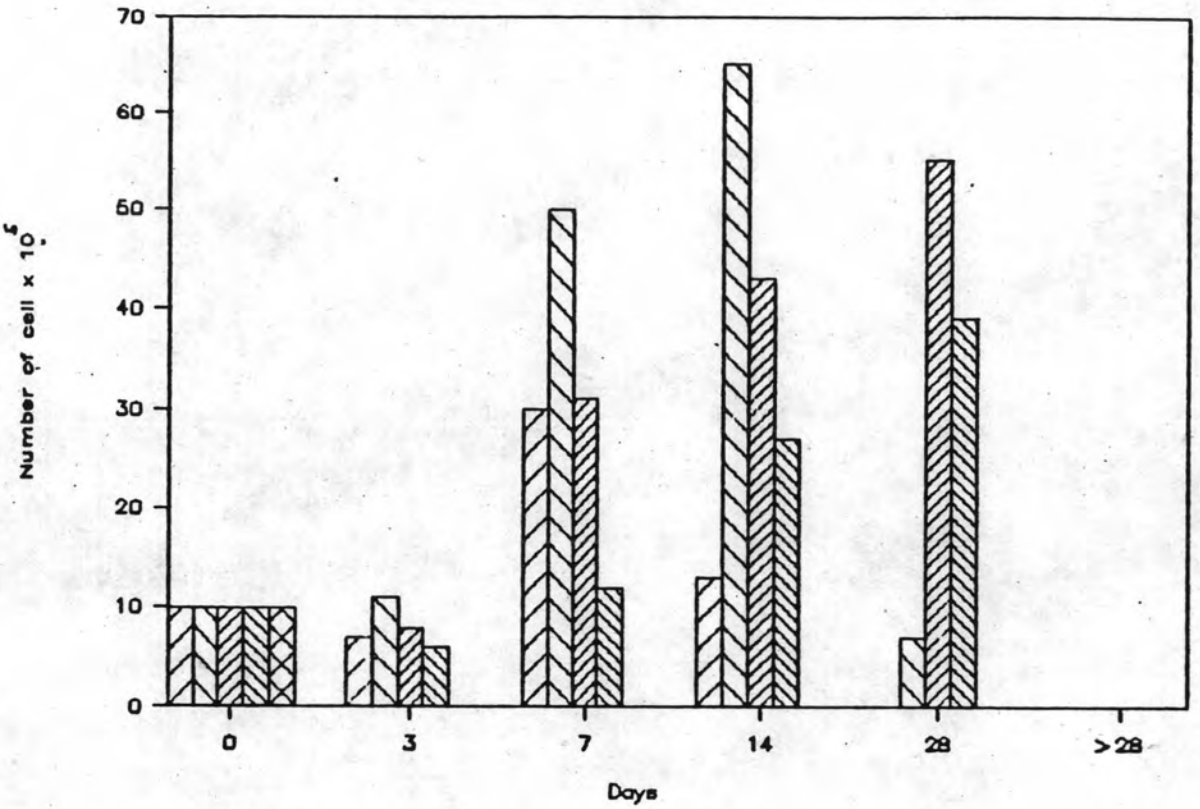

Figure 18. Growth of O. lucius cells at different incubation temperatures. Culture flasks (25 cm²) were seeded with 1x10⁶ cell/ml and divided into 5 groups, each incubated at one of the following temperatures :

37° 28°
22° 16° 4°C

2. Subculturing and Propagation of Cell Culture.

Now the cell cultures from A. testudineus were subcultivated (1:3 to 1:4) every 3 - 4 days. The cell cultures were carried through 70 serial passage when using the fishes 2-3 cm in size. But the cell cultures from the fishes 10-11 cm in size degenerated after 15-16 serial passages.

The cell cultures from C. carpio were subcultivated (1:2 to 1:3) every 3-4 days. These cell cultures did not survive more than 50 serial passages.

The cell cultures which were prepared from the caudal trunks of O. lucius were subcultivated (1:3) every 3-4 days. The cell cultures carried through 120 serial passages.

3. Virus Multiplication.

3.1 Stock of virus.

a) IPNV : This virus was obtained from Stirling University, Scotland, U.K., as frozen tissue culture fluid from infected culture of BF-2 cell lines. The virus was prepared by inoculating culture of BF-2 cells with 1 ml of approximately a 1:50 dilution of the frozen culture fluid and incubating at 22°C for 3 days. The BF-2 cell lines showed CPE (Figure 20) and the culture fluid was centrifuged at low speed, 200, g 10 min, the supernatant was dispensed in small volume and stroed at -80 °C. The virus concentration was

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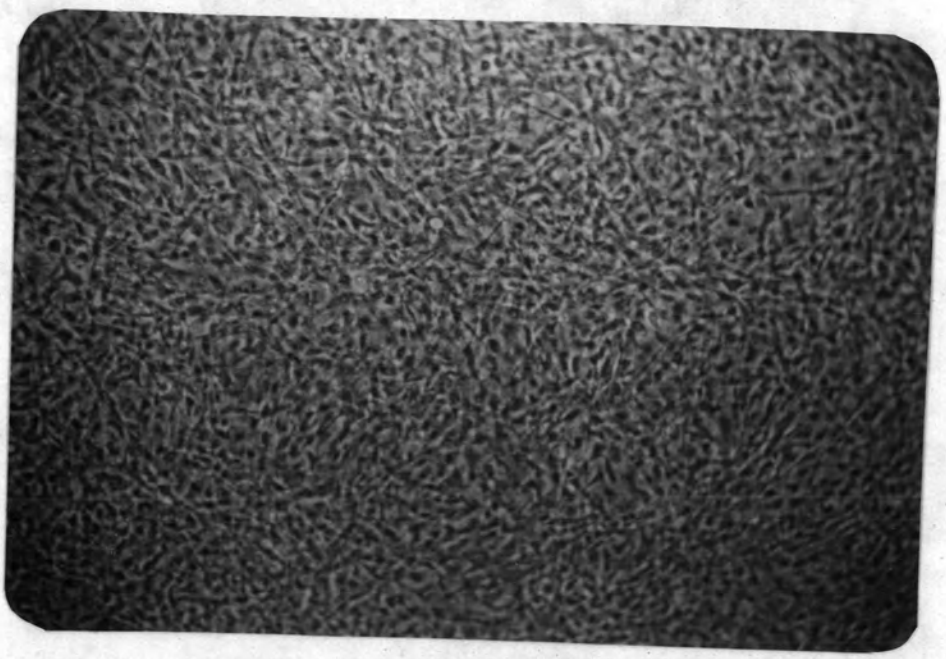

Figure 19. Photograph of a normal BF-2 cell line (phase contrast 10x).

Figure 20. BF-2 cell lines showing cytopathic effect (CPE) caused by IPNV (phase contrast 10x).

10^{-7} $TCID_{50}$/50 ul (Determined by Reed and Muench method 1938, see appendix II).

b) CV : This virus was isolated from carp which obtained from Dr. Kamonporn Tonguthai, National Inland Fisheries Institute, Freshwater Fisheries Division of Department of Fisheries and cultured in BF-2 cell lines, in 1985. In the 5th passage, the culture fluid used as stock virus was stored at -80 °C, and the virus concentration was $10^{6 \cdot 4}$ $TCID_{50}$/50 ul (Determined by Reed and Muench method, see appedix II).

c) SHV : This virus was originally isolated from the internal organs of striped snakehead fish (O. striatus) during the epizootic in 1985. The virus was prepared by inoculating culture of BF-2 cell lines and incubating them at 22 °C. Cells were observed daily for cytopathic effect (CPE). Three to five days after infection, they showed CPE. The culture fluid was centrifuged at low speed, the supernatant was dispensed in small volume at the 5th passage and stored at -80 °C. The virus concentration was $10^{5 \cdot 2}$ $TCID_{50}$/50 ul (Determined by Reed and Muench method, see appendix II).

3.2 Virus multiplication in three kinds of cell cultures.

Virus multiplication in cultures was studied in O. lucius, A. testudineus and C. carpio cells, and determined by $TCID_{50}$ analysis as described by Nime

et al. 1970, Solis and Mora 1970.

Among fish virus tests, every kind of viruses provided virus yields higher than 10^5 $TCID_{50}$/50 ul in cell cultures of O. lucius and A. testudineus (Table 10 Figure 21, 22).

On the contrary, no cytopathic effect was obtained in C. carpio cell cultures, even after five successive blind serial passages. There was no CPE after the final incubation (Table 10).

There was no statistically significant difference between the two ways analysis of variance for studying viral multiplication in three kinds of cell cultures.

Electron microgarp revealed virus particles in BF-2 cell line which had been infected with IPNV. Icosahedral viral particles were found in the cytoplasm figure 23).

Electron microgarph revealed virus-like particles in cell cultures of freshwater fish (O. lucius) which had been infected with SHV (figures 24, 25). Icosahedral virus particles and rhabdovirus-like could be found in cell culture of freshwater fish (A. testudineus and O. lucius).

Table 10 Virus multiplication in three kinds of the fish cell lines.

Cell culture	Virus detection ($logTCID_{50}$/50 ul)		
	IPNV	SHV	CV
O. lucius	8.2	6.0	7.4
A. testudineus	7.8	6.3	6.5
C. carpio	0	0	0
BF - 2	8.7	5.5	6.3

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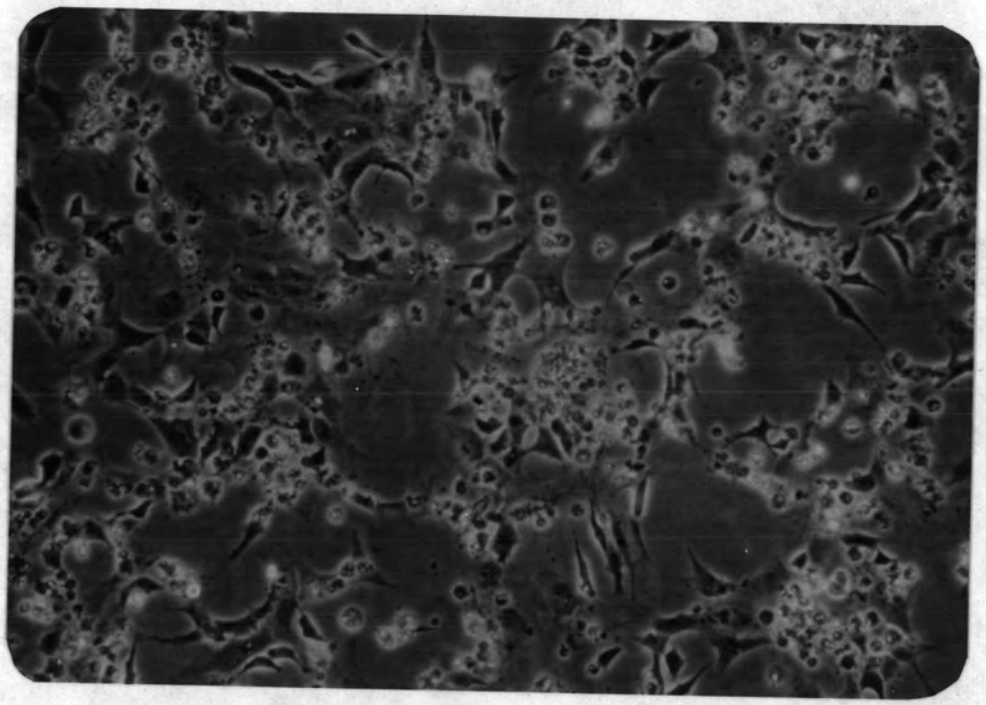

Figure 21. Cells cultured from Anabas testudineus showing cytopathic effect caused by SHV (phase contrast 10x).

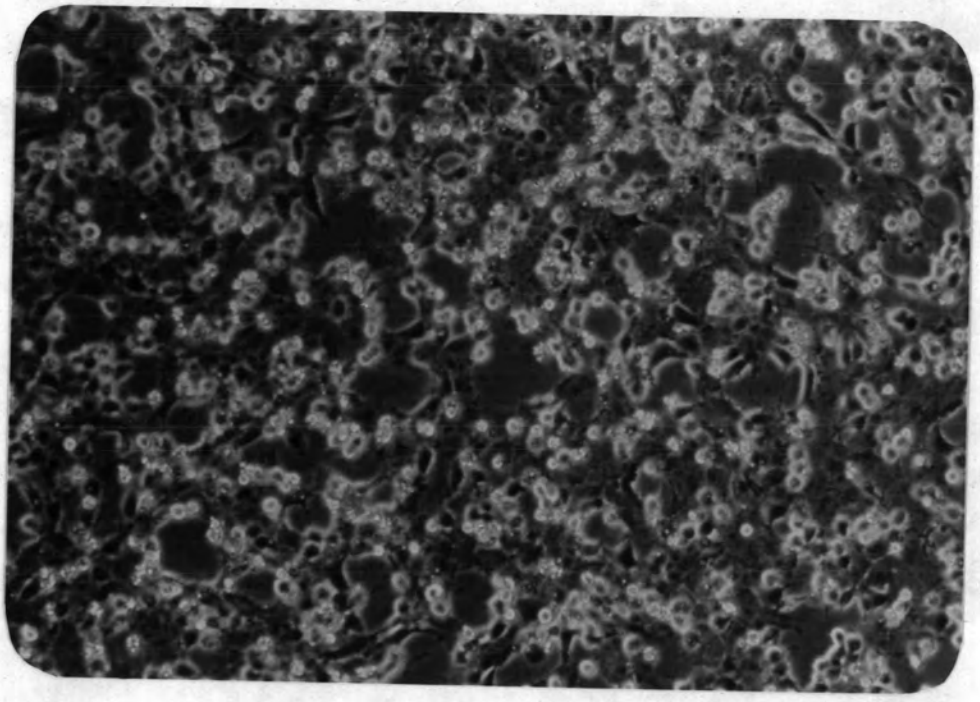

Figure 22. Cells cultured from Ophicephalus lucius showing cytopathic effect cuased by SHV (phase contrast 10x).

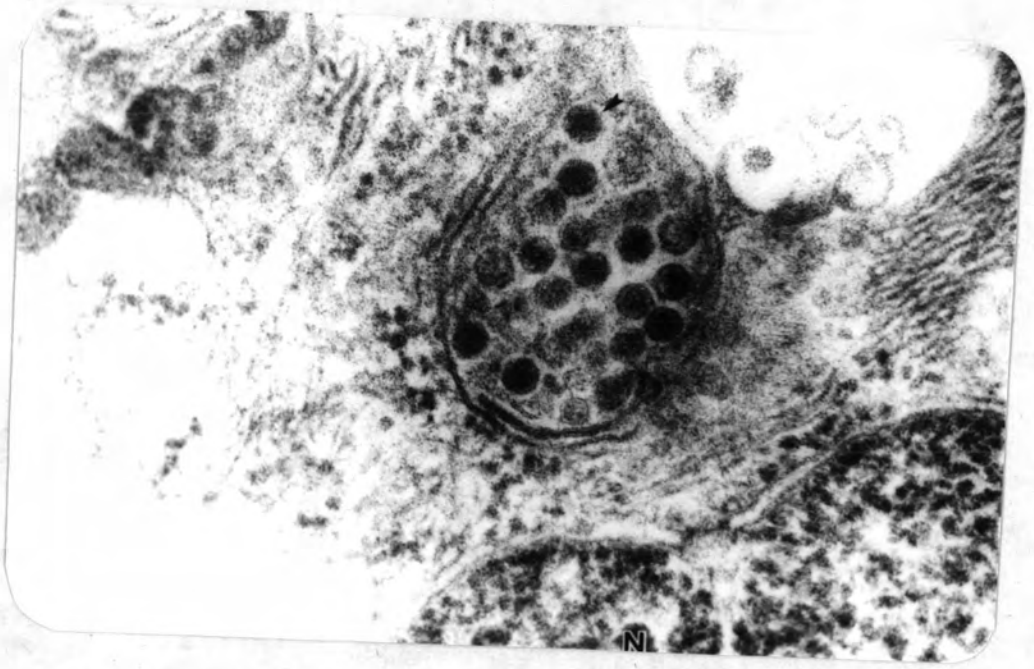

Figure 23. IPN particle in BF-2 cell lines, 3 days after infection. Virion were found in the cytoplasm.

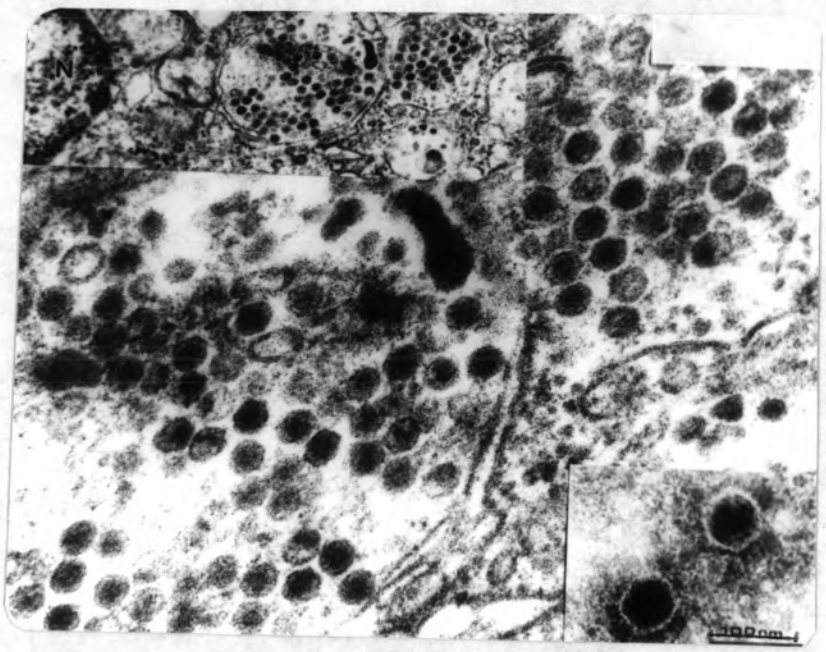

Figure 24. Icosahedral virus particles, 50-55 nm in diameter, in Ophicephalus lucius when infected with SHV.

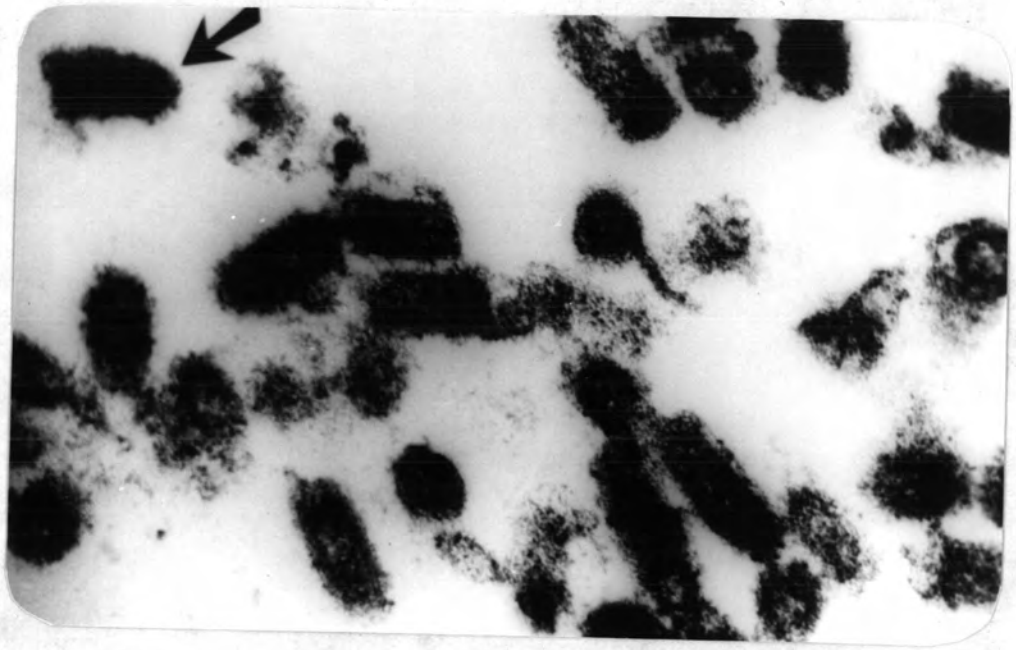

Figure 25. Rhabdovirus-like virus in cell culture from Ophicephalus lucius when inoculated with SHV.

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