STUDIES ON THE HOST-PARASITE INTERACTION BETWEEN CARP AND SAPROLEGNIA

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THESIS SUMMARY

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The thesis compares the effects of temperature and substrate on the growth of fish pathogen Saprolegnia diclina and the saprophyte Saprolegnia ferax. Studies revealed optimal growth of both species occurs at 20-25°C depending upon the substrates used. Growth is restricted at higher and lower temperatures. For both species optimum growth was recorded on medium containing 1% casein and 1% glucose, but high levels of lipid and glucose inhibited growth. However, S. diclina exhibits a higher growth rate than S. ferax at high lipid concentrations.

S. diclina produces more proteolytic activity per unit weight at 10°C than S. ferax, regardless of substrate. S. diclina also demonstrated greatest lipase activity at 10°C, particularly in the presence of casein or lipid. These factors may facilitate colonisation of fish tissues by S. diclina at low temperatures.

Carp maintained at 10°C showed greater infection by S. diclina, than carp kept at 20°C. Evidence is presented for a lack of antibody production in infected carp maintained at 10°C. Fish kept at 20°C only produced antibody to Saprolegnia antigens when they were coupled to erythrocyte carriers. During infection phagocytic macrophages and neutrophils increased; there was a decrease in the numbers of mucous secreting goblet cells in the skin, and lymphoid organs showed increased pigment deposition.

Infected fish showed evidence of physiological stress including decreased levels of erythrocytes, haemoglobin, liver glycogen and protein, and an increase in liver lipid. Ascorbic acid levels decreased in interrenal tissue.

Histological and scanning E.M. studies of skin lesions provide new information about changes in the surface during UDN disease.

Key words: Saprolegnia infection and UDN
Temperature, substrates and fungal growth
Carp (Cyprinus carpio)
Low temperature, stress and immunity.

I dedicate this work to the spirit of
my father and to my brother-in-law.

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LIST OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

In recent years fish hatcheries and farms have become increasingly important in supplementing fish stocks for leisure fishing and in food production. The commercial rearing of fish often involves stocking them at relatively high densities and gives rise to problems of stress and disease. These problems have prompted an increasing interest in aspects of fish diseases, health and immunology (Ahne, 1980; Andersson and Hennessen, 1981; Olah et al., 1981; Van Muiswinkel and Cooper, 1982; Anderson et al., 1983, Manning and Tatner, 1985). As a result, vaccines are now available against a variety of major fish diseases (Anderson et al., 1983). Vaccines against bacterial and viral diseases are now playing an increasingly important role in the management of intensively reared fish.

One continuing problem in aquaculture is infection by saprolegniaceous fungi. This is a particular problem in fish farming because it can affect fish at any stage of the life cycle from egg to adult and, since the pathogen can also live saprophytically on dead organic matter, there is always a large reservoir of infective material in the aquatic environment.

The strains of Saprolegnia spp. pathogenic to fish have been investigated by a number of anthors, and their role in ulcerative dermal necrosis of fish has been established. Aspects of their fine structure, life cycle and taxonomy have been examined by Beakes (1980), Pickering et al. (1979) and Willoughby (1977, 1978). Factors

controlling activity of zoospores derived from Saprolegnia diclina have also been studied by Smith et al. (1984). In contrast relatively little is known about the nature of the physiological and biochemical interactions which take place between the pathogen and its fish host, and knowledge of the immune response of fish to Saprolegnia infections is also sparse. The overall aim of this thesis, therefore, is to investigate these aspects in more detail.

1.1 Ulcerative Dermal Necrosis (UDN) Disease

1.1.1 Historical review

Ulcerative Dermal Necrosis (UDN), a disease affecting the skin of salmonid and cyprinid fish, made its first detected appearance in the British Isles as Salmon Disease (Smith, 1878). The first detailed account of the condition, in the rivers of the Solway area in Scotland, was recorded by Stirling in 1879 and on the Tweed, Eden and other rivers by Buckland and Walpole in 1880. However, the Report of Her Majesty's Inspector of Salmon Fisheries (England and Wales) for 1881 and 1882 (Report, 1882) provides some evidence that sporadic cases of the condition had existed for many years prior to 1868. By the early years of the twentieth century the disease was recorded on specific rivers in England, Wales and Scotland (Grimble, 1899, 1913; Patterson, 1903; Malloch, 1910; Bisset, 1946). In the early sixties, occasional fish with lesions were seen in the Waterville river system of south west Ireland (Jones, 1959; Robinson, 1963), but the disease has since spread to the rest of Ireland. Since UDN was first recognized it has spread throughout

European waters and in 1975 it was reported in Sweden for the first time (Ljungberg & Johansson, 1977).

1.1.2 Aetiological investigations of UDN

Over the years many attempts have been made to establish the aetiology of the disease, aspects of which are reviewed by Roberts (1972); Murphy (1973); Hill (1976) and Willoughby (1972). The possible roles of bacterial, viral and fungal agents thought to be associated with the disease are outlined below.

1.1.2.A. Bacteriology

Jensen (1965) was the first worker to examine diseased fish, and he suggested that UDN macroscopically resembled Columnaris Disease, a myxobacterial disease of fish caused by Chondrococcus columnaris. White (1965) made extensive efforts to substantiate Jensen's diagnosis, but found no conclusive evidence that any of the wide range of bacterial organisms isolated by him from diseased salmon was responsible. Brown and Collins (1966), however, insisted that the condition was Columnaris Disease, because of the macroscopic similarities between UDN and Columnaris Disease. A number of bacteriological studies were therefore performed in an attempt to resolve this issue by Elson (1968) in Scotland, and Carbery & Strickland (1968), who continued White's work in Ireland. However, they isolated myxobacteria from external surfaces of only 30% of UDN diseased fish.

1.1.2.B. Virology

A number of workers have failed to demonstrate viral involvement in the skin lesions of diseased fish. Carbery & Strickland (1968)

attempted to infect primary cell cultures of salmon tissues with infected fish skin but no consistent evidence of cytopathogenic effect (CPE) was obtained. Although Carbery (1968b) mentions occasional CPE, he felt that this could be due to technical factors such as contamination during handling tissues. De Kinkelin & Le Turdu (1971) repeated the experiments devised by Carbery & Strickland (1968), using both Salmon (S. salar) and mature trout (S. trutta), but they also failed to obtain any evidence for a viral aetiology from tissue culture experiments. Munro (1970), however, supported the view of Carbery (1968a) that the aetiological agent of UDN is a virus, since he claimed to have transmitted the disease using bacteria-free filtrates of preparations from diseased fish.

1.1.2.C. Mycology

Smith (1878) was completely convinced that the phycomycoete Saprolegnia ferax was the aetiological agent of UDN. Stuart & Fuller (1968) and Willoughby (1969) investigated the mycological aspects of UDN disease, and justifiably emphasized the importance of fungal invasion in the condition. Both studies stressed that only Saprolegnia spp. could be isolated consistently from lesions of diseased fish. Other workers have isolated Saprolegnia from mycotic infections of eels (Anguilla spp.) and from all stages of UDN infected Salmon (Hoshina and Ookubo, 1956; Stuart and Fuller, 1968; Carbery, 1968,a,b; Roberts et al., 1969). In addition, Stuart and Fuller (1968) and Willoughby (1968) found that a single species of Saprolegnia was involved and identified it as S. parasitica. It would seem that in Ireland, Scotland and England the same species of aquatic fungus is implicated in UDN and all mycologist studying UDN

have stressed that S. parasitica appears to be intimately involved with the condition. However, should the fungus not prove to be the primary aetiological agent, it must still be considered as much more than a mere opportunist because of its consistent and specific relationship with surface lesions.

1.1.2.D. Role of Saprolegnia in UDN

Saprolegniasis is the most important fungal disease of fresh water fish. With particular reference to the agents responsible for the disease it is an all-encompassing term which includes approximentally 3 orders, 10 genera and 22 species of fungi, naturally or experimentally infectious to fish. The term is often (erroneously) used to refer to any cotton wool-like growth of fungi adherent to skin or gills of the fish (Wolke, 1975). Little is known about the nature of the infective stage of Saprolegnia under natural conditions (Pickering & Willoughby, 1982), however, Norland-Titinger (1973) and Willoughby (1977) both implicate secondary zoospores. Direct contact with fungal hyphae is not thought to cause infections (Norland-Titinger, 1973).

The exact cause of UDN disease is still unknown (Roberts, 1972; O'Brien, 1974) but it is generally accepted that the mycotic element may perhaps be the most important component of the disease (Carbery, 1968a; Roberts et al., 1971; Roberts, 1972; Reichenbach-Klinke, 1974).

Pathogenic fungi have been isolated from a wide variety of teleost fish (Scott & O'Brien, 1962; Baudouy and Tuffery, 1973; Roberts and Shepherd, 1974; Wood, 1974; Wilson, 1976) with as many

as four different species of fungi occuring together in a mixed colony on a single fish (Stuart & Fuller, 1968; Pickering & Willoughby, 1977). Willoughby (1970) isolated six fungal genera from a population of perch (Perca fluviatilis) from Lake Windermere.

It would appear that many of the species causing Saprolegniasis are primary pathogens, i.e. they are capable of infecting fish in the absence of existing bacterial or viral diseases (Wolke, 1975; Pickering & Willoughby, 1977; Willoughby & Pickering, 1977). Primary Saprolegniasis has been reported by Hoshina & Ookubo (1956) and Hoshina & Sunayama (1960) in cultured eels which displayed no evidence of previous injury. Similarly, Kanouse (1932) has isolated Saprolegnia from fish eggs; Tiffeny (1939) has demonstrated fungal invasion in a variety of fish without any obvious prior injury. Vishniac & Nigrelli (1957) and Neish (1977) report that S. parasitica was shown to be pathogenic to both uninjured and injured fish following exposure to zoospores, thus proving its primary pathogenicity. As mentioned earlier, Saprolegniasis appears to be associated with adverse environmental conditions. For this reason investigators of fish disease have long felt that pathogenic aquatic fungi were simply secondary invaders (Wolke, 1975; Richards, 1977). Willoughby (1970) noted small translucent areas on fish from which fungus was absent. These translucent areas came to be regarded as signs of a pre-fungal stage of the disease, involving bacteria. The fungal component of the disease becomes apparent on these areas slightly later. Therefore, it was felt that the fungus only becomes established on physically damaged (Roberts & Shepherd, 1974) or necrotic tissue resulting from mechanical trauma or from a primary

bacterial, parasitic or viral infection (Egusa & Nishikawa, 1965; Willoughby, 1970). The work of Vishniac & Nigrelli (1957) who infected platy fish, following scale removal, with 16 species in 7 genera of Saprolegniales supports such a view.

On the other hand, Saprolegnia alone has been isolated consistently from lesions of diseased fish. It has been isolated externally and internally from the musculature to a depth of 1.5 cm, and occasionally elsewhere other than from visibly diseased areas (Roberts, 1982). Saprolegnia species have also been isolated at all observable stages of the disease, including the "white patches" of the presumed pre-mycotic stage (Willoughby, 1970). In salmonid fish fungal infections often involve a single strain of Saprolegnia (Stuart & Fuller, 1968; Willoughby, 1968, 1969, 1970, 1971, 1972, 1977, 1978; Neish, 1977).

The species of Saprolegnia implicated in fish pathology are probably best considered as those in which the fish is the major substratum for growth (Lewis, 1973). Brown & Collins (1966); Stuart & Fuller (1968) and Willoughby (1968, 1969) have all emphasised the importance of the role of Saprolegnia in UDN. Of 48 isolates from infected salmon, 36 were identified as S. diclina in pure culture, and the remaining 12 proved to be other Saprolegnia sp. (Pickering & Willoughby, 1977). Of 20 isolates taken from infected salmon by Neish (1977) 12 belonged to the S. diclina-parasitica complex, the other isolates being Saprolegnia sp.. In another study, thirteen isolates from a sample of fungally-infected fish all proved to be Saprolegnia sp. and the majority of these were further identified as S. diclina (Willoughby, 1978). Richards & Pickering (1978, 1979)

and Pickering & Christie (1980) also refer to the pathogenic fungus as S. diclina. McKay (1967) carried out a series of infection experiments with young Coho salmon using a fungal isolate identified as S. diclina. McKay's results indicated that the fungus could cause a primary infection and some of Neish's experiments with young Coho salmon have confirmed McKay's results (Neish, 1977). Other workers have obtained similar results using different species of fish (Tiffney, 1939; Vishniac & Nigrelli, 1957; Scott & Warren, 1964; Norland-Tintigner, 1970, 1971, 1973, 1974). An opinion that is becoming increasingly acceptable to fish pathologist is that Saprolegniaceous fungi could be the major, and perhaps the only infectious component of UDN-disease (Carbery 1968a,b; Roberts & Shepherd, 1974; Wolke, 1975).

1.1.2.E. Factors predisposing fish to fungal infection

There are a number of factors which facilitate colonization of fish skin by fungi. Temperature is of crucial importance, since the disease is most common in late autumn, winter and spring when water temperatures are lowest (Carbery, 1968a; Norland-Tintigner, 1973). However, this is also the time when the fish are spawning and likely to be in poor condition.

Species of the genus Saprolegnia are usually considered as facultative parasites so that infection is more likely to occur on fish which are injured or weakened by adverse conditions e.g. high population density and stress (Scott, 1964; Snieszko & Axelrod, 1971). Physical damage to the surface of the fish after handling or any traumatic damage to the skin may also result in infection (Roberts & Shepherd, 1974).

The incidence of infestation of brown trout, Salmo trutta L., by the parasitic fungus Saprolegnia diclina is greater in sexually mature fish compared with immature fish sampled at the same time from the same body of water (Richards & Pickering, 1978). In salmonids, fungal infections are often associated with sexual maturity of the fish (Roberts & Shepherd, 1974; White, 1975; Neish, 1977). Under certain conditions sexually mature, male brown trout and char are more frequently infected than sexually mature females. In salmonid fish, it is common for the survival rate of spawning females to be greater than that of males despite the apparently greater metabolic demand for gonadal maturation in the female. This sexual difference in mortality has been noted for the char (Le Cren & Kipling, 1963); the atlantic salmon, Salmo salar L. (Netboy, 1968); and the sea trout (Lamond, 1916). However, female perch had a significantly higher incidence of infection than did the male (Pickering & Willoughby, 1977). It is tempting to conclude that, in general, males are more vulnerable to epidermal lesions with subsequent fungal invasion. It is known that sexual dimorphism occurs in the structure of the epidermis of the sea trout, Salmo trutta L. (Stoklosowa, 1966, 1970) and hatchery-reared, brown trout (Pickering, 1977). This difference in epidermal structure consists, in part, of a reduction in the concentration of goblet cells which also results in a reduction in the concentration of secreted mucous (Pickering, 1974). The continual secretion of mucous from the epidermis is believed to protect the fish by preventing potential pathogens from colonizing the body surface (Jakowska, 1963).

A statistical comparison of the patterns of infection reveals that there are large areas on the flanks of sexually mature male brown trout, which are significantly more frequently infected than corresponding areas on the female. Equally, there are areas on the tail and ventral fin of the female, which are more frequently infected than corresponding areas on the male (Richards & Pickering, 1978). White (1975) also found that spawning female brown trout have significantly more fungal infection in the caudal area and that males were more frequently infected anteriorly. It has been suggested that infection on the tail of females results from damage inflicted during redd digging and that the pattern of fungal infection on the body of the male results from abrasions incurred during spawning behaviour and wounds received during territorial defence.

1.2. Saprolegnia Species as Fungal Parasites

1.2.1 Classification and description

Several detailed classifications and descriptions of the class Oomycetes are to be found (see for instance Coker, 1923; Kanhouse, 1932; Coker & Matthews, 1937; Alexopoulos, 1962; Stuart & Fuller, 1968; Willoughby, 1968, 1969, 1978; Neish, 1976; Neish & Hughes, 1980; Webster, 1980 and Turian & Hohl, 1981). The more important general features described here are based on these accounts.

The classification of the Saprolegnia sp. involved in UDN has been discussed by Stuart & Fuller (1968) and Willoughby (1968, 1969). Examples of difficulties in classification were demonstrated by Willoughby (1970), who isolated six fungal genera from a

population of perch (Perca fluviatilis) captured in lake Windermere. The indiscriminate use of the term Saprolegniais for any cotton wool-like growth on teleosts is not accurate as lesions may include several fungi, some of which (for exampe Leptomitus lacteus) are not even members of the order Saprolegniales (Pickering & Willoughby, 1982). Another problem is one of fungal taxonomy. Saprolegnia parasitica was first described by Coker (1923) on the basis of the substrate from which it was isolated (fish or fish eggs), and the lack of oogonia when grown under normal culture conditions. The sexual structures were later described by Kanhouse (1932) as having thin, unpitted oogonial walls; diclinous antheridia and small (18-22 μ m in diameter) subcentric oospores. Coker and Matthews (1937) incorporated Kanhouse's description into their account of S. parasitica. However, Neish & Hughes (1980) pointed out that this description was made without reference to Coker's type material. Hence, the modern concept of S. parasitica reviewed by Neish & Hughes (1980), dates from 1932 or at least no later than 1937. Many authors, however, have still used the name S. parasitica in its original sense and consequently, until quite recently, the name S. parasitica has been used in two ways: Firstly, referring to Saprolegnia isolated from fish or fish eggs and appearing unable to produce oogonia; and secondly, to Saprolegnia with thin oogonial walls, predominantly diclinous antheridia, and subcentric oospores, regardless of the source of the isolate. Neish (1976) sought to resolve this problem by proposing that mean oospore size and oospore type (centric or subcentric) were of little value in separating S. parasitica from other Saprolegnia species with predominantly diclinous antheridia.

A more detailed analysis by Willoughby (1978) describes a S. diclina-S. parasitica complex with three subspecific types based on growth characteristics and especially on the length to breadth ratio (L/B) of the oogonium. S. diclina Type 1 (L/B ratio > 2, > 13%) occurs as a parasite of salmonid fish only, S. diclina Type 2 (L/B ratio > 2, > 12%) is a parasite of coarse fish and S. diclina Type 3 (L/B ratio > 2, > 10%) is purely saprophytic.

Organisms included in the class Oomycetes which are Mastigomycotina (Webster, 1980) constitute the sub-division Eumycota (the true fungi) of the division Mycota. The order Saprolegniales may have dimorphic zoospores, i.e. two kinds of zoospore may be present. Most Oomycetes are aquatic, and although some Saprolegniales grow in soil the production of zoospores implies a dependence on water for dispersal. Subsequent germination may be by means of zoospores. Amongst fungi, Oomycetes are unusual in that chitin is absent from their cell walls. In most Oomycetes small amounts of cellulose are present, however, the principal cell-wall components are glucans with β -(1-3) and β -(1-6) glucosidic linkages (Aronson et al., 1967). In Saprolegniales the thallus is predominantly eucarpic, often composed of large diameter coenocytic hyphae. Of the order Saprolegniales, the Saprolegniaceae are the largest family and best-known group of aquatic fungi, often termed the water-moulds and fish-moulds. Members of the group are abundant in wet soils, lake margins and fresh water, mainly as saprophytes on plants and animal debris. A few species of Saprolegnia are economically important as parasites of fish and their eggs (Willoughby, 1968, 1969, 1970; Willoughby & Pickering, 1977) and may

cause significant damage to commercial or government fish hatcheries. The Saprolegniaceae are characterized by a profusely branched, coenocytic mycelium easily visible as it forms a colony around decayed plant or animal tissue in water. Septa are formed in the mycelium just below the reproductive organs, separating them from the somatic hyphae, which generally remain aseptate.

1.2.2 Life cycle of Saprolegniaceae

The life cycle of Saprolegnia is outlined in (fig. 1). The ends of mature hyphae can widen to form a zoosporangium, which is separated from the hypha by a septum. Zoosporangia are typically long, cylindrical and terminal, and on reaching maturity contain large numbers of zoospores.

Sexual reproduction is effected by zoospores which become mature prior to the formation of sexually mature reproductive organs. Zoospores in the Saprolegniaceae are biflagellate, and two zoospores types occur in this family. One is the primary zoospore which is pear-shaped with the flagella at the apex, each of the same length and slightly longer than the body of the zoospore proper. Each of these two flagella is inserted in a highly staining granule, situated at the apical and of the anteriorly located nucleus. The cytoplasm of the primary zoospore is differentiated into two parts: a central, dense mass enclosing most of the nucleus; and a vascular, less dense, peripheral zone. In S. diclina the zoosporangium releases pyriform primary zoospores which swim for a short period of time only. Such zoospores swim in a weak, erratic manner often adopting an irregular spiral course, with a rotation ususally in a counter-clockwise direction. After dispersal, the primary zoospore

cyst forms and is normally covered with single, unbranched hairs approximately 1.5 μm in length. Each primary cyst normally releases a secondary zoospore. The secondary zoospore is kidney-shaped with two rather long flagella at the concave side which are extended in opposite directions, one anteriorly and the other posteriorly. Each of the flagella is associated with the nucleus by means of a basal granular body. The nucleus is centrally located and surrounded by a rather dense mass of cytoplasm. The rest of the zoospore body consists of vascular, less dense cytoplasm. In its swimming movements, the rotation is generally clockwise. The secondary zoospore functions normally over a wider range of temperatures than the primary zoospore. In addition, it also swims faster than the primary zoospore, its slowest rate of movement being equivalent to the fastest recorded movement for the primary zoospore (Salvin, 1941). This active phase prior to a second encystment is an important dispersive and infective factor in the life cycle. Secondary cysts of S. diclina bear bundles of long hooked-hairs, each of which ends in a pair of recurved hooks. The hooked hairs are preformed in the so-called bar bodies of the primary cyst wall during the process of encystment. The secondary cysts, instead of forming further zoospores, may germinate by means of a germ tube to give rise to vegetative filaments (Webster, 1980).

In addition to vegetative reproduction, the Saprolegniales also reproduce sexually. Their sexual organs, antheridia and oogonia, develop on short branches. One or several eggs (oospheres) are formed in each oogonium. The antheridium is elongated and uninucleated. The growing antheridium approaches the oogonium and

forms a process which enters the oogonium through pores in the oogonial wall. This process enters the egg cell and, after fusion of the nuclei, a double membrane develops around the zygote which then becomes an oospore. After a period of rest, the oospore germinates by means of a hyphal tube which shortly afterwards gives rise to a zoosporangium typical of the species.

1.3 The Economic Significance of UDN Disease

Saprolegnia species are a ubiquitous component of the aquatic environment. They are capable of growth and reproduction throughout the year, and occur as saprophytes on a wide variety of substrates, including fish eggs and fish carcasses (Neish, 1977). Mycotic infections of fish by fresh water Oomycetes can develop at all stages of the fish life cycle and are therefore of considerable economic significance to a number of fish related industries. As a consequence, the need for research into Saprolegniasis has become increasingly important in the British Isles during the last decade as losses of fish stocks from epidemics of UDN have increased (Elson, 1968; Roberts, 1972). Munro (1970) estimated that total Scottish losses were in excess of 51,000 migratory salmonids in 1967 and 38,000 in 1968. These figures represent 5.7% and 6.0% respectively, of the Scottish salmon catch during those two years. Once an outbreak occurs, the presence of infected fish ensures that the spore count in the water rises dramatically (Willoughby & Pickering, 1977). Unless treated, such fungal infections are usually lethal to fish and extensive zoospore production ensures that infections spread rapidly throughout a population (Pickering & Willoughby, 1982). It would seem that Saprolegnia infections enter

hatcheries from adjacent bodies of water (Willoughby & Pickering, 1977), however, at present it is virtually impossible to eliminate pathogenic fungi from hatcheries and farms without detriment to fish stocks. In the hatchery environment, the close proximity of individuals and the considerable stress which occurs through various handling techniques cause an increase in the frequency of fungal disease. Hatchery-reared brown trout and char demonstrate a considerably higher incidence of infection when compared with wild fish stocks. Many fish, once infected, rapidly succumb to the disease within two or three days (Richards & Pickering, 1979; Johansson et al., 1982).

Saprolegniasis causes severe losses mainly during the maintenance of brood fish and incubation of eggs. It is the main cause of mortality during the incubation of fish eggs which are spawned in autumn. Saprolegniasis also causes heavy losses during the incubation of fish eggs from species which spawn in spring (Sturgeon, Pike, Perch, etc.). The disease also appears in pond fisheries, particularly amongst incubating eggs of trout and white fish. Problems with Saprolegniasis are also encountered in the factory method for the production of eggs and larvae of carp and other plant-feeding fish. Infections have also been reported during the maintenance of spawning white fish, and in over-wintering carp and other plant-feeding fishes (Neish & Hughes, 1980).

Saprolegniasis continues to be the major fungal disease of freshwater fish. The overall aims of this thesis are to gain a clearer understanding of the phyliological and biochemical interaction which occur between the fungal pathogen and its fish

host, and to examine the immune response of the host to the pathogen. Studies presented in chapter two provides a comparison of the effects of temperature and substrate on the growth rate of two species of saprolegnia. These are a pathogenic strain which has previously been classified as Saprolegnia diclina (Willoughby, 1978; Smith et al. 1984); and Saprolegnia ferax, which is non-pathogenic. In chapter three, this comparison is extended to include an investigation of substrate utilisation at different temperatures. One aim of these two chapters was to identify those aspects of S. diclina's physiology and biochemistry which might be related to its pathogenicity.

In chapter four attention is directed towards a study of the effects of temperature on fungal infection of the host. Carp (Cyprinus carpio) were chosen for these studies, since they are of commerical interest, and can readily be kept under laboratory conditions. They are also able to exist over a wide temperature range and are therefore well suited to studies of the effects of temperature on infection and immunity. Work in this chapter assesses the influence of environmental temperature on the incidence of Saprolegnia infection in carp and examines the host's immune response towards saprolegnia and saprolegnia-derived antigens.

Work presented in chapter five examines some stress related changes in the blood and tissues of infected fish and explores the possible connection between stress and infection. In chapter six, the major findings of the thesis are discussed and suggestions are made for future work.

Fig. (1) Life Cycle of Saprolegnia sp. (after Alexopoulos, 1962)

CHAPTER II

THE EFFECT OF TEMPERATURE AND SUBSTRATE ON FUNGAL GROWTH

2.1. Introduction

Previous studies concerning aquatic fungi associated with diseased fish and fish eggs have highlighted the need for further investigations concerning physiological aspects of growth in saprophytic fungi. Normal growth results in an increase in cell number and mass, and growth studies are based upon measuring both the amount and rate of growth which, in turn, are controlled by the internal and external environments. Fungal enzymes catalyse synthetic as well as degradation reactions and the processes of cell elongation and cell division are controlled by a variety of enzyme systems. Of the external factors which modify enzyme action, temperature and the concentration of substrate and products are probably the most important.

Temperature acts directly upon the vital processes including germination of spores; vegetative growth; the induction of sexual reproduction by means of zoospores and even the activity and behaviour of zoospores. Wolf & Wolf (1947); Hawker (1950) and Cochrane (1958) reported that temperature has a profound effect upon germination of zoospores and the growth of fungal mycelia.

Growth is defined as an increase in either mass of cells or number of cells. In typical curves of growth plotted as a function of temperature there are a number of distinct phases. Following a

period of no apparent growth, there is a characteristically linear growth phase in which growth increases directly with temperature. There is then an optimum temperature range for growth, which may be narrow or broad depending on the species, followed by a descending phase of no net growth and autolysis eventually leading to a decline in dry weight as the temperature becomes too high for growth. The first phase is composed of two steps: a lag phase before spore germination followed by a second step in which growth occurs but is undetectable by the experimental methods used. In the phase of rapid growth, the utilisation of essential materials occurs. The morphological basis of mycelial development is that growth occurs only at the hyphal tips. Interior cells of the mycelium do not normally contribute to the new growth directly, although they supply nutrients to peripheral cells and especially to aerial structures. There is no single temperature optimum for fungal growth, but a range exists in which growth reaches an optimum level depending upon the time of incubation, medium and the method of growth measurement. The final phase is characterised by a decline in mycelial weight and the appearance of nitrogen and phosphate in the medium. Autolysing mycelium undergoes an extensive breakdown of chitin, cellulose, carbohydrates, lipids and proteins, all catalysed by fungal enzymes. Other autolytic products include ammonia, free amino acids, organic phosphorous compounds and sulphur compounds.

Sexual reproduction is also affected by temperature and a number of investigations of the effects of temperature on formation of oogonia and antheridia in the family Saprolegniaceae have been made. Barksdale (1960) reported that the induction of sexual organs is

temperature dependent. Sexual organs were initiated at 15-20°C in many strains of Achlya; at 21°C for Saprolegnia diclina (Szaniszlo, 1965); and at 20-30°C for Saprolegnia ferax (Krause, 1960). These differences seem to be the result of a distinct morphogenic response to temperature, and are probably characteristics of the species rather than the result of culture techniques (Szaniszlo, 1965).

Temperature has been shown to affect the production and activity of fungal zoospores. Suzuki and Hatakeyama (1960) reported that zoospore production was reduced under unfavourable temperature conditions. Zoospore behaviour can also be severely influenced by temperature. Thus, in a study by Salvin (1941) on the activity of zoospores of Saprolegniaceae, primary zoospores swam abnormally at 10°C and some of them (Saprolegnia) encysted almost immediately after emerging from the zoosporangia. At 30°C the zoospores, instead of swimming in a relatively straight line, circled, spiralled and twisted with vigorous vibratory movements. Secondary zoospores, however, behaved normally over wider extremes of temperature than primary zoospores. Optimum temperature ranges for primary and secondary zoospores were found to be 15-25°C and 10-30°C respectively.

In addition to temperature, the concentration and type of nutrients in the substrate are of great importance in determining both the rate and amount of fungal growth. The substrate concentration may be varied by diluting the entire medium or by varying the concentration of one or more constituents. In general, when an entire medium is diluted, it might be expected that the

decrease in amount of mycelium produced would be directly proportional to the amount of dilution. When the concentration of one constituent of the medium is changed, the amount of growth will be proportional to the concentration over a certain range. Above a certain concentration there will be no further increase in the amount of growth.

Fungi are known to utilise a wide range of natural organic compounds, especially carbohydrates which may be of great complexity. Monosaccharides, in particular glucose, are directly assimilated across the plasma membrane, but disaccharides and large or complex polysaccharides such as starch, cellulose and lignin, are degraded by extracellular enzymes prior to uptake by the fungus. Most of the saprolegniaceae cannot utilise nitrate nitrogen under any conditions, but in some investigations it appears that some species are able to utilise ammonium nitrogen as NH_4CI or NH_4NO_3 (Sorenson, 1964). In pure culture, proteins such a gelatin, casein, keratin and egg albumen can all serve as sources of nitrogen, especially for the common saprophytic fungi. The widespread occurrence of enzymes capable of hydrolysing proteins to amino acids indicates that, in general, the simple linear peptides provide adequate sources of nitrogen for most fungi. In culture, proteins are used more rapidly if there is no other carbon source, but the liberation of ammonia under these conditions may reduce fungal growth and activity.

Many fungi are able to grow on crude or purified natural fats and oils (Garg et al, 1985). It is clear that both fats and fatty acids

are utilised and can support growth in a medium containing no other source of carbon. In general, micronutrients are essential for fungal growth whereas vitamins are not. Inorganic requirements including magnesium, calcium, zinc, manganese and iron are essential for the growth of Saprolegniaceae. Sulphate cannot be utilised, but sulphur can be supplied in organic form as cystine, glutathione, methionine or inorganic sulphide.

There is no generally accepted method to measure growth rates in all fungi. Colony diameter has been used (Volkonsky, 1933; Lee, 1962). However, this method is not sufficiently accurate since the height and other dimensions of the colony are ignored. The most widely used and, within limits, the most satisfactory measurement of growth is by determination of dry weight of the mycelium. The principle limitation is that weight may reflect the accumulation of polysaccharides or other reserve materials rather than synthesis of new protoplasm. Lilly and Barnett (1951) have found that the dry weight method objectively measures the increase in mass of the mycelium since growth in fungi is a function of the number of actively growing hyphal tips. Other workers have also found dry weight to be an adequate estimate of growth in saprolegniaceous fungi (Whiffen, 1945; Reicher, 1951). This method has therefore been used to measure the growth of S. diclina and S. ferax in the present investigation.

2.2. Materials and Methods

2.2.A. Maintenance of fungal species

Strains of S. diclina (Humphrey) and S. ferax (Gruith), Thuret (Smith et al, 1985) were obtained from the Microbiological Laboratory, University of Aston. The isolates were initially grown on glucose-yeast extract agar medium (Appendix 1) and these were sub-cultured at bi-monthly intervals. The cultures were incubated at 10°C for 72-96 hours, transferred to 5°C until the cultures were fully grown and stored in a fridge at 4°C prior to use.

2.2.B. Effect of temperature and time on growth

Three liquid media were used, 2% casein, 1% casein plus 1% glucose and 2% glucose (Appendix 1). 20ml aliquots of each of the three media were dispensed into 100ml erlenmeyer flasks and autoclaved (Appendix 1). For each species studies (S. diclina and S. ferax) groups of 6 flasks were inoculated with a mycelial disc (1cm in diameter) of fungus and incubated at 5, 10, 15, 20, 25 and 30°C respectively for 96 hours. The contents of each flask were harvested by filtration onto Whatman no.1 filter paper which had been previously dried and weighed. The combined fungal biomass and filter papers were oven-dried at 90°C for 24 hours, cooled, and reweighed to determine the fungal dry weight.

Growth rates of S. diclina and S. ferax were also monitored over a seven day period at 10°C and 25°C. 20ml aliquots of media were dispensed into 100ml erlenmeyer flasks and autoclaved (Appendix 1). Flasks were inoculated with mycelial discs (1cm diameter) cut from colonies of S. diclina or S. ferax. The flasks were subsequently

incubated at 10°C and 25°C respectively and the fungal dry weight ascertained in the manner outlined above.

2.2.C. Effect of substrate on growth

Seven media were used, 2% glucose; 2% casein; 1% glucose + 1% casein; 1% casein + 1% fat; 1% glucose + 1% fat; 0.66 glucose + 0.66 casein + 0.66 fat and 2% fat (Appendix 1). Each medium was distributed into two groups of five conical flasks (100ml capacity), each containing 20ml of the chosen sterile medium. The two groups of flasks were inoculated with a mycelial disc and incubated at 10°C and 25°C respectively. The dry weight was determined after 96 hours of incubation as described above.

To study the effect of fat on growth, two types of media were utilized, 1% glucose and 1% casein. For each medium seven concentrations of fat in the form of cod liver oil were added under sterile conditions, 0; 0.25%; 0.5%; 1%; 1.5%; 2.5% and 4%. For each concentration five replicate flasks were inoculated and kept at 20°C for four days. The contents were then filtered and, after washing the fungus biomass with large amounts of boiling water to wash out the non-utilized fat, the dry weights were determined as described above.

For all the experiments, data were analysed using 2-way analysis of variance (F-test).

2.3. Results

2.3.A. Effect of temperature and time on growth

The results are summarised in Tables 1, 2 and 3 (Appendix 2) and Figs. 2, 3, 4, 5, 6 and 7. Fig.2 shows the effects of temperature on growth of *S. diclina*. The fungal growth increases with temperature to a maximum after which it is severely reduced by higher temperatures. The maximum growth for 2% glucose is reached at 20°C while for 2% casein and 1% casein plus 1% glucose the maximum occurs at 25°C. On all three media, growth is severely restricted at 30°C. The greatest growth of *S. diclina* occurs on medium containing both casein and glucose.

Figure 3 summarises the effect of temperature on growth of *S. ferax*. As with *S. diclina*, the best growth occurs on medium containing casein and glucose. Optimum growth occurs at 25°C on all three media, and fungal growth is curtailed on all media at 30°C.

A comparison of the two fungi demonstrates that, at higher temperatures, *S. ferax* grows better on media containing glucose than *S. diclina*, while *S. diclina* can utilize casein more effectively than *S. ferax* as evidenced by the higher growth rate of *S. diclina* on medium containing 2% casein alone. Both fungi, however, show optimal growth on medium containing both glucose and casein, where *S. ferax* shows a better growth than *S. diclina* over the majority of temperatures.

Figure 4 and Table 2 (Appendix 2) shows the growth of *S. diclina* at 10°C over a period of 7 days. Growth on each of the three media

is slow for the first three days. After day 4 however, there is a sharp increase in growth which persists until day 7. Optimum growth again occurs on medium combining both casein and glucose, while the 2% glucose medium again gives the lowest values.

Figure 5 and Table 2 (Appendix 2) illustrate the effect of time on growth of S. diclina over a 7 day period at 25°C. Growth is significantly restricted when the fungus is grown on medium without casein. S. diclina exhibited optimal growth at day six with media containing casein, especially with 1% casein + 1% glucose, but by day 7 dry weight has decreased. The 2% glucose medium still produced an increase in dry weight after day 6, although the overall rate of dry weight increase is slower than with the other two media.

By referring to Figures 4 and 5 it may be seen that there is little difference between the growth rates observed on 2% glucose medium at 10°C and 25°C. However, S. diclina grows markedly better at 25°C than at 10°C on the other two media investigated.

The growth of S. ferax over a 7 day period at 10°C is shown in Fig.6 and Table 3 (Appendix 2). The growth on 1% casein plus 1% glucose is slightly higher than for the other two media. The fungus maintained a continuous increase in growth until the end of experiment.

Figure 7 and Table 3 (Appendix 2) show the effect of time on the growth of S. ferax at 25°C. The maximum growth on media containing

casein was observed at day 6, after which the growth rate subsided. On the other hand, with 2% glucose medium, the increase in growth was maintained till day 7, albeit at a slower rate.

Comparing the growth rates of S. ferax at 10°C and 25°C (Figs.6 and 7), it may be seen that growth at 10°C is slower than that observed at 25°C. At 10°C growth continued on all three media for the duration of the experiment, but at 25°C there was a decrease in dry weight on day 7 in media containing casein.

With reference to Figs.4, 5, 6 and 7, it can be seen that S. ferax grows better on 2% glucose medium at both 10°C and 25°C than does S. diclina. The two species have the same growth pattern at 10°C and 25°C when grown on 2% glucose medium, since growth continued throughout the experimental period. Both species demonstrate a similar increase in growth rate in media containing casein when incubated at 10°C. At 25°C, however, growth was severely restricted by day 6.

2.3.B. Effect of substrate on growth

The effects of substrate on fungal growth are summarised in Figs.8 and 9 and Table 4 and 5 (Appendix 2). Both S. diclina and S. ferax show similar growth patterns. At both 10°C and 25°C the best growth occurs on medium which is free from fat and contains both glucose and casein. Growth is also significantly better at 25°C than at 10°C. At both temperatures, the least growth occurred on medium containing 2% fat but which was free from other nutrients. Growth is inhibited when the medium contains fat at any

concentration, and there is no significant difference between growth in fat supplemented media at the two temperatures tested.

Since all fat concentrations used here inhibited fungal growth, a more detailed study of the effect of a wider range of fat concentrations on the growth of S. diclina and S. ferax is carried out. Growth on all media containing casein was much better than that on media containing glucose. The best overall growth occurred on media free from fat and as the concentration of fat in the medium is increased, the growth rate decreases.

By comparing the results on Figs. 8 and 9, it may conclude that at 10°C S. diclina grows better than S. ferax on fat free media. S. ferax grows better at 25°C than S. diclina on all the media except 2% casein medium. When fat is present in the medium both species show a marked inhibition of growth at both temperatures tested. It may be seen that the two fungi have the same growth pattern when grown on media with different fat concentrations. Increasing fat concentration in the medium reduces dry weight. With one exception (0.5% fat + 1% casein) S. ferax grows better than S. diclina in the presence of low fat concentrations (0, 0.25, 0.5 and 1%) on both media. While on the other hand, S. diclina exhibits a greater growth rate than S. ferax at high fat concentrations (1.5, 2.5 and 4%).

2.4. Discussion

Temperature has a major effect on all cellular activities, including spore formation and germination (Anderson, 1978; Furch,

1981), fungal growth (Wolf & Wolf, 1947; Hawker, 1950; Cochrane, 1958) and on enzymatic activity (Farrell and Rose, 1967).

In general, the best growth of S. diclina and S. ferax is at 25°C in all the experiments presented here.

Temperature is one of the most important external factors to influence spore germination. It not only affects the percentage of germination but also the length of time required for germination. It may be assumed that a favourable temperature permits certain enzymatic activities essential to germination. Certain fungi are limited by high temperature, among these are certain phycomycetes. Lilly & Barnett (1951) reported that the maximum temperature permitting spore discharge is appreciably lower than for viability of the fungus and low temperature usually merely slows down spore production and discharge. Therefore, one factor which causes the best growth of S. diclina and S. ferax at 25°C may be the increased discharge, spread and germination of spores at this temperature.

The results of the temperature studies carried out here (Fig.2 and 3) indicate that the temperature of incubation has a significant effect on the growth of S. diclina and S. ferax. In comparing the data obtained here with those of previous reports concerning growth rates of saprolegniaceous fungi, it may be seen that both species grew between 5°C and 30°C which is within the temperature limits of 0 to 36°C previously reported by Lee (1962) for S. parasitica. The temperature range for optimal growth in the present study (15 to 30°C) accords well with the results reported by James et al. (1972).

This temperature range also compares favourably with optima 17 to 26°C reported for other species of Saprolegnia and members of the Saprolegniaceae with one exception, Achlya delebsiana, which grew well between 30°C and 35°C (Reischer, 1951). The carp (Cyprinus carpio) lives in water between 10-25°C (Wheeler, 1969) which falls within the optimal temperature range for fungal growth. This suggests that both S. diclina and S. ferax have a wide temperature range for infection in Summer. However, fish are not generally infected in Summer, due possibly to the high activity of the immune system against the fungus. In Winter, at low temperatures, fish may become highly infected as the immune system is depressed and the resistance of fish against fungal invasion is less than in Summer.

The effect of temperature on the enzymatic activity of the fungus also affects fungal growth (Farrell and Rose, 1967). The rate of reactions catalyzed by enzymes increases with temperature, and there are some reports in the literature of the rate of enzymatic reactions being increased as much as five-fold by a 10°C increase in temperature. However, enzymes are most sensitive to temperature and are gradually inactivated as the temperature increases or decreases betond the optimal physiological range. This decreased activity is reflected in a lowered rate of growth at extremes of temperature.

Many fungi have latent abilities to synthesize various essential metabolities. In the absence of these compounds in the medium and after a shorter or longer period of incubation, a fungus may begin to synthesize these essential metabolites, and growth may then take

place in a normal way (Lilly & Barnett, 1951). However, the majority of fungi are dependent upon the medium or substrate for the elements and compounds they require or utilize. From these elements and compounds they synthesize their cellular constituents and obtain the energy necessary for their life processes. As Saprolegnia species are saprophytes, determination of suitable growth substrates is of great importance to evaluate the essential materials for fungal growth. The best growth of S. diclina and S. ferax was on 1% casein and 1% glucose substrate at 25°C; this indicates that the two species prefer the two materials at the same time to grow well and also concurs with previous reports from Alexopoulous (1962); Gay & Greenwood (1971) and Heath et al. (1971), who noted that glucose was the optimum source of carbon and casein the preferred source of nitrogen for members of the Saprolegnia. Carbon compounds, such as glucose, serve two essential functions in the metabolism of fungi. They supply in the first place the carbon needed for the synthesis of the compounds which go to make up the living cell-protein; nucleic acid; reserve foods; cell wall synthesis and growth. Second, the sole source of appreciable amounts of energy is the oxidation of carbon compounds, which may account for half or more of the carbon supplied to a culture. High glucose concentrations were found to be inhibiting to growth, therefore, one factor which may permit the fungus to grow on fish is the relatively low content of carbohydrate in fish tissue, since the carp has only 0.23% glycogen in the muscles (Wittenberger and Diaciuc, 1965; Wittenberger et al, 1975).

Results recorded in Figs.1-6 indicated that the lowest dry weight recorded under any conditions of temperature, substrate and time was on 2% glucose medium. This is mainly because when the concentration of carbohydrate in a medium is increased, the dry weight does not increase proportionately, although more sugar may be utilized (Cochrane, 1958). There also occurs at supraoptimal glucose concentrations, a simple failure to utilize the sugar present. For many fungi, increase in carbohydrate beyond an optimum point results in an absolute as well as a relative decrease in growth. The factor involved is almost certainly the formation of toxic metabolic products. In Entomophthora sp. the effect of high concentrations of glucose is to increase the acidity of the medium and the lowered pH resulting from organic acid accumulation is probably the most general cause of poor growth at high sugar concentrations. Therefore, the level of carbohydrate to be used should be determined by experiment for each organism, accordingly 2% glucose was the highest concentration used in all the experiments in this investigation. This figure is based on the hypothesis that 2 per cent available carbohydrate in a liquid media is an upper limit, considering the amounts of nitrogen usually recommended in media (Cochrane, 1958). Also, James et al. (1972) inoculated S. parasitica on different media containing different concentrations of glucose and then measured the growth rate. They found that the dry weight increased with increasing glucose concentration up to 2% glucose.

There is no optimum amount of nitrogen compound for a culture; the demand depends in the first instance upon the carbon supply, but

in principle any factor may change the apparent optimum concentration of the nitrogen source. James et al. (1972) concluded that 2% casein is the optimum concentration for the growth of S. parasitica, consequently 2% casein concentration was used in this investigation.

In pure culture it appears that proteins such as gelatin, casein and egg albumen can serve as sources of nitrogen for at least the common saprophytic fungi, and casein is more rapidly utilised if there is no other carbon source in the medium. However, the liberation of ammonia under these conditions is large. No single pattern of nitrogen assimilation can be described to apply to all fungi. In general, inorganic or organic nitrogen is taken up rapidly during the phase of growth. In this case, there may be a back movement of ammonia nitrogen from the cells to the medium, reflecting the permeability of the cell to ammonia. Certain enzymes also begin to appear in the medium during this period and in at least some organisms amino acids and other soluble nitrogen compounds are liberated (Morton, 1956). The major liberation of proteins and other nitrogenous compounds occurs in the phase of autolysis. Morton (1956) indicated that upon exhaustion of the utilizable nitrogen of the medium, there is a mobilization and re-utilization of at least part of the nitrogen from older hyphae to support development of new mycelium.

The percentage of protein content of carp (Cyprinus carpio) body has been variously reported as 12.10-13.84% (Atack et al., 1979); 10.0-11.1% (Jauncey, 1979); 13.9-15.8% (Zeitler et al., 1984) and

14.3% (Schwarz et al, 1985). Therefore, the fungus may have been attracted to and colonized the fish skin due to the high protein content of carp body tissues.

It seems clear that many fungi are able to grow on purified natural fat and oils. Also, both fats and fatty acids are utilized and support growth in a medium containing no other source of carbon. The first step in fat utilization is the hydrolysis of fat to glycerol and fatty acids, and this hydrolysis is catalyzed by lipase or lipases. In this investigation cod liver oil is used as a fish oil to see how the fungus can utilize and grow on it at different concentrations and temperatures. This will provide information about the ability of the fungus to inoculate on fish tissues in the natural environment as these tissues may contain high quantities of fat. For example, the fat percentage in carp has been estimated as 5.25-9.46% (Atack et al, 1979); 2.0-5.0% (Jauncey, 1979); 5.4-8.6% (Zeitler et al, 1984); 10.7% (Schwarz et al, 1985). Generally, in all the experiments reported here the smallest growth of S. diclina and S. ferax was observed on media containing glucose alone or glucose with different fat concentrations. This may be due to the effect of glucose on the liberation and activity of extracellular lipase enzymes in the growth media. This phenomenon is termed the glucose effect or catabolite repression (Keen & Holton, 1966; Patil & Dimond, 1968; Moran & Starr, 1969; Spalding et al, 1973; Weinhold & Bowman, 1974). Interestingly, S. diclina exhibited a higher growth rate than S. ferax at high fat concentrations and may therefore be better adapted to colonising fish tissues which contain notable quantities of fat.

Fig. (2) Effect of temperature on growth of S. diclina

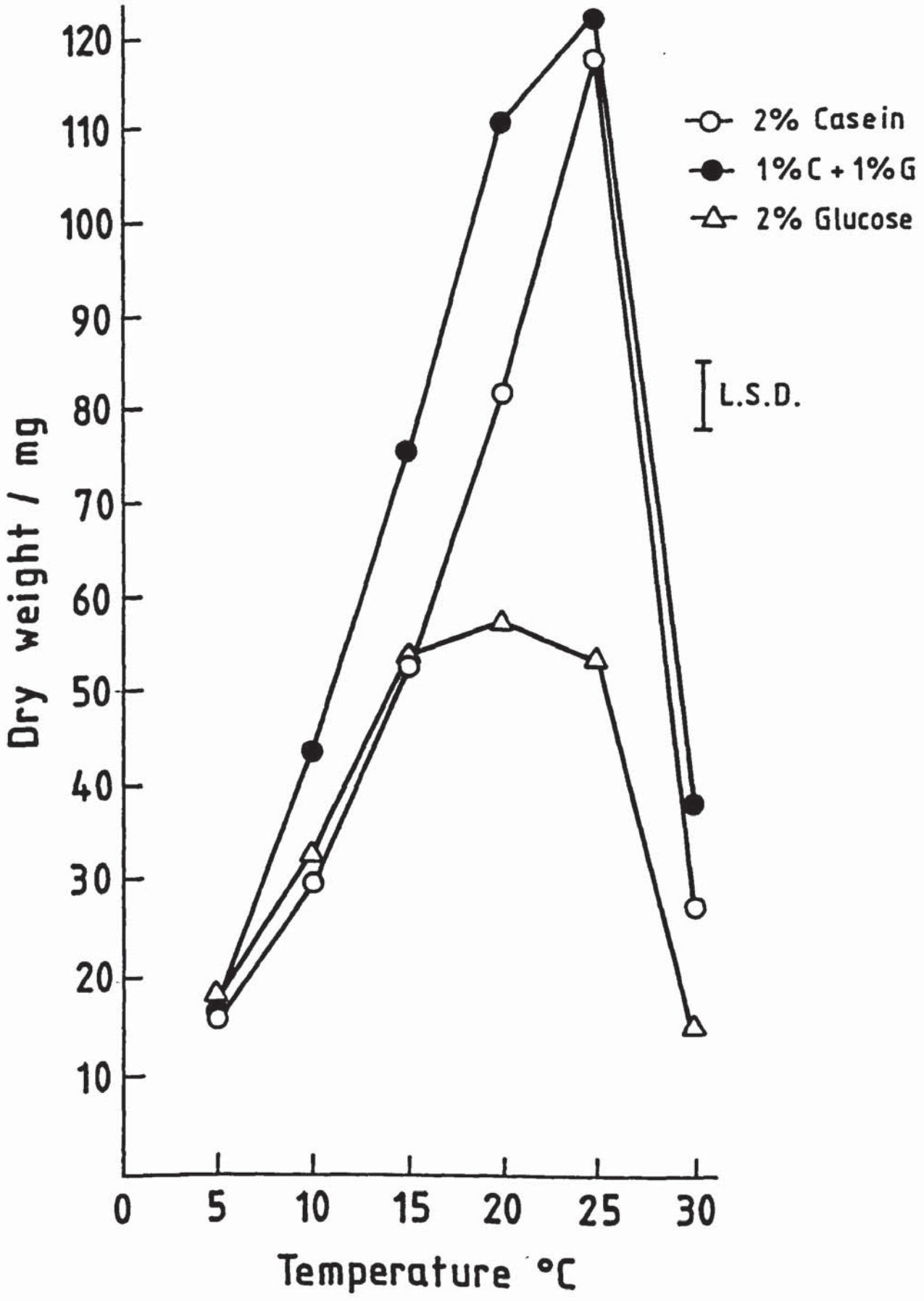

120
110
100
90
80
70
60
50
40
30
20
10
Dry weight / mg
0
5
10
15
20
25
30
Temperature °C
2% Casein
1%C + 1%G
2% Glucose
L.S.D.

Fig. (3) Effect of temperature on growth of S. ferax

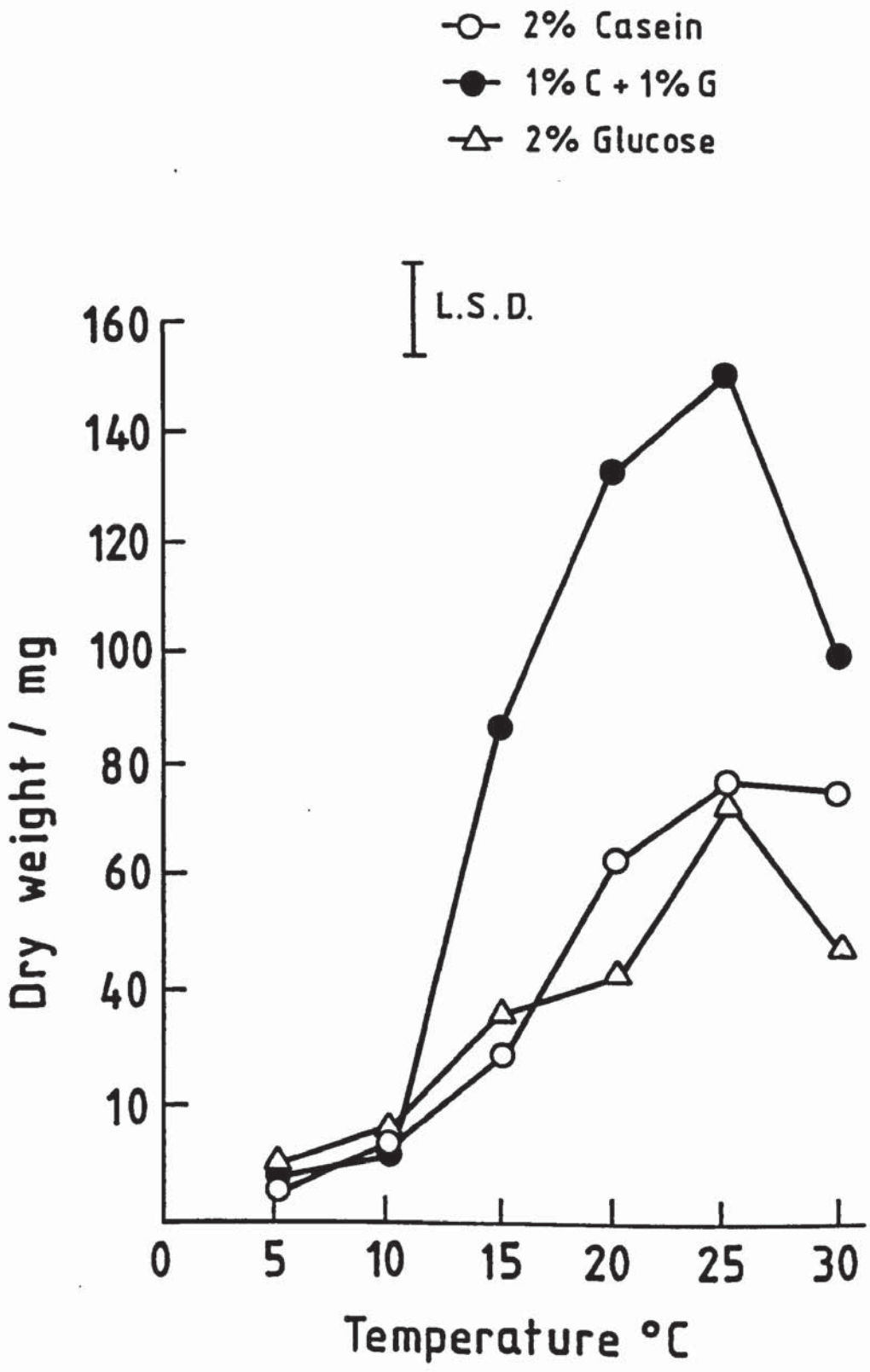

2% Casein
1% C + 1% G
2% Glucose
L.S.D.
160
140
120
100
80
60
40
10
Dry weight / mg
0
5
10
15
20
25
30
Temperature °C

Fig. (4) Effect of time on growth of S. diclina at 10°C

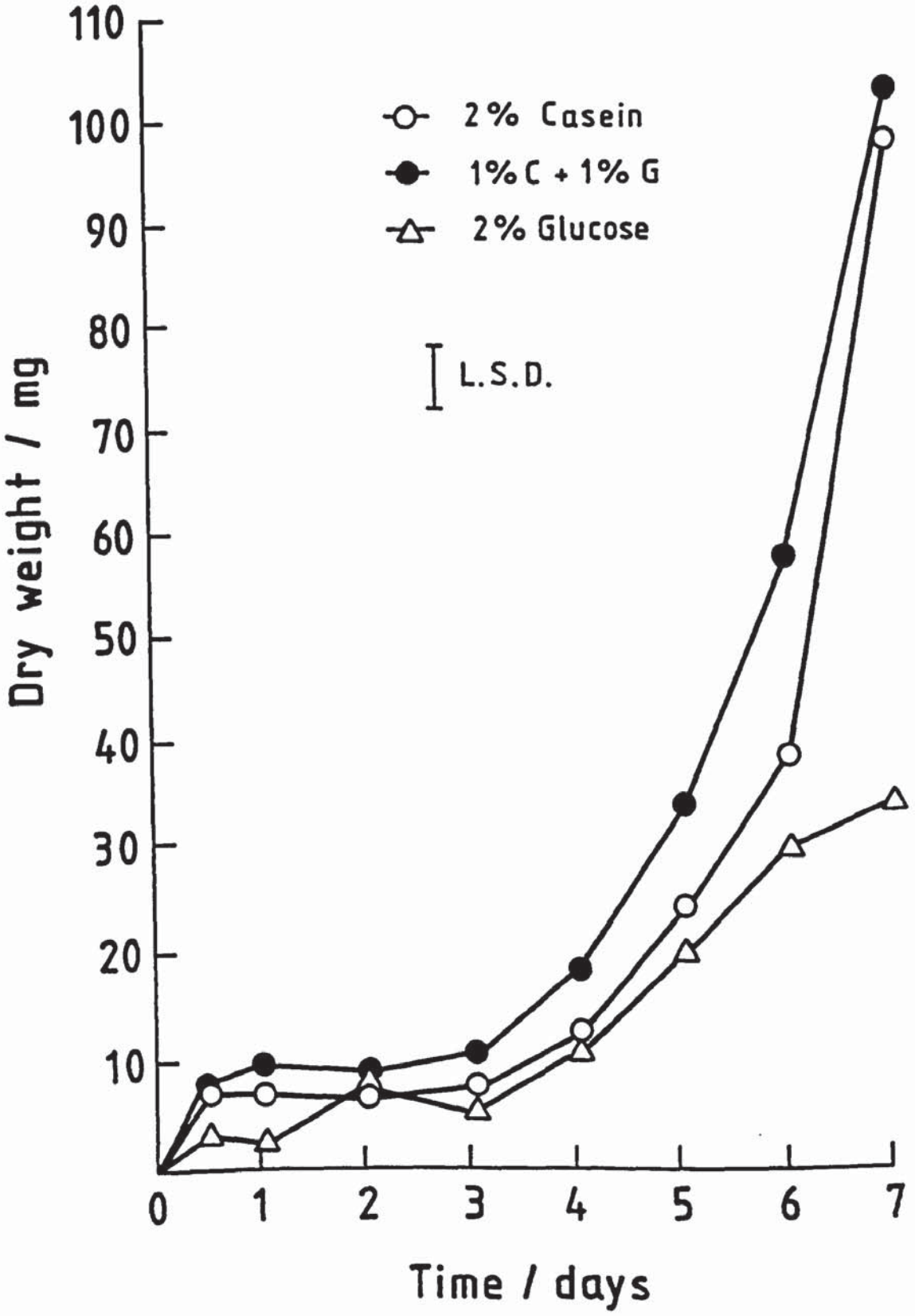

110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Dry weight / mg
Time / days
2% Casein
1% C + 1% G
2% Glucose
L.S.D.

Fig. (5) Effect of time on growth of S. diclina at 25°C

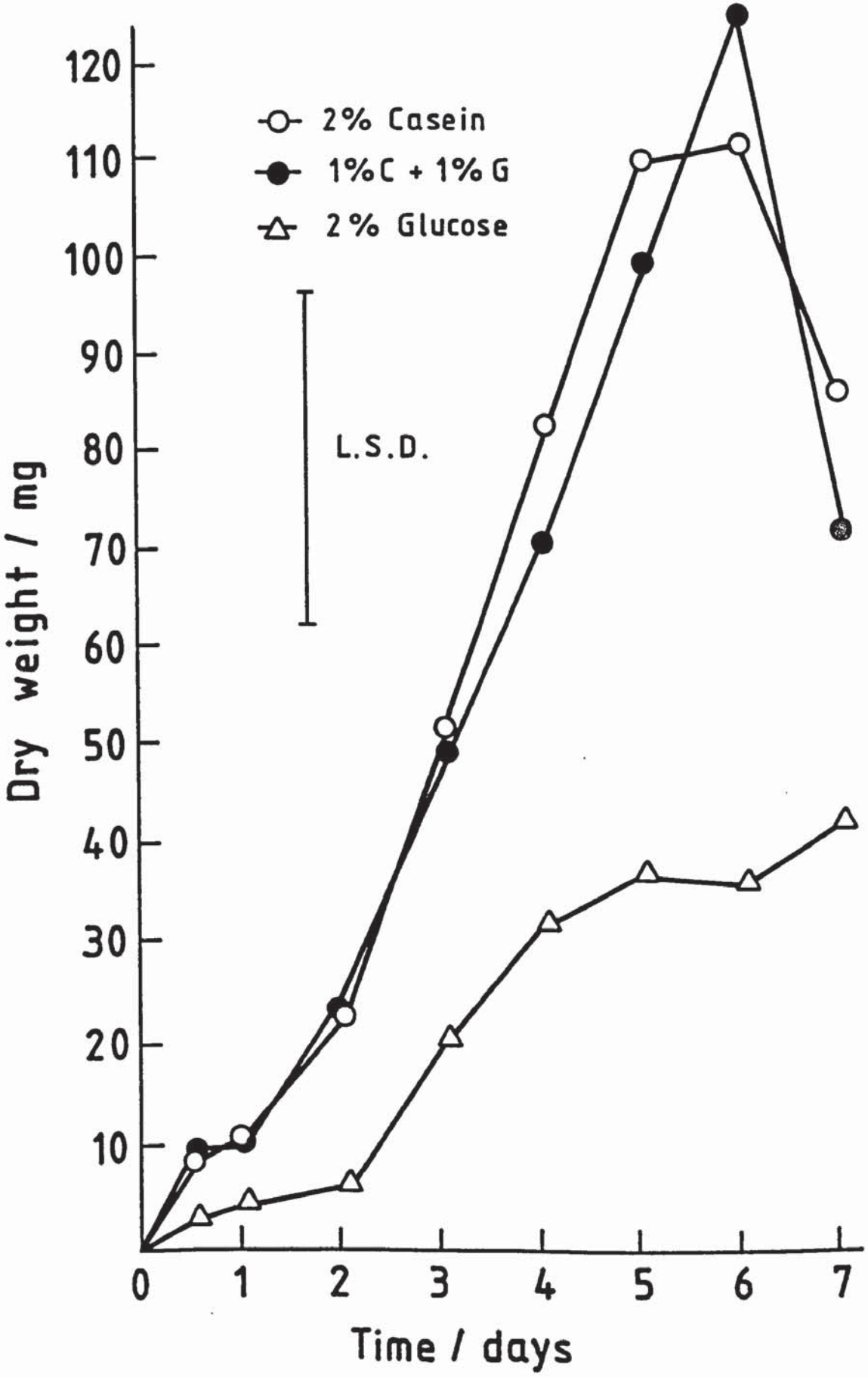

2% Casein
1%C + 1% G
2% Glucose
L.S.D.
Dry weight / mg
Time / days
120
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7

Fig. (6) Effect of time on growth of S. ferax at 10°C

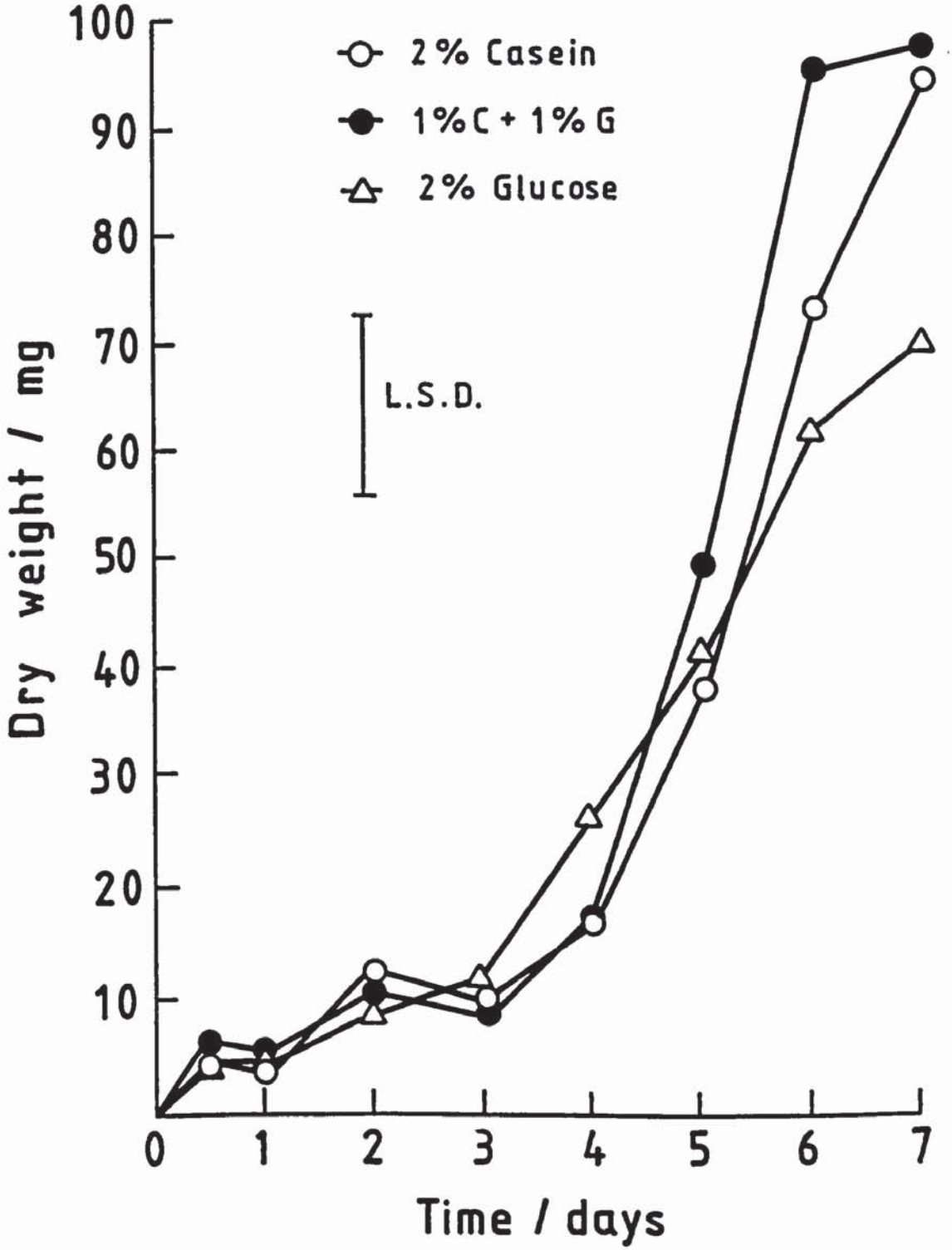

2% Casein
1%C + 1% G
2% Glucose
L.S.D.
Dry weight / mg
Time / days
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7

Fig. (7) Effect of time on growth of S. ferax at 25°C

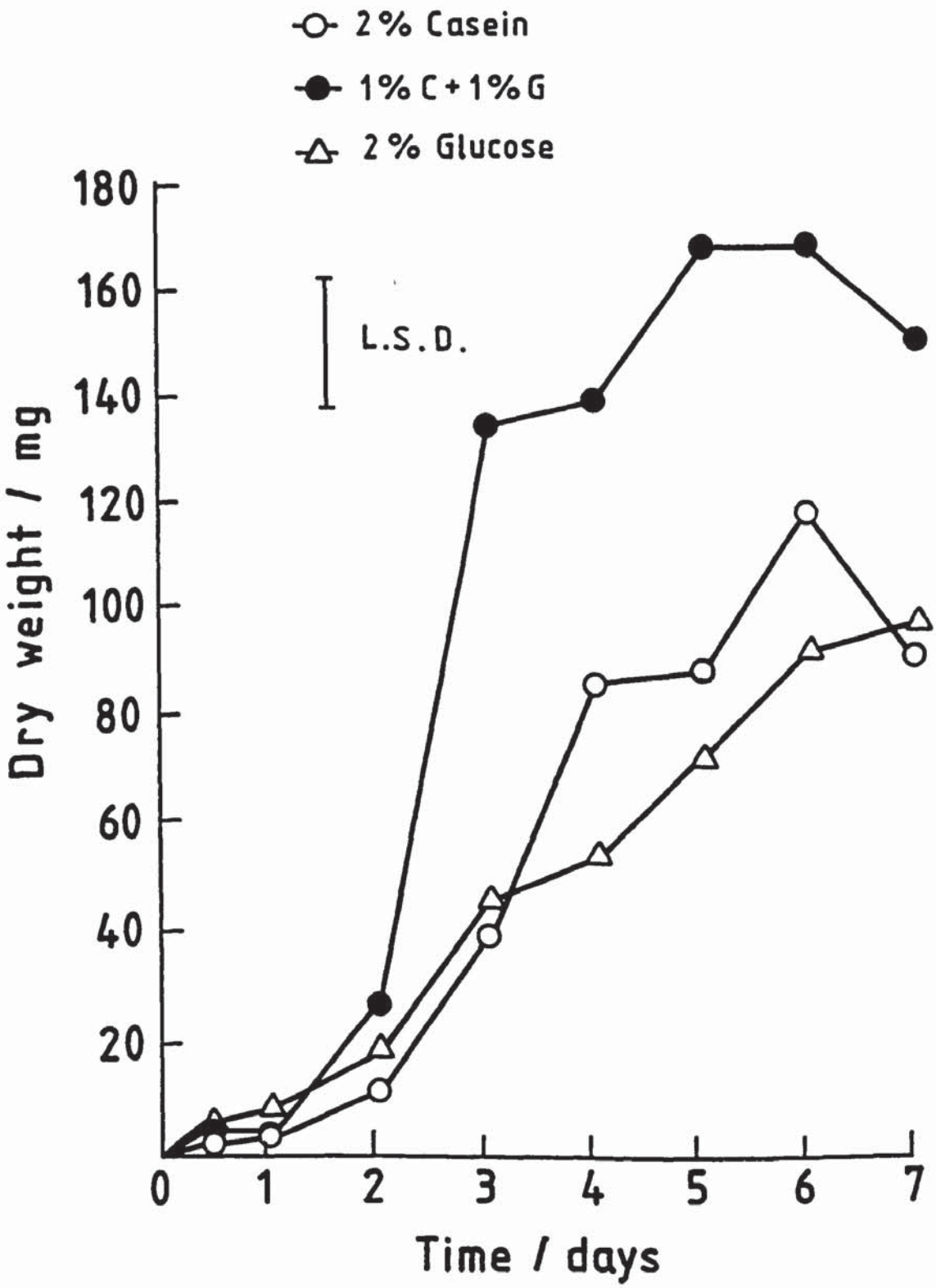

2% Casein
1% C + 1% G
2% Glucose
L.S.D.
Dry weight / mg
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Time / days

Fig. (8) Effect of substrate on the growth of S. diclina after four days of incubation

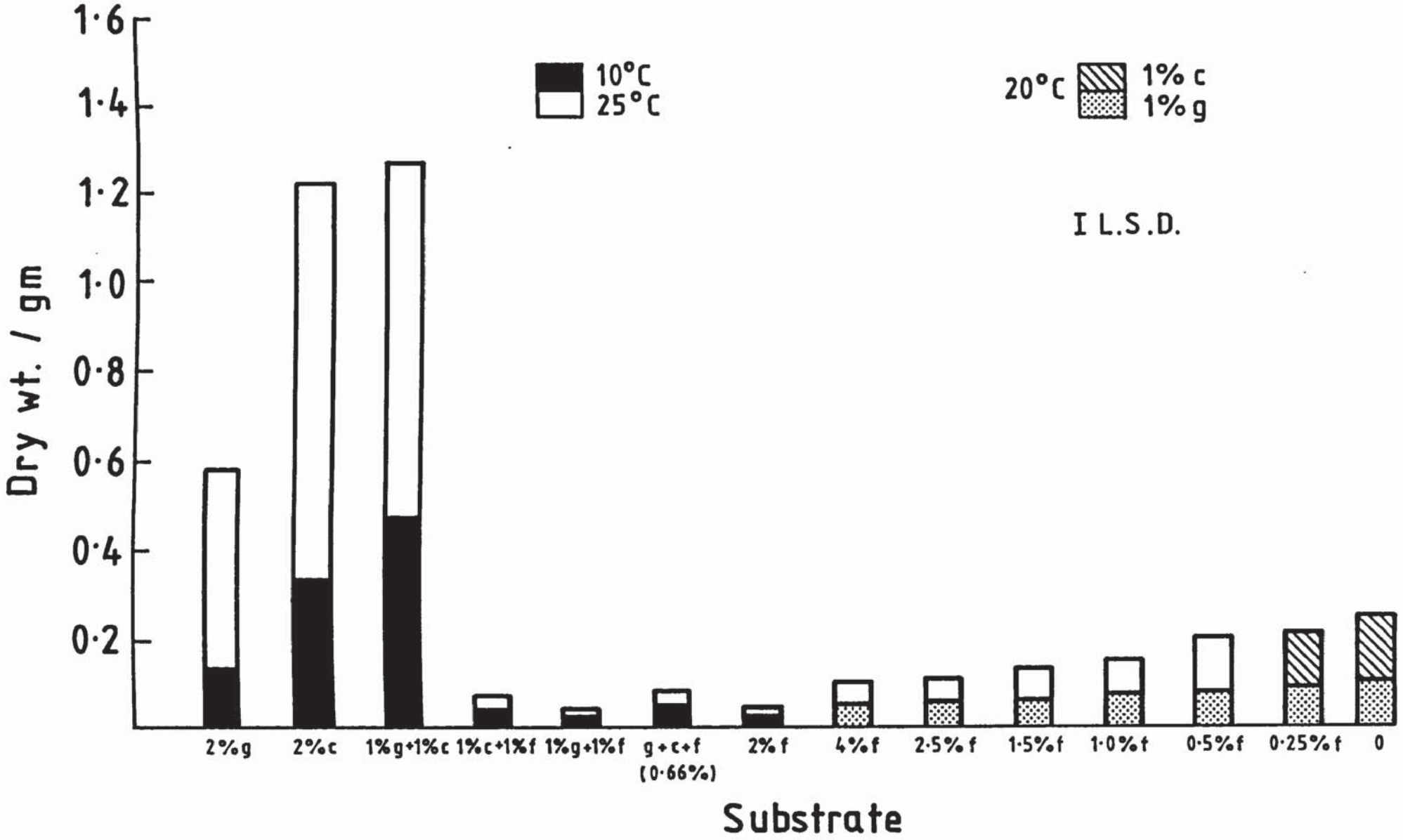
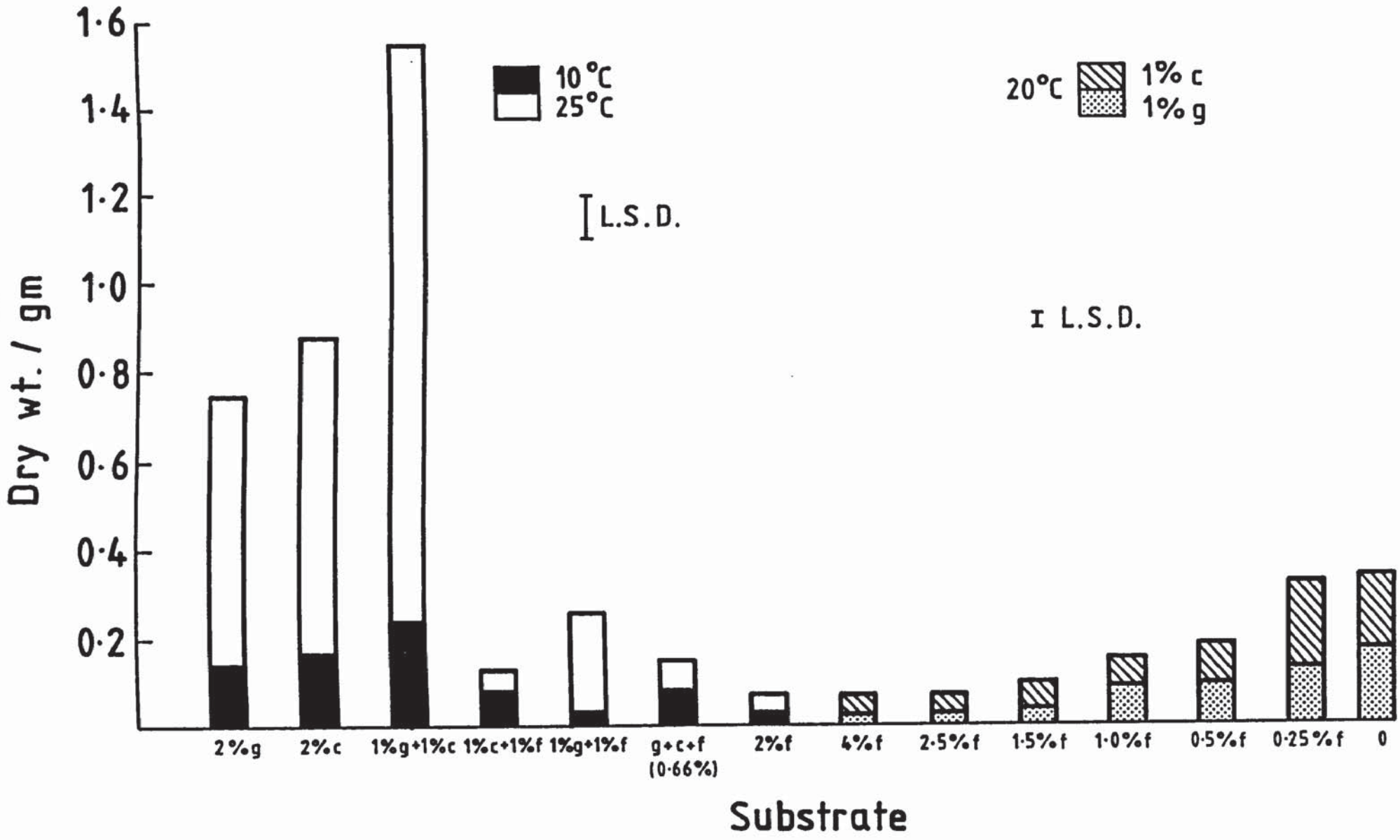

1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
Dry wt. / gm
10°C
25°C
20°C
1% c
1% g
I L.S.D.
2%g
2%c
1%g+1%c
1%c+1%f
1%g+1%f
g+c+f (0·66%)
2%f
4%f
2·5%f
1·5%f
1·0%f
0·5%f
0·25%f
0
Substrate

Fig. (9) Effect of substrate on the growth of *S. ferax* after four days of incubation



CHAPTER III

SUBSTRATE UTILIZATION AT DIFFERENT TEMPERATURES

3.1 Introduction

Earlier work in chapter II has demonstrated that both fungi can utilize both casein and cod liver oil for growth and therefore, probably produce both protease and lipase enzymes. Such enzymes produced by fungi are secreted or released into the surrounding medium and normally perform their function outside the cells that produce them, these are termed extracellular enzymes or exoenzymes (Hagihara, 1960; Kreger & Griffin, 1974; Reyes et al., 1979, 1980; Perez-Leblic et al., 1982). Exoenzymes perform the functions of digestion; i.e., the degradiation of complex food materials such as proteins, polysaccharides and lipids into simpler compounds which are soluble in water and thus able to enter the cell.

Usually, growth and enzyme formation in fungi are stimulated by supplying complex forms of nitrogen (Gomez et al., 1977; Reyes et al., 1977, 1979; Lahoz et al., 1978; Martinez et al., 1982). The production of amino acids by enzymic hydrolysis of proteins requires a mixture of proteases which can hydrolyse the protein to amino acids rather than a single enzyme which would yield only a mixture of peptides.

Two general types of proteolytic enzymes have been distinguished; endoproteolytic enzymes, acting on peptide bonds either in simple peptides or in the interior of a protein chain; and exoproteolytic enzymes, acting only on peptide bonds which are adjacent to a free α-amino or a free carboxyl group and therefore restricted generally

to hydrolysis of small peptides. The distribution of proteolytic enzymes between mycelium and culture fluid is conditioned both by age (Lahoz et al., 1976) and by the medium (Morihara, 1964; Kreger & Griffin, 1974) and the transport of free amino acids between the medium and the fungal cell interior (amino acid pool) occurs in both directions across the cell membrane. There is good evidence that some or all amino acids enter the cell by an active transport mechanism requiring an expenditure of metabolic energy (Christensen, 1955; Fry, 1955).

Lipases are primarily intracellular, but they also appear in the medium late in the culture cycle (Stern et al., 1954). The amount of extra-cellular lipase formed by Mucor muceds is influenced by the temperature of cultivation and is increased by the inclusion of a lipid in the growth medium (Stern et al., 1954).

There is no fixed relationship between incubation period and extracellular enzyme yield, it varies with the organism; enzyme and growth conditions. For most extracellular enzymes production more or less parallels growth, though there may be small differences in the times at which different enzymes appear. Maximum enzyme yield is usually reached at about the time that growth ceases, provided the medium does not become deficient in substances such as inducers which are specifically required for synthesis of the enzyme but not for growth. If incubation is continued beyond the time when growth ceases, enzyme yield may increase, remain constant, or decrease, probably through digestion by proteases produced as extracellular enzymes or liberated through cell autolysis (Rainbow & Rose, 1963;

Brunner et al., 1971; Reyes and Byrde, 1973; Lahoz et al., 1976; Reyes et al., 1980).

The first sign of UDN is the invasion of fish skin by the fungus causing local lysis around fungal hyphae. This is likely to be mediated by fungal proteolytic or lipolytic-enzyme activity (Peduzzi et al., 1976; Peduzzi and Bizzozero, 1977). It is, therefore, important to investigate the production of these enzymes by Saprolegnia species to see how the fungus can utilize materials in fish tissue.

3.2 Materials and Methods

3.2.A. Amino acid release

Aliquots of 20ml 2% casein or 1% casein/1% glucose media were dispensed into 100ml Erlenmeyer flasks. The flasks and media were sterilised at 120°C (15p.s.i.) for 15 minutes and allowed to cool before inoculation with a mycelial disc of S. diclina or S. ferax. Inoculated flasks were maintained at either 10°C or 25°C and incubated for up to 7 days. Flasks were harvested at regular intervals and the dry weight of the resulting biomass assessed in the manner described in chapter 2. The concentration of tyrosine in each filtrate was determined as follows. Aliquots (1ml) of filtrate were mixed with 1ml of 5% trichloroacetic acid (TCA) and the resulting white precipitate removed by filtration. The concentration of tyrosine in the filtrates was subsequently determined by the method of Lowry et al. (1951) with reference to a standard curve of tyrosine, Fig. 10 (Appendix 2).

3.2.B. Extracellular protease and lipase activity

Flasks containing 100ml of sterile media were made up in the following formulations:-

1) 2% glucose

2) 2% casein

3) 1% glucose/1% casein

4) 1% casein/1% lipid

5) 1% glucose/1% lipid

6) 0.66% glucose/0.66% casein/0.66% lipid

7) 2% lipid.

Each flask was inoculated with a mycelial disc of either S. diclina or S. ferax and incubated at either 10°C or 25°C for 96 hours. Flacks were harvested and the dry weights of fungal biomass determined as previously described (chapter 2). Protease activity of filtrates was determined by the method of Kunitz (1946, 1947) as subsequently modified by Wretlind and Wadstrom (1977). Filtrate aliquots of 0.2ml were added to a 4ml formulation of casein (10g casein/1mM CaCl in 0.05 M sodium phosphate buffer pH 7.4) (Appendix 2) and maintained at 37°C. Samples were subsequently incubated at 37°C for 30 minutes before the reactions were terminated by the addition of 3mls perchloric acid. The resulting precipitate was removed by centrifugation at 3000 rpm for 15 minutes. Absorbance of the clear supernatants was determined at 280nm against a blank of sodium phosphate buffer. Protease activity was assessed by comparison of filtrate absorbance with the absorbance of casein solutions incubated with known concentrations of trypsin, Fig. 11 (Appendix 2).

Lipase activity was determined by the method diescribed by Staeudinger et al. (1973) but modified by Wretlind et al. (1977). Filtrate aliquots (0.1ml) were mixed with 0.1ml of a solution of p-nitrophenyl carpylate; 7mM, in dimethyl sulphoxide with 1.0ml of 0.1M phosphate buffer pH 7.4 (Appendix 1) and incubated for one hour at 37°C. Lipase activity of the mixture was determined by measurement of the OD of the mixture at 410nM and reference to a standard curve of known amounts of lipase in equivalent reaction mixtures (Appendix 2).

3.3 Results

Proteolytic activity, as demonstrated by the amount of tyrosine released from casein, is summarised by Figs. 13 & 14 (Tables 6 & 7, Appendix 2). S. diclina appears to show most proteolytic activity per unit weight when incubated at 10°C, whatever medium is employed. In addition to the significant effect of temperature on total proteolytic activity, temperature also alters the periods over which greatest proteolytic activity appears to occur. At 25 C a sharp increase in tyrosine release may be observed after only 12 hours followed by an equally marked decline in activity. However, when cultures were incubated at 10 C the tyrosine release is again dramatic but is maintained at or near the maximum for up to three days before a more gentle decline in activity is observed.

Fig. 14 shows tyrosine release by colonies of S. ferax incubated at 10°C and 25°C. In contrast to S. diclina, S. ferax shows a greater release of tyrosine at both temperatures, furthermore S. ferax appears to demonstrate greater proteolytic activity at 25°C. Profiles of proteolytic activity also differ considerably between S.

diclina and S. ferax as the latter demonstrates maximal tyrosine release after only 12 hrs/1 day with a marked decline thereafter.

Extracellular protease activity mediated by S. diclina and S. ferax is illustrated by Figs. 15 & 16 (Table 8, Appendix 2). In common with previous observations, S. diclina demonstrates greatest release activity when maintained at 10°C, however, the presence of lipid in the medium can alter enzymatic activity as S. diclina demonstrates a marked reduction in proteolytic activity in the presence of lipid. S. ferax shows protease activity which, in common with S. diclina, is markedly reduced by the presence of lipid. Furthermore, as with S. diclina, greatest protease activity occurred in S. ferax cultures after four days of incubation at 10°C.

Lipase production by S. diclina and S. ferax is summarised in table 9 (Appendix 2) and Figs. 17 and 18 respectively. S. diclina demonstrates greatest lipase activity when maintained at 10°C, particularly in the presence of either casein or to a lesser extent, lipid. In common with S. diclina, four day old cultures of S. ferax demonstrate marginally greater lipase activity when maintained at 10°C, particularly in the presence of either casein or to a lesser degree, lipid.

3.4 DISCUSSION

The liberation of tyrosine in the growth media indicates that the fungus can break down protein by releasing proteases. The resulting amino acids present in the media may be consumed to satisfy the nitrogen requirements of the fungus; act as a carbon and energy source (Gareth-Jones, 1976); and may also act as a source of sulphur

since particular amino acids such as cysteine, cystine, glutathione or methionine are rich in sulphur and readily assimilated by fungi (Webster, 1980).

It is clear from figs. (13 & 14) that S. diclina and S. ferax are producing high levels of proteases at the beginning of the experiment. This may be due to the fact that these enzymes are involved with the initial stages of breakdown of growth substrates, and growth cannot occur until such enzymes have begun to function. The presence of free amino acids in culture medium, as a result of breakdown of casein by proteases during growth, was noted by Rao & Venkataraman (1952) for Penicillium chrysogenum. The authors found accumulation of amino acids at first, followed by their utilization during later stages of growth. The same process may be seen in this investigation of S. diclina and S. ferax after four days of incubation.

In general, a fungus produces much more of a given enzyme if cultivated on the substrate (inducer) of the enzyme. Therefore, the stimulation of protease enzyme production when the fungus grows on 2% casein medium, may be due to the inducer function of this protein. Since fish tissue contains a high level of protein, the action of the fungal proteases may be important in the process of colonization of fish skin (Pickering and Willoughby, 1982) and eggs (Smith et al., 1985). Disruption of the physical integrity of host cells or the lysis of cells by enzymatic activity (Cooke, 1977) may be important in releasing amino acids for use by the fungus. These amino acids then enter the internal pool which serves as a source for protein synthesis (Ainsworth & Sussman, 1965).

The production of enzymes requires energy and for most micro-organisms this is usually supplied in the form of metabolizable carbohydrates. As well as supplying energy, the carbon source may also fulfil the role of inducer for inducible enzyme production. In the present studies (on 1% glucose and 1% casien medium) glucose may also act as an energy source for enzyme synthesis (Vincent, 1958).

The amino acids released from the breakdown of casein by extracellular enzymes are utilized by the fungus and transported to the internal pool in the cytoplasm of the cell through the fungal cell membrane. Therefore, it is worth noting that the amount of tyrosine released and measured above is not an adequate way to evaluate the actual protease activity, since the fungus can utilize some of that tyrosine released into the growth media (Burnett, 1976). It was recorded by Pyle (1954) that the amino acids content of a medium drops as the acids are taken up by the fungal cells, and then rises again as they are liberated during autolysis. The extracellular assays carried out above should more clearly demonstrate the activity of the proteases and lipases enzymes as these assays are carried out in the absence of fungal biomass.

The formation of some enzymes is inhibited under conditions where glucose is present in the growth medium (Magasanik et al., 1959). In this investigation, the ability of S. diclina and S. ferax to produce extracellular proteases and lipases is greatly influenced by glucose which inhibits the enzymatic activity in the growth media. This phenomenon is termed the "glucose effect" which is the old term of the new name "Catabolite repression" (Keen & Horton, 1966; Patil & Dimond, 1968; Moran & Starr, 1969; Spadling et al., 1973; Weinhold

& Bowman, 1974). The glucose effect was first defined by Epps & Gale (1942) who observed that the formation of amino acids deaminases was inhibited by glucose, and by Monod (1947) who discovered that glucose prevents the formation of enzymes essential for the degradation of other sugars. Proteases were among the first enzymes whose formation was reported to be inhibited by the presence of fermentable carbohydrate in the medium. They showed that the acidity developed in the medium as a consequence of glucose fermentation, could account for the diminished production of proteases, (Cohn & Monod, 1953; Horton & Keen, 1966). Another hypothesis is that during growth on glucose the formation of glucose-degrading enzymes prevents the formation of other enzymes (Spiegelman et al., 1955). Still another hypothesis proposes that, the metabolism of glucose lowers the level of inorganic phosphate in the cell and thus prevents the synthesis of inducible enzymes (Englesberg, 1959).

When a fungus grows on the substrate (inducer) of a given enzyme, much more enzyme is produced, therefore, S. diclina and S. ferax produced more lipase enzymes when they were grown on 2% fat medium, figs. (17 & 18). These results agreed with the results of Stern et al. (1954) who stated that the amount of extracellular lipase formed by Mucor mucedo is increased by inclusion of lipid in the growth medium.

Fig. (13) Effect of temperature on tyrosine release by S. diçlina (mg/l/mg dry weight)

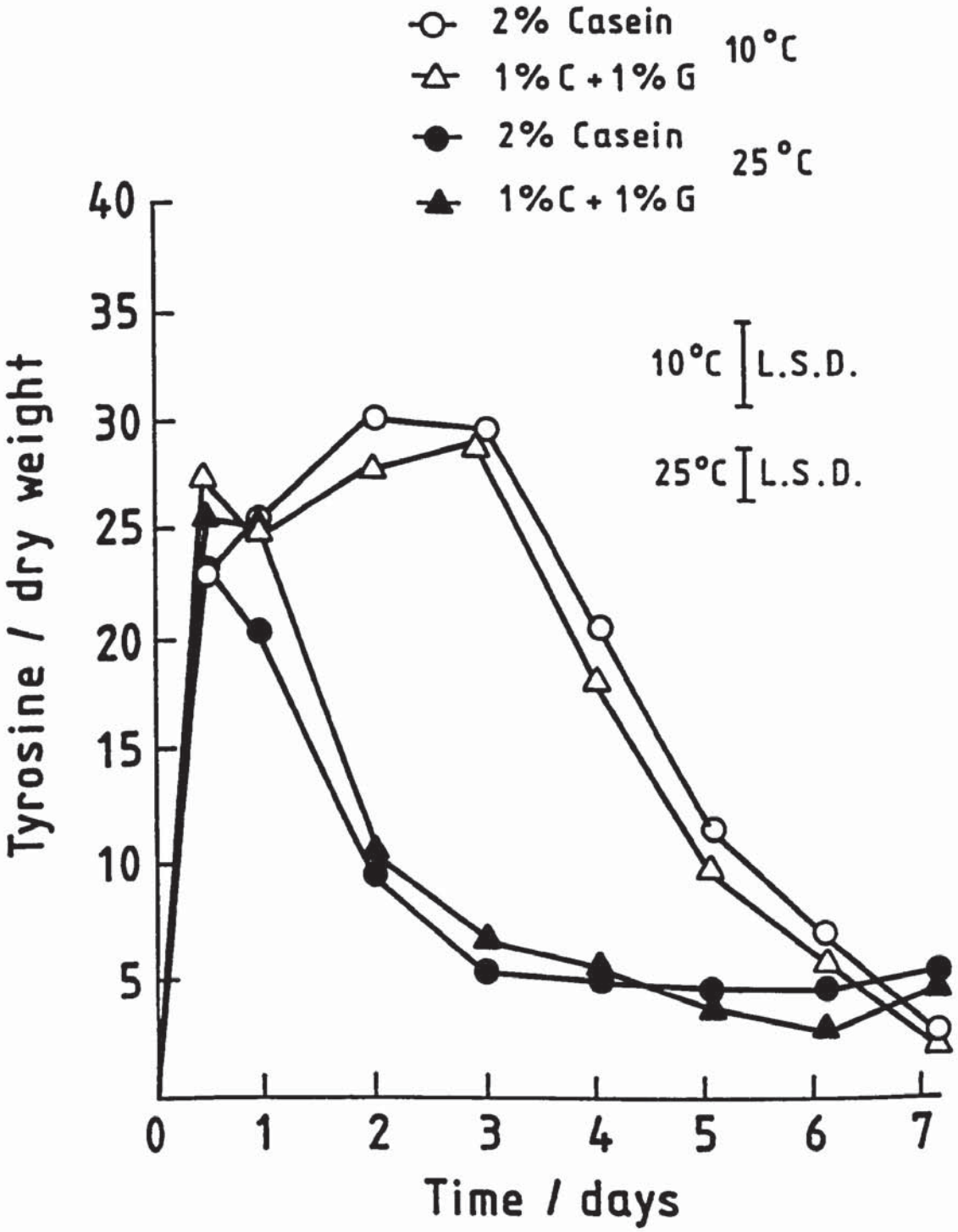

2% Casein
1%C + 1%G
10°C
2% Casein
1%C + 1%G
25°C
10°C L.S.D.
25°C L.S.D.
Tyrosine / dry weight
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
Time / days

Fig. (14) Effect of temperature on tyrosine release by S. ferax (mg/l/mg dry weight)

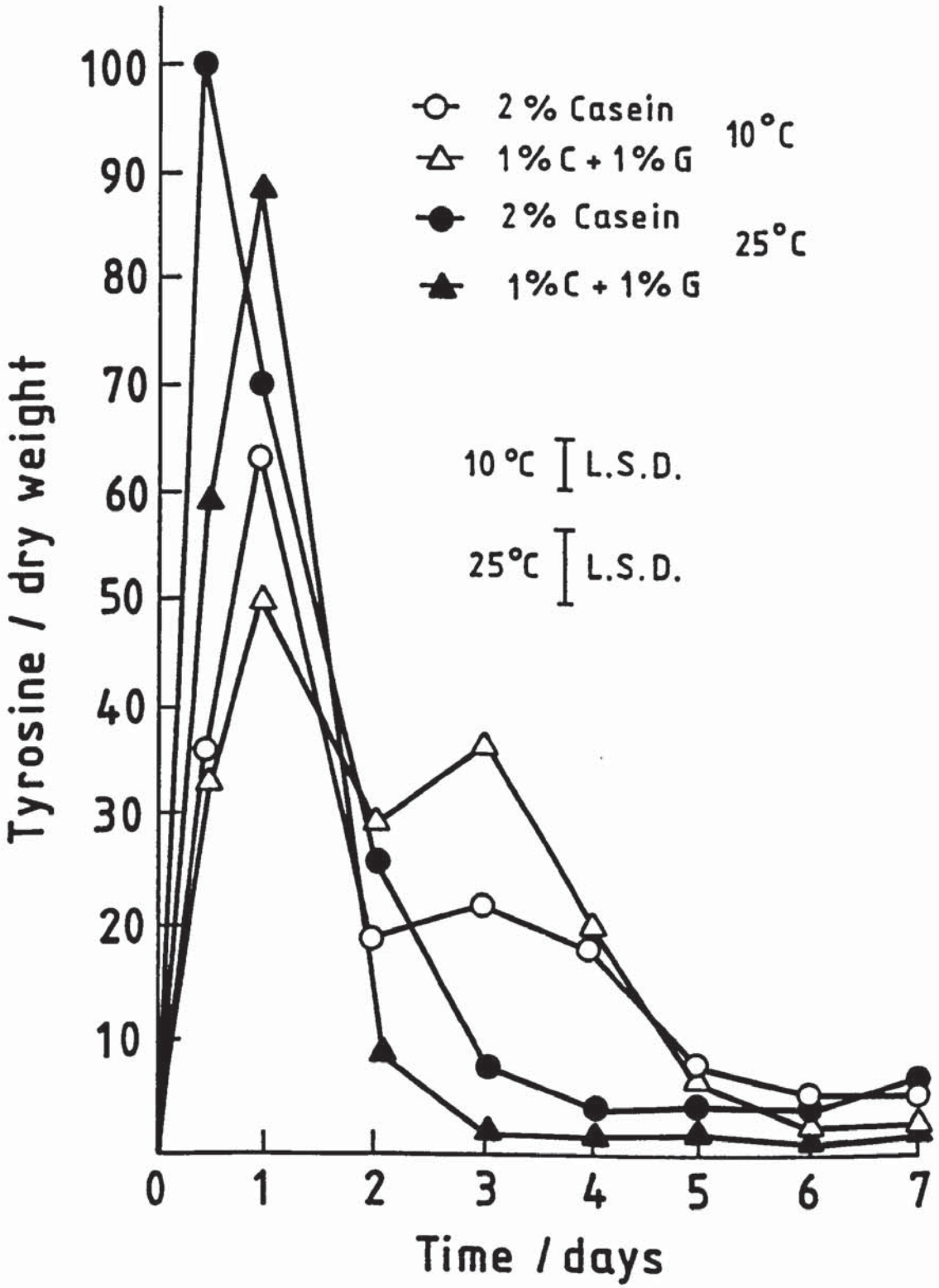

100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Tyrosine / dry weight
Time / days
2 % Casein
1%C + 1%G
10°C
2% Casein
1%C + 1%G
25°C
10 °C L.S.D.
25°C L.S.D.

Fig. (15) Effect of substrate on extracellular protease activity of *S. diclina* after four days of incubation

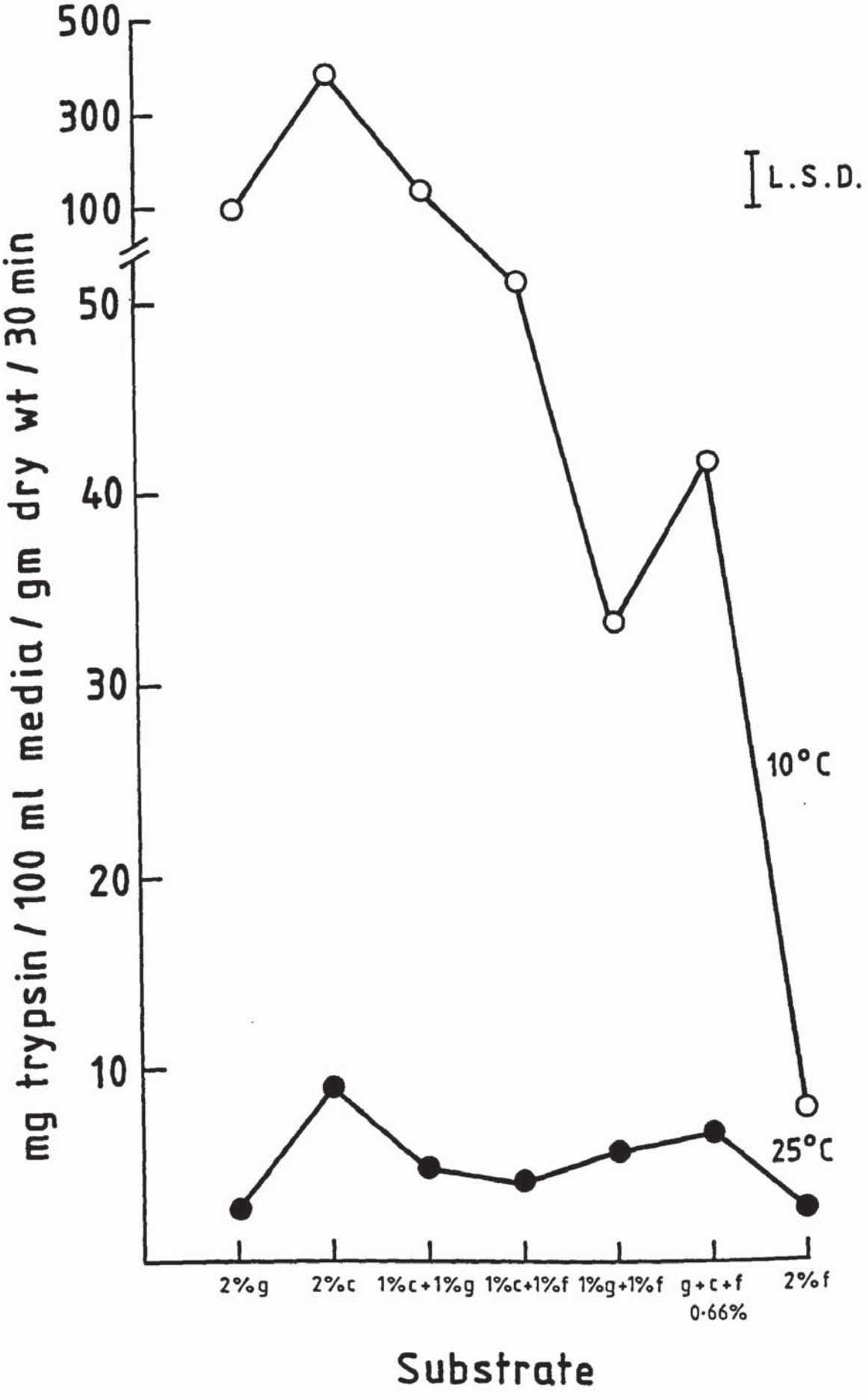

500
300
100
50
40
30
20
10
mg trypsin / 100 ml media / gm dry wt / 30 min
L.S.D.
10°C
25°C
2%g
2%c
1%c+1%g
1%c+1%f
1%g+1%f
g+c+f 0·66%
2%f
Substrate

Fig. (16) Effect of substrate on extracellular protease activity of S. ferax after four days of incubation

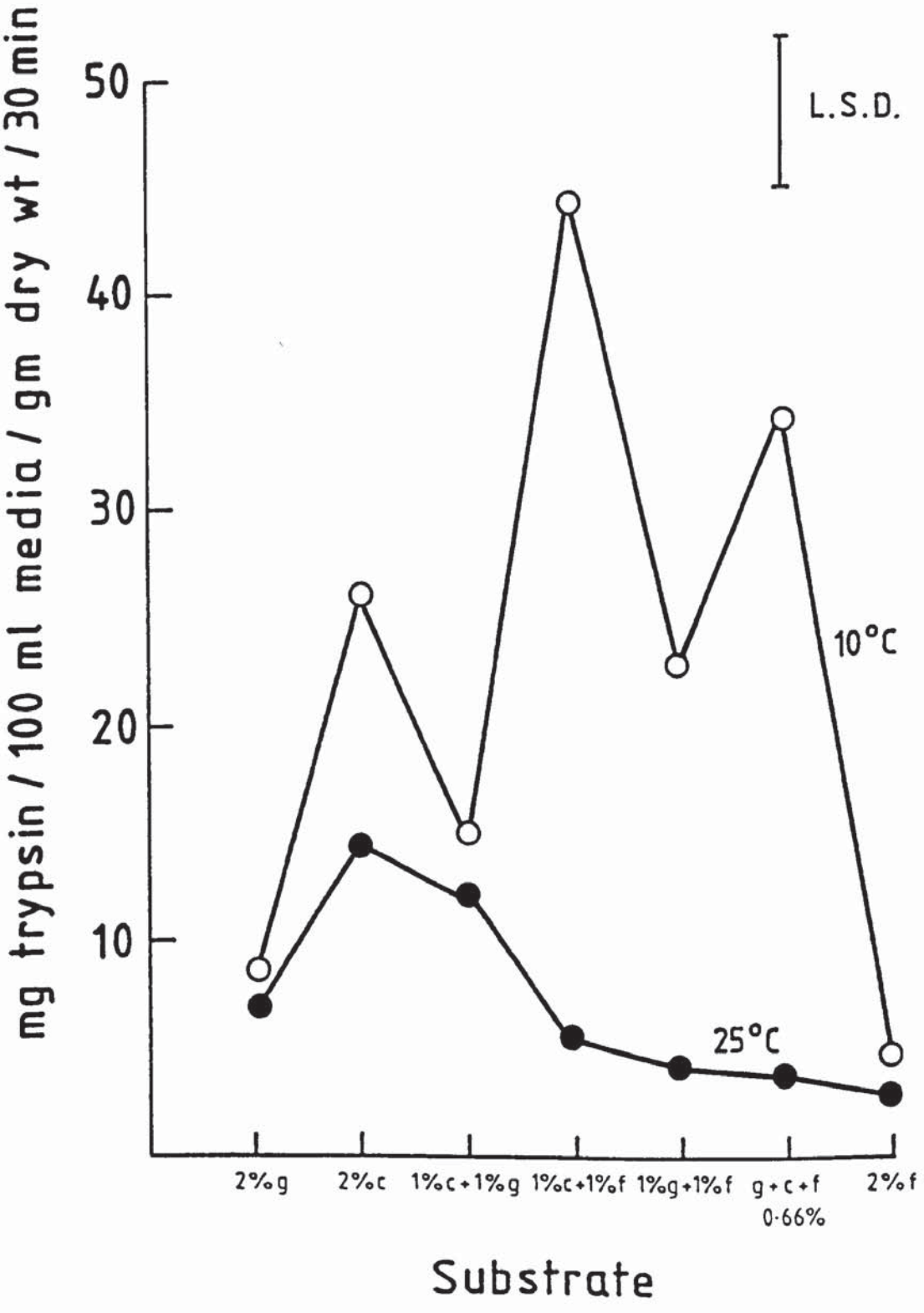

mg trypsin / 100 ml media / gm dry wt / 30 min
50
40
30
20
10
L.S.D.
10°C
25°C
2%g
2%c
1%c+1%g
1%c+1%f
1%g+1%f
g+c+f 0·66%
2%f
Substrate

Fig. (17) Effect of substrate on extracellular lipase activity of S. diclina after four days of incubation

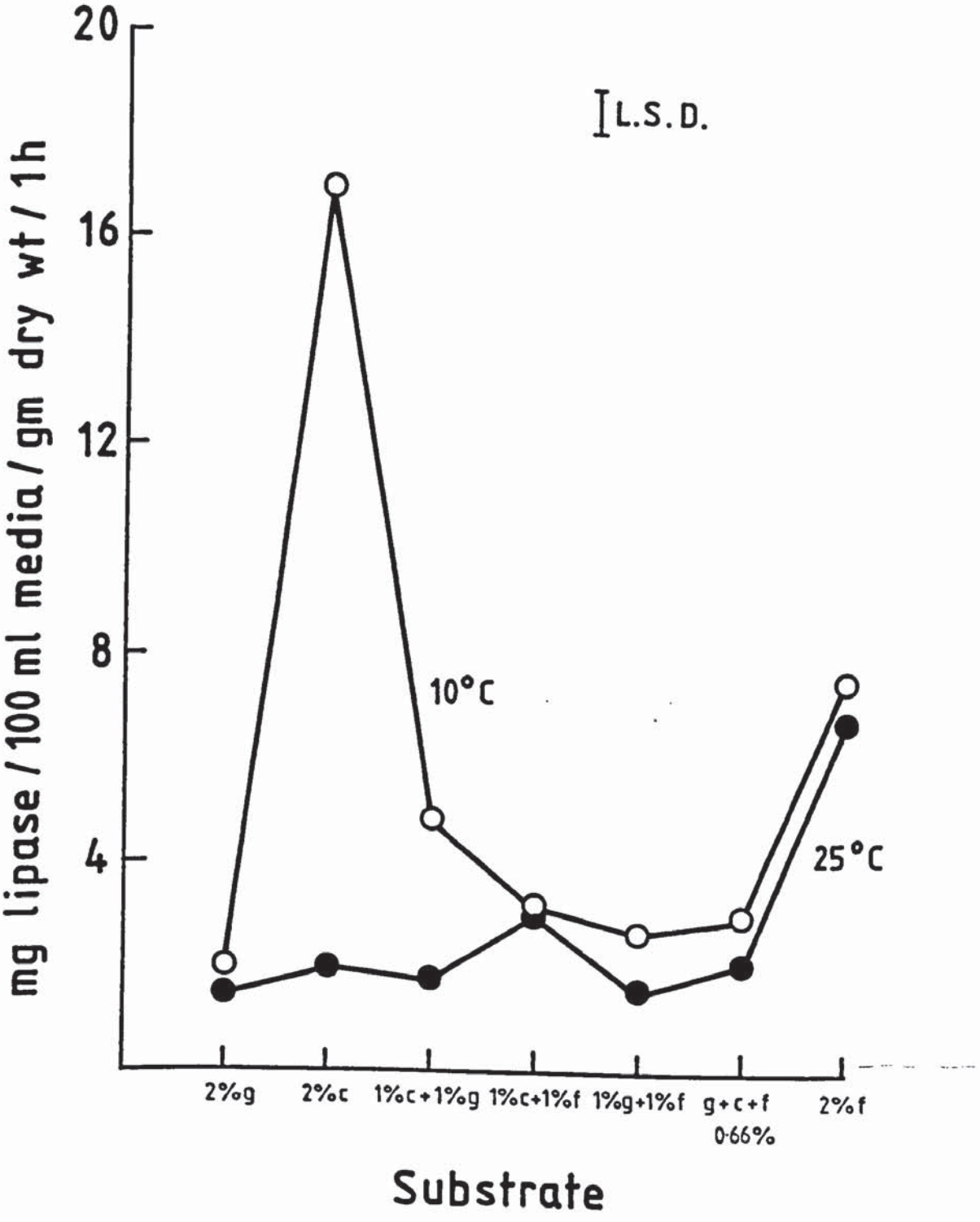
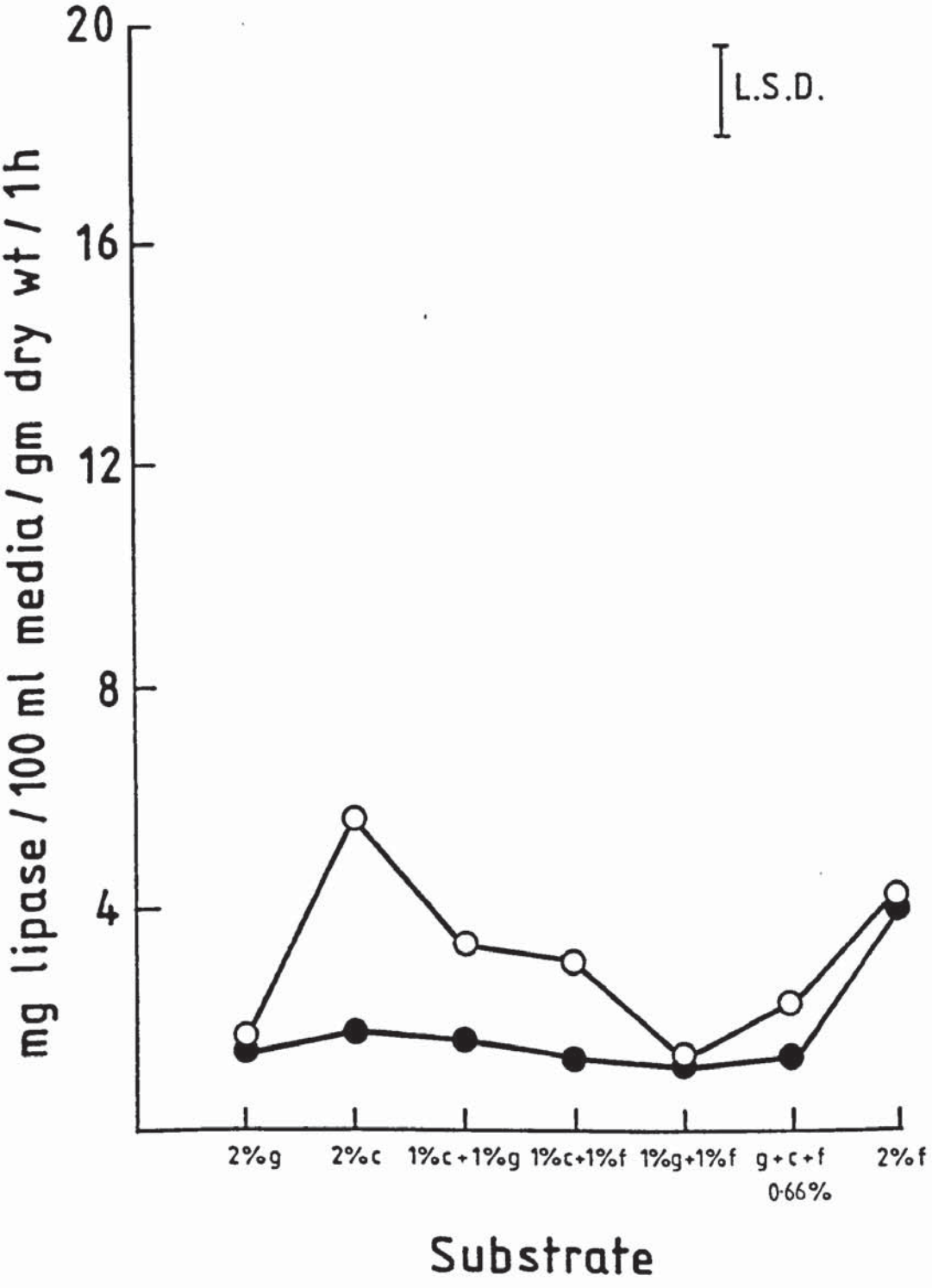

20
16
12
8
4
mg lipase / 100 ml media / gm dry wt / 1h
L.S.D.
10°C
25°C
2%g
2%c
1%c+1%g
1%c+1%f
1%g+1%f
g+c+f 0·66%
2%f
Substrate

Fig. (18) Effect of substrate on extracellular lipase activity of S. ferax after four days of incubation


20
16
12
8
4
mg lipase / 100 ml media / gm dry wt / 1h
L.S.D.
2%g
2%c
1%c+1%g
1%c+1%f
1%g+1%f
g+c+f 0·66%
2%f
Substrate

CHAPTER IV

THE EFFECT OF TEMPERATURE ON THE INFECTION OF CARP BY SAPROLEGNIA AND THEIR IMMUNE RESPONSE TO FUNGAL ANTIGEN

4.1. Introduction

It has been known since the early studies of Metchnikoff that fish are capable of initiating an immune response to recognizably foreign material (Metchnikoff, 1892, 1901, 1905; Mesnil, 1895; Noguchi, 1903). During the past decade considerable interest in the immune mechanisms of bony fish (teleost) has been generated. It was soon discovered that fish provided valuable experimental animals for study by comparative immunologists, where work has contributed significantly to an understanding of the ontogenetic and phylogenetic origins of the vertebrate immune system (Good & Papermaster, 1964; Smith et al., 1966; Manning et al., 1981; Turner & Manning, 1983; Sizemore et al., 1984; Miller et al., 1985; Gilberston et al., 1986). Much of the earlier work had been concerned with empirical attempts to produce immunity to infectious diseases but without elucidating the basic processes involved (Klontz & Anderson, 1970; Snieszko, 1970). The importance of further information on the factors influencing susceptibility of fish to infection and the economic importance of disease control in relation to fish culture have recently been emphasized by Klontz (1972b). Diseases of fungal aetiology have long been recognized in fish. Major problems often occur in identifying the fungus responsible and in determining whether it is truly pathogenic or simply a saprophyte taking advantage of an existing lesion. The

literature of fungal disease in aquatic animals is widely spread. A number of reviews have been published, but there has been an inevitable tendency either to concentrate on aquatic vertebrates (Wolke, 1975; Neish and Hughes, 1980) or to limit consideration either to marine (Alderman, 1976) or freshwater hosts only. The fungi reported as being responsible for or associated with diseases of aquatic animals belong to a wide range of classes. The most frequent in all types of host and environment are the so-called water moulds - the Oomycetes. Members of the order Saprolegniales have been found to be of particular significance in infections of fish. In this section, therefore, attention is focussed upon the immune response of fish to infection by Saprolegnia sp.

4.1.A. Fish immune system

Fish are the first group of animals in which an immune system characterized by the presence of immunoglobulins occurs. The immune system of fish is therefore considered to be of great importance in phylogenetic studies of antibody structure and function. The immune response of poikilotherms - including fish - is dependent upon environmental temperature and thus offers a unique opportunity to study the possible manipulation of immunity by variations in environmental temperature. A better understanding of the effects of temperature on the immune system of fish may aid in prophylaxis and therapy of fish diseases in fish culture.

The fish is able to protect its body from potentially harmful micro-organisms in the environment by a number of very effective mechanisms, present from birth, which do not depend upon having

previous experience of any particular micro-organisms. This innate immunity is non-specific in the sense that it is effective against a wide range of potentially infective agents. Micro-organisms which overcome the innate non-specific immune mechanisms come up against the host's second line of defence, the adaptive immune system. These two protective systems are now reviewed.

4.1.A.a. Innate immunity in fish

4.1.A.a.i Phagocytosis

Fish are probably almost entirely dependent on non-specific immunity during the period of free-living larval existence before their immune system has matured. Passive immunity transferred from the mother to the fry, has not been generally demonstrated in fish, although recently Van Loon et al. (1981) have demonstrated maternal immunoglobulin in the ova of carp. Phagocytosis has been studied in carp at 2 weeks of age (Grace et al., 1980) and is carried out by macrophages in the kidney and spleen which rapidly localize the largest amounts of phagocytosed material.

Various morphologically distinct types of cells have been described as being phagocytic in teleosts, with monocytes, macrophages and neutrophils all possessing phagocytic capacity (Ellis, 1976, 1977a; Boomker, 1981; Braun-Nesje et al., 1981; MacArthur et al., 1984). In addition to these circulating blood cells, blood thrombocytes have also been described as being phagocytic for carbon in plaice, (Ferguson, 1976b) and for bacteria in coho salmon (Lester & Budd, 1979).

The process of phagocytosis can be separated into several stages: opsonization of the particles by serum factors, attachment of the opsonized particles to the cell surface, engulfment of such particles, intracellular killing of micro-organisms, and digestion of micro-organisms and other ingested matter. Phagocytosis is facilitated by opsonins, ie. serum components such as immunoglobulin IgM and complement C3. The attachment of opsonized particles to a phagocytic cell via a receptor on the cell membrane induces the formation of pseudopods, which surround the particle while the binding of adjacent receptors to opsonins on the particle occurs (Griffin et al., 1975, 1976). Engulfment is completed when the pseudopods surrounding the particle meet and fuse to form a phagocytic vacuole or phagosome, whose wall is formed by the inverted cell membrane. This is followed by a variety of biochemical events which lead to the intracellular killing of micro-organisms (Rossi et al., 1972). Digestion of micro-organisms is established by acid hydrolases, eg. acid phosphatase, ribonuclease, deoxyribonuclease, β-glucuronidase and cathepsin, which are capable of digesting the ingested materials (De Duve & Wattian, 1966).

4.1.A.a.ii Melano-macrophage centres

Recent studies (Secombes et al., 1982a) have shown that in fish, as in homoiotherms, immune complexes (composed of antibody and antigen) are trapped in the lymphoid organs to form the melano-macrophage centres towards which macrophages containing ingested particles migrate (Ellis & De Sousa, 1974). It has been suggested in these reports that the melano-macrophage centres may be functionally analogous to the germinal centres of higher vertebrates

where the localization of antigen on the surface of dendritic cells has been implicated in the production of B memory cells (Klaus, 1978); feedback inhibition of antibody production (Stockinger et al., 1979); the switch from 19S to 7S immunoglobulin (Ig) production and increased affinity for the antigen (Blythman & White, 1977).

4.1.A.a.iii. Mucous

The fish epidermis and its layer of mucous forms an immediate interface between the internal tissues of the fish and its environment. It is generally assumed that the mucous layer somehow acts as a physical barrier to the colonization of the skin by potential pathogens. Willoughby & Pickering (1977) have shown the Saprolegnia diclina Type 1 spores can readily adhere to the surface of the brown trout and the char and that the vast majority of these spores are removed or inactivated during the first 24 hours of subsequent quarantine in clean water. It would appear that the continuous secretion of mucous by the goblet cells of the salmonid epidermis acts as an extremely effective, physical-cleansing mechanism thereby removing small particles (including potentially pathogenic fungal spores) from the surface of the fish.

The antibiotic properties of certain teleost mucous secretions have been known for a considerable period of time (Nigrelli, 1935) and more recently evidence of a range of potential antibiotic molecules in the mucous layers of fish has been reported. Fletcher & Grant (1968) and Fletcher & White (1973b) identified lysozyme in the mucous secretions of the plaice Pleuronectes platessa and in

another study (Fletcher & Grant, 1969), they induced specific haemagglutinins in skin mucous following parental administration of human erythrocytes. Antibodies have also been found in the mucous of the gar Lepisosteus platyrhincus (Bradshaw et al., 1971) and the cat fish Tachysurus australis (Di Conza, 1970). Specific agglutinin formation in the body mucous has been demonstrated following parental immunization with Vibrio anguillarum in the plaice (Fletcher & White, 1973a) and in rainbow trout Salmo gairdneri (Harrell et al., 1976).

4.1.A.a.iv Sialic acid

Sialic acids have been isolated from the mucous of many vertebrates, including the teleost fishes (Enomoto et al., 1964), and the concentration of n-acetyl neuraminic acid has been used as an alternative method for estimating the degree of mucification of the skin of the European eel, Anguilla anguilla (Lemoine & Olivereau, 1971). The mucous of the brown trout Salmo trutta L. and char Salvelinus alpinus L. was found to contain one sialic acid, n-acetylneuraminic acid (NANA), and the concentration of NANA in the epidermus was found to be directly proportional to the mucous cell concentration (Pickering, 1974).

4.1.A.a.v. Haemagglutinins and haemolysins

Natural haemolysins for heterologous erythrocytes were first reported in fish serum by Liefmann (1911). Haemagglutinins for various species of foreign erythrocyte have also been described by Hodgkins et al.(1967). The biological significance of these factors is not clear.

4.1.A.a.vi. Lysozyme

Lysozyme is an enzyme with bactericidal activity which is present in serum, mucous and phagocytic cells of many fish species (Luk'Yanenko, 1965; Fletcher & Grant, 1968; Fletcher & White, 1973b; Ourth, 1980). The molecular weight of fish lysozyme (15×10^3) is similar to that of mammalian lysozyme, the different electrophoretic mobility reflects differences in amino acid composition (Rijkers, 1982b).

Variations in lysozyme activity between individual members within one species are considerable. In general, serum lysozyme activity in carnivorous fish like pike and perch is higher than in omnivorous species like carp (Luk'Yanenko, 1965). After immunization of carp with Aeromonas punctata, the highest lysozyme activity was found to coincide with the serum antibody peak (Vladimirov, 1972).

Using immuno-histochemical techniques lysozyme activity may be demonstrated in monocytes and neutrophils (Murray & Fletcher, 1976). These cell types probably contribute to the serum lysozyme activity since the number of monocytes and neutrophils increases with serum lysozyme levels after intravenous injection of latex beads (Fletcher & White, 1973b).

4.1.A.a.vii. Complement

Complement is a group of heat labile serum components involved in both specific and non-specific immune defence. In mammals,

complement is activated by antibody/antigen interaction and is involved in a range of protective activities including lysis of foreign cells, promotion of phagocytosis, chemotaxis and inflammation (Lachmann, 1979). Its precise range of functions in fish remains unclear but it is certainly involved in lysis of foreign cells such as heterologous erythrocytes (Corbel, 1975). Fish complement is not restricted to the serum. In rainbow trout a heat labile anti-Vibrio activity was observed in mucous, presumably the result of complement and antibody acting together (Harrel et al., 1976).

4.1.A.a.viii. Transferrin

Transferrin in its many forms is the main iron transporting protein in animals and plants. In its unsaturated form, it inhibits the growth of micro-organisms by depriving them of the iron they need (Emery, 1980). Transferrin and related proteins have been found in the serum of all vertebrates and mucous secretions (Putnam, 1975).

4.1.A.a.ix. Interferon

This group of proteins interferes with the replication of viruses by stimulation of the synthesis of enzymes which interfere with RNA translation and protein sheath formation (Friedman, 1981). They also increase the activity of macrophages and natural killer cells.

Interferon has been found in fish, but only one class has been identified (Galabov, 1973) which probably belongs to either, the α-interferon and β -interferon classes.

4.1.A.a.x. C-reactive protein

C-reactive protein (CRP) is a protein which appears in mammalian serum during the acute phase of infection by micro-organisms. CRP binds to phospholyl choline residues which are present in cell wall glycopeptides of various bacteria, fungi and parasites. CRP can cause agglutination, precipitation and complement activation (Siegel et al., 1974; Pepys et al., 1980) and has been recorded in fish (Baldo & Fletcher, 1973). CRP in plaice is not an acute phase protein but a normal serum constituent which may provide the animal with a permanent line of defence against invading micro-organisms.

4.1.A.a.xi. α -Precipitins

In the sera of all healthy vertebrates there are a collection of proteins which react predominantly with carbohydrates. These have been identified electrophoretically as α and β -globulins. The α-precipitin is the one that has been most studied in fish (Alexander, 1976, 1980, 1982; Davies, 1975). It was first discovered by its reaction with extracts of UDN-infected salmon and was later shown to react non-specifically with extracts of fungi and bacteria.

4.1.A.b. Adaptive immunity in fish

When antigens (foreign molecular determinants capable of eliciting a specific immune response) on the surface of invading pathogens come into contact with cells of the fish immune system they result in the initiation of an adaptive immune response specific for the inducing antigen. In mammals two categories of lymphocyte are involved in adaptive immunity: B-lymphocytes are concerned in the synthesis of circulating antibody, while T-lymphocytes are responsible for cell-mediated immunity. Both populations proliferate following appropriate stimulation by antigen, and undergo morphological changes. The B-cells develop into plasma cells which actively synthesize and secrete antibody (humoral immunity). T-cells transform to lymphoblasts and then mature to form a variety of effector cells which do not secrete antibodies. However, they do elaborate a series of soluble factors (lymphokines) which act largely to regulate the immune response by influencing the activity of macrophages and other cells of the immune system. T-cells are also able to destroy foreign cells or infected cells by direct cell contact. This latter form of immunity is referred to as cell-mediated immunity (Roitt et al., 1985).

Fig. (19) The basic structure of IgG.

There are five immunoglobulins in mammals, IgG, IgM, IgA, IgD and IgE. The most common, IgG, is made up of two identical light chains of molecular weight 23,000 and two identical heavy chains of molecular weight 53,000. The structure of the IgG molecule represents the basic structural arrangement for the other classes of antibody. Each light chain is linked to a heavy chain by noncovalent associations and also by one covalent disulphide bridge. In the IgG molecule, the two light chain-heavy chain (L-H) pairs are linked together by disulphide bridges between the heavy chains. As shown in Fig.19, the molecule can be represented schematically in the form of a "Y" with the amino (N-) termini of the four chains at the top and the carboxyl (C-) termini of the two heavy chains at the

bottom. At each end of the arms of the antibody molecule are antigen binding sites. These are grooves or clefts formed between the variable (V) regions of the heavy and light chains. Variations in the amino acid sequence in the V region of different antibody molecules give rise to an almost infinite variety of binding sites against different antigens.

The biological activities of antibody are largely determined by the structure of the Fc-region (or tail) of the molecule. For instance, the Fc-portion of the IgG molecule appears to be the major component responsible for activating the complement system. Agglutination of particulate antigens (eg. bacteria) is brought about by the cross-linking of the two antigen binding sites of the antibody molecule. Antibody specific for particulate antigens such as bacteria plays a valuable role in defence by coating the surface and making the antigen more susceptible to phagocytosis. Macrophages and other phagocytic cells have receptors on their membrane for the Fc-region of the antibody molecule. This enhancement of phagocytosis by antibody is referred to as opsonization. Finally, soluble antigens such as toxins or extracellular products are precipitated by the cross linking activity of antibody molecules. This gives rise to molecular complexes which are more readily phagocytosed and degraded by phagocytic cells. These properties of antibody have been employed by immunologists to study their activities <u>in vitro</u> (eg. agglutination and precipitation reactions). A more detailed account of antibody structure and function is given in Roitt et al. (1985).

4.1.A.b.i. Humoral immunity

Antibodies have been demonstrated in many fish species, eg. in hag fish (Linthicum & Hildemann, 1970); nurse shark (Clem et al., 1967); paddle fish (Pollara et al., 1968) and in gar (Bradshaw et al., 1969, 1971). The striking feature of the teleost Ig is the presence of only one class. In terms of heavy chain mass, interchain disulphide bonding, amino acid and carbohydrate content, the Ig of teleost resembles mammalian IgM (Litman, 1975). The teleost IgM is mainly a high molecular weight molecule composed of 4 sub-units of 8 heavy and 8 light chains. It has a sedimentation coefficient of about 16S and approximate molecular weight of 700,000. Some species, for example, the plaice (Fletcher & Grant, 1969), and the margate (Clem & McLean, 1975) also possess a 7S monomeric form of IgM, while other species, such as salmonids and carp, only possess the tetrameric form (Shelton & Smith, 1970; Litman, 1975).

Fish antibody, in the presence of normal serum as a complement source, is capable of lysing foreign target cells (Rijkers et al., 1980). While agglutinating activity is readily demonstrated by fish, antibody precipitating activity is not always seen. Hodgkins et al. (1967) found rainbow trout IgM could detect passive haemagglutinating antibodies to foreign protein but precipitating activity was not detected. Clem & McLean (1975) found that the 16S IgM of the margate (Haemulon alluim) possessed high agglutinating activity to BSA.

An important function of antibody in mammals is its opsonic activity which is important in promoting phagocytosis of foreign cells. This is based on the steric change in conformation of the (Fc portion) of the antibody molecule on binding antigen and the subsequent binding of the antigen-antibody complex by (Fc) receptors on the surface of phagocytes enabling the complexes to be phagocytosed more readily. Recent studies by Wrathmell and Parish (1980) have been unable to find evidence of an opsonic role for fish antibody or the presence of Fc-receptors on fish phagocytic cells. Although Sakai (1984) found opsonizing activity associated with complement and antibody in salmonids.

The possible existence of a histamine-releasing function for fish antibody, an activity which is important in inflammation and allergic responses in mammals, has not been identified. The immunoglobulin (IgE) in mammals which is responsible for mediating the antigen-induced release of histamine from mast cells has not been identified in fish. But criteria for immune anaphylaxis have been reported in the gold fish where behavioural and physiological changes were induced by antigen-specific sensitization which could be transferred to normal fish by injecting serum from a sensitized individual (Goven et al., 1980).

4.1.A.b.ii. Cell-mediated immunity

T-lymphocytes are those which have passed through the thymus, or have come under the influence of thymic hormones, and effect the cell-mediated immune response.

One expression of cell-mediated immunity is the ability to reject grafts of foreign cells or tissues (allografts) from genetically unrelated donors. This phenomenon has been well studied in mammals and has been widely reviewed (McConnell et al., 1983; Roitt et al., 1985).

Graft rejection in fishes was reviewed by Rijkers (1982a) who described the cellular reactions occurring at the graft site as revascularisation of the graft, overgrowth by host tissue with vasodilation at points of graft-host contact, and invasion by lymphocytes and phagocytes. This suggests the presence of an effective cell-mediated immune system in fishes, and the probable presence of the products of a major histocompatibility system (MHS) on the surface of fish cells. Details of the vertebrate MHS have been reviewed by Cohen & Collins (1977).

In mammals, cell-mediated immunity is known to be important in the destruction of virally infected cells and also in the immune response against fungal antigens (Nahmias & O'Reilly, 1981). One way of monitoring a cell-mediated immune response *in vitro* is to measure lymphokine production by activated T-lymphocytes. Some reports suggest that fish lymphocytes also behave like mammalian T-lymphocytes in the production of lymphokines. Factors affecting the migration of leucocytes and induction of leucocyte chemtaxis have been reported by Secombes (1981). Fishes are also capable of exhibiting other cell-mediated immune phenomena including stimulation and transformation of lymphocytes by mammalian T-cell mitogens, and *in vitro* mixed lymphocyte reactions (MLRs) which are

also associated with the presence of MHS coded molecules (Klein, 1977).

4.1.A.c. White blood cells of fish

The types of white blood cells involved in fish immune responses have been studied by the light and electron-microscope and there is a close morphological resemblance between the lymphocytes, mononuclear phagocytes (monocytes & macrophages), granulocytes (neutrophils), eosinophils, and basophils of fish and mammals (Ellis, 1977a).

Lymphocytes are small round cells with a large nucleus and a small rim of basophilic cytoplasm (Weinreb, 1963; Davina et al., 1979). They have been arbitrarily divided into the categories of large and small lymphocytes, though these may represent different functional states of cells in one population rather than different functional capacities of separate populations. The small lymphocytes are the precursors of the large lymphocytes; the nucleus of the latter tends to be eccentric; occupying about 3/4 of the cytoplasm; the nuclear chromatin stains dark purple and is fairly homogeneous in appearance. The cytoplasm is non-granular and stains dark blue. The small lymphocyte accounts for most of the lymphocyte population. It is a round cell, with a high nuclear/cytoplasmic ratio. The lymphocyte nucleus is characterised by the lighter areas of inter-chromatic material (Blaxhall and Daisley, 1973). Weinreb (1963) described the presence of numerous mitochondria, rough and smooth endoplasmic reticulum, abundant ribosomes and Golgi vesicles in gold-fish lymphocytes. These structures are indicative of a

metabolically active cell, therefore, lymphocytes are highly differentiated cells which respond to immunological stimuli (Ellis, 1977a).

The nucleus of the monocyte varies from ovoid to horse-shoe shaped, the cytoplasm is slightly basophilic and it has been shown to have a phagocytic function (Thorpe & Roberts, 1972). The macrophage is a mononucleated cell with an undulating membrane and is derived from circulatory monocytes (Ellis, 1977a). The macrophage is characterised by its phagocytic activity (Ellis, 1976).

The nucleus of the neutrophil possesses several (2-5) lobes connected by fine strands of nuclear material (polymorpho-nuclear leucocytes). The cytoplasm contains fine colourless granules giving it a ground-glass appearance. In mammals the neutrophil plays a role in phagocytosis (Finn & Nielson, 1971); inflammation (Thorpe & Roberts, 1972) and in chemotaxis (Wilkinson, 1973).

The eosinophils usually have only a bilobed nucleus and many cytoplasmic vesicles and eosinophilic granules. They are also involved in phagocytosis (Watson et al., 1963); and inflammation (Jakowska, 1956). Basophils have a large eccentric nucleus and basophilic cytoplasmic granules. The function of basophils is not established. They are thought to contain about half of the histamine present in the blood, and may be involved in allergic and stress phenomena (Ellis, 1977a).

4.1.A.d. Lymphoid organs of fish

Spleen: The equivalent of the spleen in the primitive hagfish, Eptatretus stouttii, occurs in the form of an accumulation of haemocytopoietic cells in the gut tract (Good & Papermaster, 1964). The immunological significance of the spleen in the hagfish is as yet undetermined. In the lamprey, Petromyzon marinus, a primitive spleen is located in an ivagination of anterior gut tissue as a discrete structure containing haemocytopoietic tissue, and lymphoid cells (Finstad et al., 1964). In the elasmobranchs and higher vertebrates, the spleen is a discrete organ with erythropoietic and granulocytopoietic functions and contains accumulations of lymphoid cells. After antigenic stimulation the morphological equivalent of plasma cells are also detectable. Red and white spleen pulp is distinguishable in elasmobranchs, chondrosteans, holosteans and teleosts (Good & Papermaster, 1964).

In teleost ontogeny the spleen appears to be the last organ to become lymphoid and develops a lymphoid population about the time of first feeding (Ellis, 1977b; Van Loon et al., 1980; Grace et al., 1980). The arteries of the spleen are ramified into capillaries (ellipsoids) with thick walls composed of reticulin fibres and macrophages (Ellis et al., 1976). The ellipsoid capillaries open into red pulp sinuses which drain into the splenic venous system. The white pulp is rarely well developed in teleosts and contains reticulin fibres emerging from the ellipsoid sheath. Also enmeshed in these reticulin fibres are melano-macrophage centres which are belived to originate in the ellipsoid walls (Ferguson, 1976a; Secombes et al., 1982b).

Antibody-producing cells are present in the spleen (Rijkers et al., 1980). Aggregations of pyroninophilic cells (indicating protein synthesis) appear in the ellipsoid walls after antigenic stimulation (Secombes et al., 1982b).

The important role of ellipsoids is thought to be in phagocytosis and antigen-trapping. Colloidal carbon particles are rapidly phagocytosed by the ellipsoid macrophages which migrate into existing melano-macrophage aggregates or form new aggregates (Ellis et al., 1976). Bovine serum albumin (BSA) and human gamma globulin (HGG), as soluble protein antigens, are trapped as immune complexes on the reticulin fibres of the ellipsoid walls and persist for a long time (Ellis, 1980; Secombes & Manning, 1980; Secombes et al., 1980).

Thymus: The hagfish lacks (on morphological grounds) a definite thymus (Muller, 1871; Cole, 1906; Stockard, 1906; Good & Papermaster, 1964). Although in the lamprey a primitive epithelial thymus was described by Salkind (1915). In elasmobranchs, the thymus is epithelial during embryonic development, but lymphoid in adult life (Beard, 1894, 1900), and is divided into a cortex and medulla. The teleost thymus starts its development as a thickening of the epithelium in the dorso-anterior part of the pharynx without the distinct separation of any thymic epithelial buds as such. Moreover, instead of losing contact with the pharyngeal epithelium early in ontogeny, the teleost thymus retains this contact and is separated from the pharyngeal lumen by only a single layer of cells

(Grace et al., 1980; Manning, 1981). As in mammals, the teleost thymus is thought to be responsible for the production of T-lymphocytes.

The thymus of teleosts is an extremely superficial, paired organ, located immediately below the epithelium in the dorso-posterior part of the branchial cavity. It is the first organ to become lymphoid. Grace et al. (1980) who worked on rainbow trout (Salmo gairdmeni) thymus have found that the thymus is covered only by a single epithelial layer. The thymic cells are composed mainly of mitotically active and differentiating lymphocytes with small numbers of macrophages and epithelioid cells. There is little differentiation into cortex and medulla, and antibody producing cells are not present (Bogner & Ellis, 1977). No phagocytosis of foreign materials has been recorded in the thymus (Ellis et al., 1976; Ellis, 1980) and circulating lymphocytes do not migrate through the thymus (Ellis and de Sousa, 1974). Antigen-binding cells (ABC) were seen in the thymus of goldfish by Ruben et al. (1977), although antibody-secreting cells were not detected. The authors suggested that these ABC in the thymus may be T-helper cells which differentiated intra-thymically in the fish rather than extra-thymically as seen in mammals. T-helper cells in mammals are known to secrete soluble factors (lymphokines) which are important in the maturation of B lymphocytes into antibody secreting plasma cells (Singer & Hodes, 1983).

Anterior Kidney (Pronephros): In teleosts the kidney lies along the ventral sides of the vertebral column separated from the

body cavity by a tough connective tissue septum. The kidney of the fish is differentiated into two parts, the anterior or head kidney which loses its excretory function in the adult and becomes a lymphoid organ; and the mesonephros, which forms the bulk of the adult organ and contains the excretory tubules. Both parts of the kidney contain a generalized haemopoietic tissue, rich in lymphoid cells and granulocytes (Ellis and de Sousa, 1974; Ellis, 1976), which in some respects resembles mammalian bone marrow (Zapata, 1979). However, unlike bone marrow, the kidney haemopoietic tissue contains many antibody producing cells and phagocytes (Rijkers et al., 1980) and, following antigenic stimulation, many pyroninophilic cells appear, often in large clusters (Secombes et al., 1982b). Thus, the kidney tissue performs functions which are associated with both mammalian bone marrow and lymph nodes.

The kidney of the fish also contains pigmented cells. Most of these pigment-containing cells are macrophages, and Roberts (1975) has referred to them as melano-macrophages. Most of pigment is melanin but varying amounts of lipfuscin and haemosiderin are also present (Ellis, 1977a; Agius, 1979). The pigment-containing cells are either scattered randomly throughout the kidney tissue or aggregated as melano-macrophage centres (Agius, 1980). Small lymphocytes rich in RNA and antibody-containing cells are present within the melano-macrophage centres (Ellis and de Sousa, 1974).

4.1.A.e Factors influencing immunity in fish

4.1.A.e.i Temperature

Amongst the most significant factors affecting the immune response (both humoral and cell mediated) in fish is temperature (Hildemann, 1962; Avtalion, 1969, 1981; Avtalion et al., 1970, 1973, 1980). Generally speaking, the higher the temperature in the physiologically tolerated range, the faster the onset of the response and the greater its magnitude. At low temperatures there is either a prolongation of the induction period with low antibody titres being reached or a complete absence of a response. The critical temperature for development of an immune response in fish appears to be related to the natural environmental temperatures which relate to a particular species.

Shearer (1970) has carried out a detailed examination of the entire net catch on the North Esk (Angus) over the years 1967-70, which encompassed Ulcerative Dermal Necrosis (UDN) disease period on that salmon river. He showed that the highest numbers of fish with lesions occurred during the colder months decreasing with the approach of summer. Association of the UDN disease process with low temperature has also been mentioned by Carbery (1968a) in Ireland, and Stevenson (1970) in England.

4.1.A.e.ii Ascorbic acid

The influence of nutritional factors on the immune response of fish was reported by Halver (1972) who stated that ascorbic acid was essential for normal growth in salmonids, cyprinids and other types of fish. Ascorbic acid deficiency has been observed to result in

impairment of repairing tissue damage (Triplett & Calaprice, 1974; Ashley et al., 1975).

4.1.A.e.iii. Corticosteroids

The increase in corticosteroid production as a result of stress lowers the resistance of fish to infection (Wedemeyer and McLeay, 1981; Pickering & Pottinger, 1985). Elevated levels of plasma cortisol are associated with an increase in the susceptibility of brown trout (Salmo trutta) to infections of Saprolegnia and Aeromonas salmonicida (Pickering and Duston, 1983).

Little of the extensive literature on fish immunology is devoted to fungal immunity. Past studies on immunity to Saprolegnia have been largely restricted to salmonids, and have often been based upon naturally infected populations of wild fish (Hodkinson and Hunter, 1970; Roberts et al., 1972; Alexander, 1980; Davies and Lawson, 1982). As a result, much of the information concerning immune responsiveness to Saprolegnia is derived from fish with presumed past histories of infection, or from fish with pre-existing infections at the time of sampling. Serum precipitins against Saprolegnia-derived antigens have been demonstrated in salmon and trout (Hodkinson and Hunter, 1970; Roberts et al., 1972; Alexander, 1980; Davies and Lawson, 1982). However, the conclusions which may be drawn from such studies are necessarily limited. One problem in interpreting data obtained from wild fish is that the individual life histories of naturally infected fish are not known. We can, therefore, glean little information about the immunogenicity of Saprolegnia and under what conditions immunity to Saprolegnia may

develop. There is clearly a need for more precise information relating to this particular problem. A second problem in past studies has stemmed from the nature of infection. As discussed in an earlier chapter, Saprolegniasis is a vague term and Pickering and Willoughby (1977) have demonstrated that apparent Saprolegnia infections may contain several different species of aquatic fungi. Individual lesions may also contain bacterial infections. Some test antigens used in past studies have utilised homogenates of infected tissue from wild caught fish and, therefore, might be expected to contain antigenic components other than those derived from Saprolegnia (Hodkinson and Hunter, 1970; Roberts et al., 1972; Alexander, 1980; Davies and Lawson, 1982).

In addition to adaptive or specific antibody mediated immune responses to Saprolegnia, some authors have also described naturally occurring precipitins against fungal antigens in fish serum (Alexander, 1980, 1982; Davies and Lawson, 1982). However, such α-precipitins appear to be non-inducible, are non-specific (or at best, broadly specific) and have as yet no known effector function (eg. complement fixation, opsonisation etc). Their precise role, if any, in protection of fish against Saprolegnia infections remains in doubt (Ellis, 1985).

Clearly there is still much to be learned about the immune response of fish to Saprolegnia and other fungal pathogens. In examining the host-parasite interactions between Saprolegnia and carp, the aims of the present study have been to raise defined strains of Saprolegnia under laboratory conditions and to use these

both as a source of infective zoospores and as a controlled source of defined antigen. This has permitted the design of more carefully controlled investigations into the factors governing or influencing both the infectivity and growth of the fungus, and the host's immune response to it.

One important factor governing the incidence of Saprolegnia infections is thought to be temperature. As mentioned earlier, naturally occurring outbreaks of saprolegniasis are known to be associated with seasonal low temperature (Carbery, 1968a; Shearer, 1970; Norland-Tintigner, 1973). In studying the relationship between temperature and infection it is necessary to consider both the effects of temperature on different aspects of the fungal life cycle and (since fish are poikilothermic) the effects of temperature on the fish immune response (Suzuki and Hatakeyama, 1960; Hildemann, 1962; Shearer, 1970; Avtalion, 1981; Avtalion et al., 1970, 1973, 1980). Work presented in earlier chapters of this thesis has examined the effects of temperature on fungal growth and physiology, and optimal temperature ranges for the growth of S. diclina and S. ferax are described in Chapter 2. Furthermore, previous work in this laboratory has examined the effects of temperature on Saprolegnia zoospore production and motility (Smith et al., 1984). Work in the present chapter assesses the influence of environmental temperature on the incidence of Saprolegnia infection in carp. Potential differences in immune responsiveness of fish to fungal antigen are also examined in infected and antigen-injected fish held at different temperatures.

4.2. Materials and Methods

4.2.A. Fungal spore production and infection of fish

Four previously autoclaved hemp seeds (Cannabis sativa) were cut in half aseptically with a sharp razor blade and the halves placed, cut surface down, on the margin of four mycelial discs of a stock culture of S. diclina. These were maintained in approximately 15ml of sterilized tap water in a petri dish at 10°C for 48 hours to allow development of asexual structure (zoosporangia). The hemp seeds were detached from the mycelial discs and incubated at 10°C for 24 hours to produce abundant zoosporangia.

Sexually mature carp Cyprinus carpio (mean weight 150gm.) were maintained in small aerated aquaria (10 litres) and were fed daily with commercial dry pellets. Prior to the infection experiments, fish were divided into two groups of twenty, one kept at 10°C and the other at 20°C. Small injuries were made in the skin of all the experimental fish by scraping a few scales from the flank region of the body. The fish were then bathed three times with sterile water and placed in clean washed aquaria containing about 7 litres of clean tap water. Two fish were placed in each aquarium with two petri dishes, each containing ten hemp seeds covered with fungal mycelia. The petri dishes were covered with cheese-cloth to prevent direct contact between the fish and fungal mycelia. The hemp seeds were changed every week. Control groups of fish were kept at 10 and 20°C in aquaria free from fungal zoospores. Five infected fish were anaesthetized every month by immersion in 50 ppm. MS222 (Sandoz) for four minutes and the blood was collected by cardiac puncture. Samples from each of the five fish were used in each investigation.

4.2.B. Experimental design

To examine the effect of temperature on the incidence of infection by Saprolegnia diclina, groups of carp were exposed to infective zoospores at two different temperatures (10°C and 20°C) as described previously. Three separate studies were carried out, and in order to control for possible seasonal influences on infection (eg. photoperiod; hormonal status etc), experiments 1 and 3 were carried out during the summer months (May - August) whilst experiment 2 was performed in winter (November - February). Groups of control fish were kept under the same conditions as experimental fish, but were not exposed to zoospores.

The incidence of infection was recorded in each group, and at intervals post infection (see results section for details) a number of investigations were carried out. These are outlined below.

4.2.C. Confirmation of infection

Infection by S. diclina was confirmed by taking samples from four randomly selected fish each month. Samples were examined as described by Smith et al. (1984).

4.2.D. Haematological investigations

Blood samples were collected from infected fish at intervals throughout the study (see results for details). Samples were collected from anaesthetized fish using a sterile syringe and needle. Blood was collected by cardiac puncture.

In order to monitor possible changes in circulating white blood cells during infection, both total and differential leucocyte counts were made on the blood-samples collected. Total leucocyte counts were assessed by first lysing erythrocytes in the blood sample with Zaponin (Coulter Electrics Ltd). The remaining leucocytes were then enumerated using an electronic coulter counter (Coulter Counter Ltd). Differential leucocyte counts were made by preparing blood film smears on microscope slides. These were then air-dried before fixing and staining using Leishman's stain. Stained smears were examined under the light microscope using oil immersion. For each blood sample, 5 replicates of 100 cells were examined and the leucocytes (lymphocytes, monocytes, neutrophils, basophils and acidophils) counted. Individual results for each cell type were calculated from percentages of the total leucocyte count. Control counts were obtained from non-infected fish held at 10°C and 20°C.

4.2.E. Phagocytic activity in blood leucocytes

Phagocytic activity of blood leucocytes results in increased peroxidase activity in the cell due to increased phagolysosomal activity during destruction of ingested material (Griffin, 1983). Blood films were, therefore, stained for cellular peroxidase activity to study possible activation of circulating leucocytes during infection. Air-dried blood films from infected fish were prepared on microscope slides and fixed by flooding with 3% buffered formalin for 10 seconds. Staining for peroxidase activity was performed after Griffin (1983). Fixed slides were immersed in aqueous 0.01 MKH_2PO_4 (pH6.0) containing 0.008% 3, 3-dimethoxybenzidine (Sigma) and 0.003% H_2O_2 was applied as a primary

stain. After 15 minutes, slides were rinsed with de-ionised water and stained with Giemsa. A differential count was performed and the percentage of each cell type showing peroxidase staining was determined by examining 5 replicates of 100 cells in each blood smear.

4.2.F. Histochemical investigation

Since mucous is thought by some authors (Willoughby & Pickering, 1977) to play a major role in the defence against pathogens, attempts were made to assess mucous production in both infected and non-infected fish held at 10°C. Since none of the fish held at 20°C became infected, studies were made on non-infected fish only. Samples of skin, which showed no visible signs of infection, were taken from 3 fish in each group at monthly intervals from the start of the experiment. The skin samples (approx $1cm^2$) were fixed in 10% formalin and embedded in wax using standard histological techniques. Sections were cut at 5 m and stained using 0.3% alcian blue in 3% acetic acid which has been shown to stain sialic acid - a major component of fish mucous.

4.2.G. Examination of major lymphoid organs

Any immunological reaction to fungal antigen might be expected to result in changes in the major lymphoid organs. Samples of thymus, spleen and head kidney were fixed in Zenker formol fixative (Appendix 1) and stained with haematoxylin and eosin to reveal general histology.

The immune response to antigens is often accompanied by cellular proliferation within the major lymphoid organs. In the case of a strong immune response, this proliferation may lead to an increase in the size of reactive lymphoid tissues. Thymus, spleen and head kidney were, therefore, removed from infected and non-infected fish (10°C) and fish held at 20°C. The total length and weight of these organs was accurately determined directly after dissection. To account for variations related to individual differences in size between fish, the results were expressed as ratios of total length of the lymphoid organ to total body length; and total weight of the lymphoid organ to the total body weight.

4.2.H. Experiments employing injection of Saprolegnia derived antigens

Whilst experiments involving infection by live zoospores are useful in attempting to stimulate the events occurring in nature, it is difficult to ensure that each fish is receiving a standard "dose" of antigen. A second problem is that fish may also suffer from stress as a consequence of infection and this in turn may affect the immune response.

In order to overcome these problems, preliminary experiments were carried out using antigens derived from homogenised preparations of Saprolegnia.

4.2.H.a. Preparation of fungal antigen

4.2.H.a.i. Soluble antigen

Fungal cultures were grown on 1% casein + 1% glucose medium as previously described (section 2). Fungal mycelium was separated from the culture medium by filtration and washed twice with 200ml PBS (pH 7.2) by allowing it to stand for 30 minutes prior to refiltering. The washed mycelium was resuspended in twice its own weight of PBS and homogenised for 2 minutes at 14,000 rpm using a (Kinematica) homogeniser. The homogenate was left at 4°C overnight and then centrifuged at 5000 rpm for 5 minutes to remove cellular debris. The supernatant was decanted and stored at -20°C until used (Hodkinson & Hunter, 1971). The protein concentration of the supernatant was assessed by the Lowry method (Lowry et al., 1951). This antigen preparation was also used in assays for precipitating antibody (section 4.2.I.).

4.2.H.a.ii. "Crude" homogenate

The method of preparation was the same as that employed for the soluble antigen, but instead of removing the cellular debris following centrifugation, this was resuspended to a 20% v/v concentration in PBS.

4.2.H.b. Immunisation of fish with soluble antigen and crude homogenate

Eight carp received 1-5mg of the soluble antigen fraction intraperitoneally. A second group of 9 fish received 0.1ml of the crude homogenate antigen, also via the intraperitoneal route. Control fish received equivalent injections of PBS only. All fish

were maintained at 20°C in order to promote good levels of antibody production. Fish were bled at weekly intervals for four weeks post-immunisation and the serum assessed for precipitating antibodies as outlined in section .

4.2.H.c. Experiments involving Saprolegnia antigens coated onto erythrocyte "carriers"

Not all antigens provoke a strong immune response. With certain antigens it is sometimes advantageous to conjugate them onto larger protein molecules or to the surface of erythrocytes in order to improve their immunogenicity. In this experiment, soluble antigens derived from Saprolegnia diclina were coated onto sheep red blood cells and injected into two groups of carp kept at 10°C and 20°C.

4.2.H.d. Preparation of coated erythrocytes

Sheep red blood cells in Alsever's solution (Flow) were tanned and coated with antigen as described in Hudson and Hay (1980). The only difference being that 2mg ml^{-1} soluble Saprolegnia antigen was substituted for Human Serum Albumin. Antigen coated cells were stored at 4°C until used for injection or the passive haemagglutination assay.

4.2.H.e. Immunisation of fish with coated erythrocytes

Two groups of 6 experimental fish were injected intraperitoneally with 0.1ml of a 10% v/v suspension of Saprolegnia-

coated SRBC in 0.9% saline. One group was maintained at 20°C, the other at 10°C as described previously.

Two groups of (6) control fish were given equivalent injections of formalinised SRBC only. These were prepared as described above, but the cells were not incubated with Saprolegnia derived antigen. Control fish were also maintained at 10°C or 20°C. One month after immunisation, the fish were bled and haemagglutinating antibody was assayed as described in section 4.2.I.b.

4.2.H.f. Isolation of Carp Serum

Each fish was bled by heart puncture under anaesthesia (benzocaine "Sigma Co. England), the blood was allowed to clot and was held overnight at 4°C and the serum removed by a pipette, the serum was centrifuged at 3,000 rpm for 10 minutes to remove residual blood cells. The supernatant serum was removed and frozen at -20°C until required.

4.2.I. Methods used for detecting antibody

4.2.I.a. Detection of precipitating antibodies

When soluble antigens are brought into contact with specific antibodies, antigen-antibody union occurs as a result of specific recognition and combination of the antigen with its corresponding antibody combining site. Since antibody molecules are multivalent, cross-linking of antigen molecules occurs and this results in the precipitation of the antigen. This precipitation reaction forms the basis of a number of assay techniques for detecting and quantifying antibody in serum from immunised animals.

4.2.I.a.i. Double immunodiffusion (Ouchterlony technique)

In this technique, soluble antigen (section 4.2.H.a.i.) and antibody (contained in carp serum) are placed in neighbouring wells made in an agar gel layer. Antigen and antibody diffuse towards each other through the gel and where they meet at an optimum antigen/antibody ratio, a precipitation line is formed. The Ouchterlony technique is useful for identification of antigens or antibodies in serum and has the advantage that only small amounts (1-20 μl) of antigen and antibody are necessary (Ouchterlong, 1968).

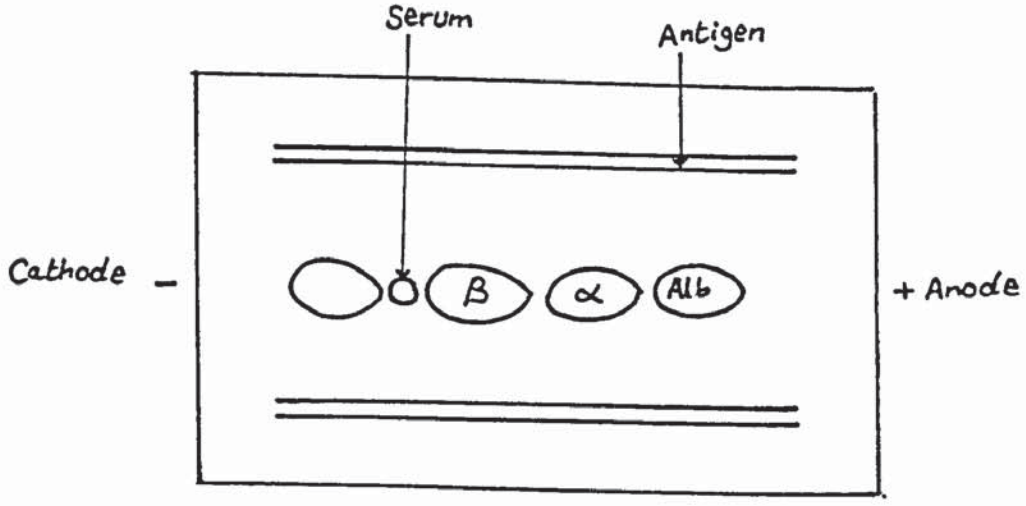
Procedure:

Clean microscope slides were coated with an even layer of barbitone buffered agar (Appendix 1) using a Shandon slide tray and gel leveller. Wells were cut in the gel using pasteur pipettes, and the distance between neighbouring wells varied to ensure optimal antigen/antibody ratios during diffusion. Various dilutions of antigen and antibody were also used in different experiments.

After filling individual wells with antigen or antibody, the slides were incubated in a humidity chamber (Shandon) for 48 hours - two weeks to allow the precipitation reaction to develop. Following incubation, slides were washed for 48 hrs in saline to remove unprecipitated proteins. The slides were then washed for six hours in several changes of water. Slides were then stained in 0.1% Azocarmine.

4.2.I.a.ii. Immunoelectrophoresis

Immunoelectrophoresis was used in the present study to determine which fraction of carp serum was responsible for any observed precipitation reactions. This was an important issue to resolve since other workers have shown apparently non-specific precipitating activity against Saprolegnia antigens in the α - globulin fraction of fish serum. It was, therefore, important to determine whether any precipitin reactions observed in the present study were due to these alpha precipitins or to immunoglobulins (antibodies). The technique employed was based on that of Grabar and Burtin (1964).



Agar coated slides were prepared as described for the Ouchterlony technique. A Shandon gel cutter was used to cut one well and two troughs in each slide as shown in Fig.20.

Carp serum was placed into the well using a fine syringe and separated into its constituent components by electrophoretic separation for 60-90 mins in barbitone buffered agar gel. (Appendix 1). The power supply was adjusted to give a potential

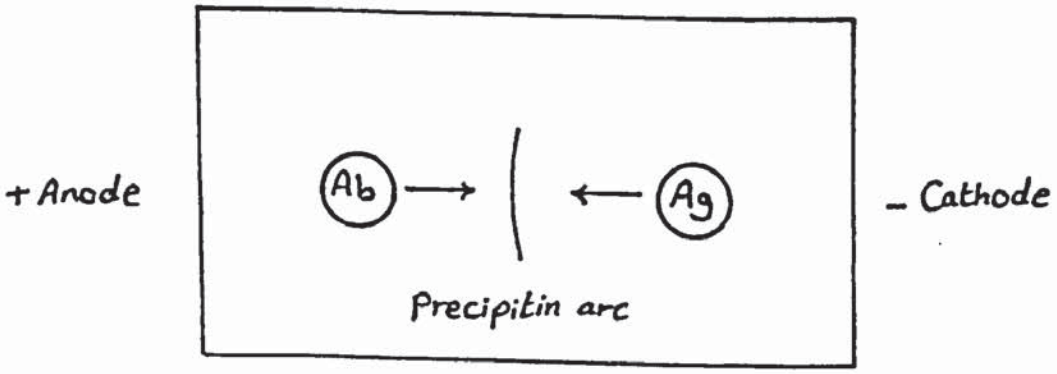
drop of 6V/cm as measured between the anode and cathode. Soluble Saprolegnia-derived antigen was then allowed to diffuse into the gel from troughs cut at right angles to the direction of electrophoresis. Any arcs of precipitation observed allow the experimenter to identify the serum fraction in which the precipitating activity resides.

Antibody and antigen were allowed to interact in a humid chamber from 12 hrs to 4 days, prior to processing and staining the slides as described earlier.

4.2.I.a.iii. Counter current electrophoresis

(Cross over electrophoresis)

The principle of counter current electrophoresis is the same as for the Ouchterlony technique. The technique is again performed in an agar gel, and the use of an alkaline buffer (pH 8.6) results in the antibody being positively charged and the antigens being negatively charged. By applying a voltage across the gel, the antigens and antibody migrate towards each other and form a precipitate at the point of equivalence. The sensitivity of this technique is reported as being 10-20 times higher than that of the Ouchterlony Test (Hudson and Hay, 1980).



Agar coated microscope slides were prepared as previously described and a pattern cut into the agar as shown in Fig. 21 above. Electrophoresis was carried out for 15 mins at a voltage drop of 6v/cm as measured between the anode and cathode. Slides were incubated and stained as described for the Ouchterlony technique.

4.2.I.b. Detection of agglutinating antibodies

4.2.I.b.I. Passive haemagglutination technique

In this technique, Saprolegnia derived antigen is bound onto the surface of sheep red blood cells (SRBC) and, in the presence of antibody to that antigen, the SRBC are cross-linked by the multivalent antibody molecules binding to the antigen and will agglutinate. The preparation of formalinised-SRBC and antigen coated-SRBC are described earlier.

The technique used here is more fully described in Hudson and Hay (1980). Carp serum from control and injected fish was serially diluted (in doubling dilutions) in physiological saline and 25 l of each dilution placed in individual wells of haemagglutination plates diluted (in doubling dilutions) in physiological saline and 25 l of formalinised-SRBC or Saprolegnia-coated SRBC was added to each well. The trays were sealed and incubated for 1-2 hrs at room temperature. A positive response is recognised by the formation of a "mat" of agglutinated SRBC on the bottom of the well. If no agglutination occurs, the individual cells roll to the bottom of the 'V'-shaped wells and form a small red pellet. The maximum dilution at which agglutination occurred was recorded for each serum tested and results were expressed as $-\text{Log}_2$ dilution.

4.2.J. Migration inhibition factor (MIF) assay

A widely used test for cell-mediated reactivity to antigens is based upon the production of a lymphokine (MIF) by sensitised T-lymphocytes. Leucocytes from peripheral blood or lymphoid organs are packed into small capillary tubes and allowed to migrate out of the tubes in vitro. The motile cells are predominantly macrophages, but their movement may be inhibited by MIF which is released from sensitised T-lymphocytes in the presence of antigen. Where migration of the macrophages is inhibited by a specific antigen, it is concluded that the cell suspension also contains T-lymphocytes which are sensitive to the antigen and, therefore, secrete MIF. Macrophages in control cell suspensions (which lack sensitised T-lymphocytes) are able to migrate normally in the presence of antigen. In this part of the thesis, the MIF assay was used to test whether lymphocytes from infected fish are able to respond positively in the MIF assay. The methodology employed is based on that of Rimmer and Gearing (1980). Infected fish and control fish with no signs of fungal lesions were sacrificed by immersion in MS222 (Sandoz). The spleens were removed under aseptic conditions and placed in cold culture medium (Iscoves medium, Flow Laboratories). The organs were teased apart using sterile watchmakers-forceps to yield a single cell suspension. The cells were transferred to sterile tubes and allowed to stand on ice for 3-4 minutes to allow larger clumps of tissue to settle out of suspension. The supernatant was then removed and the cells washed 3 times by centrifugation at 900G for 10 minutes. Viable cells were counted using trypan blue dye exclusion and the numbers adjusted to

2×10^7 viable cells/ml. Cells were then taken up into heparinised capillary tubes (Harshaw) and when full, one end was sealed using dental plasticine. The sealed tubes were then packed into flat bottomed tubes and centrifuged at 900G for 10 mins. Following this, the tubes were cut with a diamond marker pen at the cell-pellet/medium interface and placed horizontally on the bottom of individual migration chambers in Sterilin migration trays. The tubes were held in position with Vaseline and the chambers were then filled with medium alone or medium containing Saprolegnia antigen (at a concentration of 1gm fresh weight of Saprolegnia homogenised in 60ml culture medium). At least 5 replicate tubes were set up for each treatment. The chambers were then sealed using Vaseline and coverslips and incubated at 20°C for 18 hrs. Migration was measured by placing the transparent migration trays on a photographic enlarger and projecting the image of the migrating cells onto graph paper. The outline of cellular migration was traced onto the paper and the area measured. The degree of migration inhibition was calculated as follows:

$$\% \text{ migration} = \frac{\text{migration area of infected cells in presence of Ag}}{\text{migration area of control cells in medium}} \times 100$$

Statistical analysis of the data was carried out using Student's t-test to calculate P values. Differences between results were considered significant when $P < 0.05$.

4.3. Results

4.3.A. Infection of fish held at different temperatures

Temperature	Incidence of Infection		
10°C	Exp.1	Exp.2	Exp.3
Experimental Fish	5/7	20/20	17/20
Control Fish	0/6	0/5	0/5
20°C			
Experimental Fish	0/5	0/20	N.D
Control Fish	0/6	0/5	N.D

* N.D = Not done.

Table (10). The influence of temperature on Saprolegnia infection

Table 10 shows the results of three experiments which were carried out to assess the influence of environmental temperature on the incidence of Saprolegnia infection in groups of experimental fish exposed to live zoospores. There was no significant seasonal effect upon the incidence of infection [experiments 1 and 3 were carried out during the summer months (May - August) whilst experiment 2 was performed in winter (November - February)]. From the data shown, it is clear that temperature exerts a profound influence on the susceptibility of fish to infection. Forty two of

the forty seven experimental fish held at 10°C developed Saprolegnia infections, whereas none of the twenty five experimental fish held at 20°C showed visible signs of fungal attack. Furthermore, control fish which were not exposed to live zoospores remained free from fungal lesions.

Infected fish developed fungal lesions from one to four weeks after initial exposure to zoospores. The site of scale removal always became infected, but some of the fish studied (30%) also developed fungal infections at other sites, most notably the dorsal and caudal fins and the posterior part of the body. The first sign of infection did not always appear at the site of experimental injury since, in approximately 20% of fish studied, fungal lesions were first detected at other apparently non-injured sites.

4.3.B. Haemagglutinating antibodies against Saprolegnia coated erythrocytes

ANTIBODY TITRES TO

TEMP	S/SRBC	F/SRBC
20°C		
Experimental Fish	0,6,7,11,11,-(x=7)	5,0,3,3,0,-(x=2.2)
Control Fish	0,4,2,4,0,0(x=1.7)	0,2,3,4,0,0(x=1.5)
10°C		
Experimental Fish	0 0 0 0 0 0(x=0)	0,0,0,0,0,0,(x=0)
Control Fish	0 0 0 0 0 0(x=0)	0,0,0,0,0,0(x=0)

S/SRBC = Saprolegnia coated sheep red blood cells

F/SRBC = Formalinised sheep red blood cells

Each figure represents $-\log_2$ titre of Ab in one fish

- one fish died during the course of the experiment.

Table (11) Haemagglutinating antibodies against Saprolegnia coated erythrocytes

Table 11 shows that one month post-injection with Saprolegnia coated erythrocytes, fish held at 20°C gave a mean agglutinating antibody titre of 7. When sera from the same group of fish were tested against formalinised SRBC alone, the mean agglutinin titre

was 2.2, showing that the response to antigens on formalinised erythrocytes was low.

Control fish (held at 20°C) which received formalinised SRBC gave mean haemagglutinin titres of 1.7 against Saprolegnia coated-SRBC, and 1.5 against formalinised SRBC.

None of the fish held at 10°C gave detectable haemagglutinin titres to either of the antigens tested.

Studies carried out on sera from 12 infected fish held at 10°C revealed no evidence of haemagglutinating antibodies against Saprolegnia antigens in the passive haemagglutination assay. Serum samples were the same as those used for the assays to detect precipitating antibodies. Sera from 5 fish exposed to zoospores at 20°C also showed no evidence of agglutinating antibodies against Saprolegnia derived antigens.

4.3.C. Assessment of precipitation antibodies in serum of infected and non-infected fish

Temperature	Serum antibody production*	
10°C	Exp.1	Exp.2
Experimental Fish	0/7	0/20
Control Fish	0/6	0/6
20°C		
Experimental Fish	0/5	0/20
Control Fish	0/5	0/6

* As assessed by Ouchterlony test, counter current electrophoresis and immunoelectrophoresis in agar gel.

Table (12). Serum antibody production in infected and non-infected fish at 10°C and 20°C

Table 12 reveals that none of the fish utilised in the present experiments produced detectable levels of precipitating antibodies against Saprolegnia derived antigens. Fish kept at 20°C were sampled two weeks after exposure to fungal zoospores [a time when the immune response is normally well developed in carp maintained at this temperature (Rijkers et al, 1981)]. Because the immune response is normally slower in fish kept at lower temperatures, the fish held at 10°C were sampled over a more protracted time period. Blood samples were collected at monthly intervals over a four month

period post-infection. The individual fish from which serum samples were taken are the same as those referred to in Table 10. None of the infected fish held at 10°C showed any evidence of serum antibody against Saprolegnia antigens and even those individuals which remained free from infection at 10°C produced no detectable serum antibodies. Fish held at 20°C, all of which remained free from infection during the course of this experiment, also failed to show detectable titres of antibody against Saprolegnia antigens.

As a positive control to establish whether the techniques employed in these studies were appropriate for detecting precipitating antibodies in fish, a small number of rainbow trout (Salmo trutta) with Saprolegnia infections of the skin were obtained from the Aston Fish Culture Unit. Control trout with no visible external infections were also used. Serum samples from infected and control trout were used in the Ouchterlony test and in immunoelectrophoresis against Saprolegnia derived antigens. Whilst the Ouchterlony test failed to give a positive precipitin reaction, all serum samples taken from infected trout gave positive precipitin lines against the Saprolegnia antigen. Control trout serum revealed no precipitating antibody activity in either Ouchterlony tests or immunoelectrophoresis.

4.3.D. Assessment of precipitating antibodies in fish receiving injections of Saprolegnia derived antigens

	Antigenic Challenge	
	Soluble fraction	Crude homogenate
Experimental Fish	0/8	0/9
Control Fish	0/4	0/4

Table (13) Serum antibody production to injected Saprolegnia derived antigens at 20°C.

Two groups of fish were examined at weekly intervals for four weeks post-injection for their ability to respond to soluble antigenic fractions or crude membrane fragments derived from Saprolegnia mycelia. These studies concentrated on the responses of carp maintained at 20°C since earlier workers have demonstrated good antibody titres to other antigens at this temperature (Rijkers et al., 1981). No antibodies were detected to either antigenic preparation using Ouchterlony and immunoelectrophoretic techniques.

4.3.E. Changes in circulating blood cells of infected fish

Fig.22 (Table 14, Appendix 2) reveals that there was a significant decline in total blood leucocyte numbers of infected fish during the first two months of the experiment. This was followed by a sharp increase in total cell numbers during the third and fourth months of study. These changes are based on a comparison with the total leucocyte numbers measured in a group of non-infected

control fish which were sampled prior to the start of the experiment.

Examination of the differential blood cell counts reveals that the lymphocytes represented the major blood cell fraction. The percentage of lymphocytes in the blood remained unchanged throughout the course of the study. There were also no persistent changes in the relative percentages of monocytes, basophils and acidophils. Only the numbers of neutrophils and macrophages appeared to increase significantly over the four month period of study. Measurements of total leucocyte numbers and differential white blood cell counts of non-infected fish held at 20°C did not differ significantly from the data obtained for non-infected 10°C fish at the start of the experiment.

4.3.F. Phagocytic activity of blood cells

The results obtained for peroxidase activity in blood smears taken from infected fish were very variable. The number of cells showing positive staining for peroxidase ranged from very few in some fish to 35% of total blood cells in others. Of the leucocytes which showed peroxidase activity, approximately 60% were mononuclear cells (monocytes and macrophages) and the remainder were neutrophils. These were also the only cell types to show a persistent significant increase in numbers in the blood of infected fish (see previous section).

4.3.G. Histochemical investigations

Fig.23 illustrates a section through the skin of an uninfected control fish which had been held at 10°C for two weeks prior to sacrifice. The section has been stained for sialic acid (a major component of mucous) and it may be seen that the skin is richly supplied with mucous-secreting goblet cells. A substantial layer of mucous is also visible on the outer surface of the skin. In contrast, Fig.24 shows a section through the skin of an infected fish. This skin sample was taken from a site which showed no apparent signs of external damage or infection. Note that the goblet cells in skin from infected fish are considerably reduced in both size and number. Many of the goblet cells have also lost their characteristic shape and have become rounded. The surface layer of mucous is reduced in thickness when compared to that of the non-infected fish, and in some places appears to be almost absent. There was no difference in appearance between sections taken from non-infected fish held at 10°C or 20°C.

4.3.H. Changes in lymphoid organs of infected fish

Fig.25 illustrates the percentage changes in length:body length and weight:body weight ratios of spleen, head kidney and thymus from infected fish. The changes are calculated by comparison with the mean length and weight of lymphoid organs in uninfected fish which had been maintained at 10°C for two weeks prior to measurement (see Table 15 in Appendix 2). The results were very variable, but maximal decreases in the size of all three lymphoid organs were observed two months after the start of the experiment. The lengths and weights of the lymphoid organs increased during the third and

fourth months of observation but did not completely recover. There was no significant difference in the size of lymphoid organs in non-infected fish held at 20°C and 10°C.

Histological preparations of the lymphoid organs from infected fish were also examined. Fig.27 shows a transverse section through the spleen of an infected carp. One of the major changes to appear in the spleens of infected fish was the presence of large numbers of yellowish brown aggregates of cells which resembled germinal centres.

The head kidneys of infected fish were characterised by the presence of large numbers of round or ovoid bodies not present in control sections (Fig.28). These bodies are illustrated in Fig.29 and appear to contain a homogeneous non-granular substance which stained reddish-brown with haematoxylin and eosin. Large numbers of lymphoid cells are also apparent, scattered through the head kidney tissue.

Despite the decrease in size, there appeared to be no major histological differences between the thymus of control and infected carp.

4.3.I. Migration inhibition factor assay

The results obtained for this assay showed that although the cells migrated successfully in culture, there was too much variation between individual experiments to allow any firm conclusions to be drawn regarding the presence of sensitised lymphocytes in infected

fish. Further studies are planned to develop a more consistent technique.

4.4. Discussion

The present study has demonstrated that environmental temperature exerts a profound effect upon the incidence of infection in carp exposed to zoospores of S. diclina. Previous workers have reported that fish are more susceptible to saprolegniasis when seasonal temperatures are low (Carbery, 1986a; Norland-Tintigner, 1973), but this is the first demonstration of the effect of temperature on infection under controlled laboratory conditions. The temperature effects reported here did not appear to be influenced by seasonal variations in daylength, hormonal status etc, since experiments carried out in winter and summer yielded similar results. Carp proved to be a suitable model for the study of saprolegniasis since, although they were susceptible to infection by Saprolegnia zoospores, they were able to survive for many weeks after infection. This contrasts with studies on other species of teleost fish (eg. char and brown trout) which normally die within 2-3 days of infection (Richards & Pickering, 1979; Johansson et al., 1982). The appearance of fungal colonies on apparently undamaged areas of carp integument lends support to previous reports which suggest that, under appropriate conditions, Saprolegnia spp. may act as primary pathogens in other species of teleost fish including eels and sockeye salmon (Tiffney, 1939; Hoshina & Sunayama, 1960; Neish, 1977). The reasons why temperature has such a dramatic effect on infection by S. diclina zoospores are not known, but may be related to aspects of the fungal life cycle and physiology, or may be a

function of depressed immunity in fish held at low temperatures. Chapter 2 examined growth rates and subtrate utilisation in S. diclina over a range of temperatures. The fungus is able to grow well at both temperatures (10°C and 20°C) tested here, but was not seen to grow better at lower temperatures. The increased incidence of infection at 10°C is therefore unlikely to be a result of the fungus being more invasive at lower temperatures. However, studies on substrate utilisation presented in Chapter 3 have shown that S. diclina shows more proteolytic activity per unit weight when incubated at 10°C. Since proteolytic enzymes are likely to play a major role in the breakdown and colonisation of fish tissue, increased enzyme production at lower temperatures may account, in part, for the increased incidence of infection seen here at 10°C. Previous studies by Smith et al. (1984) have investigated the behaviour of S. diclina zoospores at different temperatures. At 10°C zoospore motility and germination were significantly less than that observed at 20°C. Low temperature is therefore unlikely to promote infection as a result of direct advantageous changes in zoospore activity.

It is well established that low temperatures have an inhibitory effect upon the immune responses of fish (Carbery, 1968a; Shearer, 1970; Stevenson, 1970; Avtalion et al., 1970, 1973, 1980; Avtalion, 1981). The studies presented here have concentrated largely upon the production of precipitating antibodies and α-precipitins, since earlier reports had monitored increases in both fractions in the sera of Saprolegnia infected fish (Alexander, 1976, 1980, 1982; Davies, 1975). The protective effects of maintaining the fish at

a higher temperature do not appear to involve increased production of antibodies or ∝-precipitins, since there was no evidence of antibody or ∝-precipitin titres in the sera of fish held at 10°C and at 20°C. If these precipitating factors had played a major role in protecting carp from infection, then it would be reasonable to have expected higher titres of such factors in the sera of the non-infected group (20°C) when compared to the infected group (10°C).

Precipitation reactions in gels have been widely used by fish immunologists to study the presence of serum antibodies and other precipitating factors. A number of workers have described the presence of ∝-precipitins in serum of the Atlantic salmon Salmo salar and rainbow trout, Salmo gairdneri (Alexander, 1980, 1982; Davies and Lawson, 1982; Munro et al., 1980). Such a precipitin was found in a number of salmon when their sera were diffused against an extract of necrotic tissue taken from UDN affected fish. Following electrophoretic separation, this factor was shown to have ∝-migratory properties (Davies and Lawson, 1982). Precipitins have also been described when fish sera are diffused against extracts prepared from cultures of Saprolegnia diclina and extracts of soluble starch and amylopectin (Wilson, 1976; Hodkinson and Hunter, 1970; Alexander, 1980). The protective role of these ∝-precipitins is dubious, since ∝-precipitin levels in salmon serum were not altered in fish infected with Saprolegnia diclina (Davies and Lawson, 1982). Furthermore, Ellis (1985) has recently produced evidence to suggest that the reactions observed by the workers cited above may not be genuine precipitation reactions at all. Ellis's results suggest that the precipitation observed is artifactual, and

that the precipitate is not formed by complexing of antibody and antigen, but represents the result of proteolytic breakdown of serum lipoproteins by extracellular products of the fungus. Extracts of diseased tissue and Saprolegnia culture supernatants would almost certainly contain proteases (see Chapter 3) and may explain the apparent precipitation reactions observed in salmonid fish. The present study failed to detect any precipitation reactions with any of the methods used. Even the sensitive counter current electrophoresis method failed to demonstrate precipitin lines in the agar. The lack of precipitin reactions is perhaps surprising in the light of the proposed enzymatic reaction described above, but it may reflect differences between the serum components of carp and salmonid fish.

In salmonid fish, antibody reactivity has been associated with the β_2- electrophoretic fraction of the serum (Wilson, 1976). However, Wilson reported that even where precipitating antibodies were detected, there was no correlation between the levels of antibody and the degree of infection by Saprolegnia diclina. The present study failed to detect precipitating antibodies in the serum of both infected and non-infected fish and therefore the protective role of precipitating antibodies in saprolegniasis must remain dubious. It must be remembered however that, in general, fish do not produce good precipitating antibodies and that the level of reaction appears to be dependent upon the species of fish and the antigen used (Ellis, 1982). Interestingly the only antibody reaction observed in the present study involved the use of the passive haemagglutination technique which detected agglutinating

antibodies to S. diclina antigens coated onto the surface of sheep erythrocytes. The response was only observed following in vivo challenge with Saprolegnia derived antigens bound to the surface of sheep red blood cell "carriers". Agglutinating antibodies were not detected when the passive haemagglutination technique was applied to sera from infected carp. Hart (1985) reported the presence of agglutinating antibodies in the serum of eels and carp following injection with homogenates of S. diclina in Freund's adjuvant, but his fish were maintained at a higher temperature than the infected fish tested here.

The absence of an agglutinating response to formalinised sheep red blood cells in fish held at 10°C is in line with previous observations that antibody production is severely impaired in carp held at such low temperatures (see below). The low antibody response seen to formalinised SRBC in carp held at 20°C is similar to results reported by Jayaraman et al (1979) who demonstrated that this antigen also gave rise to weak antibody titres in the fish Tilapia mossambica.

Earlier studies on the effect of temperature on the humoral immune response suggest that carp are unable to produce circulating antibodies at temperatures below 15°C (Fijnan and Cvetric, 1964, 1966; Avtalion, 1969, 1981; Avtalion et al., 1970, 1973; Avtalion and Shahrabani, 1975; Weiss and Avtalion, 1977). The optimal temperature for antibody production in carp is 25°C (Avtalion et al., 1973) and whilst carp in the present study failed to produce

precipitating antibodies when held at 20°C, they did produce agglutinating antibodies at this temperature.

The inability of fish to produce antibodies at low temperatures may be due to a phenomenon known as immunological tolerance (Humphrey, 1976). It has been demonstrated that a state of specific and long-lasting tolerance can be induced in adult carp held at low temperatures (Avtalion et al., 1980; Avtalion, 1981). Weiss and Avtalion (1977) and Avtalion et al. (1980) have obtained data which are consistent with the existence of helper and suppressor cells in carp (in mammals these thymus derived lymphocytes regulate the immune response by promoting or inhibiting antibody production). These workers have proposed that induction of helper cell function is preferentially suppressed at low temperatures and the necessary interaction between helper cells and antibody producing cells is therefore lacking.

The studies on circulating leucocyte numbers and changes in lymphoid organ size in infected fish, provide tentative evidence for a decline in both. The results of these studies must be interpreted with caution because of the wide variation between replicates (particularly in the size of lymphoid organs) and also the lack of time-matched controls. The apparent decrease in circulating leucocyte numbers during the first two months post-infection is based on a comparison with measurements taken from a group of control carp at the start of the experiment. Any observed changes are based on the assumption that the levels in control fish remained constant over the four month experimental period. The apparent

decline in total blood leucocytes and the size of lymphoid organs may have been a direct result of fungal infection or may have been caused by stress occurring as a secondary consequence of infection and/or exposure to low temperatures. The relationship between stress and disease has been well-established (Wedemeyer, 1969; Sniesko, 1974) but little is known about the mechanisms involved in this association. One well-documented component of physiological response of teleost fish to different stresses is an increase in circulating corticosteroids (Donaldson, 1981). Increases in corticosteroid levels are known to have an immunosuppressive effect in mammals and Pickering and Duston (1983) were able to demonstrate increased levels of both saprolegniasis and furunculosis in cortisol treated brown trout. It is possible that the generalised decline in leucocyte counts observed here are the result of elevated corticosteroid levels in infected fish. The precise effects of corticosteroids on circulating white cells are difficult to establish, since handling alone is sufficient to bring about marked changes in both corticosteroid levels and the numbers of circulating white blood cells (McLeay, 1970, 1973a,b, 1975b; Ellis, 1977a).

The only categories of leucocytes to show a persistent, significant change in infected fish were the macrophage and neutrophils which exhibited a steady increase in numbers throughout the observed period of infection. Histological studies in blood leucocytes of infected fish also revealed that both these cell types exhibited increased levels of peroxidase activity associated with phagocytosis. Previous reports have also identified monocytes, macrophages and neutrophils as being phagocytic in fish (Weinreb and

Weinreb, 1969; Finn and Neilson, 1971; Klontz, 1972a; Ferguson, 1975; Ellis et al., 1976; Ellis, 1977a; McKinney et al., 1977; Griffin, 1983). In the present study it is not known whether the phagocytic activity observed was directed against components of the pathogenic fungus or was involved in removing damaged or necrotic host cell components (Iwama et al., 1986). Similar increases in macrophage and neutrophil numbers have been recorded in other types of infection in fish (Finn & Nielson, 1971; Thorpe & Robers, 1972; Ellis, 1977b.

The increases in leucocyte numbers and lymphoid organ size observed during the third and fourth months of the experiment are difficult to explain, but may result from a recovery from stress or a gradual acclimation of the fish to low temperatures. An alternative explanation is that the recovery was brought about by an increase in haemopoietic activity stimulated by the loss of circulating blood cells at the site of the fungal lesions (Hiroshi, 1969; Richards & Pickering, 1979; Pickering & Willoughby, 1982). Whether or not the increase is indicative of a belated increase in immune responsiveness is not known. Both antibody production and cell-mediated responses in fish held at 10°C are known to be very slow indeed. There was no apparent synthesis of antibody following this recovery so this explanation would appear unlikely, although the possibility of a belated cell mediated immune response cannot be ruled out.

One characteristic feature observed in infected carp was the increased deposition of pigment in the spleen and head kidney. These histological changes in Saprolegnia infected carp are similar

to those seen during disease processes in other fish. Pigment deposition in lymphoid organs during infection is characteristic of a variety of fish diseases including: Aeromonad diseases of salmonid fish (Klontz et al., 1969; Thorpe & Roberts, 1972); parasitic infestations of roach (Rutilus rutilus) caused by Myxobolus pseudodispar (Roberts, 1975) and Ichthyophonus-like fungal infections of scabbard fish (Aphanopus carbo) (Agius, 1978). The significance of pigment deposition in fish disease is not known, but has been reviewed by Agius (1985) who suggests that it may be involved in direct killing of bacteria, or may occur as a consequence of tissue damage at the site of infection, followed by the peroxidation and eventual transport of unsaturated lipid components of damaged cells to melano-macrophage centres in lymphoid organs. A third proposal advanced by Agius is that the pigment deposits result from tissue damage caused by stressed fish not feeding properly - since starvation results in marked enlargement of melano-macrophage centres.

The spleen of carp (Cyprinus Carpio) contains many antibody-producing cells (Rijkers et al., 1980) and, following antigenic stimulation, clusters of pyroninophilic cells appear in the ellipsoid walls. Observations suggest that these clusters may develop into melano-macrophage aggregates (Secombes et al., 1982b). The melano-macrophage centres may be considered as foci for primitive germinal centres involved in immune reactions (Sailendri & Muthukkaruppan, 1975; Ferguson, 1976a). In mammals, similar clusters were considered possible regions for cellular interaction in the immune response (Farr & De Bruyn, 1975). The absence of

antibody titres observed in the present study makes it likely that the histological changes reported here are a consequence of infection and stress rather than the result of an active immune response. Stress related changes in infected fish are further examined in chapter five.

The fish epidermis together with its secretions has been implicated in many processes of osmoregulation, locomotion and protection against mechanical injury. One important role of the epidermis is to act as a physical barrier between pathogens in the surrounding water and the internal tissues of the fish (Pickering & Richards, 1980). It has been suggested that the continuous replacement of mucous helps to prevent colonisation of the skin by ectoparasites, fungi and bacteria. Pickering (1974) has demonstrated that the rate of mucous production is governed by the concentration of mucous-secreting cells (goblet cells) within the epidermis and the turnover rate of these cells. As mucous cells synthesise more mucous they increase in size as a consequence of mucous deposition within the cytoplasm (Harris and Hunt, 1975). Sialic acid has been isolated from the mucous of many vertebrates including the epidermal mucous of teleost fish (Enomorto et al., 1964; Lemoine and Olivereau, 1971). Histochemical analysis of the goblet cells of brown trout and char epidermis indicate that their secretions are predominantly sialic acid (Pickering and Macey, 1977), and the present study has shown that sialic acid is also present in the goblet cells of carp epidermis. A comparison of goblet cell numbers in the skin of non-infected and infected carp revealed a dramatic decline in the latter group during the course of

infection. The reasons for this decline are not known, but may be a direct consequence of fungal infection or a secondary effect brought about by stress and prolonged exposure to low temperatures. Further work is needed to clarify this issue, but if low temperatures do result in a reduction in the numbers of goblet cells in the epidermis, this could represent an important factor in promoting infection by fungal pathogens.

In view of the general lack of evidence for a humoral immune response to antigens of S. diclina, some preliminary trials were carried out to investigate possible cell-mediated immune responses. Unfortunately, these did not yield consistent results and it was therefore not possible to conclude that cell-mediated events were involved in the response of infected fish. However, further experiments are planned to establish optimal culture conditions and to determine the appropriate doses of antigen for use in vitro, The MIF assay was used by Timor (1975) in a study of chronic inflammatory disease in flatfish, and Jayaraman and Muthukkarruppan (1979) have utilised the MIF technique to study the cell-mediated immune response of Tilapia to sheep red blood cells. It is therefore hoped that this will be an appropriate method for future studies on the immune response of carp to Saprolegnia.

Fig. (22) Total and differential leucocyte counts of control and UDN-infected carp (*Cyprinus carpio*)

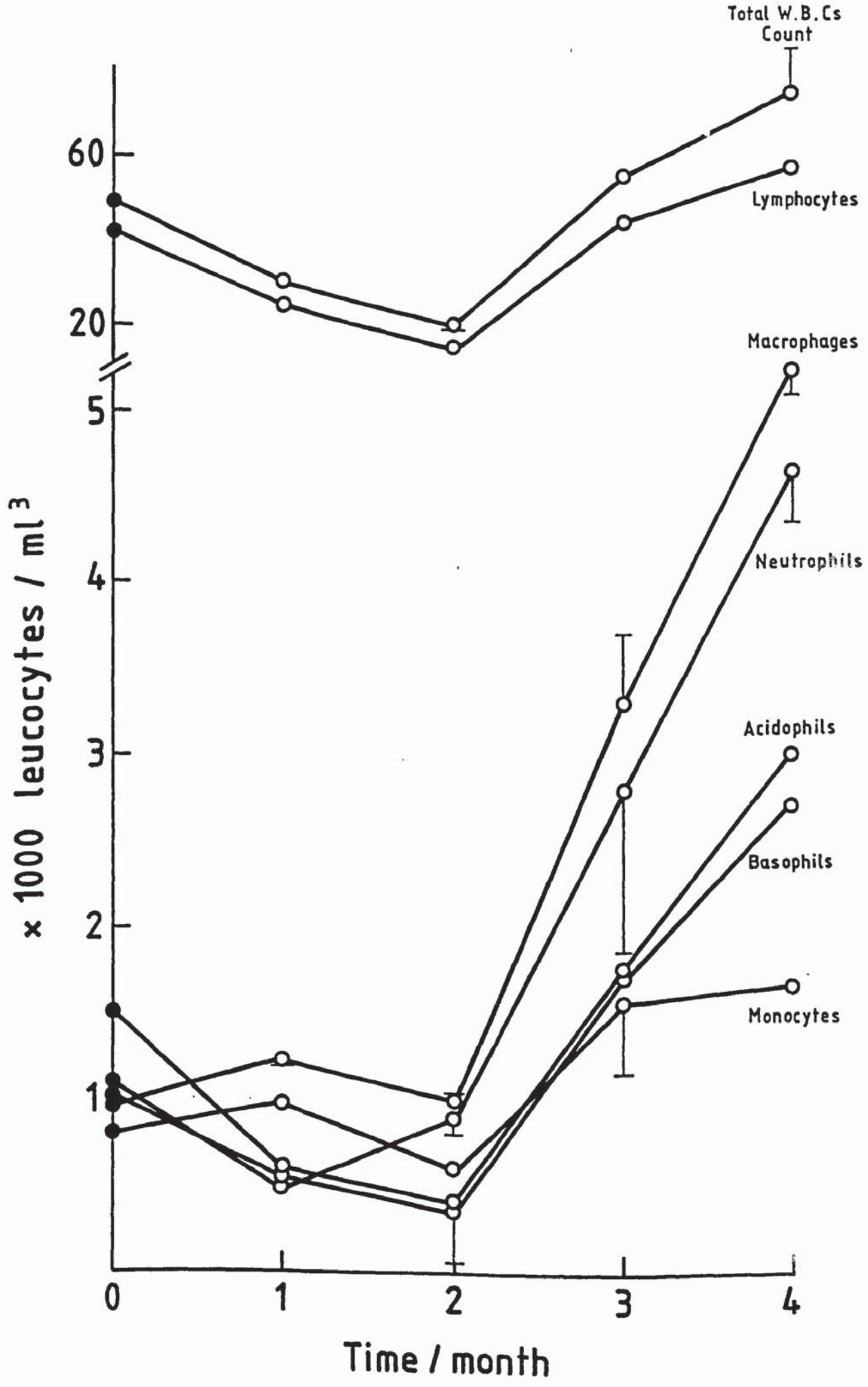
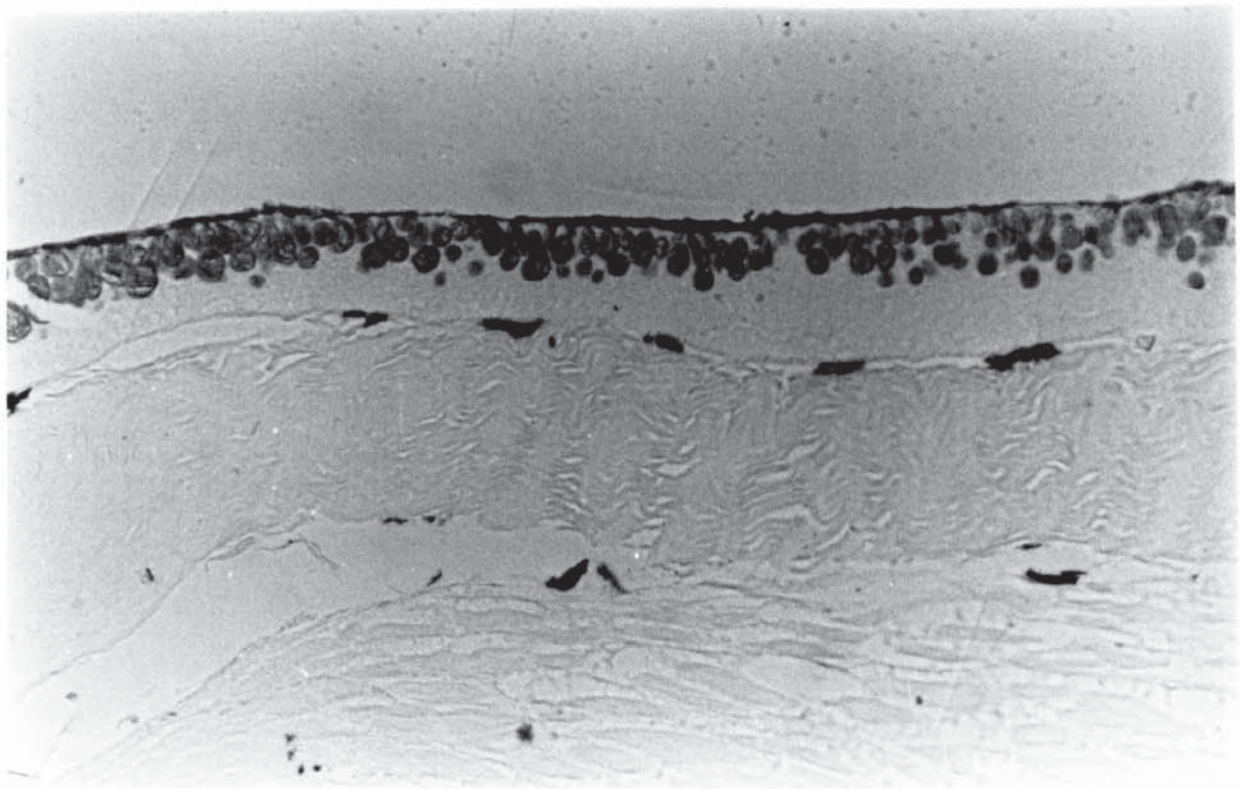

Total W.B.Cs Count
Lymphocytes
Macrophages
Neutrophils
Acidophils
Basophils
Monocytes
60
20
5
4
3
2
1
0
1
2
3
4
× 1000 leucocytes / ml³
Time / month

Fig. (23) Section through control fish skin to demonstrate the sialic acid (X100).

Fig. (24) Section through infected fish skin. Note the reduction in numbers of goblet cells together with their small size. A thin layer of mucous covers the skin surface. Some parts are devoied of mucous (arrowed) (X100).



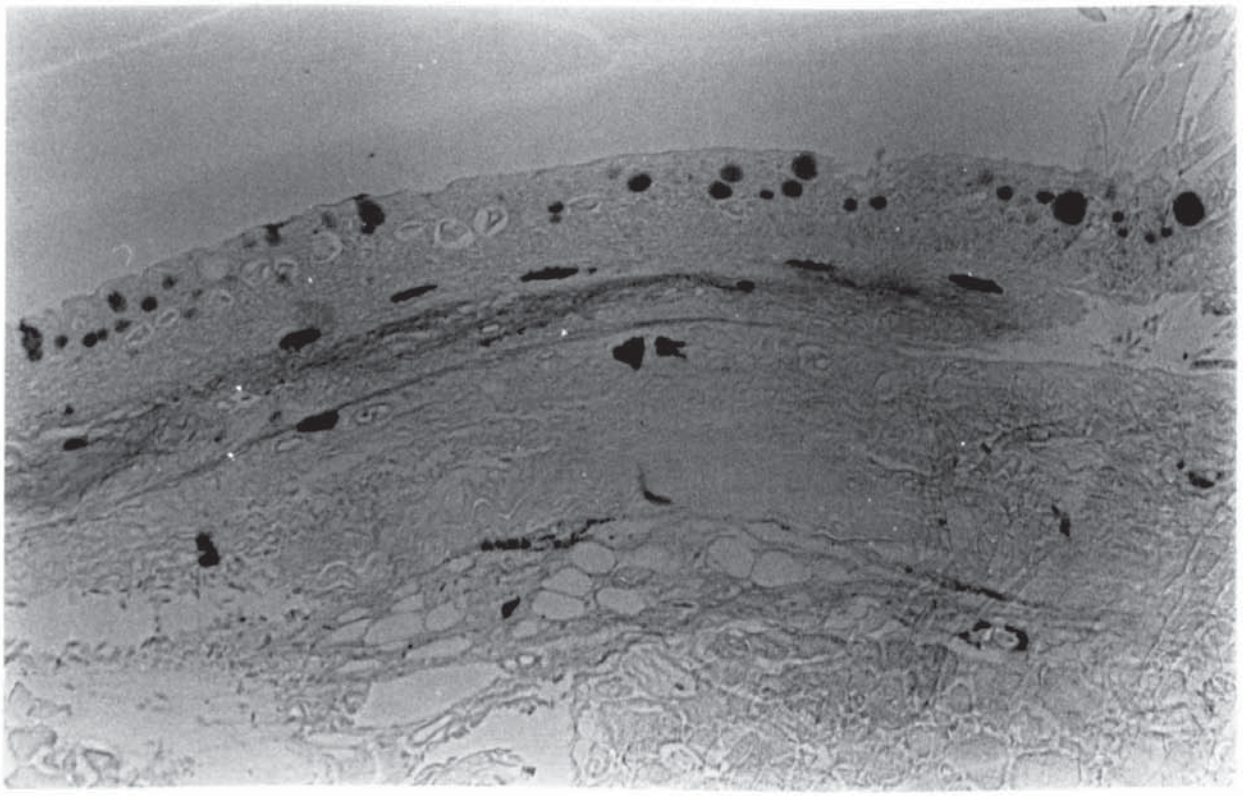


Fig. (25) Changes in length:body length (Δ—Δ) and weight:body weight (●—●) ratios of major lymphoid organs following infection

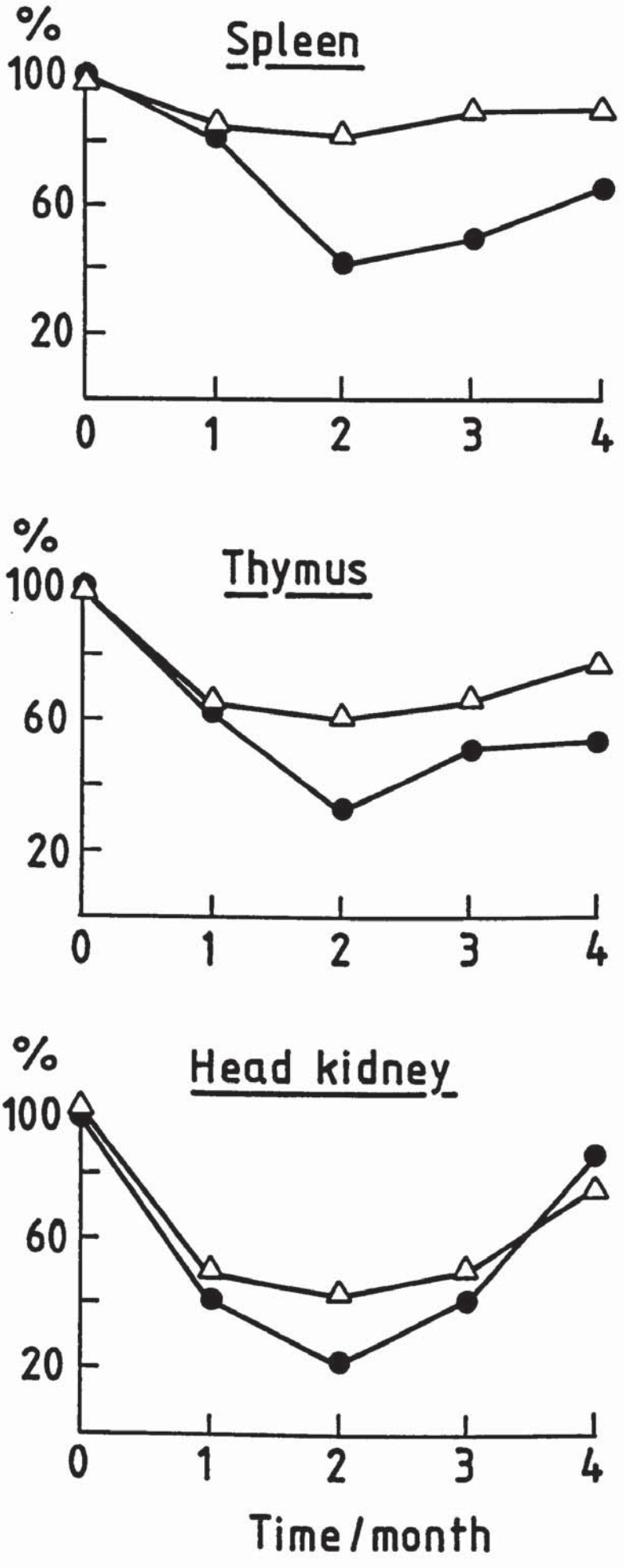
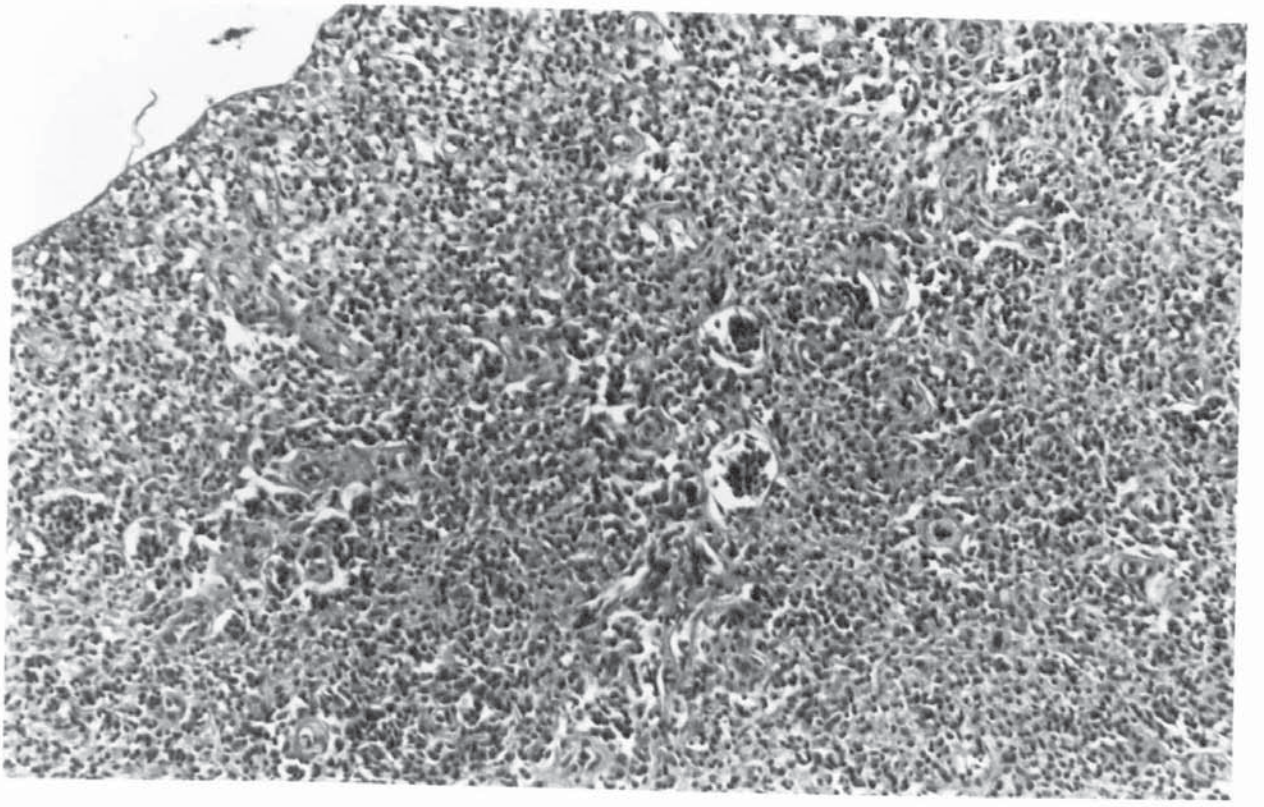

%
Spleen
100
60
20
0
1
2
3
4
%
Thymus
100
60
20
0
1
2
3
4
%
Head kidney
100
60
20
0
1
2
3
4
Time / month

Fig. (26) T.S. in control fish spleen (H.E.X 100).

Fig. (27) T.S. in infected fish spleen. Note the presence of germinal centres (arrowed) (H.E.X100).



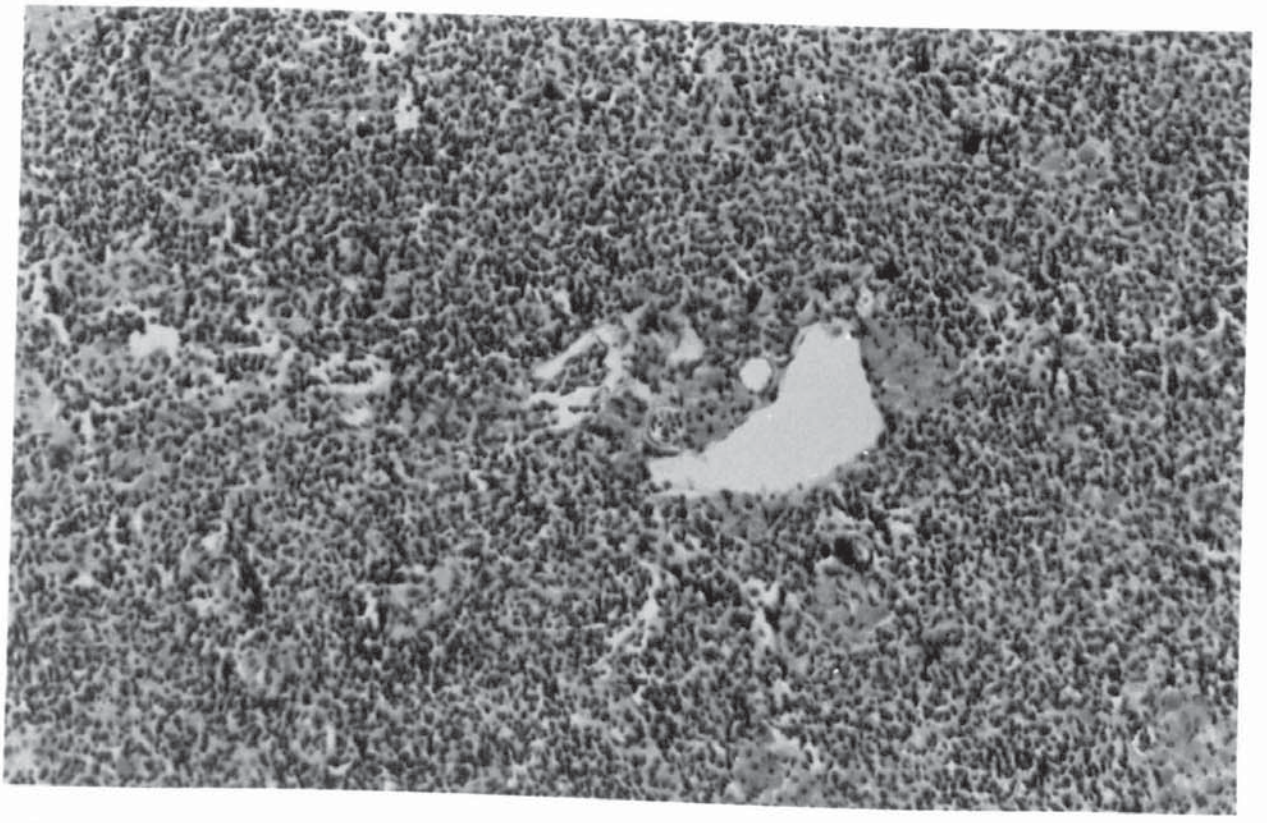
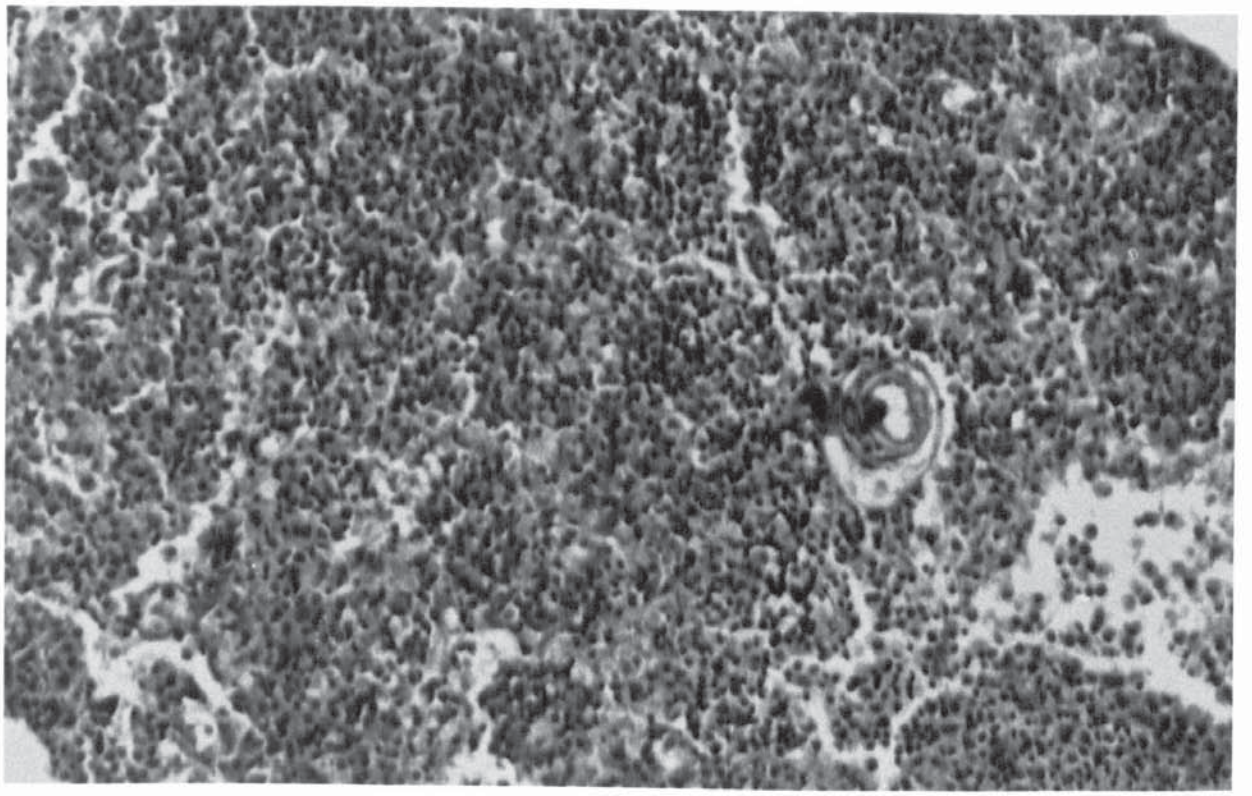
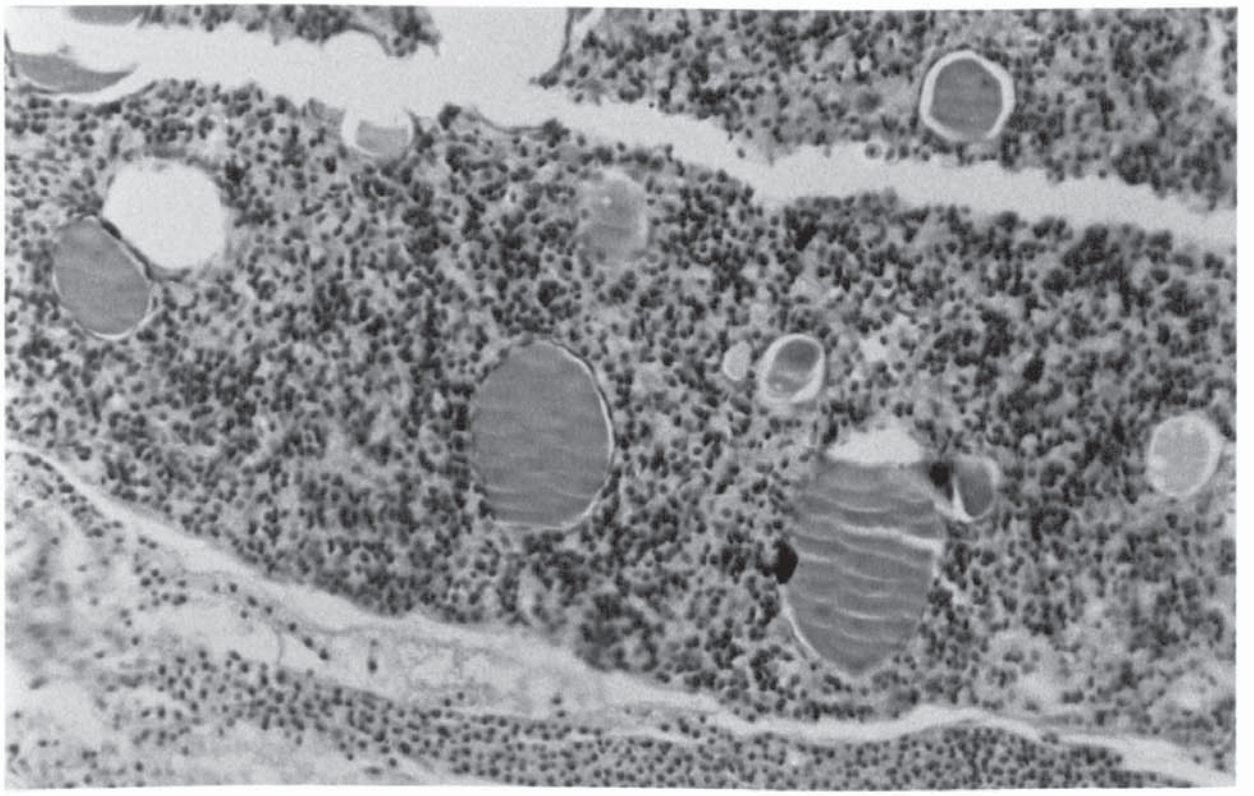


Fig. (28) T.S. in control fish anterior kidney (H.E. X100).

Fig. (29) T.S. in infected fish anterior kidney. Note the spherical bodies which surrounded by reticular fibre sheath and enclosing reddish brown pigments (H.E. X100).





CHAPTER V

STRESS RELATED CHANGES IN THE BLOOD AND TISSUES OF INFECTED FISH

5.1 Introduction

Studies in the previous chapter have revealed that carp maintained at 10°C for prolonged periods exhibit an increased susceptibility to fungal infection. Carp are eurytherms with a preferred temperature range of 19-29°C and stress may occur at temperatures below 15°C (Elliott, 1981). As discussed in chapter 4, one effect of stress may be to impair the immune response and promote infection; and infection itself may result in additional stress to the host. In fish, exposure to adverse factors is known to result in a series of physiological changes which in many respects are analagous to those of the mammalian stress response (Peters, 1979). These changes include the release of adrenocorticotropic hormone (ACTH) from the adenohypophysis and the subsequent release of "stress hormones" (catecholamines and corticosteroids) from the interrenal gland. This in turn brings about a number of secondary changes in the physiological status of the fish which may be monitored by appropriate biomedical test procedures. These have been reviewed by Wedemeyer and McLeary (1981) and include the measurement of circulating red blood cell numbers and haemoglobin content of the blood; together with measurements of tissue changes such as alterations in the levels of glycogen, protein and lipid in the liver, and ascorbic acid (vitamin C) in the interrenal tissue. In the present chapter erythrocyte counts and haemoglobin levels in the blood of infected fish have been measured using a coulter counter and haemoglobin meter.

Possible tissue changes associated with stress have also been examined in the liver and head kidney of infected fish using a range of histochemical and biochemical techniques.

In addition to the possible effects of stress outlined above, previous reports have suggested that some of the physiological changes observed in Saprolegnia infected fish may be due to loss of epitheleal integrity as a result of external damage caused by the fungus. Several authors have reported that death in Saprolegnia infected fish may result from osmoregulatory breakdown caused by the increased osmotic influx of water through the damaged integument. Haemodilution and anaemia have both been reported following infection of fish by Saprolegnia species (Hiroshi, 1969; Mulcahey, 1971; Roberts, 1972; Roberts et al., 1970, 1971, 1972; Gardner, 1974; Leivestad et al., 1976; Richards, 1977; Richards and Pickering, 1979; Pickering and Willoughby, 1982). Another aspect of the work presented in this chapter is a histological examination of infected skin at different stages post-infection. In particular, the surface of infected skin has been studied using scanning electron microscopy in order to gain further information about the nature of external changes taking place in the skin during saprolegniosis.

5.2 Materials and Methods

5.2.A General. Fungal zoospores were produced and fish infected by exposure to live zoospores of S. diclina, as described in the previous chapter. Prior to infection fish were maintained at 10°C for two weeks and control values for all the studies described in this chapter were obtained from a group of five such fish. Twenty

fish were exposed to infective zoospores and maintained at 10°C for four months. Groups of five infected fish were sacrificed at monthly intervals and the following experiments carried out on each. A group of five non-infected fish which had been maintained at 20°C were also used to provide material for comparison with the other groups described above.

5.2.B Haematological investigations. Red blood cell counts were determined by Coulter counter (Coulter Electronics Ltd.) and haemoglobin in the blood was measured using a haemoglobin meter (Corning-Eel Scientific Instruments).

5.2.C Histochemical investigations of tissues

5.2.C.a. Liver Samples of liver for glycogen studies were fixed in Bouin's fluid and stained with Periodic Acid Schiff's (PAS) reagent (Appendix I). Specificity of staining was determined by examining sections treated with diastase enzyme prior to staining (Bedi and Horobin, 1976). For studies on lipid, samples of liver were fixed in formol calcium (appendix I) and stained with Sudan Black (McMannus, 1946). Protein in the liver was studied following fixation in 10% formalin and staining with Bromophenol Blue (Chapman, 1975).

5.2.C.b. Head kidney

5.2.C.b.i. Ascorbic Acid

Since the head kidney has been shown to be the major site of ascorbic acid storage, the present study has also examined ascorbic acid levels in this tissue. Head kidney tissue from infected and non-infected fish maintained at 10°C was compared with that of non-

infected 20°C fish. Head kidney was fixed in Zenkerformol fixative (Appendix 1) and prepared for histology following the method described by Girond et al. (1936).

5.2.D Biochemical investigations of fish liver

5.2.D.a. Determination of total glycogen in fish liver

Glycogen was determined in tissues according to the method of Seifter et al. (1950).

Reagent:

a. 30% KOH Solution (30gm/100ml dist. H_2O).

b. 95% ethanol

c. 95% Sulphuric acid

d. 0.2% anthrone solution: This constitutes the working reagent, and is made by dissolving 0.2gm of anthrone in 100ml of 95% Sulphuric acid (the reagent is not stable in solution for extended periods of time and is best made up daily as needed).

Procedure:

One gram of liver tissue (immediately after dissection) was dropped into tubes containing 10ml of 30% KOH. Tubes were heated for 20 minutes in a boiling water bath. One ml of the digested solution was transferred to a centrifuge tube and 1.25ml ethyl alcohol (95%) was added. This mixture was shaken, then left in a water bath for 30 seconds to one minute, cooled and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was decanted and the tube allowed to drain on filter paper for 2 minutes, the precipitate was dissolved in 5ml distilled water. A 0.1ml of aliquot was mixed with 0.9ml distilled water in a test tube, then transferred to an

ice bath. The mixture was mixed with 5ml anthrone reagent, transferred to a boiling water bath for 10 minutes, allowed to cool at room temperature and the absorbance measured at 620nm against a blank (1ml distilled water + 5ml anthrone reagent) on an LKB 4050 spectrophotometer.

The amount of glycogen was calculated according to the following equation:

$$\frac{\text{Conc. of unknown X dilution}}{1.11 \text{ X weight of tissue}}$$

where, 1.11 = the factor determined by Morris (1948) for the conversion of glucose to glycogen.

The calibration curve was constructed from different concentrations of a working glucose standard solution containing 100mg of glucose/ml distilled water. (Fig. 30 Appendix 2)

5.2.D.b. Determination of total lipid in fish liver

The lipid content in tissues was determined by the phospho-vanillin method of Knight et al. (1972).

Reagent:

a. Phosphovanillin reagent: 0.6gm of vanillin was dissolved in 8-10ml absolute ethanol, the volume was made up to 100ml with distilled water and 400ml phosphoric acid was subsequently added with constant stirring.

b. Chloroform-methanol mixture: two volumes of chloroform were added to one volume of absolute methanol.

Procedure:

One gram of liver tissue was dropped in 19ml of chloroform/methanol mixture, immediately after dissection, and was

homogenized. The homogenate was transferred to a centrifuge tube and centrifuged at 4000 r.p.m. for 15 minutes. The supernatant was removed to a pre-weighed centrifuge tube and a few mls of saline solution added, the contents were shaken, centrifuged and the upper layer (aqueous phase) discarded, this washing procedure was subsequently repeated three times. The contents of the tubes were dried under vacuum, and to the residue one ml chloroform/methanol mixture was added. A 0.1ml lipid extract was removed and 5ml concentrated sulphonic acid added. Tubes were placed in a boiling water bath for 10 minutes and allowed to cool at room temperature. A 0.4ml aliquot was taken to which 6ml of phospho-vanillin reagent was added (shaking well to ensure mixing), then tubes were incubated in the dark for 45 minutes and the absorbance measured at 525nm against a blank (0.4ml concentrated sulphuric acid plus 6ml phospho-vanillin reagent). The calibration curve was constructed from a series of dilution of pure olive oil containing from 0-10mg olive oil/ml absolute ehtanol (Fig. 31 Appendix 2).

5.2.D.c. Determination of total protein in fish liver

Total protein was determined according to Lowry et al. (1951). To 1ml of homogenate, 1ml of 5% TCA was added, the white precipitate thus obtained was filtered. 1.0ml of Lowry E was added to 0.2ml of filtrate and left to stand for 10 minutes. 0.1ml of Lowry D was then added, stirred on a "Whirly mix" and left for 30 minutes. Finally, 5.0ml of distilled water was added and absorbance read at 740nm against a blank on a LKB 4050 spectrophotometer. A standard protein curve (Fig. 32, Appendix 2) was derived by using different concentrations of bovine albumin ranging from 0 to 1mg/ml.

Statistics:

Statistical analysis of the results was carried out using Student's "t" test.

5.2.E Histological investigation of skin

Samples of carp skin were fixed in 10% formalin for general histology and to investigate fungally infected skin (Kelly et al., 1962) sections were stained with haematoxylin and eosin. A portion of muscle tissue underlying an area of infected skin was fixed in Gomori 1-2-3 fixative (Appendix 1) and stained according to Milligan (1946).

5.2.F Scanning electron microscopy

Pieces of skin from dorsal fin, caudal fin, trunk and different infected parts of the body showing fungal lesions were removed from the fish, briefly washed in physiologicaly saline solution, and left at room temperature to dry. The dried specimens were mounted on an aluminium holder and gold was evaporated onto them in a sputter device. The samples were examined using a Cambridge SEM 150 scanning electron microscope and photomicrographs were taken on Kodak 135 Black & White film with a 35-mm camera.

5.3 Results

5.3.A Haematology

The mean red blood cell (RBC) count in uninfected control carp maintained at 10°C for two weeks was 2.84 x $10^6/ml^3$. Uninfected fish kept at 20°C showed a mean count of 2.58 x $10^6/ml^3$ which was not significantly different from the 10°C control fish. Fig. (33) (Table 16, appendix 2) demonstrates that infected fish maintained at

10°C showed a marked decline in RBC numbers throughout the four months of observation.

Fig. (33) shows that the haemoglobin content in the blood of uninfected control fish maintained at 10°C was 9.22gm/100ml blood. There was no significant difference between this value and that obtained for uninfected fish maintained at 20°C (8.90gm/100ml). However, there was a highly significant difference between the haemoglobin content of the control groups and the values obtained for infected fish. As with the RBC counts, the decline in blood haemoglobin content was apparent one month after the start of the experiment and persisted throughout the four month period of observation.

5.3.B Histochemical changes in the liver of infected fish

Comparison of figs. 34 and 35 shows that there is a decrease in glycogen content in the liver of infected fish. In the normal liver the glycogen staining is concentrated around the nucleus and throughout the cytoplasm of the liver cells. Glycogen deposits are also seen at the cell borders and throughout the intercellular spaces. During infection the liver appears highly vacuolated with degenerative nuclei. Large extracellular concentrations of glycogen are also seen, expecially near the blood vessels.

The lipid content of the liver showed an increase in quantity in the infected fish. In normal liver sections, the lipid deposits are concentrated around the nuclei of the hepatic cells, and are generally distributed throughout the intercellular spaces (Fig. 36). In infected liver (Fig. 37), the lipid is situated towards the

periphery of the cells and at the same time, the nuclei become eccentrically located. Many nuclei show degenerative nuclear changes and mitotic figures are sometimes seen. In later stages of infection the lipid granules become randomly distributed throughout the hepatic cell cytoplasm and also begin to appear in large localised deposits in the intercellular spaces. These concentrations of extracellular lipid give the liver a fatty appearance and the liver eventually loses its distinct cellular configuration.

In infected fish the levels of liver protein showed a transient decrease after two months (Fig. 38b). In normal liver sections (Fig. 38a) the protein is concentrated around the centrally located nucleus and at the periphery of the hepatic cells with a high concentration of protein in intercellular spaces and around the hepatic blood vessels. In sections from infected fish, the areas around the blood vessels are relatively free from protein. Intracellular protein deposits are also much reduced. Fig. (39b) illustrates that the level of ascorbic acid staining in the head kidney of infected fish was far less than that seen in non-infected fish kept at 10°C (Fig. 39a) or at 20° C.

5.3.C Biochemical changes in liver of infected fish

The results for total glycogen, lipid and protein analysis in the liver of infected fish are given in Fig. 40 (Table 17, appendix 2).

Total liver glycogen varies somewhat over the course of the experiment. There is a significant decline after one month followed by a partial recovery to control values at two and three months.

Liver glycogen values are lowest in infected fish four months after the start of the experiment. There was no significant difference between glycogen levels in non-infected fish kept at 10°C and fish maintained at 20°C.

Fig. 40 also reveals that total lipid concentrations in the liver of infected fish were also variable. However, despite a return to control levels at two months, the general trend observed was a rise in total liver lipid. Significantly higher values were present at one, three and four months post-infection. Once again there was no significant difference between lipid levels in the livers of non-infected fish held at 10°C and those maintained at 20°C.

Measurements of total liver protein shown in Fig. 40 also vary from month to month. There was a slight increase in protein concentration one month after the beginning of the experiment, followed by a transient decline after two months. Levels of protein measured in the liver of infected fish at three and four months did not differ significantly from the values obtained for non-infected controls held at 10°C or 20°C.

The results observed using these various methods of biochemical analysis are generally reflected in the histochemical studies reported in the previous section.

5.3.D Histology of infected skin

In normal fish, (Fig. 41) skin is composed of two major layers, the epidermis and dermis, separated by a marked basement membrane. The epidermis is a stratified epithelium some 10 or 12 cells thick, with a single cell columnar basal layer, a middle layer of rounded

cells with prominent nuclei and a surface layer of squamous cells. Mucous secreting cells were found in the middle and upper layers, and granular cells in the middle region. The dermis consists of two parts, the outer layer (stratum spongiosum) represented by a loose network of collagen and reticulin fibres which support the melanophores and scales (otenoid type), and the inner layer (stratum compactum) mainly composed of dense collagen bundles. A vascularized subcutis of loose connective tissue separates the dermis from the muscle layers of the fish. Melanophores occur between the epidermis and dermis immediately beneath the basement membrane and also between the stratum compactum and the subcutis.

In non-infected fish maintained at 10°C and 20°C the histological appearance of skin and muscle was not altered. In the fish kept at 10 °C and exposed to fungal zoospores, infection was not only found on the artificially damaged areas but also on apparently undamaged sites on the body. Special attention was directed towards the latter in order to observe the initial epidermal changes which normally occur before any ulceration or fungal infections are visible. The problems involved in keeping the superficial layers of the prepared pieces of skin intact throughout the technical procedures must be emphasized. It was repeatedly noted that surface areas with fungal infection were lost during the preparation and this must be kept in mind during the evaluation of the material.

The first signs of the UDN infection are thin dark circles in the skin of the dorsal part of the body which can be seen by naked eye. The fungal growth appears inside these dark circles after 7-10 days.

Often, the circles resemble a crack limiting the affected area from normal skin. Sometimes a blanched area appears within the dark ring before fungal growth is noticed (premycotic stage). Such areas showed only small epidermal changes, but later the cells became rounded and cell adhesion is lost over large areas, resulting in extensive intercellular spaces. The staining methods applied did not indicate any mucous in the region and only a few non-secreting mucous cells with pyknotic nuclei were observed. In the dermis, the cell membranes of melanophores were ruptured and the pigment granules were free in the dermal tissue. Cells from the dark circles became detached from the skin surface and gave rise to decayed areas of skin where skin lesions developed. The lesion becomes covered with fungal hyphae. In the centre, the epidermal layers disappear, and surrounding the lesion centre there is a region where the basement membrane is intact, but exposed or covered with two or three layers of rounded cells. Outside this region is a zone of multilayered epidermis of normal thickness but consisting of rounded cells. The changes are associated with increased volume of the intercellular spaces as a result of broken cell contacts. In regions adjacent to normal skin the flattened superficial squamous cells apparently lose their intercellular contact and show evidence of rounding up. As lesions became larger they appeared as blanched depressions in the skin, due to the radiating mycelial mass which was coloured off-white or white. The skin of the affected area and the junction between normal and affected skin were paler in colour due to contraction of malanophores.

At the later stages of infection, lesions appeared as an open sore on the flank of the fish with frequent indications of fungal infection at the centre of the lesion. All the melanophores which were still intact were very contracted. As the fungus radiates away from the focus of the infection, it first destroys the epidermis. As the infection progresses, the fungus can subsequently penetrate the basement membrane, and extend into the dermis. The stratum spongiosum was invaded by fungal hyphae and the stratum compactum appeared to be swollen, irregular, ruptured and necrotic (Fig. 42).

Extensive oedema of the hypodermis was present, radiating away from the mycelial mass into non-affected areas. The oedema extended into the muscle mass, resulting in marked myofibrillar degenerative changes, followed by swelling, loss of nuclei and myofibrillar loss. Frequently, in infected areas, changes in the superficial and deep muscle masses ranged from complete sarcoplasmic loss to mild degeneration. The inter-myotomal connective tissue was swollen and had large numbers of blood cells, with an increase in collagen fibres (Fig. 44).

5.3.E Scanning electron microscopy

The normal outer surface of the integument is coated with a high concentration of mucous arranged in elongated repetitive ridges with intervening grooves (Fig. 45). During the first stages of infection mucous begins to decrease in quantity and shrinks to form small droplets which are distributed irregularly over the skin surface (Fig. 46). During the premycotic infection stage clear white patches are seen scattered on the skin surface. Mucous cell pores may be seen perforating the lamellar surface of the skin which is

devoid of mucous (Fig. 47). During this stage of infection the fungus is able to colonise the skin and the hyphae penetrate through the epithelial cells causing the loss of epithelial integrity and continuity and resulting in lesion formation (Fig. 48). In severe infections, the epidermal layer is lost and the fungus penetrates down to the dermis and muscular layer (Fig. 49). At different areas on the body surface, what appear to be secondary spores (the infective agent) and spore cysts appear attached to fish skin (Fig. 50).

5.4 Discussion

Work in this chapter has investigated changes in a number of physiological parameters which are thought to be indicative of a stress response in fish. The results presented here indicate that there is a significant decline in both RBC counts and haemoglobin concentration in the blood of infected carp. Reductions in red blood cell numbers and haemoglobin percentage have also been found in marine fish (Anderson and Conroy, 1970) and juvenile chinook salmon infected with vibriosis (Cardwell and Smith, 1971). Lowered enythrocyte counts and haemoglobin levels are also characteristic of various spontaneous and experimental anaemias of trout and salmon (Kawatsu, 1968; 1969; Smith, 1968; Smith and Halver, 1969). Wedemeyer and McLeary (1981) have suggested that lowered enythrocyte counts in fish may be an indication of haemodilution due to impaired osmoregulation; they also suggest that this may also give rise to lowered blood haemoglobin levels or, that the latter may result from nutritional disease. Both these explanations may account for the results observed here. In fungally infected carp the integrity of

the integument is destroyed. This is clearly illustrated by the histological and scanning electron microscope studies presented here, and has been widely reported by other authors. It is therefore reasonable to propose that where the integument is breached in this manner, there will be an osmotic effect. In fresh water a net inflow of water would be expected, leading to osmotic haemodilution (Roberts et al., 1970; Roberts, 1972). Such haemodilution leads to fluid imbalance and circulatory failure due to inability to maintain circulatory blood volume. Roberts (1972) has suggested that this may be a major factor leading to fish death. The observed decline in erythrocyte numbers and haemoglobin concentrations reported here, may result from haemolysis caused by osmotic dilution of the fish blood, or by haemorrhage via infected and ulcerated areas, or a combination of both factors.

Gardner (1974), working with UDN-affected salmon, found that infected fish had significantly lower plasma osmotic pressure, sodium and protein concentrations than uninfected fish. Also, Saprolegnia infection influences the salt and water balance of the brown trout (Richards & Pickering, 1979) and a significant inverse relationship has been demonstrated between serum osmotic pressure (or Na^+ concentration) and the degree of severity of the infection. Decrease in the concentrations of K^+, Ca^{+2}, Mg^{+2} and total serum protein were also evident in infected fish. The net rates of loss of electrolytes and proteins together with the increased osmotic influx of water are functions of the extent of fungal colonization over the body surface and will determine the survival time of fish in fresh water.

An additional or alternative explanation for the decline in blood haemoglobin may be related to cessation or impairment of feeding at low temperatures. The carp held at 10° C in the present studies did not increase in weight as rapidly as the fish maintained at 20°C. This may have been a direct result of low temperatures on feeding (Elliott, 1981) coupled with the stressful effects of infection. Erythropoietic impairment due to dietary deficiency may therefore have been partly responsible for the low haemoglobin values recorded here.

The liver plays an important role in regulating the nutrition of the body tissues and a general account of its functions may be found in a number of texts (Wills, 1985). The liver is involved in carbohydrate, fat and amino acid metabolism and also has major roles in protein synthesis and as a storage organ for glycogen, protein vitamins and iron in the form of ferritin. Stress induced changes in physiology might therefore be expected to result in a number of changes in the liver tissue. In the present study changes in liver glycogen in infected fish may have arisen for a variety of reasons. Hyperglycaemia has been reported as part of a general stress response (Houston et al., 1971a,b; Wardle, 1972; Wedemeyer and McLeay, 1981). The studies reported here suggest that carp may also show alterations in glycogen content of the liver following chronic stress brought about as a result of prolonged exposure to low temperatures and/or infection by Saprolegnia. The appearance of glycogen deposits near to blood vessels in the liver of infected fish, may indicate that low glycogen levels may also have resulted from loss of blood glucose during haemorrhage from fungal lesions.

Prolonged demands on the liver glycogen stores may therefore lead to depletion in stressed and diseased fish.

The liver also plays a significant part in fat metabolism. The increase in liver lipid seen here in both histochemical and biochemical studies may be a result of poor feeding rather than a direct consequence of stress. The increase in liver lipid content of infected carp and the fatty appearance of the liver may be due to an impairment of lipid catabolism resulting in the prolonged storage of lipid in the liver (Stein and Stein, 1967). However in mammals, the fat content of the liver increases during fasting (Wills, 1985) so the fatty degeneration of the liver seen here may also be related to impaired feeding behaviour at low temperatures.

The transient decrease in protein concentration seen in the liver of infected carp may have been a consequence of disturbances in serum protein levels due to loss of serum proteins via epidermal lesions. Wilkins et al. (1970) stated that UDN-infected atlantic salmon had significantly lowered total serum protein levels. Carbery (1970) and Mulcahy (1971) also made the same finding in wild trout (Salmo trutta) affected with UDN. It would seem that the fall in total protein of the serum in UDN is due mainly to falls in the A-albumen and α-globulin fractions. The fall in the albumin/globulin ratio (Richards & Pickering, 1979) is due to a fall in the A fraction, since no other fraction shows a statistically significant change. Therefore, in the present study, conversion and utilization of liver protein may have taken place to replace the loss in plasma proteins. This may account for the transient decrease in liver protein observed in infected fish. Ascorbic acid

levels are difficult to measure biochemically and so the present study has restricted itself to a histochemical examination of ascorbic acid deposits in the interrenal gland of the pronephros. Further evidence suggestive of stress in the infected carp is provided by the reduction in ascorbic acid levels in head kidney. Levels of ascorbic acid are known to decrease in infected fish and this is thought to be associated with increased corticosteroid levels in the blood (Pickering & Dunston, 1983; Pickering & Pottinger, 1985).

The histological observations presented here, together with those of other workers, showed that the first indication of UDN is that the superficial squamous cells lose contact and that the intercellular channels develop between the underlying cells, simultaneously, the cells become rounded. The cell membranes of melanophores are ruptured and the pigment granules are often free in the dermal tissue. Clumping of melanin granules in the dermal melanophores causes the white patches (pre-mycotic stage) on the skin surface and accentuates the pale appearance of fungal infected lesion (Johansson et al., 1982).

Fungal mycelia in the present study were predominantly external. The concentration of mycelium outside the body, compared to that present in the lesion, suggests that the external environment provided a better growth medium than the oedomatous and degenerative tissues at the site of infection. A possible explanation for this may be the higher level of oxygen in the water compared to the level in the necrotic areas of the Saprolegnia-infected fish. Liu & Volz

(1976) consider low oxygen levels to be one of the major limiting factors for the growth of Saprolegnia.

In severe fungal infections, the fungus penetrated to reach to the muscle layer and disruption of muscle tissue was also seen here. Stuart & Fuller (1968) have succeeded in obtaining Saprolegnia from a depth up to 1.5cm below the surface of the lesion. The marked oedema found in muscle in the present study is thought to be a direct result of the haemodilution effect (Roberts, 1972; Norland-Tintigner, 1973), since few fungal hyphae were seen in underlying tissues. The myofibrillar degenerative changes in the muscle may be in part due to oedoma, rather than the presence of the Saprolegnia (Neish, 1977). Since, Norland-Tintigner (1973) noticed myodegenerative changes some distance from the mycelial mat in Saprolegnia infections.

The lack of an effective immune response to Saprolegnia infection at 10°C was further emphasised in these histological studies. There was no evidence of leucocytic infiltration in the area of infection, thereby confirming studies presented in the last chapter and elsewhere that carp held at 10°C give very poor immune responses.

Scanning electron micrographs showed a disturbance in the arrangement and the quantity of mucous on the skin surface, which occur at the first sign of infection. A significant factor in the development of UDN is the lack of mucous on the skin surface and the inactivity of mucous cells. According to Roberts et al. (1970) the deficiency of mucous cells is a frequent finding in the region of the lesions. In the initial phases of the disease the epidermal

cells are therefore exposed to the environment without any protective mucous layer which provides an opportunity for the fungus to inoculate on the skin. The scanning electron microscopic studies presented here also emphasise the lack of surface mucous in infected fish reported in the previous chapter. The white areas in the skin (pre-mycotic stage) are caused by the lysed melanophores with ruptured plasma membranes and pigment granules. Johansson et al. (1982) suggested that when the epidermal cells are exposed to water after rupture of the zonula occludens, the effects may also extend to the pigment cells since the basement membrane is no barrier to ions and water. The initial low melanophore index may be due to hydromineral disturbances and/or an action of metabolites released by other necrotic cells.

At the late stage of infection and as a result of heavy growth of the fungal hyphae, the skin lost its integrity and the fungus penetrated through the dermis to the muscle layer giving rise to longitudinal channels which water can go through to cause haemodilution.

The rupture of the zonula occludens and the subsequent exposure of the epidermis to the environment, favours the settling and development of fungal spores (Johansson et al., 1982). Willoughby (1972) stated that the mucous may be a vital complementary factor to activate and support the growth of fungal spores in early phases of UDN when the zonula occludens is no longer intact and the metabolites from exposed epidermal cells may trigger the onset of

the fungal growth. The release of amino acids from the damaged tissue may also attract more zoospores of S. diclina (Smith et al. 1984).

Fig. (33) Changes in erythrocyte count and Hb% values

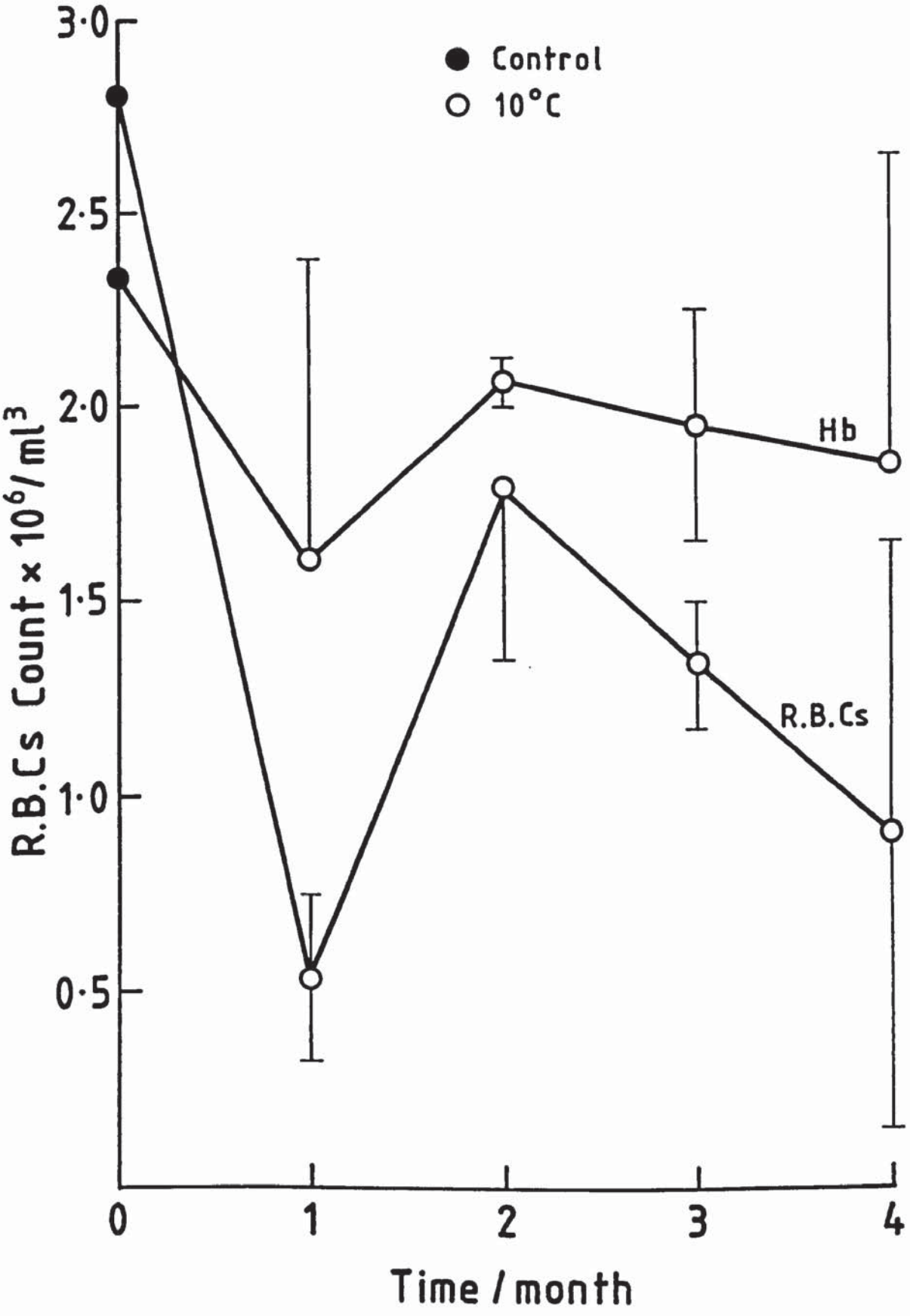
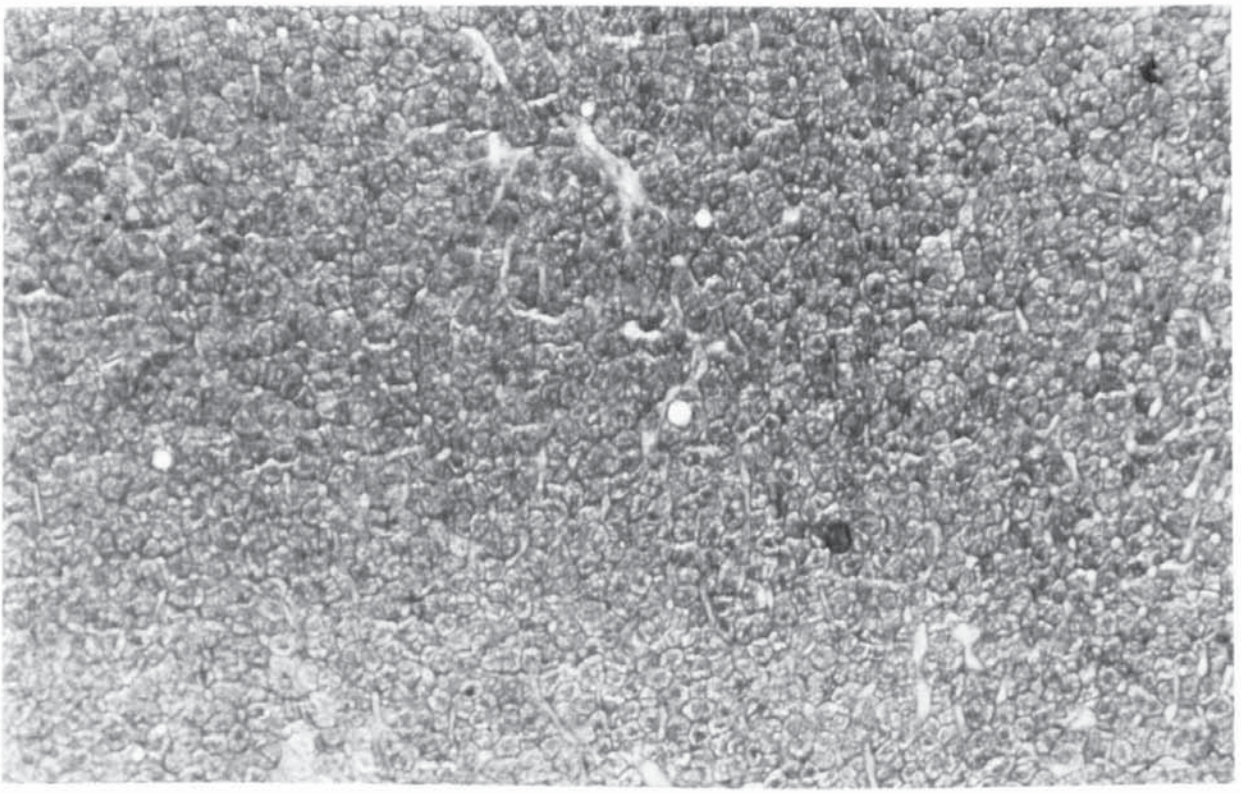

3·0
2·5
2·0
1·5
1·0
0·5
R.B.Cs Count × 10⁶/ml³
Control
10°C
Hb
R.B.Cs
0
1
2
3
4
Time / month

Fig. (34) Section of control fish liver to demonstrate the glycogen content (PAS. X48).

Fig. (35) Section of infected fish liver to demonstrate the glycogen content. Note the numerous vacuoles inside the cells due to utilization of glycogen granules. The cells have degenerative nuclei. The glycogen is mainly concentrated near the blood vessels (PAS. X48).



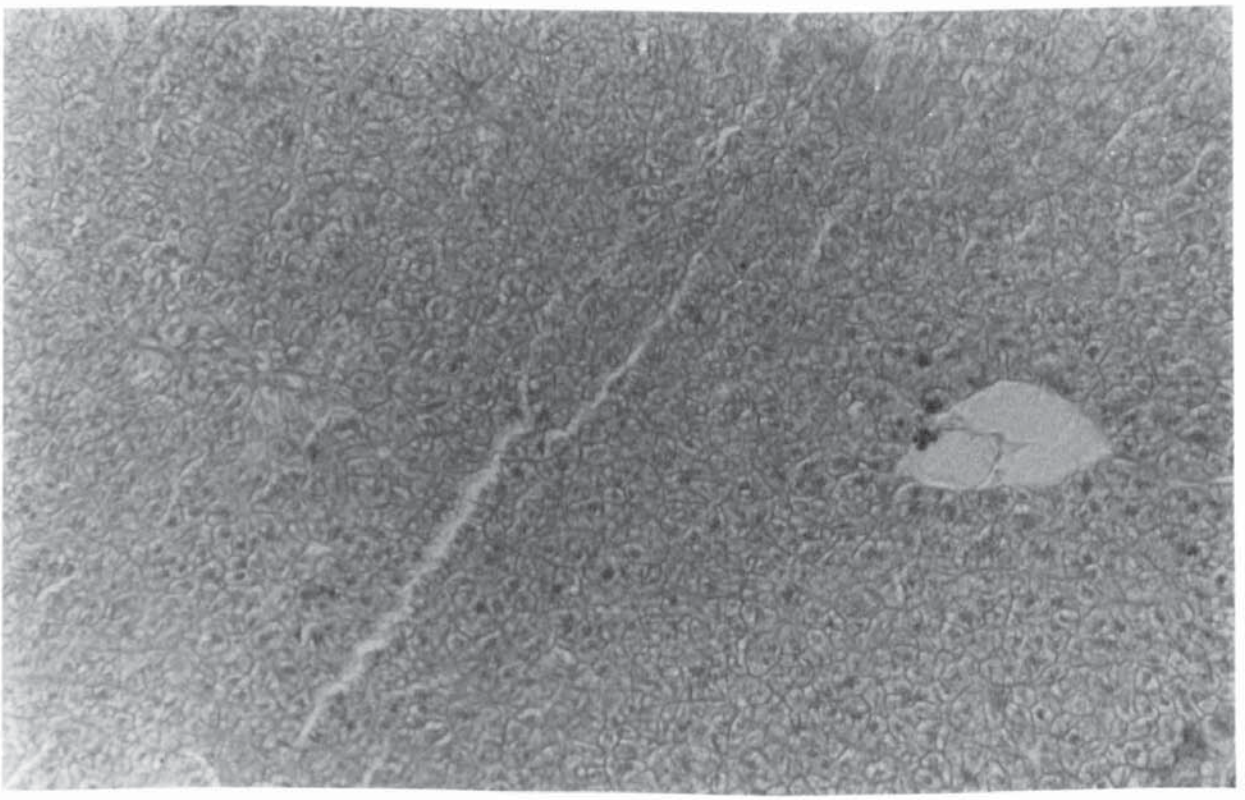
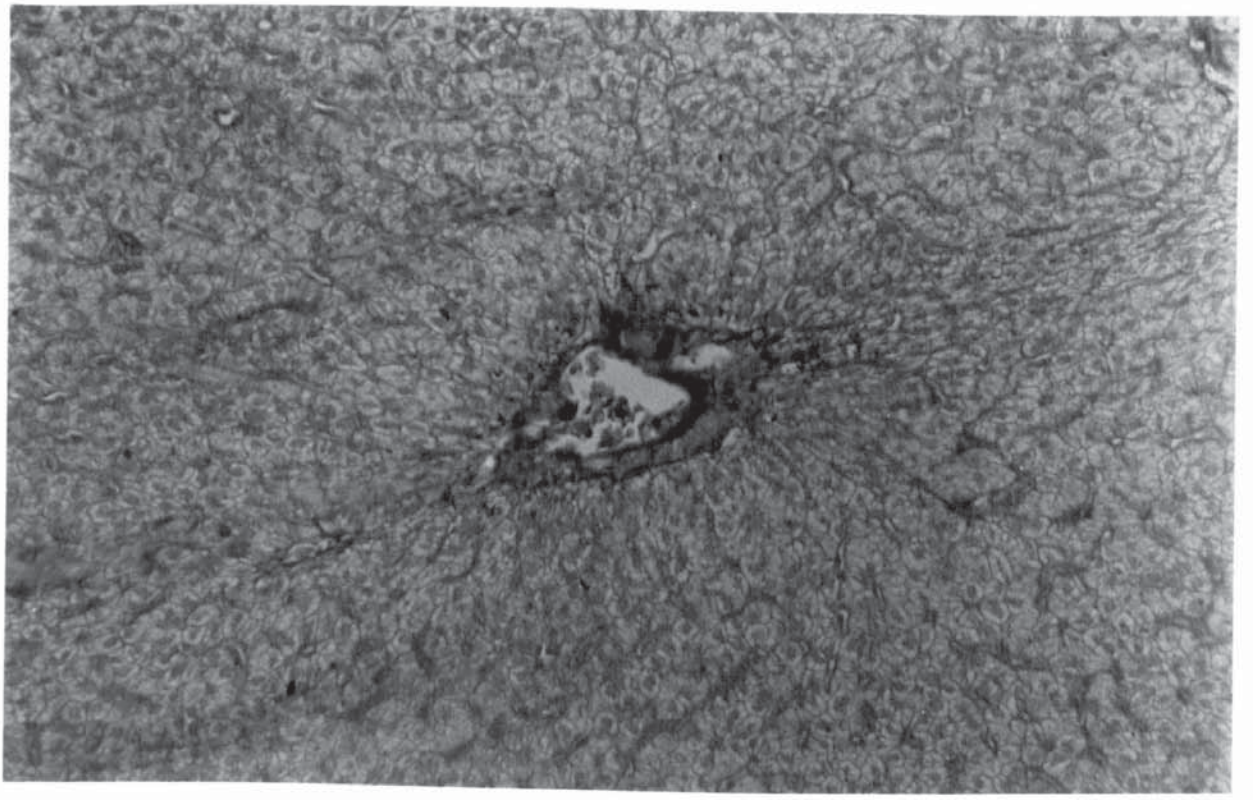


Fig. (36) Section of control fish liver to demonstrate the lipid content. Note centrally located nuclei and homogenous distribution of lipid granules. (Sudan black B.X48)

Fig. (37) Section of infected fish liver to demonstrate the lipid content. The nuclei are eccentrically located and some of them are degenerated with mitotic figures. The lipid granules are concentrated at different sites giving the liver a fatty appearance (Sudan black B.X48).



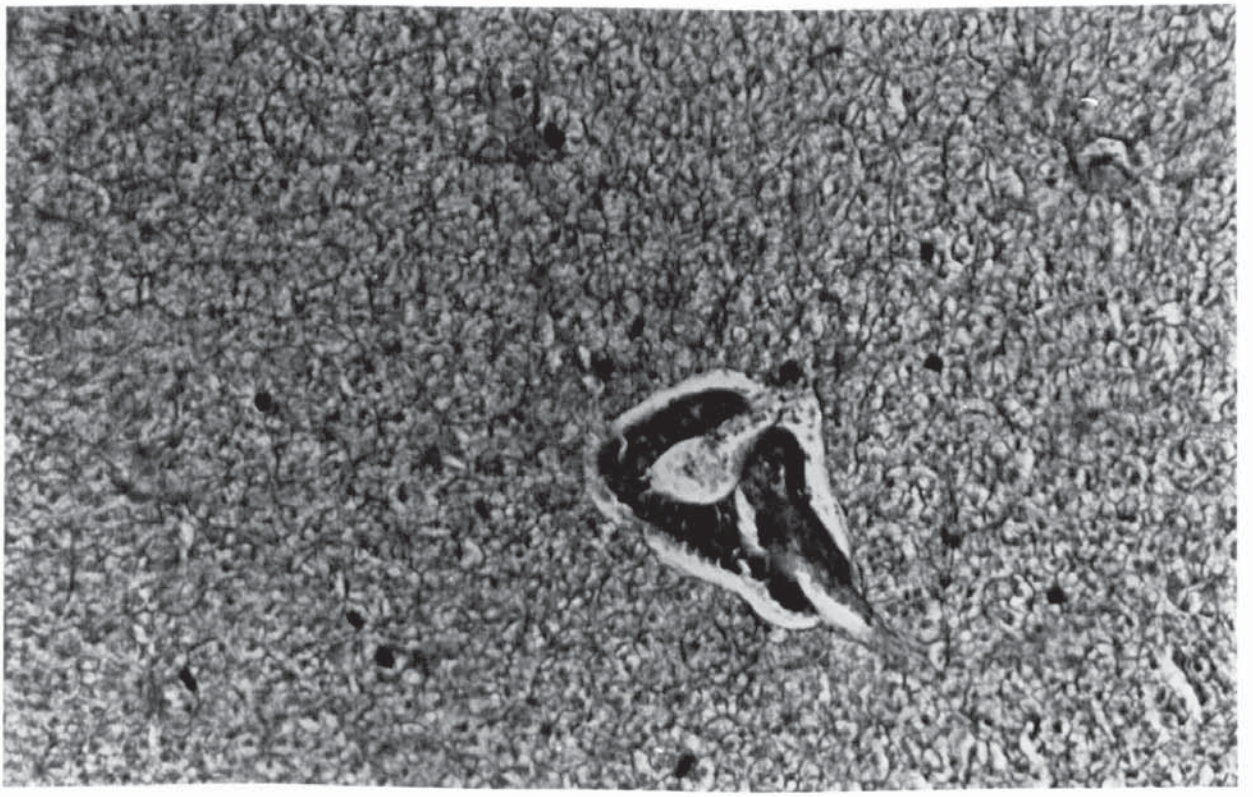
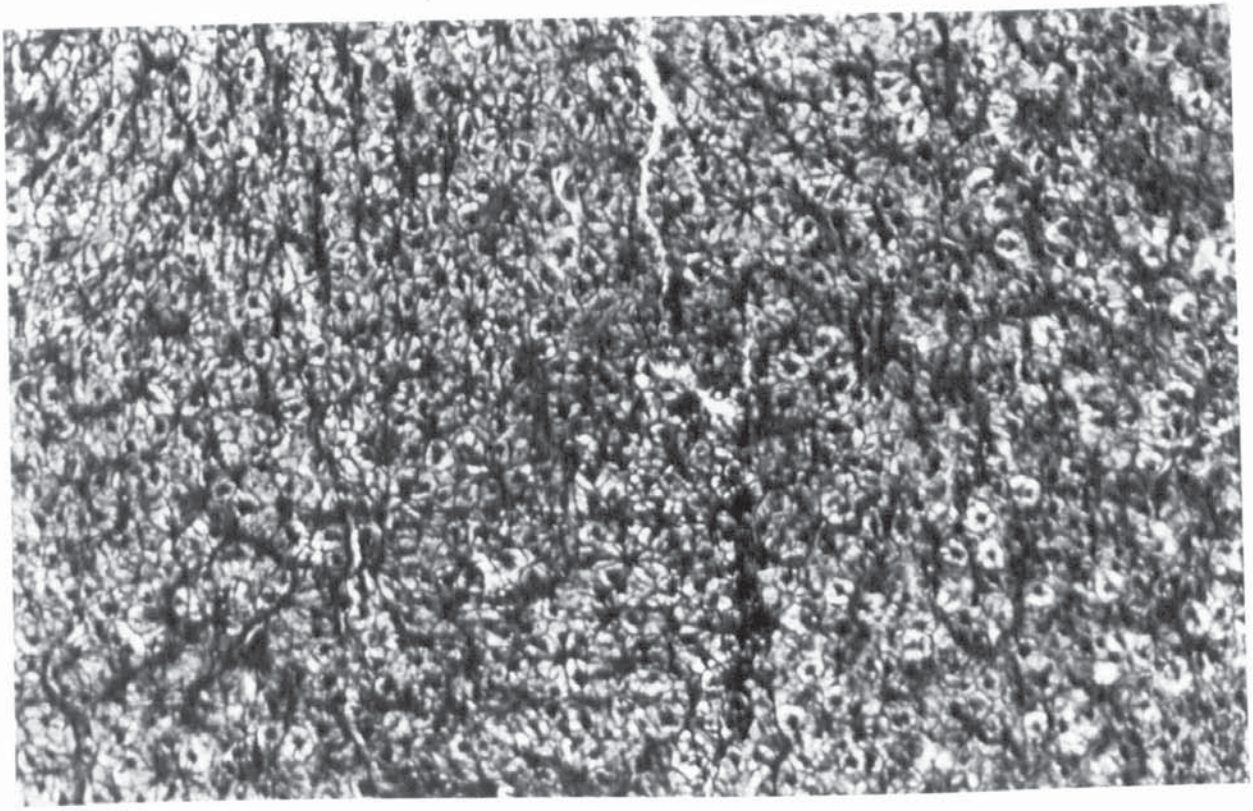


Fig. (38a) Section of control fish to demonstrate the protein content. The protein granules are concentrated around the nuclei of the hepatic cells and in the intercellular spaces. (Bromophenol blue. X 48).

Fig. (38b) Section of infected fish liver to demonstrate the protein content. The protein granules become randomly distributed through the hepatic cell cytoplasm (Bromophenol blue. X48).



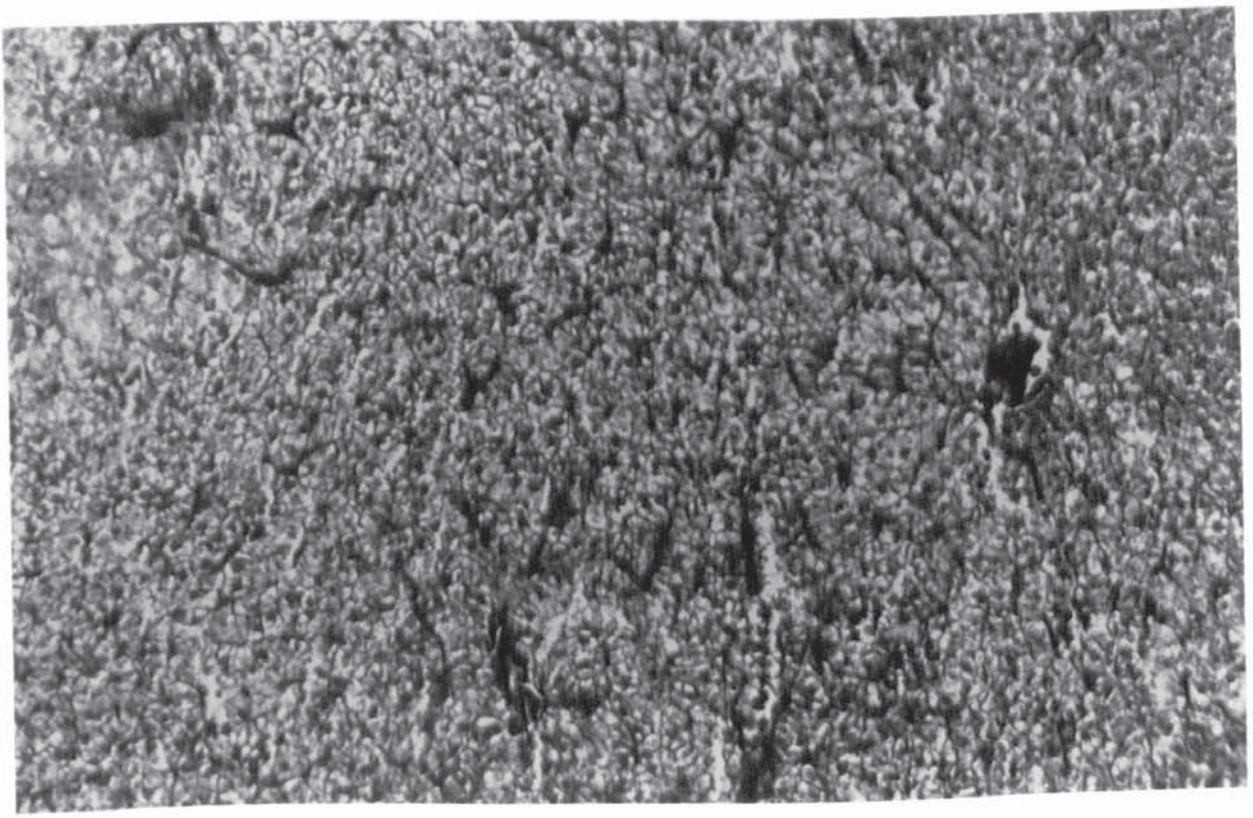
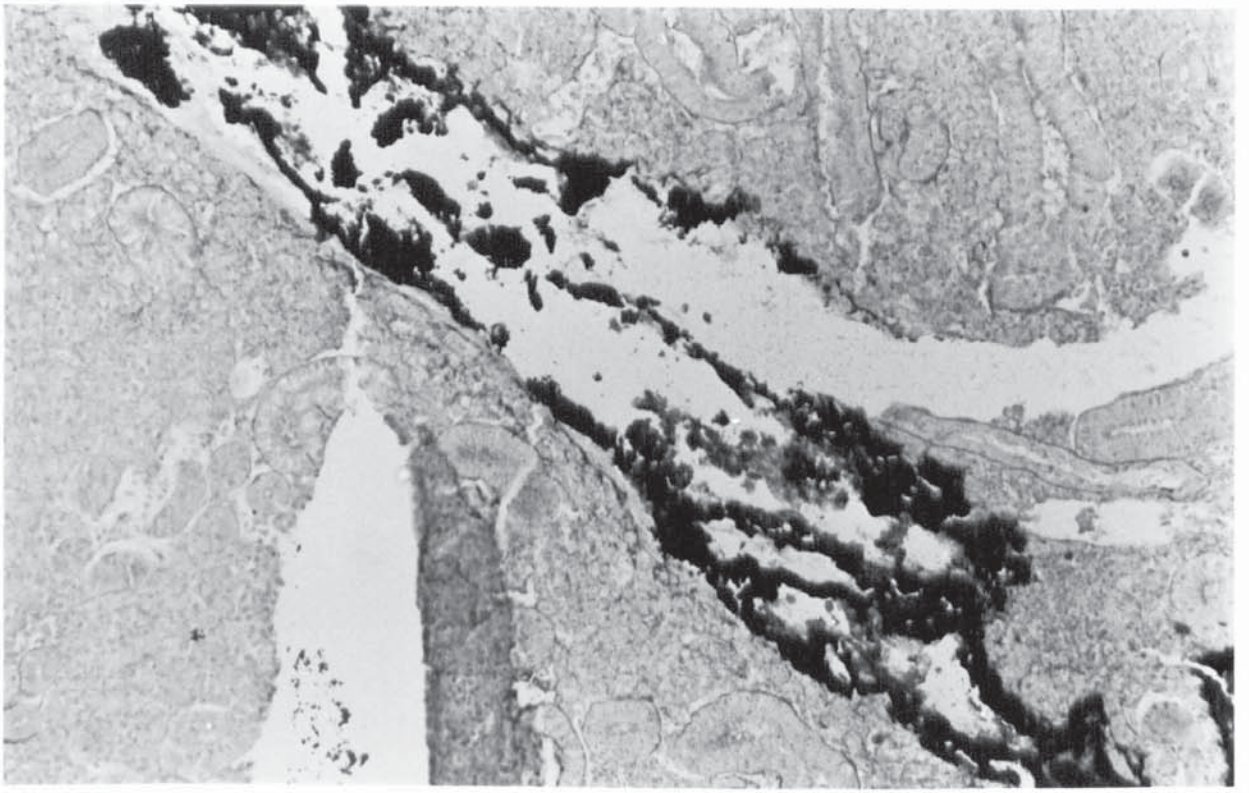


Fig. (39a) T.S. in control fish anterior kidney to demonstrate ascorbic acid (X48).

Fig. (39b) T.S. infected fish anterior kidney. Note the reduction in ascorbic acid content (X48).



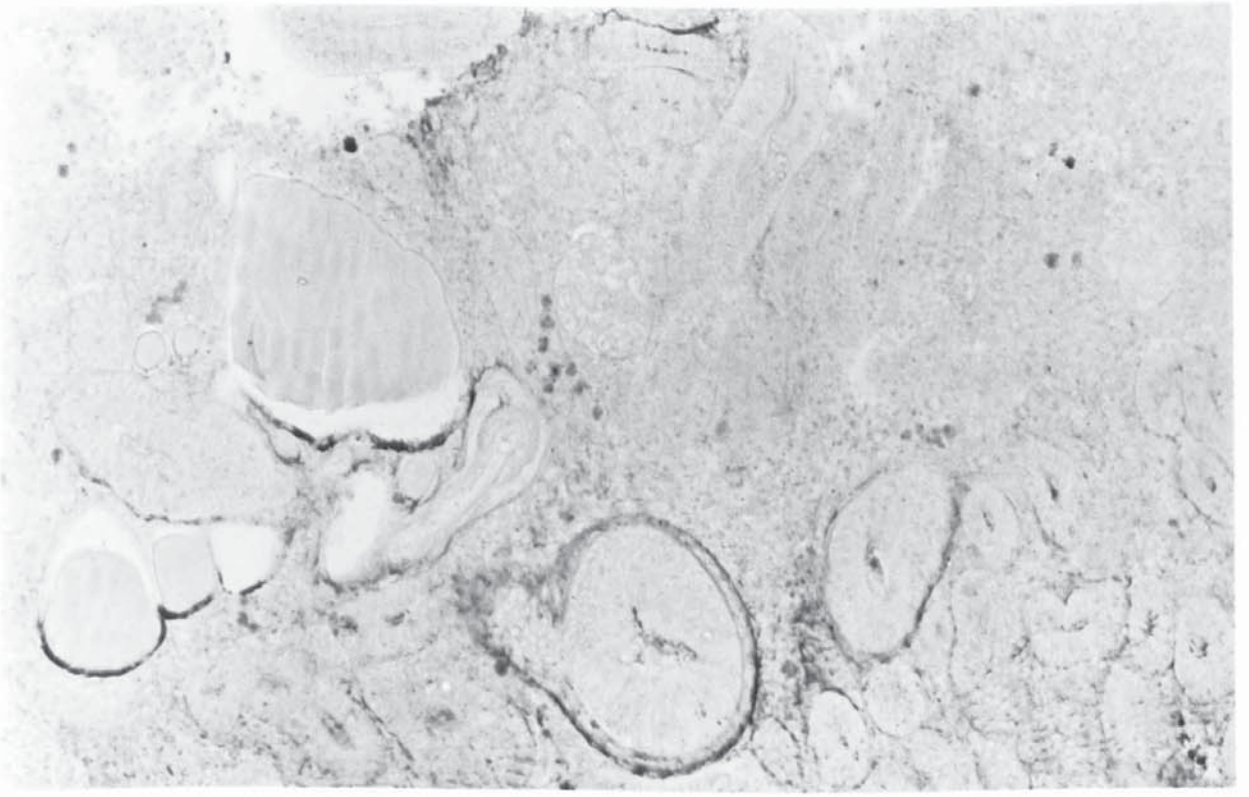


Fig. (40) Total liver lipid, protein and glycogen content during the period of infection

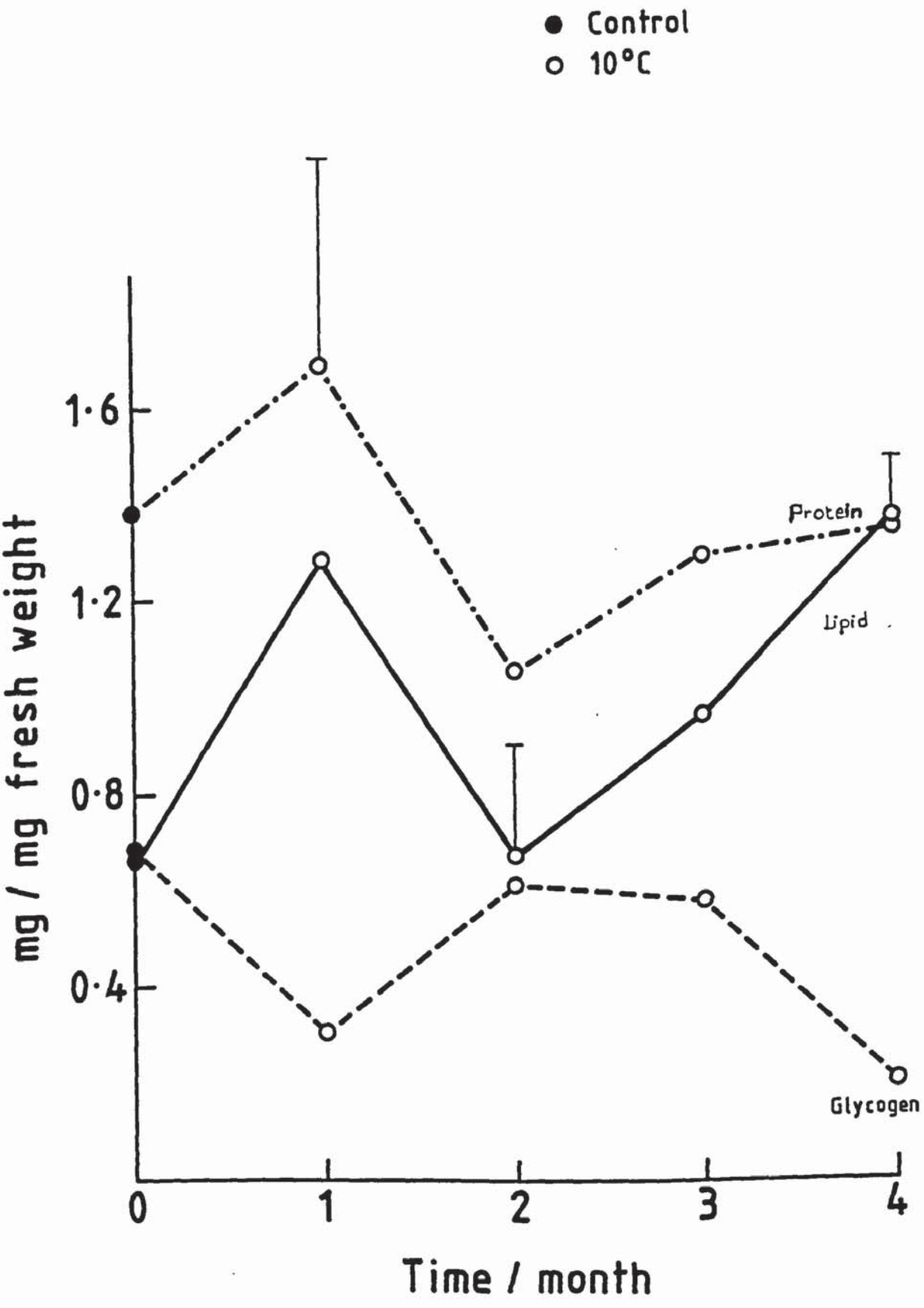

● Control
○ 10°C
1·6
1·2
0·8
0·4
mg / mg fresh weight
Protein
Lipid
Glycogen
0
1
2
3
4
Time / month

Fig. (41) Section through control fish skin to demonstrate skin structure and epidermal thickness (X48).

Fig. (42) Lesion during later stage of infection. Only a single layer of epidermal cells remains (X48).

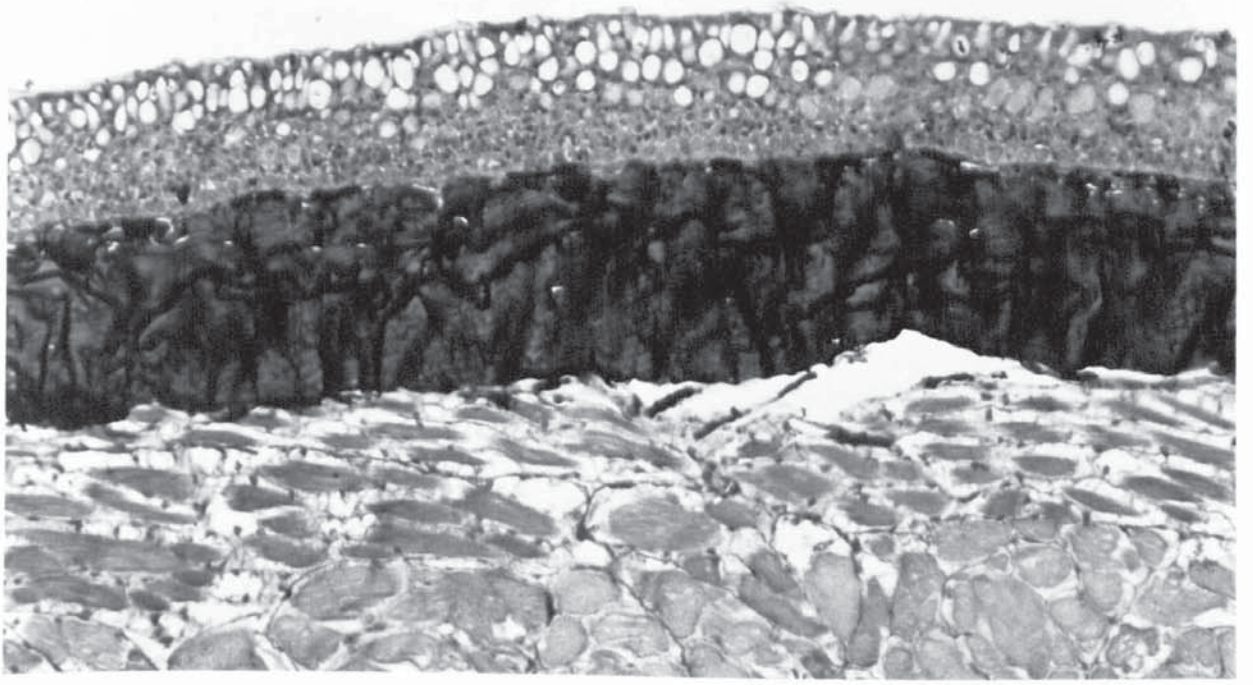
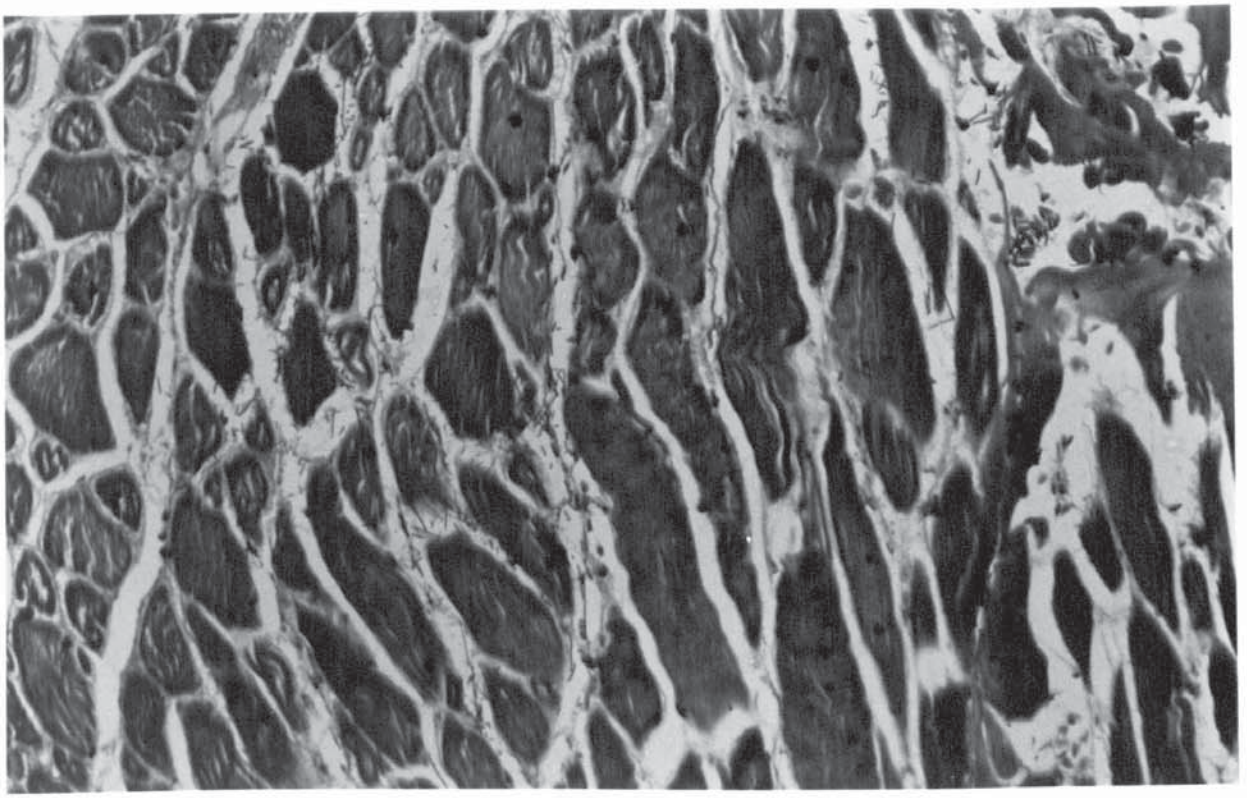


Fig. (43) Section of control fish muscle (X100).

Fig. (44) Section of infected fish muscle. Note the oedema of the muscle, myofibrillar degeneration and the presence of large numbers of blood cells (X100).



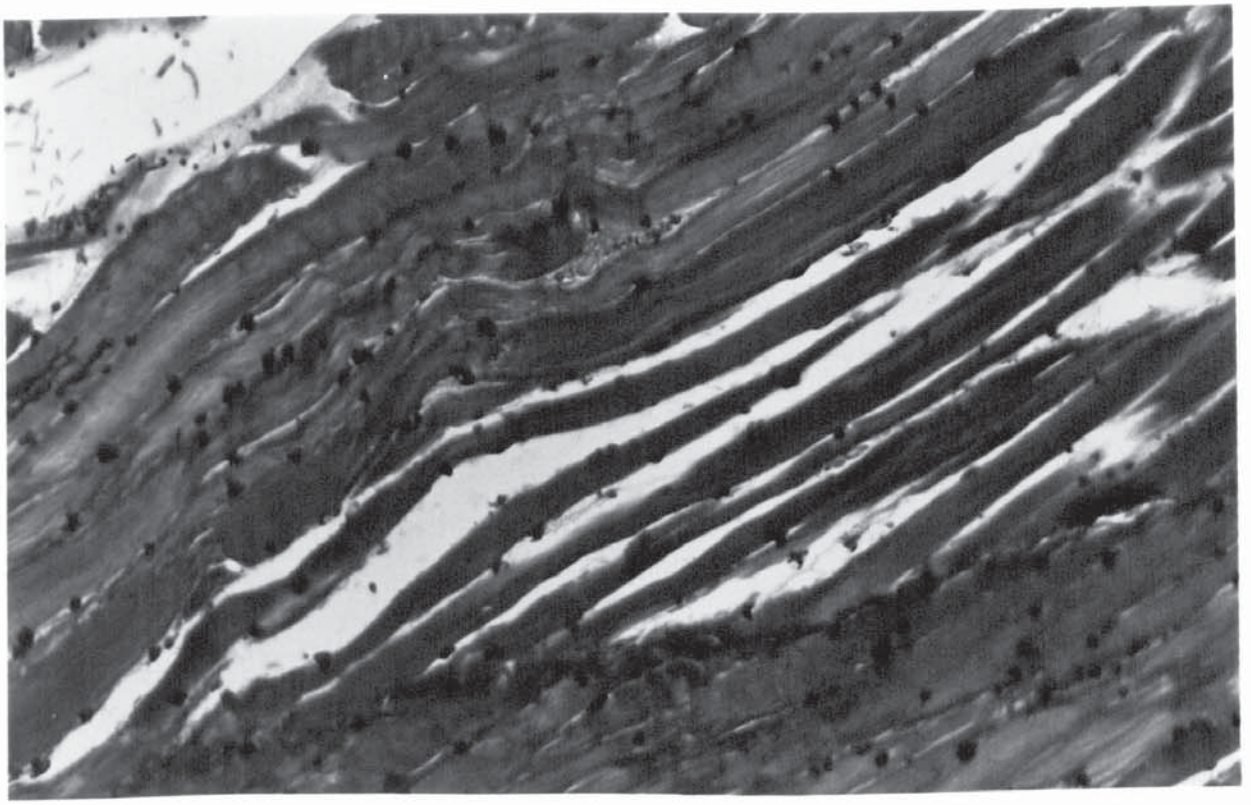
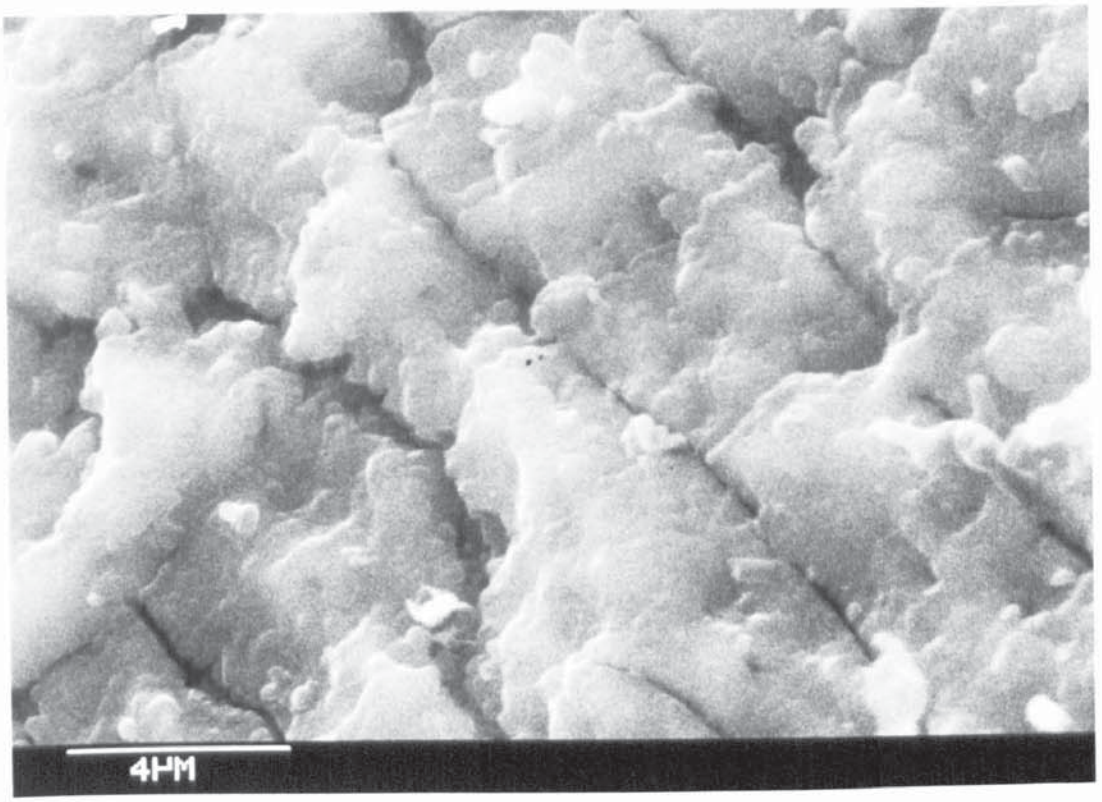


Fig. (45) Scanning electron micrograph of control fish skin. Note the high concentration of mucous and the regular ridged pattern of arrangement.

Fig. (46) Scanning electron micrograph to demonstrate the first stages of infection. Note the shrinking and irregular distribution of mucous.


4HM

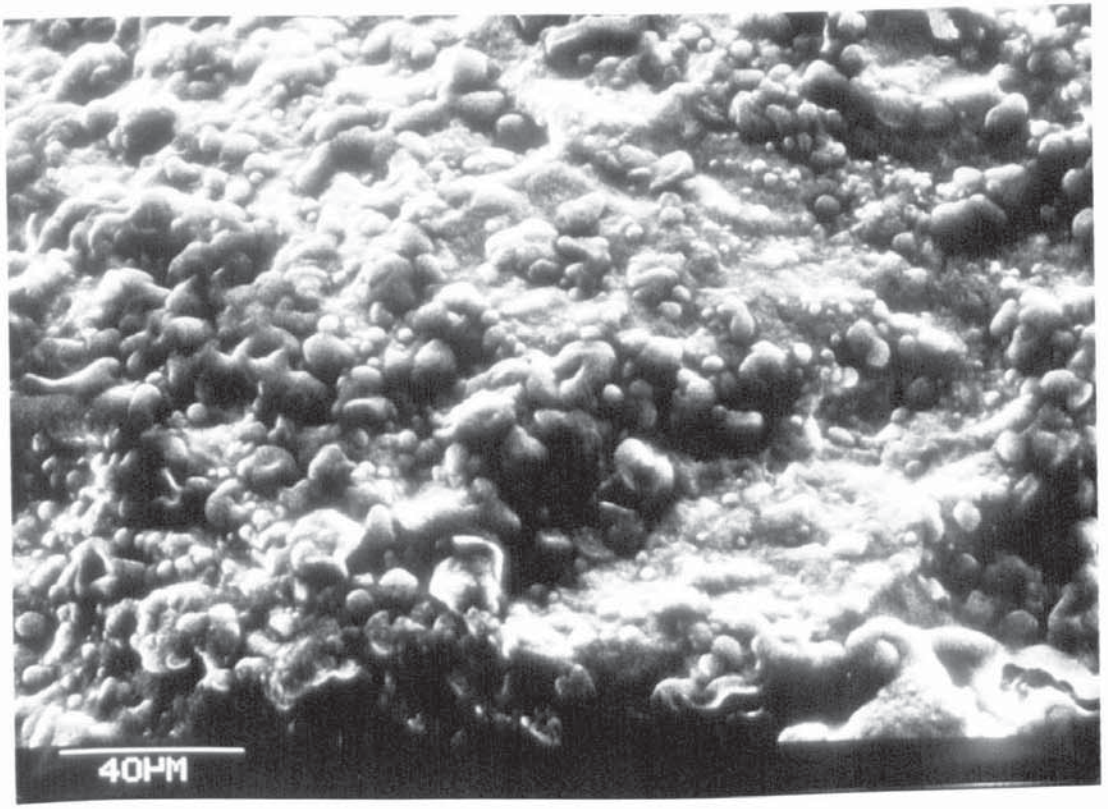
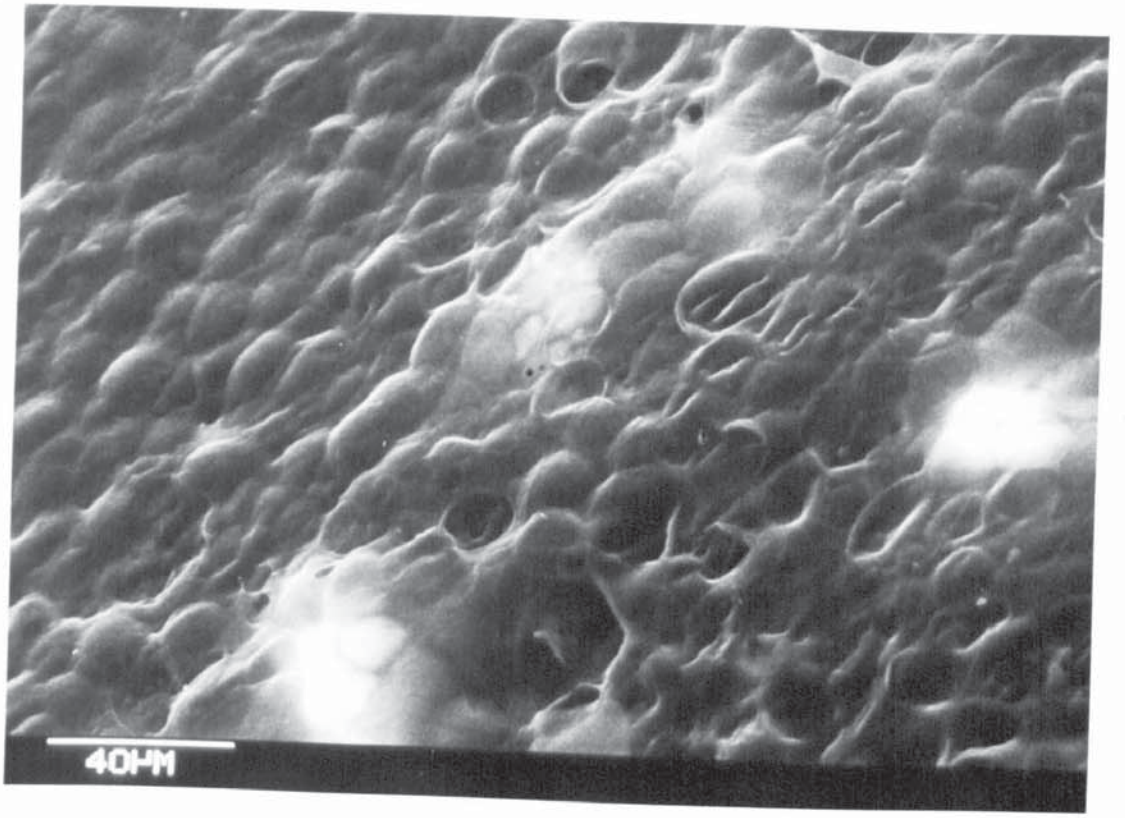

40HM

Fig. (47) Scanning electron micrograph of premycotic stage. White patches are scattered on the skin surface. Mucous cell pores (arrowed) are clearly seen in the skin which is devoid of mucous.

Fig. (48) Scanning electron micrograph of a skin lesion. The skin is lost its cellular integrity and continuity.


40HM

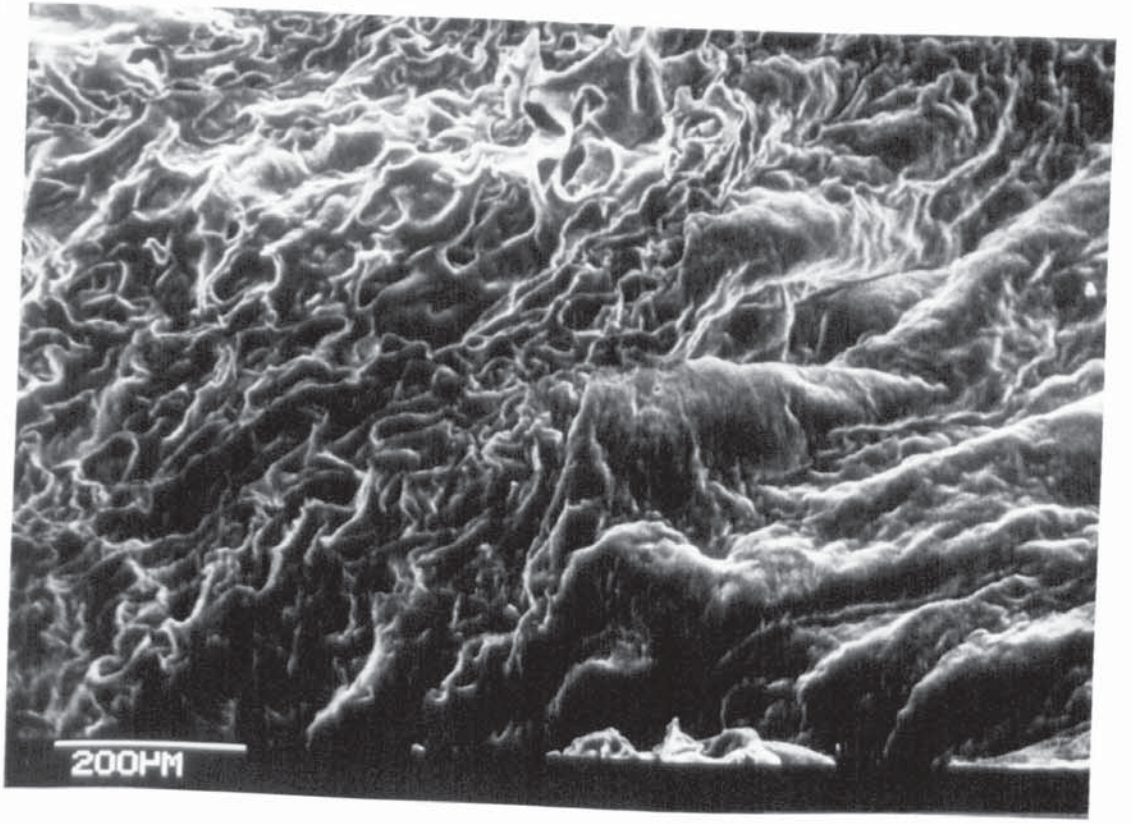
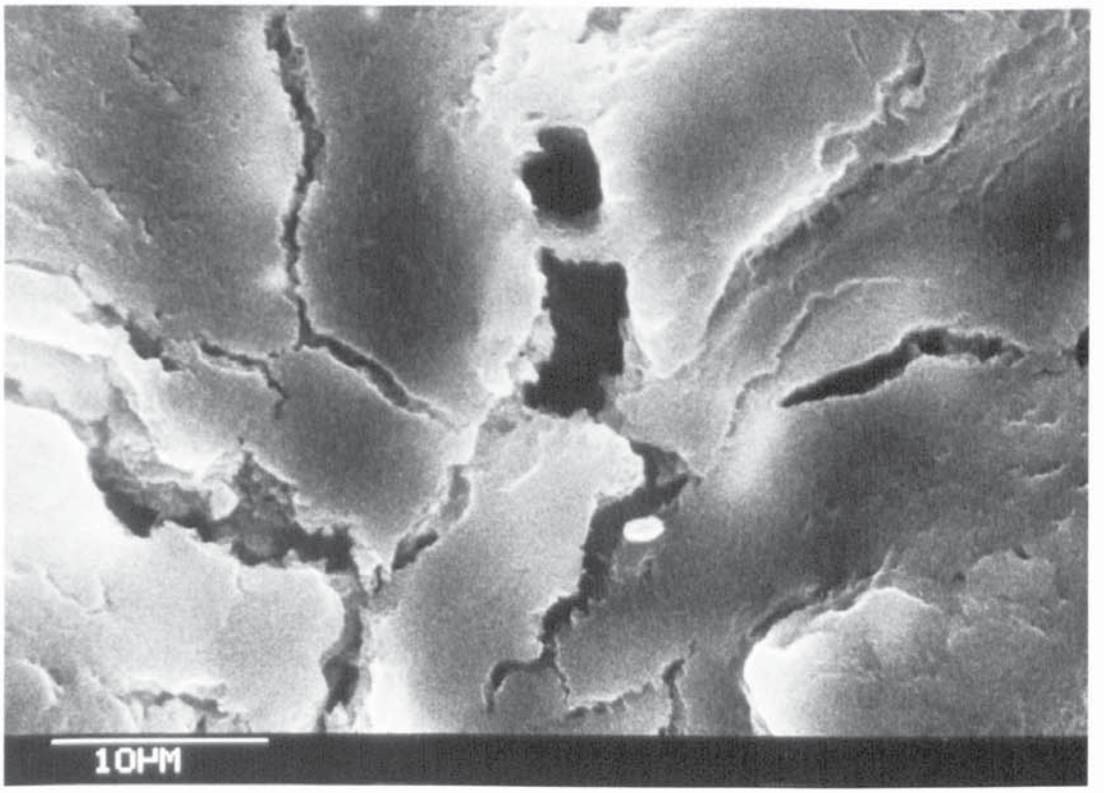
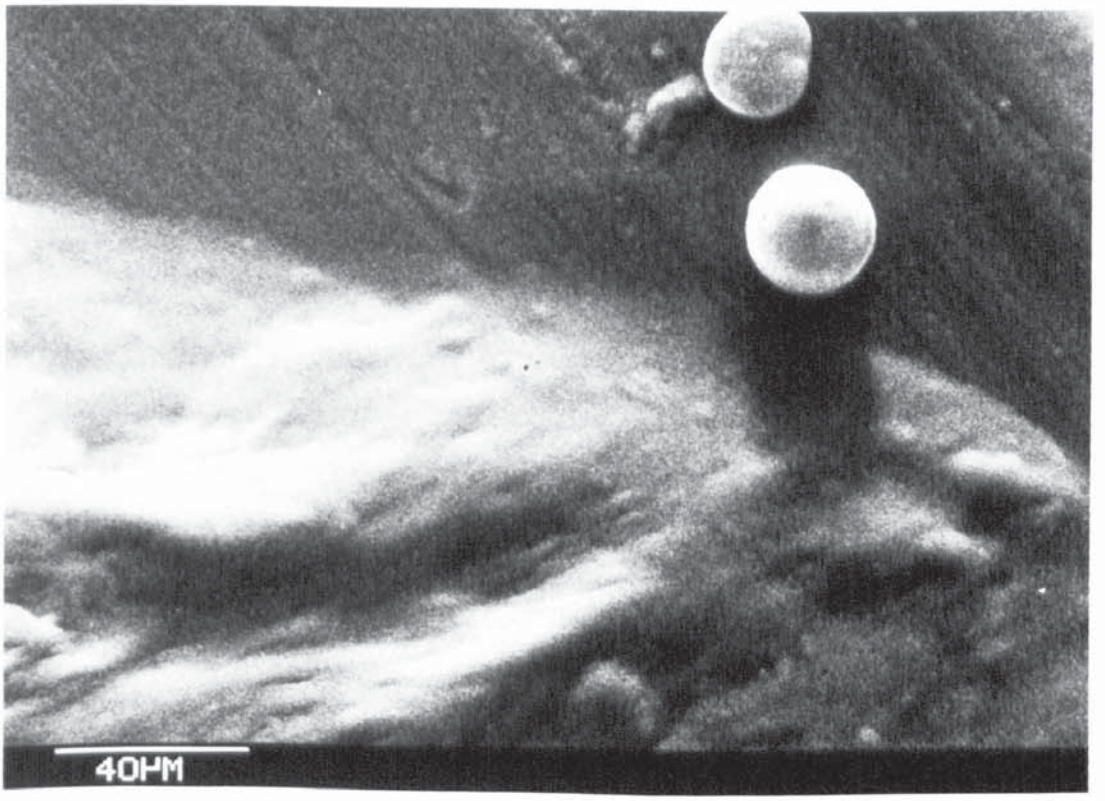

200HM

Fig. (49) Scanning electron micrograph of late lesion stage. Note the destruction of the skin by the profuse growth and penetration of fungal mycelia.

Fig. (50) Scanning electron micrograph to demonstrate the attachment of secondary zoospore (SZ) and spore cyst (SC) to fish skin.


10UM


40UM

CHAPTER 6

GENERAL DISCUSSION

Saprolegniasis continues to be the most significant fungal disease of teleost fish (Richards, 1977). The aim of the work presented in this thesis has been to try and gain a clearer understanding of the physiological interactions which occur between the fungal parasite S. diclina and its fish host.

In chapters two and three, two species of Saprolegnia were studied. Both are saprophytic but S. diclina is also a facultative parasite and pathogen of fish which has been recorded on a number of fish species (Pickering and Christie 1980; Pickering and Willoughby, 1982; Beakes and Ford 1983). S. ferax on the other hand is non-pathogenic (Willoughby, 1970; Pickering and Willoughby, 1982; Beakes, 1980).

Both species were therefore investigated in this thesis in order to identify potential differences in their physiology which might account for the pathogenicity of S. diclina. Growth of both species was monitored at different temperatures and on different substrates in chapter two. Temperature is one of the most important external factors to influence fungal growth. Optimum growth of S. diclina and S. ferax occurs between 20-25 °C, and both species have a similar wide temperature range for growth.

Both species grow best on medium containing both glucose and casein and also exhibited the same optimal growth temperature (25 °C) on this medium. Previous studies have indicated that glucose is the best source of carbon for Saprolegnia sp. and casein is a good

source of nitrogen (Alexopoulous, 1962; Gay et al., 1971; Heath et al., 1971). The least growth for both species was recorded on medium supplemented with 2% glucose. This was found to be the case at both temperatures (10xC and 25 C) tested here. The poor growth may have resulted from an increase in the acidity of the growth medium (Wolf and Wolf, 1947) which finally inhibits fungal growth. Optimal growth rates for S. ferax have been recorded at pH 5.2 (Roberts, 1963) and in the range between pH 5.8-6.9 (Krause, 1960); and for S. diclina at pH 6.4. Enzyme production by both species of fungus is severely inhibited by the presence of high levels of glucose and fat in the growth media. Previous authors have also described the inhibitory effects of glucose on fungal enzyme production (Horton and Keen, 1966; Patil and Dimond, 1968; Weinhold and Bowman, 1974). This may be due to the acidity developed in the medium as a result of glucose fermentation (Horton and Keen, 1966) or it may be that when glucose is present in the substrate it inhibits the production of enzymes necessary for utilisation of another sugar (Patil and Dimond, 1968). The low growth and enzymatic production of both fungal species on media with high levels of fat, may be due to the fact that fat is a relatively poor carbon source. An alternative explanation may be that the small amount of fatty acids produced from the breakdown of fat by fungal lipases in the growth medium are poorly utilisable or even toxic to Saprolegnia (Powell et al., 1972).

The studies presented here have shown that S. diclina and S. ferax appear to be similar in many aspects of their physiology. Studies in chapter three have shown that both species are capable of

exhibiting proteolytic and lipeolytic enzymatic activity which is greater at 10 °C than 25 °C. However, S. diclina appears to produce more enzymes at 10 °C than does S. ferax. This feature may account in part for its success as a pathogen, since it may facilitate its growth on fish tissues at low temperatures when fish resistance to infection is impaired (see chapter four).

Another feature which may aid in promoting the effectiveness of S. diclina as a pathogen is seen in the structure of the secondary zoospore which is thought to be the infective agent in saprolegniasis (Pickering and Willoughby, 1982; Willoughby et al., 1983). Infected fish may release in excess of 190.000 spores per minute (Willoughby & Pickering, 1977). Pickering & Willoughby (1982) reported that the secondary zoospore cysts of pathogenic strains bear numerous bundles of long hairs, each teminating in a pair of recurved hooks. The saprophytic strains have much shorter hooked hairs which are not aggregated into bundles. It has been proposed that the hooked hairs are attachment structures and that their greater development in the pathogenic strains represents an important adaptation to their mode of life, in which firm attachment to the host fish is necessary to ensure their parasitic existence. The long hooked hairs of pathogenic forms might also be concerned with enhanced flotation of the cyst, and thus provide a better opportunity for contact with, and colonization of, a suitable fish host. Little is known about the factors that influence the attraction of zoospores to fish skin, but it seems probable that chemotropism towards a suitable food source is an important mechanism (Pickering & Willoughby, 1982). It was found that amino

acids permit much better growth of Saprolegnia than other nitrogenous compounds (Powell et al., 1972). Smith et al. (1984) stated that the presence of amino acids, especially aspartic acid and glutamic acid, at concentrations which occur in fish tissues promotes the attraction of S. diclina zoospores towards the nutrient source thereby encouraging colonisation of the fungus on fish tissues.

Future work on the nature of the physiological and biochemical interactions between S. diclina and its host should include an investigation of substrate utilisation from fish tissue itself. The studies presented here have demonstrated that S. diclina can produce both proteolytic and lipeolytic enzymes in vitro. However, little is known about which substrates the fungus utilises in vivo. Biochemical analysis of infected and non-infected fish tissues, in addition to analysis of fungal mycelia, (using gas liquid chromatography and amino acid analysis) should provide a clearer insight into the nature of the substrates utilised by the fungus.

Work in this thesis and previous studies in this laboratory have shown that temperature has a major part to play in motility of fungal zoospores; germination and growth of S. diclina and S. ferax; and the susceptibility of fish to infection by S. diclina. Whilst higher temperatures exert a protective effect on fish in terms of a lowered incidence of Saprolegnia infection, the factors involved in this resistance have not yet been fully characterised. The studies presented here suggest that humoral immunity, as assessed by the production of precipitating antibodies, may be of limited significance in protecting against fungal attack and future work

will be directed towards extending these findings and investigating other aspects of the immune response in detail.

Work presented in chapter four suggested that there were changes in leucocyte numbers following infection. In particular, the numbers of blood macrophages and neutrophils increased over the period of study. Their lack of effect in combatting Saprolegnia infection may be temperature related, since Avtalion (1981) has shown that the phagocytic activity of these cells increases with environmental temperature. Bisset (1946) showed that blood clearance of a nonpathogenic Streptococcus was accomplished at a slower rate when fish were kept at low temperature. Barrow (1955) found that fish (perch and gold fish) kept at 5, 10 and 15 °C did not eliminate infecting trypanosomes, whereas these were destroyed when fish were moved to 20 °C. Tets (1969) mixed a sample of whole carp blood with A. punctata and observed higher numbers of phagocytic cells at 22 C than at 7 °C. Avtalion (1981) found that while the rates of ingestion of bacteria were the same at 25 °C and 10 °C, no evidence of killing could be observed at 10 °C. At 25 °C, however the rate of killing was relatively high. On the basis of this finding it may be suggested that fish became infected at low temperatures in the present studies because their phagocytic apparatus was not efficient in killing microorganisms. Phagocytosis may also be concerned with tissue repair. Phagocytes are known to produce enzymes involved in the autolysis and removal of necrotic epidermal tissue (Roberts, 1972). Impairment of tissue repair processes at low temperatures may therefore result in an exascerbation of fungal lesions.

Another aspect of the innate immune system which appeared to be altered in Saprolegnia infected carp, was the observed decline in mucous cells in the skin during infection. The reason for this decline is not known, but one explanation may involve depleted levels of glucose in infected fish. Glucose is the basic component for the synthesis of sialic acid (Peterson & Leblond, 1964). If glucose is lost from the blood in infected fish via haemorrhage or haemodilution, or on the other hand, is consumed directly by the fungus from the epidermis, then the formation of sialic acid from glucose may be interrupted, delayed, or stopped depending on the severity of infection.

The role of mucous in protection against Saprolegnia infections remains unclear. The possible physical cleansing action of mucous (Pickering and Willoughby, 1982) has been discussed earlier. The mucous of certain teleosts may also have a broad antimicrobial activity (Nigrelli, 1935; Harrel et al., 1976). Thus, a range of potentially anti-microbial agents have been demonstrated in fish mucous: lysozyme by Fletcher and Grant (1968); Fletcher and White (1973b) and Ourth (1980); Complement components by Nelson and Gigli (1968); Harrel et al. (1976); antibodies of IgM by Fletcher and Grant (1969); Bradshaw et al. (1971); Di Conza and Halliday (1971); Harris (1972); Fletcher and White (1973a); Lobb and Clem (1981a,b) and proteolytic enzymes by Hjelmeland et al. (1983). Ourth (1980) postulated that skin mucous antibodies of catfish originate by diffusion from serum or may also be produced locally by mucous lymphocytes (Cormier et al., 1984); it is also conceivable that both mechanisms could operate simultaneously.

On the other hand, some authors have suggested that the external mucous of fish represents an attractive substrate for the fungal zoospore since it has water-soluble components (at the water/mucous interface) and low concentrations of nutrients may be available. The infective agent (secondary zoospore) appears to germinate at low nutrient concentrations (Willoughby et al., 1983) and produces fungal hyphae which allow for rapid mycelial attachment. Mucous removed from the surface of fish stimulated encystment of zoospores from pathogenic strains of Saprolegnia, and promoted rapid growth of mycelia (Roberts, 1982). Epidermal mucous taken from salmonids acts as an effective growth medium for S. diclina (Willoughby, 1971; Willoughby and Pickering, 1977; Willoughby et al., 1983). Whether antimicrobial components of mucous are capable of protecting fish against Saprolegnia infections, particularly at higher temperatures where their synthesis may be increased, awaits investigation.

In higher vertebrates, the adaptive or specific immune system is of major importance in defending the host against fungal attack. As yet, little is known about the immune capabilities of fish towards Saprolegnia. There is still a need for a careful chronological examination of immunological events following exposure to infective Saprolegnia zoospores or Saprolegnia derived antigens. The present study has begun to examine these issue, but factors such as antigen dose, route of injection, and the timing and number of antigen injections, require further study. All these factors are known to influence both the kinetics and the magnitude of the immune response.

The histological changes occurring in the spleen and head kidney of infected carp are suggestive of immunological reactivity. Evidence from the participation of these organs in disease processes in fish has been accumulating. For instance similar changes to those described here have been reported in the pathogenesis of aeromonad diseases of salmonid fish (Thorpe and Roberts, 1972). Roberts (1975) reported that in parasitic infestations of roach (Rutilus rutilus) caused by Myxobolus pseudodispar, the spores locate specifically in the melanomacrophages of the spleen and kidney. Huizinga et al. (1979) noted an abundance of pigmented centres in the spleen of red-sore diseased large mouth bass (Micropterous salmoides). Mature oocysts of the coccidian Eimeria branchiphila were also observed situated within, or in close proximity to, melano-macrophage centres in the spleen of roach (Dykova et al., 1983). Spherical bodies similar to those reported here, have also been observed in the anterior kidney of Tilapia mossambica following immunisation (Sailendri and Muthukkarruppan, 1975). Lymphocytes of the spleen and head kidney of fish are known to divide following antigenic challenge, and antibody producing cells have been identified in these tissues in a range of fish species (Etlinger et al., 1978). Therefore, in future studies, cellular reactivity in the major lymphoid organs of carp should be monitored following exposure to fungal antigens. Future work would assess lymphoid cell proliferation in these organs by autoradiographical techniques. Histochemical changes (e.g. pyroninophilia) associated with immune reactivity would also be examined (Secombes, 1981). In vivo sensitization of the host immune system could also be examined by exposing host lymphocytes to

Saprolegnia antigens in culture and testing for specifically induced cell division. Tritiated thymidine uptake, as assessed by scintillation counting, could be used as an index of lymphocyte activation (Horton et al., 1976).

The lack of precipitating antibodies in the present study, indicates that alternative assays for humoral immunity ought to concentrate on techniques involving measurement of agglutination or complement fixation. The possibility of using zoospores, or foreign erythrocytes or latex particles coated with saprolegnia-derived antigens, in assays for agglutinating antibodies should be explored. Antigen coated erythrocytes could be used in serum haemolysin assays, or in plaque forming cell assays in order to determine the cellular origin of antibody production (Hudson and Hay, 1980).

Preliminary work presented in this thesis has begun to examine the possible role of lymphokines (MIF) in the immune reponse to Saprolegnia. Other in vitro assays developed for studying lymphokines in poikilothermic vertebrates include measurement of chemotatic and mitotic factors (Gearing and Rimmer, 1985a,b). These factors are thought to be of importance in inflammatory reactions since they can affect migration rates and directional movements of leucocytes and influence the division of lymphocytes. These activities are thought to be reliable in vitro correlates of cell-mediated immunity and will be further developed to study possible lymphokine involvement in the immune reaction of carp to Saprolegnia.

A further major area for future investigation would centre around the effects of temperature on the host immune response to Saprolegnia. The studies presented here have restricted themselves to an examination of infection and immunity at only two different temperatures. These experiments, together with the additional immunological studies outlined above, should therefore be repeated over a more complete range of environmental temperatures. Although temperature is widely recognised as an important element governing poikilotherm immunity, there is still some controversy regarding its precise effects at low temperatures. Some authors report that in fish held below a certain temperature (10C*x* for warm water fish such as carp) no antibody production occurs (Avtalion, 1969; Muroga and Egusa, 1969; Avtalion et al., 1980). However, other investigators have shown that low environmental temperatures result in a longer latent period prior to antibody production, but not a reduction in the magnitude of the response (Harris, 1973; Cone and Marchalonis, 1972). There is therefore a need for more long term studies on Saprolegnia infected carp held at low temperatures. The regulatory effect of temperature upon the immune response of fish is still not fully understood. However, Avtalion et al. (1973; 1981) have suggested that it is the induction phase of the primary response which is affected by low temperatures and not the antibody release from plasma cells. It has also been reported that once immunological memory has been acquired at high temperature, fish can produce increases in antibody titres at low temperatures which are known to be inhibitory for antibody production in the primary immune response (Avtalion, 1969; Weiss and Avtalion, 1977). It would therefore be of considerable interest to see whether fish which had

been exposed to fungal zoospores at 20 °C would subsequently be immune to infection when re-exposed to zoospores and maintained at 10 °C. Such studies are of potential importance in fish culture since temperature may need to be carefully regulated in devising effective immunisation protocols against pathogens.

The importance of stress related responses in fish disease was highlighted in chapter five. These studies reported changes in a number of physiological parameters which have previously been used as indicators of stress responses in fish. Thus, infected carp showed decreases in circulating erythrocyte counts and haemoglobin concentrations in the blood; together with alterations (sometimes transient) in liver glycogen, lipid and protein levels. Ascorbic acid levels in the head kidney were also reduced when assessed histochemically. Wedemeyer and McLeay (1981) have identified a wide range of environmental stressors which can act singly, or in combination, to produce physiological and behavioural alterations in fish. Many of these adverse environmental conditions (e.g, pollutants, increased sediment loads etc.) are commonly encountered in land or water development projects. Others such as water quality, crowding, handling and transportation are regular features of intensive fish culture systems (Pickering, 1981). These, and other, adverse conditions can impose a considerable load, or stress, on the animal's homeostatic mechanisms. At the individual fish level, severe stress may be lethal. Lesser degrees of stress may lead to reduced growth and predispose fish to infectious diseases, especially if pathogens are present. These effects are thought to be mediated through changes in corticosteroid levels which in turn

impair the efficiency of both innate (Fletcher, 1981) and adaptive immune systems (Ellis, 1981). Future work ought, therefore, to include a close investigation of the relationships between temperature (and other environmental factors), altered steroid hormone levels, and possible changes in immune reactivity to Saprolegnia and other antigens. The use of non-harmful antigens would be of value here, since infection by Saprolegnia may in itself prove stressful and make it difficult to distinguish between the hormonal changes brought about by environmental conditions and those resulting from infection. Thus, Pickering and Christie (1981) have demonstrated elevated blood cortisol levels following Saprolegnia infection. Methods are already available for measuring fish steroids by radioimmunosssay (Pickering and Pottinger, 1983), and could be applied to the proposed study. It is hoped that future work of this nature will throw light upon the poorly understood hormonal control mechanisms which modulate immunity in fish.

In conclusion, knowledge gained from these and similar studies will not only provide basic information about fungal physiology and fish immunity; but should prove beneficial in producing a better understanding of possible protective measures in fish husbandary and in devising methods of promoting the host's immunity to Saprolegnia by vaccination.

APPENDICES

Appendix 1

A. Media

Unless stated otherwise all media were sterilized by autoclaving at 100.342 NM^{-2}, 150 C for 15 min. at 15 lbs/sq. in. and dissolved in one litre of distilled water and 10ml of microelement was added.

1. Microelements

Calcium	8g	As	$CaSo_4$	0.069g
Iron	0.5g	As	$FeSo_4$	0.019g
Manganese	0.5g	As	$MnCl_2$	0.004g
Copper	0.1g	As	$CuCl_2$	0.002g
Zinc	0.1g	As	$ZnSo_4$	0.001g

2. Glucose-yeast extract agar

Agar	20g
Glucose	3g
Yeast	2g
$MgSo_4 .7H_2O$	0.128g
KH_2Po_4	0.0136g

3. 2% Casein

Casein	20g
Yeast	2g
KH_2Po_4	2.04g
Na_2HPO_4 $2H_2O$	0.6g

4. 1% Casein + 1% glucose

Casein	10g
Glucose	10g
Yeast	2g
KH_2Po_4	2.04g

Na_2HPo_4 $2H_2O$ 0.6g

5. 2% glucose

Glucose	20g
Yeast	2g
KH_2Po_4	2.04g
Na_2HPo_4 $2H_2O$	0.6g

6. 1% Casein + 1% Fat

Casein	10g
Fat	10g
Yeast	2g
KH_2Po_4	2.04g
$Na_2HPo_4.2H_2O$	0.6g

7. 1% Glucose + 1% Fat

Glucose	10g
Fat	10g
Yeast	2g
KH_2Po_4	2.04g
$Na_2HPo_4.2H_2O$	0.6g

8. 0.66 glucose + 0.66 casein + 0.66 Fat

Glucose	6.66g
Casein	6.66g
Fat	6.66g
Yeast	2.02g
KH_2Po_4	2.04g
$Na_2HPo_4.2H_2O$	0.6g

9. 2% Fat

Fat	20g
Yeast	2g

KH_2Po_4	2.04g
$Na_2Npo_4.2H_2O$	0.6g

B. Fixatives

1. Bouin's fluid

Picric acid, saturated aqueous	75ml
Formalin, concentrated	25ml
Glacial acetic acid	5ml

2. Formol Calcium

Calcium chloride	1g
Formalin, concentrated	10ml
Distilled water	100ml
Cobalt nitrate	1g

3. Gomori 1-2-3

Formalin, concentrated	1 part
Mercuric chloride, saturated aqueous	2 parts
Distilled water	3 parts

4. Periodic acid Schiff's

a. Periodic acid

periodic acid	0.6g
distilled water	100.0ml
nitric acid, concentrated	0.3ml

b. Schiff's reagent

basic fuchsin	1g
distilled water	85.0ml
sedium metabisulfite	1.9g
N Hcl	15.0ml

5. Zenker-formol

potassium dichromate	2.5g
mercuric chloride	4g
sodium sulphate	1g
distilled water	1000ml
formalin, concentrated	5ml

(add just before using)

C. Buffers

1. Barbitone buffer, pH. 7.6

Sodium chloride	85.00g
Barbital (5,5 Diethylbarbituric acid	5.57g
Barbital Sodium (5,5 Diethylbarbituric acid, Na salt)	2.75g
Magnesium chloride	
Calcium chloride (1.0M)	30.00ml

2. 0.05M Sodium phosphate buffer, pH. 7.4

0.05M monobasic sodium phosphate	19.00ml
0.05M dibasic sodium phosphate	18.00ml

dilute to a total of 200ml with distilled water

3. 0.01M monobasic sodium phosphate 19.00ml

0.01M dibasic sodium phosphate 18.00ml

dilute to a total of 200ml with distilled water

Appendix 2

	Sub-strate \ Temp. C°	5	10	15	20	25	30
S. diclina	2% Casein	14.216 ± 1.087	31.800 ± 0.779	54.933 ± 2.122	84.216 ± 1.792	119.716 ± 0.970	29.000 ± 5.130
	1% Casein + 1% Glucose	17.733 ± 0.779	45.650 ± 0.857	77.783 ± 3.830	112.816 ± 3.113	123.983 ± 3.729	40.016 ± 1.590
	2% Glucose	18.866 ± 1.115	39.666 ± 1.005	56.233 ± 1.988	59.600 ± 2.314	55.900 ± 2.209	16.366 ± 1.604
S. ferax	2% Casein	5.213 ± 1.006	14.503 ± 0.663	30.243 ± 2.110	67.911 ± 0.327	81.117 ± 0.446	78.341 ± 1.362
	1% Casein + 1% Glucose	7.156 ± 0.863	13.861 ± 0.842	90.500 ± 1.411	136.800 ± 4.213	153.711 ± 3.222	103.400 ± 2.176
	2% Glucose	10.860 ± 0.162	15.912 ± 1.345	37.521 ± 2.005	45.110 ± 3.012	75.511 ± 0.362	49.620 ± 1.111

Table (1) Effect of temperature on growth of *S. diclina* and *S. ferax* (mg dry weight)

S. diclina

Variation	SS	Df	MS	F
Temp.	97501.353	5	19500.270	599.614***
Substrate	14693.719	2	7346.859	225.908***
Temp. X Substrate	15576.985	10	1557.698	47.897***
Error	2926.923	90	32.521	

S. ferax

Variation	SS	Df	MS	F
Temp.	135233.032	5	27046.606	153.478***
Substrate	43507.814	2	21753.907	123.444***
Temp. X Substrate	28454.153	10	2845.415	16.146***
Error	15860.191	90	176.224	

(*** = P<0.001)

C°	Substrate \ Time/Days	12 hours	1	2	3	4	5	6	7
10	2% Casein	7.5 ± 0.867	7.6 ± 0.335	7.1 ± 0.514	8.1 ± 0.693	13.6 ± 1.098	25.3 ± 1.907	40.2 ± 1.040	105.8 ± 1.329
	1% Casein + 1% Glucose	8.0 ± 0.086	10.3 ± 0.578	9.7 ± 0.184	11.3 ± 0.751	19.7 ± 1.329	35.6 ± 3.641	59.5 ± 0.433	113.7 ± 10.867
	2% Glucose	3.4 ± 0.924	2.4 ± 0.260	7.4 ± 0.057	5.7 ± 0.289	11.7 ± 0.751	20.9 ± 0.809	31.1 ± 0.751	35.3 ± 0.404
25	2% Casein	8.8 ± 1.329	10.8 ± 0.566	23.4 ± 2.890	53.3 ± 2.658	84.2 ± 10.924	110.8 ± 8.670	112.5 ± 5.838	87.4 ± 19.13
	1% Casein + 1% Glucose	9.9 ± 0.323	9.8 ± 0.317	23.9 ± 0.982	50.7 ± 3.121	72.0 ± 1.040	102.6 ± 0.578	126.8 ± 3.815	72.8 ± 3.005
	2% Glucose	3.5 ± 0.289	4.8 ± 0.260	6.8 ± 1.560	21.5 ± 0.173	33.3 ± 3.641	38.3 ± 1.790	37.7 ± 0.867	44.1 ± 1.040

Table (2) Effect of time on growth of S. diclina (Dry weight/mg)

10 C°

Variation	SS	Df	MS	F
Time	49851.528	7	7121.646	751.856***
Substrate	9404.141	2	4702.071	496.413***
Time X Substrate	3691.890	14	263.706	27.840***
Error	454.660	48	9.472	

25 C°

Variation	SS	Df	MS	F
Time	72921.846	7	10417.407	37.010***
Substrate	22777.852	2	11388.926	40.462***
Time X Substrate	717.133	14	51.223	0.181
Error	13510.62	48	281.471	

(*** = P<0.001)

C°	Time/Days / Sub-strate	12 hours	1	2	3	4	5	6	7
10	2% Casein	4.7 ± 0.982	3.8 ± 0.578	13.4 ± 1.445	11.8 ± 0.924	17.6 ± 0.502	39.4 ± 0.132	74.7 ± 14.450	95.7 ± 3.641
	1% Casein + 1% Glucose	6.9 ± 1.156	5.9 ± 0.404	11.0 ± 0.520	9.3 ± 0.809	18.6 ± 1.791	50.9 ± 2.485	95.2 ± 7.976	98.9 ± 2.658
	2% Glucose	4.6 ± 0.115	5.1 ± 0.867	9.5 ± 0.491	12.0 ± 0.317	27.5 ± 1.849	42.6 ± 1.907	63.0 ± 14.855	71.6 ± 4.161
25	2% Casein	1.7 ± 0.433	3.7 ± 0.404	12.6 ± 1.156	42.3 ± 0.491	89.3 ± 6.994	91.1 ± 11.676	121.0 ± 10.809	93.8 ± 5.664
	1% Casein +	4.1 ± 0.566	4.5 ± 0.549	29.1 ± 0.115	137.0 ± 7.167	142.0 ± 3.121	172.0 ± 12.485	172.2 ± 10.231	153.5 ± 2.601
	2% Glucose	4.7 ± 0.346	10.2 ± 0.317	21.4 ± 0.578	49.3 ± 1.907	57.5 ± 7.745	75.1 ± 4.219	95.3 ± 14.62	100.3 ± 13.815

Table (3) Effect of time
on growth of S. ferax (Dry weight/mg)

10 C°

Variation	SS	Df	MS	F
Time	71353.566	7	10193.367	148.481***
Substrate	1377.665	2	688.832	10.033***
Time X Substrate	2062.62	14	147.33	2.146
Error	3295.24	48	68.650	

25 C°

Variation	SS	Df	MS	F
Time	171897.782	7	24556.826	170.194***
Substrate	36385.930	2	18192.962	126.088***
Time X Substrate	23571.66	14	1683.690	11.669
Error	6925.79	48	144.287	

(*** = $P < 0.001$)

	Substrate / Temp.	2% glucose	2% casein	1% glucose + 1% casein	1% casein + 1% fat	1% glucose + 1% fat	0.66 glucose +0.66 casein + 0.66 fat	2% fat
S. diclina	10 C°	0.131 ± 0.019	0.343 ± 0.020	0.482 ± 0.022	0.049 ± 0.014	0.036 ± 0.015	0.056 ± 0.016	0.032 ± 0.01
	25 C°	0.578 ± 0.055	1.225 ± 0.026	1.271 ± 0.101	0.066 ± 0.009	0.040 ± 0.001	0.085 ± 0.011	0.036 ± 0.010
S. ferax	10 C°	0.144 ± 0.033	0.170 ± 0.017	0.240 ± 0.030	0.079 ± 0.005	0.033 ± 0.009	0.082 ± 0.013	0.031 ± 0.009
	25 C°	0.748 ± 0.171	0.890 ± 0.172	1.558 ± 0.207	0.124 ± 0.017	0.269 ± 0.395	0.170 ± 0.012	0.071 ± 0.007

Table (4) Effect of Substrate on growth of S. diclina and S. ferax after 4 days of incubation

	Variation	SS	Df	MS	F
S. diclina	Temp.	1.68271	1	1.68271	1414.042***
	Substrate	8.17730	6	1.36288	1145.277***
	Temp. X Substrate	2.31742	6	0.38624	324.571***
	Error	0.06652	56	0.00119	

	Variation	SS	Df	MS	F
S. ferax	Temp.	2.95739	1	2.95739	393.269***
	Substrate	6.17647	6	1.02941	136.889***
	Temp. X Substrate	3.63661	6	0.60610	80.598***
	Error	0.42133	56	0.00752	

	Fat% / Sub-strate	0	0.25	0.5	1.0	1.5	2.5	4
S. diclina	1% casein	0.250 ± 0.073	0.210 ± 0.048	0.206 ± 0.049	0.152 ± 0.042	0.137 ± 0.023	0.111 ± 0.011	0.103 ± 0.006
	1% glucose	0.098 ± 0.025	0.092 ± 0.029	0.086 ± 0.028	0.075 ± 0.021	0.060 ± 0.008	0.058 ± 0.010	0.055 ± 0.003
S. ferax	1% casein	0.345 ± 0.024	0.327 ± 0.055	0.197 ± 0.031	0.157 ± 0.042	0.117 ± 0.007	0.078 ± 0.011	0.070 ± 0.022
	1% glucose	0.178 ± 0.013	0.132 ± 0.034	0.097 ± 0.041	0.089 ± 0.035	0.042 ± 0.002	0.030 ± 0.005	0.029 ± 0.006

Table (5) Effect of substrate (cod liver oil) on growth of S. diclina and S. ferax at 20C° after 4 days of incubation.

S. diclina

Variation	SS	Df	MS	F
Temp.	0.14951	1	0.14951	133.491***
Substrate	0.07943	6	0.01324	11.821***
Temp. X substrate	0.02254	6	0.00376	3.357***
Error	0.06292	56	0.00112	

S. ferax

Variation	SS	Df	MS	F
Temp.	0.171626	1	0.171626	207.528***
Substrate	0.420542	6	0.070090	84.752***
Temp. X substrate	0.053786	6	0.008964	10.839***
Error	0.046313	56	0.000827	

(*** = $p < 0.001$)

C°	Time/day / Substrate	12 hours	1	2	3	4	5	6	7
10°	2% Casein	23.733 ± 2.348	26.315 ± 0.869	30.563 ± 1.876	30.000 ± 1.363	20.955 ± 1.314	11.857 ± 0.409	7.338 ± 0.136	2.788 ± 0.530
	1%c + 1%g	28.125 ± 1.059	25.533 ± 1.404	28.350 ± 2.919	29.646 ± 2.901	18.781 ± 1.680	10.393 ± 0.707	6.050 ± 0.168	2.506 ± 0.197
25°	2% Casein	23.863 ± 1.950	20.925 ± 0.685	9.957 ± 0.592	5.440 ± 0.266	5.285 ± 0.577	4.738 ± 0.593	4.666 ± 0.141	5.892 ± 1.163
	1%C + 1%g	25.858 ± 0.933	26.020 ± 7.317	11.171 ± 0.512	6.863 ± 0.427	5.763 ± 0.134	4.337 ± 0.243	3.194 ± 0.207	5.151 ± 0.480

Table (6) Effect of temperature on tyrosine release by S. diclina (mg/L/mg dry weight)

	Variation	SS	Df	MS	F
10C°	Time	2461.176	7	351.596	73.494***
	Substrate	1.038	1	1.038	0.216
	Time X Substrate	27.418	7	3.916	0.818
	Error	153.11	32	4.784	

	Variation	SS	Df	MS	F
25C°	Time	1652.239	7	236.034	122.487***
	Substrate	3.192	1	3.192	1.656
	Time X Substrate	32.002	7	4.571	2.372*
	Error	61.669	32	1.927	

(*** = $p < 0.001$)

C°	Time/day / Sub-strate	12 hours	1	2	3	4	5	6	7
10	2% Casein	37.659 ± 2.920	64.473 ± 0.716	19.776 ± 1.084	23.135 ± 1.015	19.034 ± 0.811	7.944 ± 0.606	4.819 ± 0.375	6.165 ± 0.404
	1%c + 1%g	34.202 ± 3.405	51.694 ± 2.026	31.090 ± 2.442	38.172 ± 1.285	20.161 ± 1.726	7.022 ± 0.091	3.014 ± 0.077	4.095 ± 0.185
25	2% Caseine	101.764 ± 3.574	71.621 ± 8.870	27.619 ± 1.947	8.676 ± 0.445	4.479 ± 0.571	5.060 ± 0.773	4.876 ± 0.435	6.396 ± 0.369
	1%c + 1%g	63.414 ± 2.602	90.000 ± 4.546	10.137 ± 0.711	2.357 ± 0.395	2.288 ± 0.229	1.918 ± 0.088	1.573 ± 0.043	2.442 ± 0.267

Table (7) Effect of temperature on tyrosine release by S. ferax (mg L/mg dry weight)

10C°

Variation	SS	Df	MS	F
Time	5433.269	7	776.181	109.738***
Substrate	44.267	1	44.267	6.258*
Time X Substrate	252.731	7	36.104	5.104**
Error	226.341	32	7.073	

25C°

Variation	SS	Df	MS	F
Time	13800.792	7	1971.541	127.245***
Substrate	46.838	1	46.838	3.022
Time X Substrate	984.983	7	140.711	9.081***
Error	495.834	32	15.494	

(*** $p < 0.001$)

Species	Substrate / C°	2% glucose	2% casein	1% glucose + 1% casein	1% casein + 1% fat	1% glucose + 1% fat	0.66 glucose + 0.66 casein + 0.66 fat	2% fat
S. diclina	10C°	99.999 ± 66.666	384.705 ± 291.060	125.000 ± 106.066	51.612 ± 22.522	33.332 ± 18.633	41.741 ± 5.916	8.178 ± 0.614
	25C°	2.777 ± 1.963	9.280 ± 7.257	4.921 ± 1.391	4.304 ± 2.691	5.755 ± 4.101	6.844 ± 4.614	2.720 ± 0.099
S. ferax	10C°	8.620 ± 1.771	25.125 ± 8.883	14.999 ± 2.390	44.444 ± 6.761	22.727 ± 16.070	34.326 ± 4.344	4.548 ± 0.265
	25C°	6.711 ± 3.751	14.492 ± 1.464	12.121 ± 1.889	5.470 ± 1.732	4.137 ± 2.884	3.652 ± 2.222	2.665 ± 0.164

Table (8) The effect of substrate on extracellular protease activity of S. diclina & S. ferax after four days of incubation (mg trypsin/100 ml media/gm dry weight/30 min.)

S. diclina

Variation	SS	Df	MS	F
Temp.	179007	1	179007	24.714***
Substrate	256563	6	42760	5.903***
Temp. X Substrate	243647	6	40608	5.606***
Error	405618	56	7243	

S. ferax

Variation	SS	Df	MS	F
Temp.	3971.3	1	3971.3	124.492***
Substrate	3251.6	6	541.9	16.987***
Temp. X Substrate	3355.0	6	559.2	17.529***
Error	1787	56	31.9	

(*** $p < 0.001$)

Species	Substrate / C°	2% glucose	1% casein	1% glucose + 1% casein	1% casein + 1% fat	1% glucose + 1% fat	0.66 glucose +0.66 casein + 0.66 fat	2% fat
S. diclina	10C°	2.000 ± 0.707	16.940 ± 0.609	4.850 ± 0.876	3.237 ± 0.043	2.704 ± 1.407	3.075 ± 0.158	7.4499 ± 0.732
	25C°	1.721 ± 1.151	2.016 ± 0.193	1.822 ± 0.171	3.080 ± 0.160	1.625 ± 0.078	2.145 ± 0.343	6.693 ± 0.547
S. ferax	10C°	1.640 ± 1.337	5.600 ± 3.895	3.333 ± 0.961	3.038 ± 0.082	1.272 ± 0.613	2.228 ± 0.342	4.204 ± 0.277
	25C°	1.433 ± 0.973	1.776 ± 1.135	1.612 ± 0.205	1.361 ± 0.008	1.149 ± 0.197	1.289 ± 0.025	3.907 ± 0.302

Table (9) The effect of substrate on extracellular lipase activity of S. diclina & S. ferax after four days of incubation (mg lipase/100 media/gm dry weight/1 hour)

S. diclina

Variation	SS	Df	MS	F
Temp.	159.825	1	159.825	352.814***
Substrate	501.537	6	83.590	184.525***
Temp. X Substrate	425.224	6	70.871	156.448***
Error	25.361	56	0.453	

S. ferax

Variation	SS	Df	MS	F
Temp.	23.57	1	11.80	14.460***
Substrate	70.78	6	4.23	7.239***
Temp. X Substrate	25.36	6	1.63	2.595*
Error	91.49	56		

(* $p < 0.05$) (*** $p < 0.001$)

	10°C				
	Control	1 month	2 months	3 months	4 months
Total leucocyte count/ml^3	49.400	31.166	20.200	55.640	75.220
	± 3.118	± 4.217	± 0.424	±11.078	±20.475
Lymphocytes	43.867	27.239	17.897	44.512	57.919
	± 3.344	± 1.178	± 1.781	± 1.471	± 5.291
Monocytes	0.839	0.997	0.606	1.557	1.654
	± 0.083	± 0.675	± 0.514	± 0.465	± 0.628
Neutrophils	1.136	0.498	0.929	2.782	4.663
	± 0.113	± 0.269	± 0.096	± 1.112	± 0.302
Macrophages	0.988	1.246	1.010	3.338	5.265
	± 0.278	± 0.042	± 0.046	± 0.390	± 0.157
Basophils	1.037	0.560	0.363	1.669	2.707
	± 0.503	± 0.406	± 0.388	± 0.393	± 1.960
Acidophils	1.531	0.623	0.404	1.780	3.008
	± 0.498	± 0.220	± 0.045	± 0.465	± 0.075

Table (I4) Total and differential leucocyte counts of control and UDN-infected carp (Cyprinus carpio) at intervals following infection.

			10°C			
		Control	1 month	2 months	3 months	4 months
	Length	100	85	83	91	92
	%	± 11	± 12	± 12	± 14	± 16
Spleen	Weight	100	82	43	50	67
	%	± 20	± 21	± 16	± 14	± 4
	Length	100	68	58	68	77
	%	± 5	± 12	± 12	± 10	± 6
Thymus	Weight	100	66	37	51	53
	%	± 15	± 8	± 10	± 4	± 6
	Length	100	48	42	49	77
	%	± 14	± 24	± 11	± 7	± 12
Head Kidney	Weight	100	39	22	38	87
	%	± 20	± 7	±0.7	± 8	± 7

Table (15). Changes in length:body length and weight:body weight ratios of major lymphoid organs at intervals following infection.

		10C°			
	Control	1 month	2 months	3 months	4 months
R.B.C.S. Counts X $10^6/ml^3$	2.840 ± 0.720	0.540*** ± 0.235	1.790* ± 0.452	1.340*** ± 0.175	0.910** ± 0.775
Hb%	9.220 ± 0.449	6.430*** ± 0.833	8.260*** ± 0.057	7.800*** ± 0.305	7.400** ± 0.848

Table (16) Changes in erythrocyte count and Hb% values following infection.

	10C°				
	Control	1 month	2 months	3 months	4 months
Lipid mg/mg fresh wt X 10	0.672 ± 0.130	1.284 ± 0.440	0.679 ± 0.051	0.966 ± 0.413	1.364 ± 0.168
Protein mg/mg fresh wt	1.384 ± 0.153	1.693 ± 0.288	1.052 ± 0.273	1.294 ± 0.218	1.345 ± 0.429
Glycogen mg/mg fresh wt	0.691 ± 0.537	0.306 ± 0.198	0.613 ± 0.294	0.585 ± 0.148	0.213 ± 0.024

Table (16) Total liver lipid, protein and glycogen content during the period of infection.

Fig. (10) Tyrosine standard curve

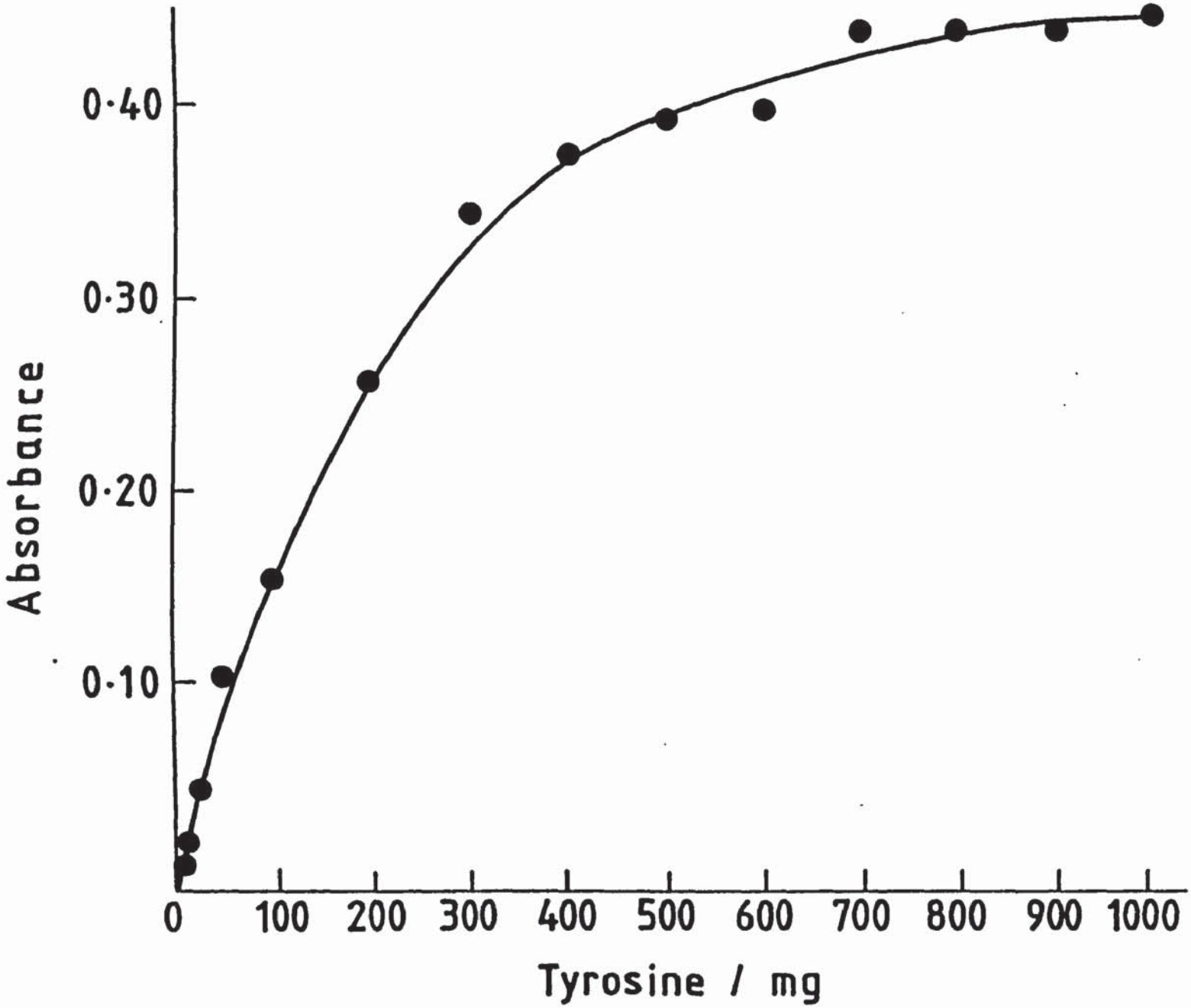

Absorbance
0·40
0·30
0·20
0·10
0
100
200
300
400
500
600
700
800
900
1000
Tyrosine / mg

Fig. (11) Trypsin standard curve

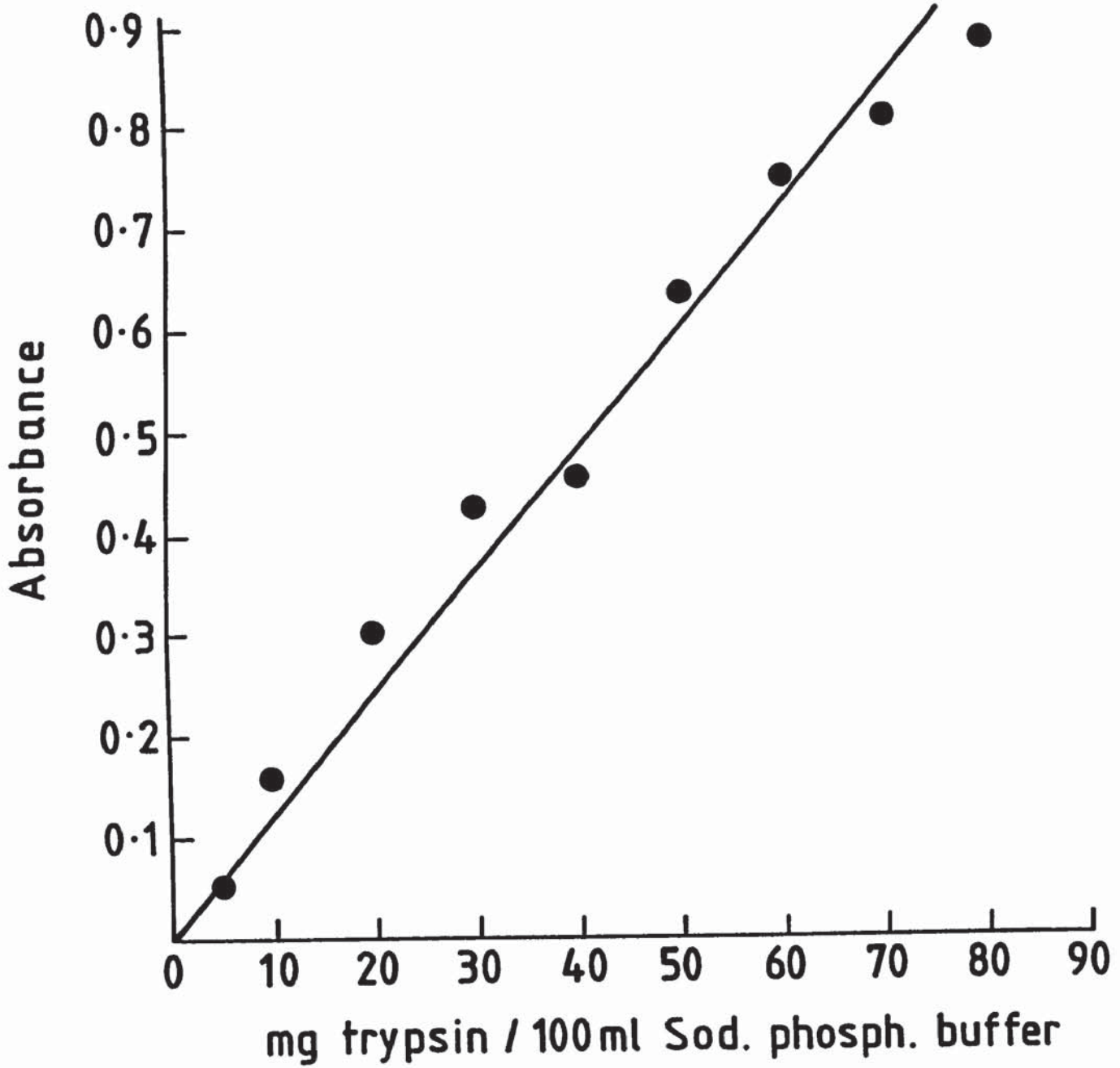
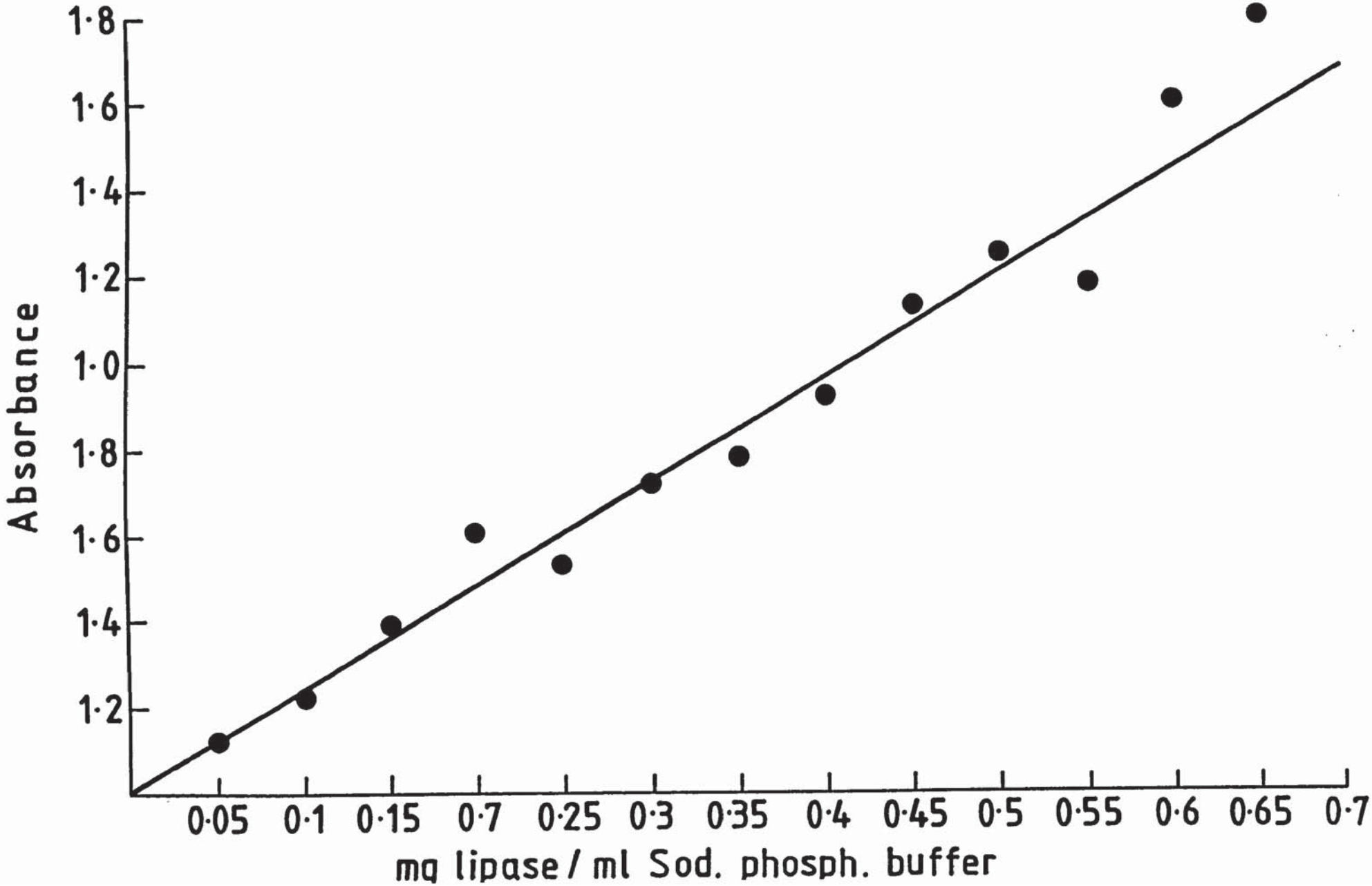

Absorbance
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
90
mg trypsin / 100 ml Sod. phosph. buffer

Fig. (12) Lipase standard curve


Absorbance
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1·6
1·4
1·2
1·0
1·8
1·6
1·4
1·2
0·05
0·1
0·15
0·7
0·25
0·3
0·35
0·4
0·45
0·5
0·55
0·6
0·65
0·7
mg lipase / ml Sod. phosph. buffer

Fig. (30) Glucose standard curve

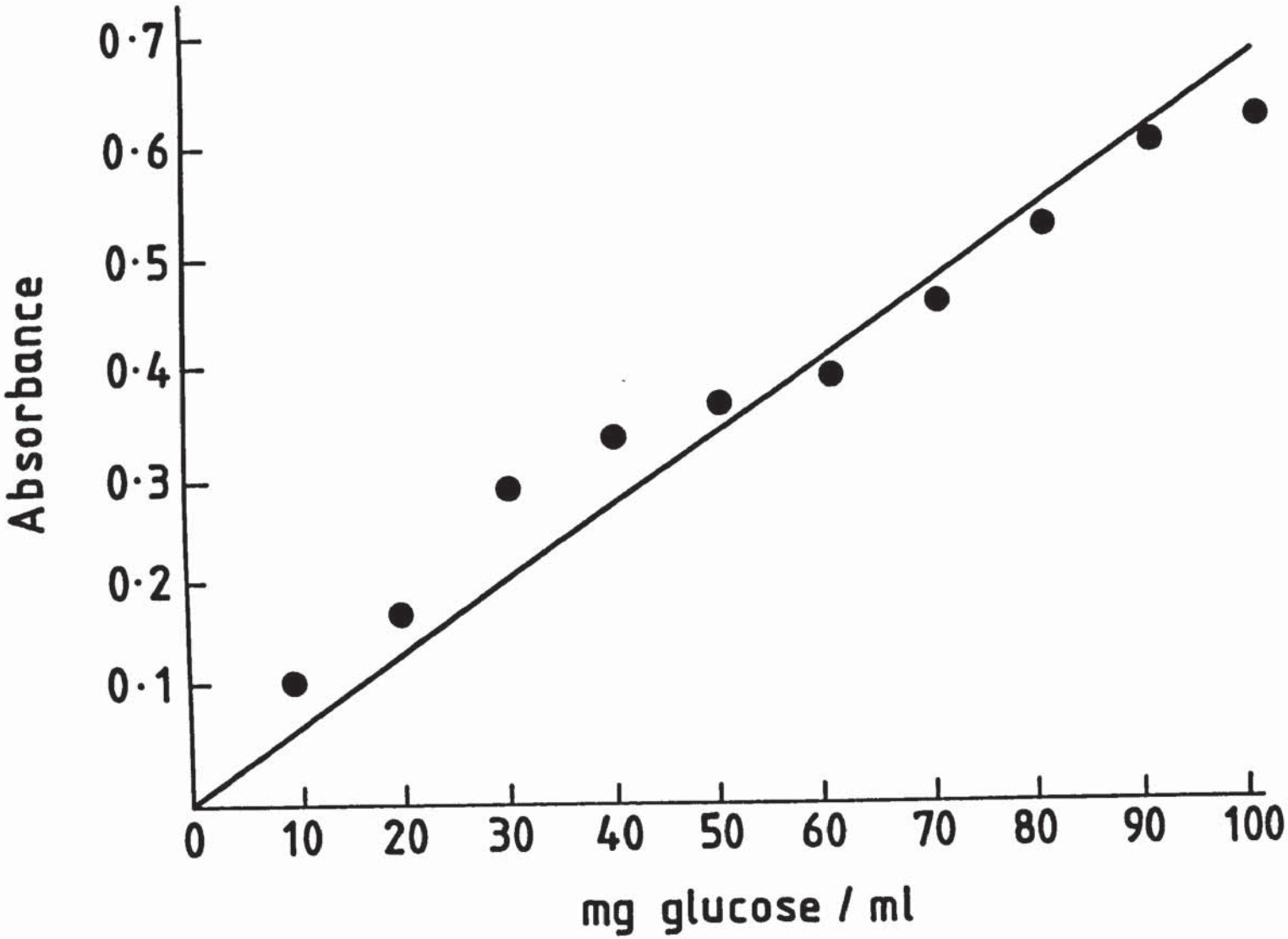
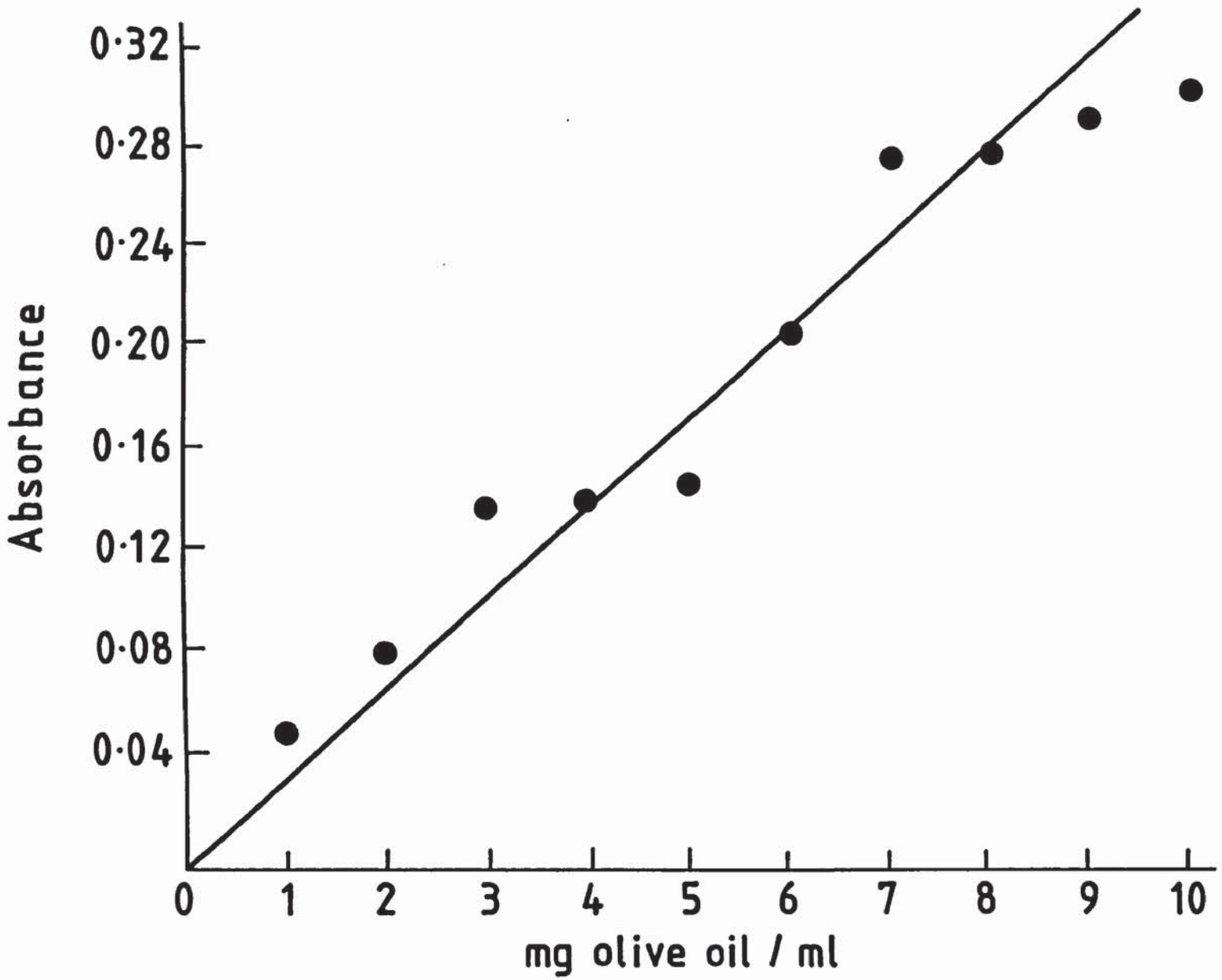

Absorbance
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
10
20
30
40
50
60
70
80
90
100
mg glucose / ml

Fig. (31) Olive oil standard curve


Absorbance
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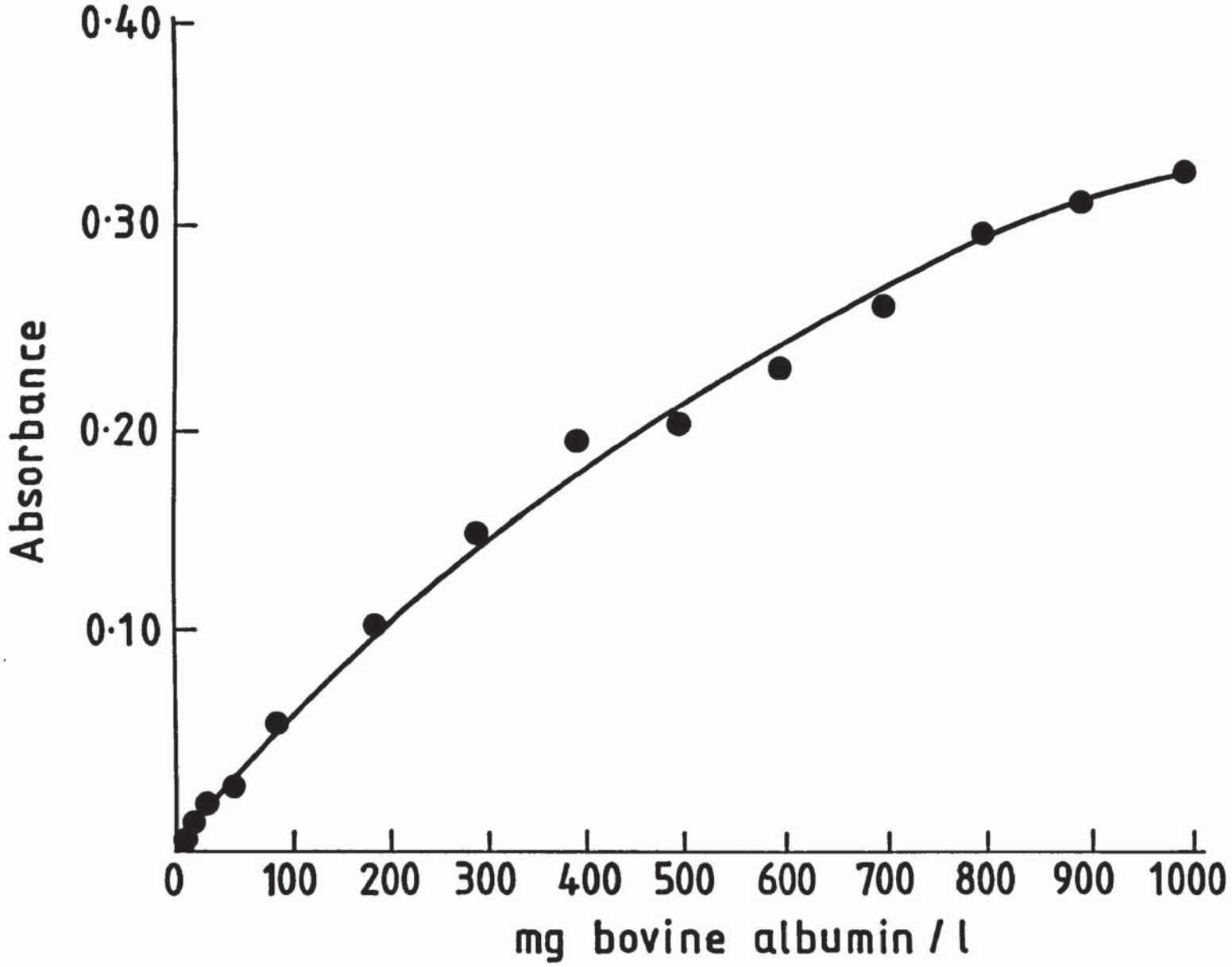
0·28
0·24
0·20
0·16
0·12
0·08
0·04
0
1
2
3
4
5
6
7
8
9
10
mg olive oil / ml

Fig. (32) Bovine albumin standard curve


0·40
0·30
0·20
0·10
Absorbance
0
100
200
300
400
500
600
700
800
900
1000
mg bovine albumin / l

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