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# Studies on the identification and characterisation of certain fish viruses with special reference to lymphocystis and piscine erythrocytic necrosis (PEN) viruses

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**STUDIES ON THE IDENTIFICATION  
AND CHARACTERISATION OF CERTAIN FISH VIRUSES**

**with special reference to**

**Lymphocystis**

**and**

**Piscine Erythrocytic Necrosis (PEN) viruses**

**by**

**David A. Smail, B.A., M.Sc.**

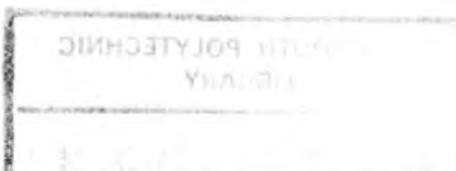
**A Thesis**

**presented to the**

**Council for National Academic Awards**

**in candidature for the degree of**

**Doctor of Philosophy**



**Plymouth Polytechnic in collaboration  
with the University College of Wales,  
Aberystwyth**

**1979**

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DECLARATION

This is to certify that the work submitted here was carried out by the candidate himself and due acknowledgement has been made of the assistance received.

Signed.....*David A. Smail*.....  
*1st Sept. 1979*

This work has not been accepted in substance for any other degree and is not concurrently being submitted in candidature for any other degree.

Signed.....*David A. Smail*.....  
*1st Sept. 1979*

To Cynthia who made this possible

To my mother and late father

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LIST OF ABBREVIATIONS

CPE	Cytopathic effect	MV	Marine Vessel
diam.	diameter	PBS	Phosphate Buffered Saline
e.m.	electron microscopy		
FRS	Fishing Research Ship	p.i.	post-infection
		proc.	procedure
G	gauge	RV	Research Vessel
g	relative centrifugal force	rbc(s)	red blood cell(s)
ID <sub>50</sub>	Infectious dose giving 50% end point	SI units such as	
		m.	metre
IU	International Units	mm.	10 <sup>-3</sup> m
		µm	10 <sup>-6</sup> m
KV	Kilovolts	nm	10 <sup>-9</sup> m
M	Molarity	TSC	Tri-sodium citrate
m-osmol.	milliosmolality (m-moles per 1000g of solvent)		
APH	Acetylphenylhydrazine		
DDSA	Dodecanyl Succinic Anhydride		
DER	Diglycidyl ether of polypropylene glycol		
DMAE	Dimethylaminoethanol		
DMP	Tridimethylamino Methyl Phenol		
EICDV <sub>s</sub>	Erythrocytic Icosahedral Cytoplasmic Deoxyriboviruses		
FHM	Fathead Minnow		
ICDV	Icosahedral Cytoplasmic Deoxyriboviruses		
MNA	Methyl Nadic Anhydride		
NEAA	Non-essential Amino acids		
NSA	Nonenyl Succinic Anhydride		
SVC	Spring Viraemia of Carp		
VHS	Viral Haemorrhagic Septicaemia		
WDS	Walleye Dermal Sarcoma		

## SUMMARY

1

Studies were performed on two types of infection of teleost fish where viruses have been observed by electron microscopy: erythrocytic infections in the Atlantic Cod (Gadus morhua) and the Common Blenny (Blennius pholis) and lymphocystis disease.

Searches were made for new isolations of these infections in British coastal waters and on shores chiefly in the vicinity of Plymouth and Aberystwyth. In the absence of disease symptoms, the blood of fish was screened for the presence of viral inclusion bodies by standard haematological methods.

PEN in cod was found in the North Sea and in the Celtic Sea off southern Eire, thus extending the previous distribution data from the Atlantic coastal waters of North America. The blenny infection was also found in new sites on shores in the vicinity of Plymouth. Moreover, the cytology of these infections was as had been previously described.

Collection data for the PEN infections showed an inverse relationship of infection incidence with age for cod sample populations but no correlation was found for blenny sample populations. In addition, no external disease symptoms were observed in either type of infection.

Concerning the recognition of the blenny infection, observations from maintaining blennies suggested the length of the natural infection might be inversely related to temperature; non-experimental longevities are quoted in this connection. The degree of infection in individual fish was estimated by light microscopy and the estimates for both erythrocytic infections cover the range 1-60% infection.

Attempts were made to propagate the viruses in vitro using fish cell and organ cultures. Primary cell cultures were originated from tissues of the Blenny, Flounder, Plaice and Dab using the protocol in the literature for marine fish cell culture. Vigorous cell outgrowth was observed in the flounder cultures and in these the time to confluence was only 3-5 days. However, established secondary cultures could not be derived from tissues of either species. Plaice and dab cultures were used for virus inoculation but the virus from the blenny infection and lymphocystis virus could not be propagated.

Organ cultures were set up using skin blocks from the Flounder. With tris-buffered maintenance medium such cultures maintained histological integrity for 15 days. However, one trial inoculation with lymphocystis virus showed no integration or multiplication of the virus in the tissue.

In connection with attempts to induce the blenny infection, the effect of high temperature in the Blenny was investigated. The infection was not induced over a 9 day holding period but lytic effects on the erythrocyte nuclei were observed. The effect of the drug acetylphenylhydrazine (APH) in the Blenny was also investigated with the aim of reproducing its reported action of anaemia induction and ensuing erythropoiesis. Marked anaemia was produced but not erythropoiesis. However, this result could not necessarily be interpreted as the effect of APH alone.

The viruses were identified and characterized with emphasis on their morphology, using ultrathin sectioning, negative staining and shadowing methods. It was concluded that the virus from the Blenny and lymphocystis virus conform to the structural measurements in the literature but negative staining indicated that both viruses display unique core structures. These are discussed in the light of the knowledge of other DNA virus cores. The position

of these viruses is further considered with respect to their classification in the virus family Iridoviridae.

## INTRODUCTION

(1) Fish Diseases

Observations of fish diseases manifested as tumours have been made since mediaeval times as Mawdesley-Thomas (1972) pointed out and the aetiology of fish tumours has recently received attention. To date, diseases in fish are known to be caused by a whole range of organisms and interest has grown in the diseases of economic fish on account of the heavy mortalities that can accompany disease (Amlacher, 1970; Roberts & Shepherd, 1974). This has been particularly true of the fish viral diseases and the modern study of fish virology has grown up over the last twenty years, starting with the first isolation of a fish virus in 1957.

Recent reviews of the subject in English by Wolf (1966 & 1972) have contributed to a wider dissemination of the literature than formerly by the reviews in German (Schaperclaus, 1969). Wolf (1976) more recently reviewed fish viral diseases in North America and research at one of the most active laboratories, the Eastern Fish Disease Laboratory, U.S.A. The progress that this subject has made can be judged from the remarks of Wolf (1976) that "the fish viruses have moved from a position of being scientific oddities to one of being recognised by virologists as part of a continuum of animal viruses".

The diseases of both marine and freshwater fish where viral aetiology has been firmly established are summarized (Table 1). It is seen that all the causative viruses, with the exception of the virus of IPN, are classifiable within the main groups of animal viruses; thus 3 rhabdoviruses, 1 iridovirus, 2 herpesviruses and one possible reovirus are listed. In addition there are many fish viruses reported by electron microscopy where viral isolations have not been performed.

**Table 1:** The diseases in teleost fish where viral aetiology has been established.

DISEASE	FISH	PRINCIPAL SYMPTOMS	VIRUS	REFERENCE
Infectious Haematopoietic Necrosis (IHN)	<u>Oncorhynchus nerka</u> (Sockeye Salmon)	Necrosis of kidney & pancreas	Rhabdo-	Amend, Yasutake & Mead (1969)
Spring Viraemia of Carp (SVC)	<u>Cyprinus carpio</u> (The Carp)	Peritonitis, enteritis, haemorrhage & oedema	Rhabdo-	Fijan <i>et al.</i> (1971)
Viral Haemorrhagic Septicaemia (VHS)	<u>Salmo gairdneri</u> (Rainbow trout)	Renal & visceral haemorrhage	Rhabdo-	Zwillenberg, Jensen & Zwillenberg (1965)
Infectious Pancreatic Necrosis (IPN)	Rainbow trout & many salmonids	Pancreatic necrosis	Ree-atypical	Moss & Gravel (1966)
Lymphocystis Disease	96 species of marine to fresh water fish	Skin nodules & tumours	Irido-	Wolf, Gravel & Malsberger (1966)
Channel Catfish Disease (CCD)	<u>Ictalurus punctatus</u> (Channel Catfish)	Oedema & necrosis of kidneys & other viscera	Herpes-	Fijan, Wellborn & Naftel (1970)
"Winthrop Rainbow Trout Disease"	Rainbow Trout	Darkening, exophthalmia, haemorrhages, anaemia & oedema	Herpes-	Wolf (1976)

Research investigations of fish viruses require much the same methodology as for other animal viruses. Tissue culture methods are used widely and cultured cells have largely replaced experimental fish hosts for virus propagation though the latter are required for pathogenicity trials and immunological studies. Several established fish tissue culture lines are available and these are propagated in the temperature range of 15-29°C, reflecting a ceiling temperature for cultured fish cells naturally lower than that for cultured mammalian cells. Although most isolated fish viruses have been propagated in cultured fish cells, it has been shown that fish viruses can exhibit a wide host range in tissue culture. Thus Clark and Soriano (1974) showed that the viruses of VHS, SVC & IHN were replicated in a variety of reptilian and mammalian cell lines e.g. WI-38 (human) and BHK 21 (hamster) and the temperature optima of these viruses in mammalian cells were the same as those in poikilothermic vertebrate cells. Likewise, viruses of homeothermic mammals exhibit a wide host range in tissue culture and it has been shown that one fish cell line can support growth of 10 out of 13 such viruses (Solis & Mora, 1970).

Recent expansion of knowledge on fish viruses has taken place in different geographical centres reflecting the widespread practice of fish farming and stocking in many different countries. SVC in Carp was first investigated by Fijan in Yugoslavia (Fijan et al., 1971), the principal viral diseases of salmonid fish have been researched since 1957 in the U.S.A. and the range of diseases in farmed trout has been investigated in Denmark (Christensen, 1972; Jørgensen, 1972).

At present the number of isolations of viruses from freshwater fish exceeds that from marine fish, reflecting the greater ease of sampling the freshwater environment and the greater resources

directed to the serious virus diseases of economic freshwater fish. However, as Sindermann (1969) has pointed out, there exist very large gaps in the knowledge of the natural diseases of marine organisms, including fish and their virus diseases, particularly concerning the role of disease in the overall ecology of marine communities.

The area of study in this thesis is confined to three virus infections in marine fish. The first is lymphocystis disease with proven viral aetiology which is associated with clear disease symptoms (Table 1). Lymphocystis is studied here in marine flat-fish hosts viz. the Plaice (Pleuronectes platessa), the Flounder (P. flesus) and the Dab (Limanda limanda). The other two are infections of blood erythrocytes, which elicit no external disease symptoms and concerning which the viral aetiology is not proven. The second is Piscine Erythrocytic Necrosis (PEN), reported in a variety of marine fish and studied herein in the Cod (Gadus morhua) and the third is a similar but distinct infection in the Common Blenny (Blennius pholis) termed simply 'the blenny infection'.

The general biology of the fish hosts will be described briefly and the different types of pathology seen in diseased fish outlined, prior to giving a detailed account of the diseases and infections under study.

## (2) Biology of marine fish hosts

### 2(a) The Common Blenny

The Common Blenny or Shanny is an intertidal shore fish of length up to 15 cm with green skin blotched with black (Plate 1b). The arrangement of internal organs for identification is shown in Fig. 1.

Its habitat is the rock pools and streams of rocky shores and it feeds on barnacles encrusting the rocks as well as polychaete

**Plate 1**

(b) Part of the cod catch on RV 'Explorer', 1st December 1975.

(a) The Common Blenny, a large male from a rocky shore collection.



a



b

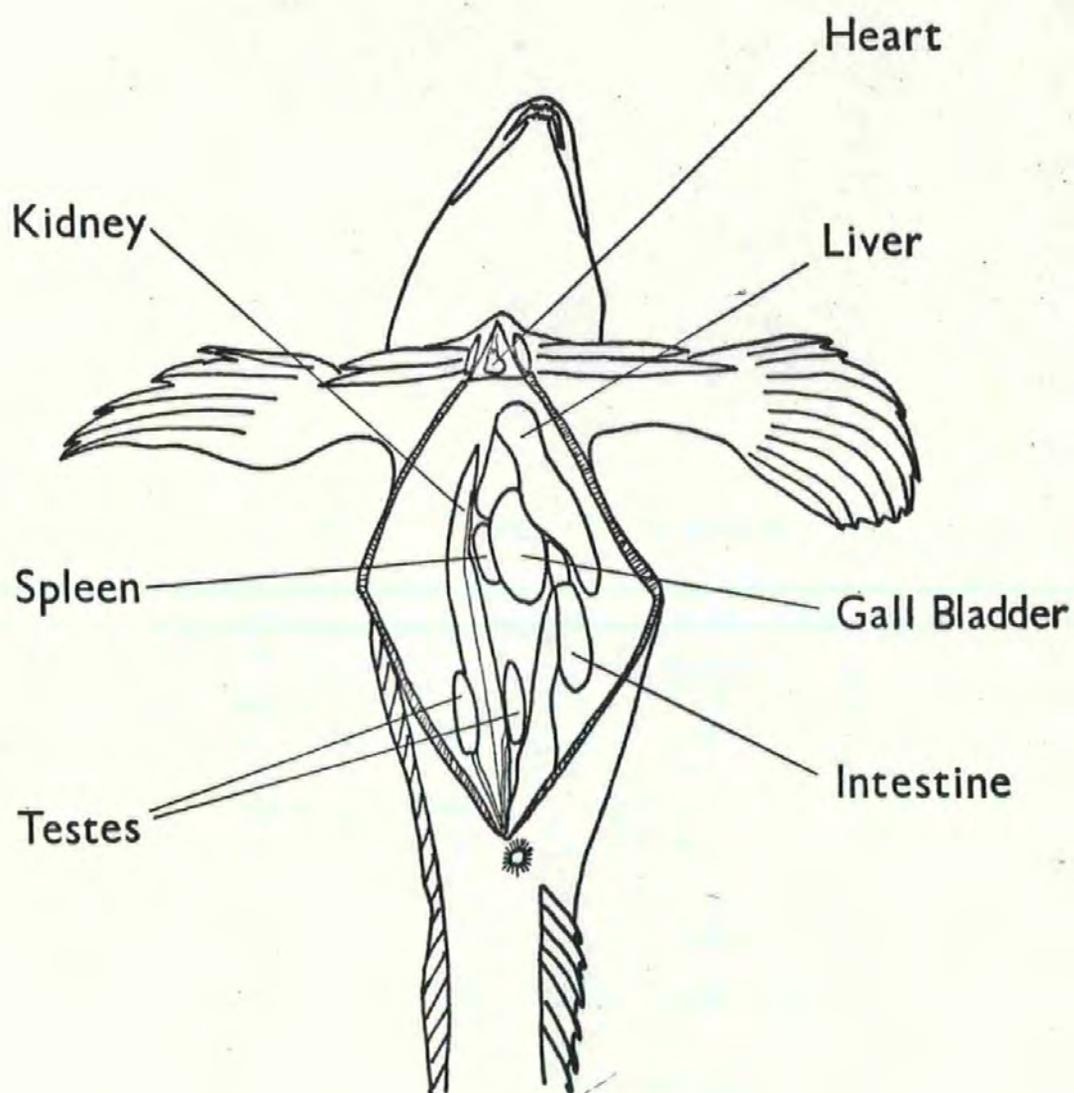


Fig. 1. Viscera of the Blenny, male, from the ventral side (3X natural size).

worms and algae in the pools (Qasim, 1957). Breeding is seasonal, mature females depositing a layer of eggs on the shore in the Spring. Fertilized eggs develop on the shore and the young larval stage hatches in the Summer to spend a feeding and growing stage in the sea. The post-larval stage then returns in mid-August to the shore where the adult life is spent.

## 2(b) The Flatfish

The Plaice, Flounder and Dab are flatfish differently adapted for life on the sea bottom. In particular the skin colour of each varies according to the nature and colour of the substratum on which it lies, but each species can be distinguished by shape, form and colour. The species are found up to 30 cm length, their habitats being sandy coastal sediments  whilst that of flounder includes also the sediments of estuaries. All species lay planktonic eggs which hatch into young larval stages on the sea bottom, the mature fish eventually spawning in deeper waters. The arrangement of internal organs in the Plaice is drawn in Fig. 2 for identification.

## 2(c) The Cod

The Cod (Gadus morhua L.) is a large fish of economic importance and is found up to 1 m in length and 10 kg weight (Plate 1b). The skin is green-yellow in colour and the Cod shows a prominent sensory barbel on the lower lip. It is carnivorous and undertakes extensive migrations associated with its search for food and for its breeding grounds (Harden Jones, 1968).

## (3) Pathology of Fish Diseases

As Wolf (1972) pointed out disease symptoms do not necessarily accompany virus infections in both freshwater and marine fish. Thus different types of infections must be classified according to the

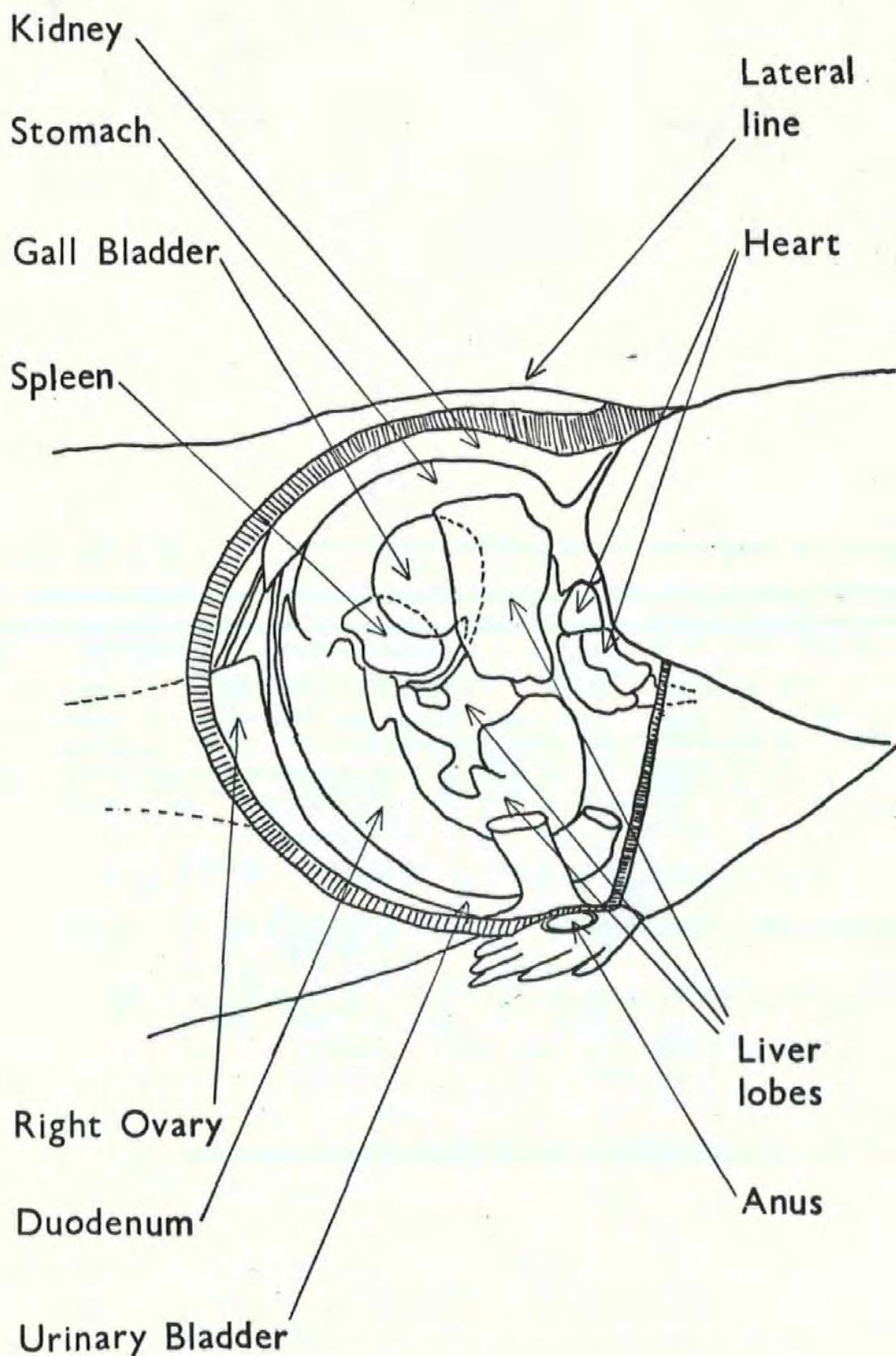


Fig. 2. Viscera of the Plaice, female, from the ocular side ( $\frac{2}{3}$  natural size). After Cole & Johnstone (1901).

degree and time course of associated disease and it is possible to draw up three states of infection from the documented examples.

Firstly, there is a state which produces overt symptoms of disease and may be lethal e.g. IHN in salmonid fish. Secondly, there is a state where the infection is latent there being no visible symptoms and this has been termed the 'carrier state'. IPN in brook trout is an overt disease but adult fish have been shown to be symptomless carriers as a result of exposure to previous infection (Wolf et al., 1968; Billi & Wolf, 1969). Thirdly, there is a state where no disease symptoms are known but which is associated with an orphan virus e.g. Epitheliocystis in the North American Bluegill (Lempomis macrochirus) (Hoffman et al., 1969). This is termed the asymptomatic condition. The essential difference between the latent and asymptomatic status is that in the former, disease symptoms may be evoked by the effect of stress or an environmental change on the host, whereas in the latter, symptoms are not known to be inducible.

Clearly this classification cannot be too absolute as the determination of the type of pathology depends on the amount of evidence available on the association of symptoms with the disease agent. There is also the difficulty in using this classification that the real symptoms of disease may not have been recognised. In other words a virus may have been isolated from fish by tissue culture and seen by electron microscopy but symptoms of disease may not have been correctly investigated. They could be purely physiological in nature and not external.

The examples quoted of latent and asymptomatic infections are those of freshwater fish but in marine fish there are few well studied examples of these distinct situations. However, investigation of the Grunt Fin Agent (GFA) in the marine blue-striped Grunt (Haemulon sciurus) suggested that GFA may be an orphan virus representing the asymptomatic condition (Clem, Sigel & Friis, 1965).

The agent was isolated from degenerative focus which developed at the sixty fifth passage in an established grunt cell culture and the supernatant fluid from this culture gave a cytopathic effect in both primary grunt and established goldfish fin cells. A plaque assay was developed using this isolate and the increase in infected cells with infection conformed to the normal virus growth curve suggesting a virus. Furthermore electron microscopic examination of the sedimented supernatant revealed ovoid particles of 100-150 nm diameter.

Diseases in fish may also give rise to the symptoms of tumour formation or abnormal growth. There is a good deal of information on tumours in marine fish and the extensive reviews of Mawdesley-Thomas (1972) and Harshbarger (1969 & 1972) describe a range of different types and locations. Both natural benign tumours and rapidly growing neoplasms have been observed in a variety of tissues. Equally, the causes of tumour formation are varied since there are several proven examples of viral aetiology as well as dietary aetiology (Ashley, 1970) and there is growing evidence that environmental carcinogens may be important.

The fish tumour viruses form a somewhat heterogeneous group, of which some examples are mentioned here and were noted in the review of Wolf (1972). Lymphocystis virus is the best known of this group (see part 4) which may also include, as possible members, viruses isolated from lymphosarcoma of the Northern Pike (Esox lucius) and papilloma of the European Eel (Anguilla anguilla). However, the establishment of viral aetiology is rarely clear cut. For some diseases the evidence is at the level of electron microscopic observations only while for others there is additional proof of virus transmission in vivo and in vitro according to Koch's postulates.

Studying lymphosarcoma in northern pike, Mulcahy & O'Leary (1970) showed that preparations of tumour tissue filtered at 220 nm transmitted

the infection to two recipients and four controls injected with filtered healthy tissue were uninfected up to 144 days after. Mortality took from 6 to 24 weeks for onset, the haematopoietic organs being the principal target. Sonstegard (1976) studied the aetiology and epizootiology of the disease in northern pike and a related species the Muskellunge (Esox masquinonay). He was able to derive neoplastic cells in vitro from tumour tissues of the Muskellunge and maintain these for up to 3 years. However, no transformation of normal cells was found on cocultivation with the neoplastic cells or on inoculation of extracts, infectious in vivo. Although cell-free transmission in vivo was recorded in both species of Esox, in agreement with Mulcahy & O'Leary (1970), Sonstegard concluded that the aetiology was not proven. Typical C-type virus particles were also reported by Sonstegard (1976) within lesions and Papas, Dahlberg & Sonstegard (1976) characterized these particles further. Homogenised tumour material sedimented on a sucrose gradient gave a band of reverse transcriptase activity at the same density as a characterised mammalian RNA tumour virus. Electron microscope examination of the particles in this fraction showed the two concentric membranes typical of a mammalian type-C tumour virus. Interestingly, the reverse transcriptase activity of the particles was maximum at 20°C, rather than the normal maximum of 35°C for C-type viruses, 20°C being the usual summer maximum temperature for the Northern Pike. However, despite the isolation of this virus, the aetiology of the infection remains equivocal.

'Cauliflower disease' or oral papillomatosis in the European Eel is also associated with a virus. Deys (1969) inferred that particles were seen in tumours by electron microscopy and Pfitzner & Schubert (1969) isolated a virus from diseased young eels in tissue culture. Wolf & Quimby (1973) later reported that a virus could be grown and plaqued in FHM cells from the Fathead Minnow

(Pimephales promelas) and Wolf (1972) commented that his own studies showed virus in some but not all of the affected specimens. Schwanz-Pfützner (1976) described virus isolation from the blood of diseased eels by passage in RTG-2 gonadal cells of the Rainbow Trout and also in FHM cells. However, the disease could not be induced in eels with cell culture-passaged virus, papilloma extracts or blood from affected eels. Similarly, Deys (1976) reported that although virus was found in the tumour, induction and transplantation could not be demonstrated. A different virus isolate from eels was listed by Fenner (1976) as a possible rhabdovirus.

There is much suggestive information that implicates artificial chemicals and pollutants as direct or indirect causes of fish tumours (Brown et al., 1973). The increase in the number of recorded fish tumours may reflect an increase in the number and carcinogenic efficacy of pollutants but certainly does reflect also a heightened interest and search.

In the North East Irish sea it was reported there was a simultaneous occurrence of three kinds of skin lesion in large plaice and dab over 250 mm length, viz., ulcers, lymphocystis and fin damage (Perkins, Gilchrist & Abbott, 1972). It was suggested that the lesions could all be linked to pollutants, especially polychlorinated biphenyl compounds, acting as irritants when the fish burrow in sandy sediments. Shelton and Wilson (1973a) however, refuted this suggestion on the grounds that a later survey revealed a lesser incidence of lesions and fin damage in the same area. They remarked that cyclical changes in infection incidence were common in the area and these were more likely associated with sheltered conditions and variable salinity rather than an increase in pollutants.

Chemical carcinogens are present in sea water and their distribution, levels and potential hazards have been reviewed by Kraybill

(1976). Moreover, as Stitch & Acton (1976) remark fish tumours have a possible use in monitoring carcinogens in the marine environment. However, further investigation will be needed to show how the known chemical carcinogens, the tumour viruses and the environment interact to cause tumours in fish.

(4) Details of the Diseases and Infections under study

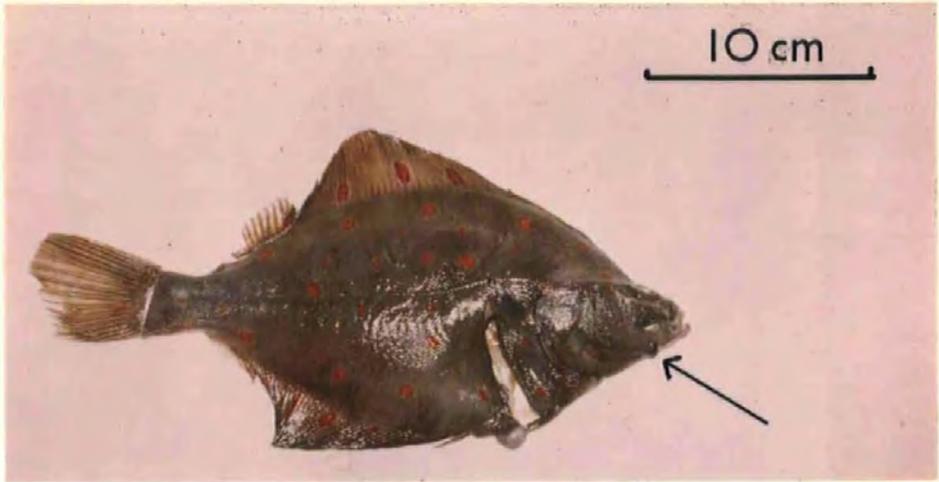
(a) Lymphocystis disease

(i) General/Historical

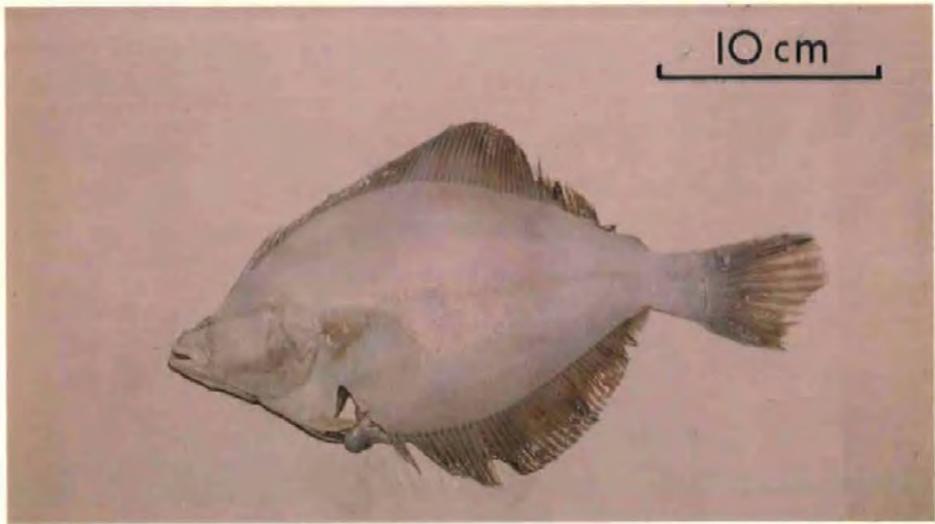
Lymphocystis disease was first described in flounder and plaice as multiple tumours by Lowe (1874). It is characterized by the development of small whitish nodules on the skin and fins (Plates 2a & 2b) and these may be found singly or as confluent clusters. Woodcock (1904) believed the nodules to be sporozoa and named the organism Lymphocystis johnstonei. This provoked some controversy among students of this disease as to the proof of the sporozoan identity and in the Paradise fish (Macropodus) it was even suggested that the nodules were fish ova (Zachieche, 1910). However, the studies of Weissenberg continued over 50 years, established the view that the nodules were greatly hypertrophied host connective tissue cells (Weissenberg, 1914, 1920). He produced histological and cytological evidence of the growth of the cells in experimentally produced tumours, examined periodically. The observation that the cells contained large nuclei, nucleoli and Feulgen-positive cytoplasmic inclusions was therefore explained wholly as a consequence of cellular hypertrophy. The same interpretation was then given to the disease in a wide range of freshwater and seawater fish from Europe and North America e.g. in the Flounder (Caussen, 1917) and in the Orange Filefish (Ceratacanthus schoepfi (Walbaum)) (Nigrelli & Smith, 1939). The latter authors observed that lesions were not confined to the skin but were found also in the spleen, ovary and

## Plate 2

- (a) A plaice lightly infected with lymphocystis showing a tumour on the anal fin. It also shows a growth on the jaw (arrow) with darkly pigmented overlying skin. Ocular side.
- (b) As above, abocular side.



a



b

gastro-intestinal tract. The disease is also recorded as occurring in the New York Aquarium, where outbreaks were commonest at the height of the Summer (Nigrelli, 1940). Nigrelli & Reggieri (1965) produced an excellent review of the spontaneous and experimentally induced cases of the disease.

Successful transmission experiments were reported by a number of authors but Weissenberg was the first to suggest the disease was caused by a virus and obtained experimental proof that the infectious agent passed bacterial filters (Weissenberg, 1951). Experimental evidence of the viral nature of the disease was also obtained by Wolf (Wolf, 1962; Wolf & Carlson, 1965).

Walker (1962) first reported the fine structure of the virus from the Walleye (Stizostedion vitreum vitreum) and Walker & Weissenberg (1965) described the composition of the lymphocystis cell in different fishes studied by light and electron microscopy. Wolf, Gravell & Malsberger (1966) reported the first isolation and propagation of lymphocystis virus in vitro; virus from the Bluegill was propagated in monolayer cultures from both the Bluegill and the related Largemouth Bass (Micropterus salmoides). Bluegills inoculated with cell-passaged virus then developed the disease and the results fulfilled Koch's postulates.

Lymphocystis disease is widespread amongst teleost fish and Lawler, Ogle & Donnes (1977) recently listed 96 species as hosts of the spontaneous disease. Other aspects of lymphocystis are discussed here under ecology, cytopathology and propagation and morphology.

#### (ii) Ecology

Lymphocystis disease has been found in fishes from a wide range of geographical areas and from freshwater, brackish and marine environments. It is reported in freshwater fish in several continents

and is found in the Walleye from North America and Russia. It was also reported in the East African lakes on the tails of cichlid fish (Paperna, 1973). It is reported in sea water fish from the Pacific and Atlantic coasts of North America and around the shores of Great Britain in specific localities. Templeman (1965) reported an outbreak in the American plaice (Hippoglossoides platessoides) on the Grand Bank off eastern North America and Shelton & Wilson (1973b) reported the disease incidence in pleuronectids in the Northern Irish Sea and in Rye Bay.

Natural infection is thought to start with an abrasion or cut of the skin surface. This could occur through penetration by an ectoparasite, through occasional damage by external objects or by abrasion in combat fighting and mating. Nigrelli believed the first alternative that parasitic copepods, protozoa or leeches could transmit the disease. McCosker (1969) reported transmission associated with combat display in the Californian Blenny (Hypsoblennius jenkinsi); lesions were noticed to correspond with the wounds inflicted during territorial combat at spawning.

The affect of the disease on the fish is a general debilitating one causing a loss in weight and the camouflage is spoiled in the worst cases making the fish more susceptible to predation. However, Nigrelli & Smith (1939) remarked that fish having had external signs of the disease and recovered, survived well in the New York Aquarium suggesting that the disease is rarely fatal in the absence of natural predation.

The host response may exert an influence on the course of lymphocystis infection and in this connection there is much evidence that fish can mount an effective immune response to invading microorganisms but this is temperature-dependent, as reviewed by Avtalion et al. (1973). Russell (1974) found that the sera of naturally infected plaice and flounder showed precipitating antibodies

to homologous lymphocystis antigen. Since 80% of the sera gave a precipitation reaction and since this test is fairly insensitive, it was inferred that the concentration of antibodies was probably fairly high.

(iii) Cytopathology & Cytochemistry

Virus infected cells enlarge enormously to a diameter of over 1 mm, resulting in one of the largest known animal cells. The nucleus and nucleolus enlarge in proportion to the cytoplasm (Plate 3b); mature cells are surrounded by a hyaline capsule (Plate 3a) and prominent inclusions are present in the cytoplasm. Weissenberg concluded the inclusions arose de novo from the cytoplasm and not via the nucleus and thus associated the inclusions with the virus effecting the cytological changes.

Preliminary electron microscopic studies of McCoy, Edwards & Walker (1970) indicated that particles enter the cell by phagocytosis and are uncoated within cytoplasmic vacuoles. Uncoating was found to be associated with the fusion of small vesicles with the vacuoles and a marked increase in the electron-density of the vacuoles. It was therefore suggested that lysosomal enzymes, contained within the Golgi-types vesicles, are responsible for uncoating.

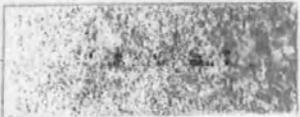
Recently it has been reported in the Walleye, that tumours of both lymphocystis and dermal sarcoma (WDS), with distinctive cytologies, can be found on the same fish and can be confluent (Yamamoto et al., 1976). By electron microscopy, it was observed that the cell types of the two kinds of tumour were associated with distinct virus particles and the distribution of each virus was limited to the characteristic cell type. In the case of WDS this was a possible RNA virus of 135 nm diameter.

The cellular changes of lymphocystis are accompanied by changes in cytochemistry.

## Plate 3

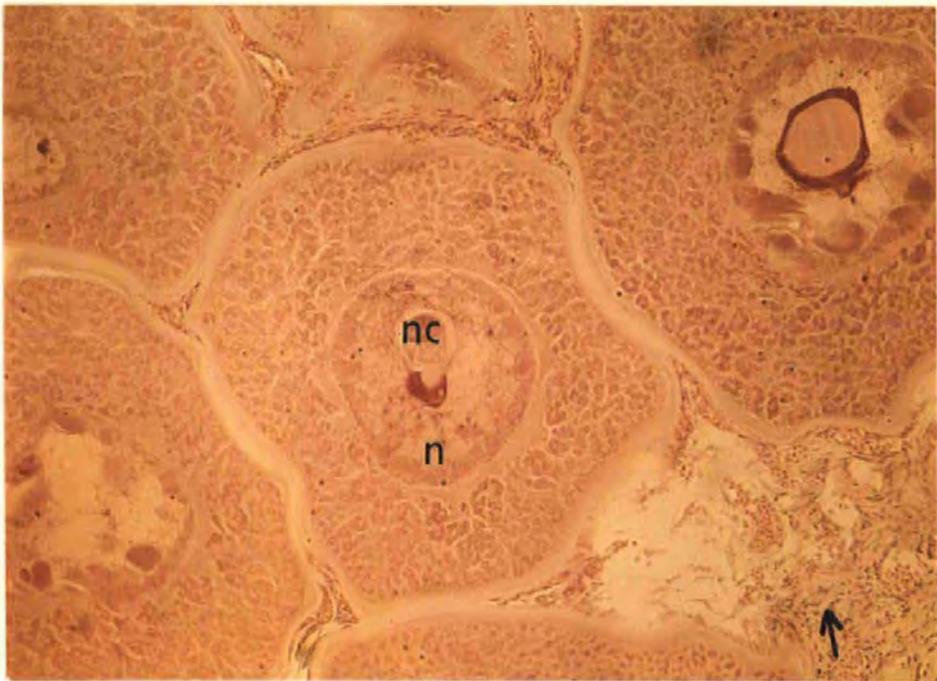
Lymphocystis tumour from flounder showing individual cells and their composition. Slides by courtesy of Mr. G. LARBALÉSTIER.

- (a) Mallory's stain, X 660. A hyaline capsule, involuted at the centre and stained light blue, surrounds the cytoplasm of each cell.
- (b) Haematoxylin and eosin stain, X 660. n - nucleus, nc - nucleolus. The degree of abnormal cell enlargement is emphasized by comparison of the nuclei of interstitial dermal fibroblasts with those of lymphocystis cells. Dermal fibroblasts are arrowed.





a



b

The inclusion bodies have been shown by cytochemical staining and autoradiography to be DNA-containing and thus are taken to be sites of viral replication (Walker, 1965; Sigel, Beasley & Launer, 1966; Pritchard & Malsberger, 1968; Midlige pers. comm.,). Walker (1965) confirmed this in lymphocystis cells of the Walleye using acridine orange (AO) and Feulgen stains and Sigel, Beasley & Launer (1966) did so using AO in infected tissue culture cells. Pritchard & Malsberger (1968) showed the capsule was positive for the Periodic-acid-Schiff (PAS) reaction indicating its content of acid mucopolysaccharides and this fact was confirmed by Howse & Christmas (1970).

Changes in cell synthesis also take place but to date there is no complete account of the biochemical composition of lymphocystis cells and no reported work on factors that inhibit cell division or prevent cell lysis. Pritchard & Malsberger (1968) showed the total composition of the cell was proteins, histones, DNA, RNA and polysaccharides. Lopez et al., (1969) reported that there was no accumulation of protein, DNA or RNA in infected cells in vitro, although increased rates of DNA and RNA synthesis were detected. Wolf (1972) commented on this work that "it seems likely ..... that accumulation must occur and that the data would reflect that change if they were reported in terms of product synthesized per cell". These studies also did not discriminate between viral and cellular nucleic acids. However the later abstract of Lopez et al. (1970) reported virus infection correlated with an increase in total DNA synthesis. In this case the base analogue 6 mercapto-purine was used for inhibiting virus growth.

#### (iv) Propagation

Lymphocystis virus from the Bluegill has been passaged in vivo and the cytological course of the disease described (Wolf, 1962; Wolf

& Carlson, 1965; Dunbar & Wolf, 1966). In agreement with the results of in vitro propagation, there was a fall in the recoverable virus titre till 6 days post infection (p.i.) then a sharp rise in titre of 2 to 3 logarithms up to 15 days p.i. with a plateau till 28.

There are many published records noted by Nigrelli & Ruggieri (1965) of the experimental transmission of lymphocystis in freshwater fish and Roberts (1976) has also reported transmission in the Plaice. For plaice held at 10°C, the skin lesion first appeared after a month, it developed slowly over 3/4 months and there were demonstrable levels of serum antibody and cell-mediated immunity prior to its termination. Roberts (unpublished) has also performed transmission of flounder lymphocystis and cross-infection between plaice and flounder. Plaice/flounder hybrids as well were found susceptible to virus from both fish but the Turbot (Scophthalmus maximus) was not. The early experiments of Weissenberg and the observation of Nigrelli & Smith (1939) also suggested that cross-transmission was only possible between closely related genera.

Lymphocystis virus from the freshwater Bluegill has been propagated in vitro in monolayer cultures from the Bluegill and the related Largemouth Bass (Micropterus salmoides) (Wolf et al., 1966) and this enabled studies to proceed on the structure and maturation of this virus isolate (Midlge & Malsberger, 1968; Zwillenberg & Wolf, 1968). To date the virus isolates studied here, those of the marine pleuronectids of British waters, have not been propagated in vitro. However, the virus from the marine Grey Snapper (Lutjanus griseus) has been propagated in established GFS cells derived from fin tissue of the marine blue-striped Grunt (Lopez et al., 1969). Indeed, this is the only lymphocystis virus isolate from a marine fish to have been isolated in vitro and the GE-1 cell line the only established line derived from a marine fish that is available from a commercial tissue culture supplier. By contrast there are many more

established lines derived from freshwater fish e.g. FHM & RTC cells (section 3).

The methods used by different authors for the establishment of monolayer cell cultures from marine and freshwater fish have in common similar requirements for the dispersion and growth conditions of the cells and tissues (Table 2). However, tissues from the marine grunts required a high sodium chloride concentration of approximately 0.2 M for growth (Clem et al., 1961; Clem et al., 1965). The recommendations of Wolf & Quimby (1969) for marine fish monolayer cell cultures also employed the medium formulation of Clem et al. (1961) and gave additional details of the required aseptic techniques.

Table 2: Conditions for the establishment of monolayer cell cultures from marine and freshwater fish and the growth time to confluence of cultures.

	MARINE		FRESHWATER (As adults)	
FISH	Yellow-striped Grunt ( <u>H. flavolineatum</u> )	Blue-striped Grunt ( <u>H. sciurus</u> )	Sockeye Salmon ( <u>Oncorhynchus nerka</u> )	Atlantic Salmon ( <u>Salmo salar</u> )
REF.	Clem <u>et al.</u> (1961)	Clem, Sigel & Friis (1965)	Fryer, Yusher & Pilcher (1965)	Nicholson & Byrne (1973)
TISSUE(S) TAKEN	Ovary, Heart & Gillcover	Fin	Embryo	Heart, Liver, Kidney & spleen
DISPERSION	Trypsin	Trypsin	Trypsin	Trypsin
CULTURE MEDIUM	Eagles MEM 0.219M NaCl	Eagles BME 0.196M NaCl	Eagles MEM, BME 0.116M NaCl	Eagles MEM 0.14M NaCl
CULTURE TEMP. (°C)	20-22.5	20	18	20
GROWTH TIME (DAYS)	10-28 (1°)	7-10 (2°)	7-28 (1°)	14-28 (1°)

MEM - Minimal Essential Medium

BME - Basal Medium

(1°) - Primary culture (2°) - Secondary culture

Successful explant cultures have also been derived from marine fish. Clem et al. (1961) found cells from grunt tissues grew well in a medium designed basically for mammalian cells. Further, Townsley, Wright & Scott (1963) obtained vigorous cell growth from explants in a basic salt-unadjusted mammalian-type medium. Several marine fish were tried including the Atlantic Cod and tissues from many different organs gave good cell growth.

(v) Morphology

Lymphocystis virus is broadly spherical in outline and is thought to conform to the icosahedral symmetry of spherical viruses described by Caspar & Klug (1962). However, there exists no report of the double shadowing proof of this symmetry for the virus (Williams & Wycoff, 1946).

The virus is amongst the largest known spherical viruses, different authors quoting the particle diameter by section as 130-250 nm (Table 3). As Kelly & Robertson (1973) emphasized, it is not known whether this variation in sizes represents different strains of the virus in the respective hosts. Another possible reason for reported variation is that authors do not state whether or not the diameter was measured between the apices. Since the sectioned outline is polyhedral there can be variation in measurement. By negative staining the particle is often reported as larger than by thin section e.g. up to 300 nm diameter (Zwillenberg & Wolf, 1968) (Table 4).

Thin sections show the virus is composed of an inner core and an outer bilaminar shell or capsid with an electron-lucent gap between. Negative staining confirms this arrangement and also reveals some detail of the structural units of the capsid. Zwillenberg & Wolf (1968) commented on "an ordered array" of morphological units in collapsed virions but this was difficult to discern from their electron micrographs. However, Midlige & Malsberger (1968) reported

**Table 3:** Iridoviridae; summarized information for viruses from different hosts, after Fenner (1976).

<u>Virus genera &amp; species</u>	<u>Host</u>	<u>Nucleic acid</u>	size (nm)	<u>Morphology</u>		symm.	<u>Reference</u>
				p.a.	c.m.		
<u>Iridovirus</u> (type species <u>Tipula iridescent virus</u> )	Various insects	ds-DNA MW-130 <sup>6</sup> 160x10 <sup>6</sup> D	130- 180	+	-	s/s	Bellett, (1968)
Iridescent virus	<u>Octopus vulgaris</u> (Octopus)	?	100- 140	(+)	-	s/s	Rungger et al. (1971)
Iridescent virus	<u>Nereis diversicolor</u> (Ragworm)	?	?	?	?	(s/s)	Devauchelle & Durchon (1974)
Iridescent virus	<u>Entamoeba histolytica</u> (Amoeba)	(ds)-DNA	80	-	-	s/s	Mattern, Hruska & Diamond (1974)
<u>Other probable genera</u>							
African swine fever virus	<u>Sus scrofa</u> (Swine) also wild pigs & hogs	ds-DNA	175- 215		+	s/s	Hess (1971) Coggins (1974)
Amphibian icosahedral viruses (type Frog Virus 3)	<u>Rana pipiens</u> (Frog)	ds-DNA MW- 130x10 <sup>6</sup> D	130	+	+	s/s	Granoff (1969)
Icosahedral Frog Virus	<u>R. pipiens</u>	?	280- 380	-	(-)	s/s	Bernard et al. (1968)
Gecko virus	<u>Gehyra variegata</u> (Gecko)	?	220	-	-	s/s	Stebbens & Johnston (1966)
Lymphocystis viruses	Many spp. of fish	DNA	130- 250	(-)	-	s/s	Kelly & Robertson (1973)

Abbreviations: ds - double stranded  
p.a. - paracrystalline array  
c.m. - cellular membrane acquired  
symm. - symmetry of virus (outer/inner)  
MW - molecular weight

Table 4: Measurements of lymphocystis virus reported from thin sectioning and negative staining.

<u>Host</u>	<u>Particle diam. (nm)</u>		<u>Capsid width</u>	<u>Gap</u>	<u>Core</u>	<u>Ref.</u>
	<u>Thin section</u>	<u>Negative stain</u>	<u>(nm)</u> <u>Thin section</u>		<u>diam. (nm)</u> <u>Thin section</u>	
<u>Lepomis macrochirus</u>	250	300	12-16	-	-	Zwillenberg & Wolf (1968)
(unstated)	192-215	200	-	-	-	Midlige & Malsberger (1968)
<u>Pleuronectes flesus</u>	130-150	-	-	-	-	Walker & Weissenberg (1965)
<u>Micropogon undulatus</u>	240-260	-	12-14	14	135-144	Howse & Christmas (1971)
<u>Stigostedion vitreum vitreum</u>	180-220	-	12	12	150	Walker (1962)
"	260	-	-	-	-	Yamamoto et al. (1976)
"	200-250 (oval particles 65-100)	-	-	-	-	Dolowy et al. (1976)

they could not resolve the capsomere structure by negative staining. Both papers reported observations of external filaments or spikes. Zwillenberg & Wolf observed long filaments apparently attached to the vertices of the virion and Midlige & Malsberger commented on a fringe of spikes forming an outer capsid layer.

It has been reported in typical lymphocystis cells from the Walleye that lymphocystis particles contain and are intermingled with "sub-viral" particles (Dolowy et al., 1976). Oval particles 65-100 nm diam. were seen and also particles of size and shape intermediate between these and the typical virus. It was hypothesized that the small and intermediate particles could have a role in the life cycle of the virus.

The broad properties of lymphocystis virus indicate its similarity to other icosahedral viruses with cytoplasmic replication. It is appropriate therefore to review these similar viruses drawing

comparisons with lymphocystis virus.

(vi) The family of viruses: Iridoviridae

Many viruses have been described in a range of hosts including both vertebrates and invertebrates, which show the common features of icosahedral symmetry, replication in the cytoplasm and virion DNA. The amphibian viruses with these properties were first termed Icosahedral Cytoplasmic Deoxyriboviruses (ICDVs) by Granoff (1969) and the abbreviation was used to encompass the range of similar viruses.

The ICDVs were then collectively reviewed by Kelly & Robertson (1973). Later the group was renamed the family Iridoviridae by the International Committee for Taxonomy of Viruses (ICTV), the stem irido- being taken from the word iridescent (Fenner, 1976). The ICTV classification included in this family as species: 19 types of iridescent viruses of insects, reviewed by Bellett (1968), Chironomus iridescent virus (Stoltz, 1971) and iridescent viruses of the Octopus (Octopus vulgaris), a polychaete annelid (Nereis diversicolor) and an amoeba (Entamoeba histolytica). Listed as probable members of the family were the following: African swine fever virus (ASFV) from swine, reviewed by Hess (1971), ICDVs of amphibians including frog virus 3 (FV3), 'gecko virus' from the reptilian lizard-like gecko and lymphocystis virus from fish (Table 3).

Complete biochemical characterization is available for only the insect Iridovirus species and FV3 but these viruses show some common characteristics (Fenner, 1976). The virion is large containing about 1500 capsomeres, a single structural unit membrane is associated with the core and the virion contains many proteins and several enzymes. The genome is a single molecule of double stranded DNA of molecular weight  $130-160 \times 10^6$  D and multiplication of the virus occurs only in the cytoplasm.

Variation between Iridovirus species is found in the particle diameter, in the degree of virus assembly during multiplication and in the presence of an additional cellular membrane.

The range of particle diameter is from 80 nm for the amoeboid virus V301 to 330 nm for the frog virus of Bernard, Cooper & Mandell (1968), the majority of insect iridescent viruses being intermediate in size at 130 nm. Enormous numbers of particles are found in insect Iridovirus-infected cells and this fact combined with suitable surface structure for regular close packing, leads to a paracrystalline array. This is not observed on the whole in the replication of the large Iridovirus species from vertebrates, although Walker & Wolf (1962) remarked on crystalline lattices in lymphocystis cells from the Bluegill.

ASFV and the amphibian icosahedral viruses show an external membrane or envelope but lymphocystis virus does not. This is known to be acquired by budding of the cell membrane around single particles and this process therefore effects virus release (Breese & de Boer, 1966). The presence of the envelope accounts for the lipid in the virions of ASFV and for its ether sensitivity. Although the insect Iridovirus species show no envelope, some of them show a small lipid content but this is thought to be contained internally rather than externally (Kelly, 1973).

Although the ICDVs can be conveniently classified together as a group, there is evidence from serology and nucleic acid homology studies that this is a large assemblage of viruses, many of which outside the insect iridescent sub-group are unrelated. Bellet & Fenner (1968) thus demonstrated by DNA-DNA hybridization methods that FV3 was unrelated to a group of insect iridescent viruses and each of these were also dissimilar to different poxviruses. Kelly & Avery (1974) similarly showed FV3 was unrelated to iridescent viruses 2, 6 & 9 by DNA-DNA hybridization techniques and by analysis of

virion proteins. They therefore suggested "rejection of the proposal that FV3 and these iridescent viruses should be classified together". Serological comparisons have pointed to other dissimilarities: FV3 was unrelated to bluegill lymphocystis virus and to ASFV (Came & Dardiri, 1969), LTI virus of R. pipiens was unrelated to bluegill lymphocystis virus (Clark et al., 1969) and various amphibian ICDVs were all dissimilar to Tipula iridescent virus (TIV) (Kaminski et al., 1969).

The genus Iridovirus shows icosahedral symmetry externally and the core is generally spherical. Electron microscopic studies of two species, Tipula iridescent virus (TIV) and Sericesthis iridescent virus (SIV) have revealed aspects of the virus construction (Wrigley, 1969 and 1970). By using different methods of virus breakdown with negative staining it was found the outer icosahedral surface of the particles showed morphological sub-units in close-packed hexagonal array. It was also found that complete breakdown resulted in the appearance of triangular, pentagonal and linear fragments. These structures led Wrigley to propose a model structure for SIV in which these fragments make up the facets, apices and edges of an icosahedron of unknown skew. It is an interesting comparison that although ASFV, lymphocystis virus and the larger icosahedral viruses of vertebrates have been studied by negative staining, the breakdown fragments noted by Wrigley have not been seen.

#### 4(b) Erythrocytic Infections of Fish & Other Vertebrates

##### (i) General/Historical

The infections under study, PEN in the Cod and the blenny infection are of interest to the virologist on account of their probable viral aetiology but they were first discovered by parasitologists during searches for blood protozoa.

Ridgway (1956) recorded granular material in the cytoplasm of

fixed red blood cells from several salmonid species, namely Chinook salmon (Oncorhynchus tshawytscha), Sockeye salmon (Oncorhynchus nerka) and Rainbow trout. Gardner & Yevitch (1969) later reported non-specific cytoplasmic inclusions in the red cells of three species of killifish (Fundulus sp.); these were seen only in wet films and not in fixed smears. Laird & Bullock (1969), in a paper reporting a variety of marine fish protozoa from blood, noted an unusual necrosis of the nuclei in red blood cells. This involved a marked fragmentation, granulation and lysis of the nucleus, and was associated with a prominent cytoplasmic inclusion in infected cells. It was most common in Atlantic cod from the coast of New Brunswick, Canada, but was also found to a lesser extent in the Sea Snail (Liparis atlanticus) and the Longhorn Sculpin (Myoxocephalus octodecimspinosus). These authors coined the term 'Piscine Erythrocytic Necrosis' (PEN) for the massive destruction of the red cells they noticed.

Walker (1971) reported electron microscopic evidence of the viral nature of cod PEN and this was confirmed by Appy, Burt & Morris (1976). Walker & Sherburne (1977) reported on ultrastructure further and gave data on the incidence and distribution of cod PEN.

PEN infections like that in cod have been reported from other seawater and anadromous fish. Sherburne (1973) described by light microscopy PEN in the Atlantic herring, (Clupea harengus harengus) and electron microscopic studies have shown that this infection is viral (Phillipon, Nicholson & Sherburne, 1977; Reno et al., 1978). Sherburne (1977) described the first record of PEN in the blood of an anadromous pelagic fish, the Alewife (Alosa pseudoharengus).

Other intra-erythrocytic infections of fish, amphibia and reptiles, some previously described as protozoa, have been studied by electron microscopy and cytoplasmic viruses observed. Thus particles have been seen in Immanoplasma lesions from erythrocytes of the Dogfish (Scyliorhinus canicula) (Johnston, 1975), in Toddia lesions of a frog

(Sousa & Weigl, 1976) and in erythrocytes from the Leopard Frog (Rana pipiens) (Bernard et al., 1968). In the last example, the frogs had been irradiated 3 days before the examination of blood but the authors mention the results of Schmittner and Ball (unpublished) who found virus in the erythrocytes of unirradiated frogs.

An infection similar to PEN, here termed the blenny infection, was reported by Johnston & Davies (1973) in the erythrocytes of the Blenny at Aberystwyth. By light microscopy, a prominent cytoplasmic inclusion was noted in wet films and fixed smears but by contrast to PEN no nuclear involvement or lysis was observed. The infection was described as 'Pirhemocytion-like' on account of its resemblance to the parasite Pirhemocytion tarentolae. P. tarentolae was named after the host in which it was first found and described as a protozoan viz. Tarentola mauritanica, a gecko from North Africa (Chatton & Blanc, 1914). Stehbens & Johnston (1966) reported ultra-structural evidence that Pirhemocytion in the Australian gecko (Gehyra variegata) was a viral and not a protozoan infection and this was the first proof of viral identity in the PEN-type infections of vertebrate erythrocytes. Cytological and ultra-structural evidence also strongly suggested the blenny infection was caused by a virus (Johnston & Davies, 1973).

Besides these 4 hosts, PEN has been recorded in 10 other genera of fish from the Atlantic coast of North America and 5 species have been listed as possible hosts (Walker & Sherburne, 1977). In total 16 PEN positive species have been described from the North American coast and the Blenny is the only host to be described from the British Isles (Johnston & Davies, 1973).

#### (ii) Ecology

Infection incidences given by Walker & Sherburne (1977) vary from 3% for the Tautog (Tautoga onitis) to 93% for the Sea Raven (Hemitripterus americanus). The largest samples, about 100, no doubt give the most accurate mean incidences but the records for cod PEN clearly show some geographical variation (Table 5). In the survey of

**Table 5:** Infection incidences reported in wild caught Atlantic cod, common blenny, alewife and herring.

<u>Host</u>	<u>Site</u>	<u>Population incidence (%)</u>	<u>Sample size</u>	<u>Cells infected (%)</u>	<u>Ref.</u>
Cod	St. Andrews to Kent Island N.B., Canada	20.0	5	-	Laird & Bullock (1969)
Cod	St. Andrews N.B., Canada	11.1	18	10	Walker (1971)
Cod	Boothbay harbour, Maine, U.S.A.	13.8	116	99 (max.)	Walker & Sherburne (1977)
Cod	Nantucket Shoals to George's Bank	16.0	256	-	"
Cod	Passamaquody Bay, N.B., Canada	6.5	523	38-90 (mature) 0.5-18.5 (immature)	Appy & Burt (1975)
Blenny	Aberystwyth Wales, U.K.	24.1	58	60-80	Johnston & Davies (1973)
Alewife	Damariscotta Mills, Maine, U.S.A.	56.1 (pre-spawning) 10.5 (post-spawning)	991	<0.17	Sherburne (1977)
Herring	Maine & New Brunswick Coast	4.0	201	6-90 (mature) 1-90 (immature)	Sherburne (1973)

cod PEN by Walker & Sherburne (1977) approximately half of the stations were negative and half positive in the largest sample. No correlation of the infection was recorded with the sex or total length of the fish but on George's Bank there was a higher incidence at depths of 55 m. With respect to the age of fish, Sherburne (1977) found that PEN in the Alewife was predominant in prespawning adult fish, less common in postspawning adults and absent in juveniles. The difference in incidence was suggested might be attributable to an activation of a latent infection during the spawning run.

In other aspects of the general ecology e.g. the natural transmission, the affect on the host and the infectivity of the virus there is little or no available evidence for the blood infections.

### (iii) Cytopathology

Infected red cells are distinguished by the presence of a basophilic cytoplasmic inclusion body around which may be present a cloud of granules, the actual virus particles. In cod erythrocytes this measures 0.3-1.5  $\mu\text{m}$  diameter, it is round, compact and Feulgen-positive confirming DNA content (Walker & Sherburne, 1977). In the Blenny the inclusion measures 1-4  $\mu\text{m}$  diam., often it is surrounded by a lightly stained 'halo' and sometimes there is more than one per cell (Johnston & Davies, 1973). The inclusion is shown by electron microscopy to be viroplasm i.e. the matrix of viral precursors accounting for the DNA content. In herring, the inclusion was reported<sup>x</sup> as 1.3-3.3  $\mu\text{m}$  diam. according to the type and stage of infection (Sherburne, 1973). Two types of inclusion have also been reported in infected herring erythrocytes by Reno et al. (1978). The type I inclusion was circular, electron dense, measured 1.5  $\mu\text{m}$ , was clearly associated with virus particles and was not membrane bound; this therefore corresponded to the viroplasm of other PEN infections. The type II inclusion was distinct to type I. It was not directly

virus-associated, measured (0.5-3  $\mu\text{m}$  diam., was electron-lucent and was surrounded by multiple membranes.

PEN infections also involve varying degrees of margination of the nuclear chromatin and its fragmentation. In cod erythrocytes pycnosis and vacuolation of the nucleus is marked in early stages of infection though the nuclear membrane is intact (Walker & Sherburne, 1977). Fragmentation of the nucleus then results in a staining pattern of dense chromatin blobs. All stages of infection were recorded; early with nuclear lesions but no apparent inclusion and late with advanced lesions and viroplasm. In the Blenny by contrast, these kinds of nuclear changes have not been described.

PEN in the Alewife shows similarity to PEN in cod (Sherburne, 1977). It was noted that the characteristic nuclear distortion was present without a visible cytoplasmic inclusion but that when present inclusions were more often found in the cells at the initial stage of infection.

PEN in Atlantic herring involves similar but distinctive changes to cod PEN (Reno et al., 1978). Firstly, the red cell surface was irregularly lobulated; secondly, margination of the chromatin was typical; thirdly, occasional intranuclear inclusions were seen by light microscopy and corresponding intranuclear granular masses by electron microscopy; fourthly, infected cells showed "conspicuous electron-dense granules" which were thought to be ribosomes. Rare lamellar bodies with dark and light bands at full repeat of 100 nm were also noted near and within the inclusion and Walker & Sherburne (1977) noted the same bodies in cod PEN. The detection of intranuclear inclusions Reno et al. suggested might indicate some nuclear involvement in replication of the herring PEN virus.

The degree of infection of erythrocytes in smears has been quantified by a number of authors (Table 5). Walker (1971) noted a 10% level of infection for cod PEN and Walker & Sherburne (1977)

reported a range of 1-99%, mature red cells being predominantly infected. For the George's Bank sample the highest degree of infection was found amongst the heaviest infected sample. Similarly, a wide range of level of infection was noted by Appy & Burt (1975) for cod PEN and a wide range of 7-96% has also been noted for herring PEN (Sherburne 1973; Phillipon *et al*, 1977). In contrast, PEN in the Alewife was recorded at very low levels of less than 1%.

(iv) Propagation

No PEN infection has been propagated in vivo and passage of the blenny infection has not been reported in detail although Davies (1973) described one experiment demonstrating passage on inoculation of infected blood. 1 survivor of 2 fish injected developed the infection and 2 control fish remained uninfected. The infection first appeared in the peripheral blood at 8 days p.i., it showed a maximum level of infection at 30 days p.i. with 50% of red cells infected and thereafter the level declined, the infection disappearing by 67 days p.i.

Neither PEN infections nor the blenny infection have been propagated in vitro.

The similar intra-erythrocytic infections of reptiles and amphibians viz. Pirhemocytos and Toddia, have been transmitted experimentally by inoculation of infected blood and the experimental reports have been catalogued by Johnston (1975). For Pirhemocytos in a lacertilian host, transmission has been achieved not only by inoculating infected blood directly but also by inoculating homogenates of a mosquito which had previously fed on infected animals. For both infections the prepatent or latent period has been recorded as 5-6 days at the least and in the case of Toddia, inter- and intraspecific transmission has been performed by intraperitoneal inoculation of infected blood.

(v) Morphology

Electron microscopy of the viruses from cod and herring PEN and the blenny infection has shown some broad similarities but other specific differences between the viruses.

Three common characteristics were revealed. Complete and incomplete particles were found in the matrix of an inclusion body and/or a viroplasmic body. The virion capsid was bilayered; for cod PEN (Appy et al., 1976) and the blenny infection this was shown from thin section and for herring PEN by negative staining. The capsid was separated from the electron-dense core by an electronlucent gap.

Distinguishing characteristics were also shown. The particle diameters of viruses varied; in thin section, from 145 nm for herring PEN, 200-300 nm for the blenny infection to 310-360 nm for cod PEN. Rare lamellar bodies found within or near the viroplasm were noted in cod PEN (Walker & Sherburne, 1977) and herring PEN but not in the blenny infection. The cytoplasm adjacent to the inclusion bodies in blenny infection and cod PEN was electron-lucent but by contrast this was not noted in herring PEN. Various core morphologies have been reported; in cod PEN a uniformly electron-dense core was noted, in the blenny infection an internal cable-like structure was indicated and in herring PEN a doughnut shape was suggested from the clearly discernible light centre of the core.

The similar ICDVs from Pirhemocyton infection (Stehbens & Johnston, 1966) and erythrocytes of the frog R. pipiens (Bernard et al., 1968), show particle diameters in the same range as those for the PEN viruses, 200-240 and 280-380 nm respectively. However, in these infections, the authors observed a particular association with cytoplasmic membranes, which has not been observed in PEN infections. Empty vesicles and flattened membranous sacs surrounded the particles in Pirhemocyton infection and trilaminar membranes encircled the

particles in R. pipiens erythrocytes. It was suggested that both membrane systems might be functionally associated with virus maturation.

The objectives are set out according to the section headings of the thesis as follows:-

(A) Ecology

- (i) To extend the distribution data for cod PEN, the blenny infection and lymphocystis disease, using standard haematological methods for the recognition of the blood infections; furthermore, to ascertain the prevalence of infection within sample populations and to correlate prevalence with the age of fish sampled and the sampling location;
- (ii) Using light and electron microscopy, to estimate the degree of infection in individual fish;

(B) Propagation

- (i) To attempt to find suitable in vitro procedures for propagating the viruses of the blenny infection and lymphocystis disease and to make use of suitable procedures;
- (ii) In connection with attempts to explore the possibility of propagation of the blenny infection in vivo, to determine the effect of two factors in the Blenny on the count of red blood cells and their morphology, viz. APH administered by intra-peritoneal injection and temperature increase;

(C) Morphology

To examine the viruses of the blenny infection and lymphocystis disease by electron microscopy, in order to clarify the morphology of these viruses and to provide information which is useful to their classification.

## MATERIALS

1. Live fish and blood samples

1(a) The Blenny Blennies were caught on many rocky shores near Plymouth and on the shore at Aberystwyth. One collection was also made at Roscoff, Finistere, France on the shore in front of the Biological Station (Plate 4).

The method of collection varied according to the nature of the rock pool and its size. In shallow pools found on rock ledges and in small streams on these ledges caused by the ebbing tide, blennies were caught by hand simply by lifting stones. In large pools with few crevices or stones the fish were caught with a large net. In rock pools with a stony bottom and crevices the pool was emptied as far as possible and an anaesthetic used to release the fish i.e. 5 ml of 20% solution of quinaldine in acetone mixed thoroughly.

The fish were transported from the shore to the laboratory in seawater without the use of an anaesthetic. No mortalities resulted when the duration of transport to the aquarium was short i.e. up to 3 hr. For longer periods of up to 48 hr the fish were held in seawater in a large plastic tank providing a high surface area to volume ratio, and the seawater was changed every 12 hr.

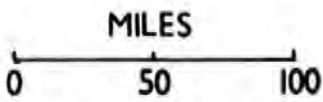
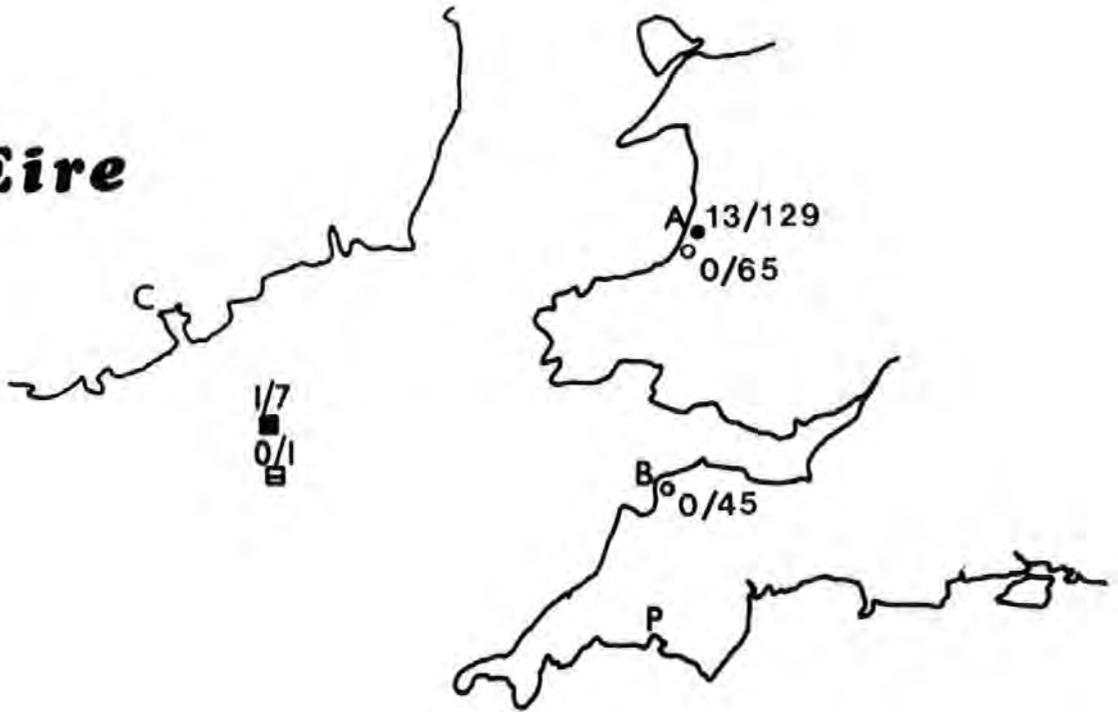
Blennies were maintained in plastic tanks in seawater circulated and filtered over charcoal. They were fed a ration of crayfish meat every 2 days and in the absence of temperature regulation the water temperature was in the range 12-18°C. Temperature regulation from 8-24°C was provided by the use of a cooling unit or an aquarium heater.

1(b) Cod Cod were caught by fishing with a 48 ft otter-trawl of small mesh cover drawn at 4 knots, on board cruises of the research vessels RV 'Sarsia' (MBA, Plymouth) and FRS 'Explorer' (DAFS, Aberdeen) (see Table 6). The location of the stations worked is shown for the Celtic Sea (Plate 4) and for the North Sea (Plate 5).

## Plate 4

Sample sites for the blenny infection on shores outside the Plymouth area (P) and those for cod PEN in the Celtic Sea. 2 uninfected cod were also caught on the Jones Bank, western English Channel (not shown). Numbers denote the fish infected and examined.

**Eire**



- |                   |              |
|-------------------|--------------|
| A Aberystwyth     | B Bagg Point |
| C Cork            | R Roscoff    |
| ■ Cod positive    | ▣ negative   |
| ● Blenny positive | ○ negative   |



## Plate 5

Sample stations for cod PEN in the North Sea off northern Scotland. Higher numbers denote the fish infected and examined. Lower numbers denote station nos. AB - Aberdeen, FI - Fair Isle. Station 58 was actually north of the position shown. Offshore contour is fathoms.

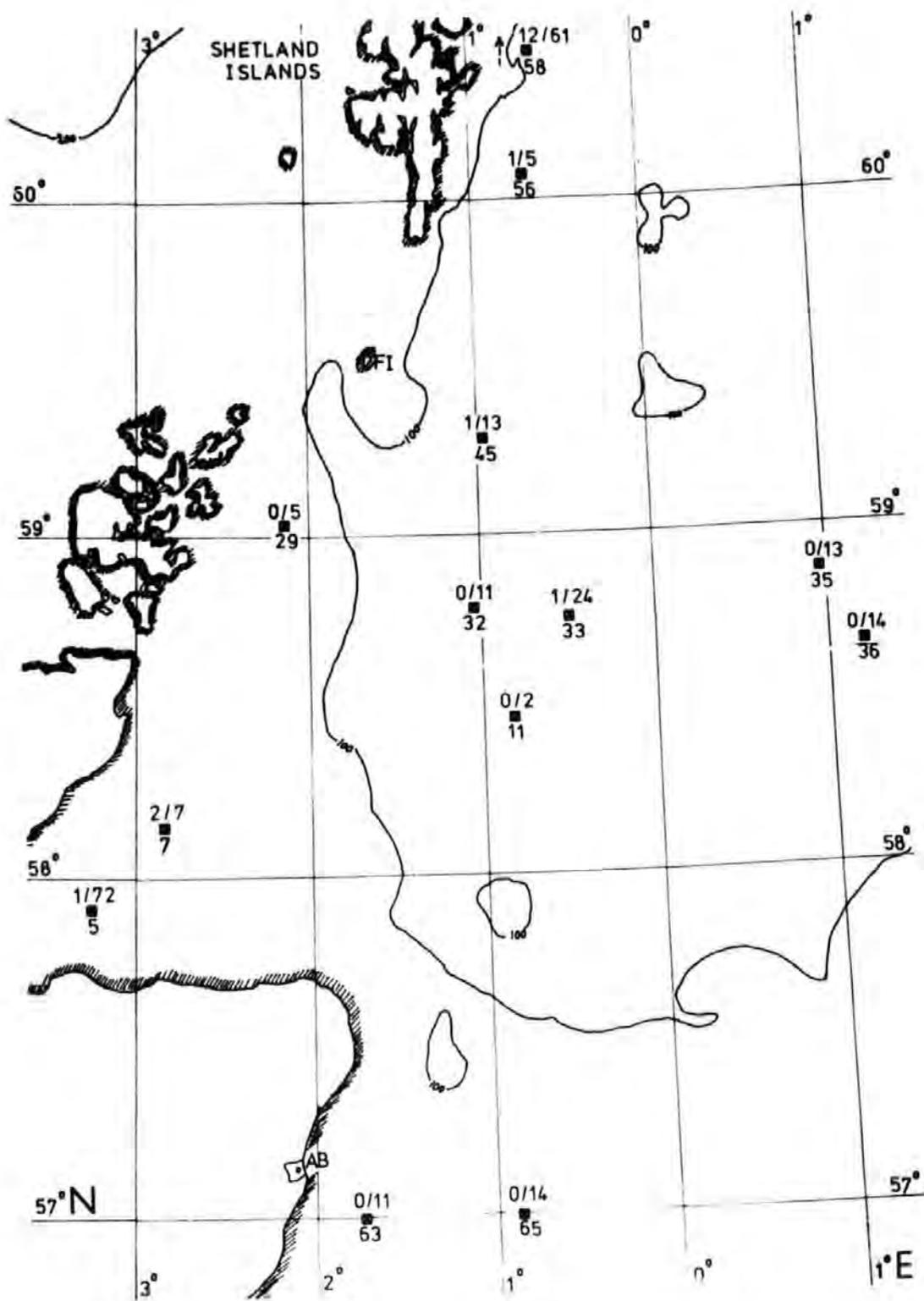


Table 6: Data for fish collected on research trawls

<u>Port of Sailing</u>	<u>Vessel</u>	<u>Auspice</u>	<u>Dates</u>	<u>Material Collected</u>	<u>Area</u>
Aberystwyth	MV 'Kay' B.B.	Geology Dept. University College, Aberystwyth	Oct.1973	Plaice & dab, lympho- cystis infected	Cardigan Bay
Plymouth	RV 'Sarsia'	Marine Biological Association, Plymouth	Apr.-May 1975	Blood smears from a range of sea fish including cod	Western English Channel & Celtic Sea
Aberdeen	FRS Explorer'	Dept. Ag. & Fish. Scotland The Marine Laboratory, Aberdeen	Nov.-Dec. 1975	Cod blood smears & blood samples	North Sea off Aberdeen

Blood smears were made from each fish, using fresh blood taken from the heart and the smears quickly air dried. They were stored unfixed.

Blood samples were taken from the heart using a 'vacutainer', with 4% TSC to prevent clotting, connected to a 19G 2" needle. The needle was inserted ventrally immediately anterior to the V formed by the shoulder girdle and at right angles to the body. By this method up to 15 ml of blood could be taken from cod that had been on the deck for up to 3 hr.

The citrated blood was stored at sea at 4°C for up to 10 days with negligible lysis and transported in an insulated ice pack to the Marine Laboratory, Aberdeen where the samples were stored for 4 days at 4°C. After the infected blood samples were identified from the smears by the procedure described in the Methods, these samples and some controls were transported to Plymouth in an insulated ice

pack. This did not result in significant visible red blood cell lysis. The samples were then stored at 0-4°C for 4 days before processing.

1(c) Flatfish and Bluegill Flounder and plaice trawled from coastal waters near Plymouth were obtained from the Marine Biological Association, Plymouth. Flounder, plaice and dab trawled from the Torbay area were kindly donated by Mr. B. Maddox of ICI laboratories, Brixham. Bluegills were also donated by Mr. Maddox.

Flatfish were maintained in circulated aerated seawater at 10-15°C and bluegills were kept in aerated tap water at room temperature.

## 2. Lymphocystis

Most of the material was obtained by other persons and it originated from either the North East Irish Sea or the River Ythan, Aberdeenshire. Both whole fish and individual lesions were received and the different materials collected are termed isolates 1 to 7 (Table 7). Infected flounder and plaice obtained from the Min. of Ag. & Fish, Food office at Fleetwood were caught by trawl at approximately 30 fathoms. The data for the fish collected by Dr P. Russell were described by Russell (1974). Isolate 7 was a lyophilized preparation of lesion material from the Bluegill and was kindly donated by Dr. K. Wolf of the Eastern Fish Disease Laboratory, U.S.A.

Infected plaice and dab (isolates 1 & 2) were caught first hand by trawl in Cardigan Bay at 10-13 fathoms depth approximately 5 miles due west of Aberystwyth.

The materials were transported to Plymouth as follows. Living infected plaice and dab from Cardigan Bay were carried in sea water for 15 hr and held in tanks overnight. The lesions were then dissected out and used immediately. Infected plaice and flounder

from Fleetwood were stored at  $-20^{\circ}\text{C}$  at Fleetwood in sealed polythene bags and then transported for up to 3 days in a 'Thermos' canister packed with ice. All the lesion areas were then dissected out as soon as possible, labelled and stored at  $-20^{\circ}\text{C}$ . Individual tumour lesions of plaice and flounder, from Dr P. Russell at Glasgow were transported on dry ice for up to 40 hr. and then stored at  $-20^{\circ}\text{C}$ .

3. Virus Inocula The methods are detailed here for the preparation of inocula used in in vitro propagation attempts            for the viruses of lymphocystis and the blenny infection. The isolate numbers of the materials used refer to those in Table 7.

(a) Lymphocystis virus

Isolate 3i 2 inocula were prepared for use in experiment 1. (Table 20). Inoculum 1 was prepared using the procedure in method B (isolate 3i- in vivo experiments) then the supernatant was further clarified by sedimentation at 700g for 5 min. Inoculum 2 was prepared by filtering 1 ml of the above clarified supernatant on a 'Millex' disc of 220 nm pore size.

Isolate 3ii Inoculum 3 was prepared as follows: 0.48g of cells was homogenised in 2.5 ml 0.1 M phosphate buffer pH 7.5 and the homogenate filtered through four times thickness dressing muslin. A 0.5 ml aliquot of the filtrate was layered on a 10-40% w/v sucrose gradient made up in the same phosphate buffer. The gradient was centrifuged firstly at 32,260g for 2 hr and secondly at 43,300g for 2 hr. The refractive bands were collected from the gradient manually and the virus sedimented from suspension by centrifugation at 95,000g for 2 hr. The pellet was then resuspended in 0.2 ml 1% ammonium acetate. The preparation was examined in the electron microscope at this stage to establish sufficient purity and concentration of virus. The suspension was diluted 20 times in salt-adjusted Hanks washing solution and filtered on a 'Millex' membrane of 450 nm pore size.

Table 7: Sources of lymphocystis infected fish and the isolate numbers of the lesion material

<u>Fish collected</u>	<u>Person/Auspice</u>	<u>Site</u>	<u>Date</u>	<u>Isolate No.</u>
Plaice	Self	Cardigan Bay off	Nov. 1973	1
Dab		Aberystwyth	Nov. 1973	2
Plaice	MAFF Fleetwood	N.E. Irish Sea off Blackpool	All 1973 Sept. 1974 Sept. 1975	3 i 3 ii 3 iii
Flounder	MAFF Fleetwood	N.E. Irish Sea off Blackpool	Sept. 197 Sept. 1975	4 i 4 ii
Plaice	Dr. P. Russell	N.E. Irish Sea off Whitehaven	Oct. 1972	5
Flounder	"	River Ythan Culterty, Aberdeenshire	Feb. - June 1973	6
Bluegill	Dr. K. Wolf	N. America	1966	6

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Isolate 5 Inoculum 4 was prepared from 0.05g of tumour material homogenised in 20 vol. FHM maintenance medium. The homogenate was sedimented at 700g for 10 min for clarification and 0.4 ml supernatant was filtered through a 220 nm 'Swinnex' membrane. The membrane was flushed with the medium giving 1 ml of inoculum.

The inoculum for organ cultures was prepared from this isolate but the homogenate was filtered through a 450nm Millipore filter.

(b) Blenny erythrocytes

(1) Infected erythrocytes for inocula were fragmented to release the virus. Different methods were used as follows:

Expt 1. Red blood cells from <sup>wild</sup> infected and <sup>uninfected</sup> control fish were taken separately to 0.5 ml 4% TSC, sedimented at 600g for 5 min. and then immediately lysed by the addition of 1 ml distilled water. The resulting lysate was agitated and filtered through a 450 nm 'Swinnex' membrane, giving approx. 0.5 ml of filtrate (See B1b p.107 & Table 16 p.108).

Expt 2. One inoculum was derived from sonicated erythrocytes and another from haemolysed erythrocytes. Approx. 0.1 ml blood was taken from an infected fish, suspended in 1.4% saline and the red blood cells sedimented and resuspended in 2 ml of FHM maintenance medium. A 1 ml aliquot was sonicated with an ultrasonic disintegrator at an amplitude and frequency calibrated to give complete cell lysis. The suspension of red cellghosts was then filtered through a 450 nm 'Swinnex' filter. Another 1 ml aliquot was centrifuged and 0.5 ml distilled water added to the cell pellet. 0.5 ml 2X maintenance medium was added to the lysate and this filtered on a 450 nm 'Swinnex' membrane ( See B1b p.107 and Table 16 p.108 ).

(4) Preparation of APH for intra-peritoneal injection.

APH was dissolved in sterile 1.4% saline to give 1 mg/ml. Each fish was weighed and the volumes for injection were calculated as given in Table 23          to give doses of 0.1 mg & 0.2 mg per 10g body weight (see Appendix p. 177).

## SECTION A

ECOLOGY OF THE INFECTIONS

(1) Identification of the Infection in the Blenny

Blood smears and organ imprints were used to establish whether inclusions were present in the red blood cells.

Fish were anaesthetized using either MS-222 or benzocaine. A small amount of blood            was then taken from the caudal vein using a glass micropipette of approximately 0.2 mm bore. Smears were made immediately by spreading (Dacie & Lewis, 1975). They were routinely <sup>air dried,</sup> fixed in methanol for 5 min, dried, stained 20 min. in 10 X dilution of Giemsa stain in buffer and differentiated in buffer up to 10 sec.

Imprints from the spleen and kidney were prepared and stained either by the method of Klontz (1972) or that of Ashley and Smith (1963) as given below.

Smears and imprints were observed for 10 min by light microscopy at a convenient objective magnification of X40.

(2) Methods for staining organ imprints

A. Method of Klontz (1972)

1. The organ was cut in half and the face to be used dried with Whatman No. 1 filter paper. Twelve imprints were made by touching the face to alcohol-cleaned slides.
2. The slides were fixed in methanol 5 min and air dried.
3. They were overlaid with 2 ml Leishman stain for 3 min, and an equal vol. of a 10 X dilution of Giemsa stain added to give a metallic sheen. This was left for 7 min. The slides were then washed in tap water and air dried.

B. Methods of Ashley and Smith (1963)

1. Imprints were fixed in fumes of 40% formaldehyde.
2. Glass slides, imprint side up, were covered with a stock solution

of Wright's blood stain for 5 min.

3. An equal volume of phosphate buffer (pH 6.5) was added for 5 min.

The stain was mixed with the buffer by gently blowing on the stain.

4. Slides were then washed with distilled water and dried.
5. Giemsa stain diluted in buffer was added and the slides stained 20 min. and finally washed in distilled water.

### (3) Fluorescent staining of blood smears

A fluorescent compound, which binds readily to DNA, was used to demonstrate DNA-containing cytoplasmic inclusions. It was 4'-5-diamidino-2-phenylindole (DAPI) (Russell, Newman & Williamson, 1975).

2 infected and 2 control blood smears from the Blenny were fixed and dehydrated by the method of Randall and Disbrey (1965), then stained in a solution of DAPI at 0.1 µg/ml in Sorenson's buffer pH 6.0 for 15 or 20 min. The smears were observed in a fluorescent microscope at 280 nm wavelength of ultra-violet light.

### (4) Method of estimating the number of infected erythrocytes in smears

For counting, a round coverglass was made to fit in the eye-piece of a binocular microscope and on this were ruled squares with black ink of 2.5 mm<sup>2</sup>. This allowed four squares to be viewed at once. A total of 500 cells was counted on each smear for estimation of the proportion of infected.

### (5) Isolation of the Infection in the Cod

The blood smears from the samples of 249 North Sea and 10 Celtic Sea fish were fixed and stained using the routine described in Methods (1) for the Blenny. The infected smears were determined and the respective blood samples isolated.

## RESULTS

The results in this section centre on the ecology of the virus infections and deal with their recognition and geographical distribution, the distribution of the virus in the host and the symptoms of the infection. These are subheaded sections A1 to A4.

### A1 Recognition

The recognition of the blood infections was complex since they are symptomless whereas lymphocystis disease does present external symptoms. Recognition of the blenny infection and PEN in cod was thus subjective in the sense that the particular appearance of infected smears had to be recognised by the observer. Therefore observations of how staining affected the recognition of the infections and details of the time course of the natural infections are mentioned here.

#### A1(a) Recognition of the Blood Infections

##### (i) The blenny infection

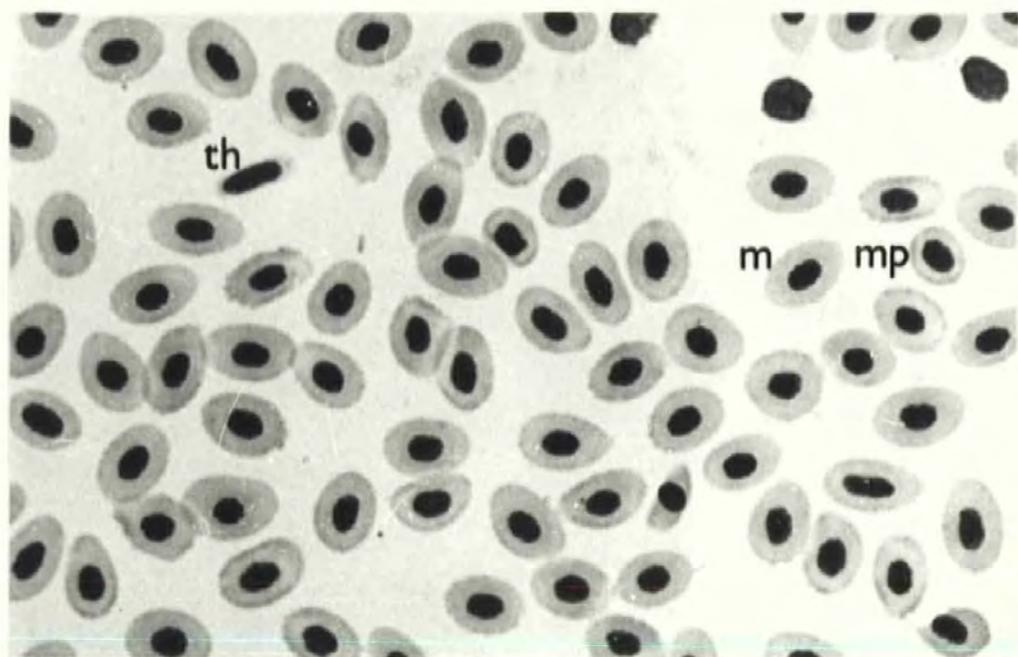
The normal blood smear from the Blenny showed the range of cell types typical of teleost fish blood (Plate 6). A range of sizes and types of erythrocyte was seen varying from mature (m) with a low nucleus to cytoplasm ratio to immature (mp-middle polychromatic) with a high ratio. Leucocytes were very sparse but fusiform thrombocytes (th) were observed more commonly, often in clusters.

The blenny infection was clearly distinguished by cytoplasmic inclusions in erythrocytes, measuring 1-1.5  $\mu\text{m}$  across (Plates 7a & 7b arrowed). They were seen either close to the nucleus or at the periphery of the cell. When near the nucleus, the inclusions were sometimes difficult to distinguish from small knob-like extensions of the nucleus.

The best staining results for the blenny infection were found

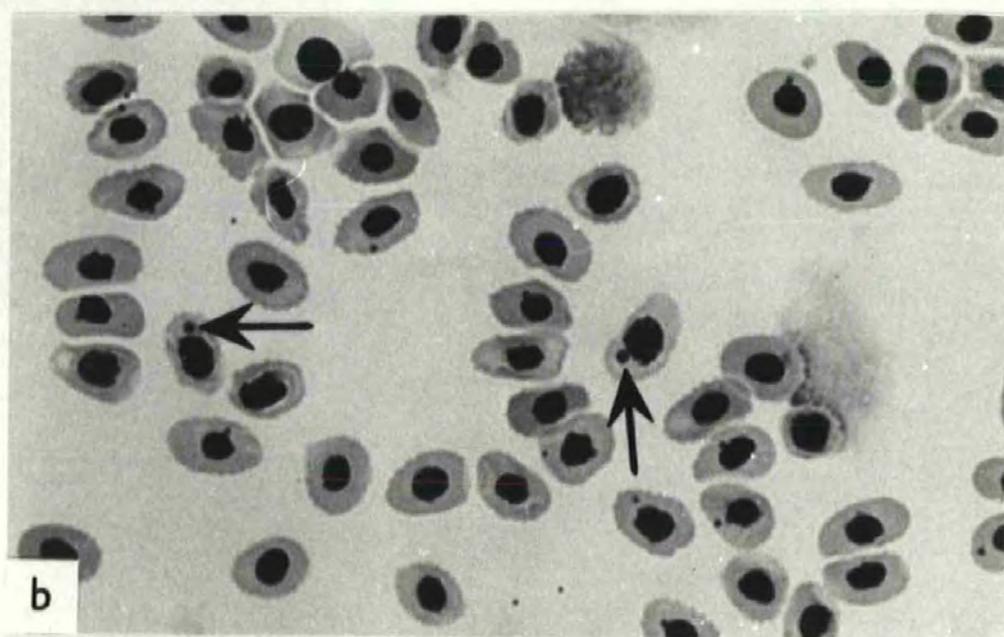
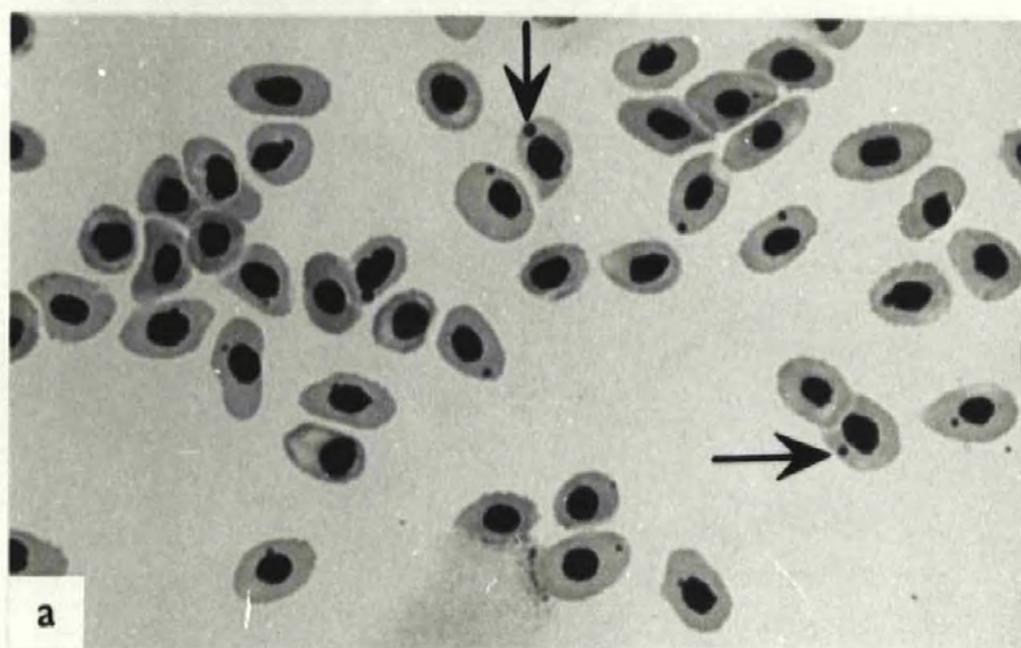
## Plate 6

Normal blood film, Common Blenny, Giemsa's stain, X 1024. Erythrocytes of different stages of maturity are seen (m - mature, mp - middle polychromatic); also a fusiform thrombocyte (th).



## Plate 7

(a) & (b). Blood film, Common Blenny, no. 29 (14th May 1975), Giemsa's stain, X 1024. Infected erythrocytes show a small spherical body in the cytoplasm (arrows).



by using the Giemsa slow staining method of Busby, House and Macdonald (1964) which is specifically recommended for staining viral inclusions and elementary bodies. Erythrocytes stained in this way showed not only the usual cytoplasmic inclusions but also occasionally a faintly stained circle or 'halo' around the inclusion (Plate 8b - arrow) and fine granules of 0.3 - 0.5  $\mu\text{m}$  close to it (Plate 8b). Smears stained by the standard method for 20 min at room temperature, rather than overnight at 37°C, never showed these granules.

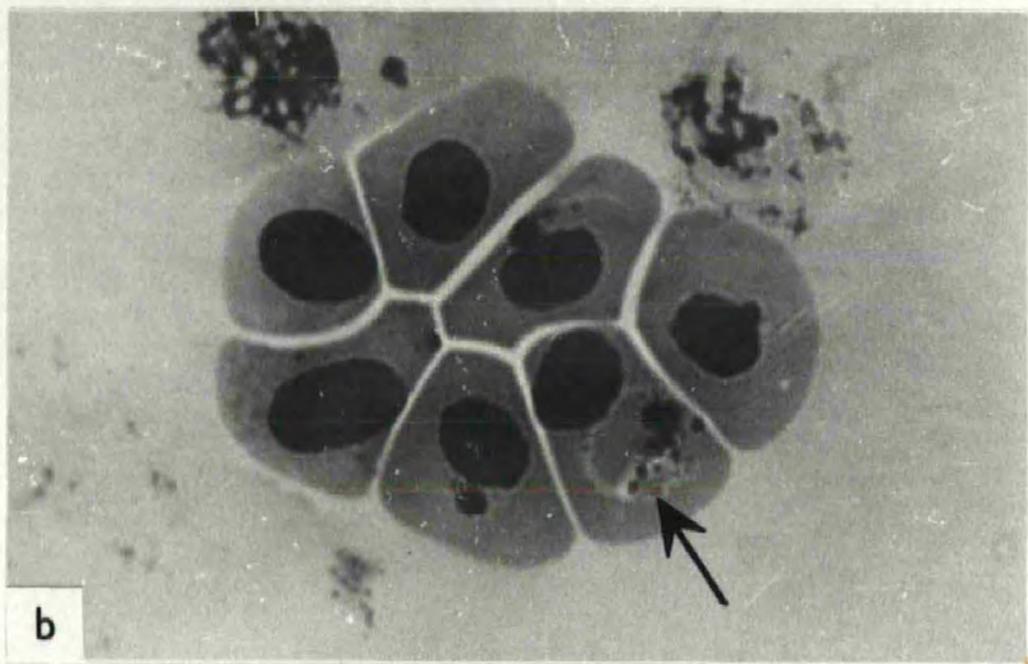
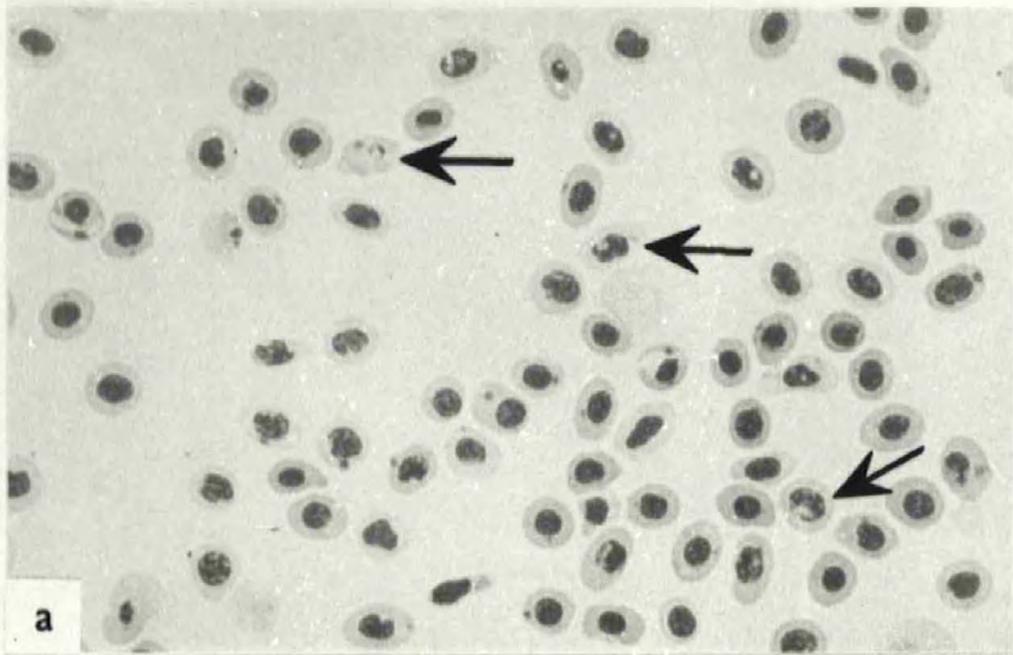
The inclusions were stained with the fluorescent compound DAPI which is specific for DNA. Their bright green fluorescence confirmed that DNA is localized at the inclusion, no cytoplasmic fluorescence being seen in control smears. At a stain concentration of 0.1  $\mu\text{g/ml}$ , the cytoplasmic fluorescence, by contrast to the nuclear fluorescence, was brightest when the staining time was 20 min. DNA content was also confirmed by staining with acridine orange using the method of Randall & Disbrey (1965).

The infection in the Blenny rarely involved lysis or breakdown of the nucleus but a few smears were seen where vesiculation and granulation of the nuclei was observed (Plate 8a). In these the nuclei of the red cells closely resembled the vesiculated nuclei of cod red cells showing PEN. The proportion of cells infected in a smear varied from 3-60% the upper range 30-60% being quite common. Inclusions were consistently observed more frequently in mature erythrocytes than in immature ones though this observation was not quantified. However, inclusions were never seen in leucocytes.

In respect to recognition, two observations suggested the length of the natural infection was inversely related to the temperature of maintenance. Firstly, for 3 naturally infected fish collected at Aberystwyth, transported to Plymouth and maintained in tanks at 20°C, at a first check 14 days after identification only 2/3 were infected and at 19 days none of the 3 were infected. Secondly, holding

## Plate 8

- (a) Blood film, Common Blenny, Giemsa's stain, X 820. Erythrocytes show cytoplasmic inclusions typical of infected films; these cells and those without inclusions also show nuclear fragmentation (arrows).
- (b) Blood film, Common Blenny, Giemsa's stain using a slow staining method, X 2560. Two of four infected cells show granules around the inclusion body and in one cell the body is circled by a halo (arrow).



infected fish at a regulated temperature of 12°C or below successfully preserved the infection for at least 60 days.

Other observations were made as follows. Wild caught blennies maintained in aquarium tanks at a density of dwelling greater than that in the natural habitat, showed the infection in the laboratory under artificial conditions but could then lose the infection in up to 11 days at 18-20°C.\*\*

\*\* (Experiments involving variation in the density of dwelling of fish were carried out under a current Home Office license concerning The Cruelty to Animals Act, 1879.)

(ii) PEN in cod

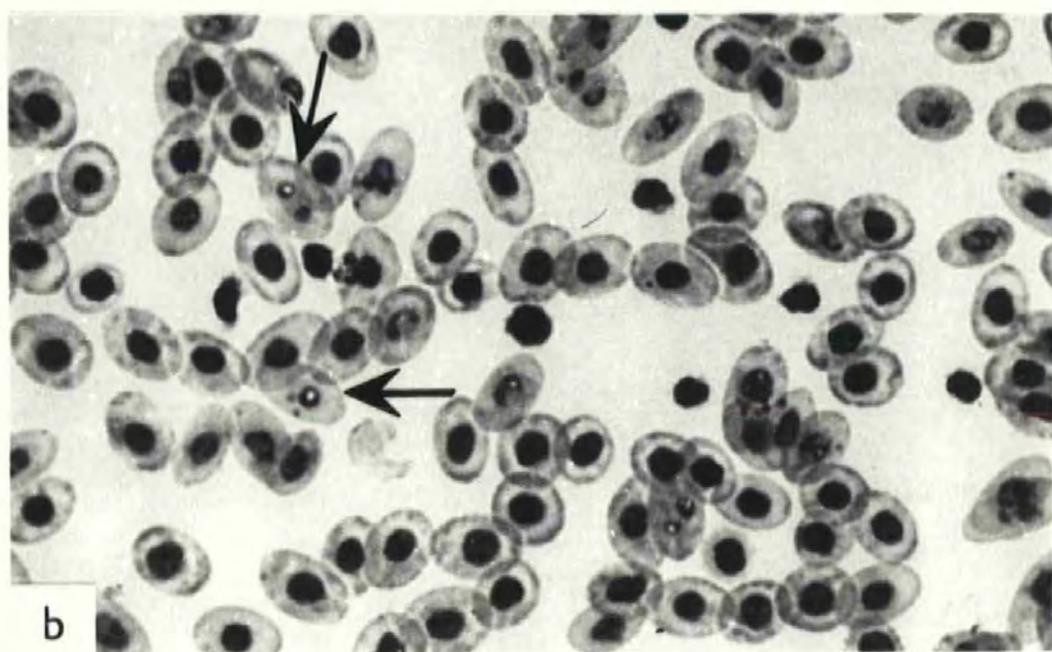
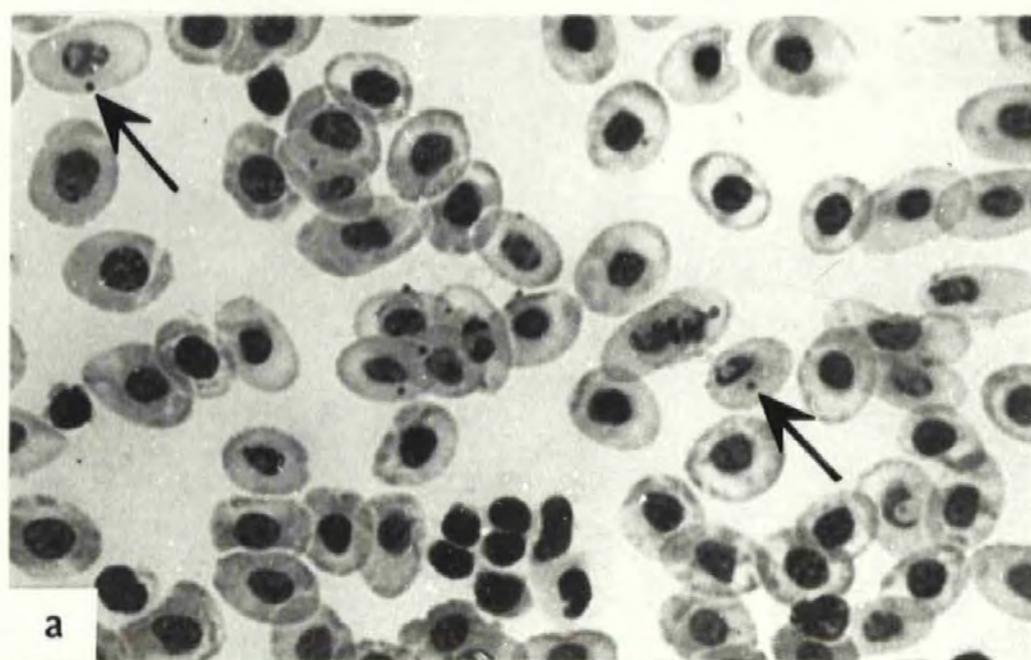
The recognition of PEN in cod was more distinct than that of the blenny infection.

The infection was observed entirely in mature erythrocytes and not in leucocytes or thrombocytes. It involved a breakdown of the nucleus and changes in the cytoplasm, in particular the presence of a Giemsa-staining inclusion body. This was round, measuring 0.6-2.0 µm diameter, and was found in a variety of positions in the cytoplasm (Plate 9a). In 5/18 positive smears examined, only the nuclear lesions were seen whilst in the other 13 both nuclear lesions and an inclusion body were observed (Table 8). The level of infection seen varied from 1-50% of erythrocytes infected.

## Plate 9

Blood film, cod, no. 4 (Celtic Sea), Giemsa's stain.

- (a) X 1024. Small spherical inclusions are seen (arrow) and the nuclei of some infected cells show fragmentation to two arcs or half-moons (centre).
- (b) X 820. The nuclei of some infected cells (arrowed) at an early stage of infection show a central vesicle.



**Table 8:** Estimated levels of infection in PEN cod smears and the types of lesion pattern seen. ( X = presence)

<u>Slide No.</u>	<u>% Mature rbc infected</u>	<u>Cytoplasmic inclusion</u>	<u>Nuclear lesion only</u>
153	2-5	X	X
143	1-5	X	X
24	< 1	X	
194	10		
43	1		X
74	1-5		X
76	< 1		X
184	< 1	X	
16	10-20	X	X
224	5-10	X	
248	5-10	X	X
82	5-10		X
211	1		X
199	1-2	X	X
191	40-50	X	X
195	20	X	
232	10-20	X	X
40	< 1	X	X

Observation of the positive smears gave the strong impression that the type of cytology seen was correlated with the stage of infection. In early stages the nuclei of infected cells clearly showed vesiculation (Plate 9b) and granularity (Plates 10a and 11b) and at later stages showed fragmentation (Plate 10b). The breakdown patterns of the nuclei were varied. A typical configuration seen at early stages of infection was two facing arcs or half-moons (Plate 9a) whilst later in infection round granules (Plate 10b) were seen as well as lobular granules connected by 'tails' (Plate 11a).

#### A1(b) Recognition of lymphocystis disease

The lymphocystis-diseased flatfish were typical with respect to the distribution of the infection over the skin. The characteristic enlarged cells and warty tumours were seen most commonly on the fins, the fin margins and the anal spine (Plates 2a, 2b). Variation in the degree of infection and the size of the cells was also seen. Plaice and flounder from Fleetwood (isolates 3 & 4) both showed very heavy infections. Both large old necrotic tumours, measuring up to 1 cm diameter, and small newly formed lymphocystis cells were seen. By contrast, plaice and dab caught in Cardigan Bay showed a relatively low level of infection and only small cells of approximately 1 mm diameter.

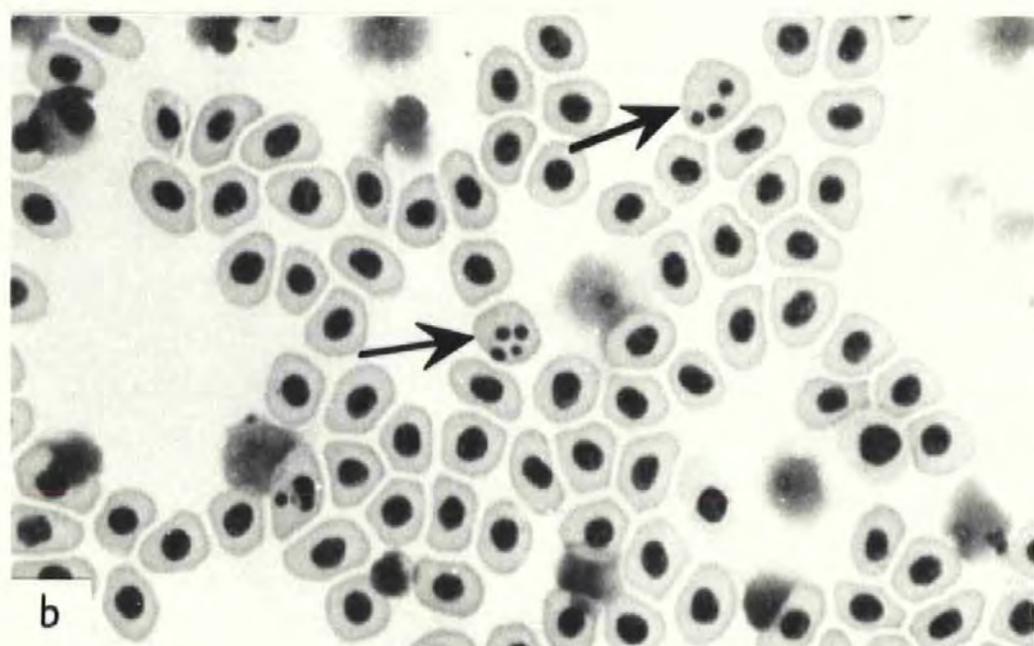
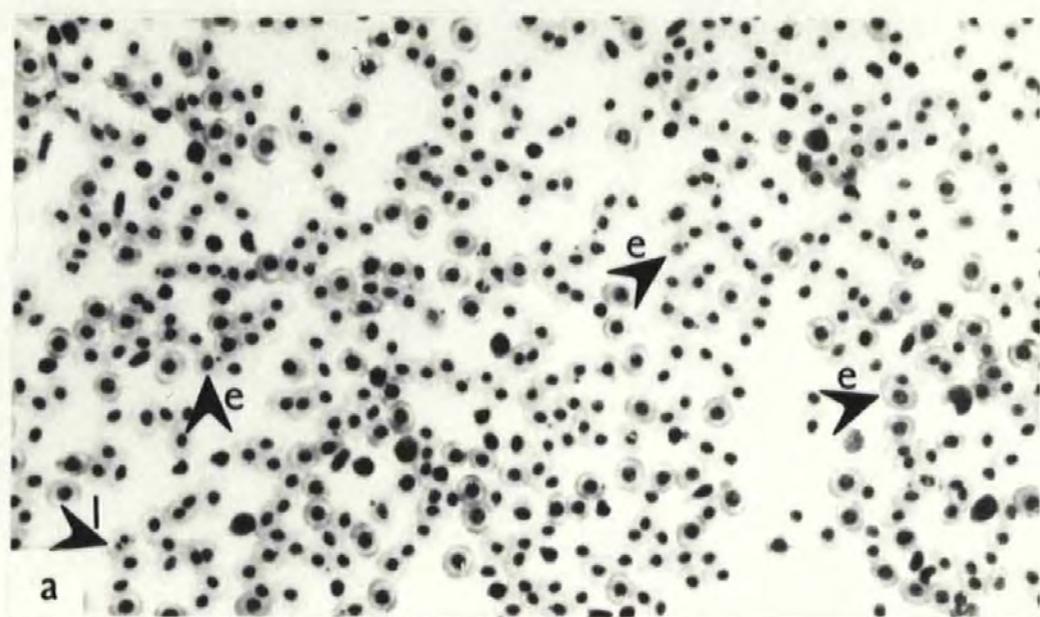
The disease may be found in commercial catches; as evidence of this an infected fish was found in a purchased batch of plaice that originated from Fleetwood (Plate 2). Interestingly a darkly pigmented growth was observed on the lower jaw of this fish (Plate 2a arrowed).

#### A2 Collection Data on the Infections

Efforts to locate the infections and to seek new sites gave new information which is subdivided in this section under geographical

## Plate 10

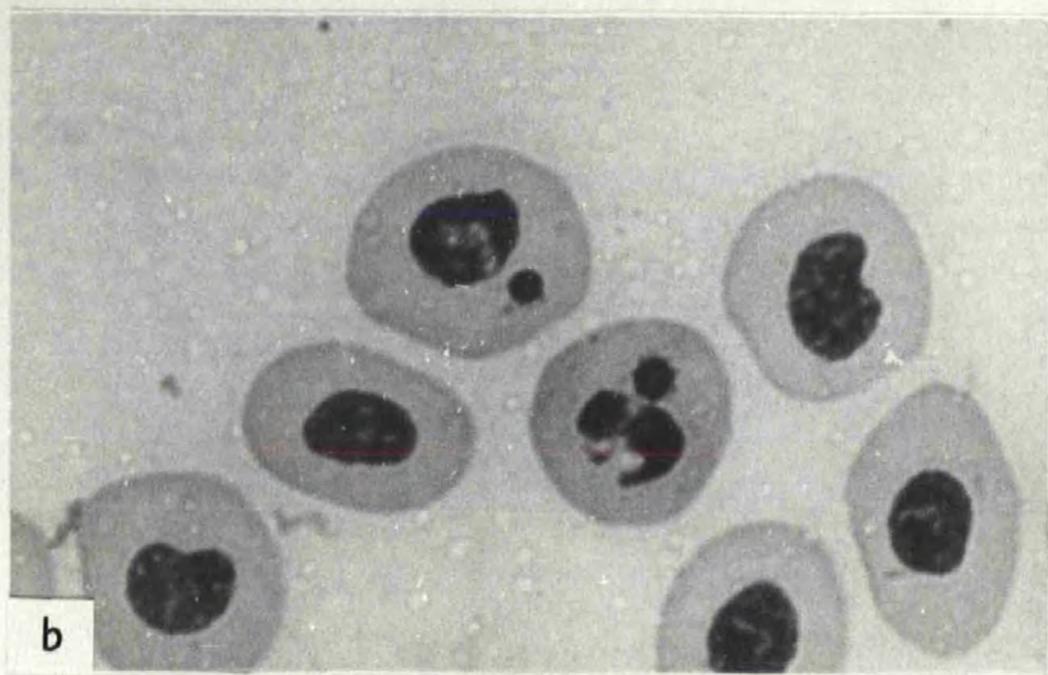
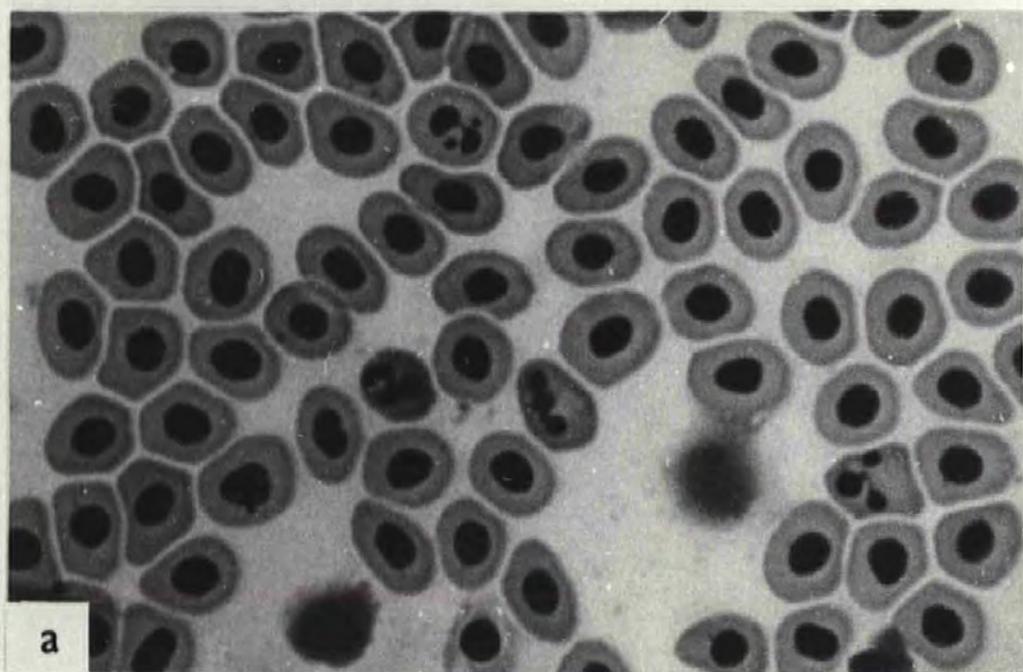
- (a) Blood film, cod, no. 153 (North Sea), Giemsa's stain, X 410. Early stage infected cells (arrowed, e) show inclusions and the nucleus in that to the far right shows fragmentation. Late stage infected cells (arrowed, l) show subdivision of the nucleus.
- (b) Blood film, cod, no. 194 (North Sea), Giemsa's stain, X 820. Late stage infected erythrocytes show fragmentation of the nucleus to round granules (arrows).



## Plate 11

Blood film, cod, no. 194 (North Sea), Giemsa's stain.

- (a) X 1024. Fragmentation of the nucleus of infected erythrocytes results in lobular granules connected by 'tails' in this smear.
- (b) X 2560. The nucleus of one infected erythrocyte shows increased granularity and another shows fragmentation.



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distribution and the correlation of infection rates with age of the fish. The data are limited mostly to the Blenny as the survey of PEN in cod was essentially only a preliminary one.

#### A2(a) Geographical distribution

All of the material from the blood infections was collected first hand as described in the Methods. This involved making use of the established infection at Aberystwyth and also extending the search to Devon and Cornwall in the case of the blenny infection but for PEN in cod collecting trips for new isolations had to be made. Most of the lymphocystis material was obtained from the MAFF office at Fleetwood and these fish had been trawled from the N.E. Irish Sea. One collection was also trawled in Cardigan Bay off Aberystwyth.

##### (i) The blenny infection

Collections in August 1973 and July 1974 confirmed that the infection was present in the shoreline population from College Rocks, Aberystwyth, as the discovery of Johnston & Davies (1973) had shown. However, there was considerable advantage in finding a source of the infection close to Plymouth for ease of keeping fish alive during transit to the aquarium and so a search was made in the Plymouth area. Infected fish were found on 9 shores in Devon and Cornwall (Fig. 3) in addition to the data for Aberystwyth and the mean infection rate for the total of 717 fish collected varied from 2.4 to 20% (Table 9). The total sample for each shore in the table represents in some cases more than one collection and the number caught varied widely from 4-70.

The records of collection clearly showed differences in the infection rate between sites and it was interesting to determine whether differences in the infection rate between sites could be explained purely by random sampling i.e. that there existed an equal

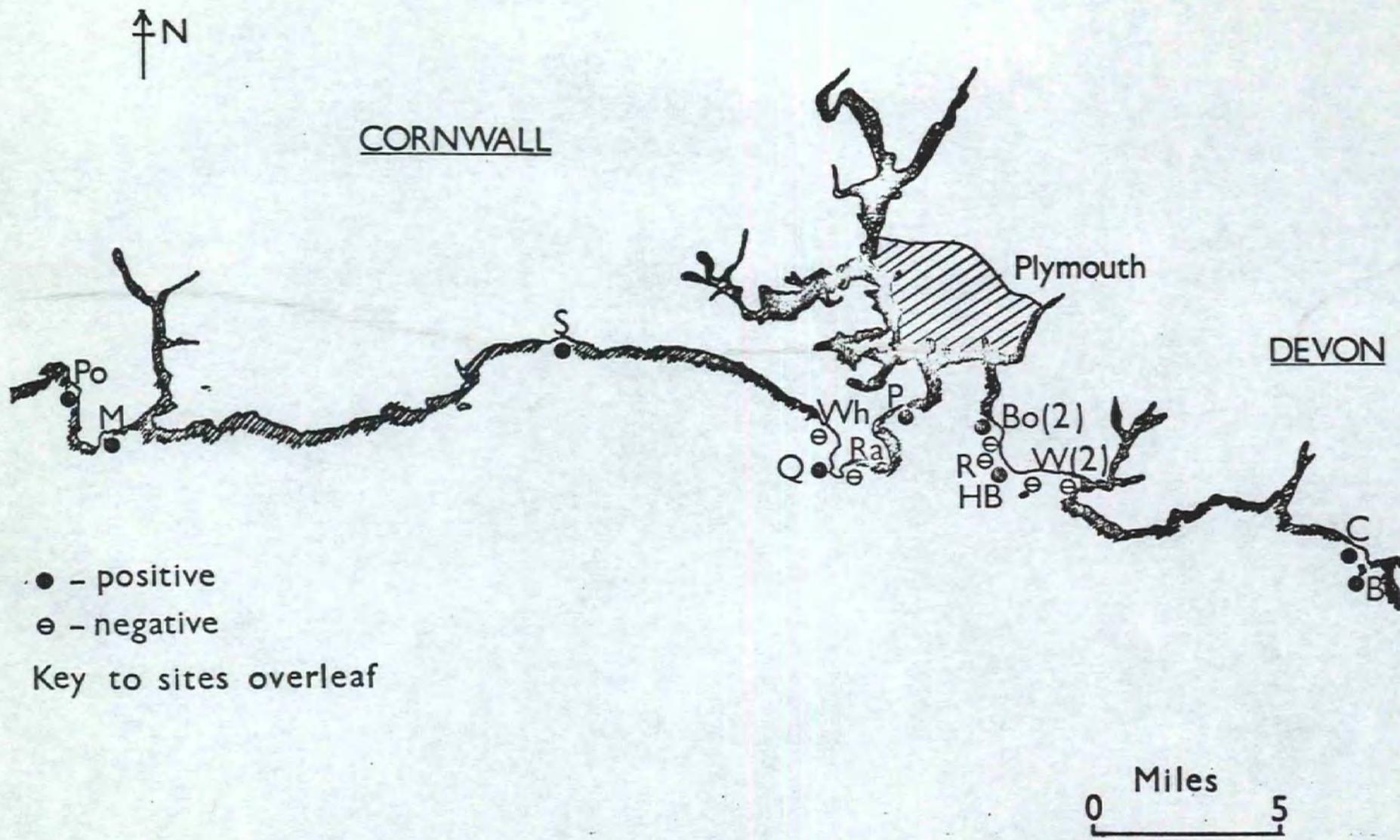


Fig. 3. Collecting sites for the blenny infection in the Plymouth area. 2 sites are shown for Bovisand & Wembury.

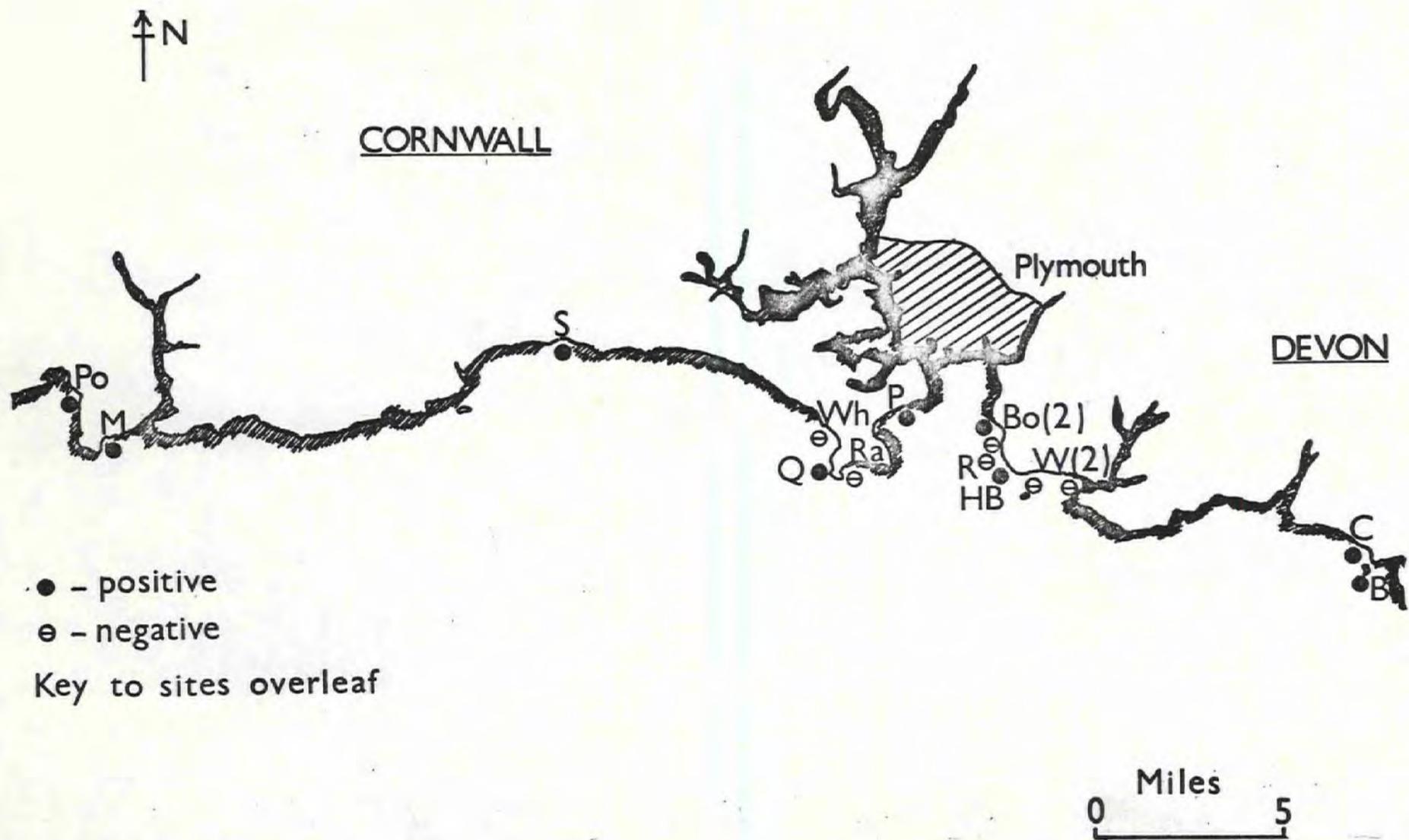


Fig. 3. Collecting sites for the blenny infection in the Plymouth area. 2 sites are shown for Bovisand & Wembury.

B Burgh Island  
Bo Bovisand  
C Challaborough  
HB Heybrook Bay  
M Menabilly Bay  
P Picklecombe Fort  
Po Polkerris

Q Queener Point  
R Renney Rocks  
Ra Rame Head  
S Seaton  
W Wembury  
Wh Whitsand Bay

Table 9: Collection data on the blenny infection.

<u>Site</u>	<u>No. taken</u>	<u>No. Infected</u>	<u>Site Score</u>	<u>Mean infection rate (%)</u>
<b>WALES</b>				
Aberystwyth	194	13	+	6.4
<b>FRANCE</b>				
Roscoff, Finistère	24	0	-	0
<b>DEVON &amp; CORNWALL</b>				
Baggy Point	45	0	-	0
Bovisand	112	4	+	3.6
Burgh Island	33	1	+	3.3
Challaborough	35	7	+	20.0
Heybrook Bay	36	2	+	5.6
Menabilly Bay	41	1	+	2.4
Picklecombe Pt.	25	1	+	4.0
Polkerris	9	1	+	11.1
Queener Pt.	26	1	+	3.8
Rame Head	14	0	-	0
Renney Rocks	14	0	-	0
Seaton (C'wall)	21	1	+	4.8
Wembury	40	0	-	0
Wembury Point	26	0	-	0
Whitsand Bay	<u>22</u>	<u>0</u>	-	0
	<u>717</u>	<u>32</u>		

probability of catching infected fish on different shores. On this hypothesis the distribution of the infected fish in samples from the population would be expected to approximate to the Poissonian distribution of a discontinuous variable as long as the probability of finding the infected individuals was low and the sample size reasonably large and of confined range. Using only the large samples in the range 20-33, the average infection rate was calculated as 4.5% so the hypothesis might be expected to apply to these data. The small variation in sample size could be ignored as a plot of sample size against infection rate showed no positive or negative correlation.

The data were arranged to show actual class frequencies, using figures from the large samples only, and these were compared with the hypothetical frequencies according to the Poisson formula, to give a value of  $\chi^2$ . Applying the important limitation of summing low hypothetical values to give a value of 5 and above,  $\chi^2 = 1.35$  and  $p > 0.5$ , or at least 50% probability that the observed deviations occurred by chance. However, if the limitation on small hypothetical numbers were ignored and the frequency classes treated individually  $p > 0.005$  i.e. a very low probability <sup>of</sup> 0.5% that the deviations occurred by chance. In this calculation, it was clear that one frequency class where  $x=6$ , relating to the collection at Challaborough, contributed 98% of the deviation. If this sample were omitted,  $p$  was then calculated at  $> 75\%$ , much the same result as for the amalgamated comparison.

Clearly, the outcome of this comparison is that the observed deviations occurred by chance and that the probability of finding the infection was equal on most shores but that this conclusion did not apply to the site at Challaborough, which was collected only once and where the incidence of infection was the highest found.

Blennies were collected from the shore at Aberystwyth on 7

occasions and experience suggested that the distribution of the infection on the shore might be patchy or non-random. Thus College Rocks close to the pier were more reliable for finding the infection than the shoreline closer to the harbour, termed 'Castle Rocks' (Fig. 4). No infected fish were found in one large sample of 65 from 'Castle Rocks' whereas 13 were found out of 129 in six collections on College Rocks, the respective mean infection rates being 0 and 10.1% (Table 10). There was not enough data to show whether frequency classes

Table 10. Collection data on the blenny infection at Aberystwyth

<u>Site</u>	<u>Date</u>	<u>Nos. taken</u>	<u>Infected</u>		<u>Mean (%)</u>
			<u>Nos.</u>	<u>(%)</u>	
College Rocks	8/8/73	41	6	14.6	} 10.1%*
" "	2/5/74	11	0	0	
"Castle Rocks"	23/6/74	65	0	0	- 0
College Rocks	22/7/74	19	0	0	} 10.1%*
" "	23/7/74	23	3	12.5	
" "	24/7/74	24	2	8.3	
" "	9/9/75	11	2	18.2	
College Rocks	TOTAL =	<u>129</u>	<u>13</u>		
	Castle Rocks	<u>65</u>	<u>0</u>		
	GRAND TOTAL =	<u>194</u>	<u>13</u>		

\* taken together

for collections on College Rocks conformed to the Poissonian distribution. In addition the sample size throughout the 6 collections varied greatly and this would limit the value of an analysis.

(ii) PEN in cod

PEN in cod was found for the first time in British coastal waters. It was discovered at a single station in the Celtic Sea off southern Eire in May 1975 and also at several stations in the North Sea off northern Scotland in December 1975 (Plates 4 & 5). The location off Eire was  $51^{\circ}09.6'N$   $07^{\circ}57.7'W$  and the trawl was at 55 fathoms depth over sandy gravel.

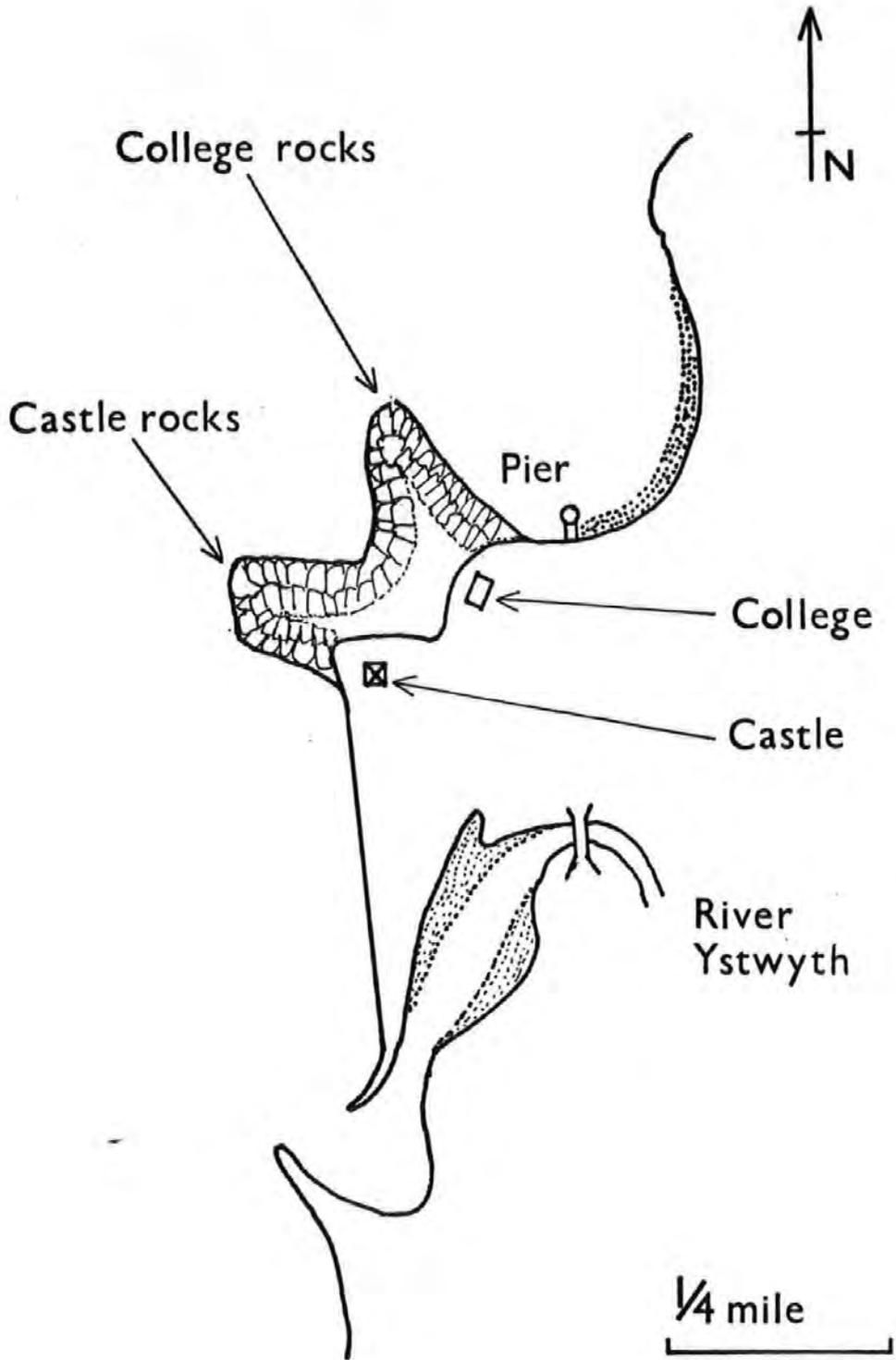


Fig. 4. The shoreline at Aberystwyth, Wales.

18/249 cod were found infected in the North Sea and 1/10 infected in the Celtic Sea, 2 cod being taken in the western English Channel and 8 in the earlier sample. The data for the North Sea showed a range of infection rate which varied from 1.4 to 28.0% in the 6/13 stations sampled where infected fish were found (Table 11). The infection incidence in only three station samples could be regarded as reliable i.e. nos. 5,33 and 58 as at these stations the sample sizes were greater than 20. The mean infection rate for these stations averaged 8.9% compared to 7.3% for all the station samples.

The catch in each North Sea trawl was extremely variable as the purpose of the cruise was a pre-recruit survey of demersal fish and not primarily a cruise for cod. Thus samples handled varied from 2-72, the latter being the largest at station 5 in the Moray Firth and this represented only 20% of the total catch. Thirty baskets of cod were caught (Plate 1b) but only six could be handled as the blood of cod began to clot after three hours on the deck.

(iii) Lymphocystis disease

Lymphocystis nodules were found on plaice and dab on at least 1% of the fish caught in Cardigan Bay in October 1973. They were caught by trawling approximately 5 miles due west of Aberystwyth harbour and at 10-13 fathoms depth.

However, lymphocystis was not seen on 100 plaice and dab taken in trawls from the western English Channel during a research cruise of RV 'Sarsia' in May 1975.

A2(b) Infection rates and the age of fish; Blenny and Cod

The data for the Cod and the Blenny were treated so as to determine a correlation of the infection incidence with age. Indices of fish growth related to age were used for this purpose. The blenny

Table 11: Distribution data for cod PEN in the North Sea, November - December 1975.

<u>Date</u>	<u>Trawling Station</u>	<u>Depth (fm)</u>	<u>Map Reference</u>	<u>Nos. Caught</u>	<u>Infected</u>		<u>Year Group</u>	
					<u>Nos.</u>	<u>%</u>		
28/11	65	37	57°00'N 00°53.5'W	14	0	0	-	
28/11	63	40	57°00'N 01°44'W	8	0	0	-	
29/11	32	67	58°47'N 01°04'W	11	0	0	-	
30/11	29	45	59°05.5'N 02°09'W	5	0	0	-	
30/11	7	22	58°11.8'N 02°49'W	7	2	28.0	0/1	
1/12	5	30	57°54'N 03°15'W	72	1	1.4	3	
3/12	11	60	58°26'N 00°51'W	2	0	0	-	
4/12	33	75	58°46'N 00°30'W	24	1	3.9	1	
4/12	45	72	59°17'N 00°58.5'W	13	1	7.7	2	
5/12	35	70	58°51'N 00°58'E	13	0	0	-	
5/12	36	60	58°39'N 01°11'E	14	0	0	-	
6/12	56	72	60°05.5'N 00°40'W	5	1	20.0	2	
6/12	58	58	60°42.5'N 00°39'W	61	12	19.7	0/1	
<b>TOTAL TOTAL</b>					<b>MEAN</b>			
=249					= 18	=7.3		

data were grouped into 6 year classes from year group 0 to 5, by fitting the length measurements done at the time of examination to the ranges of fish length for each year class given by Qasim (1957). The cod data was assigned to year classes from ring counts of sectioned otoliths.

The total number of fish in each year class screened positive and also the percentage positive are detailed (Table 12 & Fig. 5), showing two effects. Firstly, the frequency distribution of both

Table 12: Analysis of the infection rates in the Blenny and the Cod by year groups

<u>Year Group</u>	<u>Blenny</u>		<u>Cod</u>	
	<u>Nos. Taken</u>	<u>Infected</u> <u>Nos.</u> <u>(%)</u>	<u>Nos. Taken</u>	<u>Infected</u> <u>Nos.</u> <u>(%)</u>
0	167	9    5.6	22	7    31.8
1	210	13   6.2	94	10   10.6
2	131	2    1.5	65	1    1.5
3	61	2    3.3	36	0    0
4	32	0    0	22	0    0
5	29	0    0	4	0    0
	<u>TOTAL= 630</u>	<u>26</u>	<u>243</u>	<u>18</u>
NOT MEASURED & CLASSIFIED	87	6	6	-
	<u>GRAND TOTAL= 717</u>	<u>32</u>	<u>249</u>	

(See table 4 also)

population samples is similar and secondly both samples show positives only in the lower year groups. For cod, infected fish were found from year group 0 to 2 and for the Blenny from 0 to 3. For both species the first year class is the mode but when the positives are expressed as percent. the plots of infection rate against year group are markedly different (Fig. 6). Cod shows a maximum of nearly 32% for year group 0 declining to a minimum in year group 3 whereas the Blenny shows only low values in all year groups. These data

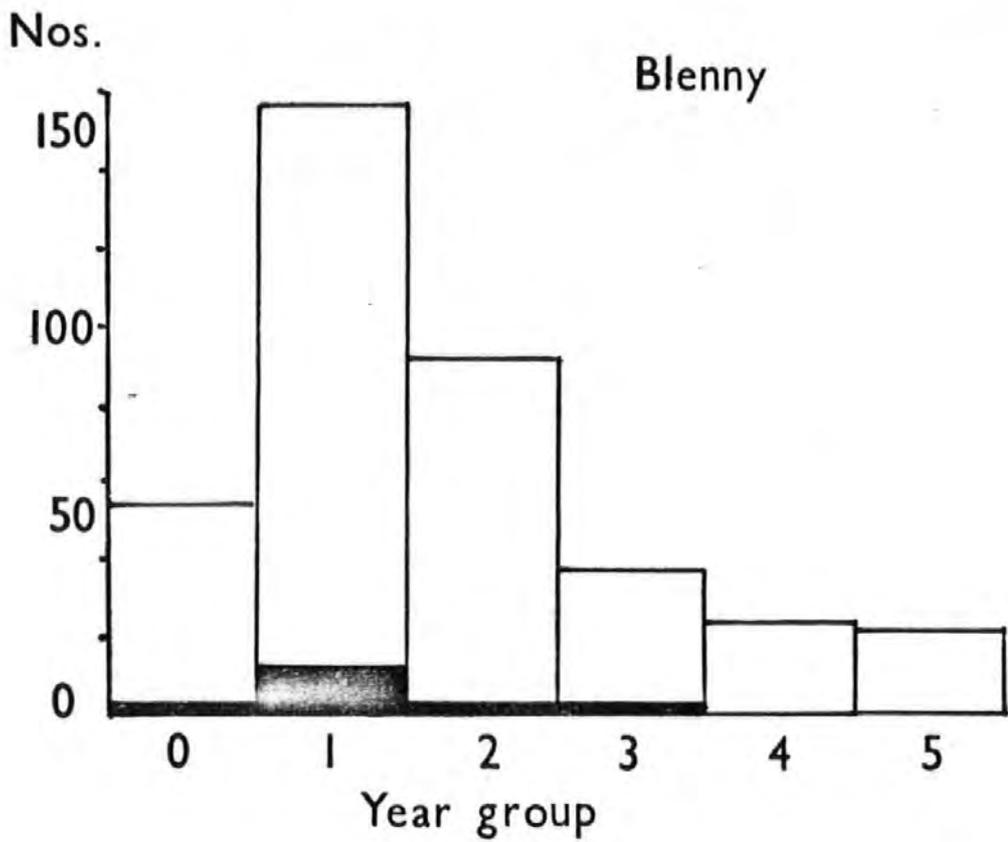
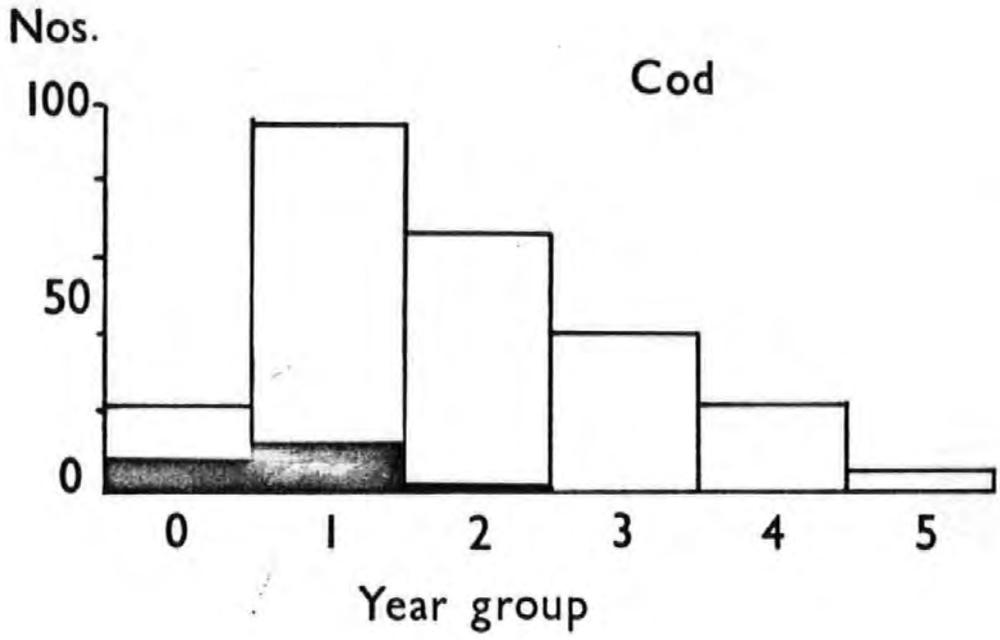


Fig. 5. Year group distributions of cod PEN and the blenny infection. (Black bars - infected, white - uninfected.)

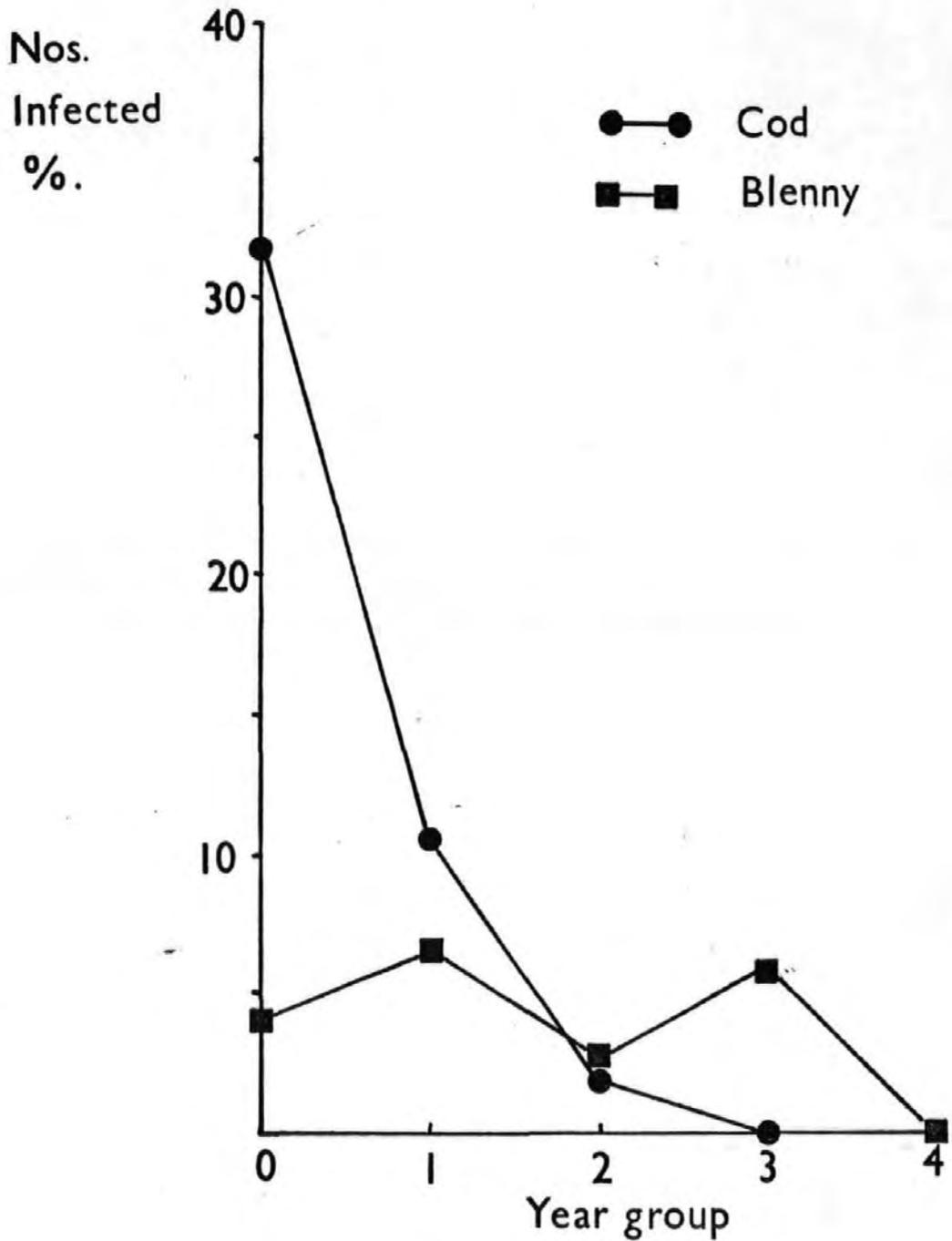


Fig. 6. Plot of infection incidence against year group for cod PEN and the blenny infection. Incidences are from total collection data.

suggested that in the Blenny the infection rate deviated little from the mean value, 4.5%. It seemed to vary with the sample size for each year group and this assumption was tested by applying chi-squared for significance of deviation of the two sets of year group data from the mean infection rates, respectively 4.5% for the Blenny and 7.3% for cod. For the Blenny, chi-squared=2.86 at  $f=1$  giving  $p=0.10$ . It was quite likely therefore the small deviation from the mean was due to random sampling and the effect of sample size. For cod, chi-squared =16.2 at  $f=2$  giving  $p<0.001$ . It was extremely unlikely therefore the deviation was random, rather it showed bias towards positives in year groups 0 and 1.

### A3 Degree of virus infection

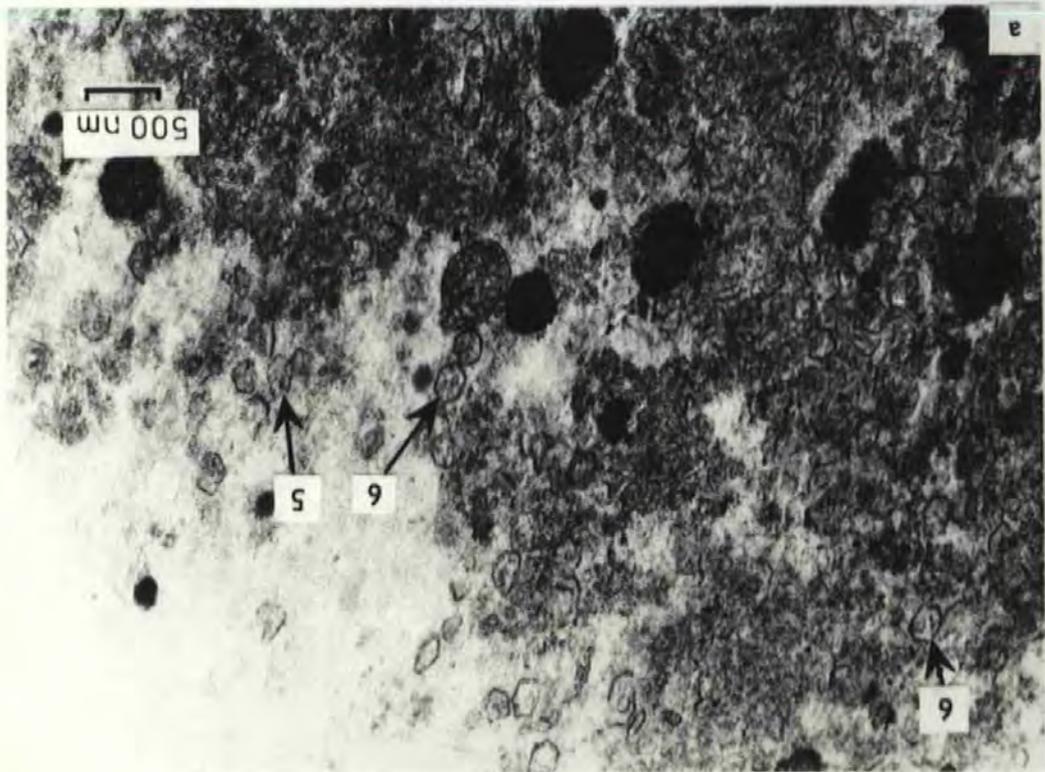
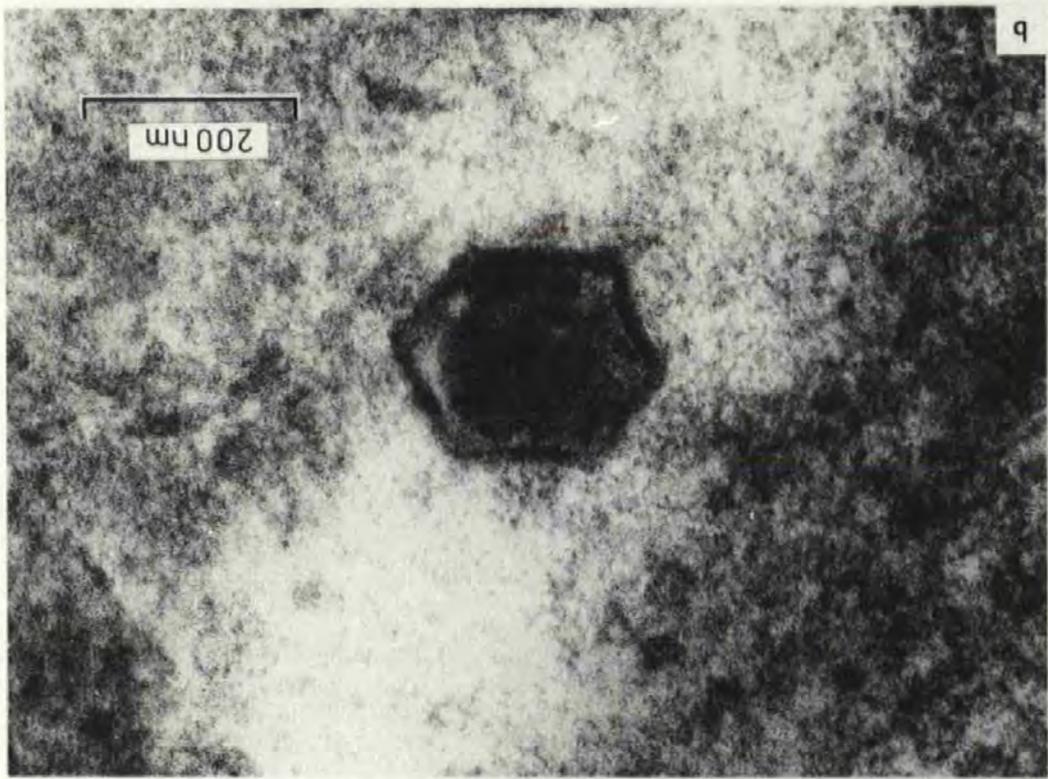
The level of infection could be estimated from ultrathin section evidence. Lymphocystis isolate 3ii showed virus particles closely spaced throughout the cytoplasm although ordered semi-crystalline array was not observed (Plate 12a). By contrast, particles were seen in blenny erythrocytes only in a discrete area of the cytoplasm and the maximum number seen in one section was ten (Plate 13a).

Limited evidence from the electron microscopic examination of the tissues of one wild caught fish suggested the blenny infection might be distributed outside the circulating blood. Thus in one wild caught infected fish sacrificed three days after capture, the spleen and liver showed virus particles present in erythrocytes (Plates 13a, 14a & 14b) but particles were not observed in the kidney.

## Plate 12

Electron micrographs, sectioned lymphocystis isolate 3ii (plaiice).

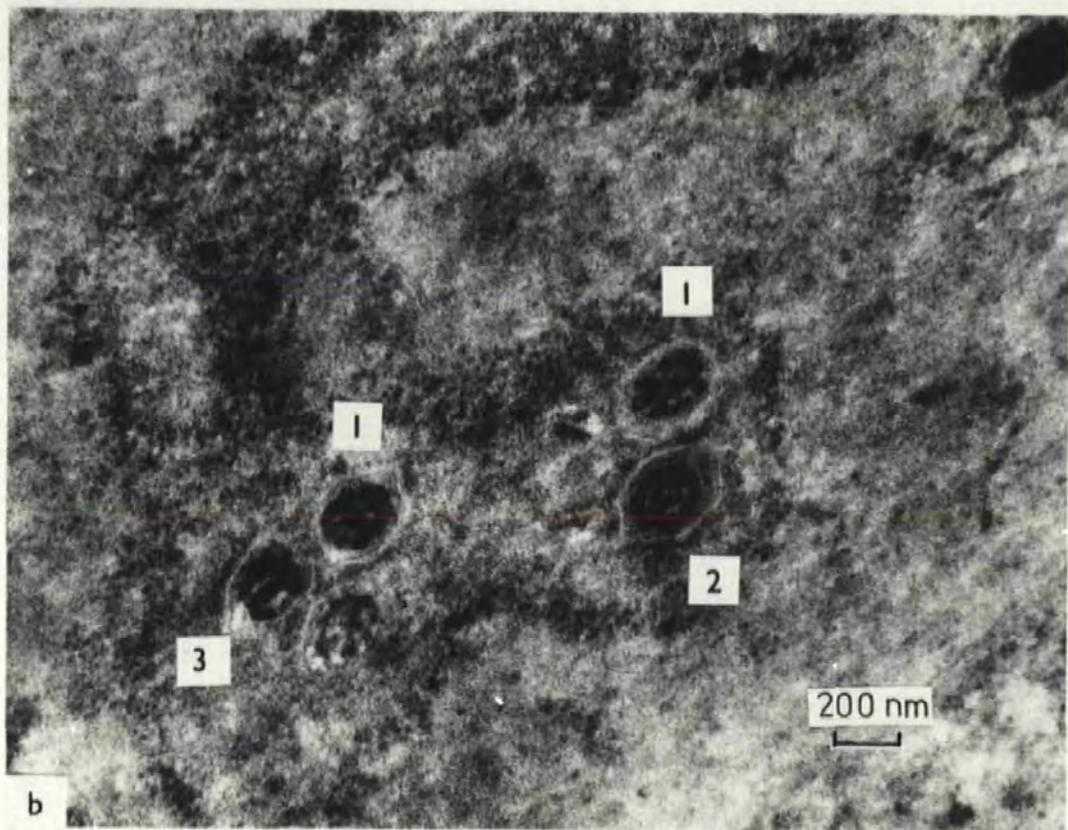
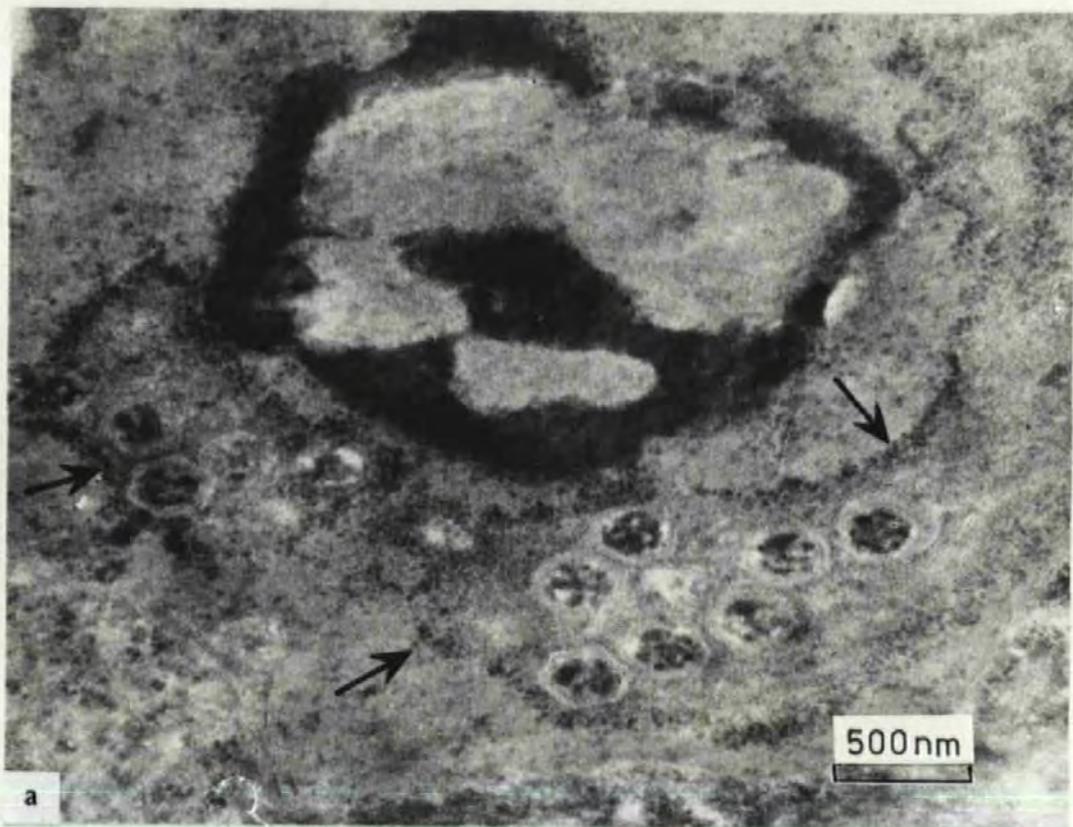
- (a) Six-sided and five-sided profiles are seen (arrows 6 & 5) and particles at top centre show internal threads.
- (b) Hexagonal capsid outline in mid-planar section. The electron-dense core occupies most of the internal space.



## Plate 13

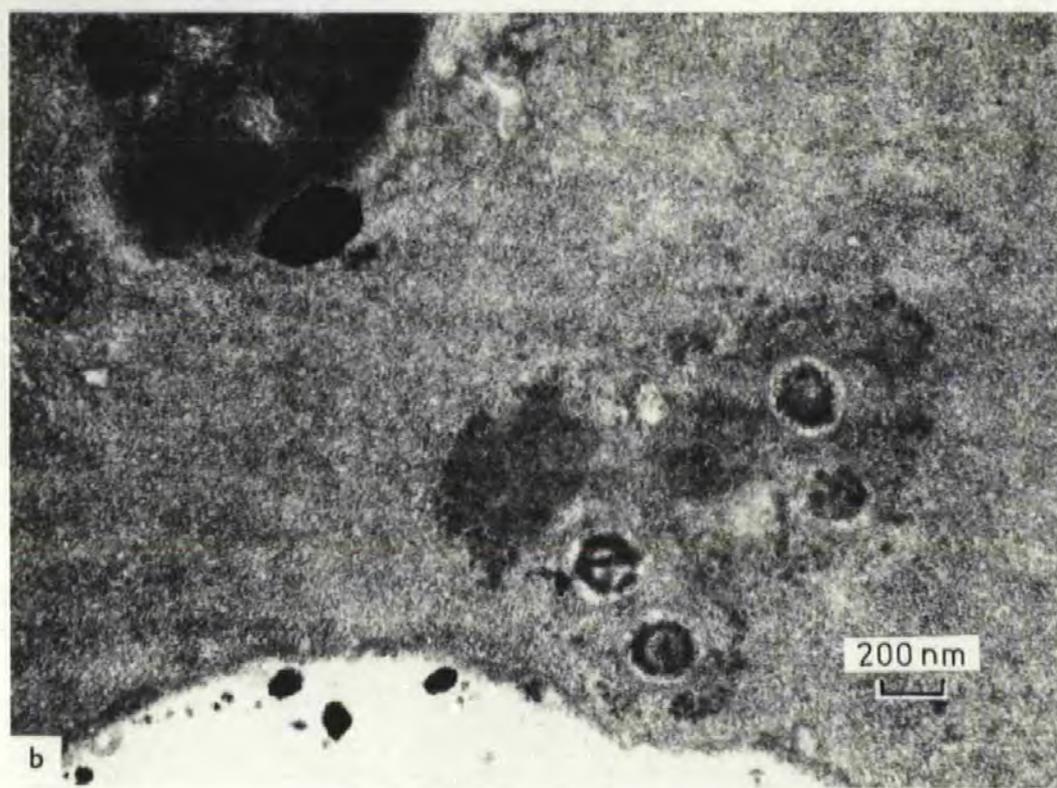
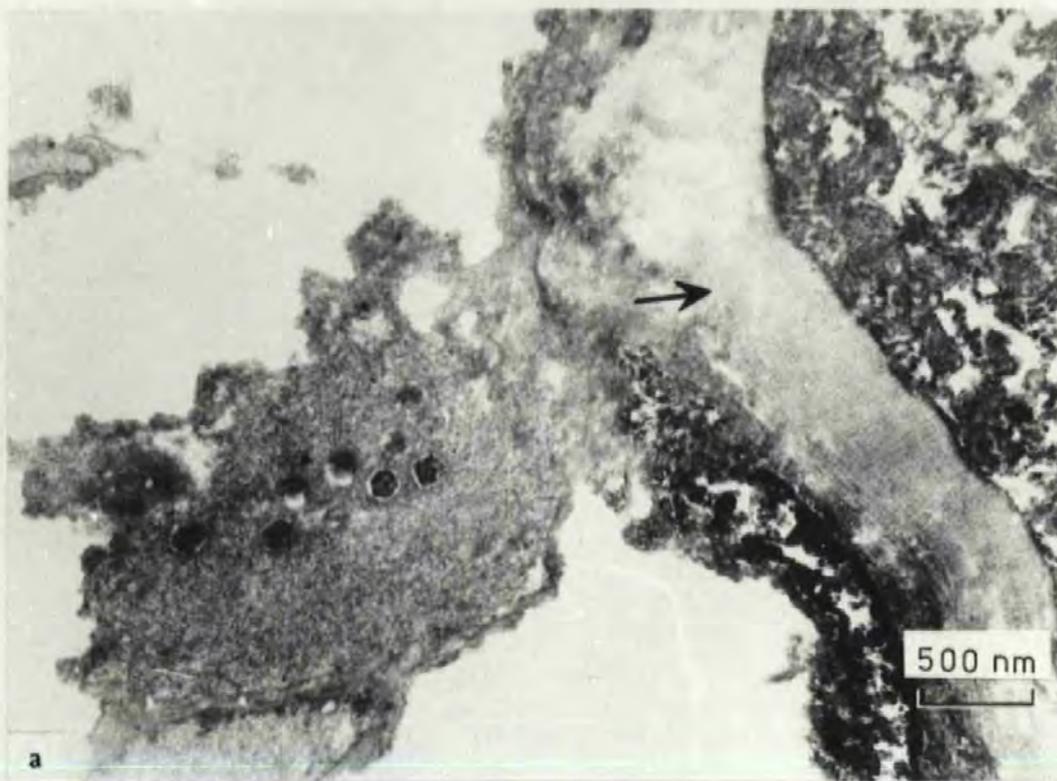
Electron micrographs, erythrocyte, sectioned spleen of a naturally infected blenny.

- (a) Virus particles are situated in an area of fine granular cytoplasm, which is bordered by a row of electron-dense granules 20 - 30 nm diam. (arrows).
- (b) The area of granular material containing the virus particles. Different staining patterns of the virus core are seen (labeled 1, 2 & 3).



## Plate 14

- (a) Electron micrograph, sectioned liver venule, naturally infected blenny. The lumen contains an erythrocyte with cytoplasmic particles and the wall consists of inner indistinct intima and media layers and an outer adventitia layer of collagen fibres (arrow).
- (b) Electron micrograph, erythrocyte, sectioned spleen of a naturally infected blenny. Cytoplasmic particles of 200 - 250 nm diam. are seen in an area of more electron-dense material.



#### A4 Effects on the host

Observations and limited collection data indicated that the blenny infection and PEN in the Cod were not correlated with poor general conditions of the fish. However, by contrast some lymphocystis-diseased fish bore a large number of ectoparasites and were in poor general condition, particularly when heavily diseased.

##### A4(a) The Blenny infection

Infected blennies showed no external symptoms of disease. With respect to their respiration, P. Milton of Plymouth Polytechnic (pers. comm.) showed that the oxygen consumption of an infected blenny was the same as that of an uninfected one. The methods used were those of Milton (1971); fish were starved for 12 hr and acclimatized for 2 hr to the dark conditions in which the oxygen uptake was measured.

##### A4(b) PEN in cod

The ranges of length and weight of infected fish were within the ranges of these measurements for the uninfected fish and did not suggest the infected fish were in poor condition (Table 13). However, in year classes 1 and 2 the ranges of weight and length of the infected fish were at the lower end of the range for the uninfected. A condition index, such as  $\text{weight}/\text{length}^3$ , was not calculated for the reason that the weights were measured to the

Table 13: Parameters of size for cod at different ages correlated with PEN

<u>Year group</u>	<u>Weight range (lb)<sup>±0.5</sup></u>		<u>Length range (cm)<sup>±0.5</sup></u>	
	<u>PEN+ive</u>	<u>PEN-ive</u>	<u>PEN+ive</u>	<u>PEN-ive</u>
0	1-2	1-5	33-42	33-54
1	1-6	1-10	33-59	30-76
2	(8)	3-15	(72)	50-84
3	(14)	5-22	(83)	62-96
4	-	11-27	-	78-103
5	-	11-23	-	76-102

(Brackets indicate a single record only)

nearest pound on a torsion balance, so such an index for comparison of the smaller fish in particular would have been meaningless.

#### A4(c) Lymphocystis disease

Lymphocystis-diseased fish bore a large number of ectoparasites, especially the flounders in which the copepod Lepeophtheirus pectoralis (Muller) was found behind the pectoral fin but also over the entire surface of the skin. Many hundreds were observed on the fins and gills of infected flounders sent from Fleetwood but interestingly the parasite was also seen on the skin of healthy flounders obtained from the MBA, Plymouth. The parasites were removed from those flounders used in transmission experiments.

### Recognition

The blood infections were recognised by the same screening method as that of previous authors (Laird & Bullock, 1969; Johnston & Davies, 1973) and the same clear cytological changes in red blood cells of the Blenny and the Cod could be recognised.

In several ways, the cytological picture of the blenny infection differed to that described by Johnston & Davies (1973). Firstly, the range of diameter of the inclusion was smaller being 1-1.5  $\mu\text{m}$  rather than 1-4  $\mu\text{m}$  and secondly the level of infected erythrocytes was from 3-60% compared to 60-80%. Thirdly, it was found that the appearance of the infection depended on the type of staining method used. Thus using the slow staining method of Busby *et. al.*, (1964), which involved incubation at 37°C overnight, a cloud of fine granules was resolved close to the inclusion bodies, whereas this was never seen when the standard fast method was used, performed at room temperature. Fourthly, some occasional infections were observed where the nuclei were clearly vesiculated and lobated (Plate 8a) the infection involving a greater degree of nuclear lysis than described by the previous authors.

Partial explanations can be offered for these differences. An important factor to be considered is that Johnston and Davies (1973) reported only blood infections from the Blenny collected at Aberystwyth whereas the remarks here refer to all the localities examined. This might explain why the Aberystwyth infection in 1971 showed such a high level of infection and a great range of inclusion size, as characteristic of the local infection. However, the data here also included a total of seven collections made at Aberystwyth in 1974 and the high level of infection was not observed. Regarding the difference with staining methods, it is most likely that the manner of staining does affect the resolution obtained, as material from the

same specimen was used for both fast and slow methods.

It is difficult also to know if the nuclear lysis observed in several cases is a natural and concomitant part of the infection. It could be that it is an unusually late stage of infection and then it closely resembles PEN in cod. However, it can only be said to approach the advanced nuclear degradation seen in PEN, and cannot be equated with it. The limited nature of this evidence and the fact that the nuclei in normal blenny erythrocytes are sometimes seen with strands of chromatin at their edges, would also favour the disassociation of the two infections.

Fluorescence staining of the smears with DAPI provided evidence, additional to that with acridine orange, that the inclusions and hence the virus particles, contain DNA. Green fluorescence with acridine orange cannot be regarded as being truly indicative of DNA in some viral infections (Kasten, 1967), but it is probable that DAPI is a more reliable stain for DNA, as Russell *et al.*, (1975) recommended its use with cell cultures for the detection of cytoplasmic DNA due to contaminants, where an absolute specificity for DNA was required. Furthermore, in uninfected control blenny smears no cytoplasmic fluorescence was seen indicating that no compounds other than DNA could have been stained.

The observation of the spontaneous occurrence of the blenny infection in captive fish suggests that it might be induced as a result of conditions of stress including increased temperature. This observation is interesting in relation to indirect evidence from PEN infections that stress factors are correlated with increased incidences of infection (Sherburne, 1973; Sherburne, 1977). In the Atlantic Herring for example the incidence of erythrocytic degeneration, shown later to be PEN, increased rapidly in captive fish held for 2 weeks at 16°C, from 6.6% to 96% of herring. The collection data on wild caught fish also showed that the infection was recorded only at a

seawater temperature of 13.8°C and above, with one exception (Sherburne, 1973). In the Alewife, distribution data on the incidence of PEN suggested that stress factors of spawning migration might be causal, since the infection was found predominantly in pre-spawning adults (Sherburne, 1977). Although the evidence for induction of the blenny infection was fortuitous the likely factors in the possible induction of the blenny infection resemble those for PEN infections mentioned above.

The circumstantial evidence gained in following the progress of the natural blenny infection in the laboratory would suggest the time course of infection is dependent on stress factors including temperature extremes and possibly other environmental variables. However, the evidence for high temperature clearance of the blenny infection cannot be corroborated with similar evidence from PEN infections.

The tentative suggestions from this kind of evidence should be qualified by saying that it is not strictly experimental and is therefore of limited implication. However, the main point is that recognition became unreliable under artificial conditions due to unregulated complicating factors. Elucidation will require firstly the correlation of ambient temperature with infection incidences to give clear ecological data and secondly proof of the experimental role of temperature under conditions of reduced stress i.e. maintenance in glass aquaria at low density.

The light microscope description of PEN in cod from the east American coast has been noted by a number of authors (Laird & Bullock, 1969; Walker, 1971; Appy et al.<sup>1976</sup>, Walker & Sherburne, 1977). Each have described a common cytology in infected cells; namely an early stage where the nucleus shows vesiculation with a pinkly-staining inclusion body clearly visible, a middle stage where the

nucleus appears lobulated, the 'crests' or 'arms' connected by fine strands and<sup>a</sup> late stage where the nucleus is fragmented in up to 6 small granules. All these cytological figures were recognised in smears from infected cod taken in British waters and it can be concluded that the infection examined is definitely PEN.

The level of infection in blood cells ranged from <1-50% and was very comparable to that in the blenny infection, 3-60%. However, Walker & Sherburne (1977) noted the range of level of infection in the Cod from <0.01-99%.

The composition of the inclusion body was suggested at the light microscope level. In smears of PEN prepared by S. Sherburne of the Fisheries Research Station, West Boothbay Harbour, USA, examined at high magnification in Walker's laboratory, a cloud of particles could be clearly resolved around the inclusion body. Groups of granules were described by Laird & Bullock (1969) and Walker & Sherburne (1977) concluded the granules were virions and the inclusion body was viroplasm i.e. a multiplication and maturation site.

Particles were not seen at this resolution in my own smears of PEN from British waters. Obviously a number of factors could have accounted for this e.g. resolution of the microscopes, differences in staining methods, differences in stage of infection and possibly real differences between the infections.

At this level there is a need for cytochemical staining of PEN, firstly to identify whether the inclusion body and the particle cloud contain DNA and secondly to map the distribution of this staining at various times after infection. There would be the difficulty to overcome of distinguishing the inclusion body from nuclear granules by fluorescent staining. Early infections would be most relevant or an experimental system in which no nuclear lysis were involved. The need for histochemical staining has also been mentioned by Reno

et al. (1978), in discriminating the two inclusion types in PEN-infected herring erythrocytes.

Recognition of lymphocystis disease was complicated by factors that are discussed here, e.g., should freshly infected nodules or older tumours be used for extracting the virus?

Studies on the course of the disease in the Bluegill have shown that maturation of the infected cell, with accumulation of virus particles, takes 28 days (Wolf, 1962) and this result has been corroborated by in vitro studies (Midlge and Malsberger, 1968). After this time the cells or nodules degenerate and the effective virus concentration declines. It is reasonable to infer that the different stages of infection, observed in the flatfish received from Fleetwood, i.e. individual nodules ranging to large necrotic tumours, probably contained different concentrations of virus particles. No direct evidence, in the form of a quantitative estimation of particle numbers was available to test this hypothesis but this would merit further investigation.

Cytological confirmation of the lymphocystis condition in flatfish was not used. This was an error in view of the number of reports of skin tumours in flatfish caused possibly by viruses other than lymphocystis or by environmental factors. For example, Wellings and co-workers have described three hyperplasic skin lesions in pleuronectid species from the Californian coast of North America, namely angioepithelial nodules, polyps and epidermal papillomas (Wellings, Chuinard & Cooper 1967; McArn et al., 1968).

Lymphocystis infected fish can also bear other lesions with which the true lymphocystis tumour might be confused. For example, ulcers have been described on lymphocystis infected flatfish from British waters (Shelton & Wilson, 1973b). A dermal sarcoma (WDS) was described in the Walleye (Walker, 1958); this tumour can be found on walleyes with lymphocystis and the two tumours may be confluent

(Yamamoto et al., 1976). It was noted that in some fish the two tumours could be recognised externally, the lymphocystis tumours being irregular in size and shape while the dermal sarcomas were paler and more spherical. However, their cytologies were clearly distinct, sections of WDS showing a disorganised arrangement of cells in a tumour mass. WDS has also been associated with a possible RNA virus of 135nm diam., smaller than lymphocystis virus.

Although this finding of confluent tumours in the Walleye raises the objection of  mixed viruses in the tumour sample, other grounds favour the conclusion that the tumours sampled on flatfish were lymphocystis. Thus confluent DS has not been recorded on flatfish of British waters, the tumours on flatfish from Fleetwood were not recognised externally as mixed in type and the virus from these tumours in <sup>ultrathin</sup> section was the typical dimension of lymphocystis virus.

It was interesting to find a lightly infected plaice in a batch for sale. Selection in nature against the disease probably operates via the grossly reduced cryptic colouration of infected fish but fishing probably exerts a selective advantage as heavily infected fish are usually thrown back. The additional handling and netting of such fish no doubt also contributes to the spread and maintenance of lymphocystis disease.

#### Collection Data

The collection data described indicate that the blenny infection is distributed outside Aberystwyth shores and the regularity of finding it at 5-10% incidence suggests it is at the level of an established infection. Clearly some patchiness occurred in the distribution data but the statistical treatment clearly showed this was explained by random sampling and differences between the total numbers of fish caught on different shores.

Preliminary conclusions can be drawn from the collection data on cod PEN and comparison made with the results of Walker & Sherburne (1977).

The data showed that the infection was present in cod in the North Sea off northern Scotland at a mean level of 7.3% and this is very comparable to the infection rate in the Blenny, 4.5%. By contrast, Walker & Sherburne (1977) found higher mean incidences in cod from three sites off the North American coast (11-16%).

Since the stations were widely distributed, it could be concluded that the infection was not localized since the furthest stations where the infection was found (5 & 58) were separated by approximately 180 miles (Plate 5). Likewise the station samples of Walker & Sherburne were widely spread.

The data for the North Sea collection showed variation between individual station samples in common with the results of Walker & Sherburne (1977). Under half of the station samples gave positives (6/13) compared to 12/25 for the George's Bank collection of Walker & Sherburne. However, some samples gave incidences above the mean, e.g. 12/61-19.7% and 2/7-28%; similarly, Walker & Sherburne found sample incidence up to 44% (11/24). PEN was found in station samples taken in depths of 22-75 fathoms and therefore it was not possible to corroborate Walker & Sherburne's finding of a higher incidence at 55 m on the George's Bank.

In view of these new geographical records, detailed surveys of the North Sea and Celtic Sea would provide useful information i.e. for PEN not only in cod, on account of its economic importance but also in other ~~demersal~~ demersal ~~fish~~ fish which are predated by cod and thus transmit PEN. The surveys would require large samples and closely spaced trawling stations, say on a grid of 10 mile squares. It would also be interesting to compare the incidence of PEN in cod from offshore waters i.e. more than 60 miles from the

coastline, to that in cod from nearshore waters, as evidence from tagging studies at Aberdeen suggests that there are discrete cod stocks in the nearshore and offshore, between which there is little interchange of individuals (Dept. Ag. Fish. Scotland - Dir. Fish. Res. Rep., 1970).

Lymphocystis disease is now recognised in marine fish taken outside the north-east Irish Sea. For example, Shelton & Wilson (1973b) described a 10.5% infection of flounder in Rye Bay and it was therefore not surprising to find it in Cardigan Bay in plaice and dab though at a lesser level. Since it was not observed in flatfish trawled on sandy banks but in exposed waters, e.g. the Jones Bank, western English Channel, it is likely that the disease is somehow enhanced in sheltered waters and bays. The reason for this correlation is necessarily speculative; sharp-edged gravel in sediments could cause abrasions of the skin permitting infection and the sheltered conditions permit the survival of sloughed lymphocystis cells in the sediments.

Infection rates and age

In short, data from the Blenny did not show correlation but data from cod did, since first and second year groups were predominantly affected. In other words the mature fish are not so susceptible to infection, a possible reason being that they gain active immunity to the infection after or during the second year. Appy & Burt (1975) recorded the same trend since cod over 70 cm length i.e. of the third year, were not found positive whereas the infection was predominant in the first and second year groups. They also found the average proportion of red cells infected was greater in young fish than old i.e. 47% versus 9% and suggested that either heavy infections are fatal to young cod or that the causative agent of PEN subsides with increasing age. By contrast, Walker & Sherburne (1977) noted no

### Degree of virus infection

The ultrathin section evidence gave the strong impression that the blenny infection and lymphocystis attain different quantitative levels of infection. In the Blenny, where ten or so particles were observed in the cytoplasm in section, this could represent no more than 100-200 particles within the area of viroplasm. This assumes that the inclusion area is approximately 2  $\mu\text{m}$  diameter and the best ultra-thin section of 60 nm thickness. If the virus is of 200-250 nm diameter then approximately 10 rows of stacked particles could be arranged together. By contrast, lymphocystis virus achieves enormous multiplicity of infection, this observation being corroborated widely. Thus in the bluegill fibroblast cell line, it has been shown that the infected cell contains  $10^5$  virus particles (Wolf et al., 1966) and in the Sunfish virus particles have been reported as being in crystalline array, evidence also of high concentration (Walker & Wolf, 1962).

### Effects on the host

The blenny infection and cod PEN were entirely devoid of external signs and in this respect they resemble the majority of infections of erythrocytes in amphibians, reptiles and fish where icosahedral particles have been identified. However, some associations with signs have been recorded. Walker (1971) mentioned that "one of the fish with PEN was moribund", though it was not implicit that this was as a consequence of PEN. Fletch and Karstad (1968) also reported that the Garter Snake (Thamnophis sirtalis), which showed an unclassified intra-erythrocytic parasite was examined as a result of lethargic behaviour and mortality. Sherburne (unpublished data) also mentioned in Walker & Sherburne (1977) that PEN-infected cod are usually darker than non-infected in wild and captive specimens. Despite the absence



blood smears from the Blenny and Cod warrants the term PEN for cod as originally coined by Laird & Bullock (1969) but that PEN should not be used for the blenny infection, as advanced necrosis of the nucleus has never been observed. However, it may be that further study of the infections will show there to be a greater range of cytological configurations and these distinctions too hard and fast. As Sherburne (pers. comm.) has mentioned, "although we initially thought that a visible cytoplasmic inclusion was associated with a characteristic nuclear pattern it is now evident that some cod have only the cytoplasmic inclusion without the characteristic nuclear pattern or vice versa."

At the cytological level, there is good reason for thinking that lymphocystis disease is dissimilar to both the blenny infection and PEN in cod, since in lymphocystis very unusual hypertrophic changes of the cell are involved with massive accumulations of cellular DNA and RNA in the absence of cell division (Lopez et al., 1969).

SECTION B

ATTEMPTED PROPAGATION OF THE VIRUSES  
AND RELATED RESULTS

(1) Method for treatment with APH to produce anaemia in the Blenny  
(Grasso, 1973).

9 fish were acclimatised to the tank conditions by holding at room temperature for 3 days prior to inoculation. Each fish was weighed and inoculated with a suitable volume of an aqueous solution of APH to give a dose of 0.1 mg or 0.2 mg per 10 g body weight, respectively dose 1 & 2. 3 fish were inoculated at each dose and 3 also with sterile saline as controls. The dose for experimentals was repeated on day 1. A sample of blood was taken, a blood smear made from each fish at day 4, 8 and 12 and the red blood cell count measured by the method of Korzhuev (1962) using a diluting pipette and a counting chamber with a Neubauer ruling.

In smears mature erythrocytes were distinguished from immature ones by eye on the basis of the staining pattern and also the nuclear-cytoplasmic ratio. Thus the cytoplasm of mature red cells stains light blue whereas that of immature cells stains dark blue. The nuclear-cytoplasmic ratio is greater in immature cells than mature cells also and the cell shape is generally round and not oval.

(2) Maintenance of blennies at elevated temperature

6 blennies were screened as uninfected. 3 fish were held in 1 cu. ft. of seawater at 9.5 - 12.5°C and 3 in the same volume at 24.5°C. A blood sample was taken for a smear on two occasions from each fish.

(3) Inoculation procedures for tissue & cell cultures

Expt 1. 0.2 ml amounts of both the unfiltered and filtered lymphocystis inocula were used (respectively inocula 1 & 2 - see Materials).  
to be inoculated  
The medium was taken off the cells and 0.2 ml of lymphocystis inoculum or 0.1 ml of the erythrocyte inoculum added carefully over the semi-confluent cell sheet. The flasks were incubated for 40 min at room temperature, fresh maintenance medium added and returned to 15°C.

Expt 2. 0.2 ml of lymphocystis inoculum 3 (see Materials) was added to one flask and 0.2 ml of Hanks balanced salt solution to another

94  
as a control. The flasks were incubated 1 hr at 15°C and maintenance medium then added.

Expt 3. 0.5 ml lymphocystis inoculum 4 (see Materials) was added to the cell sheet, gently agitated, left on at room temperature (20-25°C) for 1 hr, after which maintenance medium was added. 0.3 ml of erythrocyte inoculum and 0.2 ml of control inoculum was added to 2 other flasks and these incubated for 1 hr at 20°C, after which maintenance medium was added.

(4) Preparation and maintenance of skin organ cultures

- (i) The flatfish were anaesthetised by immersion in a lethal dose of benzocaine in seawater which was 2-10 g per litre of seawater. They were then killed with a blow to the head.
- (ii) The mucus was removed from the surface of the skin as far as possible by washing in seawater.
- (iii) The skin surface was gently wiped with a 'Mediswab' anti-bacterial pad to reduce surface contamination.
- (iv) Blocks of skin 3 mm square and approximately 3 mm thick were cut with a sterile scalpel blade.
- (v) The skin blocks were washed 3 times for 5 min. each in 'Hanks washing solution' with 3 times the normal concentration of antibiotics.
- (vi) Fine scratches were cut on the base of the culture dishes to form a grid platform. The skin blocks were carefully sited on the grids, and tris-buffered maintenance medium added to cover (see Appendix). The dishes were contained in unsealed boxes, incubated at 15°C and the medium changed every 2 days but not harvested.

To assess the maintenance of the cultures the skin blocks were then processed by conventional histological techniques. The skin blocks were processed for light microscopy as follows. The blocks were washed in PBS and fixed in 10% neutral buffered formalin at least 24 hr. They were then dehydrated in an automatic tissue

processor (Bucke, 1972 - Appendix II), infiltrated in 56°C. m.p. wax  
negative  
for 30 min under pressure and embedded in fresh paraffin wax.

Sections were cut on a sledge microtome and stained with Haematoxylin and Eosin.

Skin blocks were processed for electron microscopy as in Method C of Section C with the exception that the times for embedding differed as follows:

1:3 resin/ethanol mixture	—2 hours
2/3 " " "	— overnight
Pure resin	—24 hours
Pure resin	—24 hours

A hard resin mixture was also used i.e. ERL 4206 12g, DER 735 4g, NSA 23g, S-1 0.4 ml.

#### (5) Inoculation of organ cultures

24 hr after the explanting of skin blocks to dishes, the skin surface was lightly scarified with a scalpel blade, the maintenance medium was removed and 0.2 ml of an inoculum prepared from lymphocystis isolate 5 gently overlaid on the surface of each skin block. The inoculum was incubated 30 min. at room temperature and then sufficient medium added to cover each block.

In one experiment 6 dishes containing 3 skin blocks each were so inoculated and 6 dishes left as uninoculated controls. 1 experimental and control dish were sampled on day 0 and at days 5, 10, 15, 20 & 25 post-inoculation. The skin blocks were then processed for light and electron microscopy as described in Methods (4) 'Preparation and maintenance.....cultures'.

(6) Basic procedure for preparation and maintenance of fish primary cell cultures

The method used was based on the recommendations of Wolf & Quimby (1969).

killed with a blow to the head.

(i) The fish was anaesthetised and  $\lambda$  It was then bathed in 1% 'Chloros' solution 15 min., dried and the skin swabbed with 70% ethanol.

(ii) The internal organs were dissected out as follows. The pectoral fin was cut off from one side and the body wall was cut vertically behind the shoulder girdle. A circular cut was continued around the edge of the body cavity and the skin flap removed cleanly without touching the internal organs.

for cell culture such as the gonads, spleen and liver (Table 14)

(iii) The organs useful  $\lambda$  were then teased out and washed three times in 'Hanks washing solution' with high antibiotics, after which they were weighed.

(iv) The tissues were minced finely with scissors and the tissue fragments treated with trypsin three times to give individual cells

and cell clumps for culture. 10 vol. of 0.25% trypsin solution were added to the minced tissue and incubated 30 min. at 15°C with stirring. The large tissue pieces were allowed to settle and the supernatant decanted and discarded. The remaining tissues were trypsinized twice and the trypsin solutions kept.

(v) The cells were removed from the action of trypsin by sedimentation at 100 g for 10 min and resuspended in growth medium using a volumetric dilution of 1/100 to 1/400.

(vi) The cells were dispersed in suspension and 25 cm<sup>2</sup> culture flasks seeded with 5 ml aliquots. At the time of seeding 2 ml aliquots of the growth medium were incubated at 37°C as contamination controls.

The flasks were observed every 2 days and a note kept of the type of cells growing and their degree of confluence. The pH of the medium was checked and if too acid adjusted to near neutrality by loosening the culture flask caps under sterile conditions. Contamination was also monitored and if a flask showed cloudy medium an attempt was made to recover the cells. The medium was immediately removed, the cells treated for 1 hr with growth medium with twice the normal concentration of antibiotics and neomycin or kanamycin added to the medium at 25-50 µg/ml. Gentamycin at up to 60 µg/ml was also used.

When cells attained 75-90% confluence they were passaged at 1/2 or 1/3 ratio. 0.2 ml of trypsin/versene solution was used to take the cells off the plastic and, if required, the flask surface scraped with a small 'rubber policeman' held on an angled Pasteur pipette. The cells were suspended in 10 ml growth medium and seeded to new flasks.

## RESULTS

This section records attempts to propagate lymphocystis virus and the blenny infection by cell culture (B1) and organ culture (B2). Experiments relating to the induction of the blenny infection are described (B3) viz. the effect of high temperature and APH in the Blenny, APH having been shown to cause anaemia in the newt Triturus cristatus (Grasso, 1973).

### B1 Cell culture

Attempts were made to derive primary cell cultures from the Blenny (9 trials) and also from different flatfish, with the object of propagating both the virus of the blenny infection and lymphocystis virus. The results describe the attempts to derive primary cultures under methodology (B2a) and the negative inoculation results using these cultures (B2b).

#### B1(a) Establishment and maintenance

Standard techniques for the dispersion of tissue and the culture conditions of Clem et al. (1961) were used to derive cell cultures from the Blenny, Flounder, Plaice and Dab (Table 14).

For plaice and dab cultures ovaries alone were invariably selected for the derivation of cells and ideally immature ovaries. For flounder, it was possible to make a comparison of ovarian and non-ovarian tissues for growth. Thus 3-5 days was the normal time to confluence for cultured cells from immature ovary, whereas minced tissues of the testes or a mixture of organs such as the heart, spleen and liver did not give rise to a vigorous outgrowth of cells and confluent cell growth was not reached.

The cells observed in primary cultures were mostly fibroblast in type, especially those from dab (Plates 15 & 16), although clusters of epithelial-like cells were observed in mixed cell-type cultures from the Blenny. Cultures derived from mature ovaries of dab also showed eggs in which the nuclei were clearly seen (Plate 16a).

The pattern of cell growth was similar for cell cultures from different fish though the growth rate varied. Cells grew out from islands of tissue fragments or cell clumps and a confluent cell sheet was eventually formed. The growth time to confluence varied from 3-5 days for flounder cultures, up to 14 days for dab and plaice and 28 days for blenny.

All the cultures derived were short-lived and no established secondary cultures were originated. Cells from flounder were not passaged, some cell cultures from the Blenny were passaged at most once and cells from plaice and dab were passed at most three times, using a 1/3 splitting ratio. Only the dab and plaice cells were therefore used for inoculations.

An important limiting factor in the development of these cultures was contamination. Bacterial contamination was common in the four different trial cultures and in some cases this could be removed permanently by the use of broad spectrum antibiotics such as kanamycin and gentamycin. However, if these compounds were used for over 6 days the cells stopped growing. Fungal contamination was particularly prominent in the flounder culture and this took the form of a fan-like branching thread composed of oval cells. It grew in the culture despite the presence of mycostatin in the medium at 50 µg/ml.

A more detailed study to establish cell cultures from different organs of the Blenny was undertaken. Many variables in the dispersion of the tissues and in the growth conditions affected the results

⊛ This yeast was most probably a species of Monilia (family Moniliaceae -Fungi Imperfecti)

**Table 14:** Origination of primary cell cultures from marine fish.

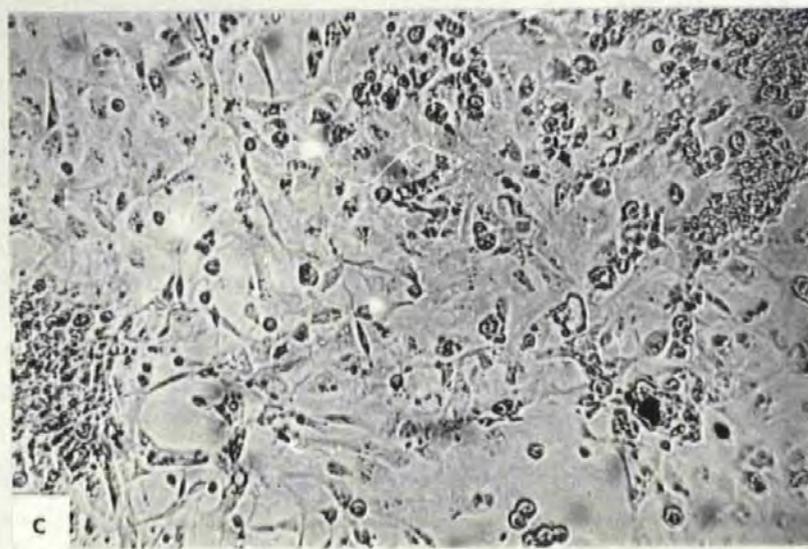
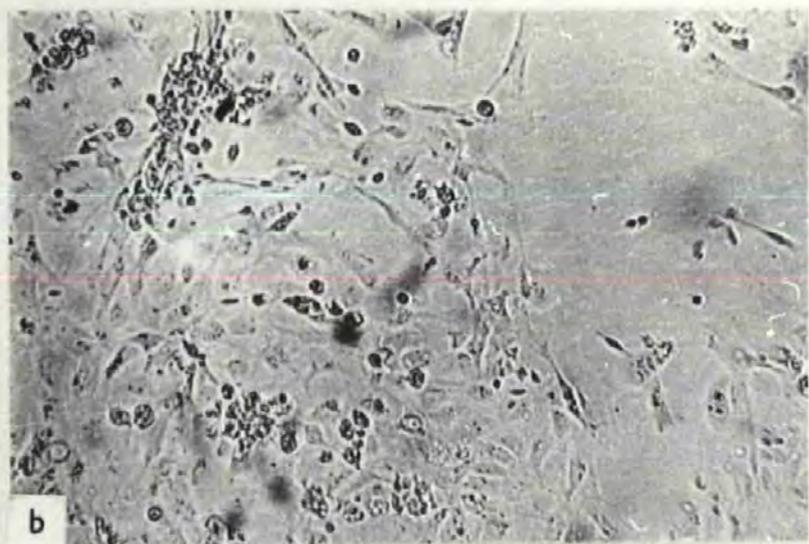
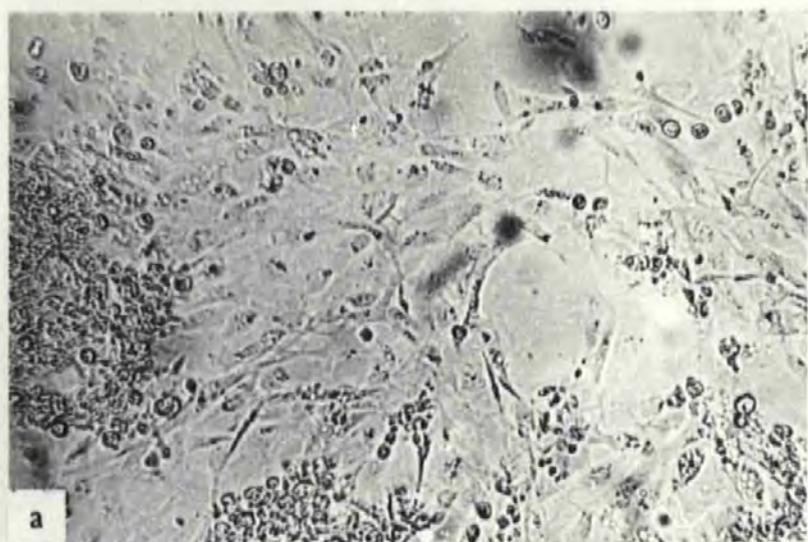
	<u>Blenny</u>	<u>Flounder</u>	<u>Plaice</u>	<u>Dab</u>
Organs taken	Gonads†, heart, spleen & liver (see Table 15)	Immature ovary <sup>+</sup> , testis, ovary & spleen	Mature & immature ovary	as left
Method of dispersion & temp.	Mincing, Tryp. 15-20°C	Mincing Tryp. 15°C	Mincing Tryp. 15°C	as left
Growth time to confluence (days)	28*	3-5 <sup>+</sup>	14	11-14
Cell type	Fibro. & epith.	Fibro.	Fibro.	Fibro.
Contaminants	Bacteria (gram -ive cocci), yeasts	Branching yeasts esp.	Some bacteria	Some bacteria
Medium	MEM-E 15% FCS 0.206M NaCl	MEM-E 10% FCS 0.206M NaCl	as flounder	as flounder

Abbreviations: MEM-E - Eagles Minimal Essential Medium + Earle's Salts  
 FCS - Foetal Calf Serum  
 Tryp. - trypsinization  
 Fibro.- fibroblast  
 epith.- epithelial

## Plate 15

Direct light micrographs, living primary culture of fibroblast-like ovary cells from dab, 7 days after seeding, X 200. Photographs by courtesy of Mrs. F. Price.

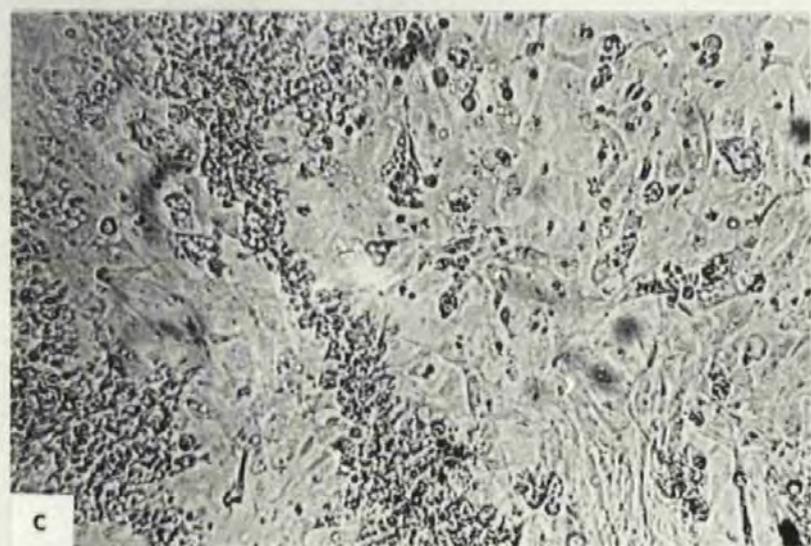
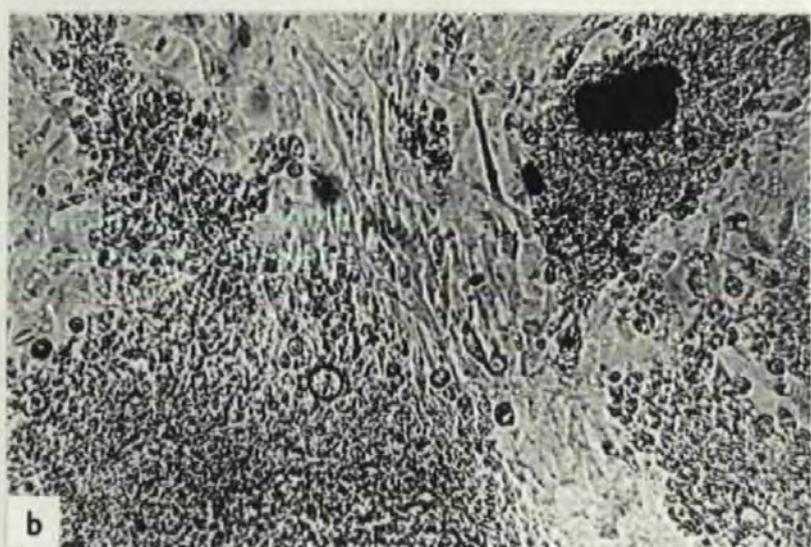
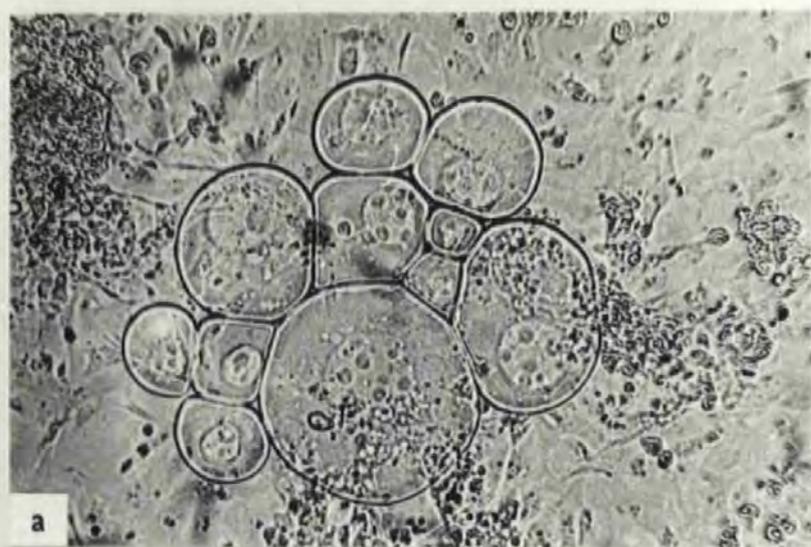
- (a) Islands of closely-packed cells are seen at the left and lower edges. These attached soon after seeding. Growing dividing cells are seen centrally.
- (b) An island of closely-packed cells is seen (upper left) with scattered growing cells elsewhere.
- (c) Islands of condensed cells are seen at the upper right and lower left.



## Plate 16

Direct light micrographs, living primary culture of fibroblast-like ovary cells from dab, 7 days after seeding, X 200. Photographs by courtesy of Mrs. F. Price.

- (a) Eggs with visible nuclei are seen clumped in this culture.
- (b) Areas of refractile cells confluent with growing fibroblast-like cells.
- (c) An area of almost confluent growing cells.



achieved (Table 15). Cultures no. 2, 7 and 8 gave the best results though no single factor was correlated with this promising growth.

In two variations of the standard technique in culture no. 7, it was found that large fragments of tissue and cell clumps provided the best starting conditions. One (UT) was derived from untrypsinized tissue fragments and the other (P) from tissues trypsinized normally and a pellet of released cells dispersed. Culture no. 8 was also seeded with an aggregate of pelleted cells released by trypsin and yielded growing cells of fibroblast type.

Some variations tried with methods of dispersion gave some indication of optimum conditions. In no. 1, trypsinization was done at 37°C twice for 40 min each while for the other attempts 15°C or 20°C was the usual temperature and trypsinization was done three times for 15 min each. Cell growth was very limited in no. 1 and also very variable for all other attempts so no conclusion could be drawn from the effect of temperature of dispersion. In no. 7 and no. 8, the volume of trypsin used varied from 25 to 100 vol. of the tissues, whereas in all other cases it was 10 vol. The larger volume gave reasonably good results so it is probable the volume of trypsin used is not important.

The total viable cells liberated were estimated in three culture attempts by the trypan blue dye exclusion test. The cell yield ranged from  $3.6 \times 10^9$  -  $1.3 \times 10^{10}$  cells per g. of tissue as starting material, showing that as much as  $10^{10}$  per g was obtainable.

For seeding the cells, experience showed that making a volumetric dilution from the final cell pellet was more practical than counting cells before seeding. In practice it was found from visual inspection that the desirable higher seeding concentration was achieved by the first method. Thus for no. 7 in particular a volumetric dilution to 100 vol. of the cell pellet was successful in 2 out of 5 variations

**Table 15** Different procedures for the derivation of nine primary cell cultures from the Blenny.

	CULTURE NO.								
	1	2	3	4	5	6	7	8	9
ORGANS USED	ov	spl, ov, t	ov (imm & mat)	ov + eggs	ov (imm)	ov&t OR ht, liv & spl	ov,t	ov,t	ov,t
DISPERSION METHOD	M Tr <sub>2</sub> x 37°C 40min f	M Tr <sub>3</sub> x 20°C 15min	M Tr <sub>3</sub> x 15°C 15min	M Tr <sub>3</sub> x 15°C 30min f	M Tr <sub>3</sub> x 15°C 30min f	M Tr <sub>3</sub> x 15°C 15min	'UT' M only Other M Tr <sub>2</sub> x 15°C 15min S→P/S	M Tr <sub>2</sub> x 15°C 15min	M Tr <sub>3</sub> x 15°C 15min
CELL YIELD (cells per g tissue)	NC	6x10 <sup>9</sup>	13x10 <sup>9</sup>	NC	4x10 <sup>9</sup>	NC	NC	NC	NC
GROWTH CONDITIONS:									
SEED CONC'N (cells/ml)	2x10 <sup>6</sup>	4-90 x10 <sup>6</sup>	0.3-2 x10 <sup>6</sup>	VdilT x100-400	2-100 x10 <sup>6</sup>	VdilT x100-1000	S&P VdilT x100	VdilT x100	VdilT x100
TEMP (°C)	15	23	15	15-25	15	15-22	15	15	15
NO. FLASKS SEEDED	12	12	12	7	10	12	12	1	5
ADD. ANTI-BIOTICS	-	-	-	1%neo-	1%neo-	-	-	-	-
BUFFER	b	b	t/h	b	b	b	b	b	b
GROWTH RESULTS:									
CELL TYPE	ep.& fib.	fib.	-	ep.& fib.	-	fib.	fib.	fib.	-
FLASKS SHOWING GROWTH	2/10	4/12	0/12	1/7	0/10	1/12	p-2/6 s-0/6	1/1	0/5
GROWTH TO CONFLUENCE (days)	-	3-4	-	--	-	-	20-30	-	-
CONTAMINATION (% flasks)	d7-40 d8-100	d3-25	d2-20 d8-25	d4-100	d2-20	d15-66			d7-100

**Abbreviations:**

b - bicarbonate  
d - days  
ep. - epithelial  
fib. - fibroblast  
f. - filtered  
ht - heart  
imm - immature  
liv - liver  
neo- neomycin

mat - mature  
M - mincing  
NC - not counted  
ov - ovary  
P - pellet fraction  
S - supernatant fraction  
s - sedimentation  
spl - spleen

t/h - TRIS-HCl  
Tr - trypsinization  
t - testis  
UT - untrypsinized  
Vdil- volumetric dilution

of the procedure. However, in no. 2, where cells were counted, growth occurred only at the lowest dilution, ( $4 \times 10^6$ /ml).

Small modifications to the medium produced no improvements in the growth rate. A change from bicarbonate to tris-HCl buffered medium (no. 3) obviated the need for gassing the flasks; the pH of the medium was then well maintained but even before the presence of contamination cell growth was poor. The addition of human serum (2.5%) similarly was ineffective in stimulating growth.

The best growth results were obtained with cultures derived from gonadal tissue, as with the flatfish, but other mixed internal organs were used. In culture no. 2, spleen, testis and ovary were used with fair growth i.e. 50% of cells attached to the plastic with some flasks showing growth to confluence, whereas in culture no. 6, the heart, spleen and liver were used and these gave poor cell attachment and no growth. Neither mature nor immature ovaries give rise to the vigorous growth that was observed using flounder material. Ripe ovaries containing eggs were used in no. 4 but the eggs appeared not to subdivide and were not susceptible to the action of trypsin.

Contamination with these cultures was also a problem. 4/12 no. 2 culture flasks showed semi-confluent fibroblast-like cells at 48 hr after seeding and confluent monolayers at 3 days. However, at the same time heavy bacterial contamination was found in three of the flasks growing cells and a fourth later succumbed. These contaminants were broadly typed as gram-positive and negative cocci and could be cleared with kanamycin sulphate at 50 µg/ml. The cells were then split but lost viability and could not be passed again. In two culture attempts no. 4 and no. 5, the incorporation of neomycin in the washing salt solution and in the growth medium also produced no marked reduction in contamination.

**B1(b) Inoculations**

Dab and plaice cell cultures were inoculated with preparations of lymphocystis virus isolates 3i, 3ii and 5 and infected blenny erythrocytes in experiments 1, 2 & 3 (Table 16 & Methods no. 4).

Lymphocystis gave a conclusive result in one case only i.e. no CPE up to 24 days post inoculation in experiment 2 since in the first and third contamination was found at 5 days after inoculation.

Infected blenny erythrocytes also gave no definite CPE up to 10 days after inoculation in experiments 1 and 3 for extracts produced by cell lysis and sonication. Lysed control red cells also gave no CPE.

Table 16. Virus inoculation of marine fish cells

<u>Expt. No.</u>	<u>Cells used</u>	<u>Inoculum</u>		<u>Result</u>
		<u>Origin etc.</u>	<u>Preparation (see Materials also)</u>	
(1)	Dab	<u>LC</u>		
	Young	<u>Isolate 3i</u>		
	Ovary	Inoc. 1	- Unfiltered	No CPE 5 - 6 days p.i.
	"	Inoc. 2	- Filtered	" "
(1)	"	<u>Blenny r.b.c.</u>	- Infected	No CPE 10 days p.i.
	"	"	- control	" "
(2)	Dab	<u>LC</u>		
	Young	<u>Isolate 3ii</u>		
	Ovary	Inoc. 3	-	No CPE 24 days p.i.
		Control	-	" "
(3)	Plaice ovary	<u>LC</u>	- antibiotic treated	No CPE 5 days p.i.
		<u>Isolate 5</u> Inoc. 4		
		<u>Blenny r.b.c.</u>	- infected, sonicated & filtered	No CPE 10 days p.i.
		"	infected, lysed & filtered	" "

inoc.=inoculum  
LC = lymphocystis  
r.b.c. = erythrocytes

(a) Establishment and maintenance

Skin blocks from the Flounder could be maintained in tris-buffered maintenance medium for 15 days as an organ culture with a change of medium every 2 days. Examination by light and electron microscopy after processing showed that the dermis was intact over the muscle layers but frequently the epidermis was absent or dis-integrated. Bacterial contamination was found in the cultures at 15 days after preparation and by 20 days approximately 50% of the culture dishes were contaminated.

(b) Inoculations

Examination of sections of inoculated skin blocks by light microscopy showed no histological changes typical of lymphocystis virus such as cellular hypertrophy in the dermis. Furthermore, by electron microscopy of the same material, lymphocystis virus was not observed in any of the blocks maintained up to 15 days. After 15 days maintenance contamination was observed as evidenced by turbid medium of low pH and the cultures could not be used for viral identification.

B3 The effect of high temperature and APH in the Blenny

The effect of high temperature on the Blenny was initially investigated with the aim of inducing the infection, as Sherburne (1973) had reported a correlation of the incidence of PEN in herring with increased temperature. An unequivocal result was not produced but a miscellaneous effect of high temperature on the red blood cell nuolei is reported.

The effect of APH was investigated with the aim of reproducing the anaemia caused by the drug (Grasso, 1973) and then trying to inoculate anaemic fish with infected erythrocytes in the ensuing phase of erythropoiesis. It was not possible to establish the

infection in this way as fish mortalities were experienced but a feature of the response in the Blenny to APH is reported.

B3(a) High Temperature

At 3 days after exposure to 24.5°C no inclusions were seen in the red blood cells of experimental fish nor in controls at low temperature. (Plates 17a & b). One divided nucleus was noticed in the smear of an exposed fish (Plate 17a, arrow). At 9 days, blood films of exposed and control fish did not show inclusions but in one smear of an exposed fish the outline of many erythrocytes was unusually fusiform and many of the nuclei were distinctly fragmented (Plate 18b).

B3(b) APH

6/9 fish inoculated died by 4 days after the first injection, these being all small fish under 10 g weight. One survivor was left in each group in the weight range 20-40 g and it was possible to take blood for an erythrocyte count and for a smear up to 12 days after inoculation.

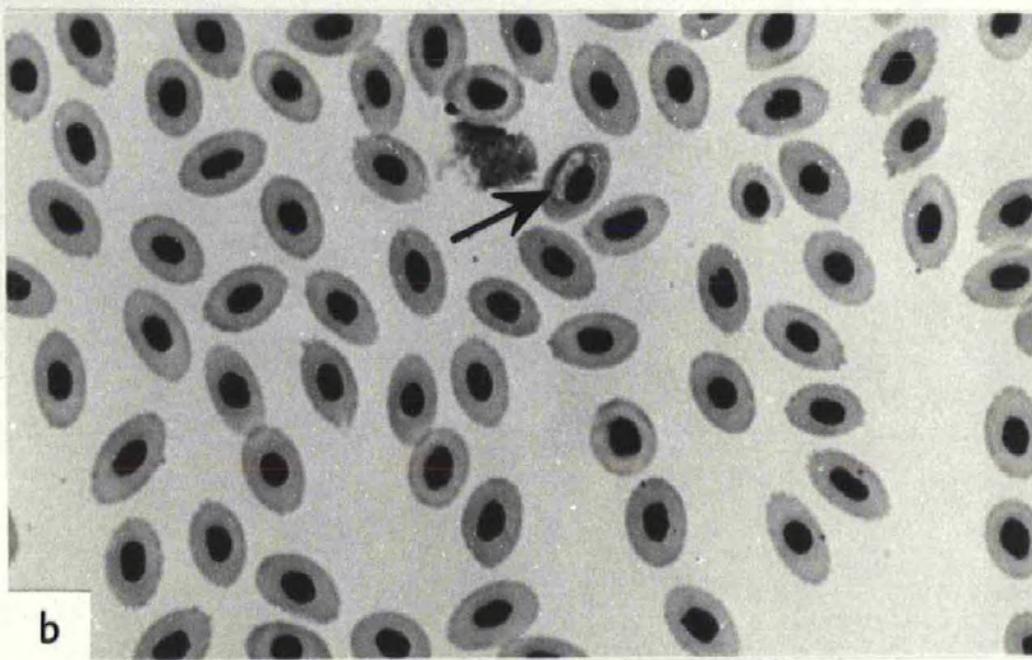
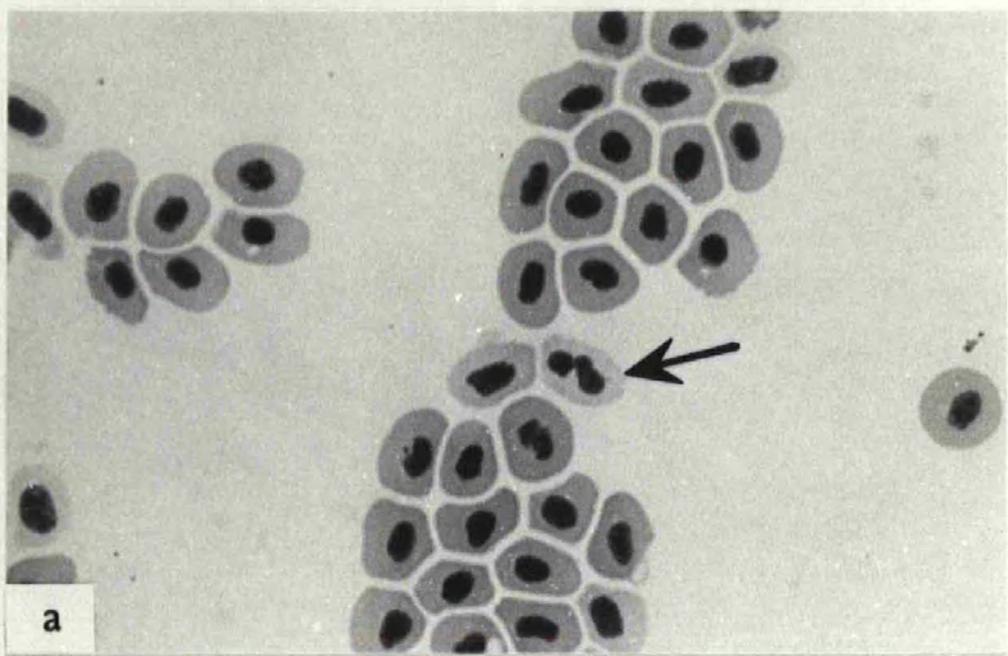
The erythrocyte count declined over the 12 day duration of sampling from 200-250 x 10<sup>4</sup> to 40 x 10<sup>4</sup> per cu. mm (Fig. 7), each value being the average of two counts on two separate blood samples from each fish. The red cell count of the control <sup>inoculated with saline</sup> also declined but unevenly. From the start of the experiment, the control and experimental fish did not feed regularly although given rations every two days. The dose 1 fish and the control fish then died at 15 days p.i.

No erythroid precursor (EP) cells were seen in smears of the inoculated blennies from day 4 to day 12. However, the proportion of immature red cells increased with the time after inoculation and this increase was greater for the experimentals than the control (Table 17). Moreover, the increase was comparable for dose 1 and 2 fish.

Plate 17

Blood films, Common Blenny, Giemsa's stain, X 1024.

- (a) Exposed 3 days to high temperature (24.5°C). One divided nucleus is seen (arrow).
- (b) Control, exposed 3 days to 12°C. The commonly seen protozoan parasite Haemogregarina bigemina infects one cell (arrow), lying close to the nucleus.

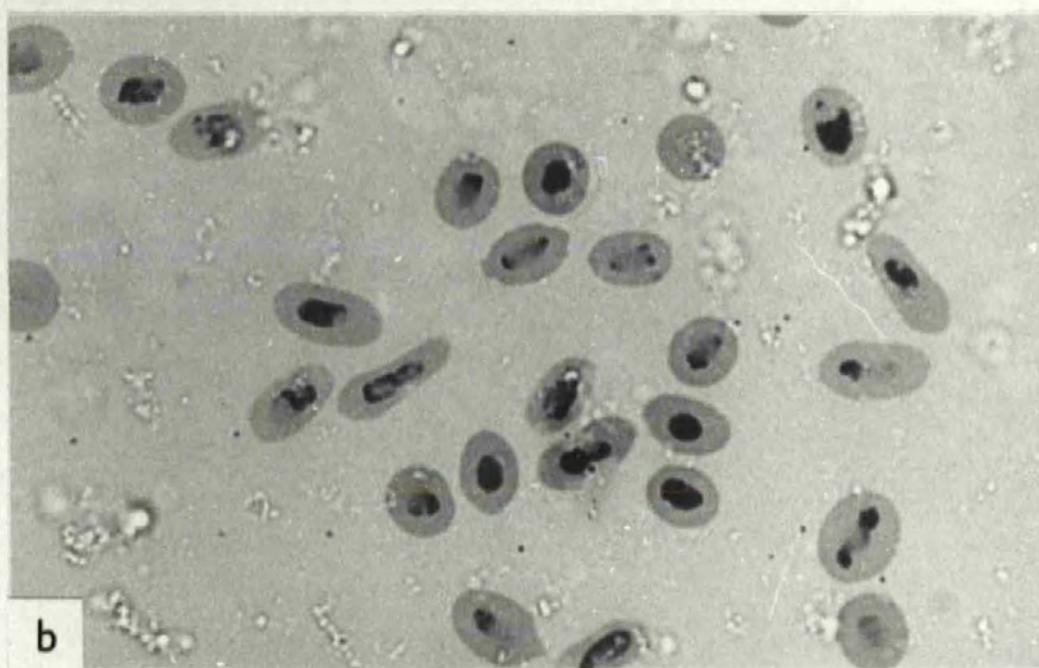
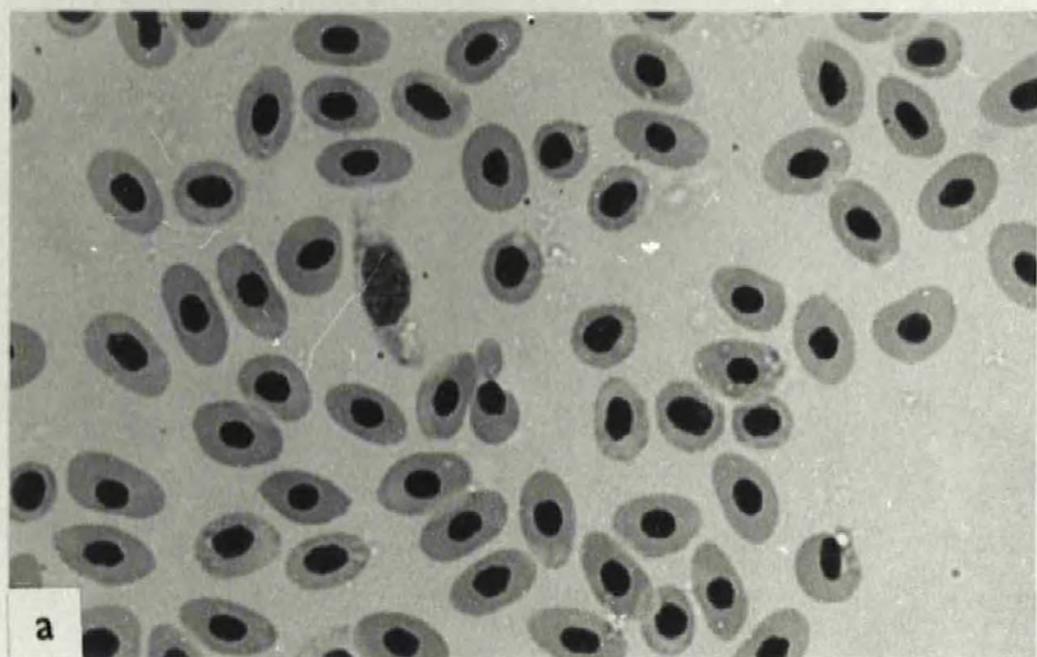


112

Plate 18

Blood films, Common Blenny, Giemsa's stain, X 1024.

- (a) Control, exposed 9 days to 12°C. No inclusions are seen.  
(Fine specks are dust).
- (b) Exposed 9 days to high temperature (24.5°C). Approx. half of the erythrocyte nuclei show fragmentation.



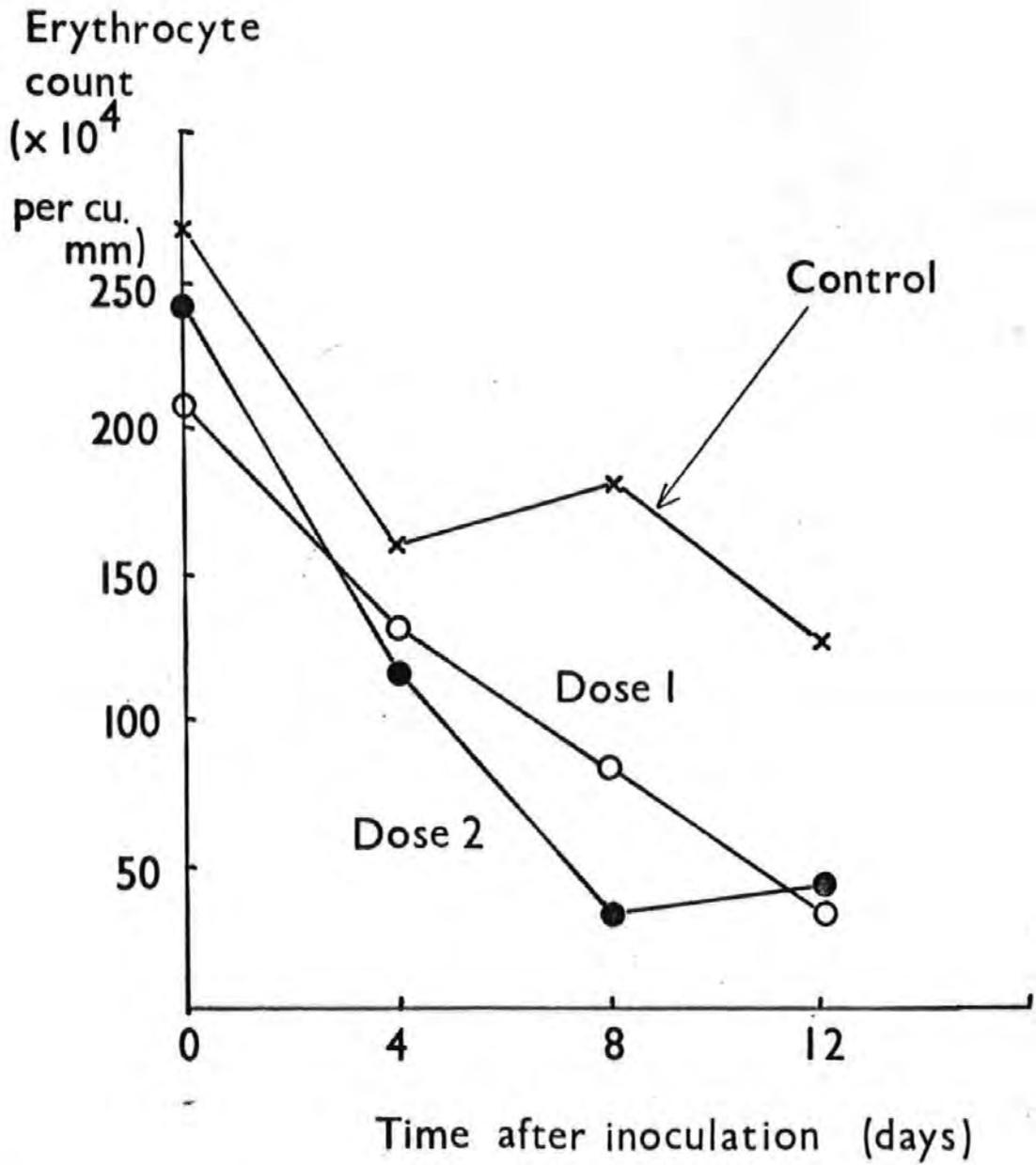


Fig. 7. Plot of erythrocyte count against time for 2 blennies inoculated with APH and for 1 control.

Table 17. The proportion of immature and mature erythrocytes in smears from the Blenny after treatment with APH.

<u>Dose Level</u>	<u>Days (After Treatment)</u>	<u>Cells counted</u>		
		<u>Immature</u>	<u>Mature</u>	<u>% Immature</u>
(mg/10g body wt) 0.1 (dose 1)	4	3	270	1.1
	8	40	320	11.1
	12	100	149	40.2
0.2 (dose 2)	4	21	463	4.5
	8	22	176	11.1
	12	95	143	39.9
Control (saline inoculated)	4	12	506	2.4
	8	18	487	3.5
	12	46	347	13.2

## DISCUSSION

### Passage in cell culture attempts

The results in preparing cell cultures would suggest that the cell monolayer technique of Clem et al. (1961), in conjunction with the recommendations of Wolf & Quimby (1969), is suitable for tissues from different marine fish viz. the Blenny, Flounder, Plaice and Dab. Experience showed that the continuity of the cultures started was hindered by contamination and this lack of continuity beyond third passage certainly prevented the establishment of secondary cultures. No recommendations can be made as to the most preferable flatfish species to be used but of those tried the cells from the Dab happened to survive for the longest.

These remarks should be qualified by emphasizing that the results of the methodology section were observations rather than experiments, as there were no true controls of the trial cell and tissue cultures. The conclusions are therefore suggestive rather than affirmative.

In a few ways the observations on methodology corroborate previous studies. All the media used for monolayers were salt-adjusted i.e. at 0.206 M NaCl and though there were no controls at the level of salt tonicity for mammalian cells i.e. at 0.14 M NaCl, it is possible additional salt was an absolute requirement for growth. Similarly, Clem et al. (1961) reported that the use of additional salt was necessary for the long term establishment of marine fish cells and Wolf & Quimby (1969) supported this for cells in primary culture. By contrast, Townsley, et al. (1963) recorded cell proliferation from a variety of explanted tissues of marine fish in a mammalian type medium, unadjusted for salt. Webb (1975) found that cells grew out from explants of external tissues from two species of goby, in both salt-adjusted and unadjusted media, but cells grew out from explants of internal tissues only in unadjusted

media. Webb therefore made the apposite suggestion that the dependence on additional salt of tissues in vitro is related not only to the nature of the tissue, and consequently its degree of contact with salt water but also the natural habitat of the fish. This is a plausible hypothesis that cannot be verified from the results here. Supporting evidence will have to be obtained by examining the in vitro growth characteristics of cells from internal and external tissues in both stenohaline and euryhaline fish.

The observations from blenny and flounder material suggested that the ovary was the best tissue for starting primary cultures. Wolf & Quimby (1969) made a similar recommendation from experience of freshwater fish, advocating the use of oviduct tissue in particular. Since the oviduct does not contain germ cells, it is likely the connective tissue, common to both, is valuable for starting growth.

Limited experience of handling fish cells indicated that contamination, possibly direct from fish material, was a major threat to the establishment and continuity of primary cultures. Wolf & Quimby (1969) recommended the use of penicillin and streptomycin at normal dose with temporary use of other broad-spectrum antibiotics such as kanamycin. They suggested fungi should be suppressed with mycostatin at 25 IU/ml. By contrast, the more modern antibiotics gentamycin (Biocult) and fungizone (Squibb Ltd) could be recommended since with fish cells they gave better sterility though no controlled studies were performed. Enhanced antibiotic characteristics of gentamycin have also been reported in mammalian cells (Schafer et al., 1972).

The inoculations performed showed that lymphocystis material and preparations of infected erythrocytes from the Blenny did not give recognisable cytopathic effects in cells from dab and plaice but indicate the need for more trials with these isolates. It would also be worthwhile to observe the cells over a minimum period of 30 days.

The maintenance and growth of fish cell cultures has furthered the propagation and characterization of the freshwater fish viruses but by contrast relatively few cell lines have been developed from marine teleost fish. Wolf & Quimby (1969) noted 4 marine fish cell lines; 2 of these from grunt species were established lines but only one GF-1, was an available certified cell line. This paucity of established cell lines explains in part the undeveloped characterization of marine fish viruses. Thus whereas lymphocystis from the Bluegill has been propagated in centrarchid fish cells (Wolf et al., 1966) and lymphocystis of marine snappers has been propagated in grunt GFS cells (Lopez et al., 1969), the virus from flatfish of British waters has not. For these marine isolates of lymphocystis, the approach of employing primary and established cultures of connective tissues cells should prove profitable. For the erythrocytic viruses however, it may be more salient to use blood culture methods already used in fish chromosome techniques (Ojima, Hitotsumachi & Hayashi, 1970).

Organ culture

The maintenance of large skin explants in mammalian-type tissue culture medium with the addition of only sodium chloride indicates the methods used for establishing mammalian organ cultures (e.g. human embryonic trachea, are essentially applicable to fish tissues.

The partial disintegration of skin blocks before 15 days could have been due to several factors. Firstly, it is possible that bacterial contaminants repressed by the antibiotics, or fungi from source material, could have been responsible. Secondly, handling during processing could be suspected of damaging the epidermis in particular. Thirdly, it is possible that an actual sloughing of the epidermis took place. D. Simpson of Plymouth Polytechnic (pers. comm.) found that skin blocks from the Blenny maintained as above also sloughed the epidermis during a 10 day period, though the dermis was well preserved. Hence it is likely that the epidermis is in any case very poorly preserved by organ culture. In addition, light abrasion of the skin during sterilization swabbing would probably serve to damage many cells.

To my knowledge there is no published account of the establishment of skin organ cultures, rather than tissue explants, from marine teleost fish. D. Simpson (pers. comm.) found that large skin explants from the Blenny could be maintained in culture up to 18 days with histological integrity of the dermis. Similar maintenance media to the one used here were employed. Simpson found that both tris- and bicarbonate-buffered media gave adequate maintenance of the skin blocks and the addition of sodium chloride to the media gave improved growth.

The negative result in the inoculation attempt should not be interpreted as a definitive result in view of the stated condition of the cultures particularly after 15 days post-inoculation. Clearly there is a need for controlled experiments to determine

the critical factors for infection in vitro e.g. degree of scarification of the epithelium required, time and temperature of inoculation etc. In view of some            success with maintenance of skin blocks in vitro, the technique would seem to be a promising one for studying cellular changes occurring during in vitro growth of lymphocystis virus.

The effect of high temperature and APH in the Blenny

A temperature rise to 24°C did not evoke inclusions in erythrocytes of the exposed fish at 9 days after temperature elevation and the controls maintained below 12°C were also negative. However, it is not necessarily valid to conclude that the infection could not be induced by temperature stress since the trial period was short.

However temperature stress did evoke the division of erythrocyte nuclei in 1/3 fish. Similarly, Sherburne (1973) noted abnormal red cells with segmented nuclei in many short-term captive herring. These cells did not show inclusions and the abnormal forms were observed only in fish held at higher than normal seawater temperatures of up to 20.5°C. Interestingly other causes of this sort of change have been described. For example in coho salmon the granulation of erythrocyte nuclei was ascribed to dietary deficiency (Smith, 1968). Although the cause of the change noticed is uncertain, the most probable explanation is that it was caused by one form of stress and that a virus was not implicated since a nuclear change was not usually seen with virus infection.

The fish injected with APH developed anaemia and there was an overall increase in the proportion of immature red cells in smears but it could not be demonstrated that the anaemia was caused by APH. It should be pointed out also that the percent. of immature cells was calculated from a red cell count in one region only of the smear. This may have introduced variability in view of the normal variation of the proportion of cell types with smear region i.e. 'head' to 'tail'. It is uncertain why 6 small fish died up to 4 days after inoculation. This may have been due to a rapid anaemic affect of the drug but more likely to a haemorrhage caused by inoculation.

Since the control as well as the experimental fish showed a fall in the red cell count it is probable that anaemia was caused by poor

feeding rather than APH. Inhibitory influences on erythropoiesis by food deprivation have also been shown in the Red Paradise fish (Macropodus opercularis) (Weinberg, Siegel & Gordon, 1973). However, at 12 days after inoculation, an erythropoietic effect was indicated by the increase in immature erythrocytes in smears of experimentals, despite the prior absence of any distinctive erythroid precursor (EP) cells as seen by Grasso (1973) in the newt (Triturus cristatus). It is possible that EP cells of the type described by Grasso are not a normal feature of erythropoiesis in teleosts.

This approach was not followed up and the anaemic fish not used for passage of the blenny infection as they were in such poor condition. The approach may prove profitable for experimental passage, if the fish can be well maintained under reduced stress during the anaemic phase, since a patent induction of erythropoiesis seemed possible. Alternatively, it could be fruitful to examine other means of direct induction e.g. u.v.- and x- irradiation.

SECTION C

VIRUS MORPHOLOGY

(1) Processing and embedding methods for electron microscopy (e.m.)

Different methods of fixation, dehydration, and embedding were used for both lymphocystis material and different tissues from the Blenny. The methods are detailed here as methods (A) to (D).

Method E for processing cod red blood cells, included the method of Gowans (1973) for pelleting the cells before processing as this obviates the problem of handling a friable pellet of fixed cells during dehydration and embedding.

Method A. Processing of lymphocystis cells with embedding in TAAB resin

Lymphocystis cells were dissected from the isolate 3ii which had been stored at  $-20^{\circ}\text{C}$ . They measured at most 1.5 mm diameter.

1. They were fixed in 3% glutaraldehyde 2 hr at  $4^{\circ}\text{C}$ .
2. Washed three times for 5 min each and left overnight in 0.1 M sodium cacodylate buffer, pH 7.4.
3. Fixed in 2% osmic acid at  $4^{\circ}\text{C}$  for 2 hr.
4. Washed in sodium cacodylate buffer as above and washed three times in tap water to remove the fixative.
5. Dehydrated by immersion in 30% and 50% alcohol 15 min each, 70% alcohol overnight and absolute alcohol 2 hr at room temperature.
6. Alcohol was removed by washing twice for 30 min each with propylene oxide.
7. The blocks were infiltrated with a 1:1 volume mixture of resin in propylene oxide. The resin formula was as shown below.\*
8. Specimens were infiltrated with resin for 1 hr at  $50^{\circ}\text{C}$ , changed to fresh resin and this hardened at  $60^{\circ}\text{C}$  for 48 hr.

\* TAAB resin 27.5 g  
 DDSA 22.2 g  
 MNA 3.7 g  
 DMP-30 0.5 g.

} All these resin components are obtainable from TAAB Laboratories, Emmer Green, Reading.

} For the proper names of these compounds see the Abbreviations p. xiv

Method B. Processing of infected blenny erythrocytes using araldite\* for embedding

1. Blood was taken by the caudal vein route and the erythrocytes suspended in 4% TSC. A pellet of red cells was prepared by sedimentation twice at 700 g for 5 min.
2. The pellet was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 1 hr at 4°C and washed three times in buffer 5 min each.
3. It was post-fixed in 2% osmic acid for 2 hr at 4°C and washed in tap water three times 5 min each.
4. Dehydration and embedding was as follows:-

30% ethanol	15 min
50% "	15 min
75% "	Overnight
100% "	60 min
Propylene oxide	30 min
Propylene oxide/araldite (50:50 by volume)	30 min
Araldite at 50°	60 min
Araldite (1.5% DMP30)	48 hr at 60°C to harden.

\* Araldite M - 20 ml  
 Resin 964B - 20 ml  
 DMP30 - 0.6 ml } For proper names and supplier see  
 the Appendix p. 181

Method C. Processing of tissues from the Blenny and lymphocystis cells using Spurr's Resin\* (Spurr, 1969)

1. Tissues were dissected out in the cold room, cut to a 1 mm cube and fixed in buffered glutaraldehyde for 30 min at 4°C.
2. Rinsed three times 5 min each in cold buffer.
3. Post-fixed in 1% osmic acid for 1 hr at 4°C.
4. Rinsed in buffer three times 5 min each.
5. Dehydrated by:-

30% ethanol	10 min
50% "	10 min
70% "	10 min
90% "	10 min
100% "	10 min
100% "	15 min

6. And infiltrated by the following steps:-

1:3 resin/ethanol mixture	20 min.
2:3 resin/ethanol mixture	30 min
Pure resin	30 min
Pure resin	30 min.

Hardened by 24 hr at 65°C

\* Medium hardness formulation

ERL 4206	- 10 g	} for the proper names and the supplier of these compounds see the Appendix p.177
DER 736	- 6 g	
NSA	- 26 g	
S-1	- 0.4 ml	

Method D. Quick processing and embedding method of Doane et al. (1974)

with lymphocystis isolate 5 using Spurr's resin

1. Large tumours were cut into small blocks of 1 mm square and fixed for 30 min at 4°C in a 1:2 mixture by volume of 2.5% glutaraldehyde in cacodylate buffer pH 7.4 and 1.5% osmium tetroxide in the same buffer.

There followed:-

2. Three rinses in buffer 1 min each.
3. Dehydration by two changes in 70% acetone for 3 min each.
4. Dehydration by three changes in absolute acetone for 5 min each.
5. Embedding in a 1:1 volume mixture of absolute acetone and Spurr's resin for 10 min each.
6. Two changes in 100% Spurr's resin 5 min each.
7. Embedding in fresh resin and hardening at 95°C for 60 min.

Method E. Processing of red blood cells from cod using the method of Gowans (1973) for pelleting the cells in agar

1. The tonicity of 0.05 M sodium cacodylate buffer was adjusted to that of cod erythrocytes with glucose by doing an osmotic fragility test. The highest concentration of glucose 0.8 M, at which negligible lysis of the red cells took place was taken as the isotonic point.

2. Red cells from an infected sample stored in 4% TSC, were sedimented at 600 g for 15 min and washed in 0.8 M glucose buffer. The cells were resuspended in 1 ml 0.05 M sodium cacodylate buffer with glucose and counted by haemocytometer.
3. The cells were fixed for 3 hr in 3% glutaraldehyde in the same buffer at 4°C.
4. The cells were washed with buffer three times and left overnight.
5. They were then fixed in 2% osmic acid 1 hr at 4°C and washed in buffer three times 10 min. each.
6. The buffer was removed and 4% pure 'Difco' Noble agar at 60°C added to the cells in the proportion of 0.03 ml agar to 10<sup>7</sup> cells. The volume of agar was estimated from the data of Gowans (1973) for the volumes contained in a fine tube of known bore.
7. The cells and agar were rapidly mixed using a 1 mm bore unheparinized haematocrit tube.
8. The mixture was then drawn up the capillary tube and allowed to solidify. When solid, the agar sausage was expelled, cut into 2 or 3 mm lengths and the agar blocks dehydrated and embedded in resin as in Method C.

## (2) Ultramicrotomy & Electron Microscopy

Blocks were rough trimmed by hand and ultrathin sections down to 60 nm cut on either the LKB 'Ultratome' or the Sorval 'Porter-Blum' automatic ultramicrotomes. Sections on grids for transmission microscopy were stained in the dark for 15 min with a saturated solution of uranyl acetate in methanol, gently washed with distilled water and stained with Reynold's lead citrate<sup>Reynolds (1963)\*</sup> for 15 min. The grids were washed with 0.1 N NaOH, washed with water, blotted dry and viewed in a Philips EM 300 electron microscope operated at 80 kV.  
\*see the Addendum of the References

### (3) Partial purification of lymphocystis virus

9 different procedures were used in attempts to purify lymphocystis virus starting with different isolates. The outline of the stages for extraction, clarification and concentration is given in Table 22, where the results of electron microscopic examinations of the preparations are shown.

An electron microscope check on each preparation was used as the principal criterion for purity and concentration. The data of Monroe & Brandt (1970) were used for estimating the actual virus concentration in the preparation from the number of particles counted on the grid squares.

The variations in the procedures used are detailed below and the final procedure for optimum purification given (procedure No.8).

#### (i) Extraction techniques

The cells were ground where stated by pestle and mortar until a white homogenous fluid resulted. Sonication was performed with the ultrasonic disintegrator. An amplitude and wavelength was used that had been found satisfactory for 99% lysis in a red cell sample from the Blenny. Douncing was performed using a Potter-Elvehjem glass homogeniser by 20 to 30 hand strokes.

#### (ii) Clarification methods

(1) Low speed sedimentation (LSS) was performed by centrifugation at 700g for 5 min using a bench centrifuge. This was sufficient to pellet all visible tissue fragments.

(2) The freeze-thaw cycle was overnight at  $-20^{\circ}\text{C}$  and subsequently at room temperature.

(3) Ammonium sulphate precipitation was performed by the addition of an equal volume of saturated solution to the virus-containing suspension.

(4) For filtering, four thicknesses of dressing muslin were used.

(iii) Concentration and purification methods

Cycles of differential sedimentation, i.e., centrifuging at low and high speeds, were used for the concentration and purification of the virus. Equilibrium density gradient centrifugation with sucrose solutions was also used. The details of these, i.e., relative centrifugal forces and times, are given in Table 18.

High speed sedimentation was performed using the MSE 'Super-speed 50' centrifuge with usually the MSE rotors with swing-out buckets (3x3 ml and 3x25 ml with adapters for 12 ml tubes). The MSE angle head rotor with 8x50 ml sockets and adapters down to 10 ml was also used.

(iv) Final method for partial purification of lymphocystis virus

- (1) 0.25 g of the tumour (isolate 6) was completely homogenised in 2 ml PBS in a glass homogeniser.
- (2) The homogenate was clarified by centrifugation at 700g for 10 min.
- (3) A cushioned sucrose solution was prepared by layering 10 ml of 20% w/v sucrose on 10 ml of 60% w/v sucrose and the clarified homogenate was then overlaid on this. The homogenate was sedimented into the lighter solution at 14300 g for 30 min.
- (4) The refractive layers in the 20% sucrose were collected by the manual methods recommended by Crawford (1969). This involved sealing the head of the tube, making a fine pinhole in the tube below the fraction to be collected, releasing the seal slightly and running out sufficient drops.
- (5) Virus in each fraction was sedimented from the sucrose by centrifugation at 42000g for 30 min. The pellet was dissolved in 1% ammonium acetate and the suspension filtered at 450 nm on a Millipore membrane. It was then further clarified by centrifugation at 700g

**Table 18.** Results of 9 procedures as attempts to purify isolates of lymphocystis virus.

PROC. NO.	ISOLATE	EXTRAC-TION	CLARIFI-CATION	CONCENTRATION & PURIFICATION	RESULTS
1	3i	G&S	LSS	(a) 2xDS (b) Supt. f. 0.45 $\mu$ & sed.	(a) Prep's contam. virus @ $3 \times 10^8$ /ml (b) virus @ $3 \times 10^7$ /ml
2	3i	G&S	LSS	(a) 1xDS. (600g/5 min, 2500g/10 min (b) f. 0.45 $\mu$ & sed.	(a) Contam. virus @ $1 \times 10^8$ /ml (b) debris, virus @ $5 \times 10^8$ /ml
3	3i	D	Freeze-thaw, LSS, 50% amm. sulphate	2xDS supt's, f. 45 $\mu$	virus @ $< 1 \times 10^7$ /ml
4	3ii	D	f.	(a) EDGS 10-40% 2x 32170g/2 hr HSS 94500g/2 hr (b) LSS $\rightarrow$ supt.	(a) 2 feint bands + debris (b) contam. virus @ $5 \times 10^7$ /ml
5	3iii	D	LSS	(a) EDGS, 5-20% 32170g/40 min (b) frac. HSS 14300g/30 min (c) pellet(a)EDGS 10-40% & HSS 14300g/30 min	(a) no bands virus $< 10^7$ /ml (b) no pellet (c) no bands
6	3iii	D	-	EDGS 10-40% 3570g/30 min	no bands
7	4ii	D	LSS	(a) Dls, 20+60% @ 1/1 vol. 14300g/ 30 min (b) Refrac. layer HSS, 42000g/ 30 min	Refrac. layer @ interface Impure little virus
8	6	D	LSS	Dls as (7). Top, middle & inter-face fr. in 20% HSS.	Top - impure middle - impure but virus @ $10^9$ - $10^{10}$ /ml Interface-no virus
9	6	D	LSS	Dls. 10-30% + 60% cushion. 14300g/30 min	u.v. absorp'n in 1st/2nd only of 12 fractions

**Abbreviations:**

D	- Douncing	HSS	- high speed sedimentation
Dls	- Discontinuous layer sedimentation	LSS	- low " "
DS	- Differential sedimentation	S	- sonication
EDGS	- Equilibrium density gradient sedimentation	sed.	- sedimented
Frac.	- fraction		
f.	- filtered		
G	- grinding		

for 10 min , prior to examination in the electron microscope.

(4) Freeze drying and unidirectional shadowing of lymphocystis virus

The method used was that of Nermut (1973) as given below and was carried out by Lynn Williams in the Department of Histopathology, NIMR, Mill Hill, London, by kind courtesy of Dr. Nermut. The machine used was the Balzers freeze-drying unit.

- 1) Copper grids were layered with 1 drop of the virus suspension and this allowed to dry. The suspension was prepared from isolate 6 using the final method of purification given.
- 2) The grid was taken in forceps and held over a Dewar flask filled with liquid nitrogen ( $LN_2$ ). The excess fluid was drained from the grid with filter paper.
- 3) The grid was dipped quickly into  $LN_2$  as deeply as possible and held for at least 10 sec.
- 4) The grid was transferred as quickly as possible onto a precooled specimen stage at  $-150^{\circ}C$  covered with Freon 22.
- 5) The specimen stage cover plate was cooled down in  $LN_2$  and put on the stage.
- 6) The chamber was evacuated and cooling of the knife arm commenced. The specimen stage temperature was adjusted to  $-180^{\circ}C$ .
- 7) The cooled knife arm was brought over the specimen stage and held there for 30 min.
- 8) The knife arm was removed and the specimen shadowed with platinum-carbon.
- 9) The specimen stage was warmed up to  $30^{\circ}C$  and heated air blown through the knife arm for 10 min.
- 10) The chamber was ventilated and the grids removed.

(5) Preparation of material for negative staining

- (i) Lymphocystis The spread-cell technique of Parsons (1963) was used

for spreading the tumour material prior to staining. By this means, Parsons (1963) showed that some tumour viruses can be well dispersed and then taken onto a grid with a droplet of the stain.

A depression slide was set on a dark matt background under a binocular microscope with an adjustable light, such that bright reflections of particles on the surface of the stain in the depression could be clearly seen. A little of the material was taken onto a 27G needle mounted on a syringe and touched to the surface of the stain in the depression. The area of spread material could be clearly seen when the light shone away from the observer.

(ii) Infected erythrocytes from the Blenny Various methods were tried to release the virus from the erythrocytes (Table 19). The best methods used were the ones involving haemolysis (trials 4 & 7).

Table 19: Negative staining of the virus from the Blenny

<u>Trial No.</u>	<u>Method of cell breakdown</u>	<u>Dispersion</u>	<u>Stain</u>	<u>Result</u>
1 -	Not done	Spreading	2% PTA pH 7.0	Poor
2 -	Freeze-sectioning	ND	3% PTA pH 6.0	Poor
3 -	Sonication	Spreading	2% PTA pH 7.0	Poor
4 -	Haemolysis H <sub>2</sub> O	ND	1.5% PTA pH 6.0	Good
5 -	Haemolysis H <sub>2</sub> O + sonication 20 sec	ND	"	Poor
6 -	Haemolysis H <sub>2</sub> O + sonication 2 min	ND	"	Poor
7 -	Haemolysis 2% ammonium acetate	ND	1% AM pH 7.0	Good

ND = not done

(a) Freeze-sectioning The method of Almeida & Howatson (1963) was used for sectioning erythrocytes when frozen. Infected cells were washed in isotonic 1.4% saline, centrifuged at 700g for 5 min and resuspended in 0.15 ml saline; 0.6 ml distilled water was then added to swell and lyse the cells. They were transferred to a small mould of 'parafilm' and the cell suspension frozen quickly to  $-20^{\circ}\text{C}$  in solid  $\text{CO}_2$  in alcohol. The frozen block was thin-sectioned at  $4\ \mu\text{m}$  on a cryostat and 30 sections collected and thawed at room temperature.

(b) Sonication (Trial 3). Infected erythrocytes were suspended in 1.4% saline and sonicated by probe in an ultrasonic disintegrator. A duration, amplitude and wavelength was used that had been found to give at least 99% haemolysis of control cells. The red cell ghosts were then sedimented at 700g for 5 min prior to spreading by the technique of Parsons (1963).

(Trials 5 & 6) The haemolysed cells were sonicated for either 20 sec. or 2 min. in a water bath sonicator.

(c) Haemolysis Infected erythrocytes were burst either suddenly, by the addition of 0.05 ml distilled water to the pelleted cells, or gradually by resuspension in a slightly hypotonic buffer.

For gradual haemolysis the solute concentration of a small sample of whole serum from the Blenny was first measured on an osmometer and was given as 500 m-osmol. Ammonium acetate was chosen as a suitable volatile buffer for electron microscopy and a 1% solution was measured as 226 m-osmol. It was therefore estimated that a 2% solution should give gentle lysis.

A small sample of blood from an infected fish was transferred to 1.4% saline, the cells washed, sedimented and resuspended in an equal volume of 2% ammonium acetate. The erythrocytes were held for 5-30 min and the ghosts then stained for electron microscopy.

(6) Negative staining procedures

(i) Lymphocystis The stains used were phosphotungstic acid (PTA) as the sodium salt (NaPT), uranyl formate (UF) and ammonium molybdate (AM) (see Appendix for titration and other details). Stains included 0.01% bovine serum albumen (BSA) which decreases surface tension forces. A 200 mesh copper grid was touched to the spread material on the stain with the formvar film surface downwards. It was dried on a Whatman no. 1 filter paper and viewed by electron microscopy.

(ii) Erythrocytes from the Blenny The stains used were ammonium molybdate (AM), which Muscatello & Horne (1968) used for contrasting red cell membranes and PTA as the salt NaPT (see Appendix for details).

The standard staining procedure, when spreading on the stain was not used, was the 'two drop method' of Haschemeyer & Myers (1972), involving superposition of microdrops of specimen and stain. Stains prepared for cell spreading included 0.01% BSA as above.

## RESULTS

This section is a descriptive account of the morphology of the viruses studied by electron microscopy. Part one describes the viruses from the Blenny and the Cod, part two describes lymphocystis virus and part three reports the processing and embedding methods used.

### C1 Viruses of the blood infections

Material from the Blenny and the Cod was available for study. Studies on ultrathin-sectioned and negatively stained virus are detailed in C1(a) & C1(b) respectively.

#### C1(a) Ultrathin-sectioned virus

(i) The blenny infection Ultrathin sections of erythrocytes in the spleen and liver of infected fish demonstrated virus particles, in which the outlines in section suggested the virus conforms to icosahedral symmetry. Ultrathin section evidence also indicated the unusual nature of the core structure.

The particles clearly showed an angular outline and despite the presence of section compression, this was in most cases clearly hexagonal (Plates 13a, & b) but some particles displayed a pentagonal outline. They measured 240-280 nm diameter between the apices and the capsid could be resolved as two electron-dense layers, 14 nm across with an electronlucent layer between (particle 2 in Plate 13b).

In spleen erythrocytes, the particles were surrounded by fine granular material with a crescent-shaped outline and this was bordered by a margin of electron dense granules, of 20-30 nm diameter (Plate 13a arrowed). Similar granules were seen throughout the cytoplasm and close to the nuclear membrane.

The core in section stained unevenly (Plate 13a) and at high magnification different staining patterns could be detected (Plate

13b labelled 1, 2 & 3) viz. a circle of eight electron-dense spots surrounding a central spot (1), an electron-dense circle with an internal projection (2) and two proximate electron-dense ovals (3).

(ii) Cod PEN With respect to methodology, erythrocytes from cod were well preserved for up to 14 days during transport with negligible haemolysis in 4% TSC at 4°C. The infected blood sample processed (No. 143) was also adequately preserved after a further 4 days. It was not possible to evaluate the effect of the glucose-modified washing buffer for fixation as there was none other for comparison. For handling red cells, the agar embedding method gave a reasonable concentration of cells in the block, sufficient for scanning cells in section by electron microscopy.

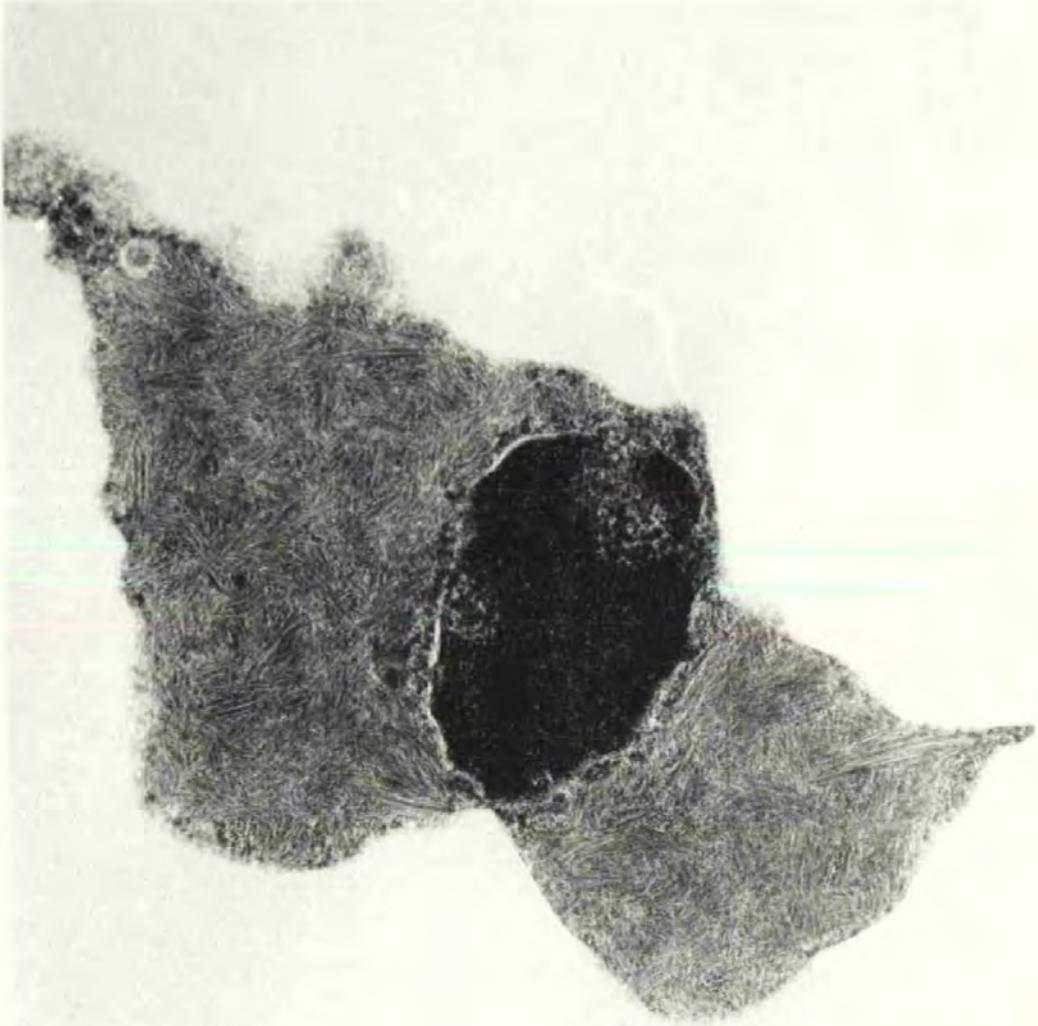
A few erythrocytes in the infected blood samples examined showed some features in common with infected cells in smears viewed by light microscopy. Vacuoles were seen within the nucleus as less electron-dense areas (Plate 19) and condensation of the chromatin was also seen but neither virus particles nor viroplasm were observed in the cytoplasm. The outline of these cells was also irregular and the cytoplasmic membrane poorly defined (Plate 19). Fine fibrils of 3-5 nm diam. were observed in the cytoplasm of all erythrocytes in the samples (Plate 19). These were shown by Thomas (1971) in erythrocytes of uninfected cod blood and probably are the normal para-crystalline form of haemoglobin in cod. In longitudinal section, the fibrils seemed to be apposed in pairs to form filaments of 10-15 nm diam. (Plate 19).

#### Cl(b) Negatively-stained virus

The negatively stained virus from the blenny infection gave information on the structure of the capsid and core. However, several variations on the negative staining procedures described in the Methods

**Plate 19**

Electron micrograph, sectioned erythrocyte, blood from PEN-infected cod no. 143 (North Sea). The nucleus shows less electron-dense areas and the cell membrane is poorly defined and irregular in outline.



1  $\mu$ m

were tried, to solve the problem of how to stain a small number of particles found in only a low proportion of the blood cells available. Therefore this section reports firstly the results of different preparative procedures and secondly the descriptive morphology of the virus.

(i) Preparative results

The results using different conditions for staining the virus are detailed (Table 19). Cell spreading was used to achieve dispersion on the stain surface of both intact infected red cells (trial 1) and lysed cells (trial 3). In trial 1 the intact cells did not spread and could not therefore be stained by touching a grid to the stain surface. In trial 3, a large number of infected cells were used for sonication, being those pooled from five infected fish but again the pelleted cell ghosts did not spread well. Very few red cell ghosts were seen adhering to the grids but these were largely obscured by much debris, including precipitates of the nuclei in the stain.

As cell spreading could not be used for staining the virus particles or the red cell ghosts and as very little material was transferred to the grids by this method, efforts were made to obtain red cell ghosts by a variety of procedures and stain these directly by the standard 'double-drop' method.

Freeze-sectioning (trial 2) was found a useful method of cell breakdown but particles could not be resolved on the resulting red cell ghosts, despite the sufficient number. 20 thawed sections from a block cut at 4  $\mu\text{m}$  gave approximately  $1 \times 10^5$  lysed cells in suspension and the drop volume was large enough for staining.

Controlled haemolysis was found the most useful way of cell breakdown for subsequent staining (trials 4 & 7). When distilled water was used to give sudden lysis, direct staining of the ghosts showed

that particles could be seen faintly within the outline of some of the ghosts but could not be resolved (trial 4). However, between the margins of two ghosts a cluster of particles could be clearly resolved in PTA (Plates 20b). In trial 7, 2% ammonium acetate buffer was used to give slow lysis of the red cells before staining and this worked very well as the buffer (452 m-osmol.) was only slightly hypotonic to the normal molality of the blood solutes (blood serum = 500 m-osmol.). By this method approximately 50% of the cells were lysed at the time of examination in the microscope.

Sonication was used in addition to haemolysis for cell breakdown, (trials 5 & 6) with the object of releasing the particles from the red cell ghosts (Table 19). After 20 sec. sonication (trial 5) particles could be seen overlying the ghost or at their margin, as in trial 4, but no particles could be seen spread outside the ghosts as these areas were largely obscured by masses of crystals. After sonication for 2 min. (trial 6), the red cell ghosts were fragmented and the areas outside the ghost were again largely obscured by much fine cellular debris, so that it was impossible to observe released virus particles.

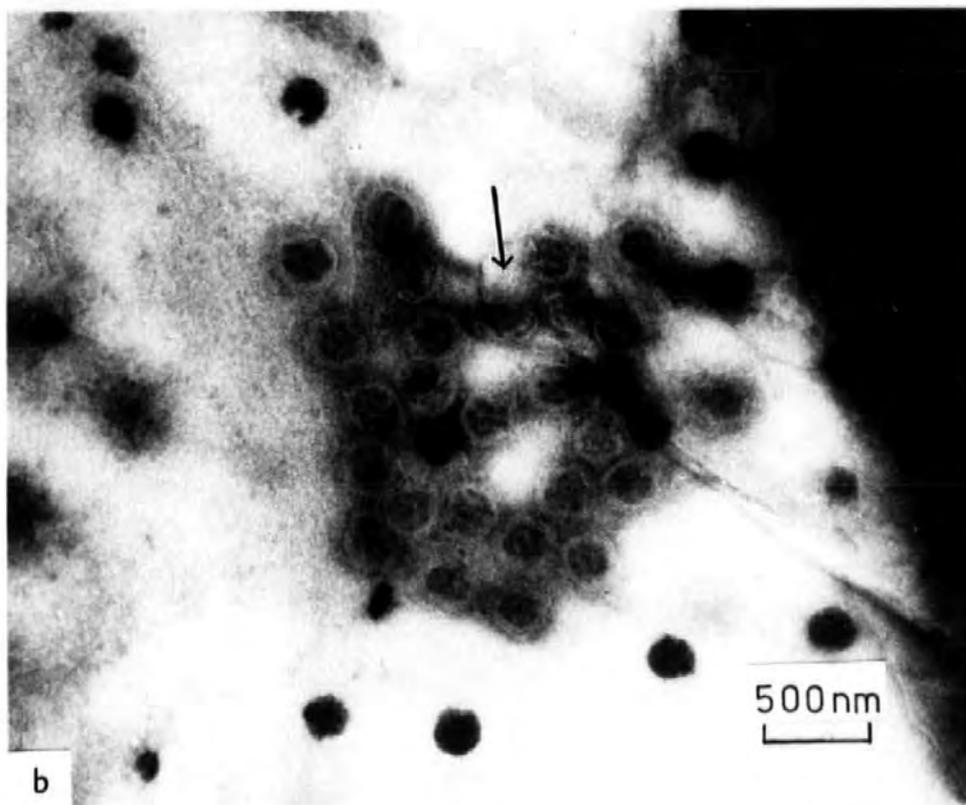
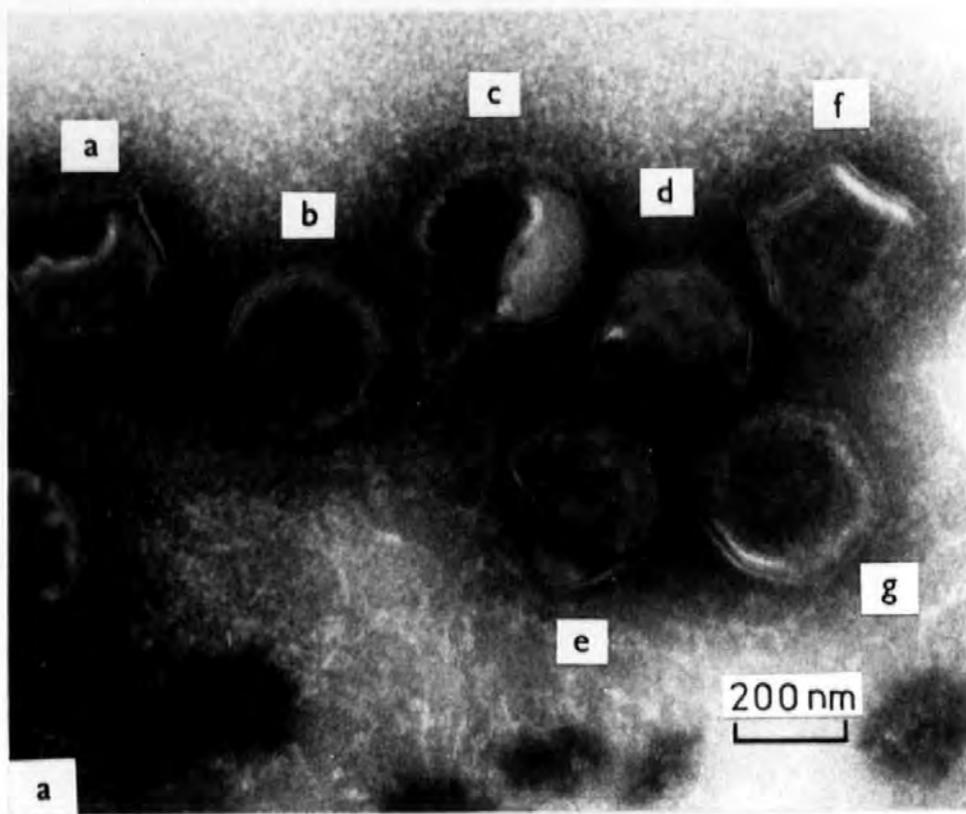
(ii) Descriptive results

Negative staining with ammonium molybdate (AM) and PTA gave new information on the structure of the virus from the blenny infection (Plates 20a, 20b; Table 20). By AM the cluster of particles (Plate 20a lettered) showed the characteristic features of the virus i.e. an angular capsid outline and a discrete core but the stained particles varied as to their condition and size. Both full and empty particles were observed and the measurements of the particles and core diameter varied. Mean measurements on single particles quoted are the average of three diametrical measurements. Thus particles (f) and (g) gave a mean diameter of 340 nm while (a) to (e)

## Plate 20

Electron micrographs, particles from the blenny infection, negatively stained.

- (a) 1% ammonium molybdate, pH 7.0. The virus particles, lettered a to g, in various states of penetration by the stain, are situated on the red cell membrane.
- (b) 1.5% sodium phosphotungstate, pH 6.0. The cluster of particles is located between two red cell ghosts. The arrowed particle shows that the capsid is composed of two layers.



**Table 20:** Particle measurements from negative staining for the virus from the Blenny

Stain	Particle diameter(nm) (A)	Core diameter(nm) (B)	(A-B)nm	capsid thickness (nm)	(capsid-core) gap width(nm)
AM	(285-320) <u>308</u>	(260-280) <u>270</u>	<u>38</u>	<u>9.5</u>	<u>9.5</u>
PTA	(270-325) <u>283</u>	(162-256) <u>221</u>	<u>62</u>	<u>10</u>	<u>20</u>

All figures are means  
Brackets denote range

gave 308 nm (Table 20). Particles (f) and (g) also showed a wide electron-lucent gap between the core and capsid which suggested irregular collapse on staining. For this reason, the lower particle diameter by AM, 308 nm, was therefore taken to be the more reliable. The mean core diameter was 270 nm (260-280 nm) in intact particles and the difference between mean particle and core diameters was 38 nm. The capsid width and the capsid to core gap were both estimated as 9-10 nm.

Using PTA, the mean particle diameter was 283 nm, slightly smaller than with AM but the core was proportionately smaller by PTA at a mean of 221 nm, a difference of 62 nm (Table 20). The capsid measured 10 nm across and the capsid-core gap 20 nm.

The situation and condition of the particles differed with AM and PTA staining. With AM, the particles were actually sited on the red cell membrane whereas with PTA the large cluster was found between the two red cell membranes (Plate 20b). By AM, 4/7 particles (a, c, d & e) were broken or disrupted whereas in PTA all 25 or so particles were unbroken.

The shape and symmetry of the virus were broadly similar by the two stains. The capsid outline was angular, in fact either hexagonal or pentagonal. In full particles, the outline of the core was the same as that of the capsid.

The capsid was well resolved by both stains and it appeared to consist of two layers although this was not clearly shown by AM. Thus by AM, some particles (d,e) showed only a single layer, especially on that side of the capsid close to the core but in others (a,c) the capsid was clearly double-layered where the core was not in contact. Particles (f) and (g) also showed a double-layered capsid; in these particles the bright electron-lucent space between the capsid and core suggested that the core contracted and collapsed under the electron beam leaving an internal space as artefactual. In PTA-stained particles the capsid was clearly separated from the core by a space and the difficulty of resolving the capsid of broken particles did not arise. In the arrowed particle there was also a strong suggestion that the capsid was a double layer.

Micrographs of the core showed some newly observed features of the virus structure. The cores of some particles penetrated by AM appeared as a relatively electron-lucent mass with no sub-structure but interestingly particles (d) and (f) gave the strong impression that the core consisted of filaments. However, no particles showed a core membrane. By contrast, PTA-stained particles showed more detail in the core and the staining pattern was different to that by AM. The prominent image seen was either a spoked wheel shape, with a large outer circle connected to an inner circle by radiating arms, or a more random pattern of filaments or spikes sometimes connected to a central circle. By PTA staining there was again no evidence of a separate core membrane.

C2 Lymphocystis Virus

The virus was studied by ultrathin-sectioning, negative staining, and freeze-drying with shadowing. This work is reported here in sections a, b and c respectively.

C2(a) Ultrathin-sectioned virus

Virus morphology was investigated in ultrathin sections from isolates 3ii and 5 of lymphocystis virus from plaice (see the Materials). The results were largely complimentary for both isolates but in some respects differed (Table 21).

Table 21: Particle measurements of lymphocystis virus from ultrathin sections

<u>Isolate No.</u>	<u>Particle diameter(nm)</u>	<u>Core diameter(nm)</u>	<u>Capsid width(nm)</u>
3ii (plaice)	(200-300) 250 <sup>10</sup>	190	(14-19) 16
5 (plaice)	Fractured (180-200) 190 <sup>10</sup>	(65-80) 72	13
	Intact 278 <sup>2</sup>	ND	18

Single figures are means. Raised figures denote nos. of particles measured. Brackets indicate ranges. ND= Not done

Isolate 3ii showed closely-packed virus particles and the profile of the electron-dense capsid was clearly either hexagonal (arrowed 6) or pentagonal (arrowed 5) (Plate 12a). The cores of these particles did not stain well and they appeared to be finely granular but in some particles internal threads or filaments were seen (Plate 12a - top centre). The diameter of ten particles measured between opposite apices at low magnification was 200-300 nm. At higher magnification the capsid hexagonal outline in mid-planar section was clearly seen and the electron dense core extended internally to the margin of the capsid (Plate 12b). The mean particle diameter for this median section was 250 nm, the mean core diameter was 190 nm and the capsid 14-19 nm across.

By contrast isolate 5 showed a high proportion of fragmented

particles of diameter 190-200 nm (Plate 21a). The capsid did not therefore show a consistent profile since at most four or five edges of the particle could be seen in section. However, the capsid was electron-dense, as in particles of isolate 3ii and appeared angular in outline. In several places it seemed to consist of two or more separate electron dense layers. The cores of these fractured particles consisted either of thread-like material (Plate 21a arrowed -th) or of more granular material that stained with a circular profile (Plate 21a arrowed c). The small cores in the shape of circles measured 65-80 nm diameter and the thread-like material approximately 10 nm across. In the same sectioned preparation of isolate 5 particulate matter was seen including small round particles of approximately 50 nm diam. (Plate 21a). These may correspond to the oval particles 65-100 nm diam. described by Dolowy et al. (1976) in tumours from the Walleye. In this material the small particles were described both intermingled among and contained within the large virus particles.

Complete particles from other regions of lymphocystis isolate 5 were larger than the fragmented ones. Two particles gave an average diameter of 278 nm, measured between opposite apices but the cores were only lightly stained and no filaments were seen (Plate 21b).

#### C2(b) Negatively stained virus

The preparative results for lymphocystis virus are detailed in section (i) and the descriptive results of virus morphology in section (ii).

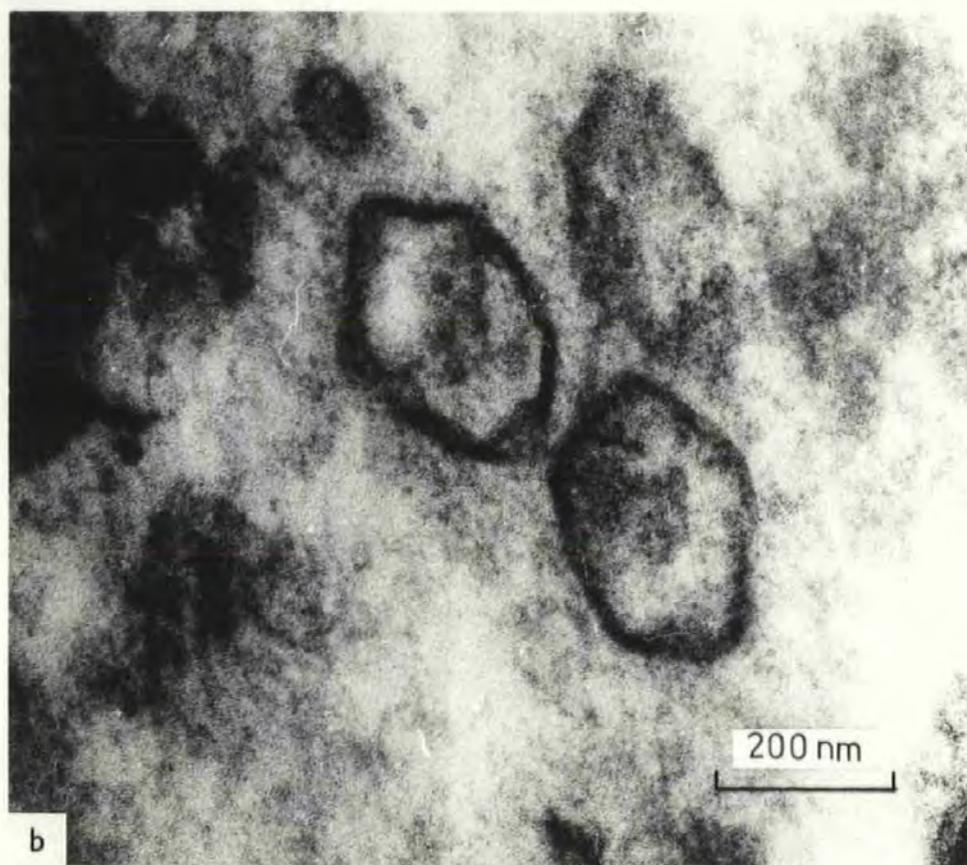
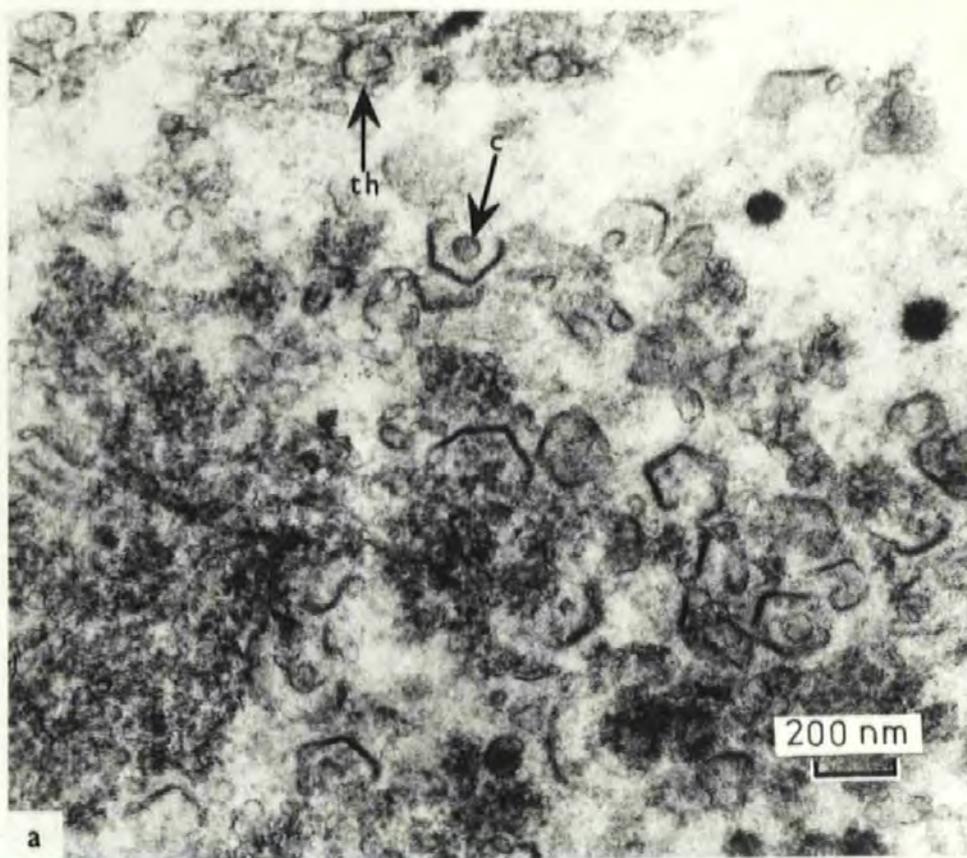
##### (i) Preparative results

With the aim of studying lymphocystis virus by negative staining, a highly purified and concentrated preparation was desired and studies were therefore made on suitable physical preparative procedures. These studies showed that the usual physical methods for purification

## Plate 21

Electron micrographs, sectioned lymphocystis tumour, isolate 5.

- (a) Fractured lymphocystis virus particles. The cores show either a circular profile (arrow, c) or a thread-like appearance (arrow, th). Small round particles of approx. 50 nm diam. are also seen (top left) unassociated with the virus capsid.
- (b) The two virus particles show a pentagonal outline. A small round particle fringed with electron-dense material is seen at the upper left.



were inadequate as sufficient virus concentration for staining could not be maintained. By contrast, the cell spreading method after Parsons (1963), which effectively involved no extraction was found sufficient to achieve a high concentration of virus for staining and gave good negative staining results.

The results achieved with different physical methods for extraction, clarification, concentration and purification of the virus in the combined procedures 1-9 are noted (Table 22).

Not only was it found that efforts to achieve purification with concentration of the virus, as far as they were taken, were unsuccessful but also the combinations of methods used in several attempts were unsuitable.

Different sizes of cells and tumours were used and it was found there was a qualitative difference in the amount of virus that each released. Lesion materials of isolates 3 and 4 (proc. 1-7) were cells of 1-2 mm diam. whereas that of isolate 6 (proc. 8 & 9) was a large tumour of 8 mm diam., weighing 0.5 g. Isolate 6 yielded much more virus than isolates 3 and 4 and the partially purified preparation of proc. 8 was used for shadowing studies as up to 100 particles per grid square could be seen. By contrast, at most 10 particles per grid square could be seen in the final preparations from isolates 3 and 4.

Sonication and hand homogenisation methods for extraction were equally efficacious and sonication gave a final virus count of over  $10^8$ /ml. However, sonication was omitted after proc. 2 as Roberts (pers. comm.) had communicated the sensitivity of lymphocystis virus to sonication.

Clarification of the homogenate was performed by a combination of sedimentation, filtration and precipitation. Low speed sedimentation was effective in removing a large amount of coarse cell debris but as Zwillenberg & Wolf (1968) found that virus from the Bluegill

could form aggregates, filtering with coarse muslin was used instead of sedimentation after proc. 3 and this was quite effective. Freeze-thawing and sedimentation, followed by ammonium sulphate precipitation gave good clarification and yielded a heavy white precipitate which dissolved easily.

Basically, three methods were used for attempted concentration and purification: cycles of differential sedimentation (DS), equilibrium density gradient sedimentation (EDGS) in sucrose solutions and sedimentation onto discontinuous layers of sucrose solutions.

DS alone was found to achieve little real purification as the electron microscopy purity checks showed many small particles both greater and larger than lymphocystis virus. Two preparations were contaminated by bacteria viz. filamentous bacilli and fusiform corynebacteria. The latter probably arose from stored distilled water and the bacteria could be removed by filtration at  $0.45 \mu\text{m}$ . Concentration of the preparation was usually poor though it was better when one cycle rather than two was used as proc. 2 gave  $5 \times 10^8/\text{ml}$  particles in filtered preparations.

Clear refractive bands were not obtained with EDGS (proc. 4-6) indicating the inability to separate virus from cell debris. Two faint bands were observed in proc. 4 but these could not be separated by further sedimentation and when collected together and stained debris obscured the virus. Separation was not possible in a less dense gradient (proc. 5) and virus could be recovered neither from the gradient by DS nor from the pellet by EDGS.

Using sedimentation in discontinuous solutions of sucrose, 20% on 60% at 1:1 volume, some separation of the virus was achieved and a reasonable concentration was maintained for staining (proc. 7 & 8). The result was an upper aqueous yellow fraction containing cell debris, a middle fraction which was uniformly opalescent and a more milky lower fraction above the 60% sucrose. From electron microscopy checks,

the upper fraction contained no virus, the middle fraction virus at approximately  $10^9 - 10^{10}$ /ml, though somewhat impure and the lower fraction little virus. The middle fraction was thus used for shadowing studies.

When isolate 6 was prepared and sedimented by EDGS (proc. 9) and the fractions monitored for absorption at 280 nm in a spectrophotometer it was found there was a single major peak at the top of the gradient (Fig. 8). Clearly there was no separation of the virus from the cell contaminants at the time and speed of sedimentation used.

#### (ii) Descriptive results

Isolates 4ii, 5 and 6 of lymphocystis virus were examined by spread-cell negative staining. Negatively stained images of virus isolate 5 from plaice were well resolved and showed interesting features in both the capsid and the core. The observations made are reported under  capsid structure, core structure and external filaments. Corroborative observations were also made on virus isolates 4ii and 6 from flounder.

#### Capsid structure

Virus from isolate 5 stained with PTA was recognised as lymphocystis virus on account of its large size and angular outline, measuring 290-333 nm diam. at a mean of 311 nm (Plate 22a & Table 22). However, some particles did show a flexuous outline, as opposed to the angular one with straight edges.

The capsid comprised two layers which were clearly distinct when stained with PTA and UF (Plates 22a, & 23b), but whereas PTA-stained particles could be well resolved UF did not offer the same resolution. By PTA the outer layer of the capsid consisted of a row of electron-lucent knobs 4-5nm diam. which fringed a thicker inner layer, the layers being separated by an electron-dense gap of 4-5nm (Plate 22b). In contrast, by UF the two layers were roughly of equal thickness (Plate 23b). The capsid measured 18 nm across by PTA and 13.5 nm

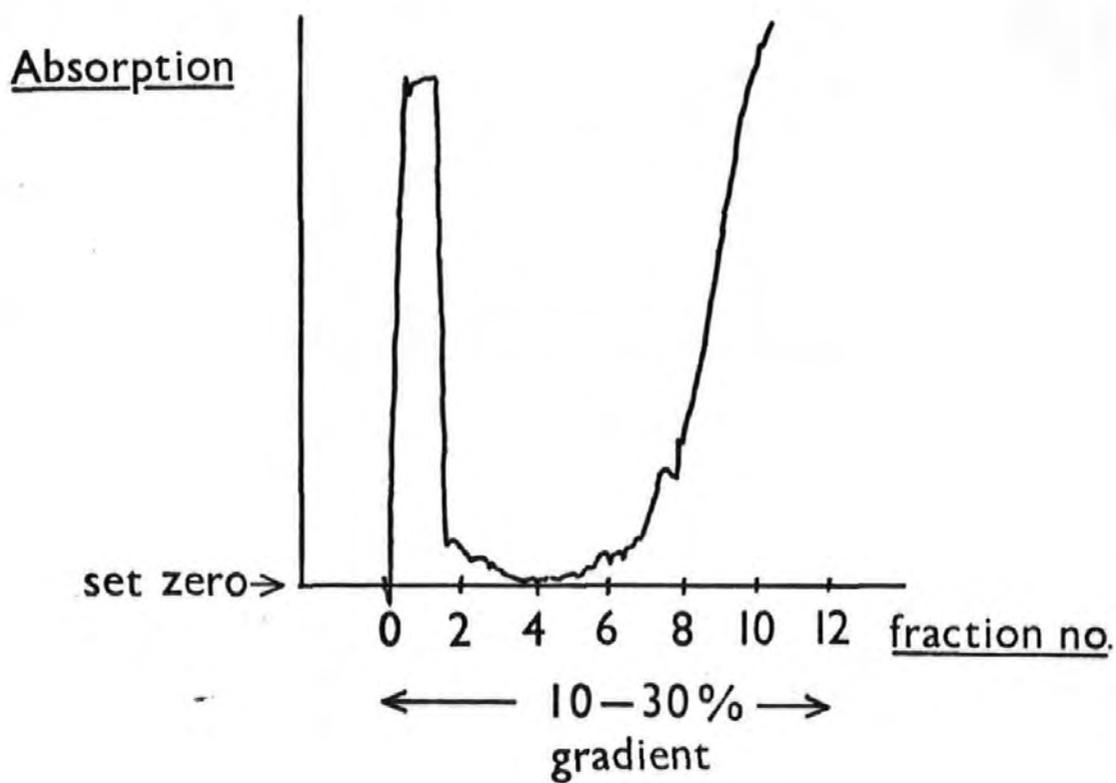


Fig. 8. Absorption at 280 nm against fraction number for lymphocystis isolate 6 sedimented in 10-30% w/v sucrose gradient on a 60% sucrose cushion.

by UF (Table 22).

**Table 22:** Particle measurements of lymphocystis virus isolate 5 from negative staining

Stain	Particle diameter (nm)	Capsid thickness (nm)	Internal Capsid Gap (nm)	Knob diameter (nm)	Internal filament width (nm)	External filament length (nm)
PTA	(290-333) 311	(14-22) 18	(4-5) 4.5	(4-5) 4.5	(13-15) 14	- 700
UF	-	(11-16) 13.5	(4-5) 4.5	5.5	(11-12.5) 12	-

All figures are means. Brackets denote ranges.

#### Core structure

In some particles stained with PTA the core was apparently absent or did not stain (Plate 22a). However, in unbroken particles the most prominent and striking feature of the core was that it comprised a filamentous structure that appeared to be kinked (Plates 23a arrowed, 23b). Filaments were clearly observed in particles from virus isolates 4ii and 6 also. It was difficult to discern whether the filaments were a continuous length and whether kinks were actually breaks. The appearance of the filaments was markedly zigzag with straight lengths of up to 200 nm and sharp kinks between.

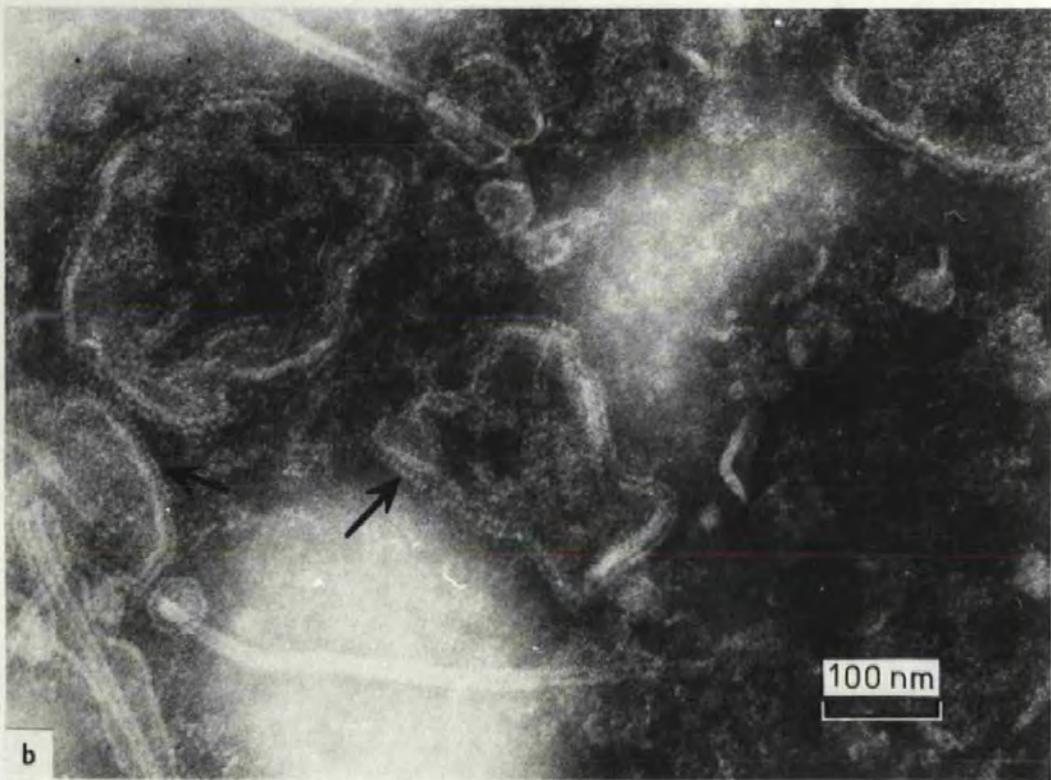
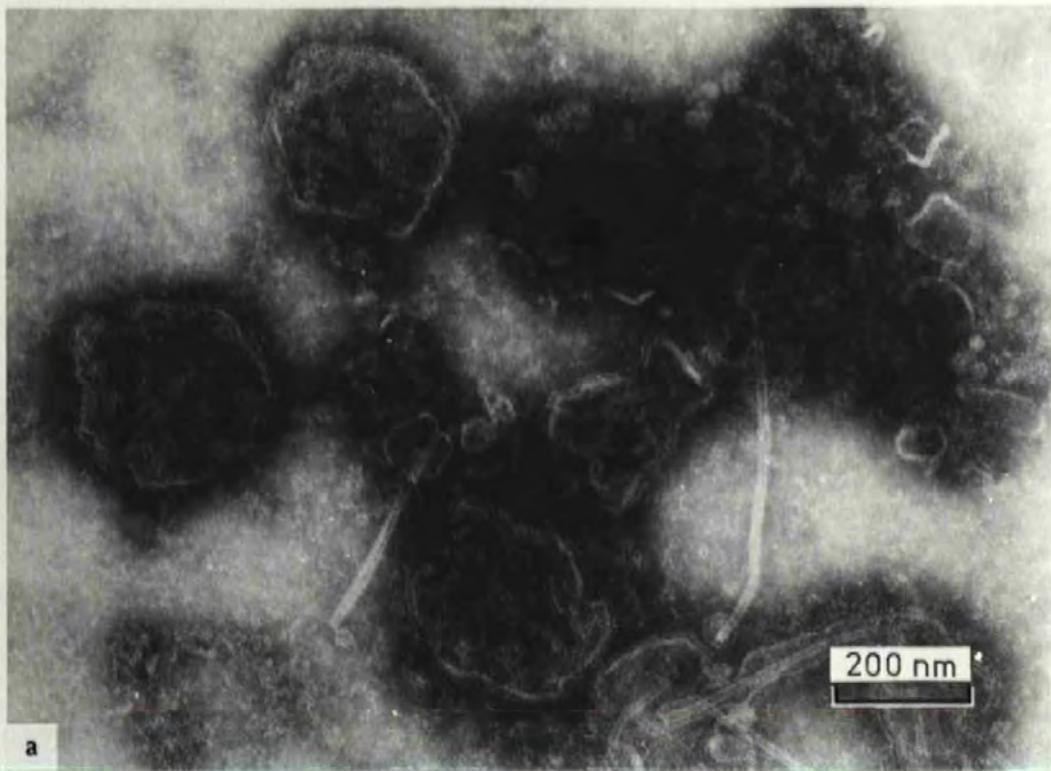
By UF also the core also appeared to consist of a filamentous structure (Plate 23b). These filaments measured 14 nm across by PTA, 12 nm across by UF (Table 26) and showed an electron-dense central line.

In PTA stained particles only, a repetitive substructure was observed along the length of the filament. Electron-lucent subunits could be resolved in the axis of the filament with <sup>an</sup> inter-subunit spacing of approximately 6-7 nm (Plate 23a arrowed). The subunits were observed not opposite one another on either side of the electron-dense line but lay on an axis at approximately 60° to that of the

## Plate 22

Electron micrographs, lymphocystis tumour isolate 5 negatively stained with 2% sodium phosphotungstate pH 7.0.

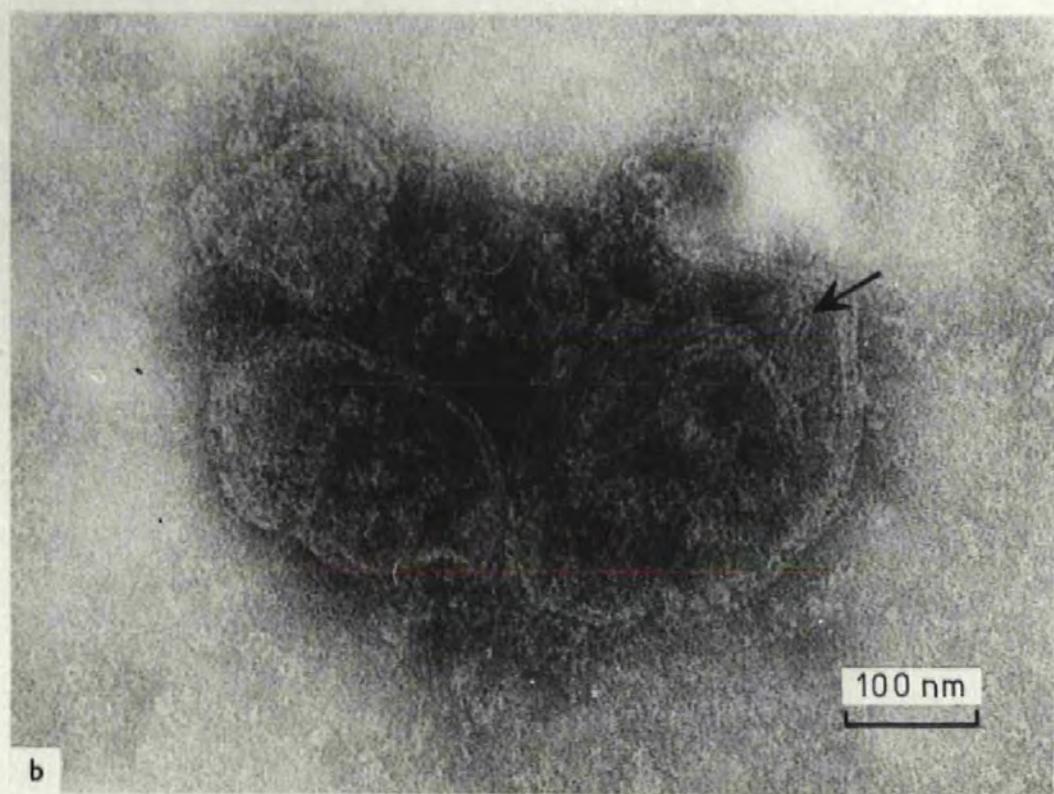
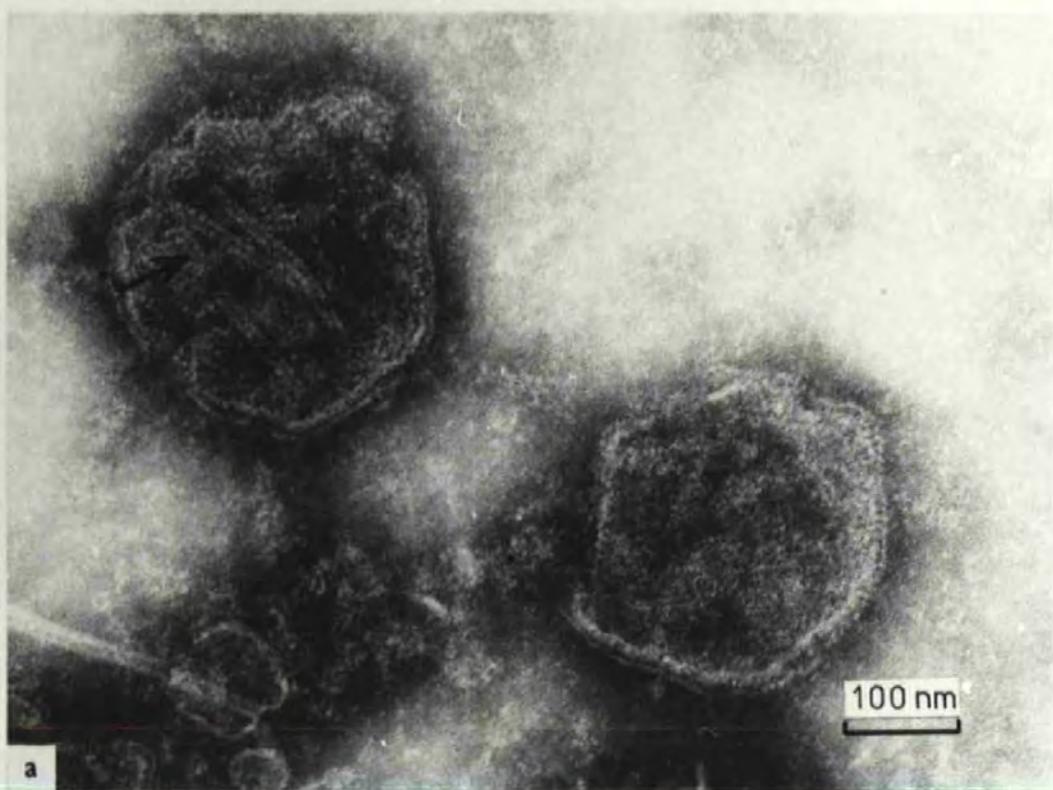
- (a) Virus particles at various degrees of collapse are seen showing both angular and more irregular outlines.
- (b) The capsid clearly comprises two electron-lucent layers; a thick inner layer and an outer layer which consists of a row of knobs each 4 - 5 nm diam. (arrowed).



## Plate 23

Electron micrographs, lymphocystis tumour isolate 5, negatively stained.

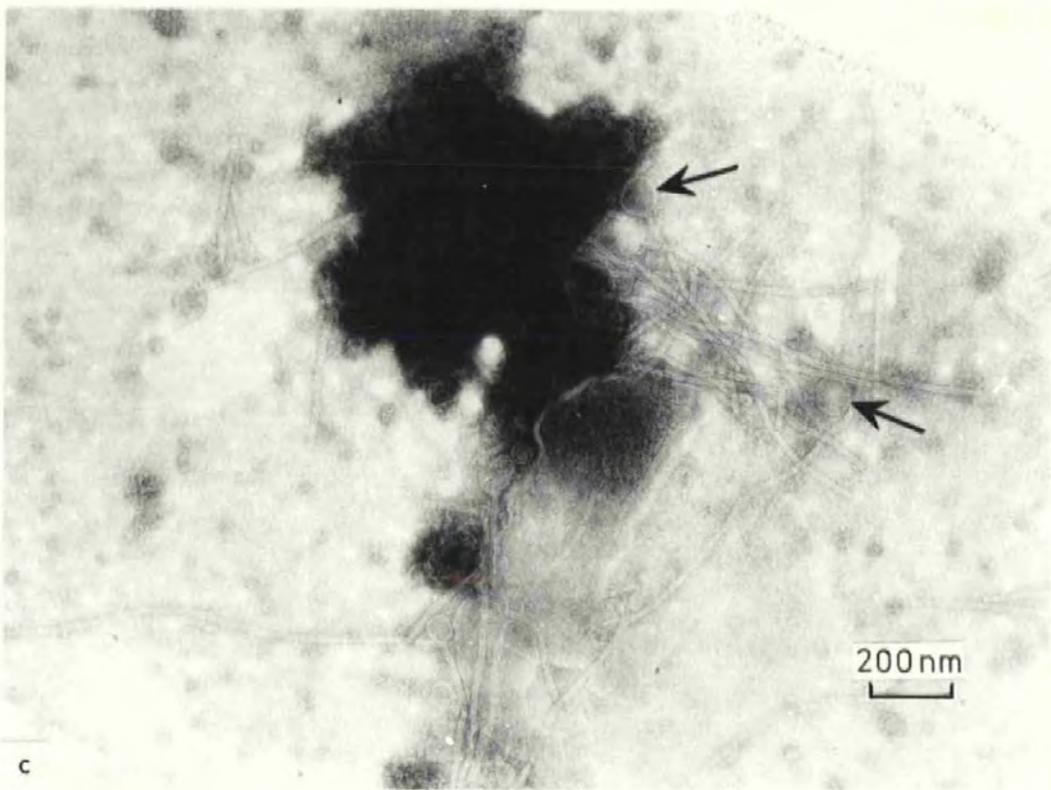
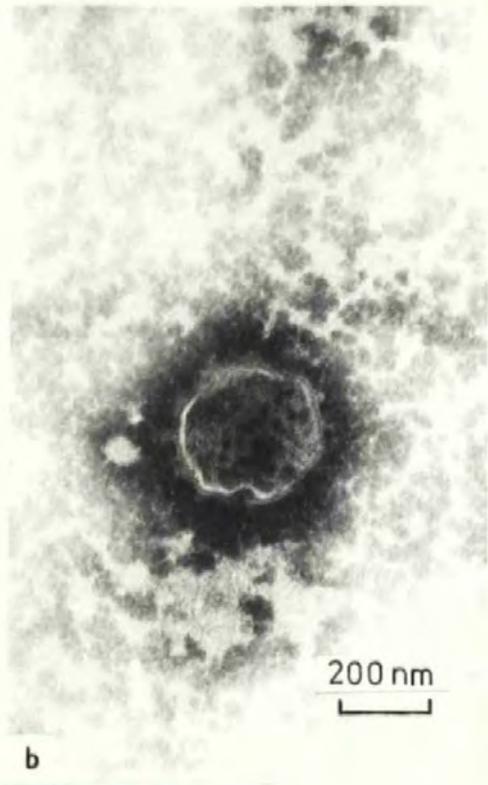
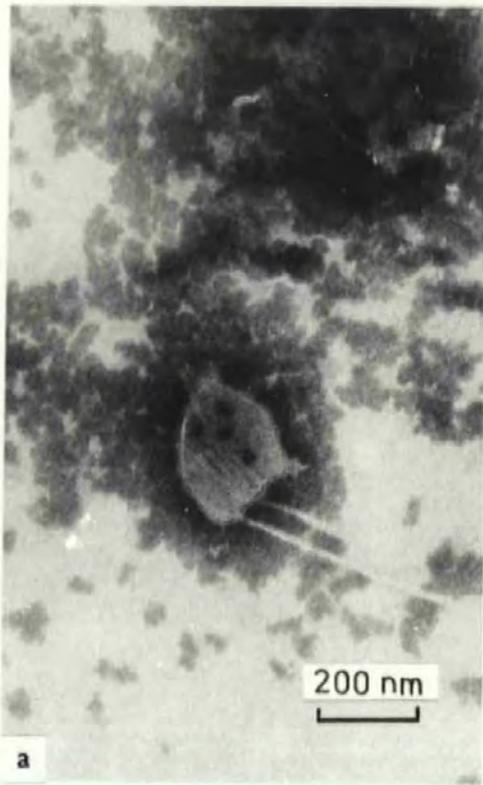
- (a) 2% sodium phosphotungstate pH 7.0. The particle at the upper left shows a kinked internal filament (arrow) with a periodicity along its length.
- (b) 1% uranyl formate pH 4.5. A kinked internal filament (arrow) is seen within the collapsed particle but no periodicity is apparent. The capsid is clearly bilayered.



## Plate 24

Electron micrographs, lymphocystis tumour isolate 5, negatively stained.

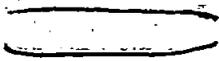
- (a) 2% sodium phosphotungstate pH 7.0. Filaments are seen outside the particle but they are clearly continuous with internal core filaments.
- (b) Staining as above. Kinked internal filaments are seen in a virtually intact particle.
- (c) 1% ammonium molybdate pH 7.4. A large number of filaments are seen unassociated with lymphocystis particles and many show terminal knobs (arrows).



filament.

#### External filaments

Negatively stained preparations of virus isolate 5 showed filaments outside the particle itself, of similar size and morphology to those described in the core and such observations provided evidence of particle breakdown. Broadly, three states of breakdown were recognised at different levels of association of the external filaments with the particle. Firstly, there was seen a state where the filaments extended beyond the boundary of the capsid but the particles were clearly recognisable as unbroken and the external filaments, as continuous with the internal filaments (Plate 24a). Secondly, there was seen a state where breakdown of the particle had clearly taken place; in this case there was no recognisable core with which the external filaments were continuous but they originated from within the particles (Plate 22a). Thirdly, a state was observed where a large number of filaments were observed unassociated with any particles (Plate 24c).

The external filaments described differed in several respects from the internal ones. In PTA-stained fractured particles (Plate 22a), the width of the external filaments was not constant but varied from 14-21 nm and the electron-dense central line within the filament  was not straight but slightly sinuous. In the AM-stained preparation where no particles were seen (Plate 24c) the filaments were of constant width 12 nm, the same approximately as the internal filaments but many showed terminal knobs up to 90 nm across, which seemed to be an extension of the filament (Plate 24c arrowed).

#### C2(c) Shadowed virus

The partially purified preparation of virus isolate 6 was freeze-dried and shadowed with platinum-carbon at a narrow shadow angle of 25°. Although few particles were seen those viewed cast a shadow that

clearly showed an angular outline(Plate 25a).

A compound shadow was also seen (Plate 25b) which is analysed in figure 9. This shows the diameters and shadow lengths of the capsid and core. Given the shadows of 144 and 288 nm and a shadow angle of  $25^{\circ}$ , the height of the core in particular is measurable by trigonometry. The core measures 134.3nm in height and this compares with 120 nm for the diameter, allowing for it to be sitting on a layer of capsid of 13.5 - 18.0 nm (taken from negative staining measurements).

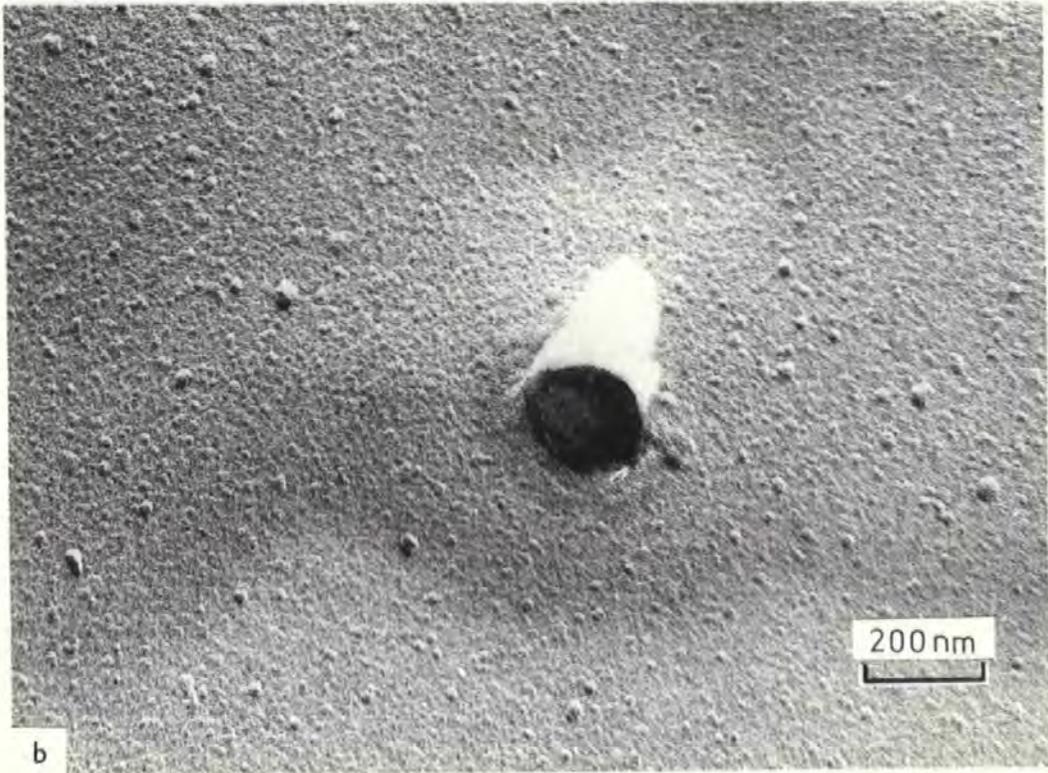
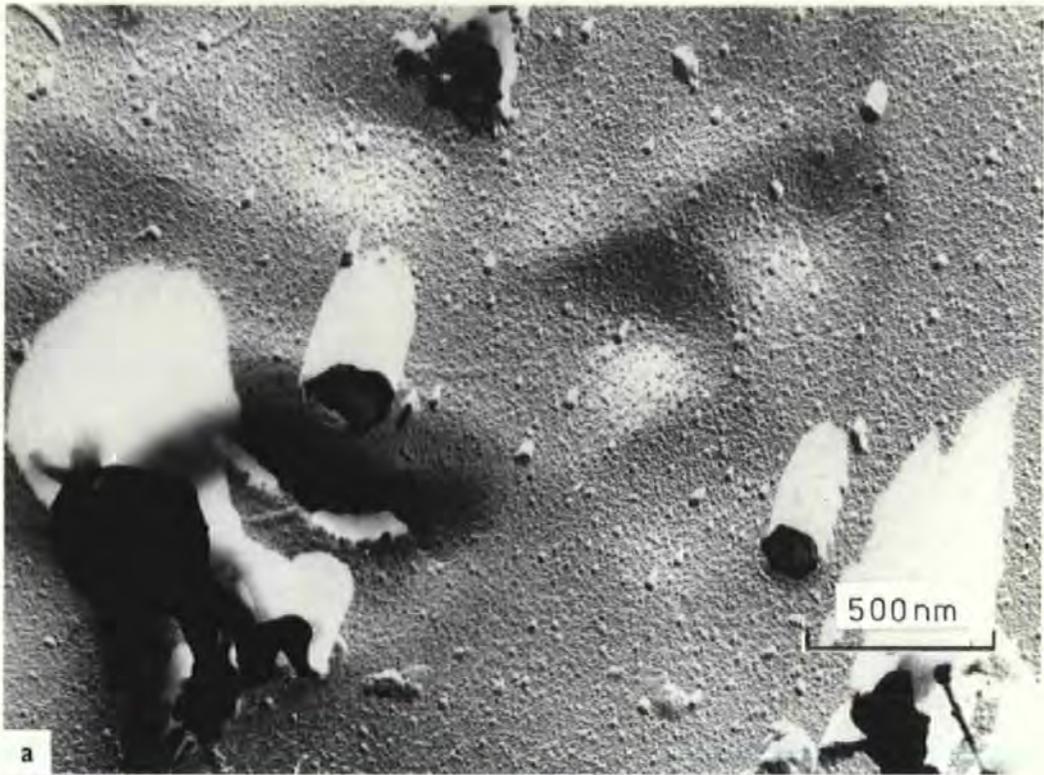
C3 Results with processing and embedding methods for electron microscopy

The recommended procedures in Mercer & Birbeck (1963) were tried at first. Lymphocystis cells (isolate 3ii), when fixed and prepared by Method A gave acceptable sectioning results with TAAB resin embedding. Method B, using araldite for embedding of erythrocytes, was not a satisfactory schedule as araldite was found brittle to section. In contrast, embedding with Spurr's low viscosity resin (Method C) was found most satisfactory for both soft and hard fish tissues with the proviso of doubling the processing times for hard tissues e.g. fish skin. The rapid processing method of Doane et al. (1974) (method D) was used for lymphocystis virus isolate 5. This was found convenient as processing was completed in 2 hr. Sectioning results were quite comparable to those with Method C, showing that the quality of fixation and embedding was not affected by shortening the processing times.

## Plate 25

Electron micrographs, lymphocystis isolate 6, particles shadowed at 25° with platinum-carbon.

- (a) The shadows of two particles are clearly angular, that to the right showing six sides.
- (b) A compound shadow of one particle comprises the relatively short shadow of the collapsed capsid and the dome-like shadow of the core.



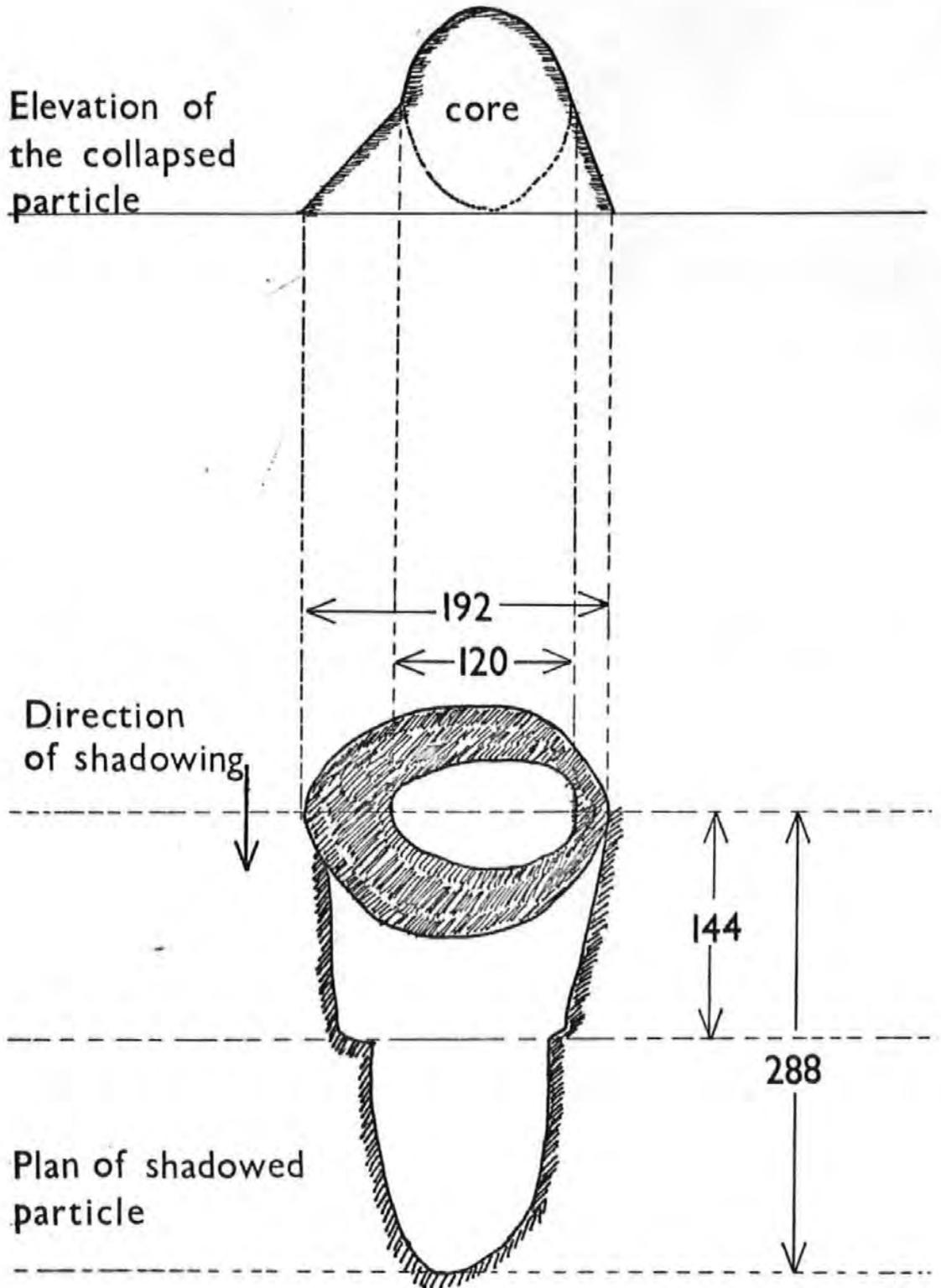


FIG.9. Shadowed lymphocystis virus indicating the elevation of a collapsed particle (numbers denote nm).

## DISCUSSION

Cod PEN and PEN in general

The results of electron microscopy were not satisfactory for demonstrating a viral infection in the erythrocytes, in so far as virus particles were not seen but some of the changes in the nucleus seen in blood smears could be identified e.g. chromatin condensation and vacuole formation.

Several facts can be adduced to suggest that in the erythrocytes of the blood sample examined, artefacts were unlikely to have been present and that there was only a small chance of seeing the virus particles in section. Thus in the respective smear of the blood sample, only 1-5% of mature erythrocytes were infected and nuclear lesions without cytoplasmic involvement were commonly seen. Furthermore the buffer was determined as isotonic to the blood cells examined and the red cells were not haemolysed before processing.

Ultrastructure of PEN in cod as described here does not permit comment on virus morphology but the virus of PEN in cod as described by previous authors can be compared to the virus of the blenny infection. Similarly, comparisons can be drawn with the virus of herring PEN (Phillipon et al., 1977; Reno et al., 1978).

The measurements of the virus from cod PEN clearly show it to be consistently larger than that of the blenny infection in capsid, core and internal space. Walker & Sherburne (1977) also remarked on the 'surcoat' that surrounds each particle from cod PEN and this has not been seen in the blenny infection. However, it may be similar to the external fibres that have been seen on the surface of some iridescent viruses (Stoltz, 1971).

The association of incomplete particles with viroplasm, typical of cod PEN (Walker & Sherburne, 1977), was also clearly shown by Johnston & Davies (1973) for the blenny infection and indicates a resemblance of these viruses with respect to their pattern of

replication. The spheroidal viroplasm is the actual replicating pool of DNA and assembly and maturation of the virus appears to take place at its edge.

The most basic difference between cod PEN and the blenny infection is the fragmentation of the nucleus. As remarked in section A under 'Recognition' this feature is present in cod red cells but mostly absent in the blenny infection. Walker & Sherburne (1977) have remarked on the integrity of the nuclear membrane of highly fragmented nuclei but on the contrary, Appy et al. (1976) remarked the nuclear membranes of infected cells were "ill-defined". It is not known of course whether this reaction is a corollary of virus infection i.e. resulting from release of virus-coded enzymes. However, investigation of biochemistry may show a fundamental difference in the replication of the viruses and the effects of the viruses on the host nucleus and cell.

Herring PEN and the virus is in some respects similar to the blenny infection and cod PEN and in other respects different. Common to all three infections is the type I inclusion body or viroplasm and common to herring and cod PEN are the lamellar body and margination of the nuclear chromatin. In three respects however, herring PEN virus is different: firstly the virus is smaller than the others measuring 145 nm by thin-section, secondly the area of cytoplasm proximate to the inclusion is electron-dense and thirdly the centre of the virus core is electron-lucent.

Comment on the significance of the changes that characterize PEN infections is necessarily speculative for the infections will have to be passaged and studied in vitro. The following questions could then eventually find answers: (1) how are the nuclear lesions caused by a cytoplasmic virus? (2) is the lytic effect on the nucleus caused by inhibition of host cell DNA synthesis? (3) how is the virus from the blenny infection different at the molecular level to that

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from cod, in involving no predominant nuclear lesions?

In the main the features of the viruses of cod PEN, herring PEN and the blenny infection reveal differences of morphology and suggest those differences of pathology in addition. Taking Walker & Sherburne's suggestion, it may be appropriate to view these three fish viruses as divergent viruses within a group of specially modified erythrocytic icosahedral cytoplasmic deoxyribonucleic acid viruses (EICDAVs).

### The blenny infection

#### (1) Preparative techniques

The results from thin-section corroborated those of Johnston & Davies (1973) but negative staining has not been done before and the results here provided useful supporting information on the morphology of the capsid and the core. There is no doubt that the methods for preparing and staining the virus could be extended to give results under a wider variety of conditions. The conclusions and suggestions for further study are therefore discussed.

As a method of dispersion it was found that pelleting red cells and transferring them direct to a grid was better than the spreading method as the cells would not spread onto the stain surface. Parsons (1963) reported differential spreading of a variety of tissues and cells, neoplastic cells and fatty tissues being better than normal cells and connective tissue. He suggested the differential adhesiveness of the plasma membrane was responsible for this effect and this would indicate the red cell membrane is quite adhesive.

Controlled lysis of the red cells was optimal for resolving the virus particles but even when sudden lysis was done it was clear that a cloud of particles was still attached to many of the ghosts or was to be found a short distance from the membrane. This indicates that the particle cluster stays intact possibly as a consequence of being surrounded by a protein matrix. Walker (1971) found a similar effect

in stained smears of cod, noting that "even in ghost cells or in residues in Giemsa smears where cytoplasm and plasma membrane are completely dispersed, the viroplasm and its adherent group of virions stay together". Walker & Sherburne (1977) also remarked on this affinity.

It was found that the two negative stains, AM and PTA, gave very different penetration of the virus core perhaps on account of their different conformations. Further study with uranyl salts could be profitable, as well as the use of a varied pH range with each. The use of AM for staining red cell membranes obviated the need for prior fixation (Muscatello & Horne, 1968) which is desirable with PTA and other stains. It would be interesting to compare the result with PTA to that using prior fixation.

Another approach that might be useful would be to try controlled degradation of the capsid layer with detergents, to prepare a naked core for staining. Unless the particles could be concentrated away from the cells, this would have to be done in situ on the red cell membrane and the additional degradative or dispersive effect of the detergents on the protein matrix would also have to be investigated.

In addition to thin-sectioning and negative-staining it will be necessary to study the virus by shadowing and possibly freeze-etching, applying the precaution of freeze-drying the specimen as it has been shown that air-drying of preparations can cause artefacts of particle collapse (Nermut, 1972).

## (2) Evidence for classification

Circumstantial evidence exists for assigning the virus of the blenny infection to the group of icosahedral cytoplasmic deoxyriboviruses (ICDV) collectively reviewed by Kelly & Robertson (1973) and more recently termed the family Iridoviridae by Fenner (1976). As the former authors pointed out, the criteria of affinity to this group

are by definition "by no means stringent" i.e. that the virus shows icosahedral symmetry, it is replicated in the cytoplasm and contains DNA.

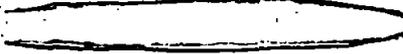
However, strictly speaking it is not possible to fulfill each of these criteria satisfactorily. Thus the symmetry has not been conclusively demonstrated by double-shadowing, rather it is implied from the observation of 6-sided and 5-sided profiles in thin section. As Stoltz (1971) has shown, the different profiles result from section in the alternative 2-, 3-, and 5-fold axes of symmetry in the icosahedron. Similarly, the virus has not been shown to replicate only in the cytoplasm, since experimental in vitro infections have not been studied but particles are formed only in the cytoplasm. This is a matter of definition but the interpretation of the sequential development of the particles remains no less conjectural than in the paper of Johnston & Davies (1973). Furthermore, the evidence that the virus contains DNA is still limited to staining, as discussed under 'Recognition' (Section A) and its isolation has not been performed.

On the whole therefore, the evidence suggests the inclusion of the virus in the ICDV group and the Iridoviridae as a probable member only and more conclusive evidence is required for classification.

The measurements by thin-section on the particle and its capsid corroborated those of Johnston & Davies (1973) but the observations on negatively-stained particles also provided evidence of a new interpretation of the core structure.

The particle diameter of 240-280 nm and the capsid width of 14 nm were very comparable to those figures previously recorded by Johnston & Davies (1973): 250 nm and 12-14 nm respectively. It was also found that the capsid consisted of two electron-dense layers and the core or nucleoid generally adopted the outline of the capsid, at least in thin-section. However, it would be inappropriate to conclude that the core therefore exhibits definite spherical symmetry.

The spleen erythrocytes, in which particles were seen, clearly showed the presence of an area of altered host cell cytoplasm.

 This was identified as somewhat resembling the inclusion body of erythrocytes but its long crescent shape in section clearly indicated that it differed from the round body of smears. It could be therefore that the pattern of infection is quite different in the spleen. Johnston & Davies (1973) also noted inclusion body areas but the large peripheral granules were not reported. Interestingly, Reno et al. (1978) also reported cytoplasmic electron-dense granules of approximately 20 nm in PEN-infected herring erythrocytes. They suggested quite plausibly that these were ribosomes, synthesized de novo under the direction of the viral genome for the production of new proteins. This may be a parallel to the blenny infection.

Results from negative staining and ultrathin sections strongly suggest the core can be interpreted as a lantern-type structure, in which there are eight outer 'arms' or threads forming semi-circular arcs about a central thread, which is hollow (Fig. 10).

There are two principal reasons for substantiating this model. Firstly, it explains how the core shows different appearances with variable orientation in thin-section and in negative stains and secondly it explains how the negative staining picture is the exact negative of the thin section appearance. Thus the model supports the appearance of the core in median horizontal and vertical section viz. respectively 'spoked wheel' and divided circle forms. Tangential and oblique sections would have given rise to the other variations shown and these were in fact seen (Plate 13b).

Interestingly a close parallel to the 'spoked wheel' forms was noted by Stehbens & Johnston (1966) in sections of the plausible virus from Pirhemocytan. They noted "irregular target and cartwheel forms" but did not propose a model structure for the virus.

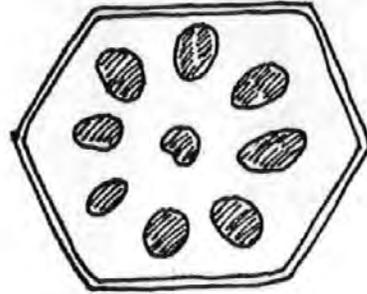
Fig. 10. Appearances of the virus from the blenny infection by negative staining and in thin section and a possible model of the core.

Negative stain



Plan

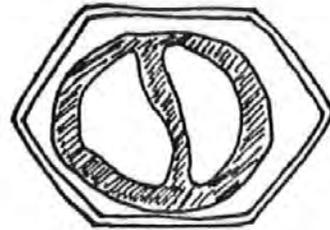
Thin section



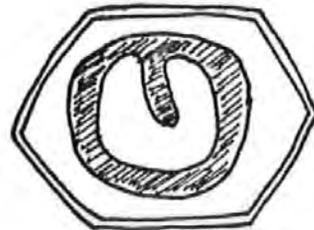
Median horizontal



Elevation



Median vertical



Oblique

Possible model  
of the core



The model was also supported by the observation that some images of the particles were seen more frequently than others. Thus since the 'spoked wheel' image by negative staining was relatively rare and its corresponding image in thin-section quite common, this would suggest the 'spoked wheel' image arises firstly only as a result of the alignment of the core axis with the electron beam and secondly only as a result of the diffraction of the beam by whole penetrated particles, which is not obtained in thin-sections. On this hypothesis, alignment other than in the axis of the beam should give a more haphazard arrangement of filaments. Orientation at  $90^\circ$  to the beam should give an arrangement of electron-lucent parallel lines but this was not seen, rather a much more random arrangement of the 'arms'. Clearly, these observations seem to be accommodated by the model.

This evidence from negative staining would suggest the organisation of the DNA proposed is unique among the Iridoviruses. Similar structures have been put forward but none is actually the same as the 'lantern' arrangement. Thus Mattern, Hruska & Diamond (1974) reported the core of a polyhedral amoebal virus V 301 was a continuous folded linear structure with orthorhombic symmetry (222) showing three orthogonal two-fold axes. The symmetry of the virus from the Blenny appears to be the same as the amoebal i.e. with three two-fold axes of symmetry but the conformation of the threads is clearly different. Mattern et al. (1974) described 19 filaments in hexagonal array in cross section and longitudinal section showed curved links between the filaments, whereas neither of these features were seen here.

Other reports indicate that DNA can be coiled around an electron-lucent centre. Furlong, Swift & Roizman (1972) suggested from thin-section evidence that the core of a herpes virus forms a toroidal structure with a central electron-lucent zone and Cummings & Wanko (1963) found this effect also in the core of T2 bacteriophage. These

studies identified a relatively discrete electron-lucent zone but no threads were observed and no conformation suggested. It appears that the virus of herring PEN may be doughnut-shaped or toroidal also, for the centre of the core is conspicuously electron-lucent in transverse and longitudinal section. Medzon & Bauer (1970) also suggested the DNA in the nucleoid of vaccinia virus was coiled in complex fashion. Tween 80-treated virions in section showed a twisted yarn appearance indicative of a thread-like organisation of the DNA. They suggested this was achieved by interaction with basic proteins and this is possibly the form of stabilization of the core of the Iridoviruses as well.

Comparative thin-section and negative staining studies on the cores of iridoviruses are few and there is therefore no baseline for comparison in this study. Some of the iridescent viruses have been studied by negative staining (Wrigley, 1969; Mercer & Day, 1965), particularly from the viewpoint of the surface structure but these studies only hinted at the presence of core filaments. Almeida, Waterson & Plowright (1967) studying African Swine Fever Virus (ASFV) showed that the nucleoid of mature particles was dense and compact by negative staining and this perhaps suggested filaments. It is speculation to say how the virus from the Blenny relates to other iridoviruses in this respect but comparative studies are clearly called for using identical conditions of staining.

Apart from the comparison of the core, previous work suggests that the erythrocytic viruses are distinct from the iridescent viruses and ASFV (Kelly & Robertson, 1973) e.g. the erythrocytic viruses do not form para-crystalline array as do iridescent viruses and budding from the cytoplasmic membrane is not observed as in ASFV infection.

Lymphocystis virus(1) Preparative techniques

The relatively conventional techniques used showed some novel aspects of the virus structure but there is no doubt that more sophisticated methods would attain greater resolution and possibly reveal new features of structure.

The quick method of embedding and fixation of Doane et al., (1974) worked well using isolate 5. However, it would be interesting to size the virus from the Bluegill using comparative methods.

Cell spreading was found to be the most practical method of transferring a reasonable amount of virus to the grid for negative staining and reproducible results were achieved with isolate 5. The efforts to purify the virus by physical methods proved fruitless and Hess (1971) also commented on the difficulty of purifying ASFV by differential and rate zonal sedimentation. With my isolates, at best only partial purification was attained by these methods and it is possible that both ASFV and lymphocystis virus are either fragile and sensitive to shearing forces or can become highly aggregated.

Three different approaches could be tried. Firstly, the substitution could be tried of a buffer with high level of sodium chloride in place of ordinary phosphate buffers, to raise the molarity of the buffer to that for sea water. In addition, as ammonium sulphate precipitation was relatively successful, a clarification step preceding purification would seem very worthwhile. Secondly, as it was possible that particle clumping was responsible for the removal of virus at low speed sedimentation, as found by other workers (Zwillenberg & Wolf, 1968), chemical agents could be used to lessen aggregation e.g. non-ionic detergents. Thirdly, different types of density gradient could be tried instead of sucrose e.g. tartrate and caesium chloride.

(2) Evidence for classification

The angular outline of the shadow indicated strongly that the particle is icosahedral though double-shadowing should strictly have been done for full proof (Williams & Wycoff, 1946). The thin-section evidence also indicated that the virus occurs only in the cytoplasm though replication was not proven. No studies here indicated that DNA was actually contained in the particle but other workers have indirect evidence. Walker (1965) showed that the inclusion zones of virus-infected cells fluoresced bright green with acridine orange indicating DNA at the 'factory site' and Midlge (pers. comm.) demonstrated by autoradiography that the inclusions of infected fibroblast cells from the Bluegill are areas of rapid de novo DNA synthesis.

(3) Further points of comparison

Lymphocystis virus will now be compared as far as possible with the virus of the blenny infection and other iridoviruses.

The thin-section evidence showed that the core symmetry was broadly the same as that of the capsid i.e. spherical in intact particles but occasionally threads were seen in broken ones. The capsid appeared to be double-layered as well. In view of the variation in the core staining, it would be interesting to try and prepare naked cores, then to fix and process them normally, to determine if there is a difference in staining between cores fixed in whole particles or cores fixed nakedly.

Negatively stained particles showed the capsid was clearly two layers and corroborated thin-section studies on the capsid. However, new observations strongly suggested that the core comprised filaments and that the symmetry of these  filaments was essentially helical and not  spherical or icosahedral. The symmetry of the core in no way

therefore reflected that of the capsid. The most important evidence for this conclusion is the clearly resolved periodicity along the length of the internal filaments. This has also been seen in isolates 5 and 6 of lymphocystis virus from plaice and flounder by C. R. Madeley of the Regional Virus Laboratory, Ruchill Hospital, Glasgow (pers. comm.).

To my knowledge there is no previous record of a virus showing a DNA core with helical symmetry akin to that of Tobacco Mosaic Virus (TMV) or the core of the Myxoviruses. However, the filament itself is not proven to contain DNA but in all probability it is a nucleoprotein helix. A possible interpretation of the arrangement of the core is drawn (Fig. 11).

The origin of the external filaments seen in negatively-stained lymphocystis preparations must be speculative for since the isolates used were not purified there is no guarantee of their origin. However, where the filament appears to leak from an internal component there are grounds for thinking that their true origin is from the core. Such filaments have also been observed by C. R. Madeley.

With respect to the capsid, my observations indicate that the model of Stoltz (1973) for the general capsid structure of all ICDVs can be corroborated. Zwillenberg & Wolf (1968) and Midlige & Malsberger (1968) both intimated the capsid was double-layered by negative-staining and furthermore the evidence here shows that the outer layer is particulate, staining as a row of knobs and the inner layer is continuous. The inner layer must therefore consist of the structural capsid sub-units as suggested by Stoltz (1973), to which the outer knobs are attached by the hypothetical thin spikes of Stoltz (1973). There was no evidence from my micrographs of spikes but as the working level of resolution of the microscope was not less than  $20 \text{ \AA}$ , thin spikes may not have been seen. The gap between these two layers described must be the dark space in the model of

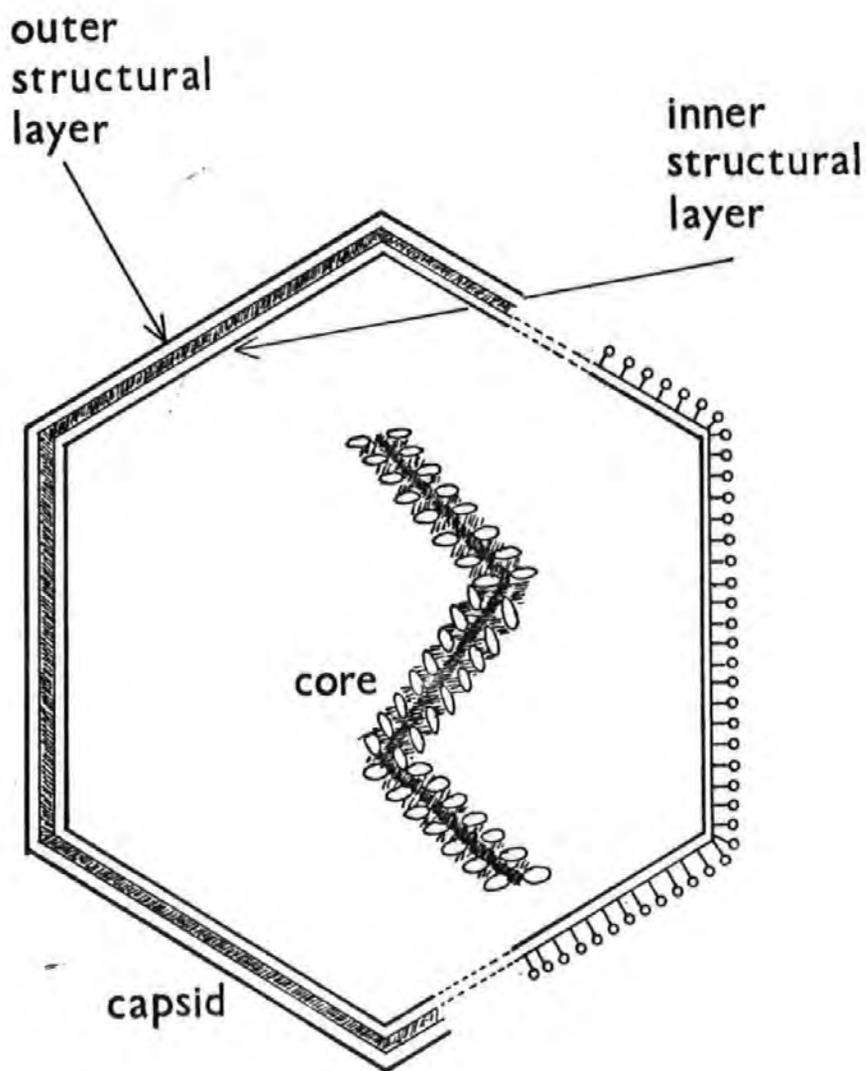


Fig. 11. An interpretation of negatively stained lymphocystis virus. At the right, resolution of the outer structural layer as knob-like sub-units is indicated.

Stoltz (1973).

It is difficult to make comparisons with the other ICDVs at this level of detail but the question remains of how similar lymphocystis virus is to the insect and amphibian ICDVs. The core structure would suggest that it is unique but other viruses of the group may be shown to have the filamentous core. It is possible that the virus was stained during particle collapse and it would certainly be more satisfactory to standardize the staining conditions for such viruses of large diameter by prior freeze drying (Nermut, 1972).

With these sort of problems of comparison, and the fact that the core filaments have been seen only in isolates of lymphocystis virus from marine flatfish taken around the British Isles and not in the Bluegill, it is not yet possible to say that lymphocystis virus is quite different morphologically to all ICDVs. However, with respect to the model proposed for the core structure of the virus from the Blenny, it is very difficult to imagine that these viruses are morphologically similar.

It should again be emphasized that as the discussion has centred on descriptive rather than experimental evidence for classification, morphological criteria form only a part of the classification evidence. Further appraisal will await the propagation of the lymphocystis virus isolates from British waters and their biochemical analysis. Nevertheless, the unusual morphologies of both lymphocystis virus and that of the blenny infection pose interesting questions in perceptual reasoning, which are significant to an understanding of the construction of viruses.

APPENDIX

The suppliers of laboratory equipment, chemicals and tissue culture media are listed here in tabular form. The compositions of tissue culture media are given in the last section.

1. Laboratory equipment

<u>Item</u>	<u>Particulars</u>	<u>Supplier</u>
Haematocrit tubes	Heparin coated also uncoated.	Gelman-Hawkesley, Haywards Heath, Sussex.
Syringes	1, 5, 10 & 20 ml capacity.	Becton-Dickinson, Wembley Park, Middx.
Hypodermic needles	19G, 25G. (Luer) 27G. (Luer)	" " Yale (Corp.), U.S.A.
'Vacutainer' system	Vacutainer tubes cap. 4.5 ml citrated. Multisample needle. Multisample vac. adapter. Luer fitting.	Becton-Dickinson, (As above)
5% CO <sub>2</sub> in Air		British Oxygen Special Gases, (Old Deer Park), Surrey.
Automatic Osmometer	{ Schuco { 'Osmette-A'	Precision Systems Inc, Newton, Mass., U.S.A.
Centrifuge & rotors	Superspeed 50 'fuge 'Swing out' head rotor 3 x 25 ml cap. 'Swing out' head rotor 3 x 3 ml cap. 'Angle' head rotor 8 x 50 ml cap.	M.S.E., London.
Laminar flow bench for tissue culture	HLP model	S.L.E.E. Co., London.
Membrane filters	Filter holders: 'Swinnex' and 'Millex' adapters. Filters: 1 & 2 cm discs of pore diam. 0.22 $\mu$ & 0.45 $\mu$ .	Millipore Corp'n

<u>Item</u>	<u>Particulars</u>	<u>Supplier</u>
Ultrasonic disintegrator	150 watt max. output	M.S.E., London.
Mediswabs		Pharmax, Bexley, Kent.

2. Chemicals  
(a) General

<u>Item</u>	<u>Particulars</u>	<u>Supplier</u>
Quinaldine		BDH, Poole, Dorset.
DAPI	4'-5 diamidino - 2 phenylindole fluorescent compound.	Hoechst Pharmaceuticals, Hounslow, Middx.
APH	N-Acetyl-N- phenylhydrazine	BDH
MS-222	Tricaine methane sulphonate	Sandoz
Benzocaine	Ethyl 4-aminobenzoate	BDH
Giemsa's stain	Gurr's improved R66	Searle Labs. High Wycombe, Bucks.
Giemsa buffer	pH 6.5	BDH
E.M. fixatives & resins	25% glutaraldehyde Osmic acid Araldite mixture TAAB resin mixture Spurr's resin mixture	TAAB Labs.
Chlorox	Sodium hypochlorite	ICI Ltd.
Noble agar		Difco (Baird & Tatlock, London).
DMSO	Dimethyl sulphoxide	BDH
Sucrose	Purified sucrose Granulated sugar	BDH Tate & Lyle
TSC (b)	Tri-sodium citrate	BDH

Buffer compositions

PBSa	NaCl 8.0g	Dissolved in 800 ml distilled water
	KCl 0.2g	
	Na <sub>2</sub> HPO <sub>4</sub> 1.15g	
	KH <sub>2</sub> PO <sub>4</sub> 0.2g	

0.1M Phosphate buffer pH7.5  
 $\text{Na}_2\text{HPO}_4$  11.56g/l  
 $\text{NaH}_2\text{PO}_4$  2.80g/l

Sodium cacodylate buffer pH7.2  
 0.2M sodium cacodylate 50 ml  
 0.2M HCl 4.2 ml  
 Distilled water 146 ml

Citrate dextrose saline (CDS buffer)  
 Dextrose 2.05g  
 Tri-sodium citrate 0.80g  
 NaCl 0.4g  
 Dist. water to 100 ml

(c)

### Stains for electron microscopy

Sodium phosphotungstate (NaPT)-A 1% solution at pH7 was the standard stain prepared from PTA. A 1.5% solution of PTA was titrated upwards to neutrality with a normal solution of NaOH and the stain was stored in a stoppered bottle at 4°C. Stains of 0.5-2% concentration and of pH range 6-8 were also used.

Uranyl formate (UF) was prepared freshly in the dark as a 1% aqueous solution and titrated upwards to pH 4.5 with dilute ammonium hydroxide.

Ammonium molybdate (AM) was prepared as a 1% aqueous solution and the pH adjusted in the range 7.0-7.4 with ammonium hydroxide or ammonium acetate.

### 3. Tissue culture materials

<u>Item</u>	<u>Particulars, storage</u>	<u>Supplier</u>
Foetal calf serum	Membrane filtered, γ-irradiated. -20°C.	Gibco-Biocult, Glasgow, Scotland.
Eagles Minimal Essential Medium (MEM) with Earles Salts. (10 x conc).	4°C	Flow Labs, Irvine, Scotland.
Hanks balanced salt solution	4°C	Flow Labs.

<u>Item</u>	<u>Particulars, storage</u>	<u>Supplier</u>
<b>Antibiotics:</b>		
Fungizone	Stored dark, 4°C	Squibb & Sons, Liverpool
Neomycin sulphate	-20°C	Sigma Co., London.
Kanamycin sulphate	-20°C	"
Penicillin - G	-20°C	"
Streptomycin sulphate	-20°C	"
Mycostatin	-20°C	Squibb & Sons.
Gentamycin	4°C	Gibco-Biocult
Trypsin	Freeze-dried solution, 4°C Dry, powdered - room temperature	Wellcome Labs. Beckenham, Kent. Difco.
Non-essential amino acids (NEAA). (100 x conc).	4°C	Gibco-Biocult
L-Glutamine 200 mM	-20°C	Gibco-Biocult
Versene	Di-sodium EDTA in PBSa. 1/50 soln. 4°C.	Flow Labs.
Tryptose phosphate broth		Flow Labs.
Tissue culture flasks	25cm <sup>2</sup> base area	Nunclon UK,
Tissue culture dishes	50 mm diam.	" "

#### 4. Media compositions

##### a) Tris-buffered maintenance medium for organ cultures.

10 X Eagles MEM Earles Salts	10 ml
100 X NEAA *	1 ml
Glutamine 200 mM	1 ml
Penicillin and Streptomycin (10,000 units/ml and 10,000 µg/ml respectively)	1 ml
NaCl 16% w/v	3.3 ml
Mycostatin 2,500 µg/ml	1 ml
Tris saline, stock 0.05M	10 ml
Distilled water	to 100 ml
Stored at -20°C	

\* Non-essential amino acids, see also the Abbreviations p. xiv.

## b) Growth medium for primary tissue cultures.

10 X Eagles MEM Earles Salts	10 ml
100 X NEAA*	1 ml
Glutamine	1 ml
Penicillin & Streptomycin (10,000 u/ml & 10,000 µg/ml)	1 ml
NaCl 16% w/v	3.3 ml
Mycostatin 2,500 µg/ml	1 ml
Sod. bicarbonate 7.5% w/v	1.1 ml
Foetal Calf Serum	10 ml
Distilled water	to 100 ml
Stored at -20°C	
* Non-essential amino acids	

## c) Maintenance medium for inoculated primary cultures.

As (b) above with the omission of foetal calf serum.  
Stored at -20°C.

## d) "Hanks washing solution".

10 X Hanks balanced salt solution	10 ml
16% w/v NaCl	3.3 ml
Mycostatin (2,500 µg/ml)	3 ml
Penicillin & Streptomycin (10 <sup>4</sup> u/ml & 10 <sup>4</sup> µg/ml)	3 ml
Distilled water	to 100 ml
Stored at 4°C	

## e) 0.25% Trypsin solution.

To: 10 X Hanks balanced salt solution	10 ml
16% w/v NaCl	3 ml
Penicillin and Streptomycin (10 <sup>4</sup> u/ml & 10 <sup>4</sup> µg/ml)	1 ml
Dist. water	25 ml
Added: Phenol red (1% soln.)	0.6 ml
Dist. water	to 100 ml
Trypsin (Difco 1:250)	2.5 g

The solution was shaken and left overnight at 4°C. It was sterilized by filtration on a 450 nm pore membrane filter and stored at -20°C.

## f) FHM maintenance medium.

10 X Eagles MEM - Glasgow modification	8 ml
Tris saline, stock 0.05M	10 ml
Sodium bicarbonate 7.5% w/v	0.04 ml
Tryptose phosphate broth	10 ml
Glutamine 200 mM	1 ml
Penicillin & Streptomycin (10 <sup>4</sup> u/ml & 10 <sup>4</sup> µg/ml)	1 ml
Distilled water	to 100 ml
Stored at -20°C	

## g) Trypsin/versene mixture.

0.1% Trypsin solution as before	1 part
0.02% Versene in PBSa	1 part
Stored at -20°C	

CONSTITUENTS OF EMBEDDING MEDIA FOR ELECTRON  
MICROSCOPY: ABBREVIATIONS OR TRADE NAMES,  
PROPER NAMES & SUPPLIERS

ABBREVIATION	TRADE NAME	PROPER NAME	SUPPLIER
Araldite M		Araldite CY212	Taab Labs, Emmer Green, Reading
DDSA	Resin 964B HY 964	Dodecenyl succinic anhydride	Ditto
	DER 736	Digycidyl ether of polypropylene glycol	"
	DMP 30	2,4,6-tridimethyl amino methyl phenol	"
	ERL 4206	Vinyl cyclohexene dioxide	"
MNA		Methyl nadic anhydride	"
NSA		Nonenyl succinic anhydride	"
DMAE	S-1	Dimethylamino- ethanol	"
TAAB resin	Epon 812 (Epikote 812)	glycerol-based aliphatic epoxy resin	"

Table 23. Doses of APH for injection and the corresponding inoculum volumes and fish weights.

Dose (mg/10g body weight)	Vol. of inoculum (ml)	Weight of fish (g)
0.1	0.20	20.2
	0.08	8.7
	0.01	1.4
0.2	0.80	40.9
	0.07	3.5
	0.05	2.2
Saline control	0.25	25.8
	0.05	5.3
	0.01	1.2

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## Observations on the Fine Structure of Lymphocystis Virus from European Flounders and Plaice

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### SUMMARY

Lymphocystis virus has been classified with the icosahedral cytoplasmic deoxyriboviruses (ICDVs) but a detailed structure of the virion has not yet been established. Virus from tumours of European plaice and flounders was allowed to decay and this revealed details of its structure. Freshly prepared virus has a hexagonal outline which breaks down to reveal surface subunits 4.5 nm in diam. and a spheroidal core with tubular material 13 nm in diam. inside it. This tubular material appears to have a periodicity suggesting a helical conformation and may be a deoxyribonucleoprotein. No triangular or pentagonal facets, as found with some other ICDVs, were seen. A possible construction of the virus is proposed.

### INTRODUCTION

Lymphocystis is a disease of a wide variety of teleost fish of which the aetiological agent is a virus classified with the icosahedral cytoplasmic deoxyriboviruses (ICDV; Kelly & Robertson, 1973). The virus is plausibly icosahedral as it appears hexagonal in section and it is found only in the cytoplasm of enlarged cells in which inclusion bodies give staining reactions characteristic of DNA (Walker, 1965; Pritchard & Malsberger, 1968). The ultrastructure of lymphocystis virus has been studied in several laboratories and evidence from ultrathin sections has shown that the morphology of the virion is essentially the same in material from different host species although particle sizes vary (Walker, 1962; Walker & Wolf, 1962; Walker & Weissenberg, 1965; Midlge & Malsberger, 1968; Zwillenberg & Wolf, 1968; Howse & Christmas, 1971). Particles seen in section showed the same features of a densely staining outer layer, an intermediate space and a densely staining core. Negatively stained virus has been examined by a number of workers (Midlge & Malsberger, 1968; Zwillenberg & Wolf, 1968) but little detail could be resolved. This contrasts with the detailed structure observed in some other ICDVs (Wrigley, 1969, 1970; Stoltz, 1973).

On the basis of structural studies on insect ICDVs, Stoltz (1973) proposed a theoretical model for ICDV capsid structure. However, there has been insufficient evidence hitherto to decide whether lymphocystis virus has a similar structure. We report new electron microscopic observations on the structure of lymphocystis virus which suggest that the structure may be different and incompatible with Stoltz's model. The observations we report were made independently in each laboratory.

## METHODS

*Source of lymphocystis.* Tumours were obtained from plaice, *Pleuronectes platessa* (L), and flounders, *Platichthys flesus* (L). Mr Peter Russell, Unit of Aquatic Pathobiology, University of Stirling, Scotland, provided plaice from the Irish Sea off the Cumberland coast and flounders from Morecambe Bay, Lancashire, and the River Ythan, Aberdeenshire. Mr J. Bonny, Ministry of Agriculture, Fisheries and Food, Fleetwood, provided plaice from Morecambe Bay.

For comparison, some lymphocystis-infected bluegill, *Lepomis macrochirus* (L) were obtained as a gift of formalin-fixed fish from Dr K. Wolf, Eastern Fish Diseases Laboratory, U.S. Department of Interior, Kearneysville, West Virginia.

*Preparation of material for electron microscopy*

*For negative staining.* Pieces of tumour were excised from infected fish and prepared for microscopy either by the cell-spreading technique of Parsons (1963) or ground up using either a Griffith's grinder or a ten Broek homogenizer with a small amount of distilled water. The resultant slurry was clarified by allowing the larger particles to settle under gravity. The supernatant was then examined directly by negative contrast or was centrifuged at 100000 g for 1 h. Following centrifugation the pellet was resuspended in 2 drops of distilled water containing 0.1% (w/v) bacitracin as a wetting agent. Equal volumes of this material and negative stain were mixed, applied to a carbon Formvar-coated 400 mesh copper grid and allowed to dry.

The negative stains used were 1% sodium phosphotungstate pH 6.5, 2 to 3% potassium silicotungstate pH 7.0, 2% uranyl acetate pH 4.5 or 3% ammonium molybdate pH 9.

*For thin section.* Blocks of 1 mm<sup>3</sup> were cut from tumours and processed using the rapid embedding method of Doane *et al.* (1974). Sections were cut using an LKB II ultramicrotome, mounted on Formvar-coated 200 mesh grids and stained with Reynolds' 1% lead citrate and aqueous 1% uranyl acetate.

*Electron microscopy.* Specimens were examined in a Philips EM 300 or 301 microscope using an accelerating voltage of 80 or 100 kV.

*Shadowing.* The preparations used were crude extracts so that conventional metal shadowing would have made it impossible to distinguish virus particles from surrounding debris. Accordingly, preparations already negatively stained were shadowed lightly with carbon evaporated at an angle of 20° in an Edwards 306 coating unit.

*Degradation of virus.* Crude extracts of tumours were subjected to the following treatments: (a) Storage at 4 °C without further treatment. (b) Treatment with nasal decongestants according to the method of Wrigley (1969) before and after storage at 4 °C. The decongestants used were: 'Afrin' (Behring Corp. U.S.A., the gift of Essex Chemie AG, Switzerland); 'Hazol' (Allan & Hanburys, London E2, England). Both contain oxymetazoline hydrochloride, but 'Afrin' also contains phenylmercuric chloride and benzalkonium chloride as preservatives.

*Microscope calibration.* The Philips 301 was calibrated at the magnification used to take photographs using the 17.2 nm lattice of glutaraldehyde-fixed beef liver catalase (Wrigley, 1968). This was kindly supplied by Dr D. L. Misell, National Institute for Medical Research, Mill Hill, London, U.K. Repeat calibrations over 2 years showed variations of less than 2%, though the actual figure was 11% less than that given by the manufacturer.

## RESULTS

Tumour material from flounders and plaice showed typical lymphocystis particles by thin section electron microscopy (Fig. 1). The appearances were indistinguishable from those described by other workers, with an outer electron-dense layer 14 to 19 nm thick giving a hexagonal outline. Inside this was an electron translucent layer surrounding a darkly staining circular core 150 to 230 nm in diam. The virus particles were 175 to 260 nm across the flats of the hexagon and 200 to 300 nm between the vertices.

By negative contrast the virus particles were less easy to distinguish from the background debris and this difficulty increased with storage as the virions collapsed and lost their hexagonal outline. However, this decay allowed details of the virus structure to become visible and our observations will be reported under different headings.

*General morphology*

In freshly prepared tumour material virus particles had a hexagonal outline which, with silicotungstate as the negative stain, appeared to have two electron translucent layers (Fig. 2). Each layer was approx. 4 nm thick and they were separated by an electron-dense layer 4.5 nm thick. The layers appeared to be homogeneous with no visible substructure. However if uranyl acetate, pH 4.5, or ammonium molybdate, pH 9, were used, only one thick featureless layer 20 nm thick was seen (Fig. 3) and this corresponds closely with the appearance in thin section when uranyl acetate was also used. With fresh virus it was also necessary to use a higher accelerating voltage (100 kV) to obtain enough penetration to see internal details. With more decayed material this was no longer necessary.

With storage at 4 °C the hexagonal outline was lost and became irregular (Fig. 4) though it was still possible to distinguish particles from other debris. This collapse could be accelerated by centrifugation at 100000 g on to the bottom of a tube in a swing-out rotor and mitigated by centrifugation on to a pad of 40% sucrose (w/v in water; C. R. Madeley & P. H. Russell, unpublished observations).

*Surface structure*

Freshly extracted virions showed a surface outline devoid of clearly identifiable subunits. Following storage at 4 °C and the loss of the hexagonal outline, it became possible to distinguish surface details. Round the periphery knob-like subunits in the outermost layer became visible (Fig. 5 and 6). These were only observed with clarity in profile at the periphery and were not seen over the remainder of the surface. They were about 4.5 nm in diam. and appeared to be connected to the rest of the virion by a narrow stalk. Treatment with the nasal decongestants did not have any visible effect and no surface detail similar to that found with other ICDVs by Wrigley (1969; 1970) was seen. Similarly, no triangular facets similar to those reported by Wrigley (1970) with *Tipula* iridescent virus (TIV) and *Sericesthis* iridescent virus were seen as the virions decayed. Such facets were, however, readily seen in a sample of TIV (kindly provided by Dr D. C. Kelly) allowed to deteriorate under similar conditions (Fig. 7).

*Cores*

Thin sections showed an electron-dense core 150 to 230 nm in diam. (Fig. 1). By negative contrast no definite core could be identified in intact particles, though those stained with uranyl acetate showed some similarities to those seen in thin section. Collapsed particles,

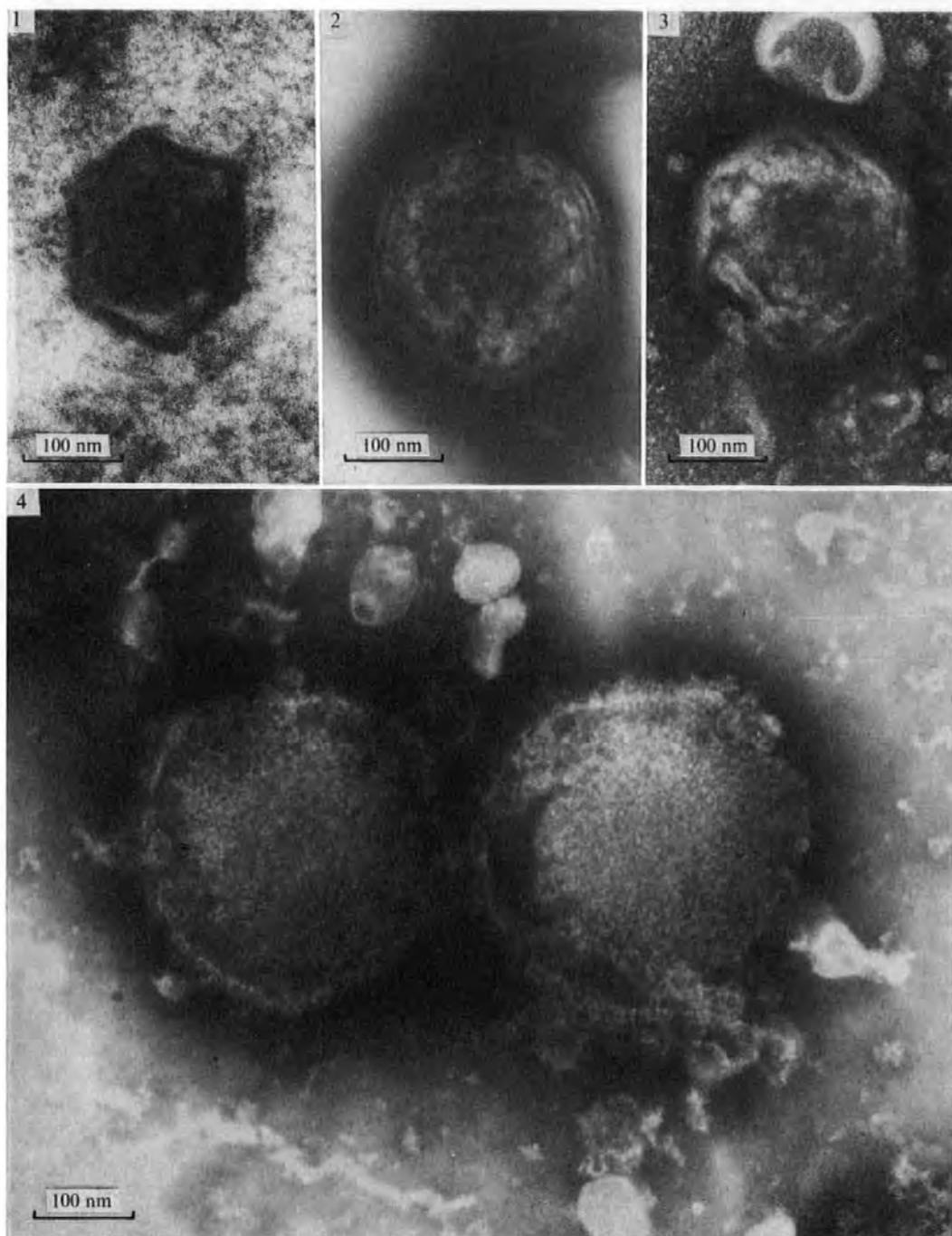


Fig. 1. Lymphocystis particle in a thin section of tumour tissue. The outline is hexagonal and a circular electron-dense core is visible. Stained with lead citrate and uranyl acetate.

Fig. 2. Freshly prepared virus negatively contrasted with 3% sodium silicotungstate, pH 7. The outline is roughly hexagonal with the periphery exhibiting two electron translucent layers separated by an electron-dense one.

Fig. 3. Fresh virus contrasted with 4% ammonium molybdate, pH 9. The periphery is seen as a single featureless layer 20 nm thick. Accelerating voltage 100 kV.

Fig. 4. Virus after storage for several weeks at 4 °C in aqueous suspension. The hexagonal outline has been lost and the periphery has become ragged. An internal core approx. 250 nm in diam. is visible inside each particle. Stained with 3% potassium phosphotungstate, pH 7. Magnification on all figures is  $\times 150\,000$ .

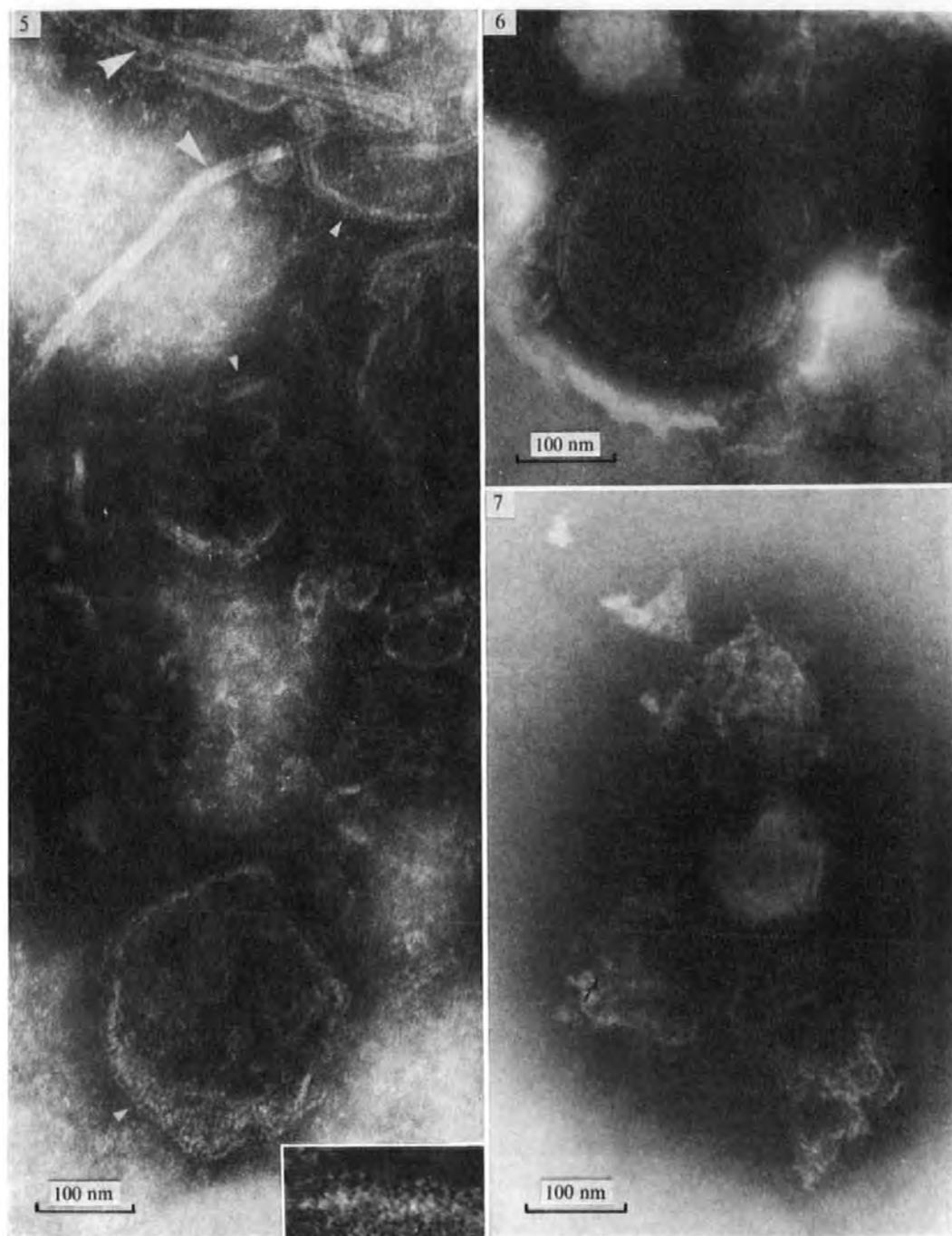


Fig. 5. Decayed virus showing spheroidal subunits 4.5 nm in diam. becoming visible on the surface (small arrows) as it breaks down. Some 13 nm filaments (large arrows) are also seen, mostly lying outside virus particles. Stain used was 1% sodium phosphotungstate, pH 6.5. Inset: part of the same area enlarged to  $\times 350\,000$ .

Fig. 6. Similar surface appearance to Fig. 5 in a preparation contrasted with 3% sodium silicotungstate, pH 7.

Fig. 7. Decayed preparation of *Tipula* iridescent virus showing release of triangular facets as described by Wrigley (1970). Stain used was 3% potassium phosphotungstate, pH 7. Magnification on all figures is  $\times 150\,000$ .

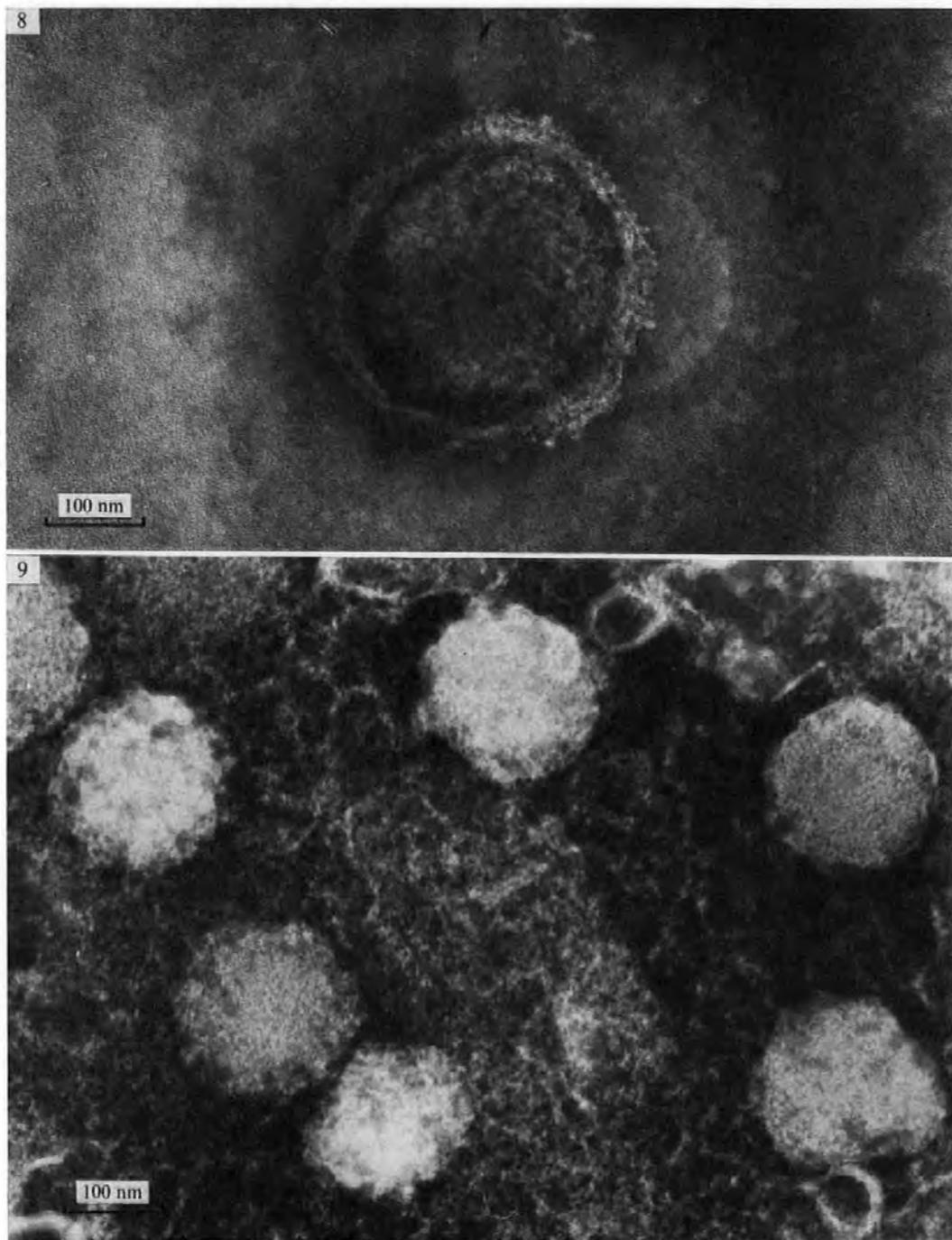


Fig. 8. Decayed virus contrasted with 3% potassium phosphotungstate, pH 7, and then shadowed lightly with evaporated carbon at 20 °C. Note the elliptical shadow cast only by the core suggesting that with the collapse of the outer surface layer(s) it retains its spheroidal configuration.

Fig. 9. Core-like structures from flounder material approx. 150 nm in diam. with an uneven surface suggesting possible contraction; stained with 3% potassium phosphotungstate, pH 7. Magnification  $\times 150\ 000$ .

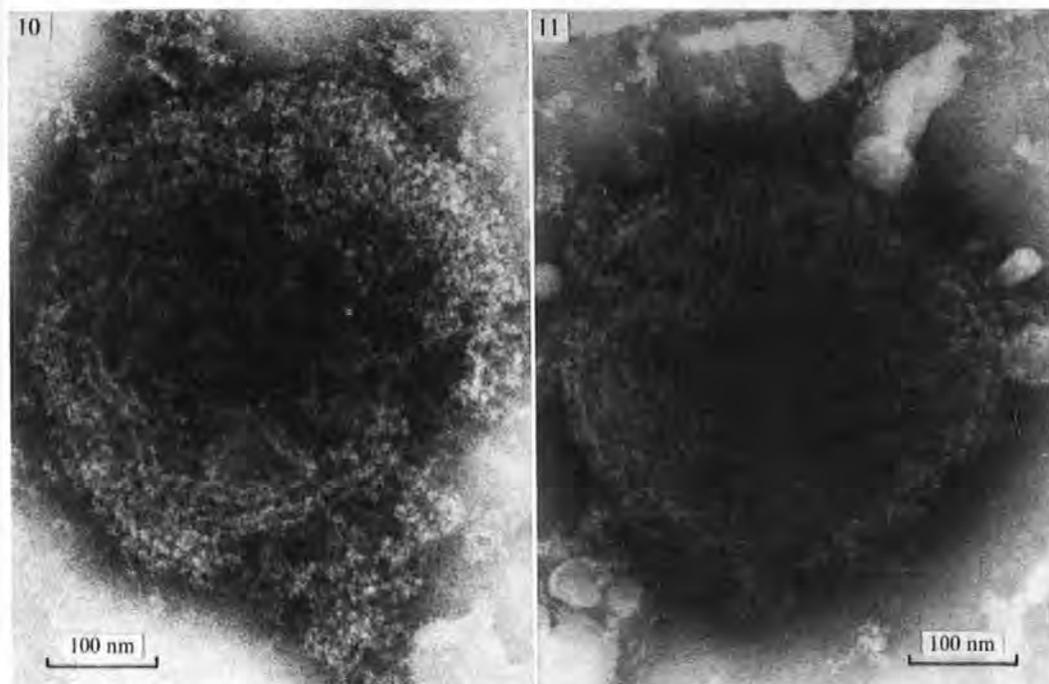


Fig. 10. Decayed virus particle showing a filament apparently lying within the periphery. It is 13 nm in width and has a central stain-penetrated canal 4 nm in diam.; stained with 3% potassium phosphotungstate, pH 7.

Fig. 11. Similar appearance from a different preparation; stained with 1% sodium phosphotungstate, pH 6.5.

Magnification  $\times 150\ 000$ .

on the other hand, showed a central mass which cast an elliptical shadow with carbon at 20 °C (Fig. 8). This technique, though simple in theory, has proved uncertain in practice and only occasional preparations revealed good shadowing and no pictures of shadowed intact particles were obtained.

One preparation of virus from a flounder tumour contained large numbers of what appeared to be naked cores (Fig. 9). At 150 nm, they were consistent in size with other evidence. They had a roughly circular or polygonal shape but no internal or surface detail was visible.

#### *Tubular material*

Following storage of virus at 4 °C and the breakdown of virus particles, a tubular component was seen regularly. It was not seen in fresh preparations but was observed within the outline of broken particles (Fig. 10 to 12). The structure was 13 nm in diam. and was variable in length. It appeared to be hollow with a central canal 4 nm in diam. into which stain penetrated. Occasionally a periodicity in the walls was detected (Fig. 12, arrowed) suggesting a possible helical construction.

In addition to this internal structure, other filaments were seen. They were found outside virus particles though adjacent to them (Fig. 5 and 13). They were 13 nm in diam. and some had terminal blebs (Fig. 13). They resembled the internal component only superficially, and no substructure was seen. The ends, as seen here, were often rounded.

No filaments similar to those described by Zwillenberg & Wolf (1968) were seen in

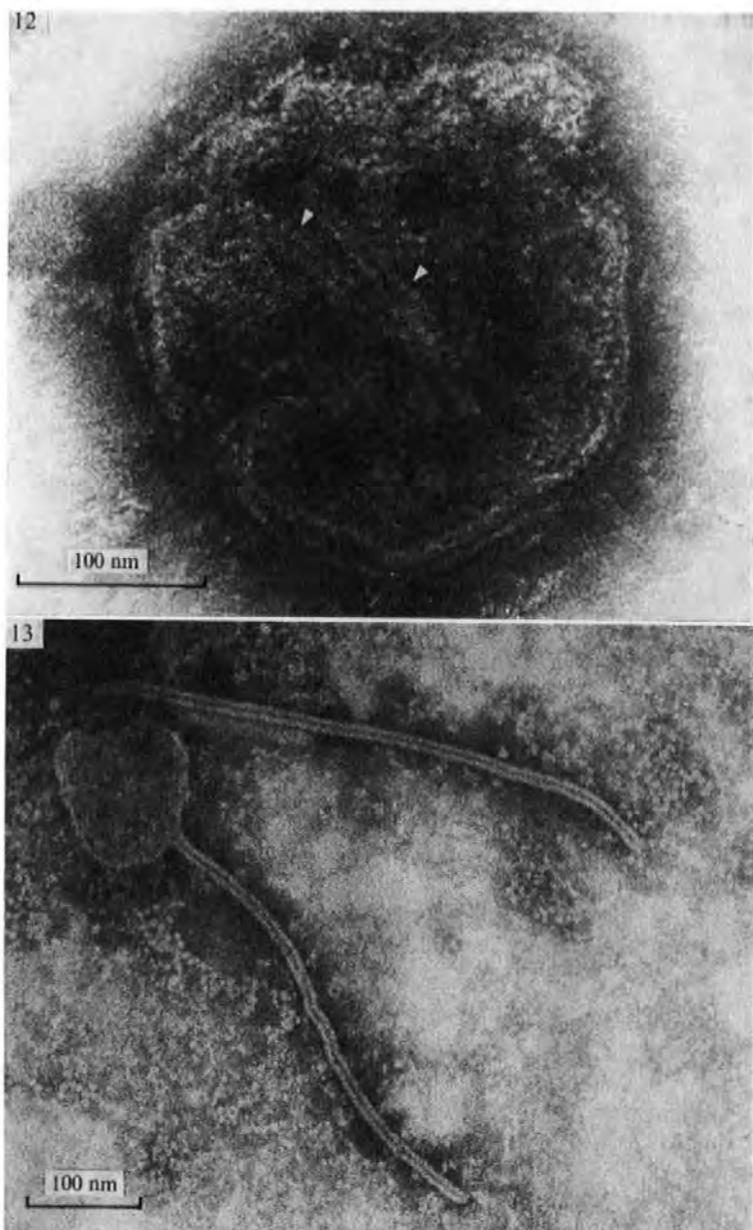


Fig. 12. Filaments inside a decayed particle with a periodic substructure (arrowed) suggesting a helical structure. The interval between turns is difficult to estimate and since it is not commonly seen this appearance may be due to loosening of inter-turn bonds. Stain used was 1% sodium phosphotungstate, pH 6.5.

Fig. 13. Filamentous material showing a terminal 'bleb' about 120 nm across. The walls of the filament do not show any substructure and are continuous with the walls of the bleb. This material is also approx. 13 nm in diam; stained with 3% potassium phosphotungstate, pH 7.

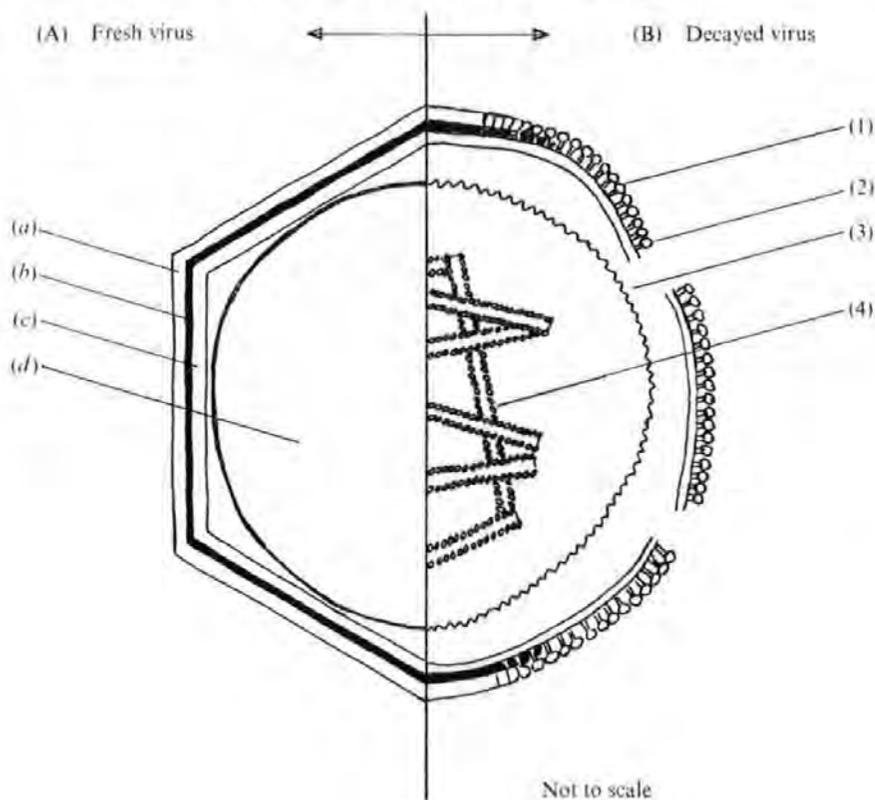


Fig. 14. Diagram of possible structure of lymphocystis virus. (A) Fresh virus: (a) outer electron translucent layer; (b) middle electron-dense layer (stained); (c) inner electron translucent layer; (d) core: circular in outline and without visible substructure. (B) Decayed virus: (1) outer electron translucent layer and middle stain-penetrated layer becoming resolved into knob-like subunits 4.5 nm in diam. as they either lose an intervening 'cement' or become less closely packed. They appear to be joined to the inner electron translucent layer (2) by a narrow stalk; (3) outer boundary of core, becoming ragged as it breaks down; (4) internal tubular structure which may be helical in structure, 13 nm in diam. and with a 4 nm central canal.

lymphocystis from bluegills. Extracts of the formalin-fixed bluegill material showed numerous virus particles but they did not break up and neither internal nor external filaments were seen.

#### DISCUSSION

Lymphocystis virus is one of the largest viruses, although estimates of its size vary considerably (Kelly & Robertson, 1973). It has been presumed to be icosahedral in form though this has yet to be proved conclusively. In thin section and freshly prepared negatively stained preparations it appears hexagonal in outline and this would be consistent with an icosahedral form. The reported variation in size may mean that not all isolates are identical and our observations can only refer to virus from European plaice and flounder. Virus from these sources have been shown by Russell (1974) to be related serologically. We found no morphological differences between virions taken from the two species.

The gross morphology of the virus in fresh preparations resembled that of other ICVDs but the structural details seen when the virus broke down suggest that the construction of

lymphocystis is quite different. As the hexagonal outline was lost, no triangular or pentagonal facets were released and the virions merely became amorphous. Decay could be accelerated by hard centrifugation, an unusual example of centrifugation causing visible damage to virus particles. The periphery of virus stained with silicotungstate usually appeared as a double layer with a total thickness of 12 to 13 nm. With uranyl acetate only a single layer was seen, 20 nm thick. This salt is used as a positive stain with thin sections and it is possible that it was acting both as a positive and a negative stain. Variations in hydration will also affect staining reactions and a similar effect has also been observed with the surface threads of vaccinia virus (Madeley, 1972).

Surface subunits were observed in *Sericesthis* iridescent virus (SIV) and *Tipula* iridescent virus (TIV) by Wrigley (1969, 1970) and their visibility was enhanced by treatment with 'Afrin'. We did not observe similar units on lymphocystis virus (LV), with or without prior treatment with decongestants including 'Afrin'. Surface knobs did become visible on collapsed LV though only at the periphery and not over the surface. Thus no conclusions as to their arrangement on the surface of the virion are possible, but at 4.5 nm they were appreciably smaller than those on SIV or TIV. Their appearance only with the loosening of the surface structure suggests either that they are normally too closely packed for individual subunits to be resolved or that collapse is associated with the loss of some form of 'cement'. We have no evidence that either of these explanations is correct.

Both thin section and negative staining techniques gave evidence of a core beneath the outer layer(s). This appeared to be roughly circular in outline in thin section and in negative staining when separated from the outer surface. Successfully shadowed, it appeared to be hemispherical when seen in the centre of a collapsed virion. Whether it is essentially spherical or conforms to the outer layer in complete intact virions is not known.

Tubular material 13 nm in diam. with a central 4 nm canal was seen inside decayed cores and appeared to be part of the virion, though no evidence of such an internal component has been seen in thin sections through the virus. It was usually seen as several short straight fragments and we do not know if it is present as one or several pieces inside an intact virion. With some evidence of periodicity suggesting a helical structure, it is tempting to believe that this is a deoxyribonucleoprotein. There is no evidence at present to confirm this though where similar structures have been found in RNA-containing viruses they have always contained the nucleic acid. The 13 nm filaments found outside the virus particles, and seen in Fig. 5 and 13, bore a superficial resemblance to this tubular internal component. Though visualized more clearly because they lay outside the virus particles, no substructure was seen, and their terminal blebs and rounded ends distinguish them from the internal component. They are probably separate structures and we cannot say if they form part of the virion.

We have attempted (see Fig. 14) to synthesize our findings into a suggested structure for this virus. It fits our observations but will need to be confirmed by additional work; in particular, it would have been useful to be able to grow the virus in cell culture. Attempts to do so in marine fish cells (D. A. Smail and S. I. Egglestone, unpublished data) have not been successful.

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