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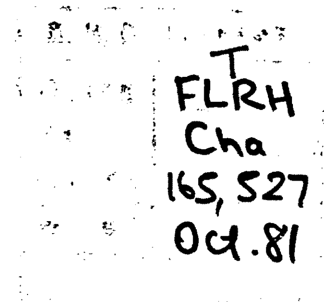
PECTINASES IN LEAF DEGRADATION BY AQUATIC HYPHOMYCETES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A Thesis submitted for the Degree of Doctor of Philosophy in the University of London.

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November 1980.

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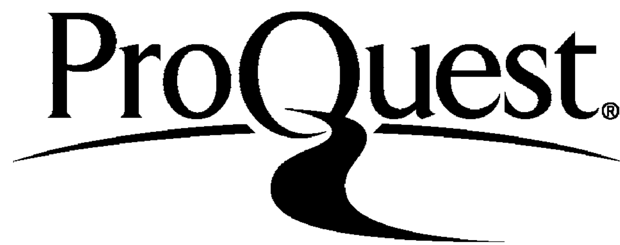

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ABBREVIATIONS.

ADCW - Acid-detergent cell wall
c. - *circa*.
cm/s - centimetre/s.
Conc. - concentration.
CPOM - Coarse particulate organic matter.
d. - day/s
diam. - diameter
DOM - Dissolved organic matter
D.W. - distilled water.
Fig/s - Figure/s
FPOM - Fine particulate organic matter.
g. - gram/s
GA - galacturonic acid.
hr/s - hour/s
l. - litre/s
m. - metre/s
M - Molar or Molarity
mM - millimolar
mg - milligram/s
min/s - minute/s
ml/s - millilitre/s
mm - millimetre/s
μg - microgram/s
μl - microlitre/s
Napp - sodium polypectate
n.d. - no data.
nm - nanometre/s
no/s - number/s
PG - polygalacuronase.
PGTE - polygalacturonate transeliminase.
PME - pectin methylesterase.
PTE - pectin transeliminase.
R. - River.
r. - radius
R.A. - Reciprocal Averaging.
ref/s - reference/s
rel. - relative
RVU - relative viscometric unit/s.
sec/s - second/s

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soln - solution
sp - species (singular).
spp - species (plural).
sub. - substrate
tt - test-tube.
UDGA - unsaturated digalacturonic acid.
Vol. - volume
v/v - volume for volume.
wt - weight
w/v - weight for volume.

ABSTRACT.

Packs of oak and alder leaves were submerged in late autumn in the River Bourne, a moderately eutrophic stream in Surrey so that the colonization pattern of aquatic Hyphomycetes on the leaves could be quantified as the leaves were degraded. The physico-chemical quality of the water was monitored over the experimental period and the inoculum available for leaf colonization was measured by filter counts of conidia in the stream. Colonization of the leaves by pectolytic bacteria was also measured. There was one series of oak packs and three of alder submerged a fortnight apart. Total spore counts/g dry wt of leaf rose to a peak for all series, followed by a decline. The time to peak colonization was slower in oak than in alder, and in the alder series was shown to depend on the level of inoculum in the stream. Pectolytic bacteria counts followed the pattern of total spore counts, suggesting the exploitation of the same substrates by bacteria and fungi. Alder I was skeletonized in 10wks., Alders II & III in 12wks. and oak in 25wks.

5g leaf packs were regarded as having a 'unit-community' of microbes. There was an association of 4 dominant species of aquatic Hyphomycetes on all leaf packs, with about 10 occasional species. Colonization involves the selection of the dominant species from the available inoculum. Within the association there is evidence of competitive interactions and a degree of substrate specificity. The species equilibrium is 14 for all series. Species numbers/pack are initially low, rise to a peak, then decline.

A quantitive analysis was made of species lists from the literature, to place the species list from the Bourne in a general context. Reciprocal averaging provided satisfactory results. It emerges that three factors influence the species of aquatic Hyphomycetes found in a stream: the geographical location; the physico-chemical quality of the water, and the plant species providing allochthonous litter.

7 aquatic Hyphomycete isolates from the stream could elaborate both polygalacturonases and pectin transeliminases. Some would grow at pH5 and 7; others only at pH7. PG's were produced constitutively and induced production of PTE's. Tricladium spendens and Articulospora tetracladia both elaborated 3 PG isoenzymes each of which was purified and characterized. Tetrachaetum elegans produced an exo-PTE and a pectin methylesterase. Mycocentrospora angulata elaborated an endo-PTE and a PME. The PTE's and PME's were partially purified and characterized.

All four species macerated strips of alder leaf completely within 9 - 12 days, utilizing PTE's mainly. Egglishaw's observation that plant degradation is most rapid in streams of high calcium concentration is likely to be due to the stimulating effect of calcium ions on microbial PGTE and PTE enzymes. Their activity increases with increasing Ca^{2+} concentration.

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LITERATURE REVIEW

A. THE STREAM ECOSYSTEM

H. B. N. Hynes, writing in 1970 on the ecology of running waters, commented on the very inadequate data then available on the trophic levels in heterotrophic stream ecosystems. Since then a great deal of experimentation has taken place in the field of freshwater stream ecology, and the data now available makes a general scheme of the structure and function of temperate-zone stream ecosystems possible (Cummins 1974, Hynes 1975). Indeed, Fisher & Likens (1973) have produced an annual energy budget for Bear Brook - a small undisturbed stream in New Hampshire. In Bear Brook it was estimated that 99% of the annual input of energy was in the form of allochthonous litter, but the degree of heterotrophy varies between and within individual streams, depending particularly on the extent of shading of the stream by the riparian canopy (Hall 1972) or from valley shading. The dependence of the stream community on energy derived from allochthonous litter has been well documented (Darnell 1964, Egglishaw 1964, Minshall 1967, Cummins et al 1973, Fisher & Likens 1973, Hynes et al 1974, Kostalos & Seymour 1976, Malmqvist et al 1978, Cummins & Klug 1979.) The litter which falls or is blown into streams consists of leaves, wood, blossoms, buds and fruits. Fisher & Likens (1973) estimated that in Bear Brook, the weight of leaves and wood entering the stream annually was about equal. In a coniferous-forest stream, wood comprises 70% of the total (Triska et al 1979). The amount and constituents of allochthonous material entering a stream and its seasonal distribution varies with forest type. The data available indicates quantities from 300 - 700 g/m2/yr (Anderson & Sedell, 1979). There is some input throughout the year, but in deciduous forests the autumn pulse represents a greater input than the three other seasons' values combined.

Stream	Forest Type	Seasonal inputs				Total
		W	S	S	A	(g/m^2/ yr)
Middle Bush N. Z.	Southern beech	34	284	136	113	567
Smith Creek, MI	Mixed hardwood	32	95	101	424	669
Coweeta, NC	White pine	22	48	52	197	319 b
	Old field	46	18	60	162	286 b
	Mixed hardwood	14	34	35	271	352 b
Doe Run, KY	Mixed hardwood	31	12	67	245	355 c
Bear Brook, NH	Mixed hardwood	10	80	195	375	660
Stampen, Sweden	Alder pasture	48	70	199	403	708
H. J. Andrew, OR	Douglas fir, Hemlock	270	150	90	220	730

b - Just litter fall, no lateral movement.
c - Leaf litter only.

TABLE 1: Comparison of allochthonous inputs to streams with various types of riparian vegetation (Anderson & Sedell, 1979; with refs therein).

Measurements of the dynamic processes involved in a stream ecosystem are complicated by the fact that input and export of nutrients, detritus and even animals, is a continuous, one-way process and not a self-contained complex. Furthermore, few streams have a uniform bed or velocity. Pools, dams, riffles, falls and rapids produce micro-environments whose substrata and micro-habitats vary, (Reice 1974). Hynes (1975) and Fisher & Likens (1973) emphasise the importance of studying a stream and its watershed as an ecological unit. Organic matter entering a stream may not be in the form of particulate matter, but as dissolved organic matter (DOM) derived from the surrounding vegetation. Water quality, as measured by dissolved nutrient concentrations, depends not only on the head-source of the stream and its geological substrata, but also on seepage of soil water from surrounding territory. Hynes (1975) quotes Hewlett & Nutter (1970) who show that in natural watersheds, overland flow of water is rare. Rainwater on a slope will tend to seep into the soil and displace water from lower levels, so that water entering a stream will have been

in contact with the soil for some time. Thus, slope of the ground, permeability of the soil and patterns of precipitation will affect the flow of a stream and influence greatly the substances in solution.

The degradation of wood debris in streams has received scant attention, though Jones (in press) has shown that some aquatic Hyphomycetes are able to rot timber blocks. The literature on the subject is reviewed by Anderson and Sedell (1979). The majority of studies are on the fate of dead leaves falling into streams, (Egglishaw 1964, 1968, Kaushik & Hynes 1968, 1971, Hynes & Kaushik 1969, Mathews & Kowalczewski 1969, Wetzel & Manny 1972, Krumholz 1972, Hayden 1973, Iversen 1973, 1975, Petersen & Cummins 1974, Hart & Howmiller 1975, Sedell et al 1975, Suberkropp, Klug & Cummins 1975, Suberkropp & Klug 1976, Howarth & Fisher 1976, Lock & Hynes 1976). The chemical composition of freshly fallen leaves varies with the age of the tree, the position of the leaf on the tree, tree species and site conditions. There are also significant differences in composition between softwood and hardwood leaves, (Ovington 1956, Coldwell & Delong 1950). Triska et al (1975) measured the carbon content of four leaf species before decomposition and their results are presented below. The acid detergent cell-wall fraction (ADCW) is made up of the most labile constituents of the leaf, including soluble carbohydrates, soluble protein, organic acids, non-protein nitrogen, hemicellulose and additional soluble organic material. Lignin fractions (L), cellulose fractions (C), and ash fractions (A) make up the leaf constituents.

SPECIES	%ADCW	%L	%C	%A
Alnus rubra	80.6	9.5	9.0	1.0
Acer circinatum	78.9	8.5	14.7	2.9
Acer macrophyllum	64.8	17.3	16.3	1.6
Pseudotsuga menziesii	59.4	24.2	14.5	1.9

TABLE 2: Carbon content of leaf species before decomposition.

(Triska *et al* 1975)

The ecological significance of the differences in chemical composition becomes apparent when rates of decomposition of leaf species are compared. The first two processes in the decomposition of leaves (CPOM - coarse particulate matter $>$ 1mm in diameter) that fall into streams occur rapidly. The first is the abiotic leaching of soluble organic matter: carbohydrates and polyphenols. Though CPOM continues to release soluble components throughout its reduction to fine particulate organic matter (FPOM - $<$ 1mm diameter), most of the leaching occurs within the first 24 hours of submersion, (Nykvist 1962, Kaushik & Hynes 1971, Petersen & Cummins 1974, McDowell & Fisher 1976). Suberkropp *et al* (1976) report a two week period for the initial leaching stage, but cannot quantify to what extent this was abiotic leaching. They report a 70 - 80% loss of reducing sugars, and Krumholz (1972) a 50 - 80% loss, in the initial two weeks. Different leaf species have differing leaching co-efficients. The leaching co-efficient, k, is the rate at which the entire leaf pack is losing weight with the final weight approaching zero, rather than the loss rate of the total leachable component.

SPECIES	k	% Loss/day
Quercus alba	0.0530	5.16
Carya glabra	0.1095	10.37
Populus tremuloides	0.2127	19.20
Salix lucida	0.2580	22.74
Cornus amomum	0.3175	27.20
Decodon verticillatus	0.1234	11.61
Fraxinus nigra	0.1145	10.82

TABLE 3: 24-hour leaching co-efficients (k) and % loss/day for selected leaf species at $5^{o}C$.

(Petersen & Cummins 1974).

At normal stream temperatures, (Augusta Creek 0.1 - 11.0°C) with relatively small temperature changes, a sizeable effect of temperature on leaching would not be expected. In laboratory experiments, Petersen & Cummins (1974) found no significant differences in the amount of leaching in hickory leaves at 4°C, 8°C and 17°C. The leaf lechate enters the pool of DOM (Dissolved organic matter - $< 0.5\mu m$).

The second process which occurs rapidly to newly-submerged leaves, is their colonization by micro-organisma in transport - cells and spores of bacteria; spores of aquatic fungi, particularly aquatic Hyphomycetes, and protozoans, (Suberkropp & Klug 1974). Microbial colonization is accomplished in a week or two, (Cummins 1974). The details of this process are poorly investigated, but certain significant changes take place in the leaves.

Kaushik & Hynes (1968, 1969, 1971), found that the initial protein and nitrogen contents of leaves varied, being high in alder and lowest in maple and beech. After leaching, the leaves continued to lose weight. This process was temperature-dependent being faster at higher than at lower temperatures, and was greatly enhanced by the presence of extra ammonium and nitrate ions, particularly where these were combined with phosphate. The rate of decay was fastest in the first few days and then continued at a lower but fairly constant rate for many weeks. The initial rate was increased if leaves were broken up, as they may be by feeding animals, but this effect did not extend beyond the first few weeks.

While decay was proceeding, the protein content of leaves declined a little in the first few days, but then rose sharply after a week at 20°C and two weeks at 10°C to a level that remained fairly constant for many weeks. During this process, no nitrogen was lost from the leaves and when extra nitrogen was available from the water it was taken up, particularly in the presence of phosphate, until a fairly constant level of leaf nitrogen was reached. The amount of nitrogen absorbed

varied with leaf species. These findings were based on laboratory experiments but the results were supported by evidence from field experiments.

Iversen (1973) observed an increase in the nitrogen content of decaying leaves in the first month of submersion in a springbrook and calculated that only 1 - 4% could be microbial nitrogen. Mathews & Kowalczewski (1969) observed the "nitrogen effect" in decaying leaves in the Thames. All these workers attributed the increase in nitrogen to the immobilization of nitrogen in micro-organisms. Suberkropp et al (1976), who also recorded the phenomenon, suggest that the observed nitrogen originates from a variety of sources. However, they only observed a 'trend' towards increasing protein values with processing time because absolute values varied greatly. They suggest that the protein may complex with plant phenolics and that this complexing of nitrogenous compounds within the leaves analyses out as part of the lignin component. Davis & Winterbourn (1977) report an increase in the percentage of protein with immersion time of mountain beech leaves in a New Zealand stream. Triska et al (1975) confirm the 'nitrogen effect' in both the streams they studied and found, too, a slight increase in phosphorus levels after initial leaf leaching. They quote Alexander (1961) who indicated that a carbon/phosphorus (C/P) ratio greater than 300 is favourable to phosphorus immobilization. All the leaf types decomposed in their two cascade streams had C/P ratios greater than 300. They concluded that P and N concentrations in stream water are theoretically limiting to the rate of leaf decomposition. Triska & Sedell (1976) carried out experiments to test whether nitrate concentration in water had a significant effect on the rate of decomposition of four leaf species. Two-fold increases in nitrogen content of leached, decomposing leaves were not affected by nitrogen concentrations in the water and they concluded that an effective mechanism for the uptake of nitrogen is more important than nitrogen concentration. Decay rates were related to the original lignin content

of the leaves and proceeded fastest in those species with low lignin. They suggest that the 'nitrogen effect' may be the result either of nitrate reduction, nitrogen fixation, or complexing of lignin with dissolved organic nitrogen or tissue nitrogen as humification proceeds. Whether the process is biological, chemical or both is open to investigation.

Carbon, nitrogen and phosphorus dynamics during leaf decay in nutrient-enriched laboratory stream micro-ecosystems were studied by Howarth & Fisher (1976). Using unenriched stream water as a control, they compared the long term (5 weeks) effects with nitrate or phosphate and nitrate with phosphate on leaf discs. The included in their experiments, measurements of microbial respiration on the discs in the water. They found that nett leaching accounted for only 35% of the total weight loss in the N+P system, compared to 51% in the control and nearly 70% in the +N and +P streams. 85% of the leached fraction remained unused in the five week period in the N+P stream, while in the other three streams, 54% - 69% of the leached material was used up in the aqueous phase. They suggest that gross leaching rates may be high in the N+P stream but that the labile substances may be quickly metabolised by disc microbes. Where N+P are available in excess, the locus of oxygen consumption (microbial respiration) is not in the water, as in the other stream systems, but in the disc complex. The leaf discs in the N+P stream showed an absolute increase in nitrogen over the five-week period accompanied by a precipitous decline in the concentration of nitrate and nitrite in the water. From days 12-34, the absolute nitrogen content and respiration rate of leaf discs rose concurrently in all four streams, suggesting that much of the nitrogen present was microbial. Howarth & Fisher found a lack of correlation between nitrogen content and respiration on discs prior to day 12, which suggests that leaching was the dominant factor controlling nitrogen up to that time; i.e. that microbial biomass was insufficient to retain much nitrogen and that leaf nitrogen was lost by leaching.

Phosphorus dynamics were found to be parallel to the observed changes in nitrogen. Howarth & Fisher's data support the 'nutrient limitation' hypothesis that where nutrient levels are ample, the locus of heterotrophic activity and nutrient accumulation is in the original source of energy - the leaf. Where one or more nutrients is in short supply, energy (organic carbon) is released and used elsewhere. However, immobilization and storage is a temporary phenomenon, The nutrients are later released, but probably in quite different physical and chemical form, e.g. as particulate organic matter which is later converted into particulate organic nitrogen or ammonia.

Meyer, (1980) found that leaves incubated in Bear Brook, New Hampshire, showed an increase in phosphorus content over the first year. She attributes this to microbial metabolism on leaves. Annual exports of dissolved P were only 70% of inputs. She found that the conversion of coarse particulate phosphorus to fine particulate phosphorus proceeded at different rates according to habitat.

Respiration rates (oxygen consumption) has been used by other workers as a measure of microbial metabolism and colonization of leaves. Triska (1970) found that leaf species which decomposed most rapidly showed the highest oxygen consumption. The pattern of respiration was parallel to fungal distribution and change in calorific value of the leaves: high initial values in winter, levelling off in the spring and rising with temperature rises in the summer. Triska found oxygen consumption in the winter to be due mainly to fungal colonizers. Kaushik & Hynes (1971) concluded from their studies that in the initial stages of leaf decomposition, fungi were more important than bacteria. Bacterial respiration declined after January, possibly due to cessation of leaching and/or low stream temperatures, but increased during summer. Iversen (1973), on the other hand, found that bacteria on beech leaves were more important than fungi initially, and Davis & Winterbourn (1977) made the same findings on mountain beech leaves in New Zealand. The

suggestion is that beech leaves, which decompose more slowly than other species, are a poor substrate for aquatic Hyphomycetes.

Hart & Howmiller (1975), working on two South Californian streams, found, too, that oxygen consumption was highest in those leaves which decomposed most rapidly. All these studies indicate an active role in leaf decomposition by aquatic micro-organisms that could only be achieved if, apart from metabolising leached sugars, they are capable of producing the enzymes necessary to utilize leaf cell-wall material.

The leached, microbially-colonized CPOM is reduced to FPOM through mechanical abrasion in the turbulent lotic environment and by two interdependent community processes - microbial metabolism and animal feeding. The rate at which this conversion takes place is dependent on temperature, on the extent of terrestrial preconditioning and the qualitative characteristics of the litter, (Cummins 1974). Little work has been done on the effects of microbial metabolism on leaf decay, and it is in this area that the present study may make a contribution. Studies on aquatic Hyphomycetes will be reveiwed separately. Studies of bacteria on leaves have mainly been limited to counts (Kaushik & Hynes 1971), though Suberkropp & Klug (1976) identified some of the bacteria associated with decaying leaves and carried out preliminary investigations of their nutritional capabilities.

Workers report that aquatic detritivores feed preferentially on microbially-conditioned leaves, (Kaushik & Hynes 1971, Iversen 1973, Petersen & Cummins 1974, Sedell _et al_ 1975, Kostalos & Seymour 1976, Ward & Cummins 1979). Baker & Bradnam (1976), investigating the role of bacteria in the nutrition of two aquatic detritivores, _Simulium_ and _Chironomus_, concluded that bacteria alone were not adequate to support these animals in their stream and that bacteria were not quantitively as important as other components of the detrital food material.

A number of studies indicate the importance of fungi in the diet of some aquatic detritivores. Bärlocher & Kendrick (1973 a, b; 1975a), working on the fungal and food preferences of *Gammarus pseudolimnaeus* (Amphipoda), showed leaf preferences in the order: ash $>$ maple $>$ oak, but if the animals were offered a choice between leaves and pure fungal mycelium, preferred the latter. The degree to which the fungi colonized the leaf species was in the same order as above. Fungal growth was accelerated by the addition of N+P to the water and this had the effect of increasing the protein content of the leaves, which was high in the case of some fungal colonizers. These findings are related to those of Kaushik & Hynes (1971), and bear out the contention that microbial protein is largely of fungal origin, and that, although there is little change through conditioning in the calorific value of a leaf, it serves as a more nutritous foodstuff than an unconditioned leaf. *Gammarus* exhibited fungal preferences as well as leaf preferences, and it was found that leaf preferences could be reversed if leaves were inoculated with preferred fungi.

It appears that it is not merely the fungus that increases leaf palatability. Changes in the leaf itself brought about by microbial excretions and secretions or by a hydrolytic agent (hot HC1) have a marked effect, probably because they macerate the leaf tissue and make it easier to crop, (Bärlocher & Kendrick 1975). The combination of the results of these two studies suggest that the rate at which a leaf species is invaded by fungi depends upon its initial composition, and that this, in turn, is related to its palatability to aquatic invertebrates. Mature oak leaves, for example, have a high tannin content (5%) and tannins have the effect of inactivating microbial enzymes (Benoit & Starkey 1968, Harrison 1971) and inhibiting insect attack, (Feeny & Bostock 1968).

In a second experiment on fungi in the diet of *Gammarus pseudolimnaeus*, Bärlocher and Kendrick (1973a) found that mycelium of an aquatic Hyphomycete proved to be much better food source for adult animals

than maple leaves with rich microbial growth. Ten fungi were isolated from leaves in streams. Young gammarids were offered maple or elm leaves or mycelium as food. The largest weight increses were found in animals feeding on four of the fungi, one of which was not an aquatic Hyphomycete. This, and the study of Minshall & Minshall (1978) show that not all aquatic Hyphomycete species are palatable to gammarids.

Marcus & Willoughby (1978), working on the diet of Asellus aquaticus, found that the animals fared equally well on diets of decaying oak leaves or Saprolegnia mycelium, but considerably less well on a diet of Lemonniera aquatica, an aquatic Hyphomycete. Similar results with Gammarus pulex were reported by Willoughby & Sutcliffe (1976). It is possible that the species of aquatic Hyphomycetes chosen were not particularly palatable to the animals under investigation and that in the natural environment there may be wide differences in fungal preferences between species of aquatic invertebrates.

Ross (1963) showed that the distribution of some stream taxa was associated with the distribution of riparian vegetation. Darnell (1964) investigated three sites. The first was a small calcareous stream; the second an estuary and the third a series of habitats in the near-marine waters of an estuary. He found that that organic detritus was consumed in abundance by macro-consumers at all three sites, but that detritivores were most prominent in the streams which were continually fed by fallen leaves from riparian trees.

Egglishaw (1964) found a close correlation between benthic invertebrates and the distribution of plant detritus in the Shelligan Burn and noted that the stream had many more animals than another which was chemically poorer. Egglishaw (1968) went on to relate the quantitative relationship between bottom fauna and plant detritus in streams of different calcium concentrations. Employing regression equations, he found that the rates of change of bottom fauna on plant detritus increased with increase in calcium ions in stream waters. Furthermore, in

experiments in which plant material was confined in nylon-mesh bags, the rate of breakdown of the plant material increased with increase in the calcium concentration of the stream water. The inference drawn is that the calcium concentration of the water influences the rate of microbial metabolism. However, Egglishaw does not conclude that calcium concentration is the controlling factor, and suggests that bicarbonate ions, nitrogen, phosphorus or pH may be limiting to the processes investigated.

Minshall & Minshall (1978) worked on two sites on the River Duddon. The upper site, Mosedale Beck, and the lower site, Crosby Gill, differed in water chemistry and in the quality of available allochthonous detritus and in the fungal flora infesting detritus. There was a marked difference in invertebrate fauna between the two areas. Experiments were carried out to determine whether water quality, the quality of detritus or the two acting together, determined the distribution of invertebrates in the River Duddon. Crosby Gill on the lower Duddon was much richer in invertebrates and detritus was processed more quickly. The water had a higher pH and higher concentrations of calcium, magnesium, sodium, potassium, bicarbonate, sulphate and phosphate. Only nitrate values were similar at both sites. Nevertheless, Minshall & Minshall concluded that chemical factors in the water operated directly to restrict the activity of benthic invertebrates in the upper Duddon, rather than indirectly through the food chain. In the case of Gammarus pulex, they maintained that potassium concentration was the limiting factor, quoting experiments by Willoughby & Sutcliffe (1976).

Cummins & Lauff (1969), in a study of the micro-distribution of stream macro-benthos, found that whereas current, the nature of the stream bottom, temperature or concentration of a chemical factor may limit the general ranges of habit tolerance, it appeared that substrate particle size or food supply were the main influences in the micro-distribution of animals.

Malmqvist et al (1978) studied the dynamics of detritus in a small Swedish stream and its influences on the distribution of the benthos. Their field experiments revealed an environmental mosaic in the stream bottom which made it difficult to elucidate how much variation in species composition was due to dynamic detritus processes. They found large fluctuations of population at some sites. However, in general, on a stony bottom, shredders and scrapers dominated. Where sedimentation was heavy, scrapers, deposit feeders and filter feeders were abundant. Downstream in the section with most extensive sedimentation, all categories, including predators, were well represented. Malmqvist et al concluded that the most two important variables influencing the animal community of their stream were environmental heterogeneity and substrate stability. They refute the claim of Egglishaw (1964) that detritus dynamics are the chief influence on the distribution of the benthos.

Recent studies of stream invertebrate communities show that a majority of benthic species ingest a component of detritus, (Hynes 1961, Minshall 1967, Coffman et al 1971, Scorgie 1974). Coffman et al (1971) found that the guts of 74 of 75 species of a riffle community contained some detritus, although only five species were classified as detritivores (> 50% of diet). For many species, the pattern was for young animals to consume detritus, but to change to algal or animal consumption on maturing. Hynes' (1961) study of a Welsh stream demonstrated the seasonal significance of detritus to certain invertebrate taxa. Growth of species active in the winter was based almost entirely on allochthonous material and these taxa accounted for about two-thirds of the productivity of the stream. Minshall (1967) investigated, by analysing gut contents, the role of allochthonous detritus in the trophic structure of a wood springbrook community. He found that of the 37 taxa of animals studied, 24 were herbivores, 5 omnivores and 8 carnivores. Detritus made up from 50 - 100% of all materials ingested by both herbivores and omnivores. 81% of the fauna was Gammarus minus with well over 90% of its diet

allochthonous detritus.

Cummins (1974), stressing that the perpetually incomplete state of taxonomic knowledge would constitute a major restraint to the development of ecological theory, has proposed the need to identify functional groups of organisms. Anderson & Sedell (1979) point out that this type of classification provides only a generalised picture of the community as most aquatic insects are opportunistic feeders and thus cannot be rigidly placed in a single functional group. Cummins & Klug (1979) classify shredders and scrapers (coarse particle feeders) collectors (fine particle feeders) and predators. Shredders chew material and scrapers scrape material off stones or rasp the softer tissues of leaves, leaving the veins intact. Fine particle feeders can be divided into filter feeders which obtain suspended materials from the water, and collector-gatherers that feed on FPOM deposits.

Although leaf decomposition can take place in the absence of shredders as is shown by studies using leaf discs and excluding animals (Triska 1970, Newton 1971, Kaushik & Hynes 1971, Bärlocher & Kendrick 1974, 1975), the presence of coarse particle feeders results in a 20% increase in the conversion of CPOM to FPOM at normal autumn and winter temperatures, (Boling et al 1973a, Petersen & Cummins 1974). Shredder feeding breaks up the leaves and increases surface areas for microbial colonization. The animal faeces enter the pool of FPOM. Triska et al (1975) report from Fenchel (1970) that amphipods digest only micro-organisms on detritus. The plant residue is undigested.

The FPOM pool for fine particle feeders is created partly through physical and biological reduction in CPOM particle size, but FPOM also enters a stream directly from the watershed. About 4% of the particulate organic input to Bear Brook was estimated to be FPOM by Fisher & Likens (1973). A significant amount of small particles enters the FPOM pool through activities associated with DOM. Lechate from CPOM as well as DOM from the terrestrial system - via surface run-off and subsurface

groundwater, plus aquatic plant and microbial excretions - are converted to FPOM by physical flocculation and microbial assimilation. This flocculation can be quite significant and is dependent on turbulence, temperature, pH and ion concentrations, (Lush & Hynes 1973).

The processing of dissolved organic matter has received some attention. Wetzel & Manny (1972), working in an experimental hardwater stream into which they introduced unleached leaves, produced a lechate ten times as concentrated as measurements in natural streams. The rapidly-formed labile organic carbon dissappeared within 72 hours, and the refractory component left had a $T\frac{1}{2}$ of 80 days. Most of the refractory material persisted unmodified for at least 24 days. Wetzel & Manny concluded that the decompositional capacity of natural streams for leaf lechates was governed by rates of bacterial utilization of dissolved organic carbon and nitrogen.

Cummins et al (1972), also working in laboratory streams, found the same initial rapid depletion of biologically labile dissolved carbon compounds, followed by a precipitous decline in suspended bacteria and a slower rate of utilization of more resistant organic compounds by bacteria. They concluded that the processing of DOM was primarily by bacteria in transport, rather than by organisms inhabiting the matrix of CPOM. They calculated an 85% removal of DOM in the experimental stream and that under natural conditions, processing should occur in one mile of stream.

Lock & Hynes (1976) dispute the findings reviewed above. They found only 10% disappearance of leaf lechate in two days, and 40% in four days in an experimental stream with natural water. Their experiments with sediment cores indicate that the major agent for the removal of lechate from stream water is the stream bottom. Within 24 hours, 85% of the initial lechate had been removed by sediment cores, compared to 10% in water alone. They concluded that uptake was due to microbial activity. They pointed out that all three studies reviewed above had

been to some extent artificial and that further field studies were necessary.

Such a field study was conducted by McDowell & Fisher (1976). They constructed a DOM budget for a small woodland-stream ecosystem - Roaring Brook. The study lasted 77 days in the autumn. They estimated that 17% of the litter input was released to the water as DOM within three days of entry. This represented 42% of the total input of DOM to the 1260m study area. The rest entered as surface or subsurface water and was reckoned to be largely refractory. Autumn uptake of DOM was 77% of the lechate and 33% of total DOM. About 88% of DOM removed from the water was retained within the system, and at least 67% degraded by consumer respiration in the autumn. McDowell & Fisher thought that suspended bacteria, being constantly in transport, were less likely than benthic bacterial assemblages to process DOM. They suggested that aquatic Hyphomycetes on solid substrates may be responsible, too, for the extraction of some dissolved organic material.

Field studies of leaf processing have been conducted, firstly in order to assess leaf processing rates and secondly to try to establish the factors influencing the different processing rates observed. Litter bags of various mesh size have been used in about half the studies, and leaf packs in others. Each has its advantages and disadvantages, but it appears that in both cases, the size of the leaf pack i.e. the weight of leaves used, is important. Reice (1974), using 1, 5, 20 and 40g packs, found a significant difference in the rates of decomposition between them. He concluded that packs of different sizes offer different degrees of protection from the elements for feeding invertebrates; that central leaves are more protected from microbial invasion in large packs and become more slowly available than in small packs, and that possibly there are differences, too, in oxygen and temperature regimes. i.e. the microclimate conditions in a leaf pack are dependent on its size and will influence the degree and rate of

microbial colonization, which would, in turn, influence the extent of animal feeding and litter breakdown.

Results of field studies on leaf-pack processing are very variable partly due to differences in methodology, timing and duration of the experiments and to techniques of processing samples; but partly due to real differences between geographic regions, species of leaf used, and to differences in the microbial flora and invertebrate populations, (Anderson & Sedell 1979). Furthermore, those who have included studies of the effects of stream substrata on leaf decay rates, (Reice 1974, Meyer 1980) have found, using analysis of variance, that the type of substratum is the most important factor governing decay rate. Reice, working with white ash in leaf packs, found that leaves were broken down least quickly on silt and most quickly on gravel and stones. Meyer, too, working with tree species of leaf in 3g bags, found decay rates were not correlated with the numbers or biomass of invertebrates (she was, though, working with fine mesh bags - 1mm.) Decay rates were slowest in areas of high sediment deposition. Fastest decomposition was in rapids $>$ pools $>$ debris dams. The work of Meyer and Reice point to community processing of leaf litter which is patchily distributed and which is dependent on heterogeneity and stability of the substratum. Their work ties in with the study of Malmqvist _et al_ (1978), reviewed above, on the dynamics of detritus in a Swedish stream and its influence on the distribution of the benthic fauna. If functional groups of aquatic invertebrates are distributed in relation to the structure of the stream bottom, it is not surprising that leaves will be processed most quickly in those areas attracting shredders i.e. stony and gravel bottoms, rather than in areas of high sedimentation which attract mainly fine particle feeders.

Other studies of leaf pack processing have concluded that temperature, leaf species and rates and extent of microbial conditioning and macro-invertebrate feeding are the most important factors governing leaf decay rates. Processing proceeds faster at higher than at lower temp-

eratures, generally, (Reice 1974, Petersen & Cummins 1974, Iversen 1975, Suberkropp et al 1975), but Reice (1974) found that some leaf pack sizes degraded quicker at lower temperatures. Triska & Sedell (1976) found quicker processing rates for four leaf species in streams with lower temperatures, and Meyer (1980) found that increase of decay rate with increase in temperature did not occur on certain substrata.

The most extensive study of processing rates of different leaf species was conducted by Petersen & Cummins (1974). Working with 15 species and using 10g leaf packs at 5°C, they found no significant difference between autumn and winter processing rates, and no site differences. Their major contribution to the quantification of species processing rates rests in their formula for the calculation of an exponential decay rate co-efficient, 'k', which can be used to calculate the % leaf pack remaining after one year's submersion.

	% remaining	k
FAST GROUP	<3	>0.010
MEDIUM GROUP	3 - 16	0.005 - 0.010
SLOW GROUP	>16	<0.005

Petersen & Cummins quote Fisher (1971) who calculated a turnover time of one year for leaf detritus with an export of 20%. They concluded from their studies that the governing factors in leaf processing rates were the type of leaf i.e. its initial chemical composition, the prevailing temperatures and the degree of microbial conditioning. As a type example they produced a processing budget for hickory leaves (Carya glabra) which had a medium processing rate at 5°C.

	% Wt loss		Time
Leaching	15		24 hours
Initial microbial processing	7		30 days
Animal-microbial processing	43	24%A 19%M	90 days
Total	65		121 days

TABLE 4: Processing budget for Carya glabra.

(Petersen & Cummins 1974).

The remainder of the leaf would continue to be processed by microbes and invertebrates, but after 120d (Sept. - Dec.), leaf packs were uncommon, so this residue would have been fragmented into another particle category subject to different processing rates.

The River Thames is not a woodland stream and though the work of Mathews & Kowalczewski (1969) is frequently quoted by workers in stream ecology, it is not strictly comparable. Using 20g of oak, willow and sycamore in litter bags, they found that all species would have disappeared after 1 yr's submersion. In coarse-mesh bags, 30% of oak and 5% of other species remained after 8 mths. Since the coarse-meshed bags with a higher invertebrate fauna were not processed any faster than fine-meshed bags, they concluded that animals did not play an important part in leaf disappearance in the Thames.

Triska et al (1975) worked with 10g leaf packs for 200d (see Table 2). The concluded that leaf-pack weight loss was highest in species with the largest fraction of ADCW. Species with initial high lignin fractions were most slowly decomposed. Lignin is refractory to microbial degradation. Suberkropp et al (1975) found that although oak leaves decreased in weight more rapidly with increase in temperature, the rate slowed down with time suggesting that refractory material like lignin accounted for a greater proportion of total weight. They provided evidence to support this conclusion in a subsequent study, (Suberkropp et al 1976).

Sedell et al (1975) found that species of leaf with high ADCW fractions were colonized by microbes more quickly than those with low amounts, which, in turn, meant that high ADCW species were more quickly subject to invertebrate feeding and to overall processing. It also meant that there is a continuum of food-supply for invertebrates in a stream over autumn, winter and spring as different leaf species are

conditioned at different rates. Sedell et al also found variations in seasonal values for 'k' for conifer leaves: Nov. - April 0.006, May - June 0.018. This difference was due to feeding in the summer months by larvae of Lepidostoma unicolor. In a stream with an impoverished shredder fauna, the 'k' value was 0.003. A simulation model is quoted by Anderson & Sedell (1979) which mimicked the field growth-pattern of L. quercina whose life cycle is geared to autumnal leaf fall. Rapid growth occurred when high-quality leaves (Alder) were available from August to November, and slow growth on low-quality leaves (Maple) for the remainder of the feeding period until February, (Grafius & Anderson 1979).

Iversen (1975), working on three streams in Denmark, used bags of 0.6mm, 1.3mm, 6.0mm mesh size and beech-leaf discs. The estimated disappearance time for 50% of leaf material was closely correlated with the mean number of invertebrates collected from 6.0mm bags. Iversen found differences between streams in the amount of breakdown caused by animals, and seasonal differences in one stream. He noted that invertebrates did not feed on newly-fallen leaves and calculated that 50% of leaf material would disappear in 190d by microbial action alone.

Benfield et al (1977) worked in a pastureland stream at 1 - 5°C with 15g packs of American sycamore, the native riparian species which were thinly distributed along the stream. It took 61 wks for some packs to be processed completely, though average weight was 40.38% of initial weight at that time. The calculated 'k' value was 0.0057 (slow - medium). The conditioning period appeared to be 70d as there was a large increase in processing after that time. They noted a marked absence of macro-shredders compared to other studies and suggest that the slow rates of decay observed, were due to a lack of diversity in riparian species which would preclude a processing continuum. Also, sycamore may be unpalatable to invertebrates, or, as a slowly processed species, may not become palatable at the critical time required to support shredder species.

Davis & Winterbourn (1977) worked in a New Zealand stream with 5 - 7g of Nothofagus leaves in 1mm mesh bags. They noted that few aquatic Hyphomycetes colonized the leaves, but there were abundant bacteria. Leaves lost about 50% of initial dry weight in 150d and only the lignified framework remained, which compares well with Iversen's values for beech leaves in Danish streams, (Iversen 1975). Numbers of shredders and other detritivores increased /g dry wt of leaf throughout the experiment. Numbers / bag were fairly constant. Although the fine mesh of the bags may have excluded larger detritivores, the numbers of these were low in the stream anyway. Davis & Winterbourn concluded that decomposition of Nothofagus leaves in their stream was due to bacterial activity and invertebrate feeding.

The general picture presented by all field studies conducted into leaf processing rates is of an overlapping continuum of litter decay arising from differences in the initial composition of leaves. These differences govern the length of time it takes for a leaf to become microbially 'conditioned' and attractive to grazing shredder invertebrates. Decay rates depend, too, on the numbers of shredders in the stream. Modifications include the temperature and chemistry of individual streams and variations in the substratum which will provide a mosaic of micro-environmental conditions.

General, conceptual models of the structure and function of a small stream ecosystem have been made by Cummins (1974) and Hynes (1975). The standing crop and trophic structure of a woodland stream have been determined by Coffman, Cummins & Wuycheck (1971) and an energy budget estimated. Boling, Petersen & Cummins (1973a) have produced an ecosystem model for small woodland streams using mathematical modelling techniques. The most complete study of a single watershed was made by Fisher & Likens (1973) who produced an energy budget for Bear Brook, New Hampshire. A diagrammatic scheme of their budget is given in Fig. 1.

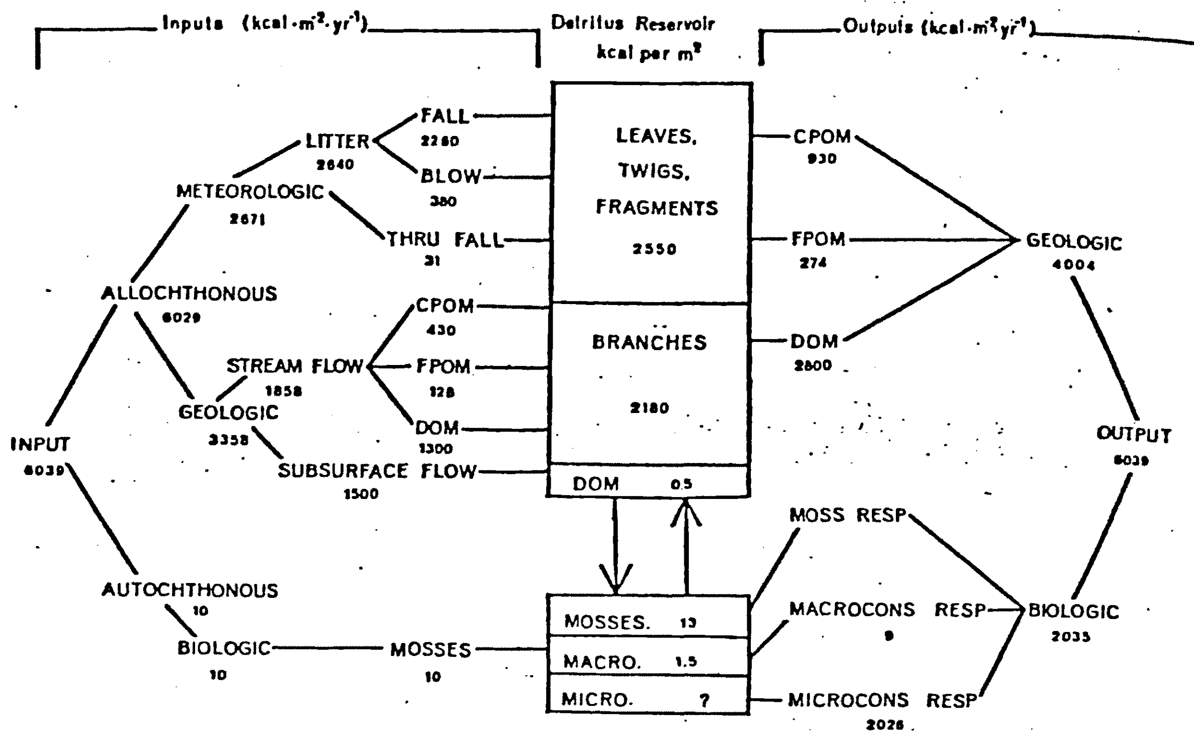


FIG. 1. Annual flux of energy in the Bear Brook ecosystem.
(Fisher & Likens 1973 after Fisher & Likens 1972.)

B. AQUATIC HYPHOMYCETES

Three substantial studies have been made to identify fungi associated with decaying leaves in streams that are active in decomposing leaf tissue at the temperatures prevailing in temperate-zone streams - 0 - 20°C.

Triska (1970) investigated the seasonal distribution of aquatic Hyphomycetes in relation to the disappearance of leaf litter in a woodland stream. He used discs cut from seven species of leaves and submerged them so that they would become unavailable to invertebrates. He found that the rate at which leaves were colonized and their mycofloral diversity varied according to leaf species. Alder, ash and birch were colonized more quickly than oak. His study lasted 1 yr, by which time the leaf discs had lost 70 - 90% of their initial weight. Triska concluded that in a year the stream microflora alone could decompose all but the most resistant leaf species. All species appeared to decompose most rapidly during the initial months - autumn and winter - with a decline in the spring followed by a marked increase in summer. Aquatic Hyphomycetes found sporulating on the leaf discs showed a seasonal succession of species. Some were dominant throughout the year, some at certain times and others appeared sporadically with no apparent pattern. The pattern of spore numbers followed that of weight loss in the leaves: high, initially, followed by a decline in spring and an increase in summer, suggestive of a direct relationship between aquatic Hyphomycete colonization and leaf weight loss. Triska concluded that stream temperature was the most important factor affecting species composition in the stream.

Bärlocher & Kendrick (1974) used leaf discs of ash, maple and oak submerged in an Ontario stream to study the dynamics of the fungal population on leaves in a stream. The mesh size of the bags was 3mm, which did not exclude detritivores. They found that the % protein in

the leaves increased with time and that there was no significant difference between pre-sterilized and unsterilized leaves. They concluded that the micro-organisms destroyed by autoclaving were inactive in the stream, or that, if they were, they were rapidly superceded by a new aquatic microflora. Measuring the length of fungal hyphae /unit area of maple leaf, they found that despite the low stream temperature (0°C), within a month of immersion there had been a great increase in hyphal growth in the leaves. Hyphal densities continued to climb as the temperature of the stream rose and as fungal growth overcame the opposing pressure of selective feeding by animals. Bärlocher & Kendrick identified all the fungi present on the submerged leaves. These included about 40 terrestrial fungi and 17 aquatic fungi, 14 of which were aquatic Hyphomycetes. Plate isolations of fungi, incubated at room and at stream temperatures, showed that the terrestial fungi present on freshly collected leaves grew better at room than at stream temperatures and on rich medium; whereas aquatic Hyphomycetes grew better at stream temperatures and on leaf or water agar. Furthermore, the terrestrial fungi failed, at stream temperatures, to cause significant weight loss or protein increment when grown on sterilized leaf discs, whilst the opposite results were obtained for aquatic Hyphomycetes isolates. Aquatic Hyphomycetes were the dominant forms observed sporulating on leaf discs. Bärlocher & Kendrick were tentative in the interpretation of their results, emphasising that the aim of their study was to distinguish between metabolically active organisms which play a part in degradative processes and those which are passively present in dormant or inactive form. The ability of aquatic Hyphomycetes to grow at very low temperatures may contribute to their dominance in the early succession on leaves, but as they also occur in the trophics, (Dixon 1959, Padgett 1976) this ability alone cannot have determined their success in well-aerated streams around the world. More probable is their ability to sporulate under water.

The third study to implicate the aquatic Hyphomycetes specifically as the dominant fungi colonizing leaf litter in streams is that of Suberkropp & Klug (1976). They placed leaf packs of white oak and pignut hickory in a small, temperate, hardwater stream and followed, by a series of complementary techniques, the fungal colonization of the leaves. They determined sporulating fungi by direct microscopic examination on sampling, and after incubation in aerated water at stream temperatures. They also used low and high temperature inorganic salts agar for incubation, and particle plate incubation on a rich medium at 25°C. They counted and identified bacteria from submerged leaves and measured total microbial biomass. The experiments were started in mid-November as stream temperatures were falling, and for half the study period, the mean weekly temperature remained below 5°C. Direct observations of the leaves at sampling time and of those incubated at stream temperatures, showed six major species of aquatic Hyphomycetes. Suberkropp & Klugg take the view from their studies that the ability of these organisms to sporulate _in situ_ is strong evidence that they are growing in the stream environment. The same species of aquatic Hyphomycetes appeared on both leaf species, but there were differences in abundance and in successional patterns. Suberkropp & Klugg compared the behaivour of aquatic Hyphomycetes with that of terrestrial fungus, _Alternaria_, spores of which were regularly observed on leaves. They found no sporulation in _Alternaria_ using the techniques and temperatures that produced sporulation in aquatic Hyphomycetes. _Alternaria_ failed to sporulate at temperatures below 10°C. The same results were obtained for other species of terrestrial fungi, and these workers came to the same conclusion as Bärlocher & Kendrick (1974), that terrestrial fungi are probably not active in leaf decomposition in streams. In support of this view Suberkropp & Klugg report that whereas terrestrial fungi occurred at fairly constant frequency and at about the same level on both oak and hickory, the aquatic Hyphomycetes showed a marked succes-

sional pattern. Moreover, the pattern agreed well with the realtive rates at which the two species were processed. There were higher frequencies of aquatic Hyphomycetes, more rapidly developed on hickory than on the more slowly processed oak. Low initial bacterial counts and high fungal frequencies associated with high measurements of microbial biomass (ATP), led Suberkropp & Klug to conclude, as did Kaushik & Hynes (1971), that fungi are the dominant micro-organisms processing leaves for the first half of the decompositional period.

Suberkropp & Klug and Bärlocher & Kendrick emphasised the need for physiological studies to explore the nutritional and enzymatic capabilities of aquatic Hyphomycetes, as well as their optimum temperatures and pH for growth and sporulation. There have been few such studies. Another field in need of investigation is that of interactions between bacteria and fungi, and between species of fungi. Bärlocher & Kendrick (1974) noted that decomposition of leaves proceeded faster when five species of aquatic Hyphomycetes were inoculated simultaneously onto leaves than when any single species was involved.

Ranzoni (1951) carried out nutritional experiments on two species of _Anguillospora_ to determine whether their morphological differences were reflected in nutritional differences. He found that both species would grow on a wide range of carbohydrates except cellulose and pectin. However, when extracted cellulose was used in an agar medium, both species grew on this.

Thornton (1963) investigated the physiology and nutrition of some aquatic Hyphomycetes. He worked with _Articulospora tetracladia_, _Flagellospora penicilloides_, _F. curvula_, _Lemonniera aquatica_, _Tricladium splendens_, _T. gracile_, _Heliscus lugdunensis_ and _Tricellula aquatica_. He found that they had optimum temperatures for growth (10 - 25°C) below those of aquatic Phycomcytes. This, he concluded, would give aquatic Hyphomycetes a competitive advantage at low temperature at which too, he had noted, they could sporulate. Nolan (1977) refutes Thornton's

conclusion, having found an aquatic Hyphomycete with an optimum temperature for growth of 20°C growing alongside a Phycomycete with a temperature optimum 13 - 19°C. Koske & Duncan (1973) investigated the temperature optima for growth and sporulation of twelve species of aquatic Hyphomycetes isolated from terrestrial habitats. The optimum for mycelial growth for most species was 20°C, and sporulation was greatest at temperatures lower than or equal to the optimum for mycelial growth. It was noted that species with a low optimum for sporulation were more seasonal in occurrence than those that sporulated well from 10 - 25°C. Neither Thornton nor Koske & Duncan report on the pH of the medium used in these experiments.

Eight species of aquatic Hyphomycetes investigated by Thornton (1963) could utilize a wide range of mono- and di-saccharides that had been identified in leaf litter from alder, oak, beech and birch, as well as starch. None of the species tested grew on methyl-cellulose. Phycomycetes were found to be more specific in their nutritional requirements. Thornton also found that the aquatic Hyphomycetes he tested had a high degree of vitamin autotrophism, which might also give them a nutritional advantage over other aquatic moulds. The aquatic Hyphomycetes were able to utilize inorganic nitrogen as nitrate or ammonium ions, a feature uncommon in other aquatic fungi. This seems significant when related to the increase in nitrogen with time observed in submerged leaves by many stream ecologists and reviewed above. Thornton (1965) also found that seven species of aquatic Hyphomycetes could utilize most of the amino acids found in leaf litter as both carbon and nitrogen sources.

Since soluble substances in leaves are leached out very quickly, it seems that they are unlikely to be the main energy source for aquatic Hyphomycetes. It is more likely that they rely for their nutrition on the degradation of the insoluble polymers that make up cell walls of

leaves. For this they would need to elaborate pectinases, hemicellulases, cellulases and lignases. If it were found that they have these enzymic capabilities, it would be strong evidence that the organisms are exploiting the native materials of the leaf - the other possibilities being that they are using the leaf as a holdfast and drawing their nutrient requirements from the water, or that they are expoliting the metabolic products of other organisms. Examination of enzymic degradation of leaf polymers before 1977 was too sparse and cursory to be taken seriously, but recently Suberkropp & Klug (in press) have reported on pectinases in Tricladium marchalianum which were associated with maceration of leaf tissue. Results of in vitro experiments were paralleled by field experiments, although the processes were slower in the latter. Increases in maceration of the leaf tissue were accompanied by maximum values for ATP and highest frequencies of aquatic Hyphomycetes sporulating on leaves.

Jones (in press) has reported cellulase activity in laboratory experiments with lignicolous aquatic Hyphomycetes. Seven isolates were tested, and the % loss of cellulose (Solka floc) after 3 weeks at 20°C was between 7.5 - 25%. However, only four of six cellulolytic species tested caused soft rot decay in wood after 13 wks incubation. Whether aquatic Hyphomycetes can and do utilize the cellulose available in leaf material is open to investigation. Pythium sp. and Fusarium sp. for example, are commonly found on decaying leaves, (Triska 1970, Willoughby & Archer 1973, Bärlocher & Kendrick 1974) and both are cellulolytic, (Park 1975, Willoughby & Archer 1973).

One would expect, in temperate zones, that aquatic Hyphomycetes would be most active at times of litter fall if allochthonous litter is indeed their substrate. Studies by Iqbal & Webster (1973) of concentrations of conidia of aquatic Hyphomycetes in the River Exe and its tributaries, show highest concentrations (up to 10,000/1) of both spores and species associated with peaks of leaf litter fall. Between the end of March and the beginning of August, there is a marked decline in

numbers of spores and species. Conway (1970) and Nilsson (1964) noted in temperate zone streams a marked increase in conidia and species of aquatic Hyphomycetes co-incident with leaf-fall in autumn.

Seasonal distribution of species noted by Triska (1970) were exemplified in Iqbal & Webster's study by the differences in concentration between Tricladium chaetocladium and Lunulospora curvula. The former was prevalent only in the winter and early spring. The latter was found only from August to the beginning of November. Webster et al (1976) investigated the differences in growth and sporulation between these two organisms in relation to temperature. Although the optima for growth and sporulation for L. curvula were some 5°C higher than those for Tricladium, they were beyond the range of river temperatures - 3 - 15°C. In that range, Tricladium showed relatively better growth than Lunulospora. When the two species were grown together, their temperature optima for sporulation was the reverse of that exhibited in monoculture, showing an influence of one upon the metabolism of the other. There was no evidence, from experiments, of mutual antagonism. As was pointed out in that study, the phenomenon of successional patterns in aquatic Hyphomycetes and the seasonal distribution of species, are probably the result of complex physiological interactions and may involve substrate specificity and/or interactions with other organisms, or mutual interactions. A further question is why, in streams with a very similar mycoflora, some species of aquatic Hyphomycetes will dominate in one stream and not in the other?

In a companion study to the River Exe and its tributaries, Iqbal & Webster (1977) looked at the conidial concentration and species of aquatic Hyphomycetes in some Dartmoor streams. There is a marked contrast between the River Exe water and the Dartmoor streams in chemical quality. The former are hardwater rivers with pH 6.3 - 9.0, high levels of dissolved ions and high alkalinity. The moorland streams are softwater streams, pH 4.05 - 6.45, with low conductivity and low alkalinity.

Nitrate and ammonium values for both are almost the same. There were no riparian, decidous trees upstream on two of the Dartmoor streams sampled where the highest spore concentrations were detected in March. At the two sited with appreciable tree cover, the maximum spore concentration was in November. Despite the general lack of seasonal input of litter, the moorland streams showed a marked periodicity of conidial concentrations, with peaks from August to March and very low concentrations from April to July. The moorland streams were found to have a distinctive mycoflora growing on the debris of Juncus, Molinia and other moorland plants, or on submerged branches. Though some of the aquatic Hyphomycetes encountered on this material were known from other substrata (e.g. Varicoporium elodeae), a number of previously undescribed species were found. Of note was the finding of relatively low spore concentrations in the Dartmoor streams (max. c. 100/1) compared to those of the River Exe and its tributaries (max. c. 10,000/1).

Suzuki & Nimura (1960a,b; 1961) in their studies of the distribution of aquatic Hyphomycetes in Japanese lakes, found a rich variety of species and high spore numbers in harmonic lakes, very few acidotrophic lakes and only V. elodeae in dystrophic lakes.

Iqbal (1972) investigated the effect of pH on growth and sporulation ofaquatic Hyphomycetes. He used phosphate buffer at various concentrations (0.25M, 0.1M, 0.06M and 0.025M). Iqbal found that the concentration on the buffer affected the capacity of the isolates tested to grow and sporulate at different pH values. A. tetracladia, for example, showed poor tolerance of high concentrations of phosphate at pH values above 6.0. At lower buffer molarity, pH tolerance went up to 8.0. Iqbal reports a range of pH tolerances for the species investigated but his data is difficult to interpret because of the effect of buffer concentration at higher phosphate values than would exist in stream waters. An interesting observation was that species found in harmonic waters could sporulate in acidic waters from Dartmoor streams.

If pH appears to be an important factor determining dominant species in a stream, another factor emerges from the ecological study of Gönczol (1975) on the aquatic Hyphomycetes of Hungary: the matter of substrate preference or specificity in these fungi. Gönczol looked at the seasonal variations of species of aquatic Hyphomycetes at two different sites on the Morgó stream. At the upper site, the main sources of leaf litter were Fagus sylvatica and Carpinus betula. The trees at the lower site were predominantly Alnus glutinosa. Gönczol found distinct and separate mycofloras at each site, as well as seasonal succession of species associations.

Padgett submerged leaf discs from five species of tropical trees in a tropical rainforest stream to determine the nature and rate of their decomposition. The leaves attracted a flora of aquatic Hyphomycetes with five species dominant. His results on the relationship between weight loss in leaves and numbers of colonizing fungi are difficult to assess, as are his data on changes in chemical composition of leaves: his results appear less clear-cut than similar studies in temperate streams. However, he noticed that fungal infection tended to be localised in the leaf discs and thin sections observed microscopically, showed progressive disintegration of mesophyll and epidermal tissues. Fungal hyphae were observed in vascular tissue - presumably those of aquatic Hyphomycetes as these fungi were found sporulating on the leaf discs.

Aquatic Hyphomycetes colonize conifer needles slowly and sparsely. Michelaedes & Kendrick (1978) suggest this is due to the thick cuticle and epidermis on the needles which present a physical barrier to fungal colonization. Leaves of colonization were greatly increased if the needles were treated with cold chloroform, methanol, hot ethanol hot water or sliced in half lengthwise. Bärlocher & Oertli (1978) were able to inhibit growth in aquatic Hyphomycetes with extracts from conifer needles. They isolated two substances, or groups of sub-

stances, from conifer needles which depressed fungal growth: one, probably free from polyphenols, which dissolves in petroleum ether as well as methanol; the other containing phenolic compounds, insoluble in petroleum ether but soluble in methanol. Polyphenols in leaf litter have been shown to lower its palatability to various invertebrates, and to depress growth of several saprophytic fungi, (Harrison 1971, Williams & Gray 1974). Many polyphenols form highly refractory complexes with proteins, e.g. enzymes, thereby immobilizing the digestive resources of predators, (Benoit & Starkey 1968, Suberkropp & Klug 1976).

Further evidence for the theory that aquatic Hyphomycetes are intermediaries of energy flow in streams (Bärlocher 1973) is that they are found sporulating not only on submerged leaves, but on wood too, (Jones & Oliver 1964, Archer & Willoughby 1969, Willoughby & Archer 1973). Jones (in press) has shown that some species of aquatic Hyphomycetes can cause soft rot in wood and Sanders & Anderson (1979) found that they can colonize sterile wood-blocks placed in a stream.

The conidial stage of aquatic Hyphomycetes seems to be essentially aquatic, though perfect stages described for some species may be terrestrial. Aquatic Hyphomycetes have been found in situations other than flowing streams, (Waid 1954, Bandoni 1972, Gönczol 1976, Singh & Musa 1977). Although they grow in terrestrial situations, they do not appear to thrive, (Webster 1977, Saunders & Webster 1978). However, Thakur (1977) found that some common species remained viable on leaves after dessication for 60 - 170 days.

The adaptation of aquatic Hyphomycetes to the stream environment is illustrated not only by the fact that conidia are produced, liberated and normally dispersed under water, but that the turbulence of the water appears itself to stimulate conidial development, (Younis 1966). The shapes of the conidia, tetraradiate or sigmoidal, Ingold (1975a), the leading authority on the subject, believes to be the result of convergent evolution in fungi in adaptation to an aquatic environment. The advantage

of the tetraradiate of sigmoidal spore over the round or ovoid spore in the turbulent aquatic environment is that the former are more easily trapped on smooth surfaces. This is probably due to the fact that a tetraradiate spore can contact a surface at three points, (Webster 1959).

The majority of the literature of aquatic Hyphomycetes is concerned with reports on new species, or with lists of species found in streams around the world (Ingold 1975b and refs therein). Most reports are from the temperate zone of the Northern Hemisphere made from foam samples. There are few quantitative studies or attempts to relate occurrence or seasonality of species to substrate or environmental conditions.

AIMS OF THIS STUDY.

It may be concluded from the evidence of the studies reviewed above, that bacteria and fungi are associated with the decomposition of leaf litter in streams. Of the fungi found on decaying leaves, it seems likely that aquatic Hyphomycetes are the most active in the aquatic environment in degrading leaf tissue. There was no proof that these fungi are physiologically capable of breaking down cell-wall polymers. If microbial colonization of leaf material is responsible for the observed increase in protein, nitrogen and phosphorus in decaying leaves with time, the possibility existed that micro-organisms were drawing on leached substances as their energy source, or were merely using the material as a hold-fast and drawing their nutrients from the surrounding water, or that they were living off the excess metabolites of other microbes.

The aim of this study was, firstly, to quantify the colonization of leaves by bacteria and aquatic Hyphomycetes in a stream with a population of these organisms. A stream with a limited number of riparian tree species was chosen so that decay of these leaf species could be

followed in the context of water chemistry and the spore inoculum available over the autumn, winter and spring period when litter-fall is at its peak and micro-organisms at their most active. It was decided to compare the colonization pattern of aquatic Hyphomycetes on a quickly-processed leaf species, alder, with that of a more slowly processed species, oak - both dominant riparian species of the stream; and to compare the colonization pattern on one species, alder, at different entry-times over the period of investigation: i.e. to compare inter- and intra- specific patterns of colonization throughout the decomposition of the leaves in the stream.

From the evidence of the field study, it was intended, secondly, to investigate one physiological aspect of the colonizers of leaves - their capacity to elaborate pectinases, production of which is a primary requisite for saprophytic invaders of non-lignified plant material. If pectinase production were proven for known colonizers, it would be strong evidence that these organisms are actively degrading leaf tissue. In vitro evidence of pectolytic capability on pure substrates would have to be supported by a demonstration that the organisms are capable of macerating leaf material by pectinase activity. The colonization pattern observed on leaves might be related to the pectolytic capacity of the aquatic Hyphomycetes investigated. The study might be placed in the general context by analysing species lists from around the world, quantitatively.

C. PECTIC POLYSACCHARIDES IN THE PRIMARY CELL WALLS OF DICOTYLEDONOUS PLANTS.

Plant cell walls are of two general types: primary and secondary. Primary cell walls are laid down by undifferentiated cells that are growing. The primary walls are those that control the rate of cell growth and which form the basic structural backbone of growing plant cells and tissues. Secondary walls are derived from primary walls by cells which have stopped or are ceasing growth and are differentiating into cells with specialized functions.

When a secondary wall is present, the following wall layers are recognized:

a) Middle lamella or intercellular substance thought to be particularly rich in pectic polysaccharides (Hall 1976).

b) Primary wall made up of cellulose, hemicellulose and pectic polysaccharides.

c) Secondary wall with cellulose as the main component, accompanied by non-cellulose substances but generally lacking pectic compounds. Lignin may be present in secondary walls. If lignification occurs, it begins in the middle lamella, extends through the primary wall and into the secondary wall, (Esau 1960).

Cellulose, hemicellulose and pectic polysaccharides are susceptable to enzymic degradation, but lignin is very resistant to enzymic attack.

The mesophyll and epidermal cells of leaves have primary cell walls only. It is in the vascular system of dicotyledonous leaves that lignification of cells occurs. Although, overall, the percentage of individual components like cellulose and lignin in leaves varies from species to species and even from leaf to leaf, (see Table 2), Albersheim and his co-workers (Darvill, McNeil, Albersheim & Delmer in press) have found that the primary cell walls of dicots studied are similar to one another, and differ from monocot primary cell walls. The polymer com-

position of the primary walls of suspension cultured sycamore is given in Table 5 below.

WALL COMPONENT	Wt % of cell wall
A. Pectic Polysaccharides	34
Rhamnogalacturonan I	7
Homogalacturonan	6
Arabinan	9
Galactan & possible Arabinogalactan	9
Rhamnogalacturonan II	3
B. Hemicellulose	24
Xyloglucan	19
Glucuronoarabinoxylan	5
C. Cellulose	23
D. Hydroxyproline-rich Glycoprotein	19

TABLE 5: Polymer composition of the primary walls of suspension-cultured sycamore cells.

(Darvill, McNeil, Albersheim & Delmer in press)

Polysaccharides capable of breaking down cell walls secreted by pathogenic fungi, have been found to be produced sequentially. Initially pectinases are produced which induce the production of hemicellulases and cellulases (Jones et al 1972). This is evidence of the sequence in which cell wall polymers are accessible to degrading enzymes. The present picture of the dicot wall, is one in which the cellulose fibres are covered in a layer of hemicellulose non-covalently bound to the cellulose. These fibres are inter-connected by the pectic polysaccharides which are covalently bound to each other. There is no known attachment between the protein and other cell wall polymers. An organism capable of utilizing cell-wall polymers as an energy source would need to start on the pectic polymers.

The most characteristic component of pectic polysaccharides are galacturonosyl residues. In addition to these there are rhamnosyl, arabinosyl and galactosyl residues. The rhamnosyl residues are closely associated with galacturonosyl residues in that both are integral components of the same polysaccharide chain. A considerable portion of the arabinosyl and galactosyl residues appear to be components of araban and galactan sidechains which are covalently attached to the Rhamnogalacturonan backbone, (Darvill, McNeil, Albersheim & Delmer in press).

Rhamnogalcturonan I isolated from the walls of suspension-cultured sycamore cells, shows a polysaccharide containing 2- and 2,4 - linked rhamnosyl residues and galacturonosyl residues in a ration 1:1:4. It is thought that the backbone of rhamnogalacturonan I is a single linear chain with a d.p. (degree of polymerization) of about 2,000, containing about 300 rhamnosyl residues and 600 galacturonosyl residues uninterrupted by regions of homogalacturonans.

The regions of homogalacturonans consist of unbranched α-4-linked galacturonosyl residues with an apparent d.p. greater than 25, but thought to be considerably longer. The carboxyl groups of the galacturonosyl residues are known to be highly methyl esterified. It is not known how the methyl esters are distributed along the polygalacturonan backbone, though it is clear that there are regions which are highly methyl esterified as well as regions which are relatively free from esterification.

Arabans have been isolated from the cell walls of many dicots An araban, free from other polysaccharides, has been isolated from a methylated primary cell wall polysaccharide fraction of suspension-culturer sycamore cells. The arabans are highly-branched polymers, and the evidence is that arabinosyl residue linkages are in the furanose ring form. Two different arabans isolated from the bark of Rosa glauca had d.p.'s of 34 and 100, and araban from willow a d.p. of 90. A number of complex pectic polysaccharides have been demonstrated

to contain arabinosyl residues, but it is not known whether these exist as relatively long arabans chains, or whether as mono-, di- or tri- saccharide sidechains attached to other pectic polysaccharides

Galactans have been isolated from citrus pectin, white willow and beech. No homogalactan has been isolated directly from primary cell walls. The pectic galactans are primarily β -4-linked polymers, with a d.p. ranging from 33-50. The presence of homogalactans in primary cell walls is inferred by the detection of large amounts of 4-linked galactosyl residues on methylation analysis of total cell walls and pectic fractions of cell walls. Also, small oligomers of β -4-linked galactosyl residues have been isolated in quite large amounts from cell walls treated with an endo-β-1,4 galactanase which can only hydrolyse galactans with 4 contigous β - 4 - linked galactosyl residues. Many of the galactosyl residues of plant polysaccharides are, however, probably not part of homogalactans, as some have been shown to occur as β-4-linked dimers in one pectic polymer and as 3- and 6-linked residues in another. It is likely that these galactosyl residues are part of an arabinogalactan.

Arabinogalactans have been isolated from the tissue of a number of dicots, but not from a source known to consist only of primary cell walls. The presence of arabinogalactans in primary cell walls is supported mainly by the results of a single study of a pectic fraction obtained from walls of suspension-cultured sycamore cells treated with endopolygalacturonase. Since no arabinogalactans has been purified from primary cell walls of dicots, its existence is not certain. Furthermore, the great variability among arabinogalactans isolated from dicots makes a generalized model difficult to achieve.

Rhamnogalcturonan II is a recently-discovered pectic polysaccharide isolated from the walls of suspension-cultured sycamore cells. Upon hydrolysis, it yields the rarely-observed cell-wall

sugars 2-O-methyl fucose, 2-O-methyl xylose and apoise. Rhamnogalacturonan II is solubilized from the cell walls by the action of endo-α-1,4 galacturonase. The polysaccharide is characterized by a wide variet of terminal glycosyl residues including T-galacturonosyl, T-galactosyl, T-arabinosyl, T-2-Omethyl xylosyl, T-2-O methyl fucosyl and T-rhamnosyl residues. The larger number of terminal glycosyl residues suggests a highly-branched molecule and Rhamnogalacturonan II appears to be the most complex plant polysaccharide known.

(Summaries from Darvill, McNeil, Albersheim & Delmer in press).

D. PECTIC ENZYMES

Maceration of plant tissue involving separation of cells along the line of the middle lamella as the result of enzymic degradation, is common in many diseases of higher plants caused by fungi and bacteria. Maceration facilities inter-cellular penetration by pathogens and exposes substrates in cell walls to polysaccharides. It is largely in the field of plant pathology that pectic enzymes have been studied, (Cooper 1974).

That pectic enzymes are responsible for maceration has been shown by treating plant tissue with pure enzymes. Endopolygalacturonases were employed by Ayers, Papavas & Lumsden (1969), Bateman (1972) and Hall & Wood (1970); endo-pectin lyases by Byrde & Fielding (1968), Hall & Wood (1970) and Garibaldi & Bateman (1971).

The group of pectic enzymes includes pectinesterase (pectin-methylesterase, PME), which catalyses the de-esterification of pectin; and depolymerizing enzymes which catalyse the splitting of glycosidic α-1,4 bonds of the D galacturonan (galacturonic acid) chain of the pectic molecule, (Rexová-Benková and Markovič 1976).

According to the IUPAC - IUB Enzyme Nomenclature (1973), pectin-esterase belongs to the carboxyl ester hydrolases (EC.3.1.1.11) and has the systematic name pectyl-hydrolase.

Demain & Phaff (1957), Neukom (1963) and Bateman & Millar (1966) classified the depolymerases into four groups depending (a) on the preference of the enzyme for pectin or pectate as a substrate, (b) on whether the enzyme cleaved the terminal glycosidic bond in the chain or operated at random. See Table 6.

Investigations of pectic enzymes conducted recently have shown this system of Neukom (1963) and other workers cited, to be inadequate. Exo-polymethylgalacturonase and exo-pectin lyase had not been found.

Preferred substrate	Action Pattern	Name	Modified[a] EC systematic name	EC No.
Hydrolases				
D-Galacturonan	random	endo-D-galacturonanase	poly-(1 - 4)-α-D-galactosiduronate glycanohydrolase	3.2.1.15
D-Galacturonan	terminal	exo-D-galacturonanase	poly-(1 - 4)-α-D- galctosiduronate glycanohydrolase	3.2.1.67
D-Galacturonan	penultimate bonds	D-galacturonandigalacturono	poly-(1 - 4)-α-D- galactosiduronate digalacturonohydrolase	3.2.1.82
Oligo-D-galactosiduronate	terminal	oligo-D-galactosiduronate		
Lyases				
D-Galacturonan	random	endopectate lyase	poly-(1 - 4)-α-D-galactosiduronate lyase	4.2.2.2
D-Galacturonan	penultimate bonds	exopectate lyase	poly-(1 - 4)-α-D-galactosiduronate exolyase	4.2.2.9
Oligo D- galactosiduronate	terminal	oligo-D-galactosiduronate lyase	oligo-D-galactosiduronate lyase	4.2.2.6
Poly (methyl D-galactosiduronate)	random	pectin lyase	poly (methyl D-galactosiduronate) lyase	4.2.2.10

[a] Modified to conform with accepted carbohydrate nomenclature.

TABLE 7: Nomenclature of Pectic Depolymerases

(Rexová-Benková and Markovič, 1976)

MECHANISM	SUBSTRATE	ACTION PATTERN RANDOM	TERMINAL
Hydrolysis	pectate	endopolygalacturonase	exopolygalacturonase
	pectin	endopolymethylgalacturonase	exopolymethylgalacturonase
β-Elimination	pectate	endopectate lyase	exopectate lyase
	pectin	endopectin lyase	exopectin lyase

TABLE 6: Classification of Depolymerizing Pectic Enzymes According to Neukom (1963).

ex Rexová-Benková & Markovič (1976)

Some exo-hydrolases as well as exo-lyases were found to degrade oligogalacturonates in preference to polymers as a rate inversely proportional to the chain length of the substrate. These were called 'oligogalacturonide hydrolase' and 'oligogalacturonide lyase'. Rexová-Benková & Markovič (1976) set out a table for the nomenclature of pectic depolymerases based on work up to 1976. See Table 7 opposite.

In this work, hydrolases are specified by the name 'polygalacturonase' and lyases by the name 'transeliminase'.

Polygalacturonases (PG's) and pectin transeliminases (PTE's) are characteristic of fungi and pectate transeliminases (PGTE's), with some exeptions, are found in bacteria. Transeliminases have a complete or partial requirement for calcium ions and a pH optimum c. 9. Polygalacturonases usually have a pH optimum c. 5 and are unaffected or inhibited by calcium. The requirement for esterified or non-esterified residues in the pectic chain may be absolute (Cooper 1974).

Pectin esterase, by present accounts, is an enzyme highly specific for the D-galacturonan structure. Even a partial reduction of the carboxyl groups of pectin brings about a marked inhibition of pectin esterase activity, (Solms & Deuel 1955). Enzymic de-esterification of the methyl esters of pectin proceeds linearly along the chain, giving rise to blocks of free carboxyl groups (Heri, Neukom &

Deuel 1961). Half the activity of a pectin esterase isolated from tomatoes was found to be initiated near the reducing end of highly-esterified pectin and the remaining activity occurred at some secondary locus, probably free carboxyl groups, (Lee & Macmillan 1970). In a second study it was found that exopectate lyase was inactive on highly-esterified pectin without pectin esterase and it was concluded that in this case all the activity was at the reducing end of the molecules, (Miller & Macmillan 1970). The pH optima for pectin esterases vary. Generally, for those from higher plants the optimum lies between pH 6-9.5, and for those of fungal origin, in the acid region, but this generalization does not always hold. Univalent and divalent salts produce a twofold increase in activity of pectin esterases of microbial origin, (Rexová-Benková & Markovič 1976, Table III). Pathogenic fungi and bacteria produce pectin esterases which pass into the culture medium together with other pectolytic and cellulolytic enzymes. Microbial pectin esterases are inducible in the presence of pectin or pectic acid.

Polygalacturonases catalyse the hydrolytic cleavage of the glycosidic α-D 1,4 bonds of non-esterified D-galactopyranosiduronic residues. Hence pectic acid and pectates having a low degree of esterification are the preferred substrates. Random splitting of internal bonds of the D-galacturonan chain, catalysed by endo-polygalacturonases results in a swift diminution in the viscosity of a substrate solution at a low degree of splitting of glycosidic bonds. Beckman (1966) quoted 1-2% splitting of glycosidic bonds with 50% reduction in viscosity. The primary products are higher oligo-D-galactosiduronates with an increase in lower oligomers as the reaction proceeds. These features are common to all endopolygalacturonases, but their mode of action is not identical. Differences are displayed not only by enzymes of different origin, but even by enzymes produced by one organism. For the endopolygalacturonases from the culture filtrate

of Coniothyrium diphodiella, Endo (1964) found that a 50% decrease in the viscosity of a sodium polypectate solution corresponded to the hydrolysis of 3, 4 and 10% of the glycosidic respectively. The rate of splitting of glycosidic bonds by endopolygalacturonases decreases with the shortening of the substrate chain, (Phaff & Demain 1956). Whether restriction of enzyme activity by the degree of polymerization of the substrate is significant in vivo is difficult to determine. Keegstra et al (1973) considered that neutral sugars in the backbone of pectic polymers effectively reduce the number of contiguous galacturonide units in sycamore cell walls to c. 8. But Darvill et al (in press) consider that sycamore homogalacturonan has a d.p. considerably longer than 25.

Exo-polygalacturonases catalyse the hydrolytic cleavage of terminal α-D-1,4 bonds of D-galacturonan chains releasing galacturonic acid. With high-molecular substrates, the action-pattern is manifest in a substantial rise in reducing groups, accompanied by a slow decrease in the viscosity of the substrate solution. A 50% decrease in viscosity corresponds to a 30-45% cleavage of glycosidic bonds, (Rexová-Benková & Markovič 1976). All known plant and microbial exopolygalacturonases degrade the substrate from the non-reducing end of the chain and the rate of cleavage of glycosidic bonds is either independent of, or inversely proportional to the d.p. of the substrate.

Transeliminases work by β-elimination, which involves the removal of the hydrogen atom from C5 of the galacturonic molecule This is activated by the electron-withdrawing group at C6 in the presence of a suitable proton-acceptor acting as a catalyst. The unstable, intermediate ion formed is stabilized by splitting of the C-O bond in the β position. The loss of substituents at C4 and C5 gives rise to a double bond. The free carboxyl groups at C6 cannot sufficiently activate the hydrogen atom at C5 in an alkaline medium and hence the non-enzymic β-elimination, in contrast to the enzymic

is restricted to esterified D-galacturonans and the degree of degradation is determined by the degree of esterification, (d.e.). In the course of degradation of non-esterified or low-esterified substrates by pectate lyases, the glycosidic bonds can be split either terminally or at random. By contrast, pectin lyases, operating on highly esterified substrates, are only known in the random action-pattern. The end product of pectate or pectin lyases is unsaturated di-galacturonic acid, (Rexová-Benková & Markovič 1976). In pectin lyases, the extent of degradation of the substrate and the affinity of the enzyme decrease with decreasing d.e.. The rate of splitting of glycosidic bonds of pectin decreases with decreasing d.p.. Calcium ions activate most pectin lyases, their effect depending on pH and d.e.. pH optimum is itself dependent on d.e.. All the pectin lyases so far described are of fungal origin and are secreted extracellularly. Pectate lyases are produced constitutively in pathogens but are inducible in saprophytes, (Rexová-Benková & Markovič 1976).

MATERIALS AND METHODS.

A. THE FIELD STUDY.

1. The Stream

The stream under investigation is the River Bourne which flows from the falls of Virginia Water, a series of lakes fed by streams, to the River Thames. The first stretch of the stream is lined by alder, birch and some oak, with an undercover of rhododendron. There are macrophytes in this part of the stream including *Nuphar lutea* and *Nymphaea alba*. The stream opens into a small lake on the eastern side of which is deciduous woodland with rhododendrons. The western side has fewer trees. These include alder, oak and willow. Macrophytes on the edge of the lake include *Nymphaea*, *Sparganium* and *Iris pseudocorus*.

The run-off from the lake passes over falls of about 1.5m. The sampling point was about 20m downstream from these falls, (Grid ref. OS sheet 170: SU987678). The stream at the sampling site is, on normal flow, 6m wide and 30cm deep at centre. The current speed in average conditions is 1m/5secs. The riparian vegetation around the falls and sampling point is beech, oak, ash, lime and alder with rhododendron. The substratum of the stream is a mixture of sand and clay.

Preliminary analyses showed that the nutrient levels of the water at Virginia Water falls were a little different from those at the sampling site, so it was decided to do independent analyses of water at the sampling site. Analyses were to cover those chemical and physical factors known to be of importance in aquatic ecosystems: nitrate, phosphate, oxygen, DOM levels, pH and temperature. Alkalinity was taken as an average of values for Virginia Water falls.

Preliminary examination of foam from below the falls at the sampling site showed an extensive aquatic Hyphomycete spora.

2. Leaves and Leaf Packs.

Alder, (Alnus glutinosa (L). Gaerth) and oak (Quercus robur L.) were chosen for the leaf processing experiments because both are reported to attract aquatic Hyphomycetes, (Triska 1970), and both are dominant species along the stream banks. Also alder is reported to be more quickly processed than oak, (Petersen & Cummins 1974, classify alder as being in the Group II, medium, processing group and oak in Group III, slow.). If the rates at which each were to be decomposed in the Bourne followed reported values, it would allow a comparison to be made between aquatic Hyphomycete colonizers of each of the two leaf species. It was decided to place three sets of alder leaves in the stream at fortnightly intervals to allow intraspecific comparisons to be made.

Newly-abscissed leaves were collected daily in nets raised above one oak tree and a stand of alders. This method minimised leaching and infection by terrestrial microbes. The leaves were air-dried at room temperature and stored in paper sacks.

Field studies of leaf processing in streams have been conducted with leaves in litter bags or with leaf packs lashed to masonry bricks, (Petersen & Cummins 1974). The latter are considered to be analagous to natural leaf accumulations and are appropriate for measuring overall processing rates by all agencies. In the present study, it was considered that the very friable quality of alder leaves would make leaf packs less appropriate to a study of the aquatic Hyphomycete flora, and litter bags were used.

The bags were made of two pieces of nylon mesh sewn together with nylon fishing line. The diamond mesh size was 3mm and the bags 19 x 15 cm without hems. They were filled with 5g leaves, labelled and lashed in sets of four with two monofilament nylon lines (12lbs breaking strain) to masonry bricks so that the bags did not overlap, and so that they would remain parallel with the stream bed. Bags

were placed in about 20cm depth of water along the edge of the stream where leaves naturally collect. It was found that the bags did not become logged with silt and that they were large enough both in size and mesh to allow water to circulate over the surfaces of the leaves.

3. Leaf-pack sampling.

Leaf packs were removed fortnightly from the stream, placed in sterile polythene bags and taken to the laboratory. Before the leaves were rinsed in stream water to remove sediment and invertebrates, ten discs were cut with a flamed #5 cork borer (1cm. diam.) for bacterial counts and placed in sterile distilled water. The remaining leaves were placed in shallow trays and covered with distilled water. The trays were placed in a growth room maintained at 20°C and left for a week to allow for sporulation of aquatic Hyphomycetes colonizing the leaves. The water level was maintained daily. Although most aquatic Hyphomycetes will have sporulated under these conditions, there is no certainty that all species will have done so. The cultured isolate of Tricladium splendens, for example, will only sporulate in aerated water. However, as alder leaves are very friable, it was felt that aeration might break them up completely, or possibly lead to recolonization of leaf material.

4. Bacterial counts. after Suberkropp & Klug (1976).

10 leaf discs each from Alder I and Oak packs were dried on sterile filter paper and homogenized in 125 ml sterile distilled water for 8mins on top speed setting. The suspension was allowed to settle and 10ml were withdrawn to be stock for subsequent dilutions. Sterile water and glassware were used throughout this procedure. Of each dilution, 0.1ml was spread on plates (3): nutrient agar plates were used to give total bacterial counts, and plates specific for the detection and enumeration of pectolytic bacteria, (Hankin, Zucker & Sands 1971). The mineral medium with 0.5% sodium polypectate and 0.1% yeast extract was used and the plates were left to dry for a day

before use to prevent smearing of bacterial colonies. All plates were incubated at 30°C for 72hrs. Counts (mean of 3) are calculated as a total per gram wet weight of leaf, or as numbers per disc.

5. Fungal spore determinations. after Willoughby (personal communication).

(i) About 1g incubated leaves were placed in 80mls distilled water in a strong polythene bag and paddled for 15 secs in a stomacher, (Colworth 400) to remove spores but prevent excessive fragmentation of the leaves. The liquid was decanted into a measuring cylinder and allowed to stand for 5mins to allow grosser particles to sediment out. The leaf material was placed in evaporating dishes in an incubator at 90°C and left for 24hrs to dry. 2ml of the decanted liquid was gently pipetted into each of two perspex sedimentation tubes and allowed to stand for 30mins. Spores were identified and counted using an inverted microscope, (Evans 1972). A preliminary count of 20 tubes showed that there was no more than a 5% error between counts of the first two and subsequent tubes using the same sample. Results are calculated as:

(a) percentage frequency of total, for individual species.

(b) total spores /g dry weight of leaf.

(ii) 41 of stream water were collected at the time of leaf sampling. 500mls of this was passed through an 8μm millipore filter which was then stained overnight with freshly-made cotton blue in lactic acid. Aquatic Hyphomycete conidia were identified and counted using a light microscope, (Iqbal & Webster 1973). Results were calculated as:

(a) percentage frequency of total for individual species.

(b) total spores /1 of stream water.

(iii) Foam samples (scum) were taken in the early stages of the field study as they provide information of species present in the stream. However, quantitatively, foam samples are unreliable as some spores are trapped more readily than others. Foam samples were used for the isolation of aquatic Hyphomycetes.

6. Isolation and culture of fungi.

0.1ml of foam sample containing aquatic Hyphomycete conidia was plated onto Willoughby's GYS medium (see D1a below) with antibiotics (0.05g/100ml each of sodium benzylpenicillin and streptomycin sulphate added after autoclaving). The plates were incubated at 15°C for 24hrs. With the aid of a dissecting microscope and a sterile needle, germinating spores were plated onto GYS medium without antibiotics and allowed to grow at 15°C until a well-developed colony was established. Pieces of the colony + agar were then submerged in distilled water to induce sporulation which allowed for identification of the species. Colonies which did not sporulate under these conditions were placed in distilled water in flasks and aerated, (see D5 below). All isolated pure cultures were maintained on GYS medium at 15°C.

B. WATER ANALYSIS.

1. When leaf packs were collected from the stream at 10.00 am on Mondays, fortnightly, 4 1 of stream water was collected in a clean flask. Water temperature was read with a mercury-in-glass thermometer at this time. The same day, the following analyses were conducted on the water:-

2. Oxygen determinations: a modified Winkler technique.

At sampling time, two glass bottles, 120ml, were filled with stream water and stoppered so that no air-bubbles were trapped. These samples were fixed immediately with (a) 0.6ml MnCl soln - 100g MnCl in 200mls distilled water.

(b) 0.6ml Winkler's reagent - 100g KOH and 60g KI in 200mls distilled water.

Stoppers were replaced and the sample shaken well. In the laboratory, 0.6ml conc. H_2SO_4 was added to the samples and the bottles shaken well. 50mls of sample were placed in a conical flask and titrated against

N/80 sodium thiosulphate to an endpoint indicated by the addition of starch glycollate towards the end of titration. The titration was done twice for each bottle fixed.

The oxygen concentration is calculated as 2x mg/1, where x is the titre of sodium thiosulphate.

Percentage saturation of oxygen was calculated from a nomogram for oxygen solubility derived from Mortimer (1956) and corrected after Montgomery, Thom & Cockburn (1964).

3. Conductivity in $\mu\Omega$.

This was measured using a Dionic water tester, temperature-compensating conductivity tube.

4. pH.

The pH of the water sample was measured with an E.I.L. Model 23A, direct-reading pH meter calibrated with buffer solutions pH4.0 or pH 9.0.

5. Phosphate (orthophosphate PO_4-P) mg/1.

Reagents:		
	Ammonium Molybdate	(3% w/v soln.)
	Sulphuric acid	(7:45 v/v)
	Antimony pot. tartrate	(0.136% w/v soln.)
	Ascorbic acid	(5.4% w/v soln.)

The first three reagents were mixed in the ration 10:25:5. Just before analysis, 10mls freshly-made ascorbic acid soln were added to the other reagents. To 50mls of membrane-filtered (0.45μm) sample was added 5mls reagent mixture. The blue colour was allowed to develop for 12mins and its optical density read in a 5cm cell against a reagent blank in an EEL absorptiometer using filter no. 609. Concentration of phosphate as PO_4-P was read off from a calibration curve, and expressed as mg/l.

6. Nitrate (NO_3-N) mg/l.

100mls of membrane-filtered (0.45μm) stream water was placed in a conical flask and evaporated to dryness in an oven at about 100°C. 2mls phenoldisulphonic acid was added to the residue in the flask,

wetting it completely, and allowed to stand for 10mins. About 50mls distilled water was added, followed by 2mls magnesium sulphate (10% w/v) soln. A 40% (w/v) soln of sodium hydroxide was added drop by drop until a heavy precipitate had developed. The soln + ppt was filtered through Whatman 42 filter paper, and the filtrate made up to 100mls with distilled water. The optical density of the solution was measured against a reagent blank in a 10cm cell in an EEL absorptiometer using filter no. 601. The concentration of nitrate as NO_3-N was read off from a calibration curve and expressed as mg/1.

7. Dissolved Organic Matter. (DOM) mg/l.

50mls of membrane-filtered (0.45μm) stream water was placed in an evaporating dish and evaporated to dryness in an oven at about 100°C, cooled in a dessicator and weighed. The residue was ashed at 500°C for at least an hour, cooled in a dessicator and reweighed. All organic material should have been combusted in the furnace and only inorganic material remain. The difference between the pre-muffle total weight and post-muffle total weight is taken to represent the DOM of the sample finally expressed as mg/l.

8. Alkalinity mg/l as $CaCO_3$.

This was not done, but a value was considered necessary when pectin lyase production by fungi was detected, as this is stimulated by calcium ions. Alkalinity is measured weekly in this department for water samples from the Virginia Water falls, and varies little. An average value is given for these samples.

C. QUANTITATIVE ANALYSIS OF SPECIES LISTS FROM THE LITERATURE.

1. Sources of data

82 stands were compiled from species lists reported in the literature. The original list is given in the text under Results. A list of 93 species was drawn from C.T. Ingold's "Guide to Aquatic Hyphomycetes" (1975) with some additions. This species list is also

given in the text under Results. Species names, in older papers, that have been changed, were altered to present nomenclature. Species illustrated as unknown that have subsequently been named were included.

2. Agglomerative Classification (Williams et al 1966).

This heirarchial classification allows a nearest-neighbour sorting by calculating euclidian distances between individuals and arranging these distances in ascending order. A polythetic agglomerative classification can be produced as a dendrogram. The method is non-overlapping i.e. an individual cannot belong to more than one group. The euclidian distance, e, is given by:-

$$e(j.k.) \doteq \left[\sum_h (X_{hj} - X_{hk})^2\right] \qquad h = 1,..........p$$

p = no. of individuals in a stand; X_{hj} or X_{hk} are the no. of species 'h' in quadrat j or k. The above formula defines a distance between two quadrats as a sum of p squared.

The primary data consisted of species lists from world-wide samples. Each list was regarded as a stand composed of a number of species. Species were regarded either as present (1) or absent (0). Thus a data matrix, T, of stands and species scores was constructed. by transposing T - T', a reverse classification, of species, can be made.

Where 'presence-or-absence' data are used, they can be represented in 2 x 2 tables:-

Quadrat j

Quadrat k		1	0
	1	a	b
	0	c	d

a no. of species common to 2 quadrates.

b no. of species in k but not j.

c no. of species in j but not k.

d no. of species in neither

$$e = \left[\frac{b + c}{p}\right]^{\frac{1}{2}} = \left[1 - \frac{(a + d)}{p}\right]^{\frac{1}{2}}.$$

where $\frac{a + d}{p}$ is the Sokal matching co-efficient, (Sokal & Michener 1958). The programme uses this form of calculation. The calculations were performed using the programme AGGLOM from the permenent file on the CDC6600 computer at the U.L. Computer Centre. AGGLOM can accomodate 100 individuals and 120 attributes.

3. Ordination: Reciprocal Averaging. (Hill 1973)

This eigen-vector technique produces an ordination of stands and of species and presents the data as loadings for each on up to 10 axes. Two-dimensional scatter diagrams can be made using the loading values on various axes. Stands and/or species will either fall in a curvilinear sequence or into clusters. Stands of similar species composition would be expected to clump together, and so would species from similar stands. This could indicate cosmopolitan species as well as those important in separating stands from well-defined geographical areas.

The key feature of this technique is that species ordination is used to give stand ordination and *vice versa*. It uses an iterative procedure to improve the estimation of vectors. The advantage of the method is that it gives good species co-ordination to match stand ordination.

The second axis is related to the presence or absence of extreme species and the fit is a general average of the species complement. Scores in the second axis which are high in relation to the first axis require interpretation in terms of a weak second axis of variation. As the data is in the form of 'presence-or absence', it cannot be multi-

variate, normally-distributed, so that the two axes are not orthoginal to each other, though the scores are represented as rectangular co-ordinates.

This ordination method uses presence-or-absence data, which are represented by an incidence matrix.

$A = (a_{ij})$ ($i = 1........m$; $j = 1........n$)

of 0's or 1's

Rows = species
Columns = stands

Row, column totals are given by:-

$$V_i = \sum_j a_{ij}$$

$$C_j = \sum_i a_{ij}$$

The reciprocal averaging procedure is:-

$$x_i = \sum_j a_{ij} y_j / v_i \qquad (i = 1........m)$$

$$y_j = \sum_i a_{ij} x_i / C_j \qquad (j = 1........n)$$

(Hill 1973)

Each species is assigned a score of 0 or 100. Stand scores can be estimated as average stand scores. New species scores are then calculated using the average of the stand scores in which they occur. The new scores are scaled from 0-100 and the process repeated until the scores stabilize. Extreme species will have extreme scores and intermediate species' scores will lie between extreme and ubiquitous species. The term 'reciprocal averaging' refers to the relationship that stand scores are averages of species scores, and, reciprocally species scores are averages of stand scores. The final species score does not depend on the initial score and the number of iterations may be high to reach stability if the initial choices are distant from the final value. The stand and species scores so determined will give a one-dimensional ordination of both species and stands. The second axis is formed by using a set of scores near to the final scores of the first axis. Before iteration the scores are adjusted by subtracting a multiple of the first axis.

The computation, based on matrix algebra, was performed using RECAV from the permanent file on the CDC6600 computer at the U.L. Computer Centre. RECAV can accomodate 100 individuals and 105 attributes.

D. LABORATORY CULTURE EXPERIMENTS.

1. MEDIA

a. Plate medium. Willoughby's GYS medium (Willoughby, personal communication)

In g/l:

10	glucose
5	soluble starch
2	yeast extract
0.6	$Na_2HPO_4.12H_2O$
2.04	KH_2PO_4
20	agar.

b. Flask medium

In g/l:

2	yeast extract
0.6	$Na_2HPO_4.12H_2O$
2.04	KH_2PO_4

With 1% (w/v) appropriate carbon source, i.e. Sodium polypectate, pectin (apple) or galacturonic acid.

Galacturonic acid - BDH

Sodium polypectate (Napp) - Nutritional Biochemicals Corp. Cleveland, Ohio. Batch 2837

Pectin (apple) 250 grade - BDH. Methoxy content about 6% on dry material.

In making-up flask media with pectin or Napp, the pH was adjusted with 1M HCl or 1M NaOH before the addition of the pectic or Napp to prevent gelling. Pectin and Napp were then dissolved into the medium on a mechanical stirrer with low heat. The medium was then sterilized in 250ml aliquots.

c. Pectinase assay plates. (Hankin & Anagnostakis 1975)

These are a rapid and reliable method for detection of pectinase activity.

(i) The full medium given was used for the fungal culture experiment (Exp. II) to detect extracellular production of pectinases

by 7 species of aquatic Hyphomycetes at pH5 and pH7.

500mls mineral salts soln containing /l: 2g$(NH_4)_2SO_4$, 4g KH_2PO_4, 6gNa_2HPO_4, 0.2g$FeSO_4.7H_2O$, 1mg$CaCl_2$, 10μgH_3BO_3, 10μg$MnSO_4$, 70μg$ZnSO_4$, 50μg$CuSO_4$, 10μgMoO_3.

1g yeast extract,

5g pectin at pH7 for pectin-transeliminase detection, or Napp at pH5 for polygalacturonase activity.

15g agar

500 mls distilled water or buffer.

Plates are developed with hexadecyltrimethylammonium bromide, 1% (w/v) soln which precipitates intact Napp and pectin. A clear zone around a colony in an otherwise opaque medium indicates pectinase activity. The width of the clear zone is proportional to the activity of the enzyme.

(ii) Cup-plate assays.

PG's have a pH optimum around 5 and polygalacturonate is the preferred substrate. Assay medium was made up in a litre of buffer at pH5 in which 5g/l of Napp and 15g of agar was dissolved. After autoclaving at 121°C for 15min, the medium was poured into sterile petrie dishes (25ml/plate). For use, cups (1cm diam.) were cut in the gel with a flamed #5 cork-borer. For assays, cups were filled with enzyme sample. Plates were incubated for 18hrs at 30°C, and developed with hexadecyltrimethylammonium bromide as above.

Fungal transeliminases generally have a pH optimum around 9, and pectin is the preferred substrate. PTE activity is stimulated by calcium ions. Assay medium was made up in 1l buffer at pH9 with $CaCl_2$ (1mM) in which was dissolved 5g pectin and 15g agar. The procedure otherwise was the same as for PG cup-plate assays, but the plates were developed with 5M HCl, and a precipitated, white zone around the cup is indicative of PTE activity. (These plates also showed up PME activity as a clear zone around the well.)

2. BUFFERS.

The buffers used were:-

Acetate - acetic acid (0.1M and below) - pH3.6 - 5.6.

Citric acid - sodium citrate (0.1M and below) - pH3.0 - 6.2.

Na_2HPO_4- KH_2PO_4 (0.67M and below) - pH5.29 - 8.04.

McIlvaine's. Citric acid - Na_2HPO_4 (0.15 and below) - pH2.2 - 8.0.

Tris - HCl (0.025M) - pH7.19 - 9.10.

3. SILICONIZED FLASKS.

Matched 100ml flasks were used for all laboratory culture experiments. These were siliconized before use to minimise fungal growth on the glass. Flasks were soaked in a 2% (v/v) soln of silicone, (Hopkins & Williams MS 1107) in ethyl methyl ketone for 20mins, then baked for 2 hrs at 180°C. Cotton-wool stoppers were used to allow maximum air diffusion.

4. STERILIZATION

Where sterilization was required, it was carried out in an autoclave at 121°C for 15mins.

5. PRODUCTION OF SPORES FOR INOCULUM

Aquatic Hyphomycetes can be induced to sporulate if mycelium is placed in water and aerated for a few days. It was decided to use sterile spores rather than mycelium for an inoculum, firstly, because one is working with the chosen organism, and secondly, because evidence is provided of the capacity of the spore to germinate in given conditions.

Mycelium from agar plates was cut aseptically into small pieces and placed in 150mls of sterile water in a sterile 250ml conical flask fitted with a cotton-wool plug through which was passed a pasteur pipette attached to a length of rubber tubing holding a cotton-wool filter. This tubing was attached to a glass manifold which was, in turn, attached to an air-filter containing a millipore filter (0.22μm). The entire apparatus up to the millipore filter was sterile. Air was

pumped by a compressor pump through the filter and thus through the water in the flask. Pressure was maintained sufficiently to keep the mycelium in suspension. Thus, after 5 - 7 days, sterile spores were produced by all the organisms used. The inoculum consisted of c. 10,000 spores/ml. The apparatus was housed in a growth room kept at 20°C.

6. SHAKE CULTURE.

100ml siliconized conical flasks containing 25mls medium and 2mls inoculum were placed on an orbital shaker and kept at 15°C and 180rpm in a cold room at 10°C.

7. CENTRIFUGATION.

The contents of the flasks were centrifuged under refridgeration to separate mycelium from supernatant. This was done on a MSE Superspeed 40 centrifuge (8 x 25ml) at 10,000rpm for 20 mins. The supernatant was decanted off and kept, and the mycelium resuspended in distilled water.

8. pH.

The pH of supernatant fluids was measured on an E.I.L. Model 23A direct reading pH meter previously calibrated with buffers pH4.0 or 9.0.

9. VISCOMETRY

The viscosity of 10mls of supernatant was measured in 'Cannon-Fenske' size 200 viscometers suspended in a water bath at 25°C. % viscosity loss was calculated from the following equation:-

$$\frac{Vo - Vt}{Vo - Vs} \times 100$$

Vo = Flow-time in secs at t_0.

Vt = Flow-time in secs at subsequent times.

Vs = Flow-time of 10mls distilled water i.e. 100% viscosity loss.

10. DRY-WEIGHT MEASUREMENT

Numbered Whatman glass-filter papers (GF/C) were dried overnight in an oven at 90°C, and cooled in dessicators. The papers were then weighed accurately. Mycelium suspended in water after separation from culture filtrates by centrifugation (see above), was filtered onto the papers under vacuum. Papers + mycelium were dried for 18hrs in an oven

at 90°C, placed in a dessicator to cool and then weighed accurately.

Wt of paper + mycelium - wt of paper = dry weight of mycelium.

E. PECTINASE INDUCTION, PURIFICATION AND CHARACTERIZATION.

(after Fanelli, Cacace & Cervone 1978)

1. ENZYME INDUCTION

Napp was used as a substrate to induce extracellular production of both PG and PTE although pectin is the preferred substrate for PTE activity. Pectin was found to hydrolyse spontaneously at neutral to alkaline pH, particularly after autoclaving and to produce a very acid medium inimical to fungal growth.

2 litres of flask medium (see D1b above) were made up in 0.1M citrate buffer, pH5, for PG induction, and in medium adjusted to pH7 for PTE induction. 1mM $CaCl_2$ was added at this stage to the PTE medium. The pH5 medium requires strong buffering, otherwise fungal metabolism brings about a rise of pH which induces production of the PTE rather than PG's. 1% (w/v) Napp was added to the medium and dissolved on low heat. The medium was then distributed in 250ml aliquots between 8 x 1l conical flasks. Flasks were stoppered with cotton-wool and autoclaved at 121°C for 15mins. To the cool, sterile medium was added 20mls of sterile spore inoculum.

Preliminary culture experiments established that 11d shake culture at 15°C and 80rpm produced the maximum yield of enzyme and brought about a 90% reduction in viscosity of the medium. The mycelium was filtered off through four layers of butter-muslin, and 1500mls of the culture filtrate was centrifuged under refridgeration in an MSE Superspeed 65 centrifuge (6 x 250mls) at 20,000rpm for 2 hrs. The decanted sample was assayed for pectinase activity. The assay was always positive. If necessary, the sample was stored at -12°C. It was found that enzyme samples could be frozen, unfrozen and refrozen without loss of activity, whereas samples stored at 3°C were liable to

contamination and loss of activity.

2. PURIFICATION

a. Ultrafiltration.

This was carried out in a cold room at 10°C. An Amicon stirred ultrafiltration cell (Model 402) with reservoir was used. As the molecular weights of pectinases are generally c. 30,000, two types of ultrafiltration membranes were used:

UM 10 which filters out substances of MW $<$ 10,000

PM 10 which filters out substances of MW $<$ 17,000

The sample was concentrated in this way from 1500mls to approximately 150mls. Both sample and effulent were assayed for enzyme activity. The enzymes appeared only in the sample.

b. Dialysis

The ultrafiltered sample was placed in a visking dialysis bag and dialysed extensively against the column starting-buffer (see below). Dialysis was carried out in a cold room at 10°C.

c. Ion-exchange chromatography.

The entire apparatus for this was housed in a cold room at 10°C.

The cation exchanger used was CM-Sepharose CL - 6B (Pharmacia)
The anion exchanger used was DEAE-Sepharose CL - 6B (Pharmacia)
The column used was a K26/40 (diam. 2.6cms; length 40cms) with adjustable applicator. (Pharmacia)
Starting buffers were: 0.02M acetate at pH5.0 or 4.0 for cation exchange.

0.025M Tris-HCl at pH7.2 for anion exchange.

The gel bed of c. 130mls of packed exchanger was first equilibrated with starting buffer. Flow-rate throughout was controlled at 15mls/hr by a Watson-Marlow peristaltic pump. The dialysed sample was run onto the gel bed, and the protein fractions eluted with a 0 - 1M linear NaCl (in

buffer) gradient; about 400mls. 5ml fractions were collected automatically with an LKB Typ 340 3B fraction collector attached to a chart instrument (LKB Typ 6520-6) which recorded fraction number and the concentration of protein passing through an ultraviolet monitor, (LKB Uvicord II control unit 8300 and absoptiometer detector type 830 3A). Fractions were collected until no protein was detected for at least 3hrs, (9 or 10 fractions).

Cup-plate assays were carried out on every alternate fraction containing protein. Fractions with high enzyme activity were pooled and frozen if necessary. Protein estimations (see below) were carried out on enzyme samples.

d. Polyacrylamide gel electrofocusing.

In order to obtain an approximate pI value for an enzyme for further purification using narrow-range ampholines, PAG plates were used for electrofocusing, (PAG ampholine plates from LKB, pH 3.5 - 9.5). The apparatus used was an LKB Multiphor 2117, maintained at 6°C. The procedures recommended by the manufacturers were followed. Samples were applied in triplicate. A surface electrode (LKB 2117-111 multiphor electrode connected to an E.I.L. Model 23A direct-reading pH meter) was used to establish the pH gradient across the gel after electrofocusing. A graph of pH against gel width was drawn.

The gel was then cut into strips and placed on pectinase assay plates which were incubated at 30°C for 18hrs and developed. Enzyme activity was thus detected and an approximate pI value obtained from the graph. Electrofocusing was found to be unsuitable for PTE's. The ampholines react with pectin to give spurious results and Napp plates gave no reaction to these enzymes.

e. Preparative iso-electric focusing.

The apparatus used was an LKB Multiphor 2117 cooled by a constant flow of tap water. Ultrodex (LKB 2117, 510 Ultrodex) was the preparative gel, swollen before use on a boiling water-bath for at least

2hrs and cooled. Samples were added to the gel after cooling. Narrow-range ampholines were Pharmalyte from Pharmacia, mixed where necessary. Procedures recommended by the manufacturers were followed. pH gradients were established as above (f) across a fractionating grid which divided the gel into 30 strips. These strips were eluted with buffer in elution columns into small test tubes. Samples were assayed by cup-plates and the pI taken as the pH range over which enzyme activity was detected. Fractions with enzyme were pooled and concentrated by ultrafiltration where necessary. Samples were stored at -12°C.

f. Protein estimation

The protein concentration of samples was measured in the course of purification. This was done by the method of Lowry, Rosenberg, Farr & Randall (1951).

Reagent A : 2% (w/v) Na_2CO_3 in 0.1M NaOH

Reagent B : 0.5% (w/v) $CuSO_4.5H_2O$ in 1% (w/v) sodium or potassium tartrate made by mixing equal amounts of 1% $CuSO_4$. $5H_2O$ and 2% tartrate made up in water.

Reagent C : Mix 50mls reagent A with 1ml reagentB. Discard after 1 day.

Reagent D : Commercial Folin reagent diluted 1:1 with water.

To 0.5 ml sample was added 2.5mls reagent C and stood for 10 mins at room temperature. 0.25ml of reagent D was added with stirring. The mixture was allowed to stand for at least 30mins at room temperature, and the colour density measured at 750nm on a UNICAM Sp.500 spectrophotometer, against a water blank or sample and water blank where the sample was coloured. Transmission readings were converted to μg protein from a standard curve made with bovine serum albumin.

3. CHARACTERIZATION.

(i) PG.

a. Washed Napp. (Cooper 1974)

In order to purify the substrate for enzyme

characterization as far as possible and to free it from low MW sugars, the Napp was washed before use. This was done by washing the product with acidified (0.05M HCl) 70% (v/v) ethanol until no pigment or reducing sugars were detectable in the washings. The solid was collected by filtration under reduced pressure and washed extensively with 70% (v/v) ethanol. The residue was further washed in 95% (v/v) and finally absolute ethanol. It was then dried at 60°C and stored at room temperature.

To establish the approximate degree of polymerization (d.p.) of the washed Napp, reducing-group determinations (Miller 1959) were carried out: firstly on a 1% (w/v) soln of dissolved Napp and secondly on a 1% (w/v) soln buffered to pH5 and treated with a 1% (w/v) soln of commercial PG. 9mls of substrate were incubated with 1ml enzyme for 24hrs at 30°C, at which time it was assumed that all available bonds in the polymer would have been hydrolysed. (This procedure was necessary as Napp is not hydrolysed by acids). By dividing the results of the first determination into those of the second, the d.p. was found to be c.25.

b. Viscometry for pH optimum.

Substrate solns were made up in 0.075M McIlvaine's buffer to cover a pH range 4.0 - 7.0. Solns contained 1% (w/v) washed Napp and were adjusted, where necessary, with 1M HCl or 1M NaOH.

A 'Cannon-Fenske' 200 viscometer was suspended in a water-bath maintained at 30°C. Reaction mixtures generally consisted of 9mls substrate soln and 0.5ml enzyme (exact volumes given under individual enzymes in Results). Percentage viscosity loss was calculated as in D9 above.

For each pH value, a graph was plotted of % viscosity against time, and the time read off for a 50% reduction in viscosity. The reciprocal of this time x 10^2 or 10^3 (see individual enzymes) was designated enzyme activity in RVU (relative viscosity units).

RVU was plotted against pH value to give a graph showing pH optimum.

c. Reducing-group analysis for Km and % hydrolysis at t_{50}.

(Miller 1959)

Dinitrosalicylic acid reagent (w/v) :

1% dinitrosalicylic acid dissolved in

1% sodium hydroxide soln.

To the reagent, just prior to use, was added 0.05% (w/v) sodium sulphate. 3mls reagent were placed in a tt; 3mls sample were added and the mixture placed in a boiling water-bath for 15mins. 1ml of 40% (w/v) Rochelle salt soln (sodium potassium tartrate) was then added to the hot mixture. The colour intensity of the soln was measured, before cooling, on a UNICAM sp. 500 spectrophotometer at 575 nm against a reagent blank. It was found that on cooling, the Napp gelled and became cloudy so readings had to be made swiftly with hot solns. The standard curve, prepared from galacturonic acid, was also made with uncooled solns. Reducing group values were obtained from this standard curve.

The substrate was the soln as in b. above which represented the optimum pH for enzyme activity. The proportions of substrate to enzyme used were the same as in b. above and the reaction was carried out on a water-bath at 30°C. One sample assayed was substrate only, and the reducing-group value for this was subtracted from enzyme/substrate values to give reducing groups released by enzyme activity, within a given time.

For % hydrolysis of the substrate at t_{50} (from viscometry, b. above) the total value for hydrolysis of all available bonds in the polymer was that obtained in a. above.

d. Paper Chromatography. Nasuno & Starr (1966)

Reaction products were analysed by descending paper chromatography. Whatman No. 4 paper was used. The standard was 75mM galacturonic acid. 25μl samples were taken at intervals with a microsyringe from the reaction mixture (as in b. and c. above) and directly

spotted onto the paper. Substrate only was also sampled. Total reaction time was 24hrs. The sovent used was 1-butanol - acetic acid - water (4:2:3) and the solvent front was run to the bottom edge of the paper which was then dried overnight. The chromatogram was developed by spraying it with a pH indicator, 0.04% bromophenol blue in 95% ethanol adjusted to pH 7.0 with 1M NaOH. Acidic substances appear as yellow spots on a blue ground.

Colour-intensity standards to give approximate values for acidic spots were made. The limits of detectability of uronic acids was about 8.5 g. Below are given the colour intensity standards (as numbers) and the molar concentrations and μg of galacturonic acid they represent.

CIS	mM GA	μg GA
1.	1.6	8.5
2.	3.2	17.0
3.	6.25	34.0
4.	12.5	68.0
5.	25.0	135.00
6.	50 - 75	270 - 400.

One thin-layer chromatogram was made to detect galacturonic acid production only from the reaction mixture. The plate was a 20x20, plastic-backed F1500 silica gel plate from Schleicher & Schull, Germany. The method used was that described by Menzies (1978). 5μl of sample was used.

(ii) PTE.

(a) Washed pectin.

Pectin used in the characterization of PTE enzymes was washed as in 3(i)a above. The d.p. of the pectin was established in a similar way to that given in 3(i)a, but total hydroysis of bonds was made with a PTE derived from *Mycocentrospora angulata* in a 1%(w/v) pectin soln with 1ml enzyme, incubated at 30°C for 7d. Ca^{2+} conc 1mM/1. D.p. c. 50 where the end-product is unsaturated digalacturonic acid.

(b) Viscometry

This method was used only to determine t_{50} for a

given substrate, because reaction times were slow. Otherwise the methods were as in 3(i)b above, but reaction substrates were 0.6 (w/v) soln of pectin made up with 1mM $CaCl_2$/1.

c. Thiobarbituric acid assay for pH optimum and Km.

Quantich (1978) after Ayers *et al* (1966)

To determine the pH optima for PTE's, solns of washed pectin (1% w/v) were made up in Tris-HCl buffer ranging from pH 7.2 - 9.8. 1g pectin was dissolved in 50mls buffer + $CaCl_2$. 50mls buffer + $CaCl_2$ were then added and the pH adjusted where necessary with M NaOH. Reaction mixtures consisted of 4mls pectin soln with 2mls enzyme (*Tetrachaetum elegans*) or 5mls pectin soln with 1ml enzyme (*Mycocentrospora angulata*). The final concentration of Ca^{2+} was 1mM/1. Reaction mixtures were incubated for 1hr exactly on a water-bath at 30°C. The substrate soln was treated in the same way for assay, as pectin hydrolyses spontaneously in neutral-alkaline conditions.

1ml sample was added to 5mls thiobarbituric acid (0.04M) and 2.5mls HCl (M) in a test tube and mixed well. The tube was covered with a metal cap and placed in a boiling water-bath for 30mins. Before cooling, the transmission of absorbance of the mixture was read against a reagent blank at 550nm on a UNICAM Sp. 500 spectrophotometer. Cooling resulted in clouding of the mixture. The standard curve for unsaturated digalacturonic acid was made using the same procedure. (The unsaturated digalacturonic acid was the kind gift of Prof. R. H. Vaughn of the University of California, Davis.) Enzyme activity was expressed as release of UDGA from values obtained from the standard curve. Graphs of pH optima are plotted as % of maximum yield against pH.

To determine whether pectin or Napp is the preferred substrate of the PTE's isolated, an identical assay was carried out at optimum using washed Napp as a substrate.

Assays to give a Km value for PTE's were identical to the procedure used for pH optima, but samples were withdrawn at

fixed time intervals in the course of incubation of the reaction mixture. An assay was also carried out to establish the % hydroysis of the substrate by enzyme activity at t_{50} (measured viscometrically).

For all assays, substrate alone was incubated at the same temperature and for the same time as rn mixtures. This was assayed and the values obtained subtracted from those of the rn mixture to give a value for enzymic degradation.

d. Paper Chromatography.

The procedures described for descending paper chromatography in 3(i)d above were followed. Unsaturated digalacturonic acid was used as a standard and incubated substrate was sampled at the end of the total incubation period which has up to 55hrs.

(iii) PME. (Pectin methylesterase)

The assay used for this enzyme was the rapid titration method described by Delincée & Radola (1970). An unbuffered soln of washed pectin (1% w/v) was used adjusted to pH9.0. To 20mls of soln was added 6mls enzyme. The mixture was incubated on a water-bath at 30°C. Untreated substrate was incubated simultaneously under the same conditions. pH values for mixture and substrate were measured every 10mins from t_0 - t_{30}. Changes in pH due to enzyme activity take into account pH changes in the substrate due to spontaneous hydrolysis of pectin in alkaline conditions. Enzyme activity is given as Δ pH/min.

F. LEAF MACERATION.

The capacity of the four aquatic Hyphomycetes from which pectinases were induced in vitro, to macerate alder leaves was tested as follows:

Entire, abscissed alder leaves were collected and air dried at room temperature. Before use, they were soaked in tepid water for 2 hrs. Two identical leaf strips were cut around a copper template 4.5 x 2cms, from either side of the midrib of the lamina. Matching strips were

notched for identification. These leaf strips were surface-sterilized with propylene oxide.

To 1 litre of stream water was added the following mineral salts:- 1.0g KNO_3, 1.0g KH_2PO_4, 0.5g NaCl, 0.2g $MgSO_4.7H_2O$, 0.1g $CaCl_2$. The soln was unbuffered and adjusted to the pH of the stream water. The soln was sterilized by membrane filtration (0.45μm).

25mls of sterile soln was pipetted into sterile, matched 150ml conical flasks. Flasks were paired and 3 sterile leaf strips were placed in one flask and the matching pairs of leaf strips in the other. One flask, which was to act as the control, was uninoculated; into its pair was pipetted 2mls of sterile spore-inoculum. The flasks were placed in pairs on an orbital shaker maintained at 15°C in a cold room.

The tensile strength of the pairs of leaf strips was measured every alternate day of incubation. This was done on an Instron tensiometer at the National College of Food Technology, Weybridge. On a graph chart, calibrated to give a full-scale deflection of 1 kilogram, it recorded, very precisely, the weight required to cause the leaf strip to split when it was held between two rubber grips. The operation of the tensiometer caused the arms holding the grips to apply tension to the leaf strip, and the extent of this tension was recorded up to breaking-point as a deflection on the chart.

The tensile strength of the inoculated strip is expressed as a percentage of that of the matching control strip. Preliminary measurements made on 10 pairs of uninoculated leaf strips showed that, on average, the tensile strength of one of a pair of leaf strips was above 90% of its matching strip. On the basis of these results it was decided to accept any decrease in tensile strength below 80% of the matching control as significant evidence of leaf maceration by fungal activity. Sampling was carried out until inoculated leaves were skeletonized.

On the day of sampling, the pH of the supernatant fluids was

measured, and the fluids were assayed for enzyme (pectinase) activity by cup-plate assay. Both controls and inoculated samples were assayed for PG, PTE and PME activity.

					F						F	
Sampling Date	5/12	18/12	3/1	17/1	30/1	13/2	27/2	13/3	4/4	18/4	3/5	15/5
OXYGEN mg/l	12.4	12.8	11.6	12.0	12.5	12.5	11.6	10.8	10.6	12.0	11.2	11.2
% sat.	95	100	92	90	94	93	94	94	90	100	97	104
CONDUCTIVITY $\mu\Omega$	300	280	300	300	325	325	330	300	325	310	300	275
PO_4-P mg/l	0	$<$ 0.005	0	0	$<$ 0.005	0.03	0.015	0.028	0.019	0.006	0.04	0.08
NO_3-N mg/l	0.49	0.35	0.50	0.50	0.64	2.50	0.78	0.84	0.66	0.52	0.56	0.70
Dissolved mg/l Organic Matter	16.6	10.6	10.4	5.4	3.0	6.0	10.8	10.4	14.2	8.0	8.8	8.6
pH	6.7	7.2	7.0	7.5	7.8	7.1	7.2	7.4	8.6	7.0	7.5	7.4
Temp °C.	4.0	5.5	5.0	3.0	3.0	2.5	6.0	8.5	7.5	8.0	9.5	12.0
Alkalinity mg $CaCO_3$/l	Average alkalinity at Virginia Water Falls = 35.											

TABLE 8: Values for physical and chemical properties of the River Bourne made over the period of the field study at leaf bag sampling dates. Water samples taken at the experimental site.

F = Floods.

THE FIELD STUDY

A. RESULTS

The results of the water analyses conducted on the River Bourne over the experimental period are presented in Table 8 opposite. Percentage saturation of oxygen was 90% and above; nitrate levels were unlikely to be limiting or inhibitory, but phosphate levels were undetectable or very low from December to February and may have been limiting to microbial growth. pH values ranged from 6.7 - 8.6 but were generally neutral to slightly alkaline. Alkalinity values at Virginia Water falls are due predominantly to calcium carbonate concentrations.

Species of aquatic Hyphomycetes found in the River Bourne between 10th October 1977 and 15th May, 1978 are given overleaf. The source of each species is given: from scum samples, from filtered water or from experimental leaf packs. It is noteworthy that some species, e.g. Articulospora inflata, Lunulospora curvula and Tricladium patulum were detected only in scum samples; Tricladium angulatum and T. anomalum were found only on filters and Lemonniera terrestris appeared only on experimental leaf packs. Details of scum samples are given in Appendix Table 1; filter counts in Appendix Table 2 and details of species and spore counts for leaf packs are presented in Appendix Table 3.

Scum samples are useful for identifying species present in a stream but for quantitative and comparative estimates are unreliable as some species are more readily trapped than others. However, as Anguillospora longissima and Mycocentrospora angulata are very similar in size and shape and were not easily distinguishable from one another under the inverted microscope, a count was made from a foam sample of their relative concentrations. 200 conidia of these species were counted, of which 49% were M. angulata and 51% A. longissima. It was decided to designate the two species 'Filiform' in microscopic counts, and combine the species.

Species of aquatic Hyphomycetes found in River Bourne

from 10th October 1977 - 15th May 1978.

S = Scum F = Filters. L = Experimental leaf packs.

Species	S	F	L
Alatospora acuminata Ingold	S	F	L
Anguillospora longissima (de Wild.) Ingold	S	F	L
Articulospora tetracladia Ingold	S	F	L
A. inflata Ingold	S		
Clavariopsis aquatica de Wild.	S	F	L
Clavatospora longibrachiata (Ingold) Nilsson ex Marvanová & Nilsson	S	F	L
C. stellata (Ingold) Nilsson ex Marvanová & Nilsson	S	F	L
Culicidospora aquatica Petersen	S	F	L
Flabellospora Sp I Ingold (1975)	S		
Flabellospora curvula Ingold	S	F	L
Heliscus lugdunensis Sacc. & Therry		F	
Lemonniera aquatica de Wild.	S	F	L
L. terrestris Tubaki			L
Lunulospora curvula Ingold	S		
Mycocentrospora acerina (Hartig) Newhall	S	F	
Mycocentrospora angulata (Petersen) Iqbal	S	F	L

Species	S	F	L
Tetracladium elegans Ingold	S	F	L
Tetracladium marchalianum de Wild.	S	F	L
T. setigerum (Grove) Ingold	S	F	
Tricladium angulatum Ingold		F	
T. anomalum Ingold		F	
T. eccentricum Petersen			L
T. patulum Marvanová & Marvan	S		
T. chaetocladium Ingold	S	F	L
T. splendens Ingold	S	F	L
Triscelophorus monosporus Ingold	S	F	
Triscelophorus sp.		F	L
Varicosporium elodeae Kegel	S	F	L

Total species = 26

Scum = 22 spp.

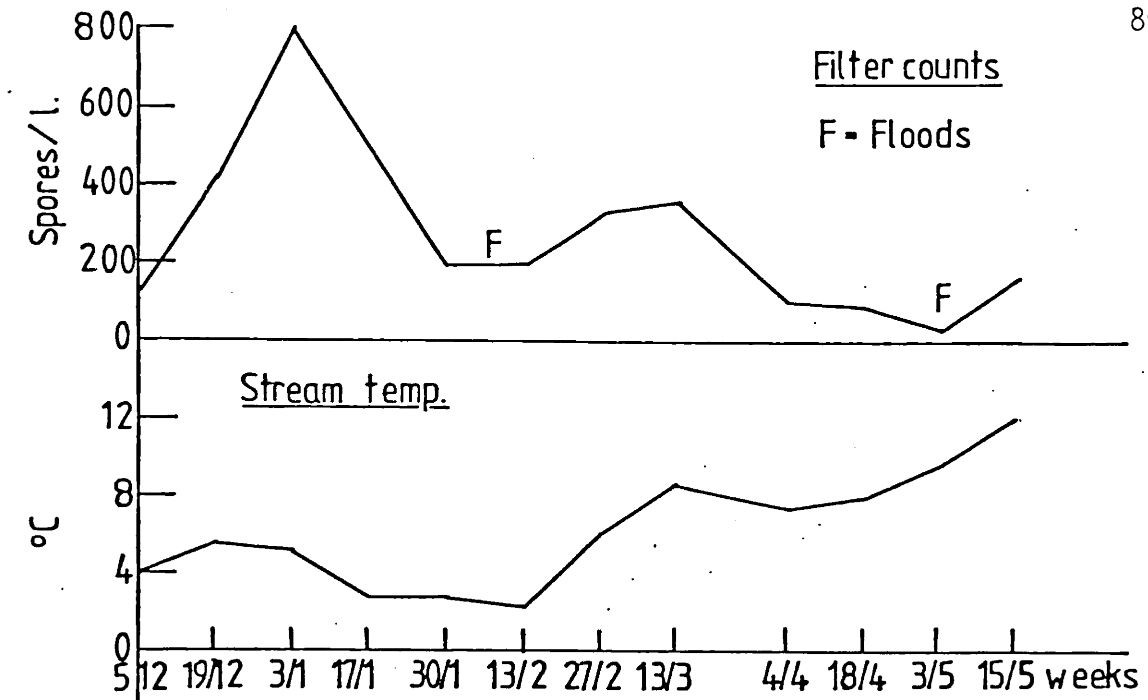
Filters = 22 spp.

Leaves = 18 spp.

Filter counts from 500mls of stream water give a more reliable quantitative estimate of species present in the stream at a given time. But it is a highly random method and can only provide crude comparative data. Total spores/litre estimated by this method are presented in Figure 2, and show fluctuations over the period surveyed. From a level of about 100 spores/litre at the beginning of December, the concentration rises steeply within a month to a peak of 800 spores/litre at the beginning of January reflecting the peak period of the processing of autumn-shed leaves which fell that season chiefly in November. The steep decline in spore concentration in January was exacerbated by floods, the diluting effect of which is clear both at the end of January and later at the beginning of May. Although spore concentrations increase after floods, there is an overall steady decline from the peak at the beginning of January to the beginning of May (despite increasing stream temperatures from mid-February). The decline reflects the depletion of resources and it will be shown that the pattern of spore concentrations drawn from the stream at large is paralleled by data from experimental leaf packs.

It is interesting from Appendix Table 2 to observe that filter counts detect on average 13-14 species at any sampling. The patterns of distribution of the species found most commonly on filters is shown in Figure 3. For some species, the rise in temperature at the end of February (see Figure 2) appears to influence their percentage frequency. The filiform species, consisting largely of A. longissima and M. angulata, are dominant together with Tetrachaetum elegans from the beginning of December to mid-February. Frequency of T. elegans drops off very sharply suggesting that, as Triska (1970) observed, it is a very early colonizer of freshly-fallen leaves. As frequency of these species declines, that of C. aquatica and A. acuminata increases. T. chaetocladium and C. longibrachiata were not detectable until the beginning of January. A. tetracladia, though detectable only at low

FIGURE 2: Showing filter counts of conidia/l and stream temperature over the sampling period. The lowest figure shows plots of spore numbers/gram dry weight of leaf at sampling dates for Alder I ●——●; Alder II x----x; AlderIII o----o; Oak O——O.



Spores /g. dry wt. leaf

10^5

10^4

10^3

5/12 19/12 3/1 17/1 30/1 13/2 27/2 13/3 4/4 18/4 15/5 weeks

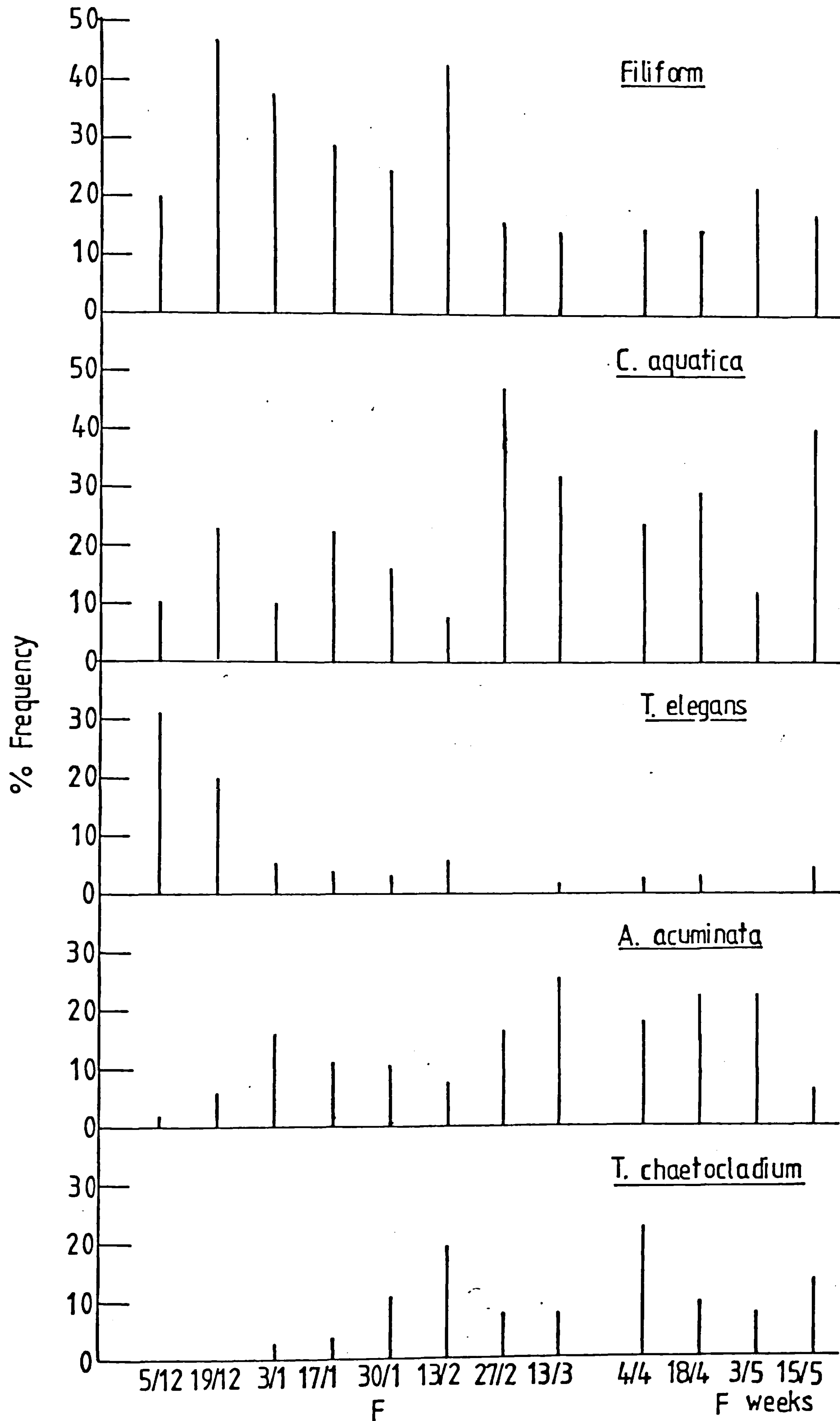


FIGURE 3: Legend and continuation overleaf.

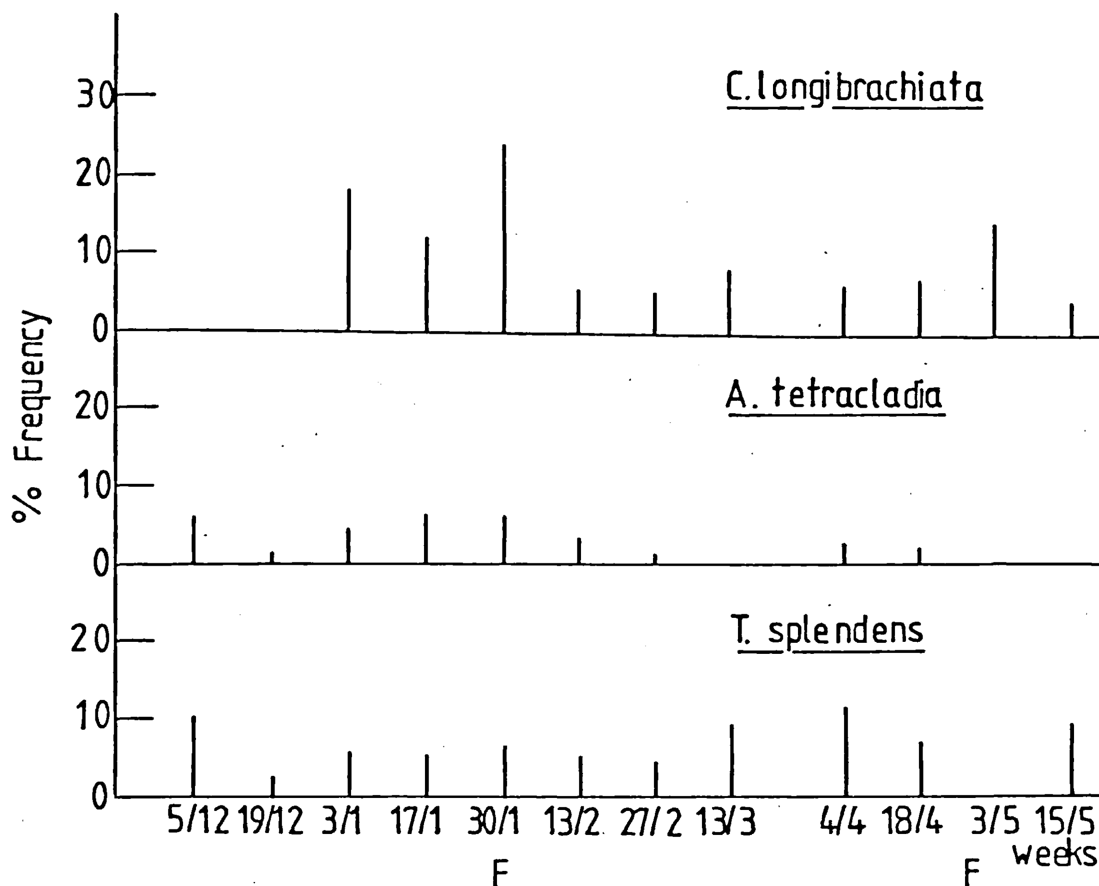


FIGURE 3: Continued. Histograms showing percentage frequency with time of conidia of individual species of aquatic Hyphomycetes commonly found in filtered stream water (500mls). 'Filiform' includes *Anguillospora longissima*, *Mytocentrospora angulata* & *Flagellospora curvula*. The first two species were dominant.

% frequency, seems to prefer the lower temperatures prevailing in December and January. Tricladium splendens shows little change with time in the pattern of its distribution and is usually present at low frequency. In addition to these 10 most common species, 11 others, given in Appendix Table 3 were detected infrequently in low concentrations.

The litter bags designated Alder I were placed in the stream on 21st October, 1977; Alder II on 5th December, and Alder III on 18th December. Bags containing oak leaves were submerged in the stream on 21st October. The first sample of Alder I and oak, made on 5th December, were lost, as the water in the sample trays dried up. The first results for these bags are given for 19th December along with Alder II. Data for leaf counts from all samples of the leaf pack experiments are presented in Appendix Table 3. Invertebrate feeding was not prevented and animals were regularly found in sample packs. Alder I leaves were skeletonized to petioles and midribs in 10 weeks and Alder II and III in 12 weeks. These differences might be explained by the fact that lower stream temperatures prevailed during the processing of Alders II and III than for Alder I (Figure 2). This figure also gives values for total spore counts with time for experimental leaves expressed as spores/gram dry wt of leaf. Oak packs were processed in 25 weeks, and may be compared with results for the leaves in Alder I. The graph of the total spore count probably represents the model for leaf pack decay. The pattern of Alder I is paralleled over a longer time scale by that for Oak overall. The sample for 18th April represents a pack whose processing rate was not in step with the general rate and emphasises the influence of micro-environmental factors on leaf processing rates. The overall pattern for Oak and Alder I is of low initial levels of colonization, reflected in total spore production, with a rapid rise which must reflect increases in mycelial growth and possibly additional colonization by new species, though this is not

necessarily the case. In Alder I maximum spore production is associated with highest species counts, but highest spore counts for Alder III are associated with only 3 species. The time from initial submersion to peak spore production may be taken as the 'conditioning' time of the leaf pack:

<u>Conditioning times</u>

Alder I	=	6 weeks.
Alder II	=	4 weeks.
Alder III	=	2 weeks.
Oak	=	12 weeks.

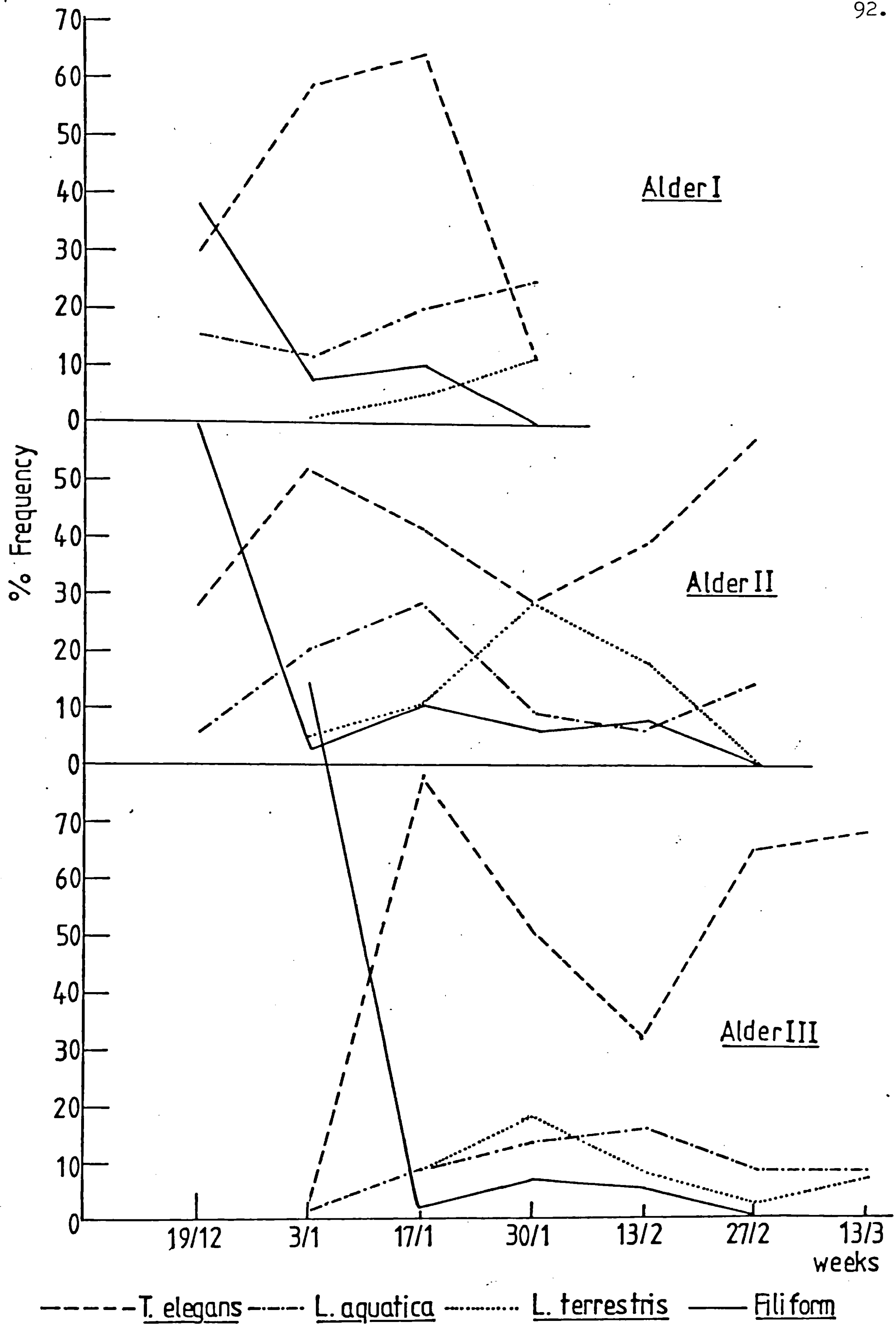
The conditioning period, if the alder packs are compared, appears to depend on the inoculum levels in the stream, and also, if oak and alder are compared, on the leaf species.

The total spore curve for Alder II is initially parallel to that of Alder I. The last two samples, like that of Oak 18/4 may represent randomly sampled packs whose overall rate was slower than the others, due to microenvironmental factors. Alder III appears to miss the initial low level of inoculum, but this is not doubt due to a quicker conditioning period resulting from high inoculum levels. Had a sample been taken after one week of submersion it is probable that a lower level of total spore counts parallel to Alder I, II and Oak would have emerged.

It is noteworthy that maximum spore counts for Alder (Alder III) are about the same for the maximum in oak (140,000/g dry wt leaf) but the resources exploited by micro-organisms from alder are more readily utilized than those of oak. The evidence for this is the longer time scale required in the case of oak for maximum spore values to be realised and for these values to decline.

Figures 4 and 5 taken from the data given in Appendix Table 3, show percentage frequency of the four species of aquatic Hyphomycetes dominant on sampled leaf packs of Alder I, II, III and Oak.

FIGURE 4: Plots of percentage frequency with time of conidia of aquatic Hyphomycetes dominant on submerged alder leaves. 'Filiform' includes *A. longissima* and *Mycocentrospora angulata*.



70
60
50
40
30
20
10
0
Alder I
50
40
30
20
10
0
Alder II
70
60
50
40
30
20
10
0
Alder III
% Frequency
19/12
3/1
17/1
30/1
13/2
27/2
13/3
weeks
T. elegans
L. aquatica
L. terrestris
Filiform

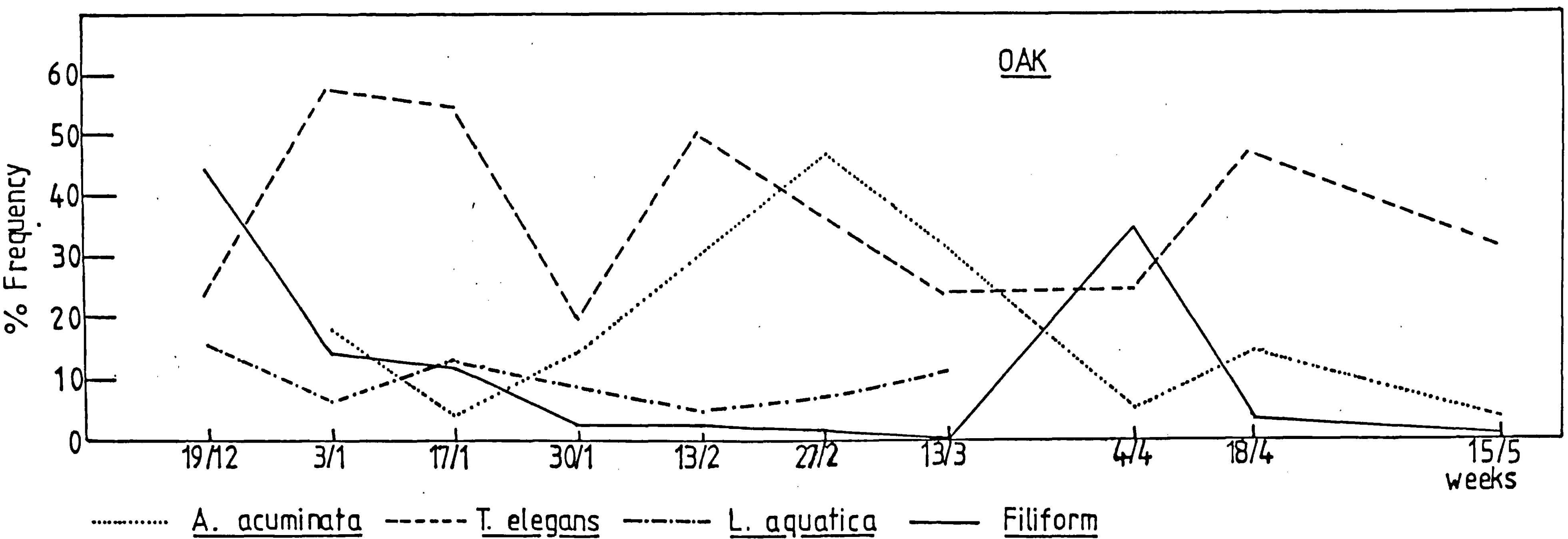


FIGURE 5: Plots of percentage frequency with time of conidia of individual species of aquatic Hyphomycetes dominant on submerged oak leaves. 'Filiform' included A. longissima & Mycocentrospora angulata.

The features in common between Alder and Oak are:

a. The dominance of Tetrachaetum elegans in all samples but the first which is dominated by the two filiform species A. longissima and M. angulata.

b. The rapid decline in the filiform species which remain depressed whilst T. elegans is dominant.

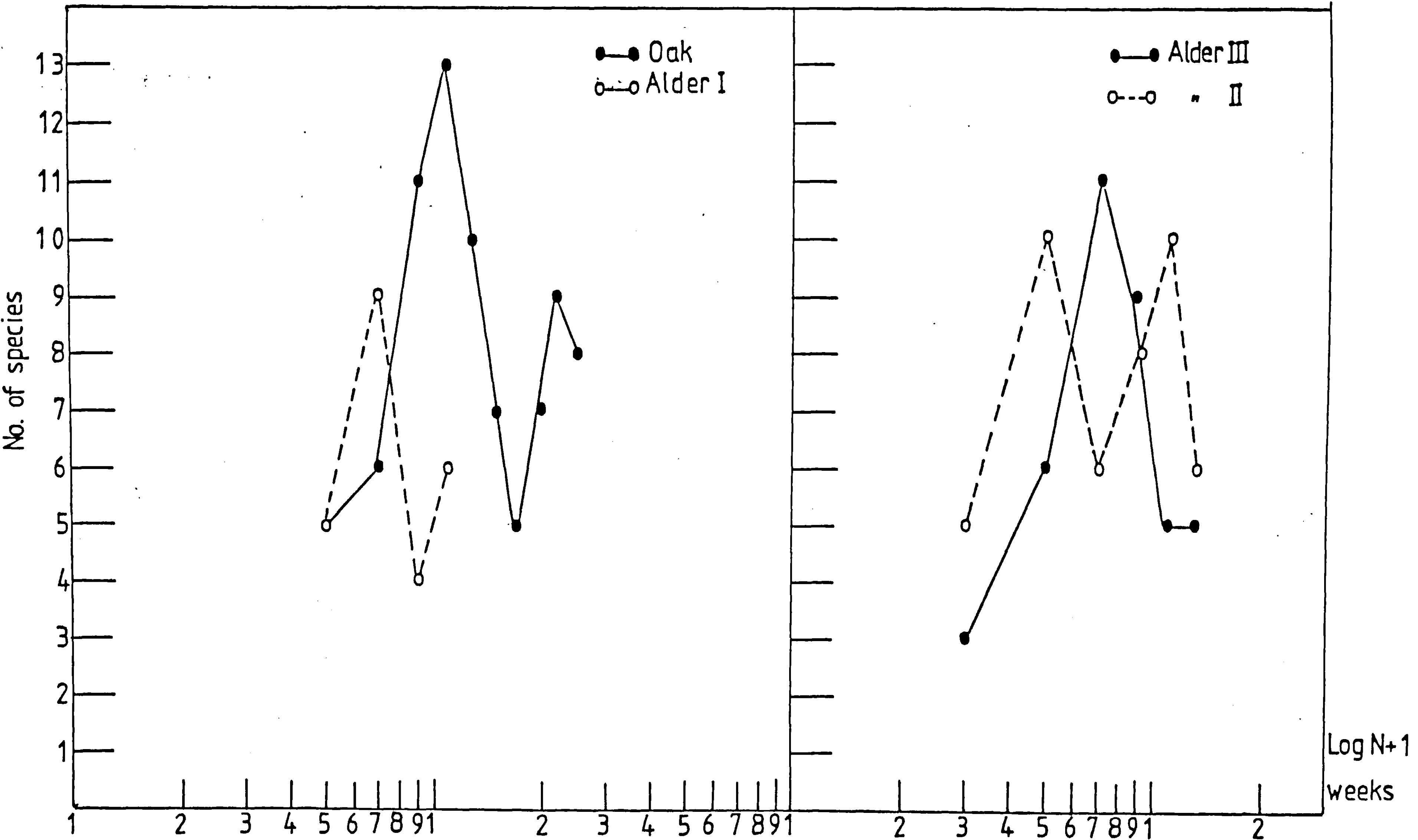
c. The levels of intermediate species (Oak: L. aquatica and A. acuminata. Alder: L. aquatica and L. terrestris) which tend to increase in frequency as levels of T. elegans drop.

d. The presence of four dominant species, 3 of which are common to Oak and Alder, and about 11 other species in each series that appear sporadically at low levels.

The initial high levels of T. elegans and the two filiform species can be explained by the high spore concentrations of these species in the stream at the time of submersion (see Figure 3) but this alone cannot explain the continuing dominance of T. elegans in all series over the entire sampling period and the rapid decline in the filiform species. Furthermore, some species dominant in the stream e.g. Clavariopsis aquatica at the same time appear rarely at very low levels on leaf packs; whilst L. terrestris appears at comparatively high levels on all three Alder series but turned up rarely on Oak and not at all in foam or scum samples. Lemonniera aquatica too, was found infrequently on filters but was amongst the dominant species on both Oak and Alder. A possible explanation is that T. elegans, an early colonizer, once established exerts an antagonistic effect on other species and that accompanying this is a degree of substrate specificity in species which give rise to associations.

The data on species-number/sample given in Appendix Table 3 is presented in Figure 6 as a plot of species number against time. The common feature of all plots is of low initial number of species

FIGURE 6: Plots of species numbers found on submerged leaf packs, against time (Log N + 1 weeks of submersion).


Oak
Alder I
Alder III
" II
No. of species
Log N+1
weeks

followed by a rapid rise to a maximum value and a rapid decline. This decline is maintained in the case of Alder III, but the other three series show increases and fluctuations after decline. This is probably due to the fact that no two packs will have identical floral compositions and that random sampling picks up species composition intermediate between the lowest and highest values. From Appendix Table 3 it can be seen that there is a general conservation of species and that any final rise in species numbers is not due to the addition of new species, but to combinations of species present on earlier samples. Of the 26 species identified in the stream 17 turned up on Oak packs and 16 on Alder packs with 14 spp common to both.

The conservation of species is well illustrated in Figure 7 which shows the cumulative number of species as new species are added, plotted against the log of cumulative spore numbers. This represents an aspect of what Whittaker (1972) has described as α diversity or the diversity of species within a community or habitat. Values tend to depend on the length of sampling time as can be seen from the differences between Alder I (10 weeks) and Oak (25 weeks). Nevertheless, a value for species equilibrium for 5g leaf packs can be ascertained by this method. This value is 14 species. 14 species was about the average number of species detected in filter samples. From Figure 7 it is possible to give an approximation in cumulative spore totals for the upper limit of productivity of a 5g leaf pack: c. 500,000 spores/g dry wt. of leaf.

The concept of species conservation can be explored by β diversity which is a measure of taxonomic similarity between stands. The co-efficient of similarity (C_s) between stands of the same series, and between the samples of different series made after the same experimental period, was calculated using Sørensen's method (Sørensen 1948 ex Southwood 1978).

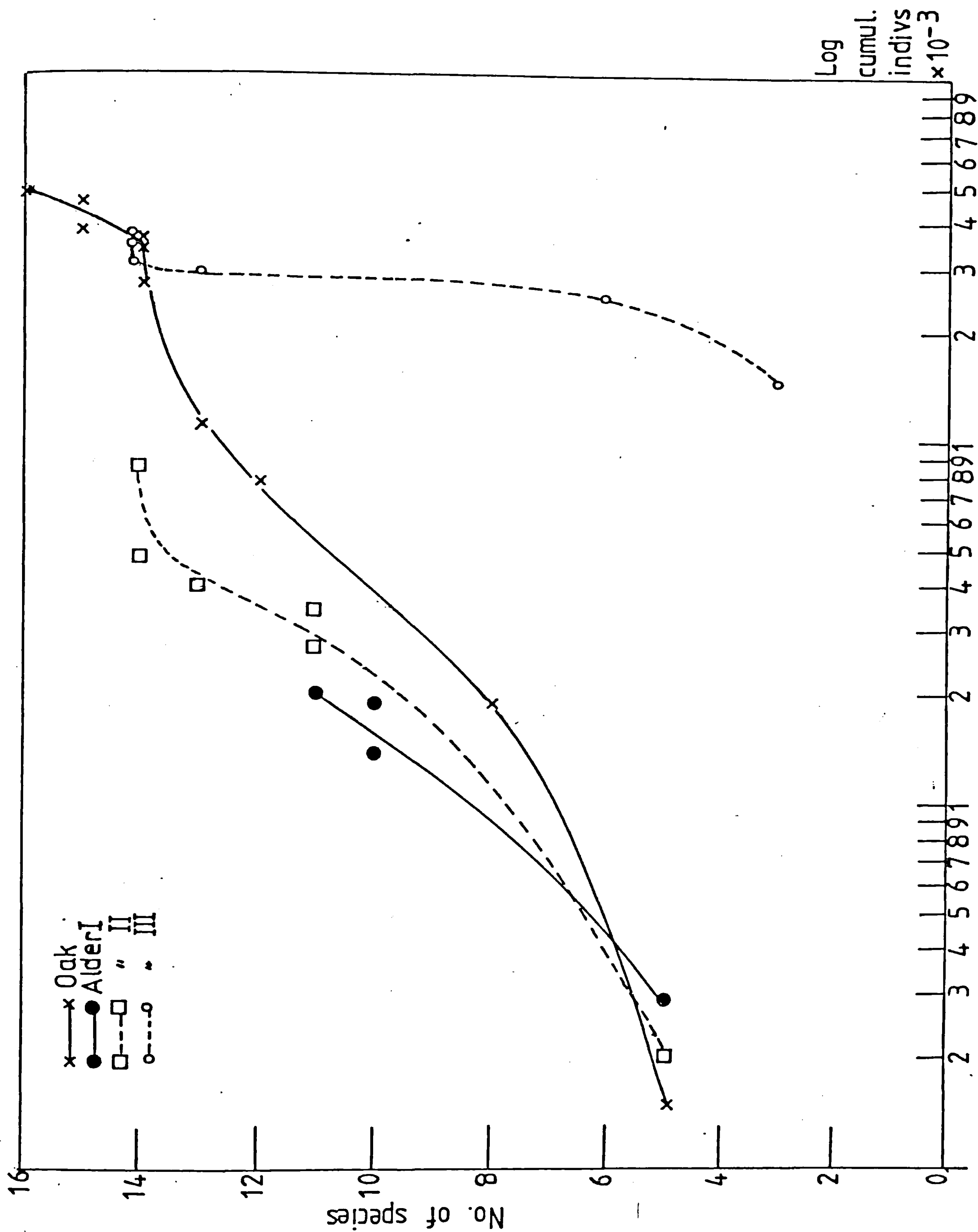


FIGURE 7: An aspect of α diversity. Plot of species number (by addition with time) against cumulative total of conidia (Log_{10} x 10^{-3}) for Alder I, II, III and Oak.

$$C_s = 2j / (a + b) \times 100$$

Where j = species in common and (a+b) = total species. The co-efficients are classified by dendograms given in Figure 8 a, b, c, and d with co-efficient values opposite. Although much information is lost by this method as only highest values are utilized, the dendrograms illustrate in the case of the three Alder series a moderately close similarity in species composition between stands, with a 60% similarity in each overall. Only Alder III shows a neat pattern of relationships between successive stands.

In Oak there appears to be a closer similarity in the first six stands (excepting 2) which group together, and the last four stands. This is due to an element of species succession which has been noted on leaves which are slowly processed. There is not enough time for a succession of species to develop on quickly-processed leaves like Alder. There is, nevertheless, an overall similarity of 67% between stands of Oak.

Co-efficients of similarity were calculated for matching stands between series of Alder. The values are presented in Table 9 below.

	Alder I & II	I & III	II & III
Stand 1	-	-	75
2	67	55	63
3	67	80	59
4	67	62	71
5	63	55	69
6	-	-	55
$\bar{x}$	66	63	65

TABLE 9: C_s between Alder series for matching stands

The evidence of β diversity calculations set out above is of a fairly high degree of toxonomic similarity between stands in each

Alder I

	1	2	3	4
1	-	57	67	54
2		-	62	67
3			-	60
4				-

(a)

Alder II

	1	2	3	4	5	6
1	-	53	55	62	40	73
2		-	75	56	50	63
3			-	57	75	67
4				-	56	57
5					-	63
6						-

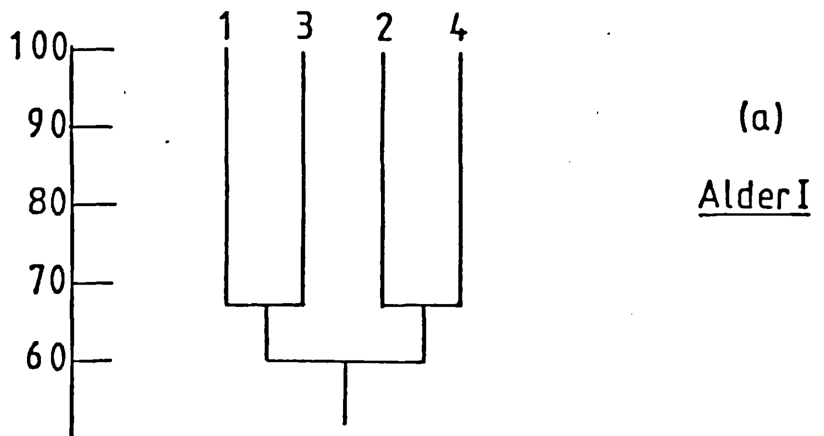
(b)

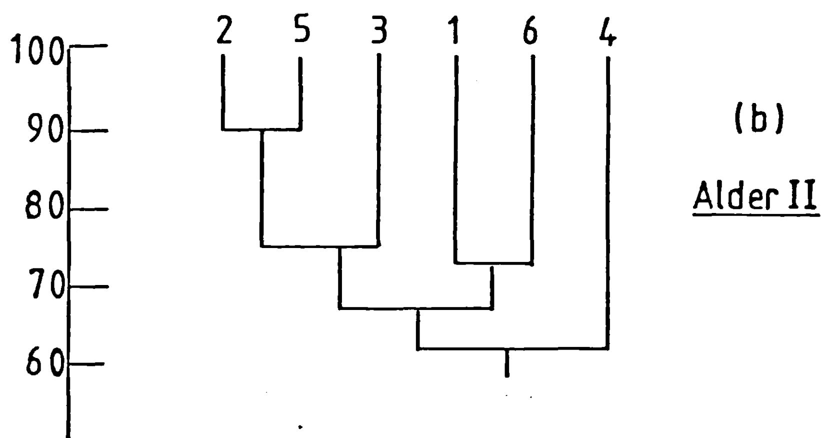
Alder III

	1	2	3	4	5	6
1	-	67	43	50	50	50
2		-	47	58	55	55
3			-	80	50	50
4				-	57	57
5					-	100
6						-

(c)

Trellis diagrams (a), (b), (c) for dendrograms in Figure 8 opposite





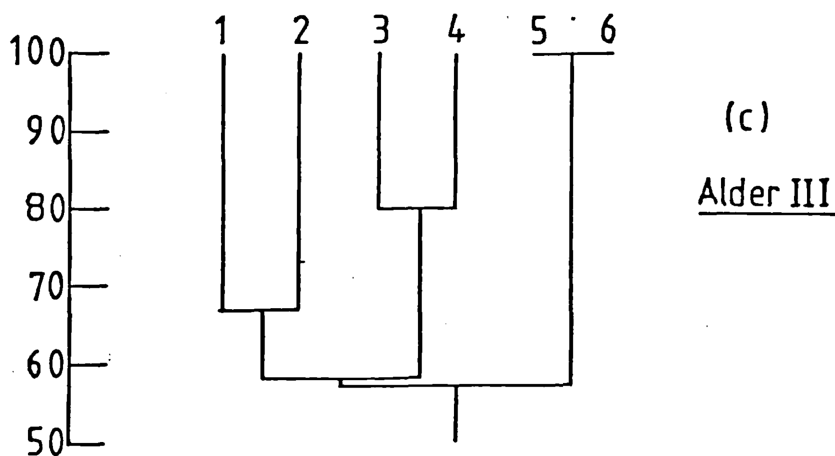


FIGURE 8 (a), (b), (c): Dendrograms of β diversity to show species similarity between successive samples of Alder I, II, III. Co-efficient of similarity used: Sørensen (1948) ex Southwood (1978). $C_s = 2j/(a + b) \times 100$ where j = spp in common & (a + b) = total species. Tables opposite.

OAK (d)

	1	2	3	4	5	6	7	8	9	10
1	-	55	50	56	67	83	40	67	57	31
2		-	70	63	50	62	55	46	53	43
3			-	83	67	67	50	56	70	42
4				-	78	70	36	60	72	48
5					-	82	67	71	74	56
6						-	67	71	75	62
7							-	33	43	62
8								-	88	53
9									-	70
10										-

Table (d) for diagrams in Figure 8 opposite.

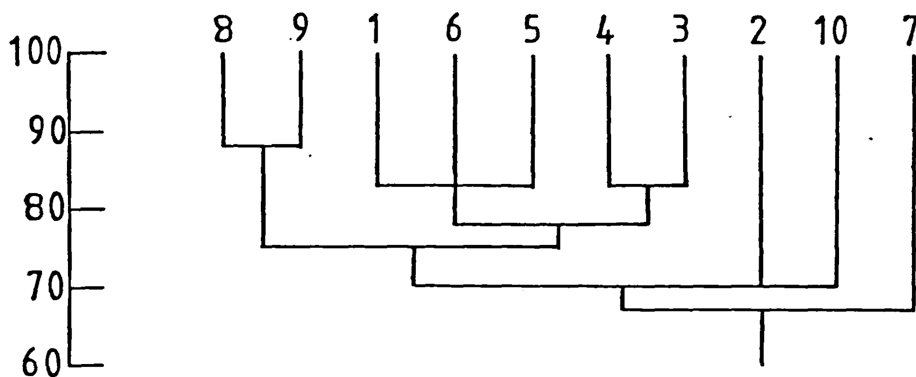


FIGURE 8 (d): Dendrograms of β diversity to show species similarities between successive samples of oak. Trellis diagram opposite.

series which is based largely on the existence and persistence of a few dominant species, associated with a greater but limited number of subsidiary species. This degree of similarity exists too in matched stands between series of Alder.

Results of plate counts for pectolytic bacteria on leaves of Alder I and Oak are presented in Figure 9. The curves can be compared with those of total spore counts given in Figure 2. Pectolytic bacteria reach a maximum at the same time as total spore counts and show a similar sharp decline in numbers. The association is emphasised by the values for Oak on 18th April which represents a sample pack more slowly processed than previous packs and at the same stage as that sampled on the 18th February. The association is not necessarily one between fungi and bacteria but probably between each and the available resources, a point brought out in the slower build up of bacterial numbers in Oak compared to the more quickly processed Alder; a point evident, too, in total spore counts. As with total spore values, there appears to be an upper limit to bacterial numbers which is common to Alder and Oak. This value is about 3.5×10^8/g wet weight of leaf. At maximum, pectolytic bacteria represent about half the population of bacteria detectable on nutrient agar plates.

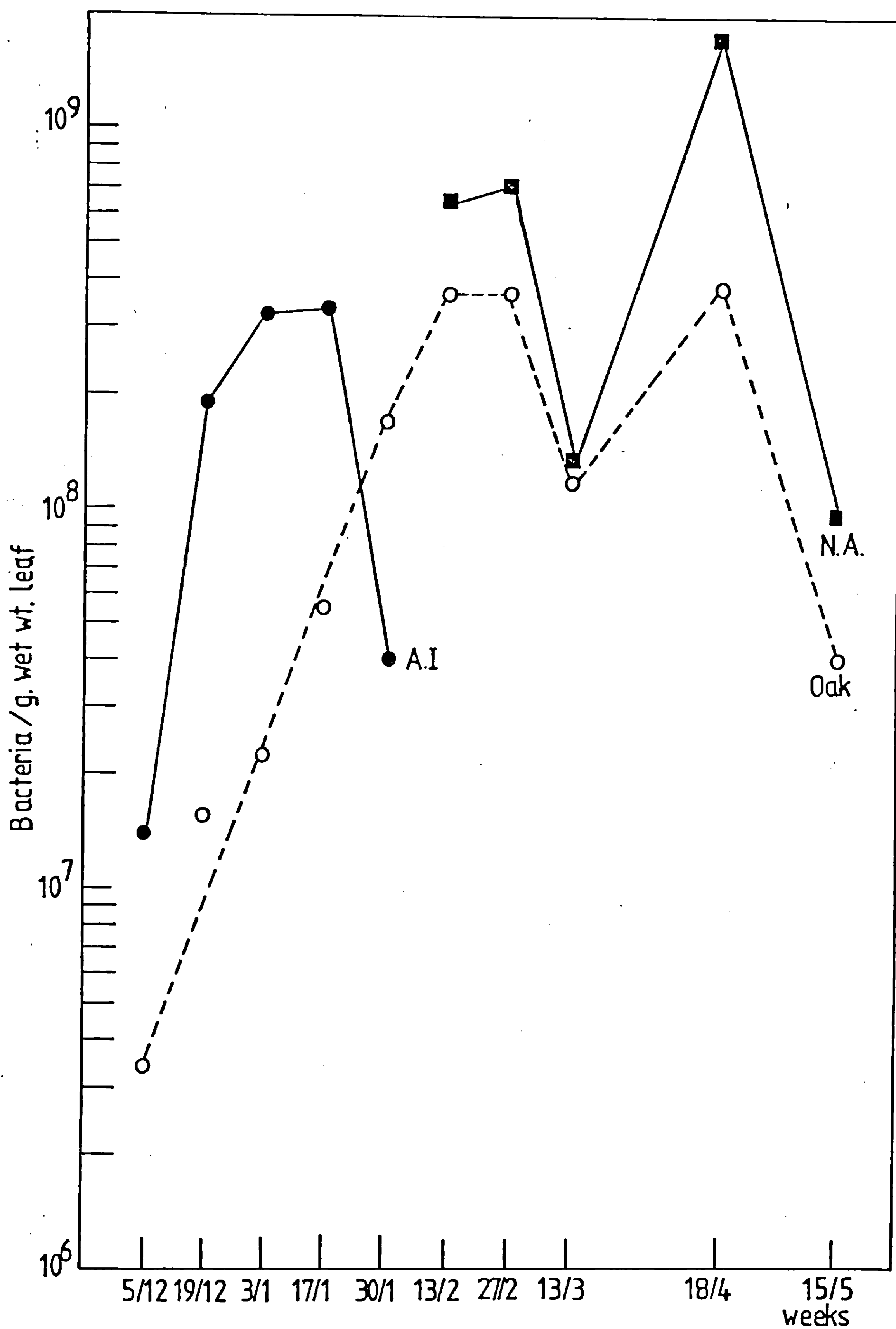


FIGURE 9: Plots to show pectolytic bacteria counts on Alder I, & Oak against submersion time. Total bacterial counts were made on nutrient agar plates (N. A.).

B. RESULTS OF QUANTITATIVE ANALYSIS OF SPECIES LISTS FROM THE LITERATURE

In order to 'place' the group of species of aq. Hyphomycetes found in the River Bourne in a wider context, it was decided to conduct a quantative analysis of as many species lists as possible given in the literature. Such an analysis might provide evidence of groups of related stands and species.

A numbered list was drawn up of published species lists (stands). This list is given on pages 104 - 107. At the same time a numbered list of species was compiled, drawn from Ingold's (1975) "Guide to Aquatic Hyphomycetes" with a few additions. This master list is given on pages 108 - 111. Individual stands were referred to the master-list and species marked present in or absent from that stand.

All 82 stands were processed by Agglom (Agglom I. See Materials and Methods) and the resulting values constructed as a dendrogram. As this produced no clear groupings, it was decided to rerun the process eliminating stands of 10 species and fewer, and stands of above 40 species. This gave a list of 59 stands for Agglom II. The dendrogram from Agglom II (stands) is presented in Figure 10. It can be split into 3 groups. However, when these groups are located (pages 113-4), interpretation of the results is not easy. There seems no obvious or expected rationale behind the group geographically; e.g. in Group 2 are the Subarctic (45), Cuba (39), East New Guinea (57) and Yorkshire (33). Analysis of species numbers shows an average of 20 in Group I, 16.5 in group 2 and 15.5 in Group 3 which opens up the possibility that groupings are based on numbers of species in common between stands. However, Chamier (6) and Alder leaves (21) have 16 species in common and are in Group 1 and 3 respectively.

STANDS

AGGLOM I NUMBER	AGGLOM II NUMBER	R. A. No.	LOCATION	REFERENCE
1	-	-	Nigeria	Alasoadura S O (1968)
2	1	47	Hawaii	Anastasiou C J (1964)
3	2	1	Leningrad Region	Arnold G R N (1970)
4	3		Danube	Banhegyi J (1961)
5	4	20	Ontario	Bärlocher F, Kendrick B (1974)
6	5	50	Wyoming	Baxter J W (1960)
7	-	-	Wyoming,Indiana	Baxter J W (1962)
8	6	2	Surrey	Chamier A C (1980)
9	7	36	Florida	Conway K E (1969)
10	8	3	Central N. York	Conway K E (1970)
11	9	38	Australia	Cowling S W & Waid J S (1963)
12	10	21	Australia	Cowling S W & Waid J S (1963)
13	11	51	N. Appalachian Highland	Crane J L (1968)
14	-	-	Ghana	Dixon P A (1959)
15	-	-	Ukraine	Dudka I A (1974)
16	12	27	Toulouse & Pyrenees	Durrieu G (1970)
17	13	35	Iowa	Dyko B J (1976a)
18	14	4	Tennesee	Dyko B J (1976b)
19	15	48	Kentucky	Dyko B J (1976b)
20	-	-	N. Carolina	Dyko B J (1976b)
21	16	5	Georgia	Dyko B J (1976b)
22	17	45	N. Ireland	Fenton A F (1950)
23	18	6	Hungary I	Gönczöl J (1971)
24	-	-	Hungary II	Gönczöl J (1975)
25	-	-	Hungary III	Gönczöl J (1975)

26	-	-	Hungary	Gönczöl J (1976)
27	19	46	S. Africa	Greathead S K (1961)
28	20	7	Jamaica	Hudson H J & Ingold C T (1960)
29	21	55	Alder leaves	Ingold C T (1942)
30	-	-	Nigeria	Ingold C T (1956)
31	22	8	S. Rhodesia	Ingold C T (1958)
32	23	37	Uganda	Ingold C T (1958)
33	-	-	Nigeria	Ingold C T (1959)
34	24	22	Kent	Ingold C T (1975)
35	25	44	Shropshire	Ingold C T (1975)
36	26	9	Scotland	Ingold C T (1979)
37	27	31	Pakistan	Iqbal S H, Sulatana K, & Shaheen Farzana (1973)
38	28	10	R. Creedy, Devon	Iqbal S H, Webster J (1973)
39	29	32	R. Exe, Devon	Iqbal S H, Webster J (1973)
40	30	11	Dartmoor, Cherry Brook	Iqbal S H, Webster J (1977)
41	31	30	Dartmoor, R. Cowsic	Iqbal S H, Webster J (1977)
42	32	12	Dartmoor, R. Swincombe	Iqbal S H, Webster J (1977)
43	33	41	Yorkshire	Jones E G B (1965)
44	34	13	Linconshire	Lindsey B I & Glover B J (1976)
45	35	12	Toulouse	Lorrillard M (1974a)
46	36	14	S Spain	Lorrillard M & Merce J (1975, 1976)
47	37	42	Pau	Lorillard M (1973)
48	38	15	France	Lorillard M (1974b)
49	-	-	Finnish Grasses	Mäkelä K (1973)

50	39	33	Cuba	Marvanová L & Marvan P (1969)
51	40	16	Czechoslovakia	Marvanová L & Marvan P (1963)
52	41	39	Ontario I	Michaelides J & Kendrick B (1978)
53	-	-	Ontario II	Michaelides J & Kendrick B (1978)
54	42	23	Okinawa, Japan	Miura K (1973, 1974)
55	43	49	Hokkaido, "	Miura K (1973, 1974)
56	44	17	Nagano, "	Miura K (1973, 1974)
57	45	34	Subarctic	Müller-Haeckel A, Mavanova L (1979)
58	46	18	Sweden	Nilsson S (1964)
59	47	57	Puerto Rica	Padgett, D E (1976)
60	48	29	N. America	Petersen R H (1962, 1963)
61	49	54	California	Ranzoni F V (1953)
62	-	-	Ohan } Hawaiian Islands	Ranzoni F V (1978)
63	-	-	Kawai } Hawaiian Islands	Ranzoni F V (1978)
64	-	-	Mawi } Hawaiian Islands	Ranzoni F V (1978)
65	-	-	Hawaii } Hawaiian Islands	Ranzoni F V (1978)
66	50	24	Devon	Sanders P F & Anderson J M (1979)
67	51	59	Virginia	Scott W W & Umphlett C J (1963)
68	52	28	S. W. Michigan	Suberkropp K F & Klug M J (1974)
69	-	-	Michigan	Suberkropp K F & Klug M J (1976)
70	-	-	Harmonic lakes of Mt. Hakkoda	Suzuki S & Namura H (1960a)
71	-	-	Acidotrophic Lakes of Mt. Hak.	Suzuki S & Namura H (1960a)
72	53	11	Harmonic Japanese Lakes	Suzuki S & Namura H (1961).
73	-	-	Dystrophic Japanese Lakes	Suzuki S & Namura H (1961)

74	-	-	Acidotrophic Japanese Lakes	Suzuki S & Namura H (1961)
75	-	-	Fukushima, Japan Acidotrophic Lakes	Suzuki S & Namura H (1960b)
76	-	-	Fukushima, Japan Harmonic Lakes	Suzuki S & Namura H (1960b)
77	54	19	Pittsburgh	Triska F J (1970)
78	55	43	Japan	Tubaki K (1957)
79	56	25	N & Central Japan	Tubaki K (1960)
80	57	40	E. New Guinea	Tubaki K (1965)
81	58	26	Lake District (Foam)	Willoughby L G & Archer, Jane F (1973)
82	59	56	Lake District (Wood)	Willoughby L G & Archer. Jane F (1973)

SPECIES LIST

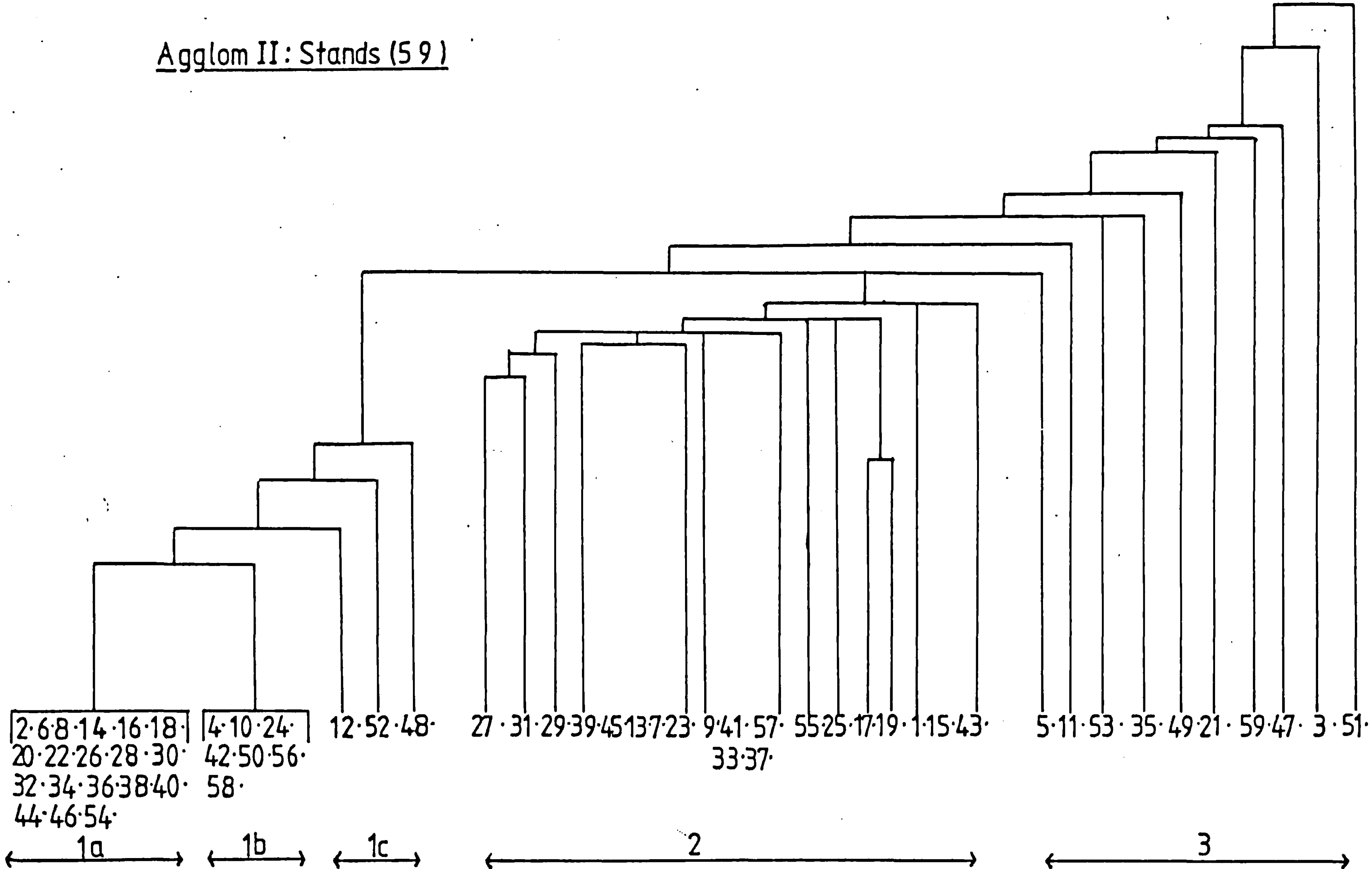
AGGLOM I		AGGLOM II, R. A.	% Frequency
1.	Actinospora megalospora	1	29
2.	Articulospora tetracladia	2	76
3.	A. inflata	3	25
4.	A. grandis	4	5
5.	Campylospora chaetocladia	5	25
6.	C. parvula	6	3
7.	Clavariopsis aquatica	7	73
8.	C. brachycladia	8	7
9.	Culicidospora aquatica	9	19
10.	C. gravida	10	15
11.	Dactylella aquatica	11	32
12.	D. submersa	12	9
13.	D. appendiculata	13	3
14.	Dendrospora erecta	14	24
15.	D. juncicola	15	2
16.	Dimorphospora foliicola	16	9
17.	Flabellospora crassa	17	7
18.	F. verticillata	18	3
19.	F. tetracladia	19	2
20.	Gyoerffyella craginiformis	20	7
21.	G. speciosa	21	5
22.	G. tricapillata	22	3
23.	G. entomobryoides	X	0
24.	G. biappendiculata	23	2
25.	Pleuropedium tricladiodes	24	5
26.	Isthmotricladia sp.	25	10

27.	Tridentaria glossopage	X	0
28.	Jaculispora submersa	26	10
29.	Lateriramulosa uni-inflata	27	7
30.	Polycladium equiseti	28	5
31.	Pyramidospora casuarinae	29	2
32.	P. constricta	X	0
33.	Speiropsis irregularis	30	12
34.	Tetrachaetum elegans	31	64
35.	Tetracladium marchalianum	32	83
36.	T. setigerum	33	66
37.	T. maxilliforme	34	14
38.	Tricladium splendens	35	64
39.	T. angulatum	36	49
40.	T. gracile	37	46
41.	T. eccentricum	38	7
42.	T. anomalum	39	9
43.	T. patulum	40	9
44.	T. chaetocladium	41	3
45.	T. terrestre	42	3
46.	T. attenuatum	X	0
47.	T. caudatum	43	3
48.	T. giganteum	44	9
49.	Tripospermum myrti	45	7
50.	T. camelopardus	46	5
51.	Varicosporium elodeae	47	46
52	V. delicatum	48	3
53.	V. giganteum	49	3
54.	Alatospora acuminata	50	81
55.	Clavatospora longibrachiata	51	46
56.	C. stellata	52	34
57.	C. tentacula	53	22

58.	C. filiformis	X	0
59.	Heliscus lugdunensis	54	66
60.	H. submercus	55	2
61.	Lemonniera aquatica	56	78
62	L. terrestris	57	44
63.	L. centrosphaera	58	2
64.	L. cornuta	59	15
65.	L. filiforme	60	9
66.	Margaritispora aquatica	61	24
67.	Trisulcosporium acerinum	62	2
68.	Volucrispora aurantica	63	9
69.	V. graminea	64	12
70.	Anguillospora longissima	65	76
71.	A. crassa	66	46
72.	Calcarispora hiemalis	67	2
73.	Centrospora acerina	68	29
74.	C. aquatica	69	2
75.	Flagellospora curvula	70	58
76.	F. penicillioides	71	20
77.	Lunulospora curvula	72	54
78.	L. cymbiformis	X	0
79.	Casaresia sphagnorum	73	5
80.	Camposporium pellucidum	74	3
81.	Pleiochaeta setosa	X	0
82.	Diplocladiella scalaroides	75	5
83.	Tetraploa aristata	X	0
84.	Triscelophorus monosporus	76	61
85.	T. magnificus	77	3
86.	Anguillospora curvula (Iqbal)	78	5
87.	Pseudoanguillospora stricta (Iqbal)	79	7
88.	Scorpiosporium minutum (Iqbal)	80	2

89.	Mycocentrospora clavata (Iqbal)	81	2
90.	Acaulopage tetraceros (Drechsler)	82	2
91.	Dicranidion fragile	82	2
92.	Mycocentrospora angulata	83	7
93	Taeniospora gracilis (Marvanová)	84	3

FIGURE 10: Dendrogram from Agglom II classification of stands (59).
Reference list for numbers given in Text.


Agglom II : Stands (59)
0·5
0·4
0·3
0·2
0·1
0
2·6·8·14·16·18·
20·22·26·28·30·
32·34·36·38·40·
44·46·54·
4·10·24·
42·50·56·
58·
12·52·48·
27·31·29·39·45·13·7·23·9·41·57·
33·37·
55·25·17·19·1·15·43·
5·11·53·35·49·21·59·47·3·51·
1a
1b
1c
2
3

GROUPINGS FROM AGGLOM II

Group 1a

Stand No.	Location	Species No.	R. A. No.
2	Leningrad Region	28	1
6	Chamier: Surrey	25	2
8	Central N. York	19	3
14	Tenessee	17	4
16	Georgia	11	5
18	Hungary I	21	6
20	Jamaica	16	7
22	S. Rhodesia	11	8
26	Scotland	31	9
28	R. Creedy, Devon	18	10
30	Dartmoor Stream	18	11
32	Dartmoor Stream	11	12
34	Linconshire	19	13
36	S. Spain	23	14
38	France	16	15
40	Czechoslovakia	16	16
44	Nagamo, Japan	28	17
46	Sweden	34	18
54	Pittsburgh	23	19
		$\bar{x}$ = 20.2	

Group 1b

Stand No.	Location	Species No.	R. A. No.
4	Ontario	16	20
10	Australia	10	21
24	Kent	24	22
42	Okinawa	21	23
50	Devon	22	24
56	N & Central Japan	13	25
58	L. District (foam)	28	26
		$\bar{x}$ = 19.0	

Group 1c

Stand No.	Location	Species No.	R. A. No.
12	Toulouse & Pyrenees	14	27
52	S. W. Michigan	11	28
48	N. America	34	29
		$\bar{x}$ = 19.6	

Group 2

Stand No.	Location	Species No.	R. A. No.
31	Dartmoor Stream	16	30
27	Pakistan	21	31
29	R. Exe, Devon	13	32
39	Cuba	11	33
45	Subarctic	12	34
13	Iowa	11	35
7	Florida	13	36
23	England	13	37
9	Australia	11	38
41	Ontario I	12	39
57	E. New Guinea	13	40
33	Yorkshire	31	41
37	Pau. France	14	42
55	Japan	13	43
25	Shropshire	25	44
17	N. Ireland	19	45
19	S. Africa	17	46
1	Hawaii	16	47
15	Kentucky	11	48
43	Hokkaido, Japan	29	46
		$\bar{x}$ = 16.4	

Group 3

Stand No.	Location	Species No.	R. A. No.
5	Wyoming	12	50
11	N. Appalachians	21	51
53	Harmonic Jap. Lake	11	52
35	Toulouse	12	53
49	California	18	54
21	Alder leaves	18	55
59	Lake District (wood)	21	56
47	Puerto Rico	15	57
3	Hungary	12	58
51	Virginia	15	59
		$\bar{x}$ = 15.5	

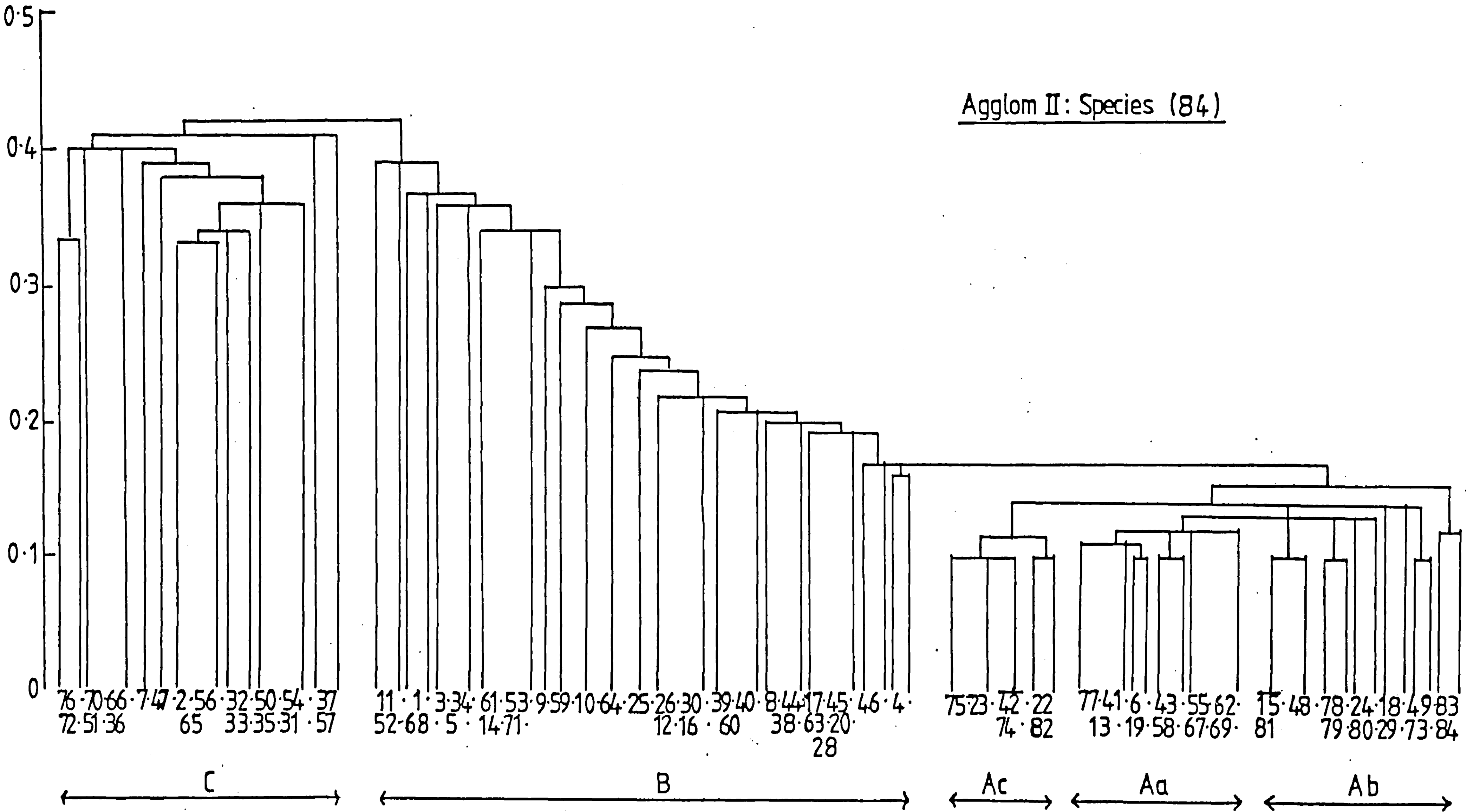
Agglom I for species (93) revealed species which though on record did not appear in any list. These were removed and the remaining species renumbered to give a total of 84. Agglom II for species, Figure 4, is easier to interpret. The species can be divided into 3 groups, A, B and C. The basis for these groupings is the percentage frequency in all stands (see pages 117-9).

Group A contains rare species with a percentage frequency of 3-5 with a few 7% where there is species association, such as 83 (Mycocentrospora angulata) and 84 (Taeniospora gracilis) which occur together in two stands: 45 (Subarctic) and 50 (Devon). Association may be based on common occurrence in one stand only e.g. 43 (Tricladium caudatum) and 58, Lemonniera centrosphaera occur only in stand 43 (Hokkaido, Japan). Group Ab has gathered species particularly associated with the Dartmoor streams (stands 30, 31 & 32) i.e. 15 (Dendrospora foliicola) and 81 (Mycocentrospora clavata), 48 (Varicosporium delicatum), 78 (Anguillospora curvula), 24 (Pleuropedium tricladiodes) 80 (Scorpiosporium minutum) and 79 (Pseudoanguillospora stricta).

Group B consists of intermediate species ranging from 5%F on the 'A' side to 34%F on the 'C' side species. Associations appear to depend on a higher number of stands in common than those of Group A. e.g. 68 (Mycocentrospora acerina) and 1 (Actinospora megalospora) occur together in 5 stands.

Group C consists of the most common species, above 40%F. A significant association here is that isolated for 76 (Triscelophorus monosporus) and 72 (Lunulospora curvula) which of all common species are world-wide in distribution being common in the tropics, and in summer in the temperate zones. This association suggests that the other species associations in Group C are significant.

FIGURE 11: Dendrogram from Agglom II classification of species (84). Reference list for numbers given in text.


Agglom II: Species (84)
0·5
0·4
0·3
0·2
0·1
0
C
B
Ac
Aa
Ab

Species Agglom II (84): GROUPINGS

	Aa	% F
6	Campylospora chaetocladia	3
19	Flabellospora tetracladia	2
77	Triscelophorus magnificus	3
41	Tricladium chaetocladium	3
13	Dactyella appendiculata	3
43	Tricladium caudatum	3
58	Lemonniera centrosphaera	2
55	Heliscus submersus	2
62	Trisulcosporum acerinum	2
67	Calcarispora hiemalis	2
69	Mycocentrospora aquatica	2

	Ab	% F
15	Dendrospora foliicola	2
81	Mycocentrospora clavata	2
48	Varicosporium delicatum	3
78	Anguillospora curvula	5
79	Pseudoanguillospora stricta	7
24	Pleuropedium tricladiodes	5
80	Scorpiosporium minutum	2
18	Flabellospora verticillate	3
29	Pyramidospora casuarinae	2
49	Varicoporium gigenteum	3
73	Cesaresia sphagnorum	5
83	Mycocentrospora agulata	7
84	Taeniospora gracilis	3

	Ac	% F
42	Tricladium terrestre	3
74	Camposporium pellucidum	3
75	Diplocladiella scalaroides	5
23	Gyoerffyella biappendiculata	2
22	" tricapillata	3
82	Aculopage tetraceros	2
82	Dicranidion fragile	2

B

No.	Species	% F
4	Articulospora grandis	5
21	Gyoerffyella speciosa	5
46	Tripospermum camelopardus	5
27	Lateriramulosa uni-inflata	7
17	Flabellospora crassa	7
45	Tripospormum myrti	7
63	Volucrispora aurantiaca	9
20	Gyoerffyella craginiformis	7
28	Polycladium equiseti	5
8	Clavariopsis brachycladia	7
44	Tricladium giganteum	9
38	Tricladium eccentricum	7
39	Tricladium anomalum	9
40	Tricladium patulum	9
60	Lemonniera filiforme	9

No.	Species	% F
26	Jaculispora submersa	10
30	Speiropsis irregularis	12
12	Dactyella submersa	9
16	Dimorphospora foliicola	9
25	Isthmotricladia sp.	10
64	Volucrispora graminea	12
10	Culicidospora gravida	15
59	Lemonniera cornuta	15
9	Culicidospora aquatica	19
14	Dendrospora erecta	24
71	Flagellospora penicilloides	20
53	Clavatospora tentacula	22
61	Margaritispora aquatica	24

No.	Species	% F
3	Articulospora inflata	25
34	Tetracladium maxilliforme	14
5	Campylospora chaetocladia	25
68	Mycocentrospora acerina	29
1	Actinospora megalospora	29
11	Dactyella aquatica	32
52	Clavatospora stellata	34

Species AGGLOM II groupings.

C

		% F			% F
2	Articulospora tetracladia	76	76	Triscelophorus monosporus	61
56	Lemonniera aquatica	78	72	Lunulospora curvula	54
65	Anguillospora longissima	76			
			70	Flagellospora curvula	58
32	Tetracladium marchalianum	83	66	Anguillospora crasse	46
33	Tetracladium setigerum	66	51	Clavatospora longibrachiata	46
			36	Tricladium angulatum	49
50	Alatospora acuminata	81			
54	Heliscus lugdunensis	66	37	Tricladium gracile	46
35	Tricladium splendens	64	57	Lemonneira terrestris	44
31	Tetrachaetum elegans	64			
47	Varicosporium elodeae	46			
7	Clavariopsis aquatica	73			

Species AGGLOM II groupings.

In view of the difficulty of interpreting the results of Agglom II (stands) it was decided to employ a different sorting method which might give a clearer relationship between species and stands. The method chosen was Reciprocal Averaging (Materials and Methods C3). The results of Ordination I are shown as scatter diagrams Figure 12 a and b. Here it can be seen that the majority of stands and species form a close knit clump with fewer intermediate stands and species and very few outliers. Stand 23 is from Okinawa, Japan, a tropical location with a unique species 19 (Flabellospora tetracladia) and a distinctive aquatic Hyphomycete flora. Placed as it is so far from stand 34 (Subarctic) suggests that the ordination is effectively separating tropical from temperate stands. Stands 11, 12 and 30 are the three Dartmoor streams which are the only acidotrophic streams in the list. They, too have a distinctive aq. Hyphomycete flora and the species particularly associated with them and mentioned above can be seen in the corresponding area of the species diagram. Species 18, 81, 48, 78, 80 & 81 are unique to those streams. Species 24 (Pleuropedium tricladiodes) and 44 (Tricladium giganteum) and 79 (Pseudoanguillospora stricta) are rare species associated with the Dartmoor streams.

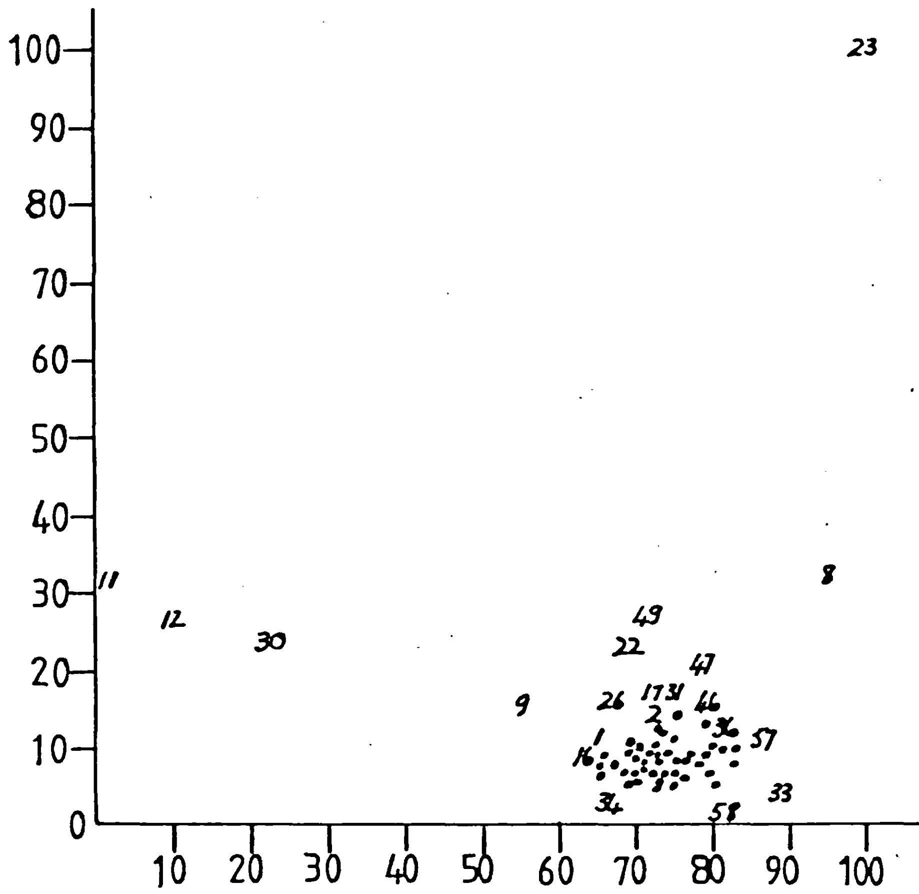
These four stands (11, 12, 23 and 30) and their unique species (15, 19, 48, 78, 80 and 81) were removed from the lists which were then rerun as Ordination II in order to 'spread' the clumped central group of stands and species. The results of Ordination II are presented as two scatter diagrams in Figure 13 a and b.

The tendency towards a tropical/temperate axis is evident once again. Outliers in the tropical zone are 57 (Puerto Rica), 33 (Cuba) and Southern Rhodesia (8). Again, the basis for the separation has been that of distinctive floras and unique species. Species 29 (Pyramidospora constricta) and 18 (Flabellospora verticillata) are tropical species also found in West Africa. Species 62 (Trisulcosporum acerinum) is a monotypic genus from Africa. At the other extreme of the axis is 34

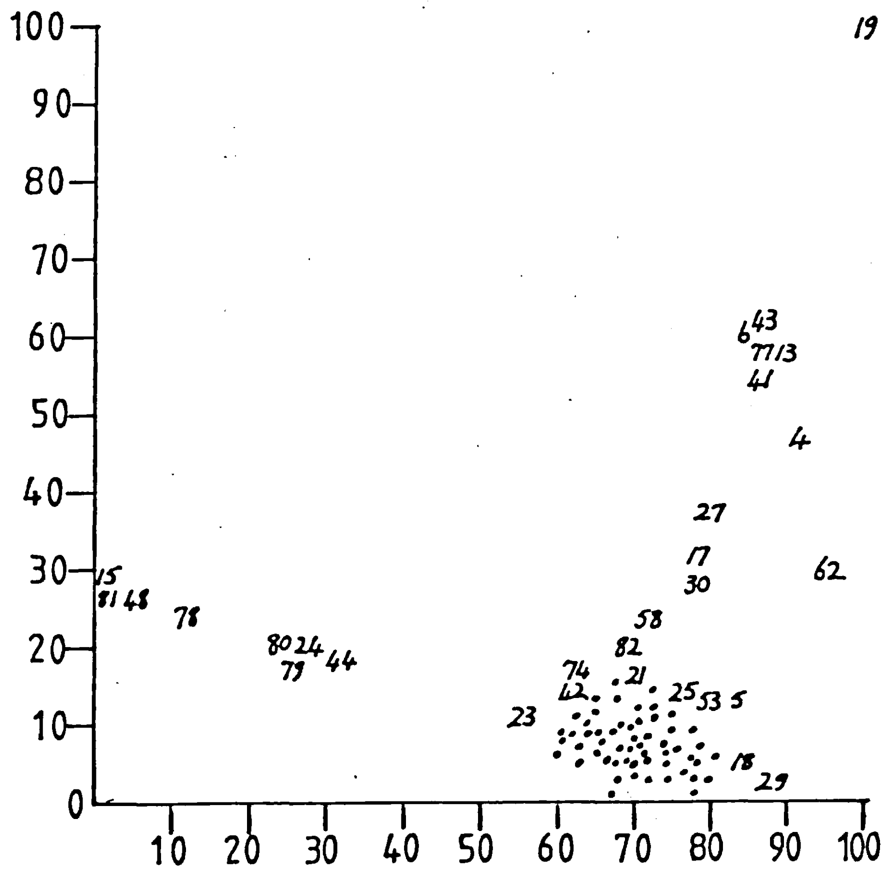
FIGURE 12: Scatter diagrams of Reciprocal Averaging Ordination I.

(a) Stands (59). Reference list for numbers given in text.

(b) Species (84). Reference list for numbers given in text.

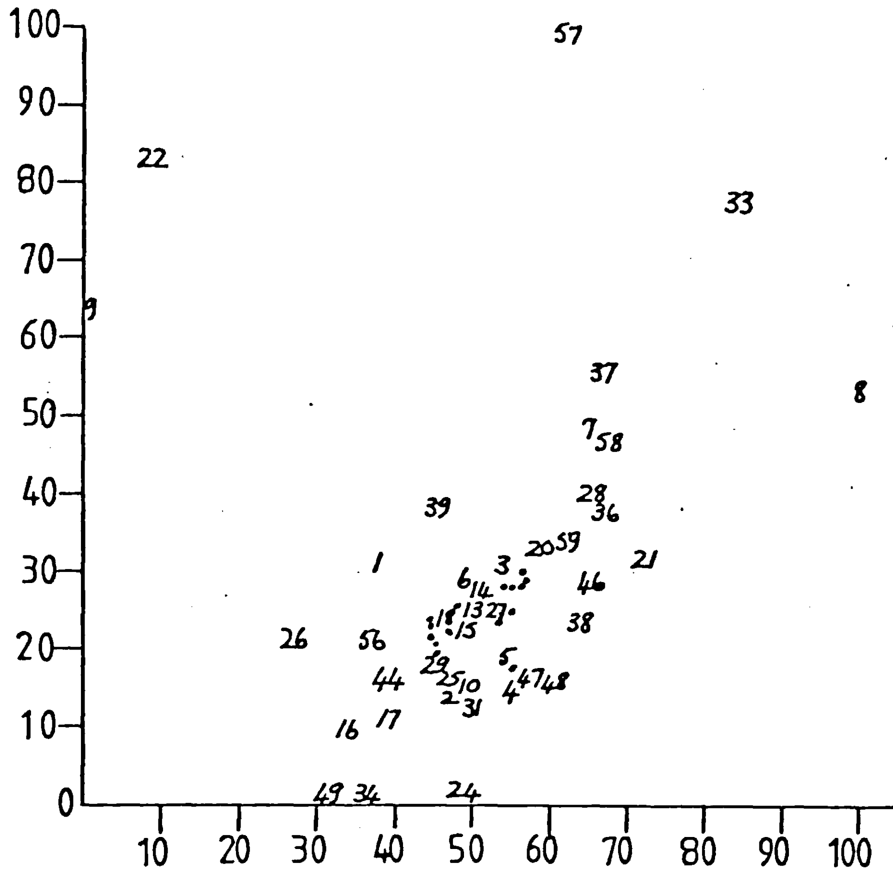


(a) R.A. Ordination.I: Stands (59)



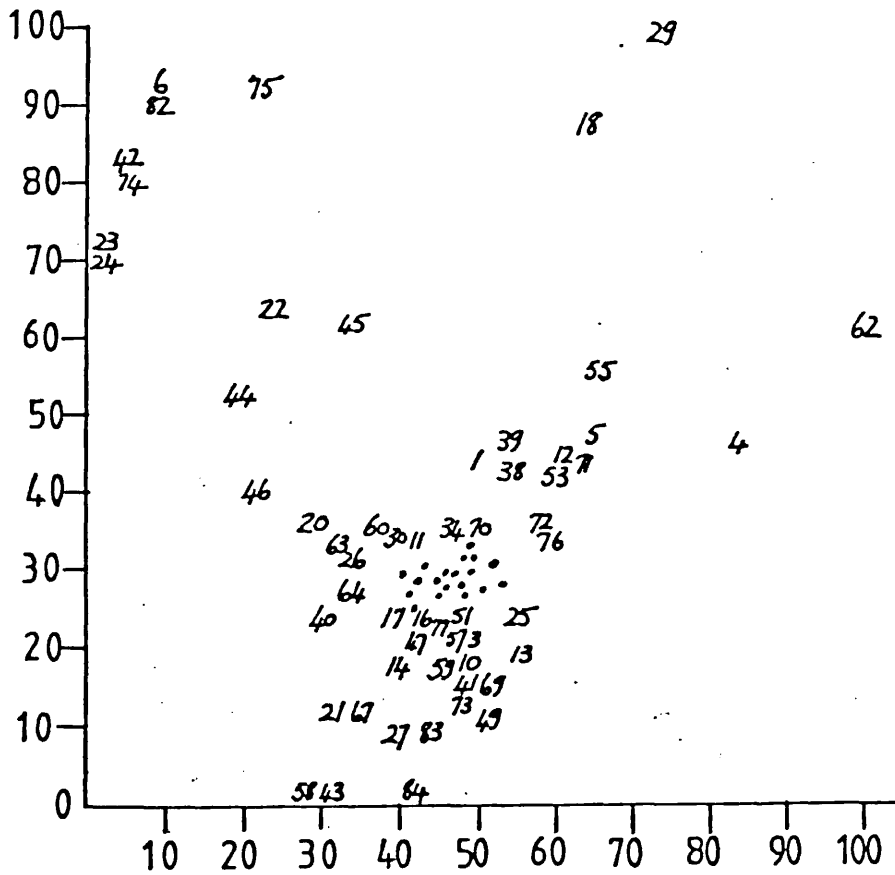
(b) R.A. Ordination I: Species (84)

FIGURE 13: Scatter diagrams of Reciprocal Averaging Ordination II.
(a) Stands (59) minus 11, 12, 23, 30.
(b) Species (84) minus 15, 19, 48, 78, 80, 81.
Reference lists for numbers given in text.



Dots:—
19, 32, 35, 40, 41, 42,
43, 45, 50, 51, 52,
53, 54, 55.

(a) R.A. Ordination II: Stands 1-59 (−11,12,23,30)



Dots:—
2, 7, 8, 30, 31, 32, 33,
35, 36, 37, 50, 52, 54,
56, 61, 65, 66, 68, 79.

(b) R.A. Ordination II Species 1-84 (−15,19,48,78,80,81)

(Subarctic). Drawn on the axis towards the tropical extremes is 37 (Uganda), 7 (Jamaica), 36 (Florida) , 21 (Australia), 46 (S. Africa) and 38 (Australia). There is a geographical consistency in the grouping of 5 (Georgia), 35 (Iowa), 51 (N. Appalachia), 48 (Kentucky) and 4 (Tennessee) but this may be due to stands having been described by one worker, Dyko. Stands 9 and 22 (Scotland and Kent) appear as extremes. This may be due to their having been thoroughly investigated over a number of years by Ingold, the leading authority on these fungi. Both represent recent publications (1979 and 1975) so that rarer species would have come to light and recent identifications included. Worker-consistency may account too for the close proximity of stands 10 (R. Creedy, Devon) and 31 (Pakistan) both worked by Iqbal. Another important separation can be made through the 50 unit line of the lower axis. To the right of this, stands are predominantly N. America, African, tropical and sub-tropical. To the left are European and temperate Japanese stands. Only very early reports from America 29, (1962) and Wyoming (1960) appear to the left of the 50% axis. An apparent anomaly is 58 (Danube) which is drawn close to Jamiaca for no obvious reason. More characteristic of Hungary is stand 6, close to France (42) and Sweden (18).

Subarctic (34) and Devon (24) are drawn together by species 83 and 84 (Mycocentrospora angulata and Taeniospora gracilis), a combination found only in these stands. Stand 2 (Chamier, Surrey) finds its place between N. America (29) and stands 34 and 24 through species 83, and the R. Creedy, Devon (10) through 15 and other species in common. It is also placed close to stands 43 and 25, early reports from Japan by Tubaki (1957 and 1960). As one would expect, it is also close to the list given by Ingold for Alder leaves (55) with 16 spp in common. On the edge of the central cluster of stands, it may be regarded as a fairly typical, Northern latitude, temperate stream.

The species ordination is distributed according to the percentage frequencies observed in Agglom II (species) with the most common species in the central grouping, the rare species on the perimeters and the intermediate species in between. The improvement in the R. A. method is that species can be more obviously related to stands. A significant feature is that species 76 (T. monosporus) and 72 (L. curvula) though common species are rather specially so, being common both in the tropics and in temperate streams, and are placed on the tropical side of the common temperate species.

C. DISCUSSION OF THE FIELD STUDY

As Swift (1976) has pointed out, any attempt to discuss the species composition of a microbial community is fraught with dangers. The methods of determination and detection of microbial species tend to be selective. In this study the bias is in favour of those species of aquatic Hyphomycetes inhabiting leaves that could sporulate in unaerated water at 20°C within a week of submersion. 20°C is about the optimum temperature for sporulation for many aquatic Hyphomycetes, (Koske & Duncan 1974) and most species will sporulate in unaerated water (Ingold 1975). It is possible that species found to be dominant by this method may not, in fact, be dominant under natural conditions, and that those rarely encountered may have a more important status in the stream than the methods allow for. The isolate of Tricladium splendens, for example when grown on agar medium, will only sporulate under aeration, but aeration of partially decomposed alder leaves would lead to their total disintegration within a short time and no result would be possible. However, species like the Articulospora tetracladia isolates which sporulates readily in unaerated distilled water when grown on agar medium, was not detected on leaves in high numbers of conidia. The Lemonniera aquatica isolate behaves likewise and was detected in

higher frequency than A. tetracladia. Direct examination of leaves taken from the stream would show up early colonizers and those swift to sporulate, but quantification of the colonization process would be extremely difficult - indeed, workers using this method have attempted only presence-or-absence data, (Triska 1971, Sanders & Anderson 1979), although Suberkropp (1976) constructed a 'relative importance' index for each species observed. They found that incubation of leaf material in aerated water at stream temperatures agreed very closely with results obtained by direct examination, but produced higher frequencies and density of sporulation.

It could be argued that spore counts give no account of fungal biomass in the leaf; but the number of spores produced by a mycelial colony must, for each species, be related to the size of the colony, and this direct method of determining species will detect only active colonizers. Plating methods do not distinguish between the active decomposer and the inactive resting spore. The chitin assay technique for estimating fungal biomass in tissues has been found to be of limited value, (Sharma, Fisher & Webster 1977) and would not, in any case distinguish between the inactive mycelium to be found in leaves from terrestrial fungal infection, aquatic Hyphomycete mycelium and the mycelium of other aquatic fungi. Estimations of mycelial area based on microscopic measurements are very tedious and too time-consuming for the purposes of this study and are open to the criticisms made above in not distinguishing between active and inactive colonies.

The advantage of spore counts in determining the composition of the aquatic Hyphomycete community on submerged leaves, is that spores can, in most cases, be identified to species and that the numbers of spores provide a quantative means of comparing the reproductive success of active species, which can reasonably be assumed to based on the extent of mycelial colonization by the species. The assumption made

throughout, which may not be justified, is that the results obtained by the methods employed are a true representation of the balance between species on oak and alder leaves in the natural environment.

The other factor in any analysis of the structure of a microbial community that Swift (1976) emphasises, is the scale, in time and space, of the work. Sampling submerged leaf packs at fortnightly intervals was the method used by Suberkropp & Klug (1976) in their study, but that lasted 6-8 months compared to 3 months for the present study. Ideally, it might have been better to have sampled the alder weekly, but practically this would have been difficult for one worker to manage. The results of Suberkropp & Klug (1976), those of Sanders & Anderson (1979) and those of the present study, indicate that a fortnight is a suitable time interval between samples for observable changes in the microbial community to be followed. In order to enable direct comparisons to be made between total spore counts of successive samples, it was necessary to convert results into nos/g oven dry weight of leaf material. The amount of leaf used was, on average, about 0.25g, so the scale-up is about x4. This scale-up is in proportion to the resource - 5g of air-dried leaves - under investigation. Since Reice (1974) found significant differences in the rates of decomposition of leaves according to the pack size, and since Gönczol (1975) presents evidence of resource-specificity in aquatic Hyphomycetes, it is important that the results presented and discussed here, refer to the microbial communities of 5g packs of oak and alder leaves, specifically. Parallel experiments would need to be conducted with different species of leaf and different sized of leaf-pack before general conclusions could be reached about species composition and balance in leaf degradation by aquatic Hyphomycetes.

Three factors appear to influence the species of aquatic Hyphomycetes present in a stream. The first is geographical location; the second is the physical and chemical qualities of the water and the

third, the species of riparian vegetation whose litter falls into the stream. Evidence that these factors operate is provided by this study.

The analysis of published species lists by Reciprocal Averaging gives a clear-cut separation between temperate, and sub-tropical and tropical regions. There is evidence, too, of a separation between temperate regions, with Europe and Japan on the one hand and North America on the other.

The 'nearest neighbour' to the R. Bourne on the stands scatter-diagram is the R. Creedy in Devon. Of a total of 18 spp they have 15 in common. Though the Creedy is a medium-sized river with tributaries, and the Bourne a stream flowing from lakes, the published data of the physico-chemical quality of the water of each shows marked similarities.

	R. CREEDY	R. BOURNE
Current speed (m/s)	0.25-0.33	0.25
Temperature (^{o}C)	2.5-20	2.5-20
pH	6.4-9.0	6.7-8.6
Conductivity ($\mu\Omega$)	247-440	275-330
Alkalinity mg$CaCO_3$/l	27-127	35
Nitrate (mg/l)	0.8-3.9	0.35-0.84

TABLE 10: Physico-chemical data of the water of the Rivers Bourne & Creedy.

Phosphate and oxygen values for the Creedy are not given. The very low phosphate levels in the Bourne in December and January may represent the mopping-up of available phosphate by microbes active in leaf-processing over that period which co-incides with highest concentrations of aquatic Hyphomycete spores in the stream. DOM levels were not given for the Creedy. The levels for the Bourne seem high compared to values given by McDowell & Fisher (1976) for Roaring Brook - average about 3.0mg/l; and by Fisher & Likens (1973) for Bear Brook - c 3.0mg/l. The method used in the present study is probably too crude for any but comparative purposes. Highest values for DOM coincided with the leaching

period for allochthonous litter in early December and fell off in January. Values tended to rise after floods, probably due to underground seepage.

Overall, the two rivers may be described as moderately eutrophic and their similar aquatic Hyphomycete flora contrasts markedly with those of the acidotrophic Dartmoor streams. Suzuki & Nimura (1960a, 1961) found marked differences between the fungal flora or harmonic (eutrophic) and acidotrophic lakes in Japan. Of the most common and abundant species detected on filters, only two are the same in both streams: A. longissima and C. aquatica. T. elegans was found to be a dominant species in eutrophic Japanese lakes by Suzuki & Nimura (1960a) and it is possible that its dominance in the Bourne is due to its capacity to cope well in a lacustrine environment.

The significance of a specific resource in the species composition of the aquatic Hyphomycete flora of a stream is illustrated by the close proximity on the R.A.II scatter diagram of stand 55, from Alder leaves (Ingold 1942), to stand 2 (Chamier, Surrey). The Bourne is lined with many alders from the Virginia Water lakes to the sampling site. The two species' lists have 16 species in common from a total of 18. Between the Creedy and the Alder list, there are only 11 species in common.

Comparison between filter counts taken from the Creedy and from the Bourne shows some similarities. Both illustrate a marked overall decline in spores/litre from a peak in early winter to low levels in late spring, following the pattern of litter fall. In both streams there is a fairly consistent level of species numbers over the autumn/winter period. However, at peak, spore levels in the Creedy in early autumn are an order of magnitude higher than in the Bourne. The number of species detected in the Bourne was an average 13 compared to 18 in the Creedy. These differences can be explained by differences in the catchment area leading to the sampling site. The Creedy has a

large catchment area with tributaries and probably very mixed riparian species along the catchment area. By a process of accumulation, species and spore numbers would build up along the course of the river. The catchment area for the Bourne is about half a mile from the V. W. lakes to the sampling site, with a restricted variety of riparian trees and shrubs. The more restricted the species composition of the deciduous trees, the more pronounced is the peak season of aquatic Hyphomycete activity, and the more resource-specific the species are likely to be.

The rate of processing of the leaf packs can be compared with results obtained by other workers. The results obtained in this study have been calculated to give 'k' vaules for alder and oak.

	Oak k	50% decay	Gp	Alder k	50% decay	Group
Chamier	0.0052	c.90 d	Med.	(I) 0.0129	35 d	Fast
				(II&III) 0.0108	42 d	Fast
Triska (1970)				0.0075		Med.
Matthews & Kowalczewski (1968)	0.0044		Slow			
Kurcheva (1960) soil	0.0057		Med.			
Sedell et al (1975)					40 d	Fast

TABLE 11: Comparison of reported decay rates of oak and alder leaves.

The values for 'k' for oak vary from slow to medium. The results of Matthews & Kowalczewski and Chamier are not strictly comparable. The former worked on the Thames, a great river, and their results are dissimilar generally from those obtained subsequently for streams. In a soil environment open to microbial activity and invertebrate feeding - circumstances similar to those obtaining in the Bourne - the decay rate of oak leaves is very similar.

Triska excluded invertebrates from his experimental leaves and leaf-degradation rates were slower than those reported from the present study. Sedell et al worked at temperatures comparable with those prevailing in the present study and their decay rate for alder leaves is markedly similar (50% in 40d).

The differences between the decay rates of oak and alder leaves can be accounted for by the differences in their composition at abscission. Triska et al (1975) report a very high labile component (ADCW) in the make-up of Alnus rubra, 80.6% with 9.5% lignin. Whereas oak, with 40% lignin, (Hering 1967) must have, correspondingly, a much lower labile component. As lignin is very resistant to microbial breakdown (Suberkropp et al 1976) and ADCW a fraction readily available to digestion by microbial enzymes, oak leaves are more slowly degraded than alder leaves. In addition, freshly-fallen oak leaves have a high tannin content (5%), (Feeny & Bostok 1968). Harrison (1971) found that oak-leaf tannin had a significantly inhibitory effect on the growth of many fungi. The slower rate of fungal and bacterial colonization of oak leaves compared to alder leaves is evident in this study, and lends support to the conclusion that microbes are active in the processing of leaf litter. The conditioning period of leaves by microbes depends not only on leaf quality but on the level of stream inoculum. The length of the conditioning period determines the initiation of shredder feeding, the second important factor in leaf degradation and whilst microbial colonization is reported by many workers (see Literature Review) to enhance the palatability of leaves to animals, it is also reported that the polyphenols in leaf tannins form highly refractory complexes with proteins which may immobilize the digestive resources of grazing animals, (Benoit & Starkey 1968). It may be that the high tannin content of oak leaves not only inhibits microbial colonization, but animal feeding as well.

It is useful in the discussion of the microbial communities

found to be associated with decaying oak and alder leaves, to invoke Swift's (1976) concept of the 'unit-community' and also the concept of 'association' between microbial species. In an analysis of the microbial community found on branches of different size, Swift points out that each branch has a community of micro-organisms of varying diversity depending on its size, comprising several species of fungi, plus bacterial and other microbial components. Such partitioning could, he presumed, be demonstrated for other resource types such as roots, fruits and leaves. This type of community he terms a 'unit-community' which may be regarded as having a functional integrity. The 'unit-community' resembles an 'association' but in the former the degree of constancy of species may be low, whereas in the latter it is high.

"The 'unit-community' should be regarded as a spatial unit within the whole microbial community which is determined solely by the spatial, temporal, and size-class distribution of the resource units. The 'unit-community' provides a convenient basis for the analysis of community structure and function." (Swift 1976). In the present study, a 5g leaf pack may be regarded as a 'unit-community' and evidence is provided both of 'associations' between fungal species, and of species diversity within that community. Comparisons will be made between the present study and that of Sanders & Anderson (1979) who investigated the colonization patterns of aquatic Hyphomycetes on wood-blocks of varying sizes.

If total spore counts are taken as a measure of aquatic Hyphomycete colonization, a sequence is observed, in time, in alder leaves between leaves which fall early in the autumn when spore inoculum in the stream is comparatively low (Alder I), to those falling later when the inoculum is high, (Alder III). Alder II represents an intermediate stage. One would expect the rate of degradation, if aquatic Hyphomycetes were the sole agents in the process, to proceed fastest in Alder III with the highest degree of fungal colonization. But it does not. Alder II

and III proceed at the same rate and Alder I is degraded fastest. Prevailing stream temperatures were warmest when Alder I was processed and coldest for Alder III. Factors other than aquatic Hyphomycetes metabolism, which is probably affected by temperature levels - though the range is but a few degrees in winter - must be involved. These may include the numbers of grazing aquatic invertebrates in the leaf packs and their rates of feeding. When resources are low there is likely to be more intensive feeding by the fauna than when resources are abundant. Bacterial metabolism on leaves may be depressed, too, by low stream temperatures.

From total spore counts for both alder and oak it is possible to abstract a model for colonization patterns of aquatic Hyphomycetes on 5g leaf packs, illustrated particularly by Alder I and oak. The pattern is of a comparatively low level of initial colonization, followed by a rapid rise to a peak and a subsequent, more gradual, decline. The overall process is slower in oak than in alder. The validity of this pattern is strengthened by a parallel pattern in the filter samples of spore concentrations in the stream over the period of experimentation. If the period of autumn leaf-fall is short due to restricted number of riparian species, most leaves would be processed by aquatic Hyphomycetes over a concentrated period, so that the spore concentration in the stream would reflect the spore production pattern of individual resource units. Triska (1970), using direct microscopic examination of discs of 7 species of leaf, observed this two-phase pattern. He found that spore numbers were highest shortly after initial infection followed by a gradual abatement during the spring. Triska found a similar pattern for species numbers which is in general accord with the findings of the present study. The pattern of bacterial and fungal colonization of leaves is similar to that observed by other workers for increases in protein, nitrogen and phosphorus levels in leaves submerged in streams, (see Literature Review). Though there may be complexing of microbial

protein with polyphenols with time, there seems little doubt that the observed increases are initially due to immobilization by microbes colonizing leaf litter.

The productivity of the resource in terms of spore production can be estimated from maximum individual total spore counts and from total cumulative spore counts. For oak and alder these are c. 140,000 and c. 500,000 respectively/g oven-dry wt of leaf. (Leaf packs were made up of 5g air-dry weight). The finite nature of the resource can also be related to the cumulative total of species making up the cumulative spore totals. The species equilibrium is 14, which is within the range of 6, 9, 12, 16, 19 species which Sanders & Anderson (1979) found for wood-blocks ranging from 5x5 mm - 80x80 mm.

From % frequency for individual species it is obvious that there is an inequity of distribution between the species, and the moderate degree of similarity between stands of individual series and between matched stands of alder series, points up the existence of 4 dominant and persistent species in association; together with about 10 species of low % frequency which represent the element of species diversity between unit-communities. This accords with the findings of Sanders & Anderson (1979) who found six species of aquatic Hyphomycetes on wood blocks of all sizes, a further seven intermediate species on all but the smallest blocks and another six occasional species on the largest blocks only. They concluded not only that the species composition of aquatic Hyphomycete communities is closely related to the size of the available resources, but that once a species assemblage is established, other species may be excluded. There is strong evidence in the present study to support this conclusion. For alder, the assemblage or association noted for all series is between _T. elegans_, _A. Longissima_ and _M. angulata_ - the filiform species - _L. aquatica_ and _L. terrestris_. The same association is found for Oak, but _A. acuminata_ replaces

L. terrestris.

It appears that the establishment of a dominant association is a selective and competitive process. Selectivity is exemplified by the fact that the species involved in the dominant assemblage are not necessarily those in high concentration in the stream inoculum. C. aquatica would otherwise have featured as a dominant species on the leaf packs, but it was rarely encountered. L. aquatica turned up rarely on filters and L. terrestris was found only on leaf packs. Sanders & Anderson (1979) made the same observation. They found that the percentage frequency with which a species was found on wood-blocks bore no relationship to its mean conidial concentration in the stream.

Evidence of competitive interactions between species is provided by all the experimental series. The common feature is for the filiform species to establish early dominance within a fortnight of submersion, with T. elegans present but at lower frequency. Within the next fortnight, T. elegans establishes a dominance at the expense of the filiform species which persists throughout. The other two species making up the association appear at intermediate to low frequency. The competitive interactions between species is further illustrated by the fact that where levels of T. elegans drop, other members of the association rise in importance rather than new species.

A similar interpretation could be made of the pattern of colonization of aquatic Hyphomycetes on 5g leaf packs of oak and hickory observed by Suberkropp & Klug (1976). Only six persistent species were found and they were common to both species of leaf. Of these, 3 were dominant species, the other 3 occurring at intermediate to low frequency. F. curvula established early dominance and whilst it was dominant, other species were at low levels. It was two of these species, not newcomers, that took over dominance when F. curvula declined, though there were, as between oak and alder in the present study, one or two species that appeared to do better on one leaf type than on the other. This suggests

that there is an element of resource specificity operating to determine dominant species as well as competitive interactions between species of aquatic Hyphomycetes.

In competition for the resources available in leaf tissue, not only would interactions between fungal species be important, but between fungi and other members of the microbial community. The numbers of pectolytic bacteria associated with decaying oak and alder leaves are not insignificant, and follow a similar pattern to total spore counts from the same samples, suggesting that the same resources are being exploited by both fungi and bacteria. Iversen (1973) found that plate isolations gave a much lower account of bacterial numbers than direct counts, but the latter would include individuals which may be inactive. Suberkropp & Klug (1976) found discrepancies between direct counts and viable plate counts of bacteria associated with hickory and oak leaves. They found, too, consistently fewer bacteria on oak than on hickory. In the present study, bacterial counts for alder are higher than those for oak over the same period of submersion in the stream, following the slower processing-rate for oak leaves, but peak values for pectolytic bacteria for both are very similar: c. 7×10^6 /disc or 3.5×10^8/g wet wt of leaf. This was from a peak count for total bacteria of c. 2×10^7/disc, a value which compares well with 2.6×10^7 as a maximum value for March given by Kaushik & Hynes (1971) for leaf samples from the R. Nith, and lies within the peak range/disc given by Suberkropp & Klug (1976) for hickory and oak, from plate counts. These workers detected an overall increase in bacterial numbers from December to May, whereas the present study detected a decline in bacteria on the final samples.

It was the purpose of the laboratory studies which follow, to investigate some of the physiological factors which may be involved in competitive interactions - the pectolytic capability of individual species of aquatic Hyphomycetes in particular.

Lawson (1978) used a quantative approach to analyze the dis-

tribution of seaweed floras of the tropical and sub-tropical Atlantic Ocean. He used cluster analysis, based on an agglomerative procedure similar to AGGLOM, reciprocal averaging and indicator-species analysis. The results of his cluster analysis produced a dendrogram which made more sense, geographically, than the dendrogram groupings in the present study.

With reciprocal averaging, he found an East-West distribution on axis 1 and a temperature gradient along axis 2. The present separation showed similar trends. Because R.A. works out a simultaneous ordination of stands and species which can be plotted to give a two-dimensional distribution that can be easily interpreted by eye, to show groupings, if they exist, or the gradually-changing continuum of stands and species, Lawson found it the most satisfactory method of the three. Furthermore, R.A. weights the data so that groupings do not rely too heavily on a single species, or a few species, and thus cuts down the margin of error due to misidentifications. As was the case in the present anlysis, Lawson's stands varied greatly in size and species number, though, he too, cut out stands of 10spp or fewer, and some of his data was from old records. Nevertheless, he found that when he used R.A. on an old record from 1960 and supplemented it with more recent data, the new information did not appreciably alter the general distribution given by the first.

However, it would appear from the present R.A. analysis that the most recent species lists, produced by an expert worker on the same stream over a long period and including recently-named species, do separate out from what is, basically, a fairly homogenous group of stands. The conclusion that may be drawn from the R.A. analysis of species lists of aquatic Hyphomycetes, is that most are probably incomplete and represent random samples in which the commoner species, identified and named years ago, obviously predominate in older records. More complete lists can be obtained by regular sampling over a long period of time

where scum, water samples and leaf material are all examined. R.A. points up, too, the paucity of species lists from tropical and sub-tropical areas. It would be interesting to do a further analysis to try to determine which indicator species give rise to the 50 unit-line separation of America on the one hand, and Japan and Europe on the other.

Within the limitations of the methodology and the data available, R.A. analysis gives a useful guide to the context of a study such as this. It is helpful to be able to compare the species-composition of a stream and its physico-chemical quality with 'nearest neighbours', and if all workers provided the physico-chemical data for their sampling sites, the significance of their species' lists would be much enhanced.

PECTINASES IN AQUATIC HYPHOMYCETES

RESULTS AND DISCUSSION

A. Preliminary Experiments

Experiment I

The aim of this experiment was to compare growth and pectinase production of Tricladium splendens on a glucose substrate, with pectic substrates.

25mls of flask medium at pH5 containing 1% (w/v) Glucose, Napp and galacturonic acid(1:1 ratio), or Napp were sterilized in siliconized 100ml conical flasks. 2ml of spore inoculum was added aseptically. The flasks were placed in an orbital shaker maintained at 15°C. After 5 days, three flasks of each carbon source were sampled daily. The dry weight of mycelium and the pH of the supernatant were measured; percentage viscosity of the pectic media was calculated and pectinase activity was assayed on cup-plates at pH5 (PG) and pH9 (PTE).

The results are presented in Figure 14. They show that T. splendens grows as well on a polypectate substrate as on a simple monomer, glucose. Lower growth rates on GA/Napp are attributable to lower initial pH, 4.3. The production of polygalacturonase in a glucose substrate indicates that this enzyme is constitutive. Production of PG on a pectic substrate however is substantially greater, indicating that the presence of a specific substrate stimulates the constitutive enzyme. No PTE production is detectable on the glucose substrate and there is little change in pH values of the supernatant in this medium. In the pectic media the pH of the medium rises, probably due to the accumulation of metabolic products - there is a slight rise in the glucose medium after d6. At pH 6.5 and above, the production of PTE in the pectic media is induced. The activity of PTE on 50/50 Napp/GA is slight - possibly due to catabolic repression by the galacturonic acid. By contrast, there is a strong activity of PTE on the polymeric substrate. The

less viscous Napp/GA medium had lost 100% viscosity by d6, and the Napp medium 98% by d5 and 99.4% by d10. This indicates the depolymerising activity of the pectinases that accompanies growth of the organism.

Experiment II

This experiment was designed to test the capacity for growth and pectinase production by seven species of aquatic Hyphomycetes at pH values corresponding to those of acidotrophic waters (pH5) and harmonic waters (pH7). Pectinase assay plates were used (See D1(c); materials and methods) using the full medium and Napp as a carbon source. Replicates of three plates were used for each organism at both pH values. Discs of mycelium were cut from fungal colonies growing on GYS agar plates using a flamed #5 Cork borer (10mm diameter). One disc was placed aseptically in the centre of each assay plate. The plates were incubated at 15°C for 11 days, then developed with Cetrimide 1% (w/v) solution.

The results are presented in Table 12 below.

SPECIES	pH5 PLATES		pH7 PLATES	
	Colony * diameter mm	Radius of pectinase activity mm	Colony * diameter mm	Radius of Pectinase activity mm
Tricladium splendens	25	22	25	5
Varicosporium elodeae	36	2	38	3
Articulospora tetracladia	16	3	25	3
Lemonniera aquatica	12	20	16	10
Mycocentrospora angulata	13	5	23	5
Tetracladium setigerum	10	8	33	2
Tetrachaetum elegans	10	15	23	10

* includes original 10mm core. All replicates gave identical results.

TABLE 12 for Experiment II: Pectinase production and growth of aquatic hyphomycetes on solid Napp medium at pH 5 and 7.

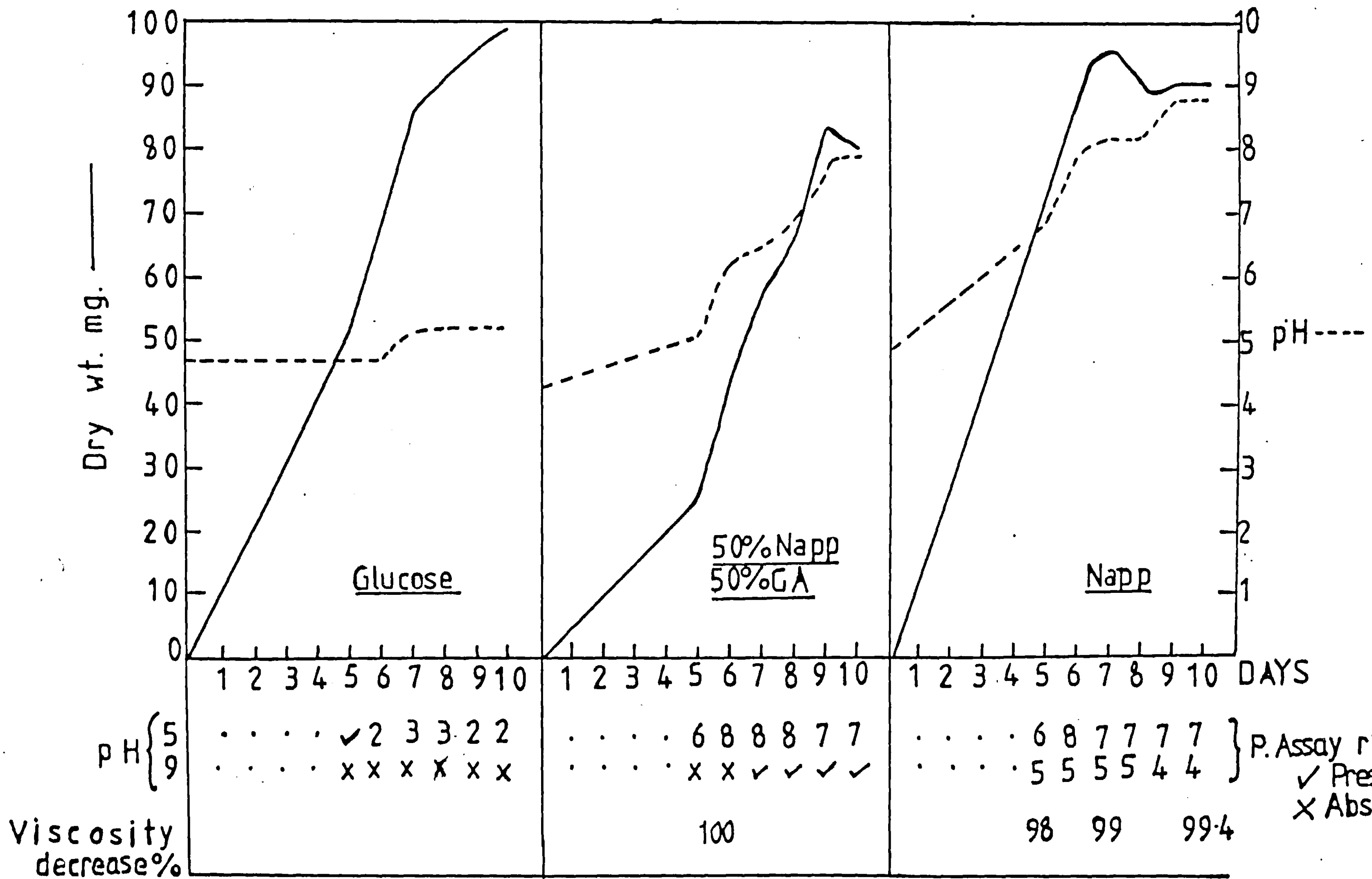


FIGURE 14: From Experiment I. *Tricladium splendens* grown on 3 carbon sources; in shaker culture at 15°C.

To test whether an organism that did not grow at pH5, but produced a polygalcturonase nevertheless, was simply reacting in response to a pectic substrate, or whether the enzyme was produced constitutively, the following experiment was conducted. A 10mmd. core of T. setigerum grown on GYS plate medium was placed on a sterile millipore filter on a GYS plate. The mycelium was thus prevented from penetrating the plate medium as the organism grew. The plate was incubated at 15°C for 6 days. The filter was removed and a disc (10mm d.) cut from agar under the filter was assayed for pectinase on plates pH5 and pH9. The diffusate showed no pectinase reaction at pH9 but a 3mm radius ring developed on the pH5 assay plate. From the results of this experiment and those of Experiment I it is concluded that polygalacturonases active at c. pH5 are constitutively produced by the aquatic hyphomycetes tested. The fact that some organisms do not grow on Napp medium at this pH must be due to physiological factors other than enzymic capability.

Experiment III

To test the validity of Experiment II, and to ascertain which species would be most suitable for larger-scale production of pectinases for purification and characterization, a further experiment was conducted.

To ascertain the most active species for polygalacturonase production on the basis of Experiment II, T. splendens and V. elodeae were grown on medium pH5. All other species were tested on medium pH5 and pH7. 25mls of flask medium at pH5 or 7 with 1% (w/v) Napp were pietted into siliconized 100ml flasks. 2mls of spore inoculum was added. (No Ca^{2+} ions were included in the pH7 medium and as spore concentrations were not identical in all inocula, the results between species are not strictly comparable). Flasks were incubated on an orbital shaker at 15°C. They were sampled, in triplicate, at appropriate intervals indicated in the results. Dry weights of mycelium and pH of the supernatant were measured at each sampling and the percentage viscosity loss of the supernatant was calculated. Pectinase

activity was assayed on cup plates at pH5 and pH9. The results are presented in Figures 15 and 16. For dryweight values see Appendix Table 4. On the basis of these results, graphs were drawn of dry wt/viscosity - loss, as a measure of enzymic activity (Figs. 17, 18, 19). The experiments on A. tetracladia were repeated to check the reproducibility of the results.

CONCLUSIONS DRAWN FROM EXPERIMENTS I, II AND III

From the results of Experiment II, it appeared as if only T. splendens and V. elodeae could function as efficiently on PG activity as on PTE activity; (Gp. I). T. elegans, L. aquatica, M. angulata and T. setigerum appeared to metabolise more effectively off PTE activity at pH7 than on PG activity at pH5; (Gp. II). A. tetracladia was intermediate between these two groups metabolising somewhat more efficiently at pH7 than at pH5; (Gp. III).

However the results of Experiment III do not precisely accord with those of Experiment II. Of the four organisms in Gp II tested on shaker culture, only T. elegans would not grow at an initial pH c. 5, but grew well at pH 6.5-8.4.

Mycocentrospora angulata shows markedly similar behaviour at pH5 and pH7, though growth is slow and there is, unlike other species, no rise in pH as growth rate increases.

Tetracladium setigerum and Lemonniera aquatica grown at pH5 show rapid growth associated with a rapid rise in the pH of the supernatant, so that, as with T. splendens in Experiment I they appear to launch out on PG's, and above pH 6.5 PTE's are induced into action. Tetracladium setigerum however appears to decline or indeed to stop growing at pH values much above 7.5. This is evident from the graphs of T. setigerum grown at pH7.

FIGURE 15: Experiment III. Species grown on 1% Napp c. pH5 --- c. pH7 ——

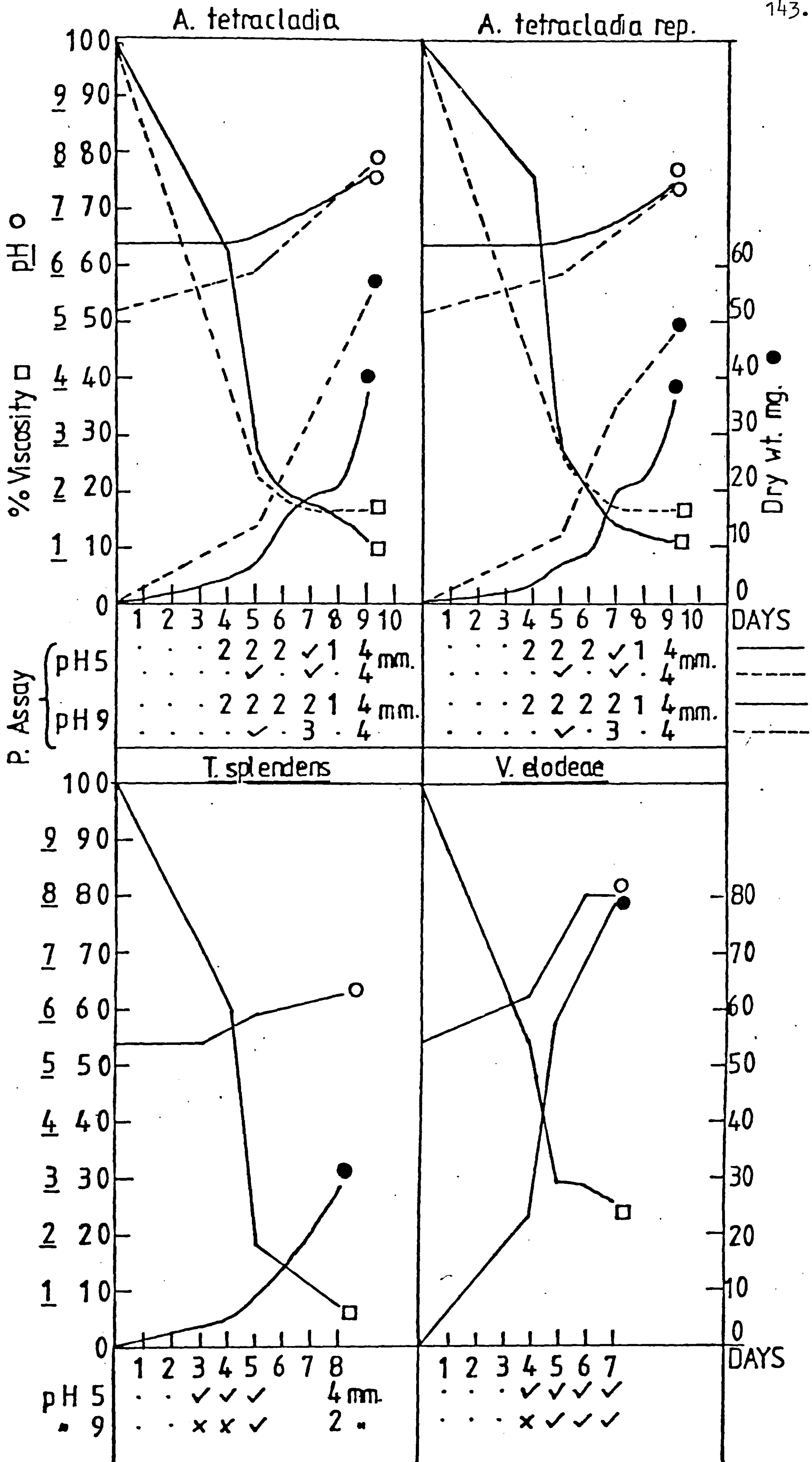
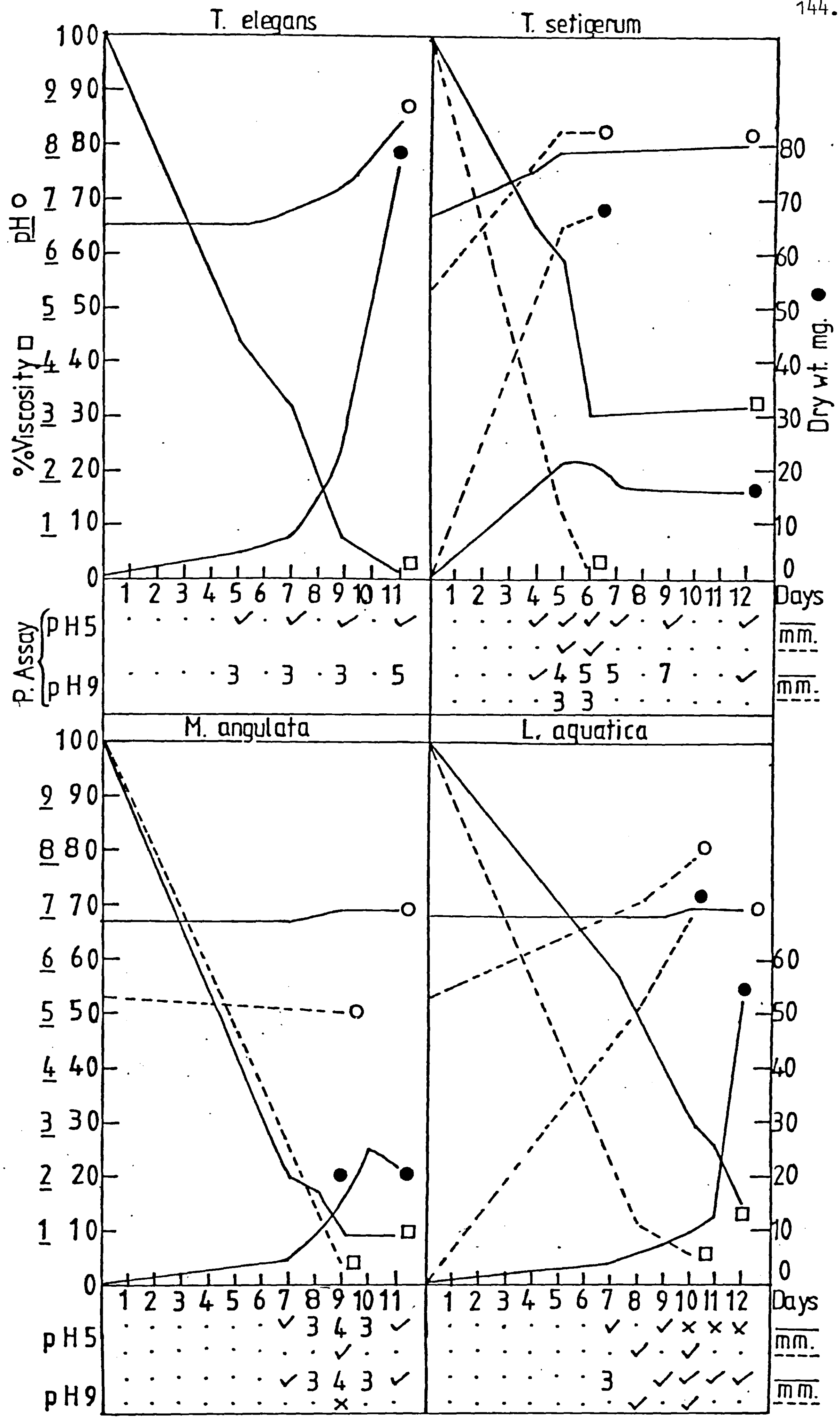

A. tetracladia
A. tetracladia rep.
T. splendens
V. elodeae
pH o
% Viscosity □
Dry wt. mg. ●
DAYS
P. Assay
pH 5
pH 9
4 mm.
2 mm.

FIGURE 16: Experiment III. Species grown on 1% napp c. pH5 ---
c. pH7 —



T. elegans
T. setigerum
M. angulata
L. aquatica
pH o
%Viscosity □
Dry wt. mg. ●
Days
P. Assay
pH5
pH9
mm.

FIGURE 17: Experiment III. *A. tetracladia* initiated on 1% (w/v) Napp *c.* pH7. Plot of dry weight against viscosity loss of medium to evaluate efficiency of pectinase production.

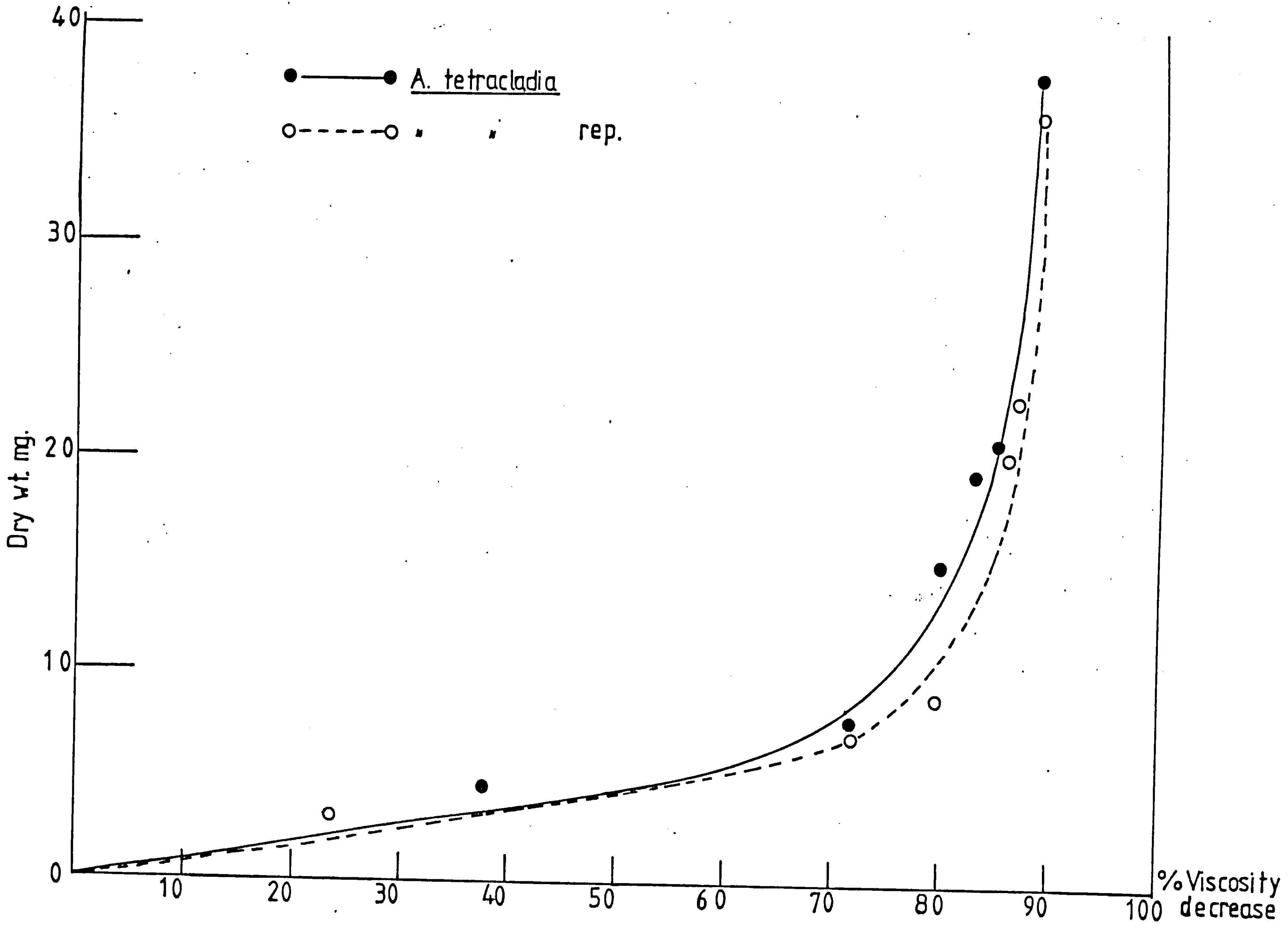
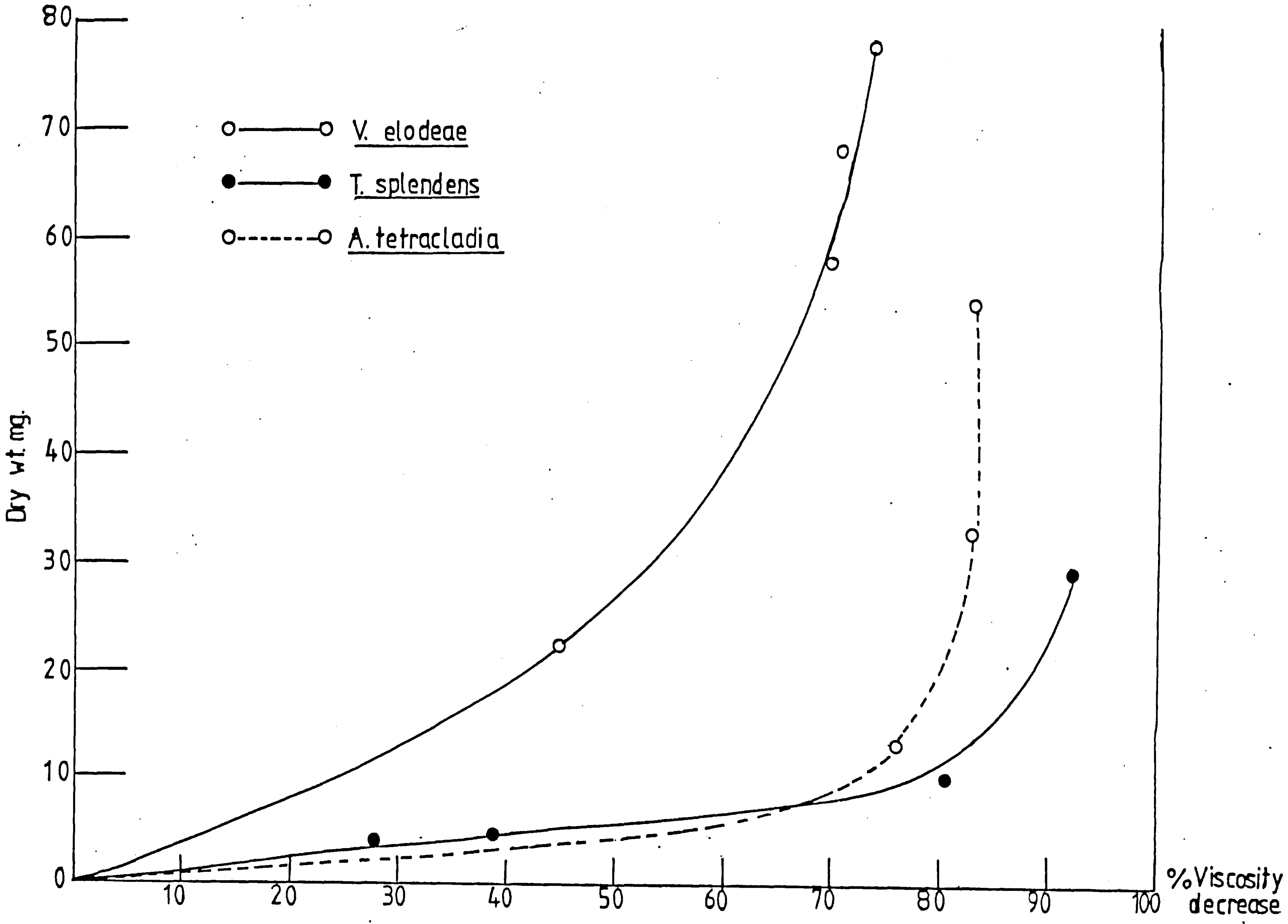
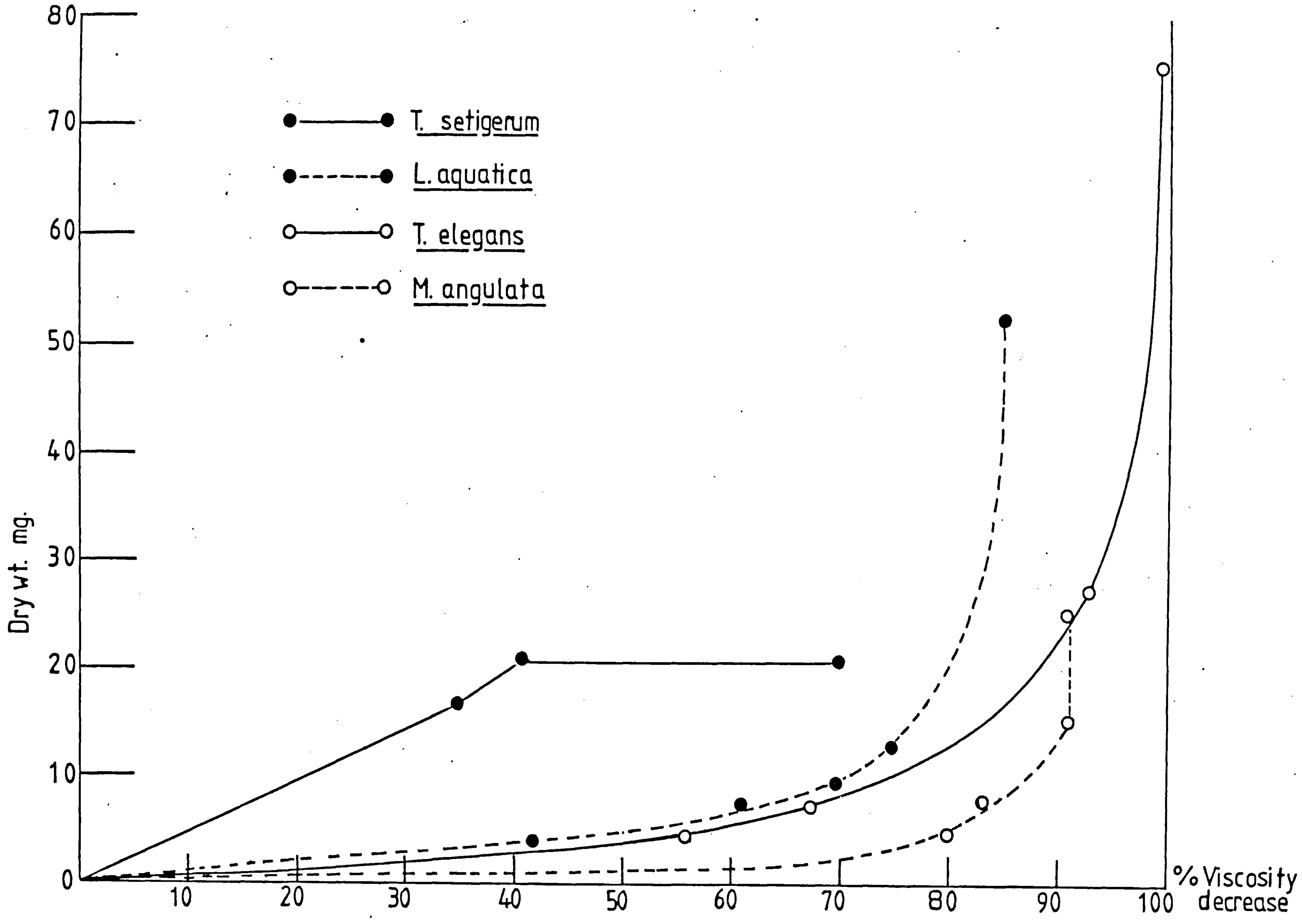

A. tetracladia
" " rep.
Dry wt. mg.
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
% Viscosity decrease

FIGURE 18: Experiment III for species of aquatic Hyphomycetes initiated on 1% (w/v) Napp pH *c.* 5. Plots of dry weight against viscosity loss of medium to evaluate efficiency of pectinase production.



V. elodeae
T. splendens
A. tetracladia
Dry wt. mg.
0
10
20
30
40
50
60
70
80
90
100
% Viscosity decrease

FIGURE 19: Experiment III. Species of aquatic Hyphomycetes initiated on 1% (w/v) Napp *c.* pH 7. Plots of dry weight against viscosity loss of the medium to evaluate efficiency of pectinase production.


T. setigerum
L. aquatica
T. elegans
M. angulata
Dry wt. mg.
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
% Viscosity decrease

The comparatively slow growth rate of the organisms of Gp II at pH7 is probably due to the lack of Ca^{2+} in the medium. Some PTE's have an absolute requirement for Ca^{2+}; in others, activity is greatly stimulated by calcium ions.

Articulospora tetracladia behaved in Experiment II on shake culture in a very similar way to Experiment II on solid medium, but its performance at pH5 was better on shake culture. Increases in growth were accompanied by rise in pH of the supernatant at both initial pH values. It is clear from the data, that growth is quicker and more abundant when the full enzyme complement is expressed. All organisms initiated on medium around pH5, which would stimulate PG production, and where metabolic products resulted in a rise in pH with consequent PTE and PME induction, showed notably higher dry weight values than the same organisms initiated around pH7.

In all organisms tested in Experiment III growth was accompanied by a sharp decline in the viscosity of the supernatant medium indicating the production and activity of extra-cellular, depolymerising pectic enzymes. These enzymes, PG's and PTE's were demonstrated, too, on assay plates. In all cases PG's were produced at the initial pH of both media suggesting that they are constitutive enzymes whereas PTE's were detected only at pH values higher than 6.0, suggesting that they are induced by PG activity. These results concur with those of Experiment I.

The differences in behaviour of Group II fungi on solid media (Experiment II) and in the shaker culture (Experiment III) may be due to a number of factors. The possibilities are exemplified by the behaviour of T. setigerum.

1. The solid medium offers a static pH value to the organism, whereas the supernatant of the liquid medium allows for dynamic change of pH with growth. T. setigerum failed to grow on plates at pH5, but on shake culture the pH of the medium rose within 2 days from 5.3 to 6.5 at

which stage to lyase would have been induced. One cannot know without purification and characterisation of the enzymes involved, the optimum pH for their activity but the probability is that in the case of the PG's of T. setigerum it is nearer pH 6.0 than pH5. Secondly, on solid medium with static pH, T. setigerum grew very well at pH7. However, in liquid medium, when the pH rose to pH 7.9, growth stopped. PTE's normally have an optimum activity around pH8-9 so that this phenomenon is inexplicable without knowledge of the enzymes involved and their Ca^{2+} requirements. In a stream pH values do not vary greatly and it would be difficult to determine, in flowing waters, to what extent the metabolism of an organism can affect the pH of its substrate environment. It seems likely that conditions would be rather more similar to the solid substrate of Experiment II than to the flask medium of Experiment III.

2. The nutrient requirements of some organisms may not have been met or have been inhibiting in one or other of the media used in experiments II and III. Higher phosphate concentrations have been found experimentally in this work in inhibit some aquatic Hyphomycetes and concentrations were considerably higher in solid medium than in flask medium. Yeast extract concentration was higher in flask medium than in the solid substrate but as aquatic Hyphomycetes show a degree of vitamin autotrophism (Thornton 1963) this factor is unlikely to have been limiting to growth. Aquatic Hyphomycetes tested by Thornton (1963) were able to use ammonium nitrogen as efficiently as nitrate nitrogen so that the ammonium sulphate in the plate medium was unlikely to have inhibited growth. In the flask medium, the nitrogen source is organic nitrogen from yeast extract. Thornton (1965) found that the aquatic Hyphomycetes he tested could utilize a number of amino acids as a nitrogen source but it was found in the course of this experimentation that 2g/1 of asparagine inhibited the growth of T. splendens. Ca^{2+}

was included in low concentration in the plate medium (0.5mg/1) which is well below the concentration generally used in PTE culture (0.01M-0.001M/1 or 1.0-0.1g/1) so that the difference in effect of Ca^{2+} between solid and flask media must have been minimal.

Summing up: when the very close similarity of results of the replicated experiments of A. tetracladia in liquid medium, carried out at different times with different concentrations of spore inoculum, are considered, it appears from Experiments II and III, that the individual species tested have differing degrees of physiological adaptation to conditions of acidic and neutral-to-alkaline pH values. These differences may be partly due to differences in enzymic capability or to optimal pH for enzymic activity. However, the relationship in each case of increased growth of the organism with loss of viscosity of the medium, associated with the presence of active extracellular pectinases, suggests that physiological differences other than those of enzymic capability are involved.

Experiment IV

This experiment was designed to ascertain the best mineral salts complement to stimulate PTE production, measured as dry weight of mycelium, of Tetrachaetum elegans. The treatments were made up:

A Flask medium + distilled water (Control)	Ag as A + 0.025% glucose (w/v)
B Flask medium + filtered stream water	Bg as B + 0.025% glucose (w/v)
C Yeast extract+ 50% D. W, 50% mineral salts solution*	Cg as C + 0.025% glucose (w/v)
D Flask medium + 0.5m M. $CaCl_2$	Dg as D + 0.025% glucose (w/v)
E Flask medium + 1m M. Na Cl	Eg as E + 0.025% glucose (w/v)

* The mineral salts solution is given in D1(c)i in Methods.

The glucose treatment was made to test whether a low concentration of a simple soluble sugar, such as may be leached from a leaf, would act as a stimulant to enzyme production. The carbon source in all cases was

1% Napp which was added after the medium had been adjusted to pH7. Three replicates were made of each treatment. 25mls of medium was autoclaved in siliconized 100ml conical flasks. 2mls of sterile spore inoculum was added aspectically to each flask. Flasks were incubated for 10 days at 15°C on an orbital shaker. Dry weight measurements were then made of the mycelium in each flask. Values are given in Appendix Table 5.

Results were analysed by a standard, one-way anova.

	A	B	C	D	E	Ag	Bg	Cg	Dg	Eg
Σx mg	137.1	230.0	94.2	206.3	104.1	153.7	197.9	39.7	156.4	166.2
$\bar{x}$ mg	45.7	76.6	31.4	68.8	34.7	51.2	65.9	13.2	52.1	55.4

$\Sigma\Sigma x = 1485.5$mg

$T_T = 83,471.4$ $T_o = 73,566.9$ $T = 83,756.9$

Source	DF	SS	MS	F
Treatments	10-1= 9	9904.5	1100.5	77.1 ***
Error	20	285.5	14.28	
Totals	30-1=29	10,190		

H_o: $\mu A = \mu B = \mu C$ etc.

H_1: $\mu A \neq \mu B \neq \mu C$ etc.

F ***: $\therefore$ H_o rejected and H_1 accepted.

Tukey's w - procedure (Steel and Torrie, 1960) was then applied to the data to give the honestly significant difference (hsd) at Q = 0.01 level between means of 3 replicates.

Q 0.01 values are obtained from a table (Steel and Torrie, 1960)

$w = Q\ 0.01\ (P, N_2)\ S\bar{x}$

P = number of treatments

N_2 = error degrees of freedom

$S\bar{x} = \sqrt{\frac{EMS}{3}}$

w = 13.3

i.e. there is no significant difference between means with a subtracted value less 13.3

Table of differences

	$\bar{x}$	Cg	C	E	A	Ag	Dg	Eg	Bg	D
B	96.6	63.4	45.2	41.9	30.9	25.4	24.5	21.2	10.7	7.8
D	68.8	55.6	37.4	34.1	23.1	17.6	16.7	13.4	2.9	
Bg	65.9	52.7	34.5	31.2	20.2	14.7	13.8	10.5		
Eg	55.4	42.2	24.0	20.7	9.7	4.2	3.3			
Dg	52.1	38.9	20.7	17.4	6.4	0.9				
Ag	51.2	38.0	19.8	16.5	5.5					
A	45.7	32.5	14.3	11.0						
E	34.7	21.5	3.3							
C	31.4	18.2								
Cg	13.2									

All differences below the stepped line are not significant at 0.01 level.

The differences between 73% of treatments are significant at 0.01 level. The highest dry weight values were obtained with filtered stream water. Those values were significantly different from the Control but not significantly different from the values obtained with calcium ions. Glucose does not affect growth significantly in the Control or the filtered stream water, but does have an effect in the case of the mineral salts solution and the calcium ions solution where it depresses growth, probably

by catabolic repression of enzyme expression. In the presence of sodium ions, however, the glucose enhances growth. The overall tendency of the glucose treatment was to give dry weight values that were not significantly different from the control. The mineral salts solution without glucose inhibited growth compared to the control, but sodium ions without glucose had a less inhibiting effect on growth. The mineral salts solution contained a high concentration of phosphate and in subsequent experiments, T. elegans would not grow on a 0.067M concentration of phosphate, so perhaps the phosphate concentration in the salts solution proved inhibiting.

The F value for the anova (significant at 0.001 level) supports the evidence of the differences table that most treatments give rise to significantly different growth conditions for Tetrachaetum elegans. The organism shows a marked sensitivity to the ionic environment in its capacity to breakdown and utilize a polymeric pectic substrate. The ionic environment must affect either the capability of the fungus to produce pectinases or affect the activity of the enzymes. T. elegans was the dominant species found on oak and alder leaves in the field study and from the evidence of this experiment seems finely adapted to the chemical quality of the stream water. The ions in the stream water can be identified by this experiment to be stimulatory are Ca^{2t}. The calcium concentration in stream water with an alkalinity value of 35 would be about 14mg/1. The experimental concentration was 0.5mM/1, or 20mg/1, which is a close approximation to the stream value. Though both PG and PTE enzymes were identified from supernatant in this experiment, the latter showed greater activity, as would be expected at pH7-8. The PG is probably the constitutive production. Calcium ions have a stimulating effect on pectin lyases with alkaline pH optima and it seems probable that the concentration of calcium ions in stream water is an important factor controlling the rate of activity of extracellular

pectic lyases. This would regulate the rate of degradation of leaf material at neutral to alkaline pH values. On the evidence of this experiment it would seem that soluble sugars leached from leaves are more likely to have an inhibiting than stimulating effect on pectinase production. Catabolic repression of pectinase production by sugars is known to occur in plant patogens.

CHOICE OF EXPERIMENTAL AQUATIC HYPHOMYCETES FOR PECTINASE PRODUCTION

The results of four preliminary experiments reported above were reviewed so that suitable organisms could be chosen for pectinase production on a large scale that would yield sufficient enzyme for purification and characterization. From results of the flask experiment (Exp. III) plots were made of mycelial dry weight against viscosity loss to evaluate the most efficient species in pectinase production, Figures 17, 18, 19. The frequency of occurrence of species in experimental leaves in the field study was also taken into account. *Tricladium splendens*, which was rarely found on leaves in bags in the stream, was chosen for PG production. *Articulospora tetracladia*, which was moderately frequent on experimental leaves was also chosen for PG production. Although both these organisms produce PTE's as well as PG's, they appeared, from the experiments conducted, to produce particularly active PG's. *Tetrachaetum elegans* and *Mycocentrospora angulata* were chosen for PTE production as they were the dominant species found on leaves in the field experiment. *T. splendens*, *A. tetracladia* and *T. elegans* all occur in over 50% of species lists reported. *M. angulata* is probably less rare than lists suggest as it is easily taken for *Anguillospora longissima*.

B. INDUCTION, PURIFICATION AND CHARACTERIZATION OF PECTINASES

1. Polygalacturonases of Tricladium splendens

The methods used for enzyme induction and purification are given in E. 1 and 2, Materials and Methods. 1500mls of supernatant from Tricladium splendens culture at pH5 on 1% ($^{w}/v$) Napp buffered medium, were centrifuged under refridgeration to remove solids from the sample The sample was then ultrafiltered to 150mls containing substances $>$ 10,000 MW. Pectinase assays showed PG activity in the sample but none in the effluent. The sample was dialysed for 24 hours against starting buffer and run into a CM-sepharose column equilibrated with 0.02M acetate buffer, pH5. The column was then eluted with a 0-1.0M NaCl gradient. Flow rate was 15ml/hr. 5ml fractions were collected, until no protein was detected in the eluent by the UV monitor. Fractions containing protein were assayed by cup-plate for PG activity. Alternate fractions from 12-103 were sampled. An elution profile is given in Figure 20. It shows three separated zones of PG activity. Enzyme I (EI) was not adsorbed onto the column and passed through before the salt gradient was applied. Enzyme II (EII) came off in fractions 48-61 and Enzyme III (EIII) in fractions 66-77. Fractions showing peak activity were pooled and concentrated by ultrafiltration where necessary and stored at -12°C.

The fractions containing EI were rerun on the column equilibrated with 0.02M acetate buffer, pH4. Fractions 15-78 were assayed for PG activity. The enzyme was adsorbed and came off the column in fractions 30-36. An elution profile is given in Figure 21.

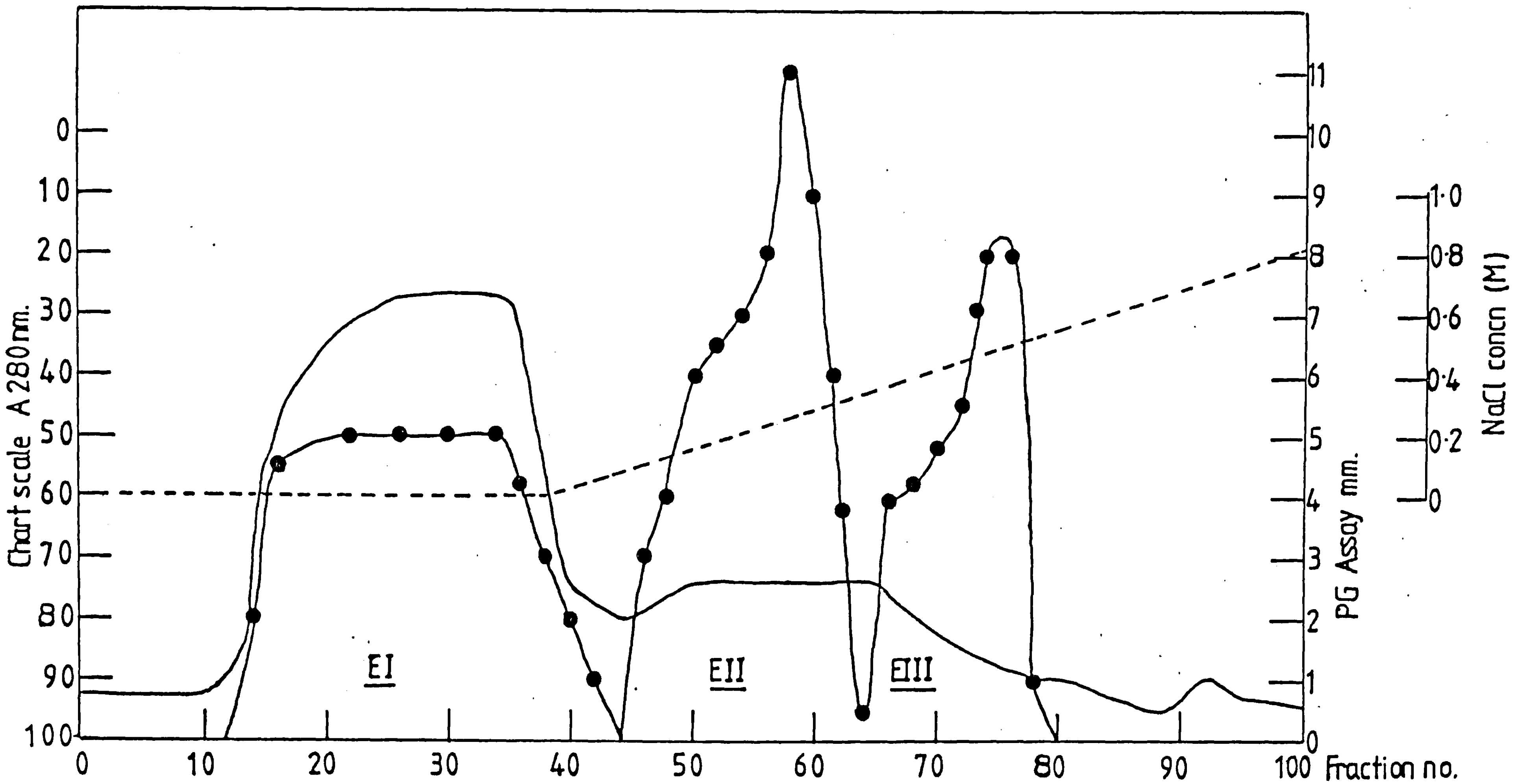


FIGURE 20: Elution profile of PG supernatant of Tricladium splendens grown on buffered 1% Napp (w/v) at pH5. Separation carried out on CM-Sepharose column. Buffer 0.02M acetate at pH5. Flow rate 15ml/hr. Fractions, 5ml, were assayed by cup-plate for PG activity. Protein levels A280nm —— ; PG activity ●—● ; NaCl concentration ---.

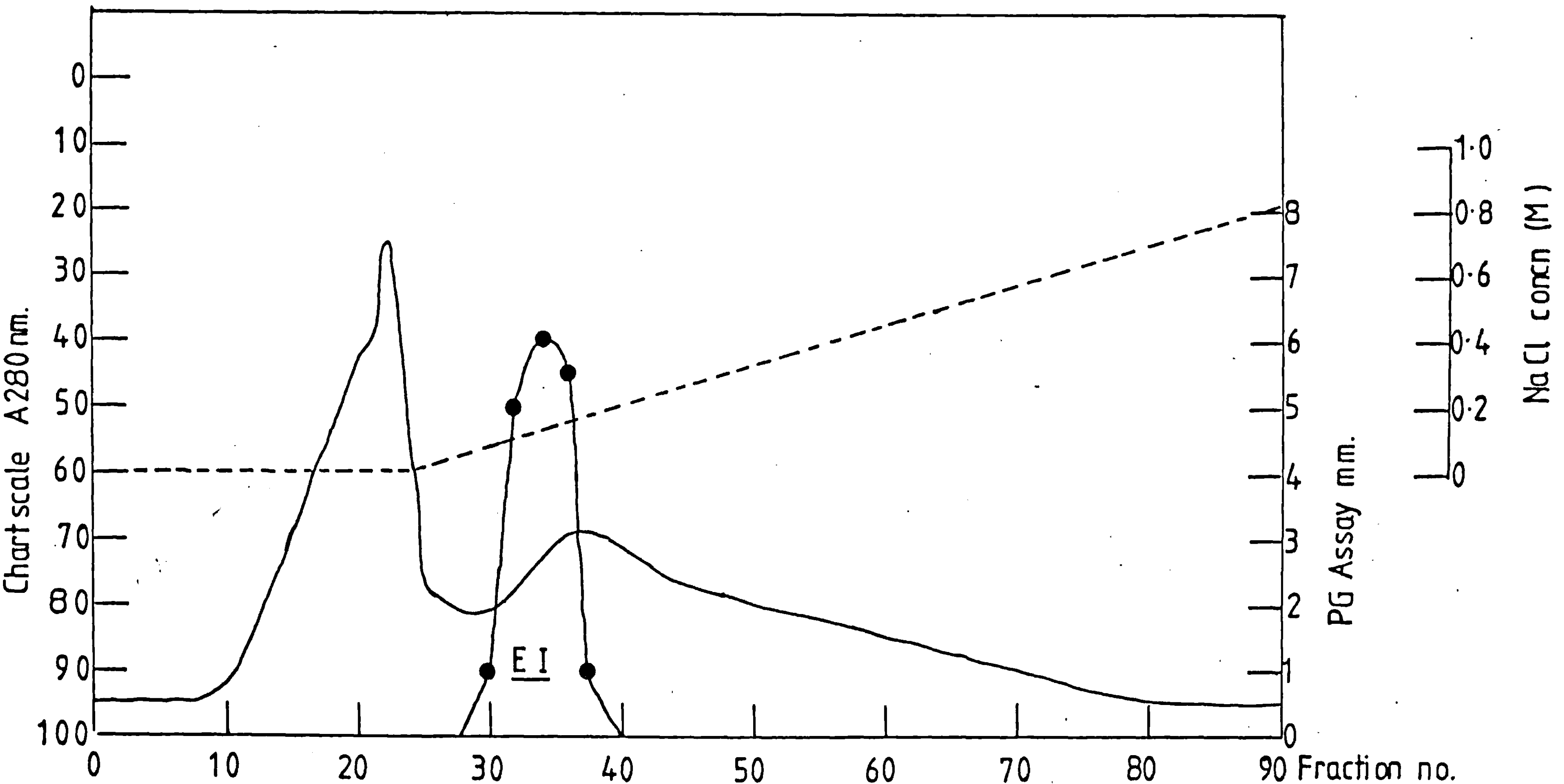


FIGURE 21: Elution profile of fractions 16 - 37, EI from column of Figure 20, Tricladium splendens. CM- Sepharose column buffered at pH4, o.o2M Acetate. Flow rate 15ml/hr. Fractions, 5ml, assayed by cup-plate for PG activity ●—● ; Protein levels A 280nm —; NaCl concentration ---.

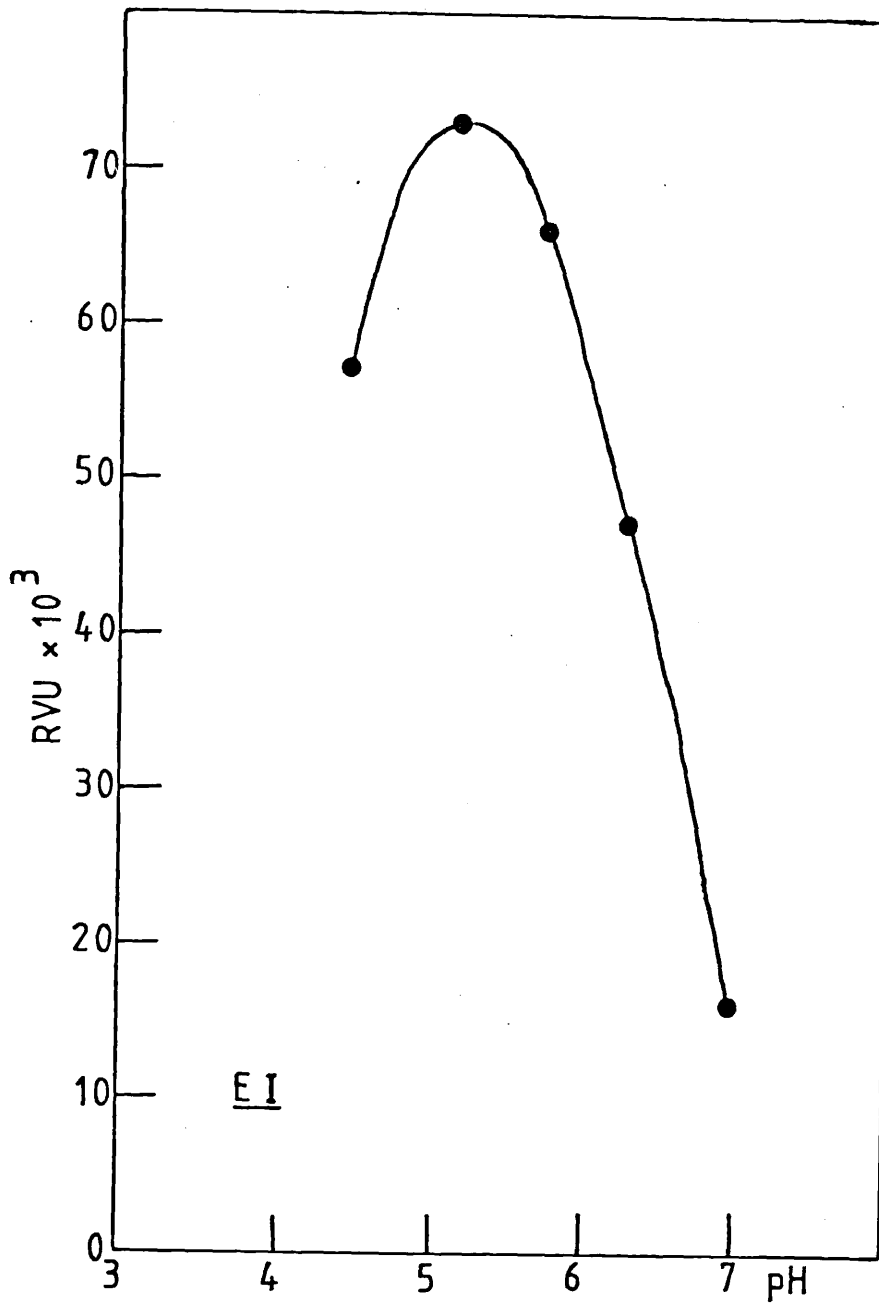


FIGURE 22: pH optimum curve for Enzyme I measured by viscometry. Relative Viscosity Units $1/t_{50}$. Reaction mixtures were 9mls 1% (w/v) soln of Napp in buffer and 0.5ml enzyme.

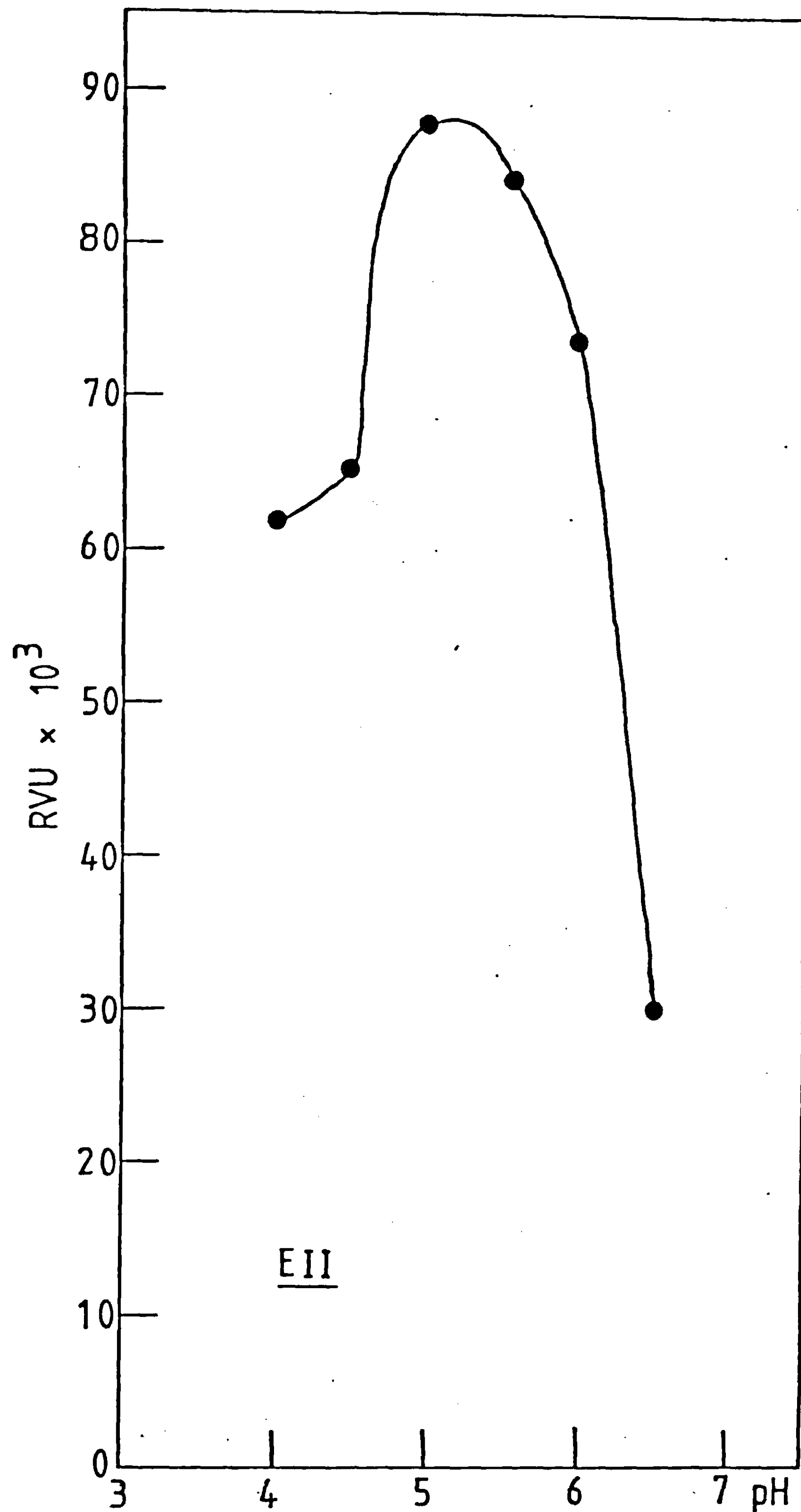
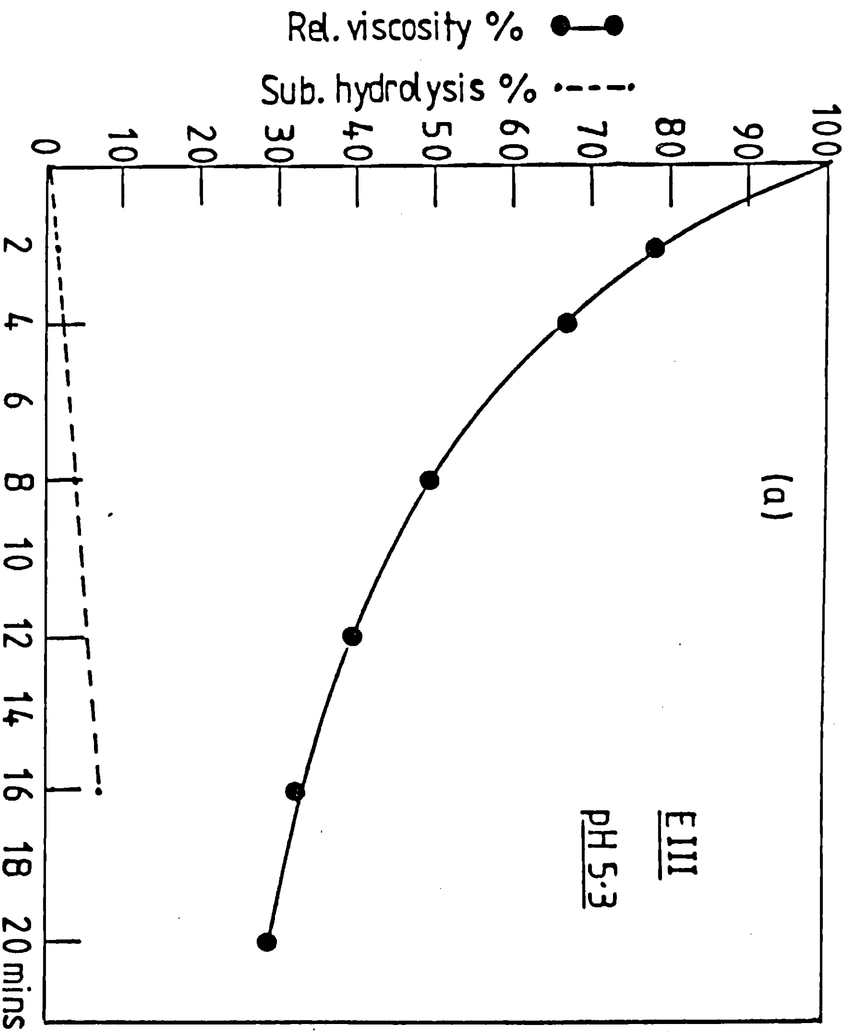


FIGURE 23: pH optimum curve for Enzyme II measured by viscometry. Relative viscometric units = $^1/_{t50}$. Reaction mixtures were 9mls 1% (w/v) soln of Napp in buffer & 0.1ml enzyme.



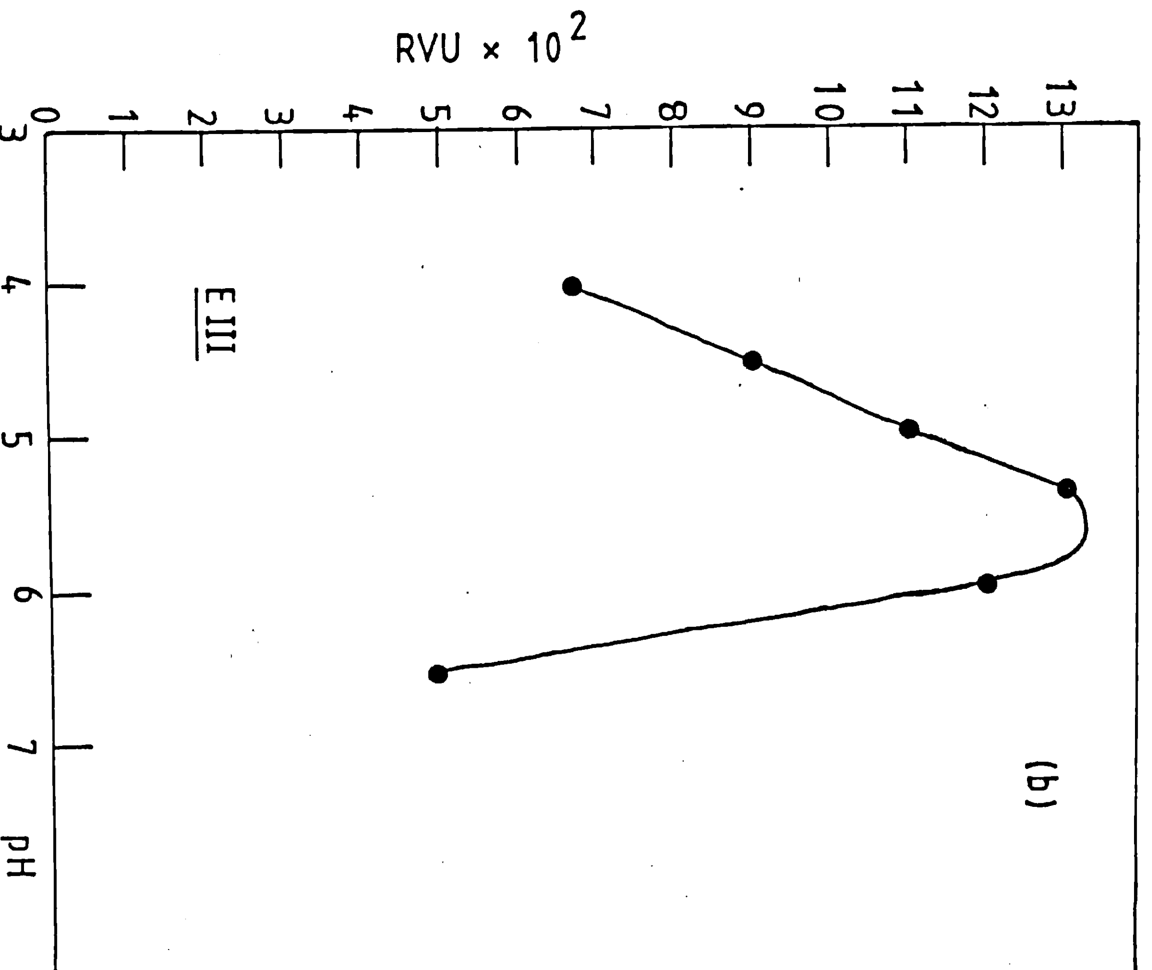


FIGURE 24 (a), (b): (a) Relative viscosity decrease & % hydrolysis of substrate by Enzyme III as a function of time at optimum pH. (b) pH optimum curve for enzyme III measured by viscometry. Relative viscosity units = $1/t_{50}$. Substrate 1% (w/v) soln of Napp in buffer. Reaction mixtures contained 9mls substrate & 0.5ml enzyme.

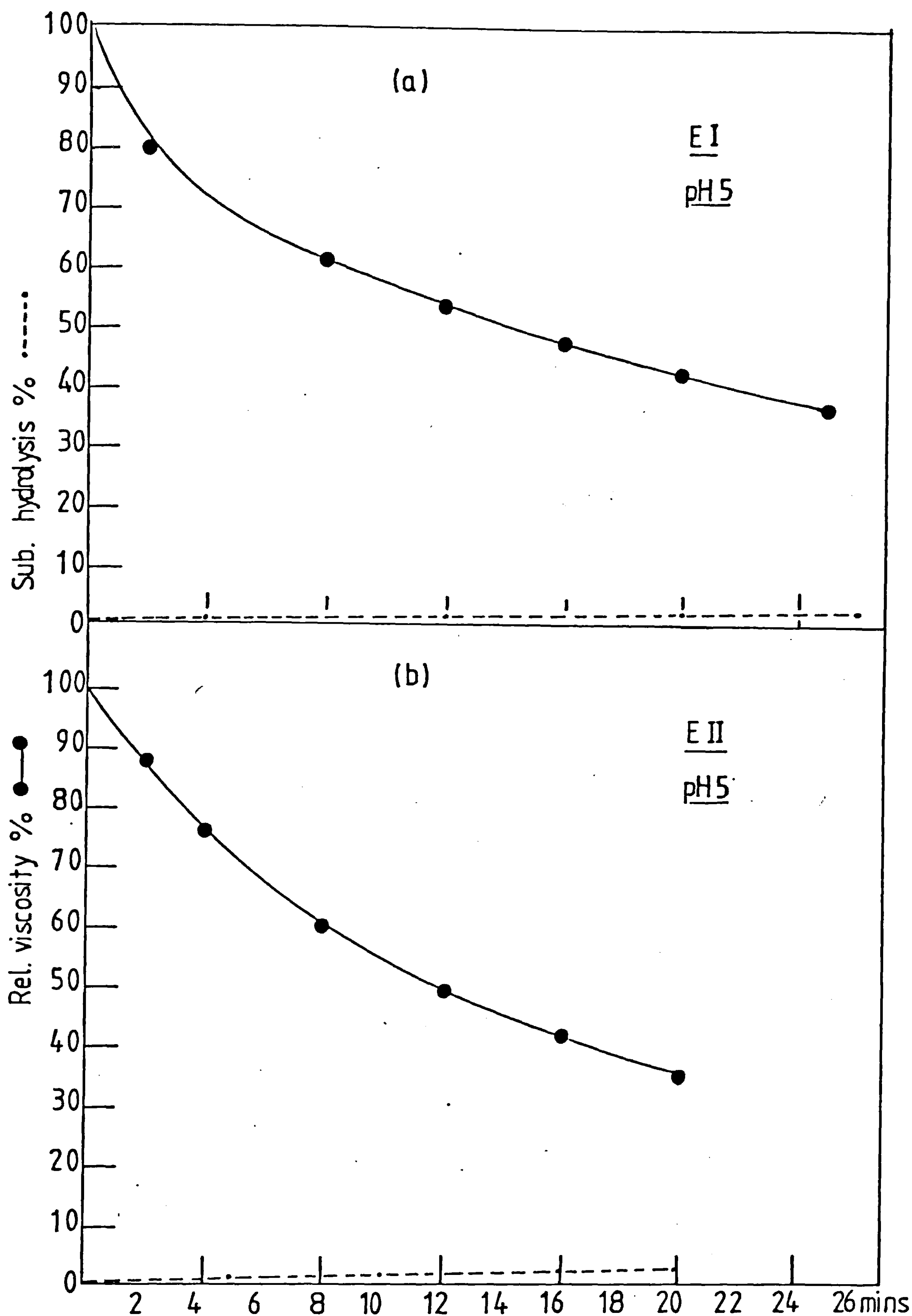


FIGURE 25 (a) & (b): Relative viscosity decrease & hydrolysis of substrate by Enzymes I, II as a function of time at optimum pH. Substrate 1% (w/v) solution of Napp in buffer. Reaction mixture contained 9mls substrate & 0.5ml Enzyme I or 0.1ml Enzyme II.

Approximate pI values for Enzymes I, II and II were obtained by electrofocussing on polyacrylamide gel. Narrow-range ampholines, covering the range of the approximate pI values of each enzyme, were used for purification by preparative gel iso-electric focussing. Accurate pI values were obtained by this method: EI, 8.4 $\pm$ 0.8; EII 5.2 $\pm$ 0.15; EIII 7.3 $\pm$ 0.3. Eluted fractions showing PG activity were pooled and concentrated by ultrafiltration where necessary and stored at -12°C.

The procedures used for characterizing PG's are given in Materials and Methods E3. The pH optimum of each enzyme was obtained by viscometry. Graphs are given in Figures 22, 23, 24 for pH optima of enzymes I, II and III. Relative viscometric units (RVU) are the reciprocal of the time taken at each pH value for a 50% reduction in the viscosity of the substrate (t_{50}) at 30°C.

Substrate	Enzyme (ml)	t_{50} at pH optimum	% Hydrolysis at t_{50}	RVU
EI 9mls 1% (w/v) solution of washed Napp, pH 5.25	0.5 ml	13.9 mins	1.8%	0.07
EII 9mls 1% (w/v) solution of washed Napp, pH 5.0	0.1 ml	11.4 mins	2.0%	0.088
EIII 9mls 1% (w/v) solution of washed Napp, pH5.6	0.5 ml	7.8 mins	4.0%	0.13

TABLE 13: Data on viscometric assay of Enzymes I, II & III

Graphs are given in Figures 24 and 25 for % viscosity loss and % hydrolysis of substrate at pH optimum for Enzymes I, II and III. It is noteworthy that Enzymes I, II and III showed little activity at pH7.

EI	36% viscosity loss at 45 mins.	pH7
EII	14% viscosity loss at 45 mins.	pH7
EIII	35% viscosity loss at 25 mins.	pH7

Values for % hydolysis of the substrate at t_{50}, for K_m and for V max for enzymes I, II and III were calculated from reducing group

analysis using the dinitrosalicylic acid method given in E3(i)c. Substrates and enzymes were those given in Table 13 above for viscometry at pH optimum, incubated at 30°C. Samples were withdrawn at fixed time intervals for reducing group analysis. Reducing group values for substrate only, were subtracted from enzyme/substrate values to give reducing groups released by enzyme activity within a given time. K_m and V max values for Enzymes I, II and III were calculated by the Lineweaver-Burk equation. The graphs obtained are given in Figure 26. Final values for V max are based on mg of enzyme protein in the reaction mixture.

	Mg enzyme protein	K_m (mg/ml)	V max (µg gal. acid equiv. /min /mg protein)
EI	0.6	4.3	23.8
EII	0.18	2.5	277.7
EIII	0.13	1.34	296.2

The data reported thus far on the purification and characterization of Enzymes I, II and III is summarised in Table 14.

Reaction products of Enzymes I, II and III were analysed by decending paper chrometography (Materials and Methods E3(i)d.) to ascertain the mode of activity of the enzymes. The reaction mixtures were those given in Table 13 above. Results are given in Figures 27, 28 & 29.

The three PG's of Tricladium splendens are shown to have a very similar action pattern; that of random hydrolysis of the pectate polymer. The detectability in all three of oligouronic acids from the monomer to the pentamer after 2(EII) hrs, 4(EIII) hrs and 7 hrs (EI) of reaction is evidence of a basically random action pattern. This evidence is in accord with the data on the low level of the percentage hydrolysis of the substrate at t_{50} and the swift reduction in viscosity that accompanies enzyme activity. These enzymes can be classified as endo-polygalacturonases

(poly-(1→4) - α - D - galactosiduronate glycanhydrolase: EC. No. 3.2.1.15)

However, though the action-pattern is basically random cleavage of glycosidic bonds, it is not entirely so. When the chromatograms were developed it was found that the monomer was detectable in all cases after 30 minutes reaction, and its concentration increased with time in a way that could not be due to a simple random process of cleavage. To test whether this was due to monomer in the substrate and to check on the results given by paper chromatography, a parallel TLC analysis was done on Enzyme I, which detected galacturonic acid only and not oligomers. The plate was photographed and appears as Plate 1. The results of the paper chromatogram are shown by TLC to be acceptable. In both, there is no evidence of monomer in the substrate, nor after 6 mins incubation; but after 30 mins. galacturonic acid is detectable in the reaction mixture. This means that a terminal molecule must be cut off more regularly than random clearage of bonds would allow and that the designation 'endo'-polygalactronase does not strictly hold. Cooper (1974) made a similar observation in his work on fungal PG's. The strongest terminal activity is exhibited by Enzyme III (0.13mg protein) where oligomers are slow to develop and are more quickly degraded than those of Enzyme II (0.18mg protein). This observation is borne out by the higher percentage hydrolysis of EIII at T_{50} (4%) compared to Enzyme II (2%). The chromatograms of Enzymes I and II illustrate the difference in V max and K_m between the two. The products of EI (0.6mg protein) appear much more slowly than those of EII (0.18mg protein).

Initial Volume mls.	Total Protein mg.	Vol. after ultra-filtration	Ion-Exchanger	Buffer and pH	NaCl gradient	5ml Fractions Assayed	PG	Fractions
1500	250	150mls	CM-sepharose	0.02M acetate pH5	0-1M	12-103	I*	16-37
							II	48-61
							III	66-77
*EI 120	40.3	-	CM sepharose	0.02M acetate pH4	0-1M	15-78	I	32-36

PG	Final vol.mls	mg/ml protein	Total Protein mg	Enzyme sample ml	pH optimum	% Hydrolysis	pI	Km mg/ml	V max (μg GA/Min /mg protein)
EI	6	1.2	7.2	0.5	5.25	1.8%	8.4 ± 0.8	4.3	23.8
EII	6	1.8	10.8	0.1	5.0	2.0%	5.2 ± 0.15	2.5	277.7
EIII	20	0.26	5.2	0.5	5.6	4.0%	7.3 ± 0.2	1.34	296.2

TABLE 14: Data on purification and properties of three polygalacturonases produced by *Tricladium splendens* grown on 1% ($^{w}/v$) Napp at pH5.

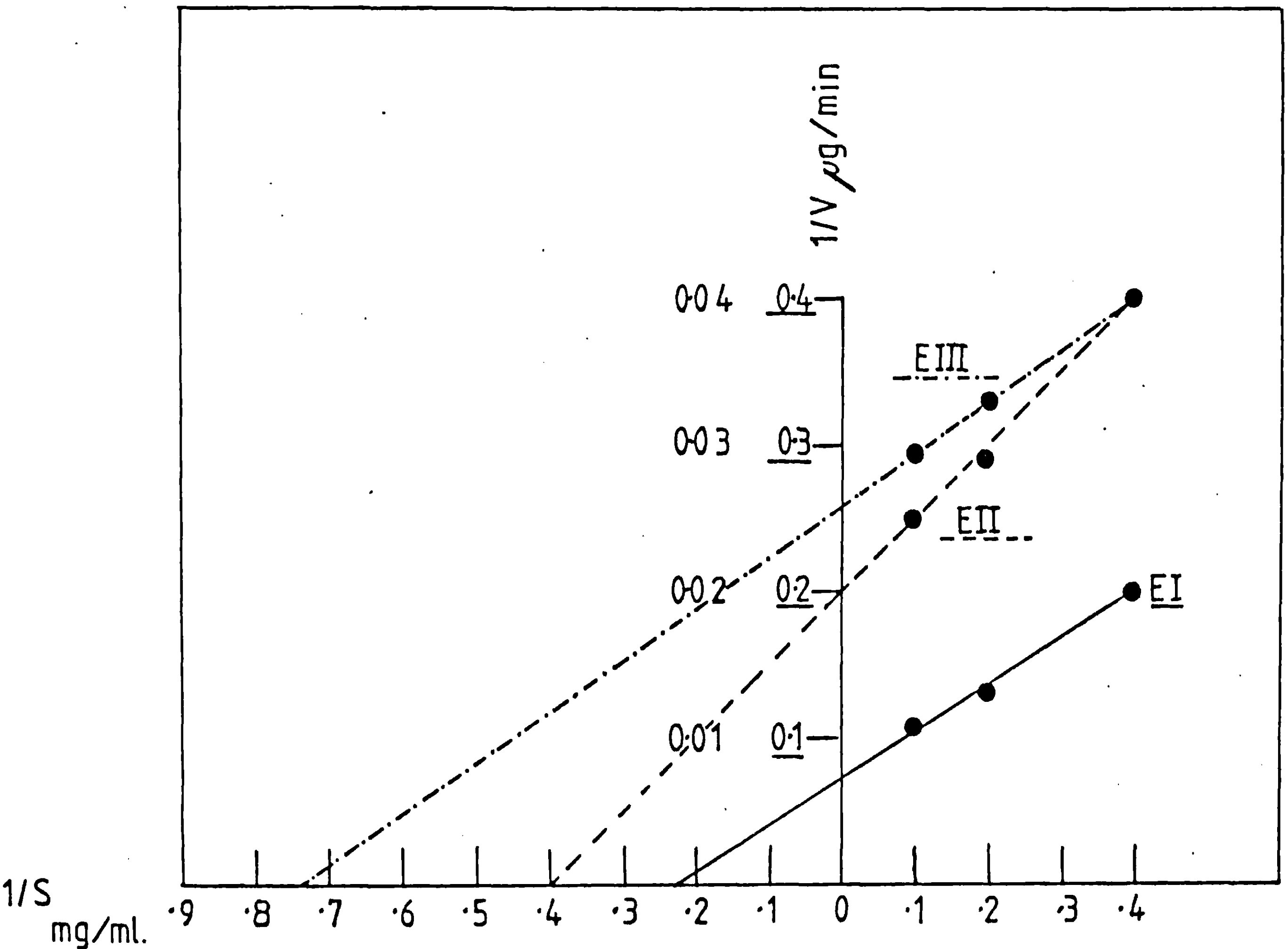
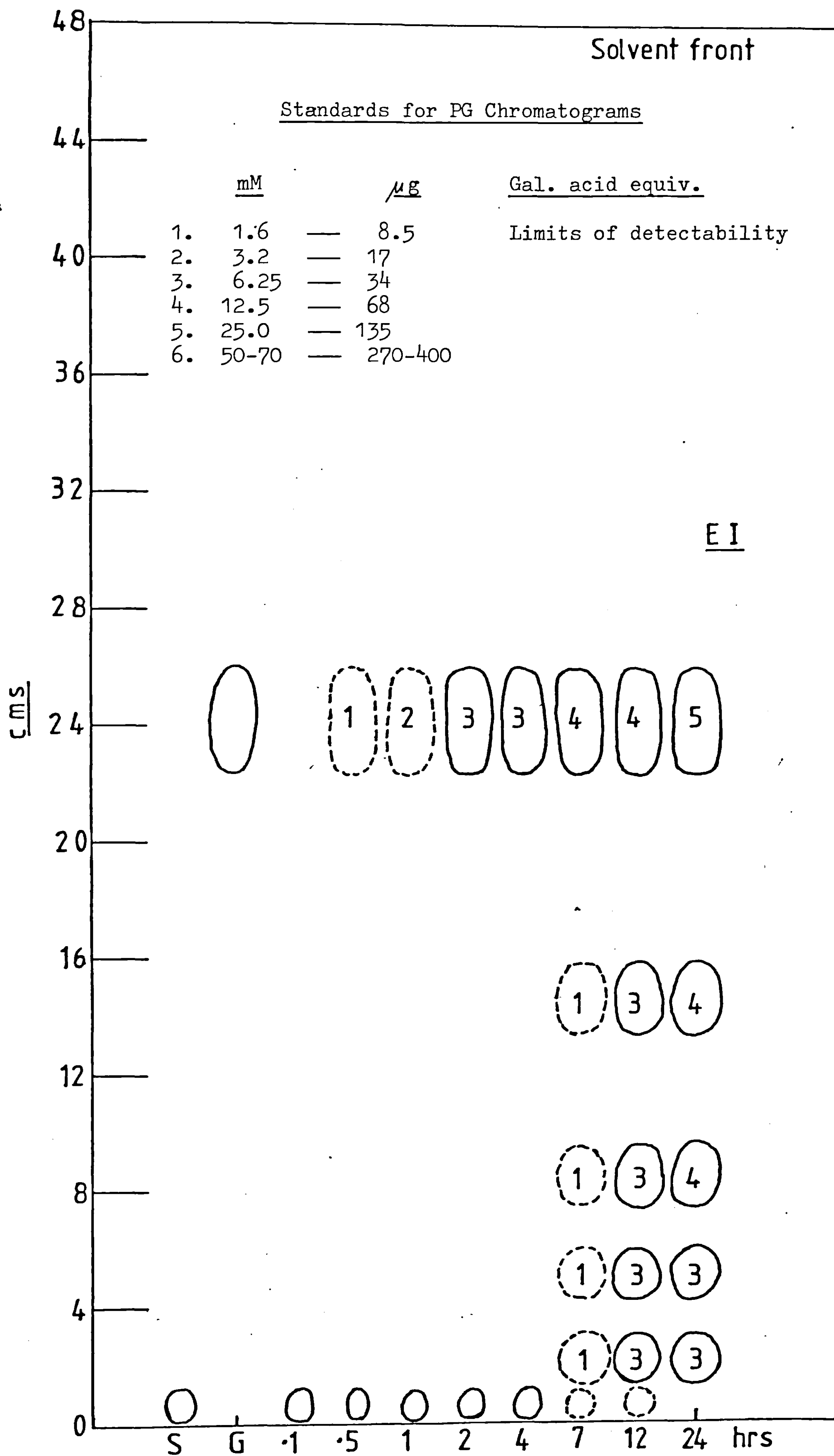
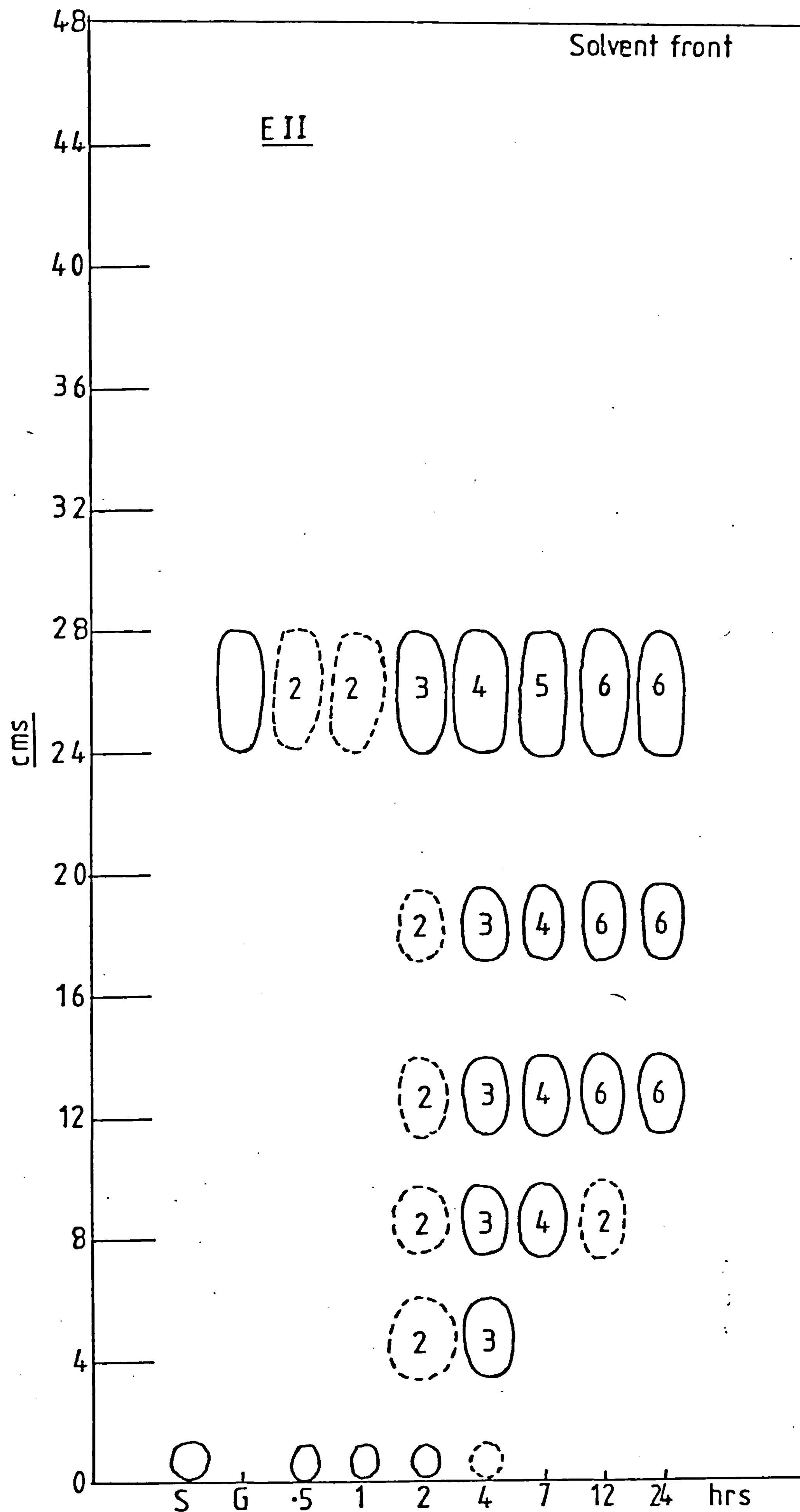


FIGURE 26: Lineweaver-Burk calculation of Michaelis constant (Km) & V Max for enzymes I, II, & III of *Tricladium splendens*. Reaction substrates 1%. o.5% & 0.25% (w/v) Napp solns in buffer at optimum pH. 9mls substrate in reaction mixture with 0.5mls EI or EIII, & 0.1M EII. 1/V values for EI are underlined.


Solvent front
Standards for PG Chromatograms
mM
µg
Gal. acid equiv.
1. 1.6 — 8.5
2. 3.2 — 17
3. 6.25 — 34
4. 12.5 — 68
5. 25.0 — 135
6. 50-70 — 270-400
Limits of detectability
E I
cms
48
44
40
36
32
28
24
20
16
12
8
4
0
S
G
·1
·5
1
2
4
7
12
24
hrs


Solvent front
EII
cms
48
44
40
36
32
28
24
20
16
12
8
4
0
S
G
·5
1
2
4
7
12
24
hrs

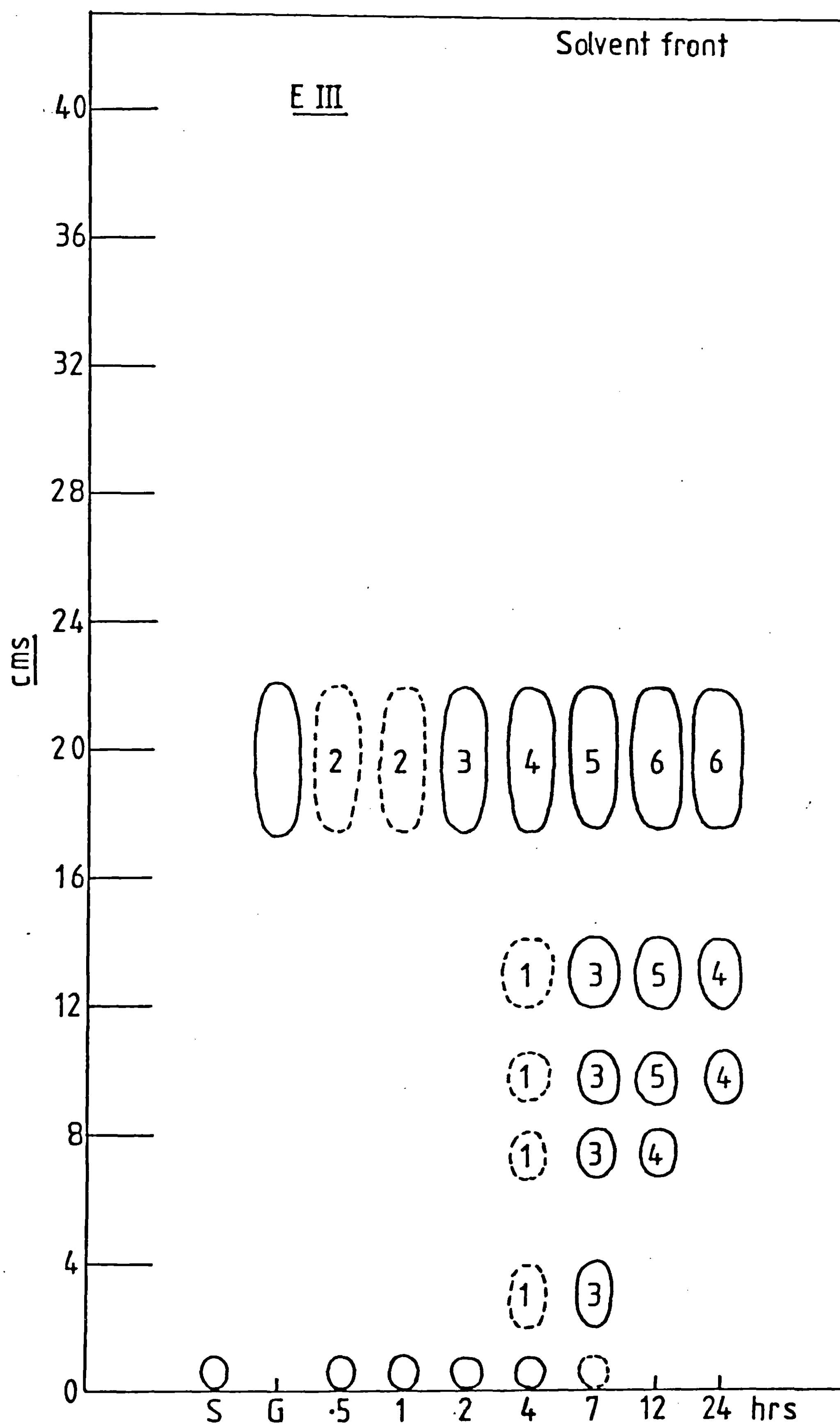


FIGURE 29: Chromatogram of reaction products of EIII with time. S = Substrate G = Galacturonic acid standard. Samples 25μl. Reaction mixture, 9mls (1% (w/v) soln of Napp in buffer, pH5.5 & 0.5ml enzyme incubated at 30°C.

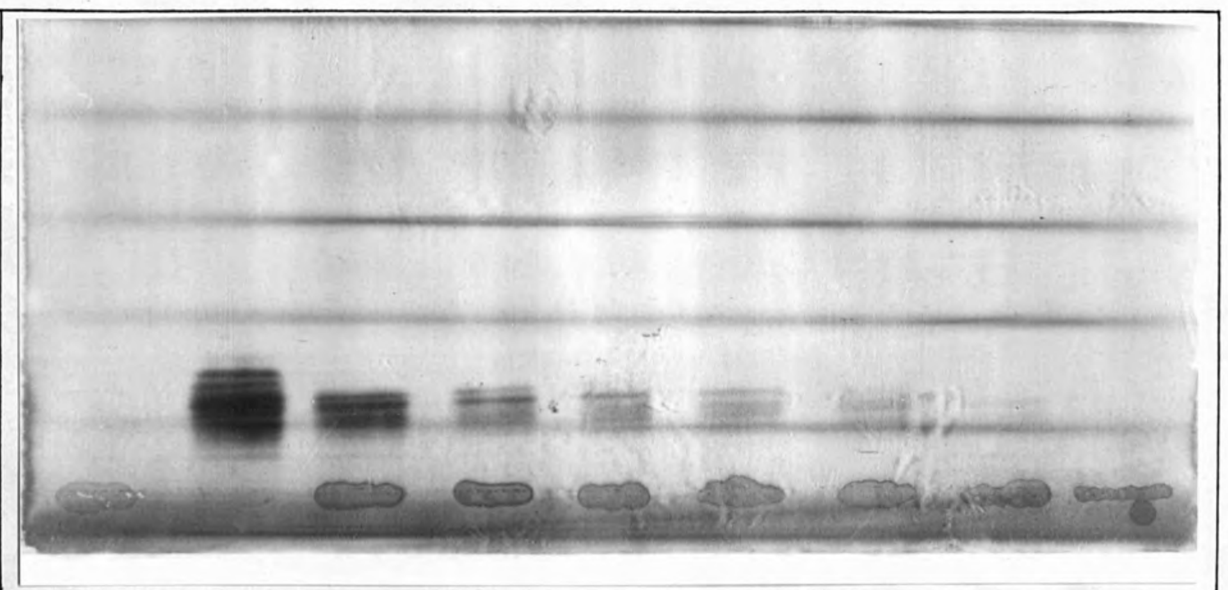


PLATE 1. TLC of release of galacturonic acid (GA) from 9mls 1% (w/v) substrate soln. of washed Napp, pH 5.25, by the activity of 0.5ml purified Enzyme I of *Tricladium splendens*. The method detects monosaccharides only. Sa - Sample. S - Substrate. (Menzies *et al*, 1978.)

2. Polygalacturonases of Articulospora tetracladia

The procedures used for the induction, purification and characterization of PG's of Tricladium splendens were employed in the supernatant fluid of Articulospora tetracladia grown on 1% (w/v) solution of Napp buffered to pH5.

Elution profiles of cation-exchange column separation at pH5 and pH4 are given in Figures 30 & 31. From these it can be seen that Enzyme C (EC) was adsorbed onto the column at pH5 and separated from Enzymes A and B, which were not adsorbed. Enzyme B (EB) was separated from Enzyme A (EA) at pH4 though EA was not adsorbed. All three enzymes were further purified by preparative gel- iso-electric focussing. pH optimum curves are given in Figures 32 & 33 and viscosity loss at optimum pH with percentage hydrolysis of substrate for Enzymes A, B and C are given in Figures 33 & 34.

	t_{50}	RVU	
EA	3.8	0.26	at pH optimum 5.5
EB	6.4	0.156	
EC	5.0	0.20	

Lineweaver-Burk plots for K_m and V max of Enzymes A, B and C are given in Figure 35. The results of purification and characterization of PG A, B and C are presented in Table 15 below.

The reaction products of Enzymes A, B and C were analysed by descending paper chromatography. The results are presented in Figures 36, 37 & 38. From these it can be seen that EA and EB have a somewhat different action pattern from EC. EC is endo-polygalacturonase with an action pattern very similar to that of Tricladium splendens but with an even stronger exo-element as can be seen from the lower concentrations and quicker breakdown of oligomers compared to the increase in concentration of the monomer. The predominantly random attack of the polymer is supported by the low degree of hydrolysis at t_{50} (2.5%0 and the

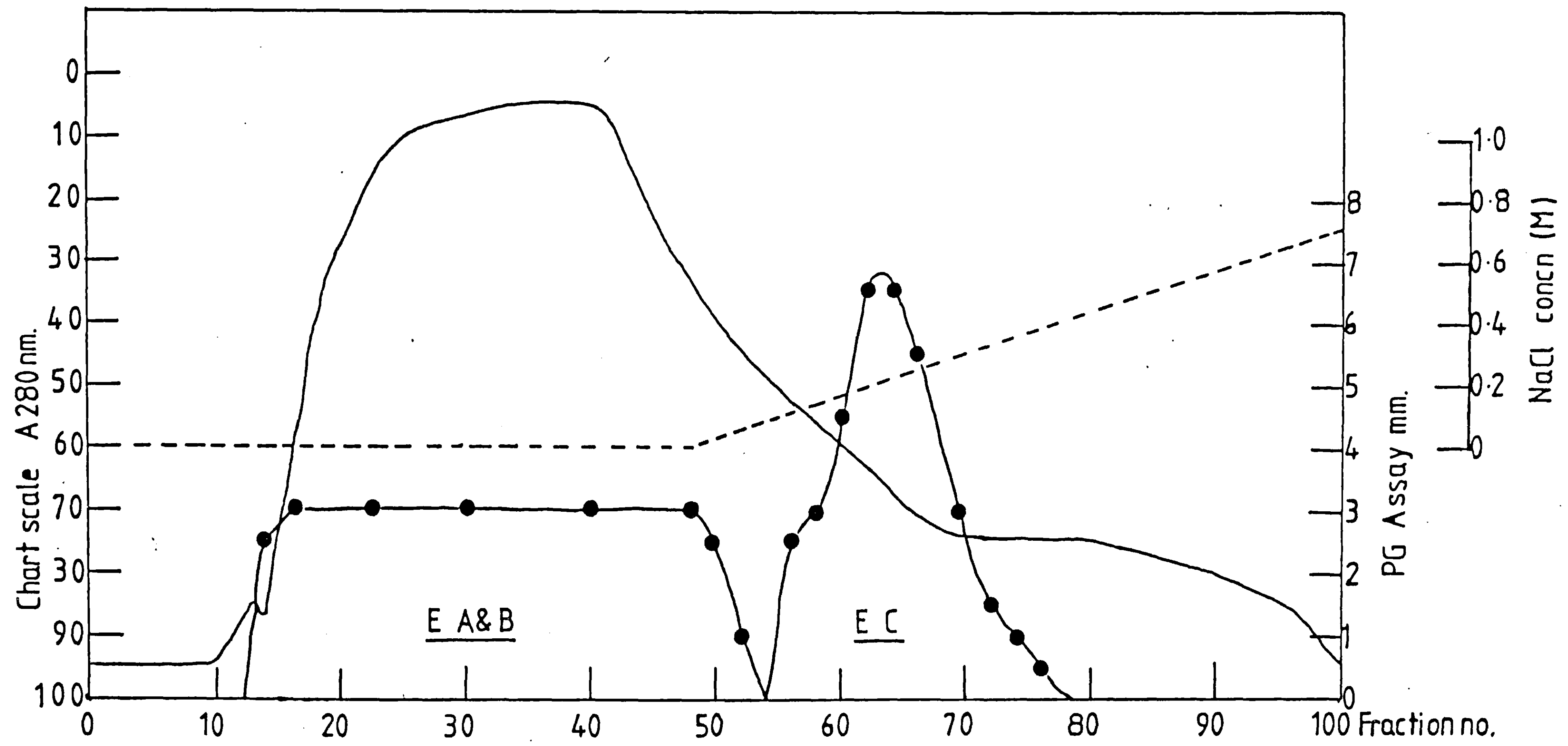


FIGURE 30: Elution profile of first PG separation from supernatant of Articulospora tetracladia grown on buffered 1% Napp (w/v) at pH5. Separation carried out on CM - Sepharose column. Buffer 0.02 Acetate at pH5. Flow rate 15ml/hr. Fractions, 5ml, were assayed by cup-plate for PG activity ●—● NaCl concentration --- Protein levels A280nm —.

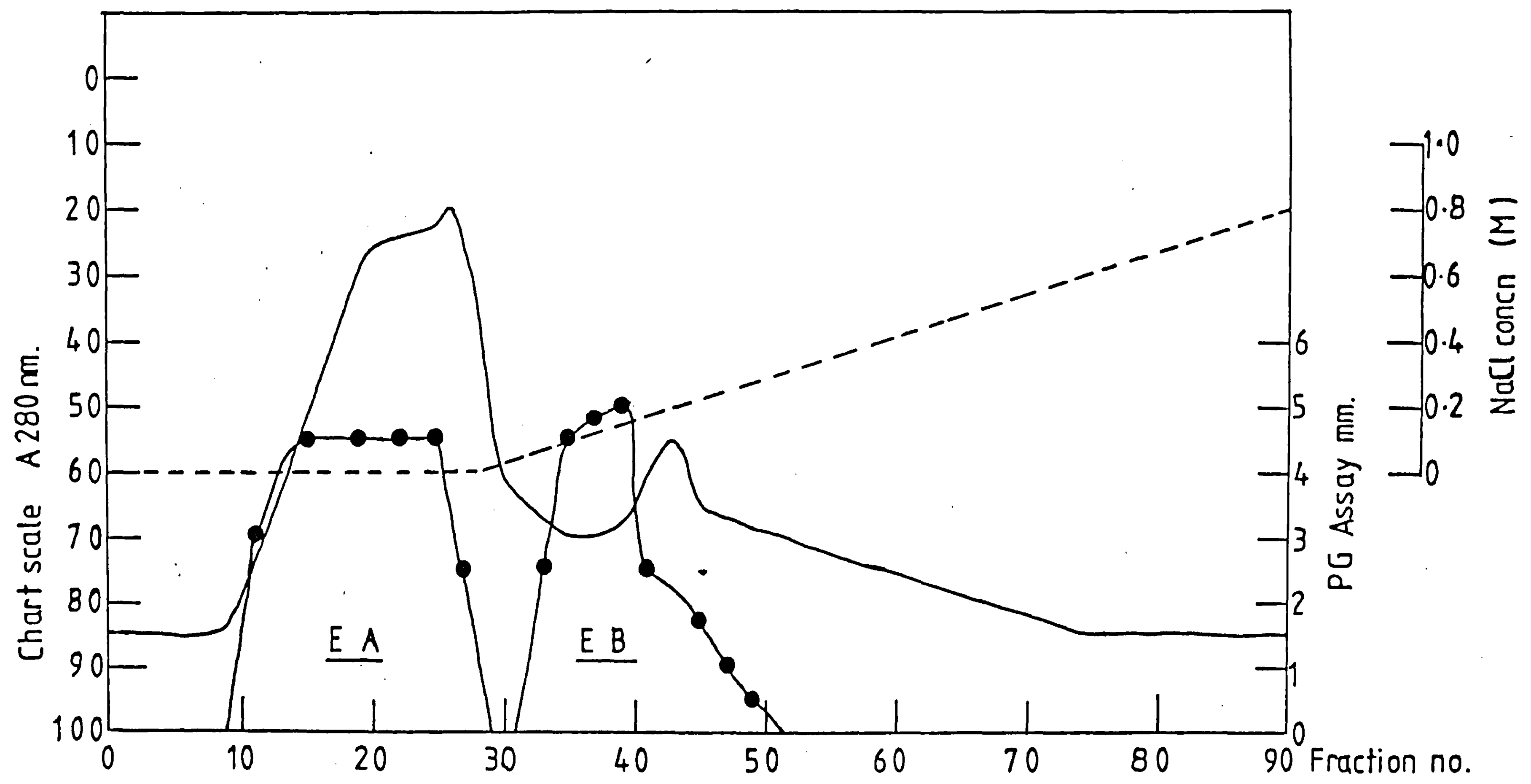
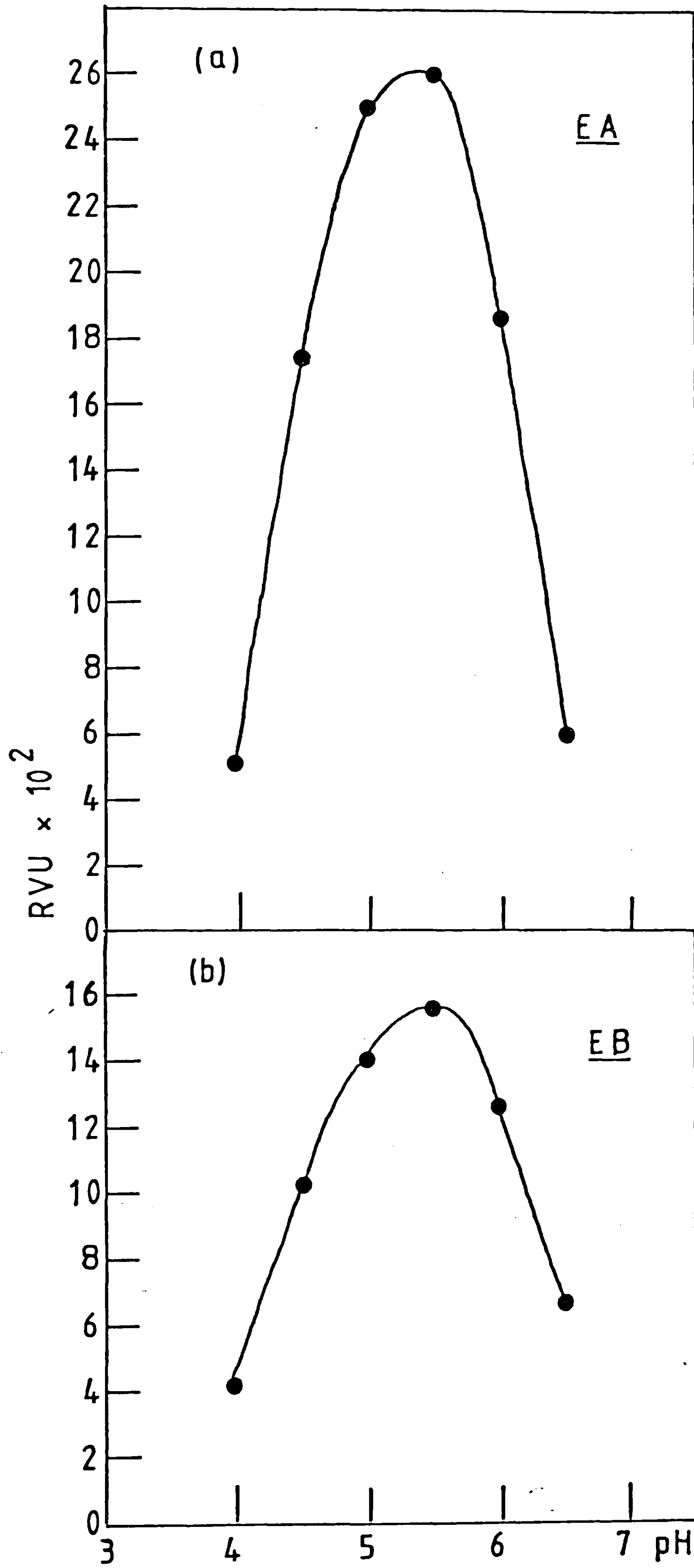


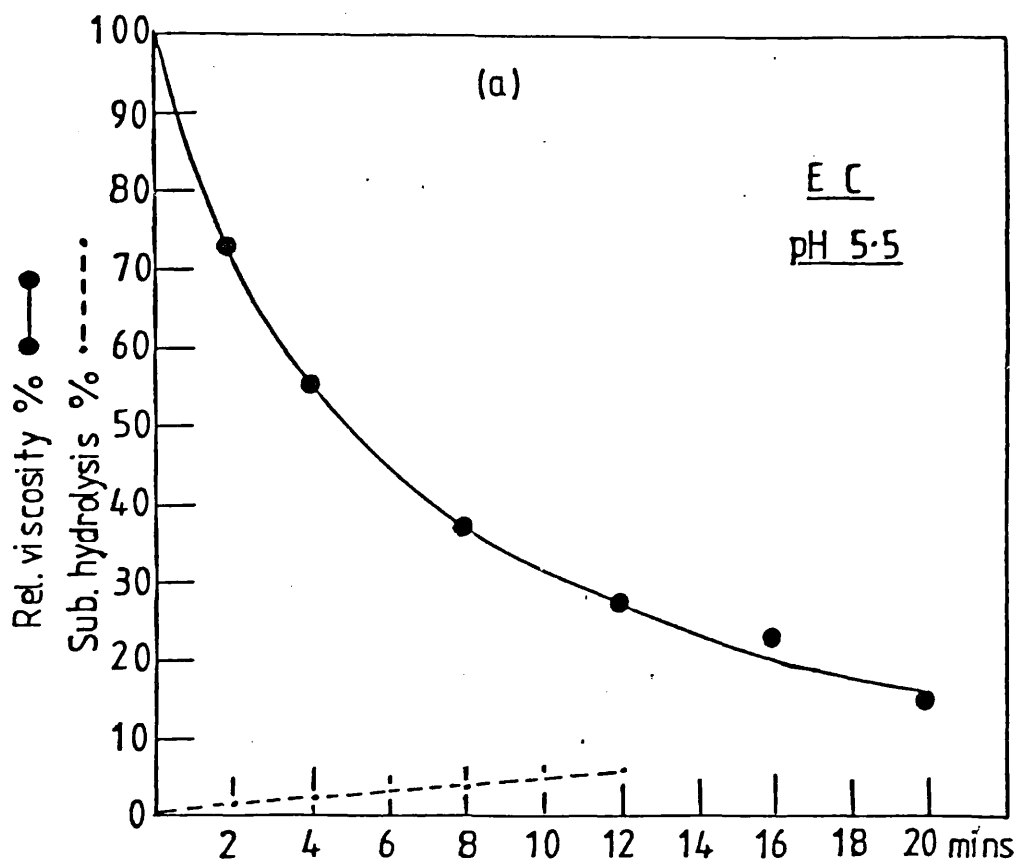
FIGURE 31: Elution profile of fractions 14-50, EA, EB from column of Figure 30; *Articulospora tetracladia* CM-sepharose column buffered at pH4, 0.02M Acetate. Flow rate 15ml/hr, fractions, 5ml, assayed by cup-plate for PG activity ●—●. Protein levels A280nm ——. NaCl concentration ---.

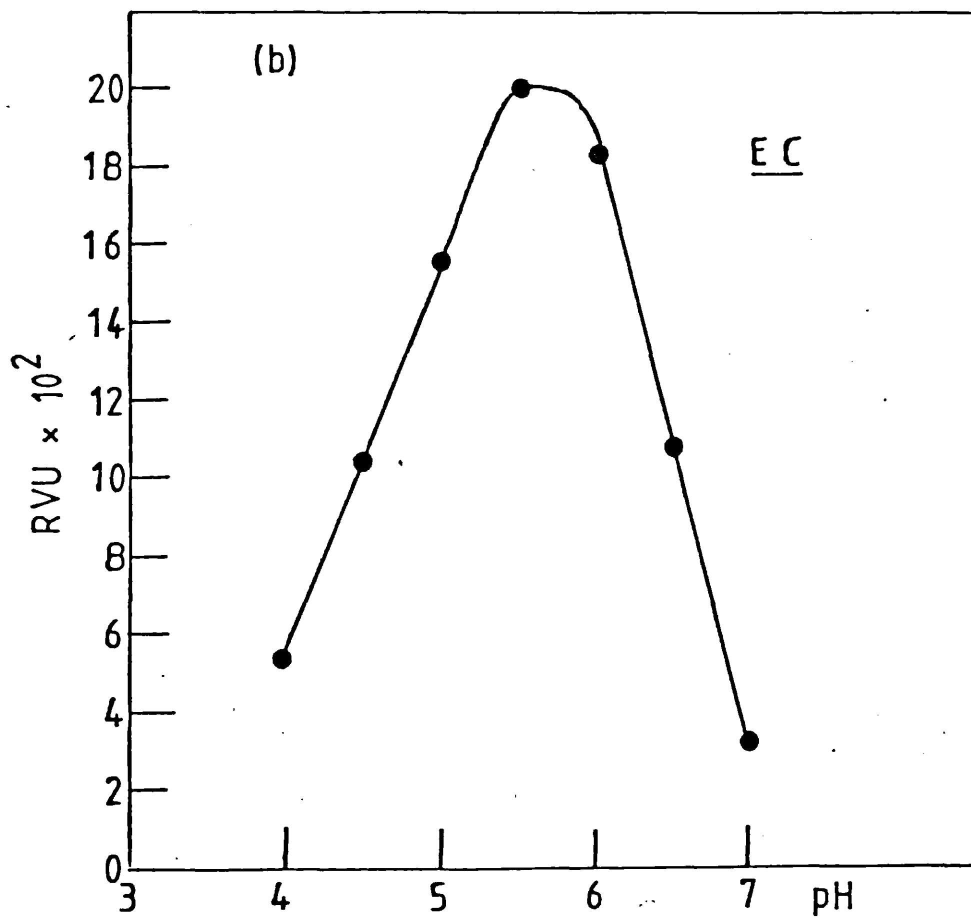
FIGURE 32 (a) & (b): pH optimum curves for Enzymes A & B, measured by viscometry. Relative viscometric units = $^{1}/t_{50}$ Reaction mixtures were 9mls 1% soln of Napp in buffers and 0.5ml enzyme.

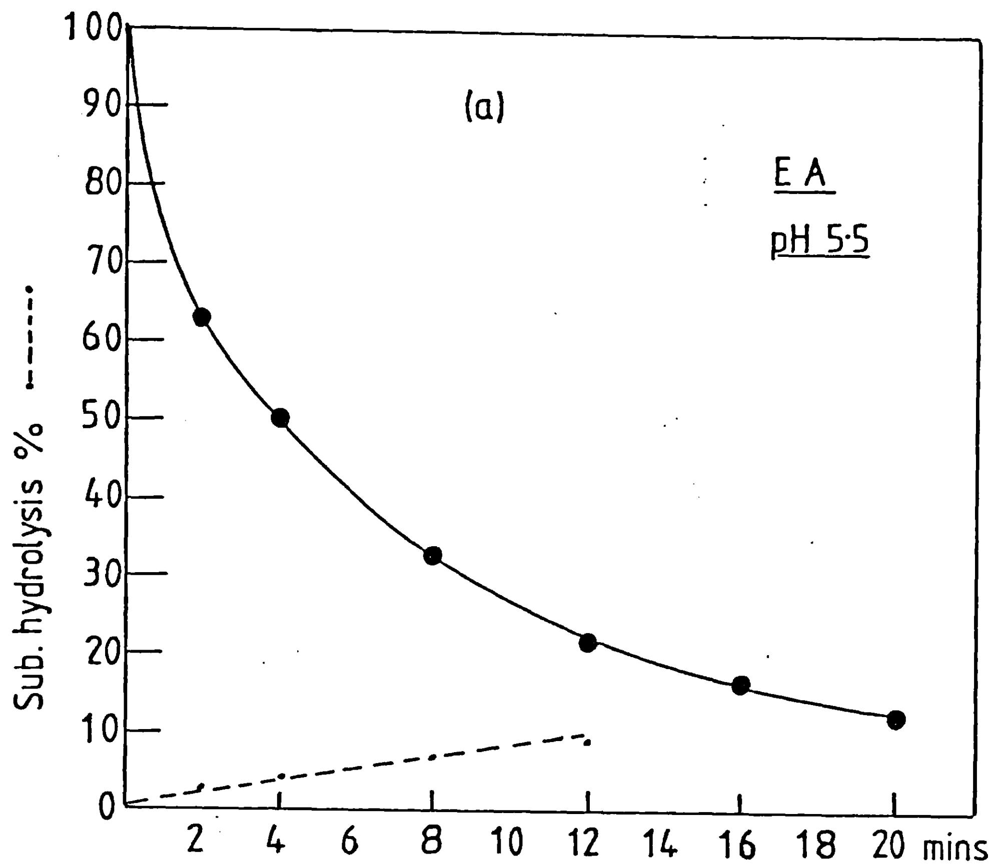


(a)
EA
(b)
EB
RVU x 10^2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
3
4
5
6
7
pH

FIGURE 33 (a) & (b): (a) Relative viscosity decrease & % Hydrolysis of substrate by Enzyme C as a function of time; at optimum pH. (b) pH optimum curve for Enzyme C measured by viscometry. Relative viscosity units = $1/t_{50}$. Substrate 1% (w/v) soln of Napp in buffer. Reaction mixtures contained 9mls substrate 0.5ml enzyme.


(a)
E C
pH 5·5
Rel. viscosity %
Sub. hydrolysis %
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20 mins


(b)
E C
RVU × 10²
20
18
16
14
12
10
8
6
4
2
0
3
4
5
6
7
pH



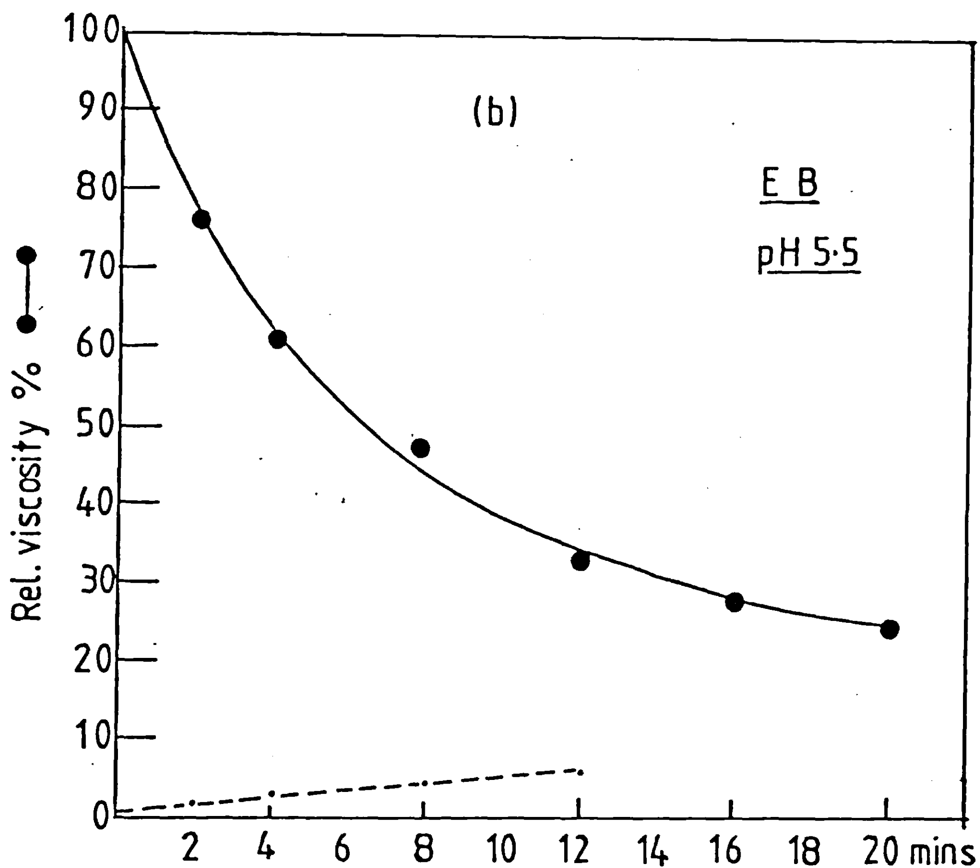


FIGURE 34 (a) & (b): Relative viscosity decrease and hydrolysis of substrate by Enzymes A & B as a function of time at optimum pH. Substrate 1% (w/v) soln of Napp in buffer. Reaction mixture contained 9mls of substrate & 0.5ml of enzyme

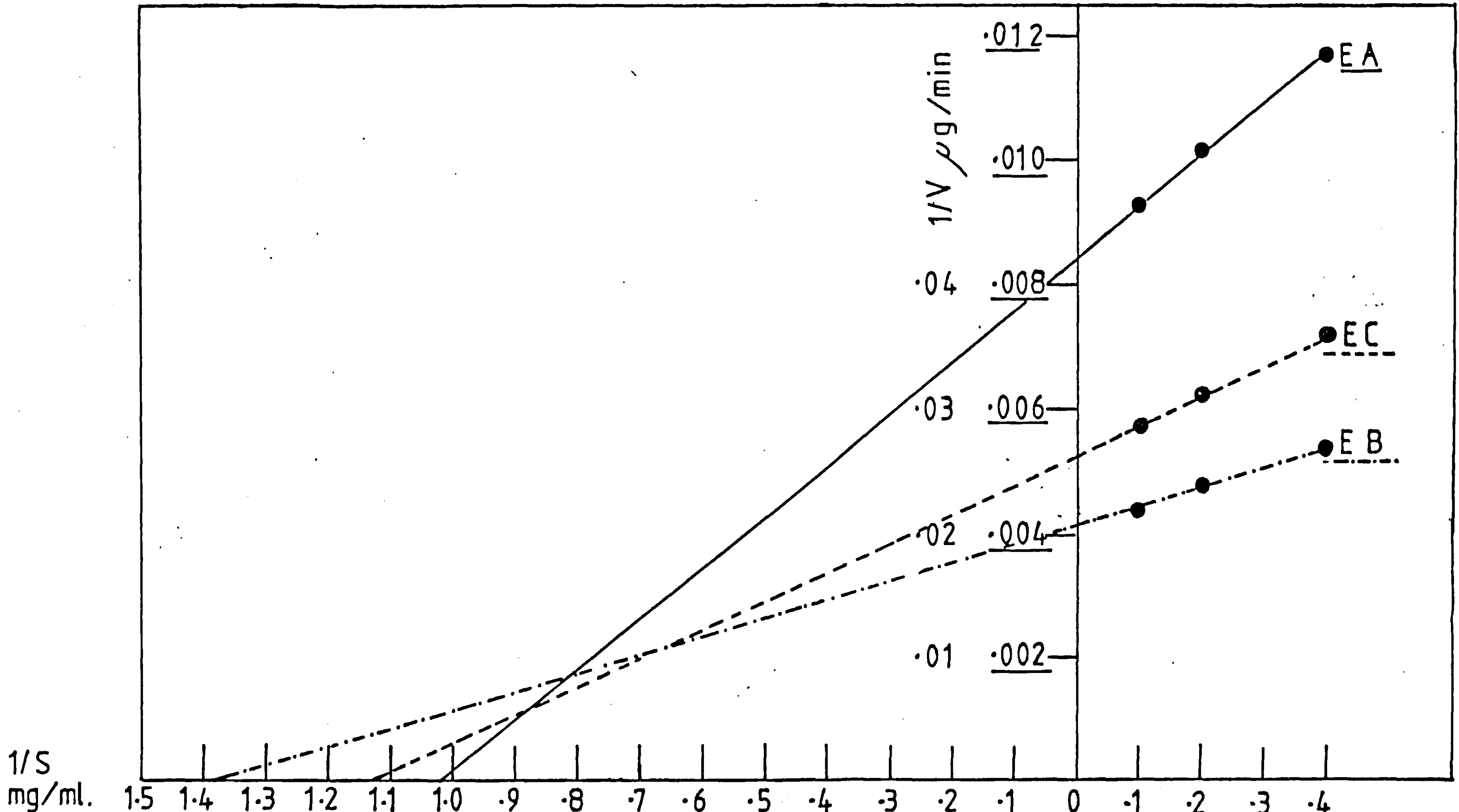


FIGURE 35: Lineweaver-Burk calculation of Michaelis constant & V max for Enzymes A, B, C of *Articulospora tetracladia* Reaction substrates 1%, 0.5%, 0.25% (w/v) solns Napp in buffer at optimum pH. 9mls substrate in reaction mixture with 0.5ml enzyme. ^{1}/V rates for EA underlined.

Initial Volume mls	Total Protein mg.	Vol. after ultra-filtration	Ion-Exchanger	Buffer and pH	NaCl gradient	5ml Fractions Assayed	PG	Fractions
1500	205	150	CM-sepharose	0.02M acetate pH5	0-1M	12-104	A & B*	14-50
							C	58-70
*A & B 140	38.6	-	CM-sepharose	0.02M acetate pH4	0-1M	9-84	A	11-26
							B	33-43

PG	Final Vol.mls	mg/ml protein	Total protein mg.	Enzyme sample mg.	pH optimum	% Hydrolysis t_{50}	pI	Km mg/ml	V Max (μg GA/Min /mg protein)
EA	15	0.62	9.3	0.5	5.5	3.5%	4.4 ± 0.6	0.98	383.9
EB	10	0.48	4.8	0.5	5.5	3.0%	7.5 ± 0.1	0.72	198.4
EC	10	0.38	3.8	0.5	5.5	2.5%	8.0 ± 0.1	0.89	198.3

TABLE 15: Data on purification and properties of three polygalacturonases produced by *Articulospora tetracladia* grown on 1% (w/v) Napp at pH5.

swift loss of viscosity in the reaction mixture; t_{50} = 5 mins.

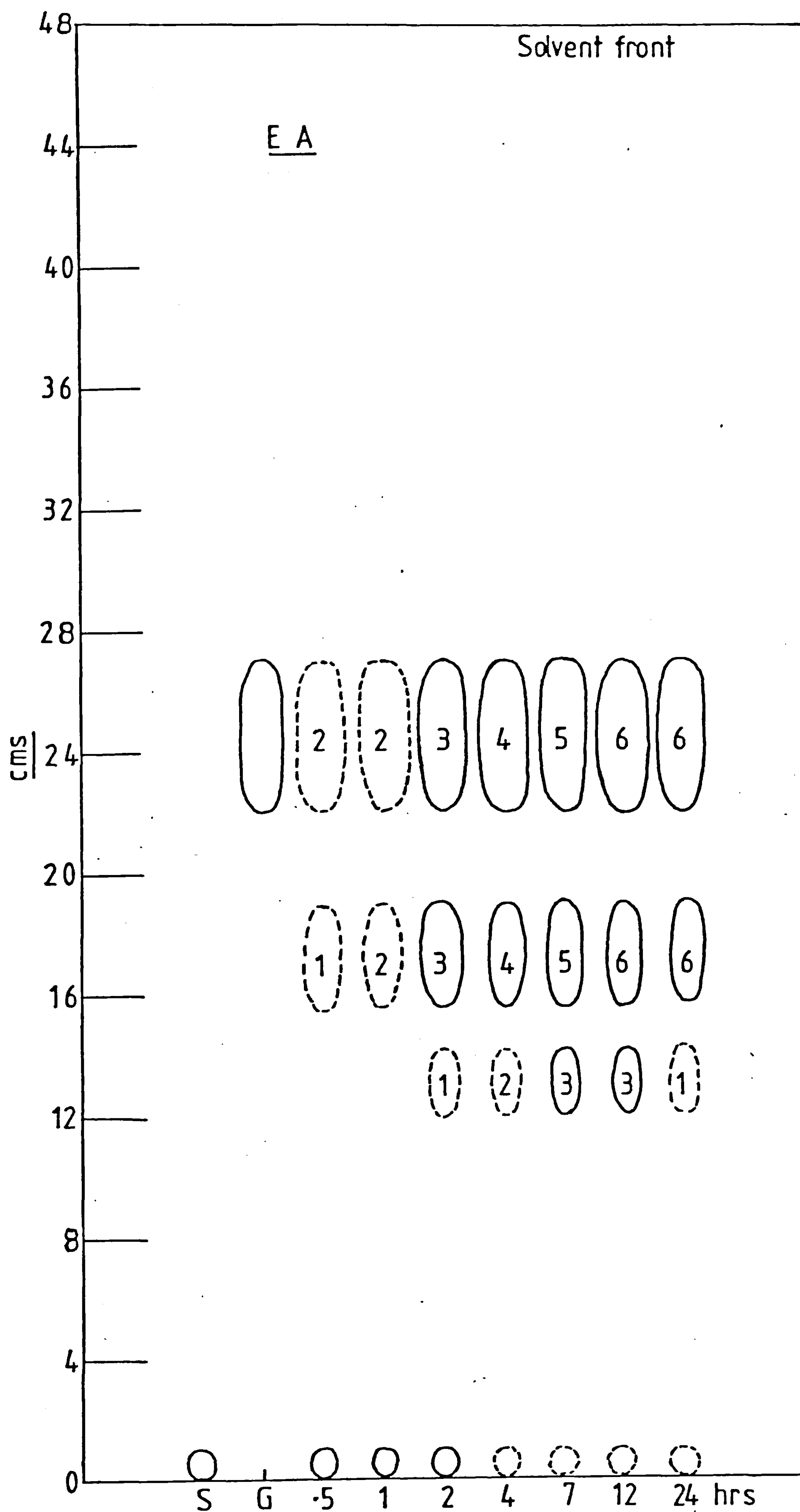
EA and EB produce only monomer, dimer and trimer as reaction products, and of these the dimer and monomer predominate as the enzyme degrades the trimer with time. Superficially, these are the characteristics of an exo-enzyme, but in Enzymes A & B t_{50} is swift (3.8 mins and 6.4 mins respectively) and is accompanied by a low % hydrolysis of the substrate (3.5% and 3.0%), both characteristics of endo-polygalacturonases. This action is unusual, but is recorded by English et al (1972) for an endo-D-polygalacturonase produced by Colletotrichum lindemuthianum. They suggested that the enzyme attached to the substrate molecules, initially, at random. The attack then progressed from that site along the chain, releasing monomer, dimer and sometimes trimer, until it reached a barrier such as a branch point or a neutral sugar residue; the enzyme would then attack another molecule. Evidence of a similar 'multiple attack' by glycosidases is given for amylases. In extreme cases the enzyme completely hydrolyses one substrate molecule before reacting with another. The subject of multiple attack is reviewed by Thoma, Spradlin and Dygent (1971).

Enzymes A, B and C can be classified as endo-D-galacturonases. (Systematic name: poly - (1 $\longrightarrow$ 4) α -D-galactosiduronate glycanohydrolase) EC No. 3.2.1.15

Discussion of endo-D - galacturonases of T. splendens and A. tetracladia

Enzymes I, II and III of T. splendens are isoenzymes as are Enzymes A, B and C of A. tetracladia. The differences in properties, particularly PI values, between the isoenzymes within a species suggests that they are genetically independent proteins, the products of separate genes. However, properties of the enzymes when compared between species suggest that each group of isoenzymes may have arisen from a common ancestor followed by independent variation. The Km values for Enzymes I, II and III for example would appear to be arithmetically

FIGURE 36: Chromatogram of reaction products of Enzyme A with time. S = Substrate. G = Galacturonic acid standard. Samples 25 μl. Reaction mixture, 9 mls 1% (w/v) soln of Napp in buffer, pH 5.5, 0.5ml enzyme, incubated at 30°C.



Solvent front
E A
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48
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8
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6
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1
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3
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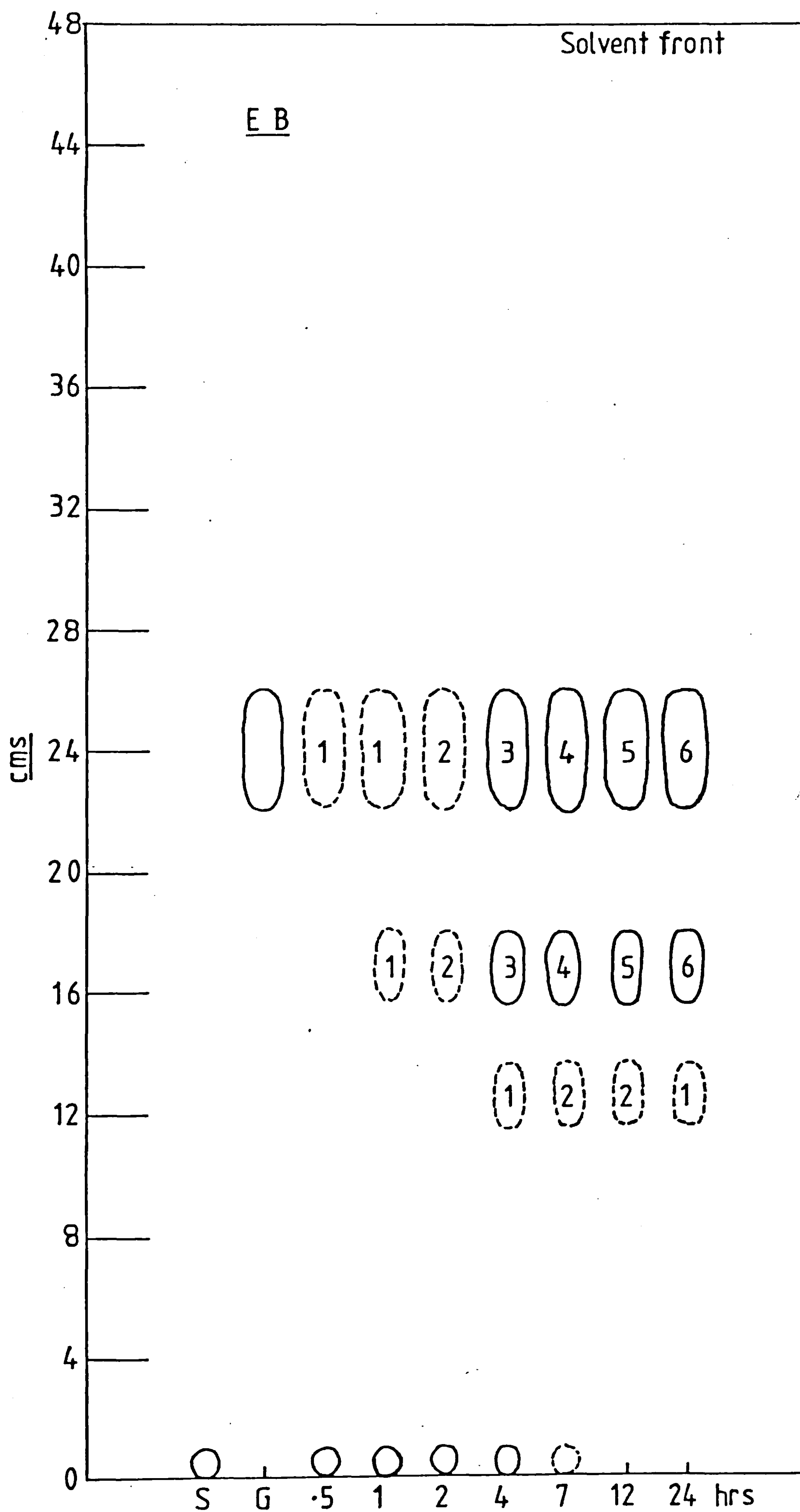
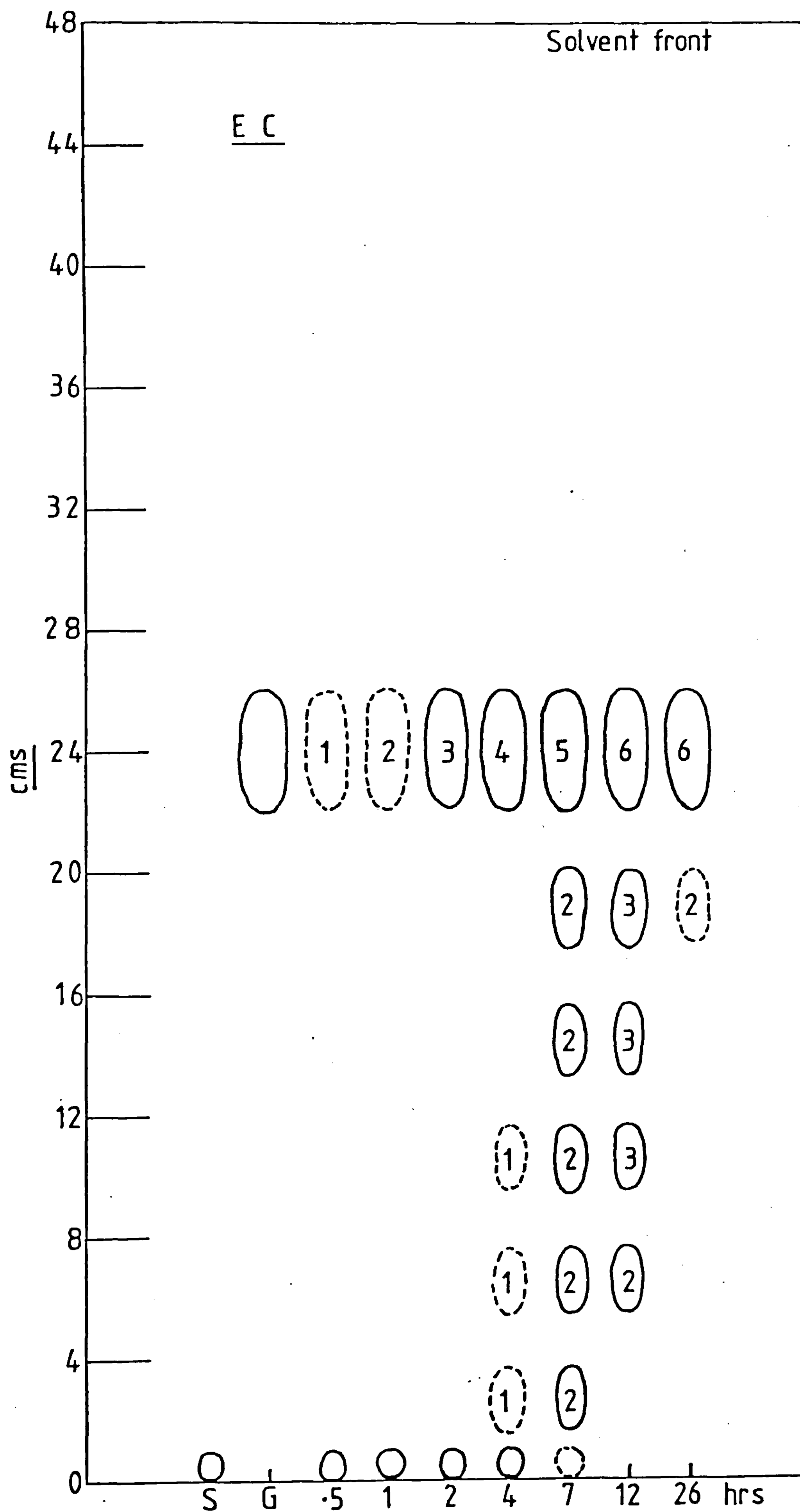
6
1
2
3
3
1
S
G
·5
1
2
4
7
12
24
hrs

FIGURE 37: Chromatogram of reaction products of Enzyme B with time. S = Substrate. G = Galacturonic acid standard. Samples 25μl. Reaction mixture, 9mls 1% (w/v) soln of Napp in buffer, pH 5.5 & 0.5ml enzyme, incubated at 30°C.



Solvent front
E B
cms
48
44
40
36
32
28
24
20
16
12
8
4
0
1
1
2
3
4
5
6
1
2
3
4
5
6
1
2
2
1
S
G
·5
1
2
4
7
12
24
hrs

FIGURE 38: Chromatogram of reaction products of Enzyme C with time. S = substrate. G = Galacturonic acid. Samples 25 μl. Reaction mixture, 9mls 1% (w/v) soln of Napp in buffer, pH 5.5 & 0.5 ml enzyme, incubated at 30°C.

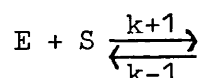

Solvent front
E C
cms
48
44
40
36
32
28
24
20
16
12
8
4
0
1
2
3
4
5
6
6
2
3
2
2
3
1
2
3
1
2
2
1
2
S
G
·5
1
2
4
7
12
26
hrs

related, the value for EII being almost the arithmetic average of the values for EI and EIII. Furthermore, if the V max value for EI is subtracted from that of EIII the result is almost exactly the value for EII.

The isoenzymes of A. tetracladia appear to have a similar relationship, though of a different arithmetic range. The Km value of Enzyme C is very nearly the arithmetic average of the values for Enzymes A and B; and the difference in V max values between EA and EB gives almost exactly the value for EC.

Structural hybrids are reported for lactate dehydrogenase and mamalian alcohol dehydrogenase (Dixon & Webb, 1979) in which the enzyme appears as isoenzymatic hybrids which have properties intermediate between the 'parent' forms. The mathematical relationship between the Km and V max values for the isoenzymes discussed above may be coincidental in one case, but for the same relationship to be found in both sets of isoenzymes tempts the investigator to propose an explanation for the data. The values for Km and V max are to some extent interrelated; the Km value being the substrate concentration at half V. max. Km values are established by measuring initial velocities of reaction at varying substrate concentrations and represent the reciprocal of the affinity of the enzyme for its substrate, in a simple equilibrium condition. As steady state conditions do not apply,

Km measures the equilibrium dissociation constant for the equation:-



$$E + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}}$$

V max represents a measure of the rate of the breakdown of the enzyme - substrate complex ES

$$ES \xrightarrow{k+2} E + \text{products}$$

Inhibitor studies of enzyme kinetics show that these effects can be, to some extent, separated and in the case of the isoenzymes under investigation, it may be possible that the factors influencing Km, are intermediate in a hybrid; whereas the factors influencing V max, may

only be inhibiting where there is identity between the two 'parent' forms. Since pI values do not accord with 'intermediacy' in these isoenzymes it may be that the overall balance of charges is not involved in Km and V max values, only the nature of the active centre. As Enzyme C has a somewhat different action pattern from Enzymes A and B this might be evidence of lack of relationship between the three isoenzymes. However, it could be argued that in the hybrid the factor for multiple attack had been supressed and only the more common random attack on the substrate expressed.

If Km and Vmax values for each isoenzyme complex are compared, it appears that PG's of T. splendens would be active over a wider range of substrate concentrations in co-operation than those of A. tetracladia, but at lower velocity. The isoenzyme complex of A. tetracladia would be active over a narrower range of substrate concentration but at much higher overall velocity.

Enzyme I, T. splendens, with very low V max and high Km and wide range of PI values seems to require some explanation when compared to the other isoenzymes investigated. That its effect with time is not negligible can be seen from the chromatogram in Figure 27. Nevertheless it appears anomalous. Its low activity might be explained by degeneration due to a number of alternative causes. Firstly, it may have been handled inappropriately; but since all three isoenzymes were treated to freezing and unfreezing processes, comparatively, this is an unlikely explanation. The wide range of PI for Enzyme I repeated with identical results on four gel electrofocussing runs, indicates instability in the net change of the protein supported by the fact that with the PI values given by these procedures, theoretically, the enzyme should have been adsorbed to the column at pH5 rather than at pH4. It is possible that in vitro, the enzyme was expressed early in the induction process when substrate concentration is higher and degenerated with time, so that

by the eleventh day of growth, when the enzymes were harvested, it had lost a large measure of activity. It cannot be that enzyme concentration was too high for the substrate involved as 0.5ml enzyme sample produced more rapid reduction in viscosity of the substrate at pH4-5 than 0.1ml enzyme sample. It is possible that genetically, the expression of this enzyme has degenerated with time.

3. Pectin transeliminases in Tetrachaetum elegans and Mycocentrospora angulata

Materials and methods used for specific induction of PTE's are given in that chapter, section E1. Initially, the ultrafiltered dialysed sample from T. elegans (150mls from 1500mls) was run onto a cation-exchange column (CM- sepharose) at pH5, as for PG's. Fractions containing protein were assayed on pectin cup-plates at pH9 to detect PTE activity. An election profile of this chromatography is presented in Figure 39. Fractions showing PTE activity were not adsorbed onto the column but an enzyme which gave a clearing reaction on the plates after development was eluted in Fractions 76-92. These fractions were pooled. The pooled fractions with PTE activity were concentrated by ultrafiltration. To try to establish the identity of both enzymes, a thiobarbituric assay was conducted with each enzyme, independently, on a 1% (w/v) solution of washed pectin. Samples were withdrawn at fixed time intervals; up to 20 mins. The products of the PTE assay showed a steady increase in absorption at 550nm, but markedly less absorption at 510nm, thus confirming the production of an unsaturated product of the enzyme reaction.

With the second enzyme there was no more activity in the reaction mixture with time than could be accounted for by spontaneous breakdown of the substrate under alkaline conditions. Absorption was greater at 510nm than at 550nm, showing that the products were not unsaturated, and gave markedly lower readings than those for PTE at 510mm, an indication of very low levels of hydrolytic activity, which distinguished it from a polygalacturonase.

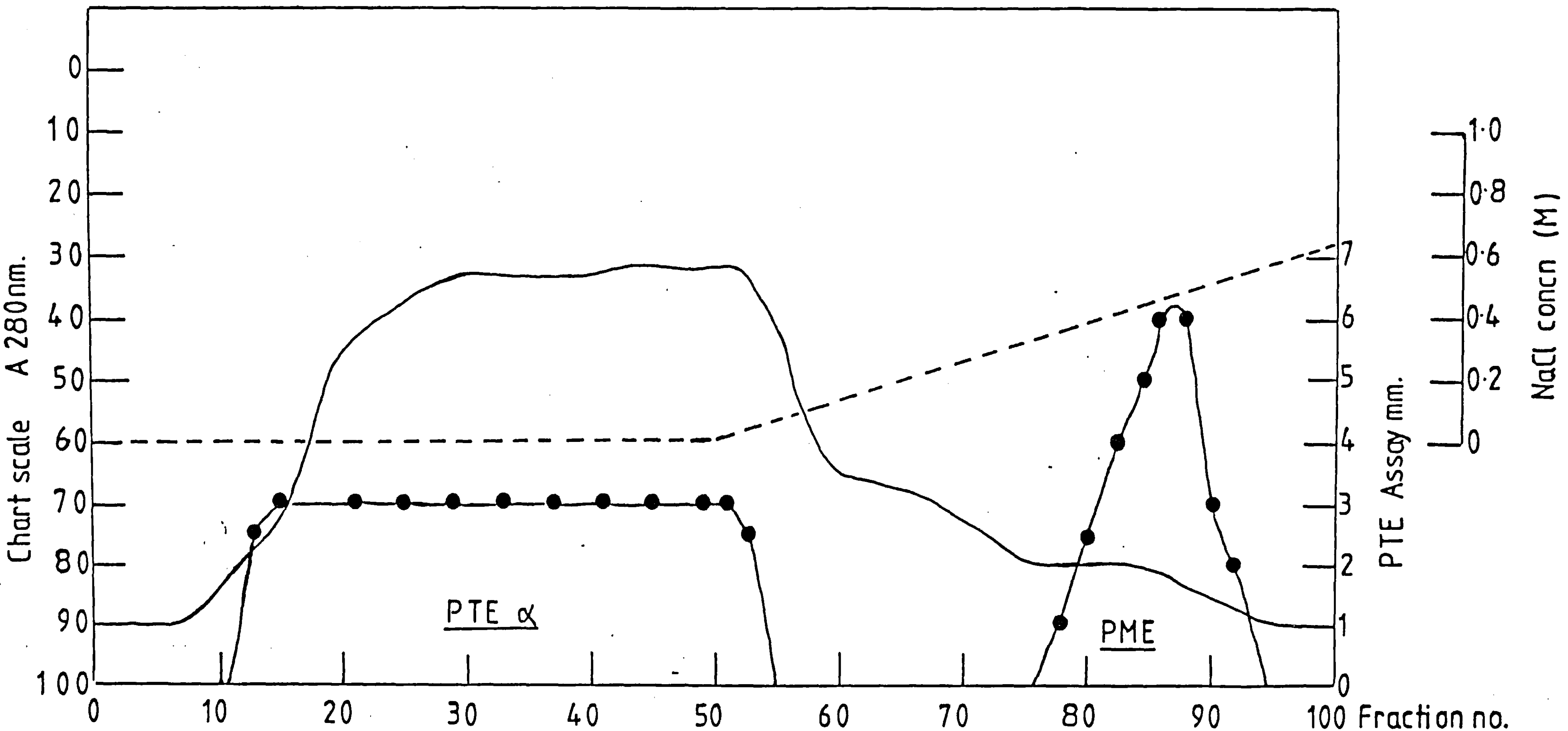


FIGURE 39: Elution profile of supernatant for *Tetrachaetum elegans* grown on 1% (w/v) Napp at pH7. A CM-sepharose column buffered at pH5, 0.2M Acetate, was used for the separation. Flow rate 15ml/hr. Fractions, 5ml, were assayed for PTE & PME activity ●—● . Protein levels (A280) —. NaCl concentration ----.

It was thought that this second enzyme might be a pectin methylesterase (PME) and a specific assay for PME activity was conducted. (Materials and methods, E3(iii)). 6mls of enzyme in 20mls unbuffered pectin solution had an initial reaction velocity which brought about a pH reduction of 0.075/min. Protein content was 150μg/ml. This is the equivalent of Δ pH 0.5/mg protein/min.

The PTE sample was run onto a CM-sepharose column at pH4 but assays showed that it had failed to be absorbed. Iso-electric focussing was done in an attempt to purify the PTE, but it was found that the ampholine carriers reacted positively with the pectin assay plates and as the extensive dialysis required for everyone of 30 eluted samples was time-consuming and laborious it was decided not to use electrofocussing on PTE's.

A fresh batch of enzyme was induced and the ultrafiltered dialysed sample applied to a DEAE-Sepharose column equilibrated to pH 7.2 (0.025 Tris-HCl buffer). The column was eluted with a 0-1M NaCl gradient. 4.5ml fractions were collected every 20mins. (Flow rate 13.5mls/hr). Fractions 23-130 were assayed by cup-plate for PTE activity which was positive in Fractions 73-80 which were pooled and designated PTEα. An elution profile of this separation is given in Figure 40.

Figure 41 presents the elution profile of an ultrafiltered, dialysed sample of supernatant from M. angulata induced and treated in the same way as T. elegans above. Fractions 18-140 were assayed for PTE activity, by cup-plate, and Fractions 93-102 gave a positive reaction and were pooled and designated PTE β .

As the elution profiles show, PTE α and PTE β were eluted at high protein levels. Some proteins were precipitated in the fractions pooled and were removed by centrifugation under refridgeration. The precipitate was dissolved in buffer and assayed for PTE activity. The assay proved negative. No other pectic enzymes were detectable in the

remaining PTE samples and though the protein levels were high (0.7mg/ml PTE α and 0.65 mg/ml PTE β) it was decided to characterize the samples without further purification.

The TBA assay used to obtain pH optima and Km values for PTE α and β are given in Materials and Methods E3(ii)c. pH optima plots are given in Figures 42 & 43. Km plots (Lineweaver-Burk) in Figure 44. The pH optima of both PTE α and β was 9.0 on the substrate and at the calcium ion concentration used in these determinations. Rexová-Benková and Markovič (1976) report lower pH optimum values for PTE's depending on the degree of esterification of the substrate. With decreasing d.e., the pH optimum is lowered. Figures 42 & 43 give a % maximum value for PTE's assayed under identical conditions and concentrations on Napp. (PTE α, 33% of pectin maxima. PTE β, 52% of pectin maxima). The preferred substrate of the enzymes is highly esterified D-galacturonans. Voragen et al (1971)* found that 1/Km values for a pectin lyase from a commercial preparation decreased as the d.e. decreased. V max values did not depend on d.e. Values of 1/Km increased with decreasing pH.

A viscometric assay was made of PTE α and β to establish t_{50} and the percentage hydrolysis of the substrate at that point. For PTE α, 8mls of a 0.6% (w/v) solution of washed pectin and 2mls of enzyme with a final $CaCl_2$ conc. 1m M, pH9, constituted the reaction mixture. For PTE β, 9 mls 0.6% (w/v) pectin solution with 1 m M $CaCl_2$ and 1ml enzyme, pH9, was the reaction mixture. Both were incubated at 30°C.

	t_{50}	% Hydrolysis at t_{50}
PTE α	6 hrs.	41%
PTE β	1 hour	3%

For analysis of reaction products, descending paper chromatography was

* ex Rexová - Benková & Markovič (1976)

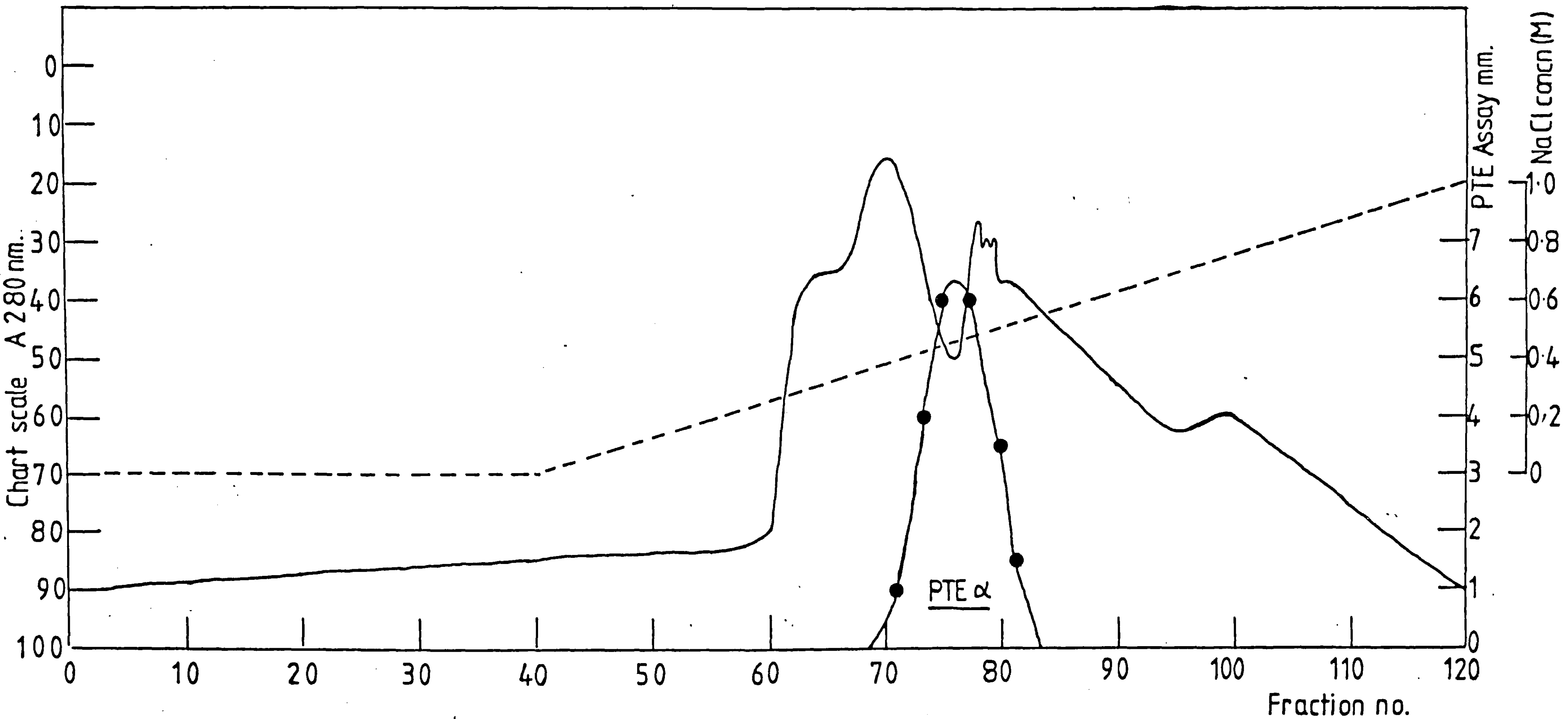


FIGURE 40: Elution profile of supernatant from Tetrachaetum elegans grown on 1% (w/v) Napp at pH7. The separation was carried out on a DEAE – Sepharose column buffered with 0.025M Tris - HCL to pH 7.2. Flow rate 13.5 ml/hr Fractions, 4.5mls, were assayed for PTE activity ●—●. Protein levels (A280) —— . NaCl concentration - - -.

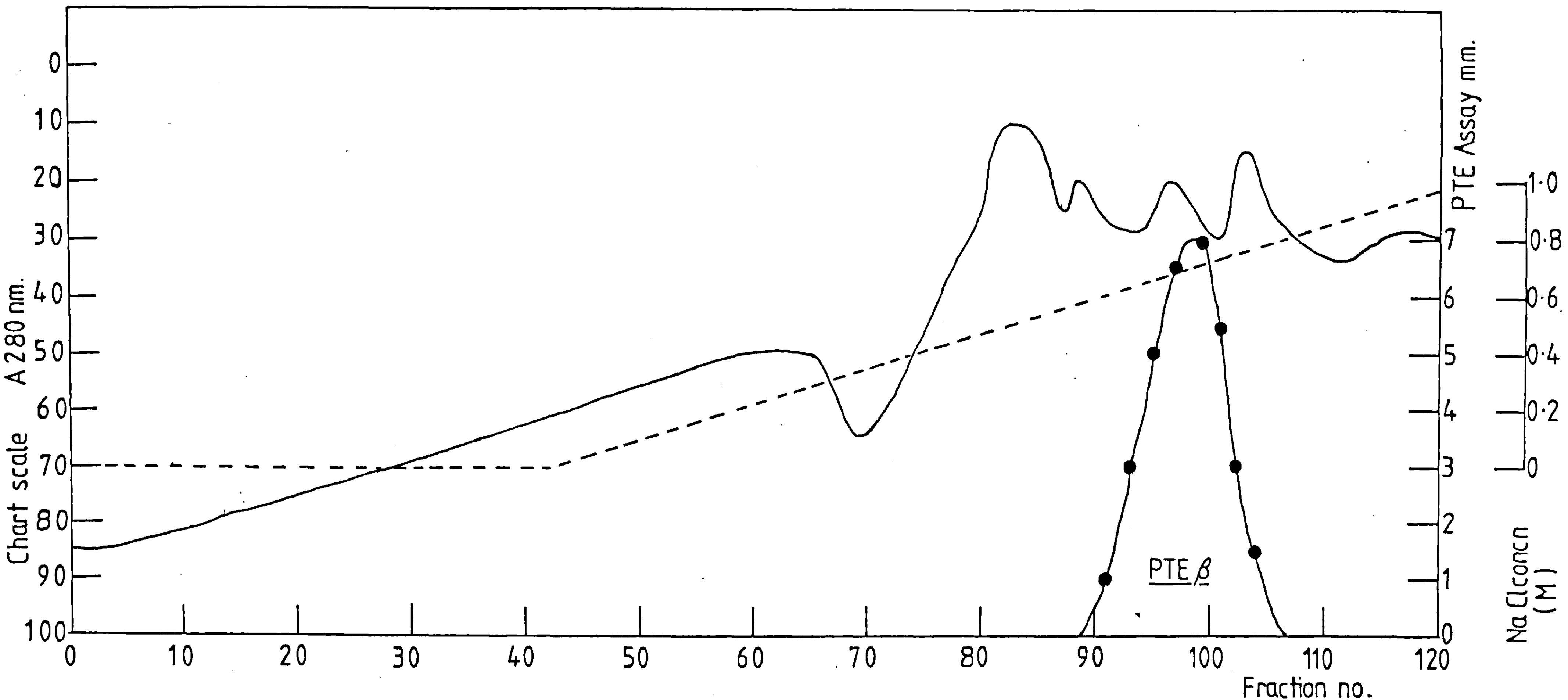


FIGURE 41: Elution profile of supernatant from Mycocentrospora agulata grown on 1% (w/v) Napp at pH7. Protein separation carried out on a DEAE Sepharose column buffered to pH7.2 with Tris-HCl 0.025M. Flow rate 13.5ml/hr. Fractions 4.5ml were assayed by cup-plate for PTE activity ●—● . Protein levels (A280nm) ——. NaCl concentration ---.

used (Materials and Methods E3(i)d). The standard was unsaturated digalacturonic acid and colour values are given in the resulting chromatograms presented in Figures 45 & 46.

For these results it appears that PTE α is an exo-pectin lyase, as no intermediate products between unsaturated digalacturonic acid and the sample were detected in 55 hours; whereas PTE β appears to be an endo-pectin lyase, since oligomers were detected after 24 hours incubation and the intensity of the oligomer concentrations increased with time. In this case, the early appearance of UDGA in the products can be ascribed to breakdown in substrate by spontaneous hydrolysis at alkaline pH. The streaking of sample in PTE α at 31 and 55 hours, probably represents shortened substrate chains that have not been reduced into detectable oligomers.

Although there is no official recognition of exo-pectin lyases in the classification of enzymes, Cooper (1974) induced a PTE with this action pattern with galacturonic acid from Verticillium albo-atrum. The standard action pattern for pectin lyase is that exhibited by PTE β .

The evidence of chromatography is supported by the results of the viscometric assay. Exo-enzymes cleaving only terminal bonds are slow to reduce the viscosity of the substrate, a 40% hydrolysis of the substrate at t_{50}. Endo-enzymes, cleaving bonds at random, produce a much swifter reduction in substrate viscosity, associated with a 2% hydrolysis of available bonds.

Table 16 summarises the data on the partial purification and properties of PTE α and β . The calculation of V max will be underestimated as the amount of enzyme protein in the total protein concentration could not be measured.

Calcium - ion concentration can affect the activity of pectin and pectate-lyases and these results have been obtained throughout on a Ca^{2+} conc. of 1mM/l. This level was adopted as a working mean

FIGURE 42: pH optimum curve for PTE α of *Tetrachaetum elegans*. Values represent percentages of maximum activity assayed by TBA method. Reaction mixtures were 4mls 1% (w/v) soln of pectin in buffer & 2.0mls enzyme + 1mM $CaCl_2$ conc. A value is given for an assay at pH optimum on an equivalent Napp soln.

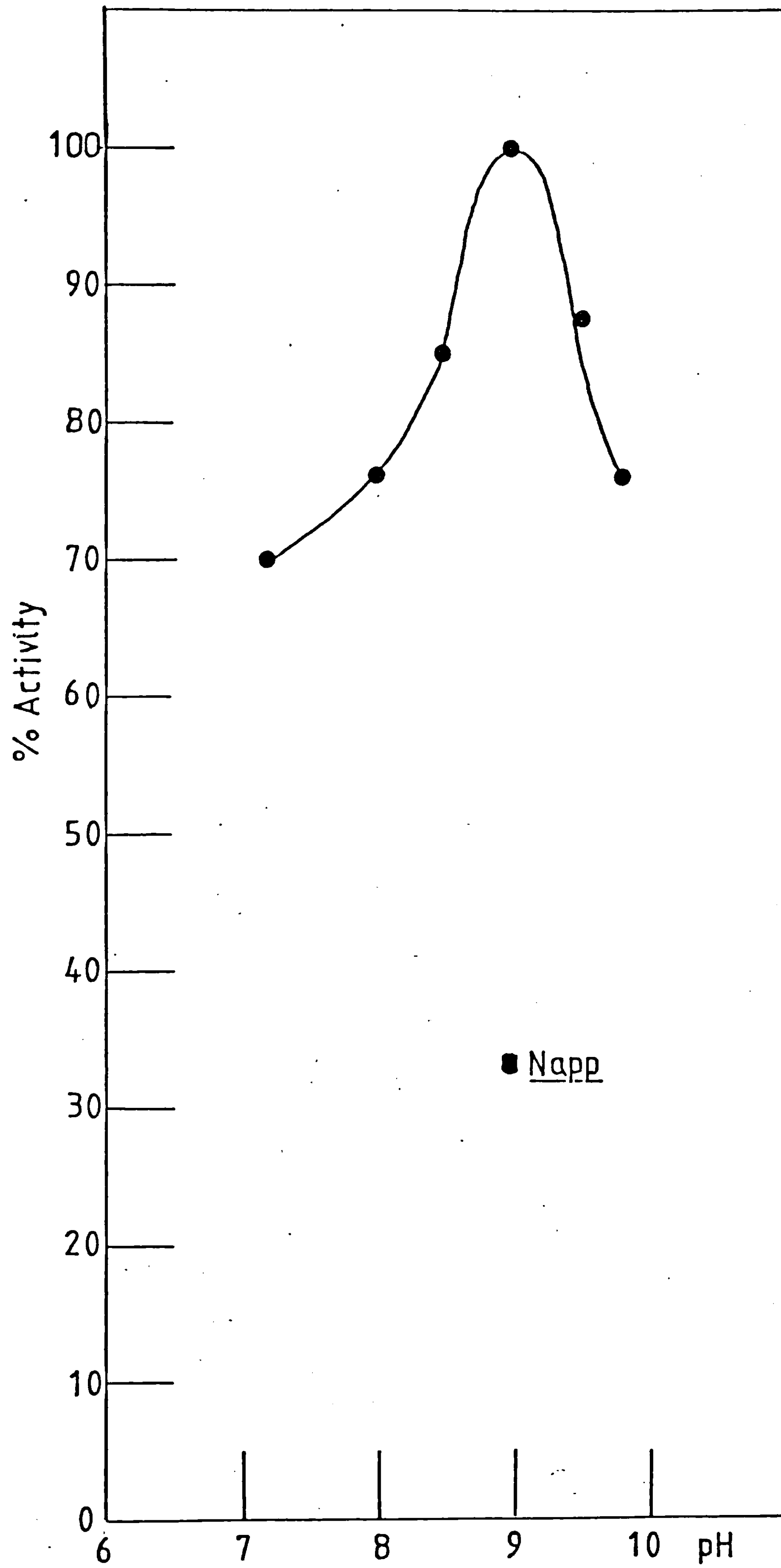

100
90
80
70
60
50
40
30
20
10
0
% Activity
6
7
8
9
10
pH
Napp

FIGURE 43: pH optimum curve for PTE β of *Mycocentrospora angulata*. Values represent percentages of maximum activity assayed by the TBA method. Reaction mixtures were 5mls 1% (w/v) soln of pectin in in buffer & 1.0ml enzyme; 1mM $CaCl_2$ conc. A value is given for an assay at pH optimum on an equivalent Napp soln.

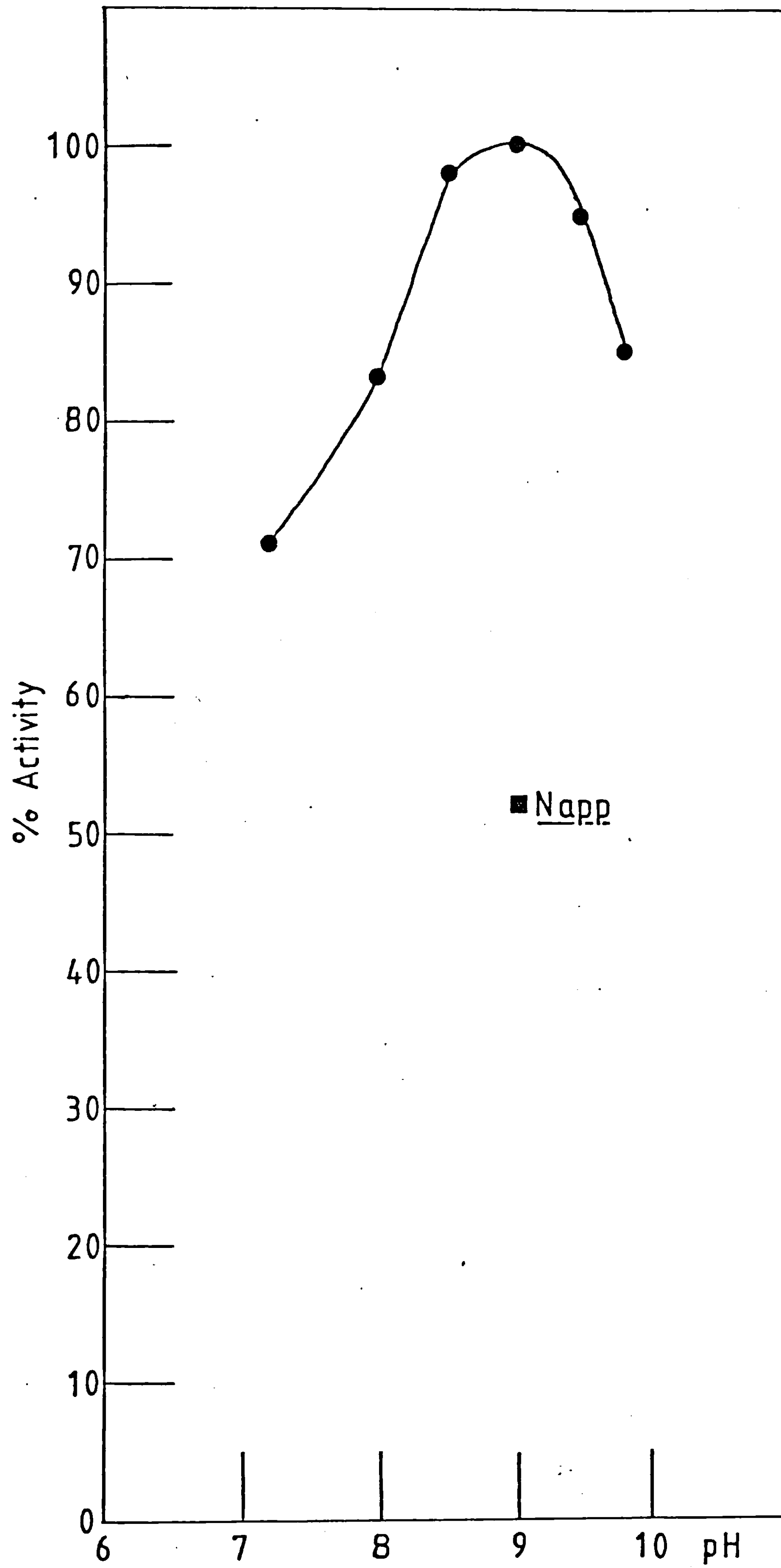

% Activity
100
90
80
70
60
50
40
30
20
10
0
6
7
8
9
10
pH
Napp

FIGURE 44:
Lineweaver-Burk calculation of Michaelis constant (Km) for enzymes PTE α & PTE β.
Reactions assayed by TBA method using pectin as substrate.
Reaction mixtures for PTE α were 4mls of sol of pectin in buffer (pH9) with 1mM $CaCl_2$ conc. & 2mls enzyme. Reaction mixtures for PTE β were 5mls of sol of pectin in buffer (pH9) with 1mM $CaCl_2$ conc & 1ml enzyme.

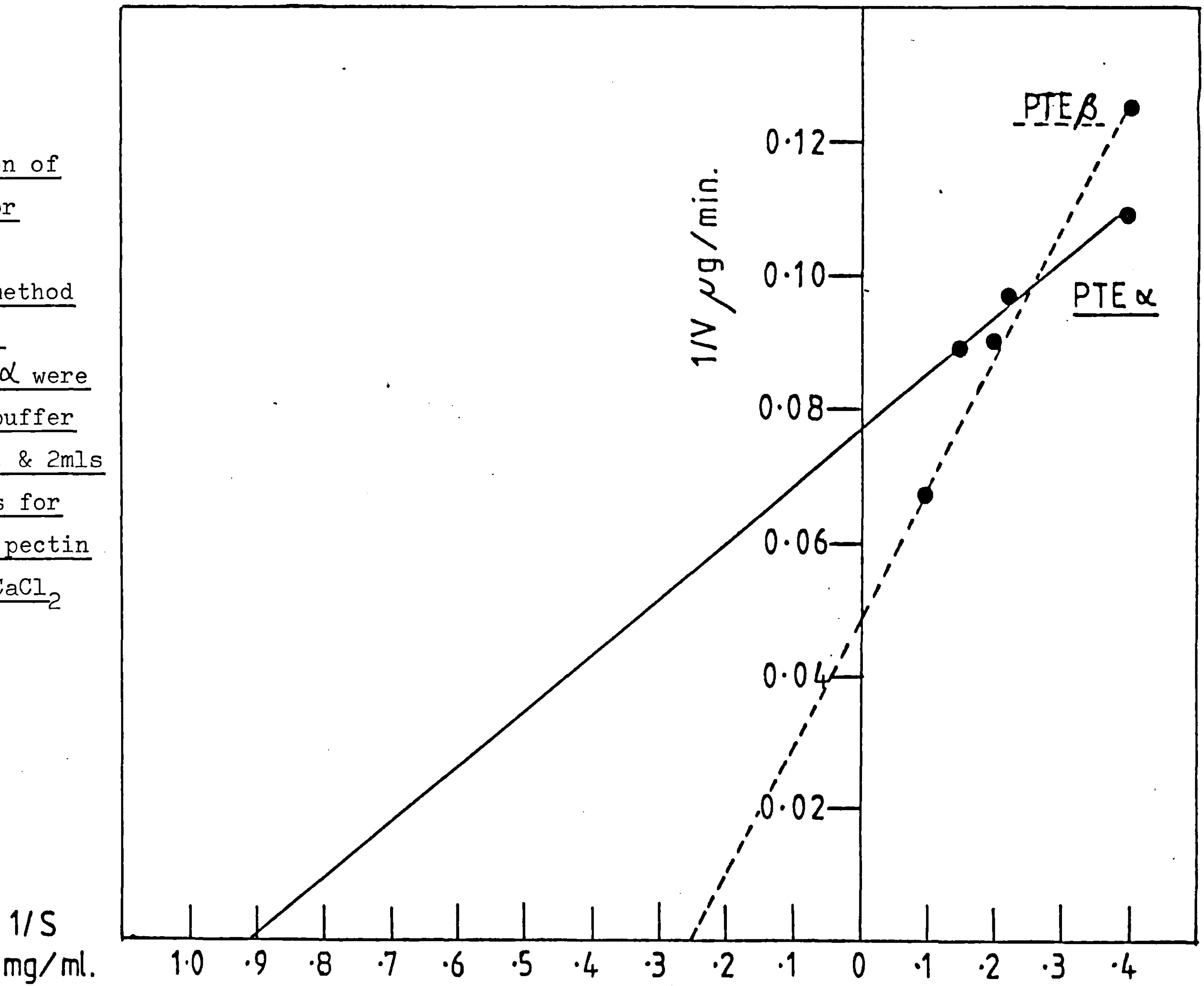


FIGURE 45: Chromatogram of reaction products of PTE α with time. S = Substrate. UDGA = Unsaturated digalacturonic acid. Samples 25 μl. Reaction mixture was 4mls 1% (w/v) soln of pectin in buffer, pH9, with 1mM $CaCl_2$ & 2mls enzyme. Incubation was at 30°C.

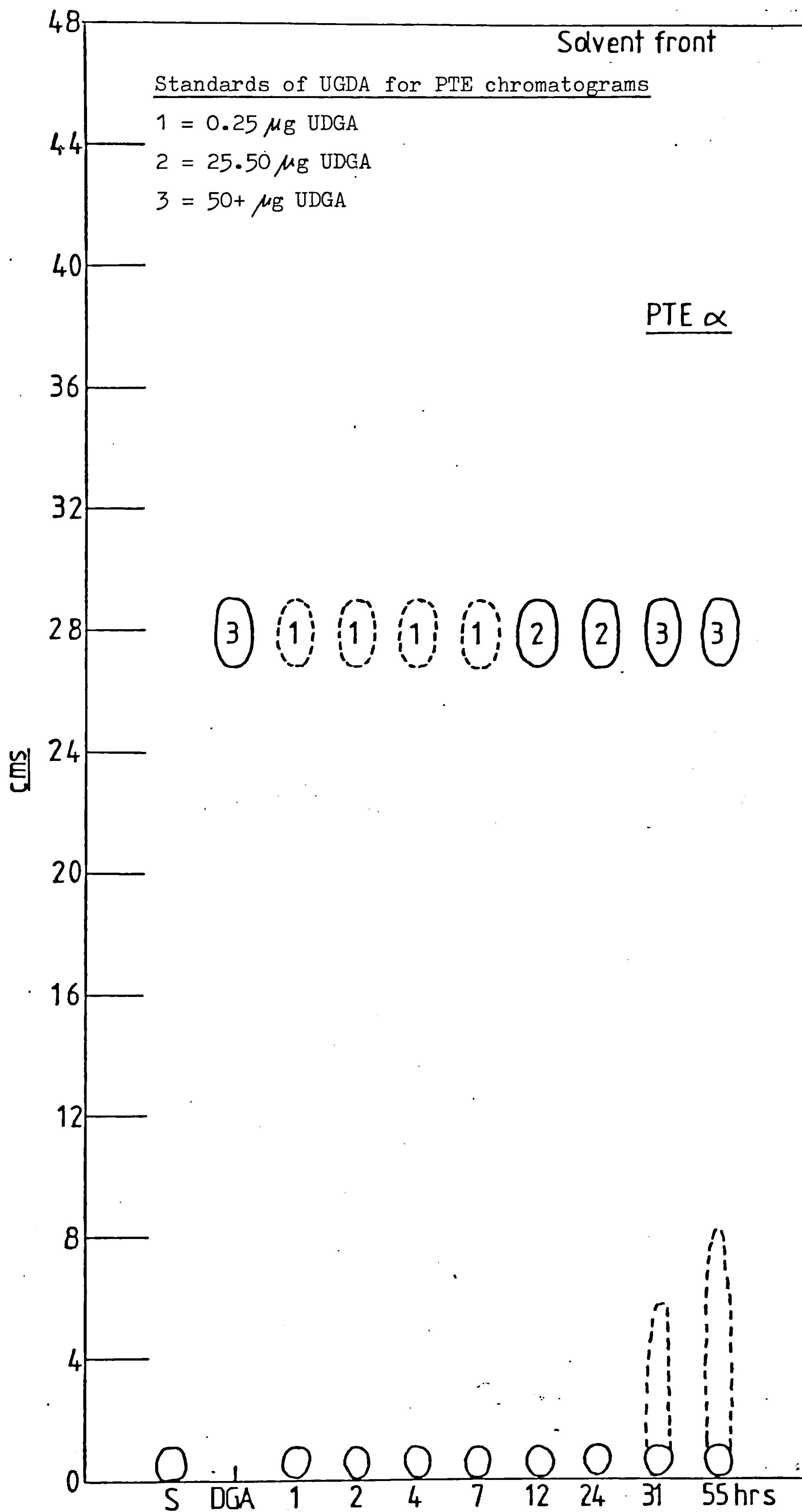
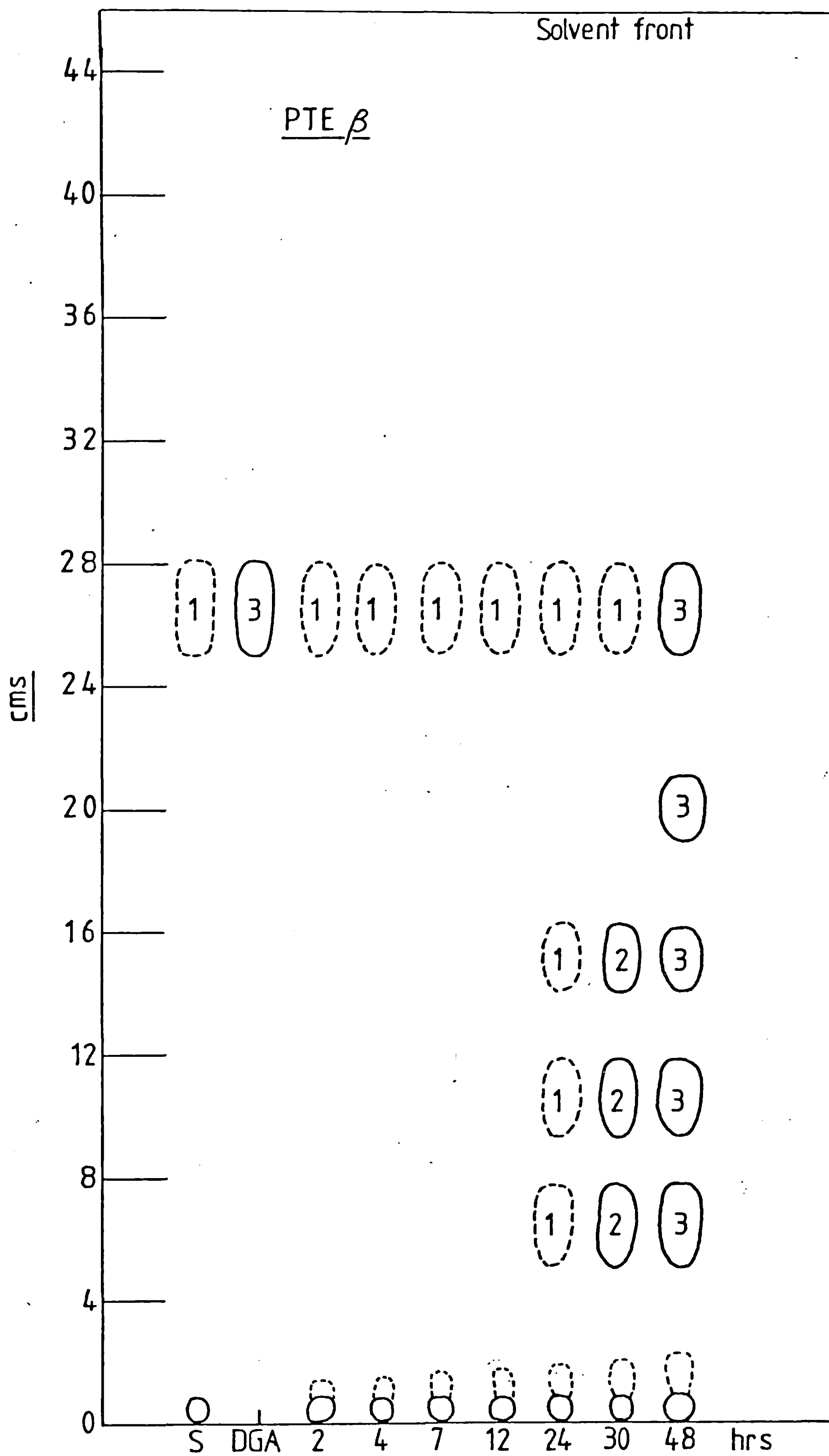

Solvent front
Standards of UGDA for PTE chromatograms
1 = 0.25 μg UDGA
2 = 25.50 μg UDGA
3 = 50+ μg UDGA
PTE α
cms
48
44
40
36
32
28
24
20
16
12
8
4
0
3
1
1
1
1
2
2
3
3
S
DGA
1
2
4
7
12
24
31
55 hrs

FIGURE 46: Chromatogram of reaction products of PTE β with time. S = Substrate. UDGA = Unsaturated digalacturonic acid. Samples 25 μl. Reaction mixture was 9mls 0.6% (w/v) soln of pectin in buffer, pH9, with 1mM $CaCl_2$ & 1ml enzyme. Incubation temperature $30^{o}C$.


Solvent front
PTE β
cms
44
40
36
32
28
24
20
16
12
8
4
0
1
3
2
S
DGA
2
4
7
12
24
30
48
hrs

Organism	Initial vol.mls.	Total protein mg.	Vol. after ultra-filtration	Ion Exchanges	Buffer & pH	NaCl gradient	4.5ml fractions assay	PTE	Fractions
Tetrachaetum elegans	1500	220	150mls	DEAE - sepharose	0.025M Tris-HCl pH7.2	0-1M	23-130	PTE α	73-80
Mycocentrospora angulata	1500	210	150mls	DEAE - sepharose	0.025M Tris HCl pH 7.2	0-1M	18-140	PTE β	93-102

PTE	Final vol. mls	mg/ml protein	Total protein mg.	Enzyme sample mg.	pH optimum	% Hydrolysis t_{50}	pI	Km mg/ml	V. max. (μg UDGA equiv./min/mg protein
PTE α	30	0.7	21	2.0	9.0	41%	n.d.	1.1	13.
PTE β	32	0.65	19.5	1.0	9.0	3%	n.d.	4.0	21.

TABLE 16: Data on partial purification & properties of PTE α & β from *T. elegans* & *M. angulata* grown on 1% (w/v) Napp at pH7.

because higher concentrations produced a very viscous substrate that inhibited induction and investigation.

PTE β can be classified as pectin lyase (poly [methyl - D - galactosiduronate] lyase) EC no. 4.2.2.10. This enzyme is defined as having a random action pattern. There are no official records for a pectin lyase with a terminal action pattern.

4. Leaf maceration by Pectinases of aquatic Hyphomycetes

Keegstra et al (1972) reported that purified polysaccharidases of Colletotrichum lindemuthianum were unable to degrade isolated host cell walls. To test whether the pectinases expressed in in vitro experiments are active on the leaf material used in field experiments at stream pH, experiments measuring maceration of alder leaves by the organisms used in vitro were conducted. Materials and methods are given in that Chapter (Section F). Results of changes in tensile strength of inoculated leaves, with time, expressed as a percentage of uninoculated leaf strips, are presented in Figures 47 and 48.

The initial pH of the experiment was that of the stream water. pH values of the supernatant and enzyme assay results of the uninoculated leaf strips are given below:-

Organism: Tetrachaetum elegans (a)

Day	pH		PG assay		PTE & PME assay	
	Control	Culture	Control	Culture	Control	Culture
1	7.1	7.1	X	X	X	X
3	6.7	6.5	X	X	X	X
6	6.7	7.1	X	2mm	X	6.5mm
8	6.45	6.8	X	1mm	X	4.5mm
10	6.7	7.2	X	1mm	X	6.5mm
12	Inoculated leaf strips had disintegrated; control strips were intact.					

Organism: Mycocentrospora angulata (b) -

Day	pH		PG assay		PTE & PME assay	
	Control	Culture	Control	Culture	Control	Culture
1	7.1	7.1	X	X	X	X
3	6.5	6.4	X	X	X	X
6	6.3	6.8	X	2mm	X	4.0mm
8	6.5	7.0	X	1mm	X	9.0mm
10	6.6	7.0	X	1mm	X	10.0mm

Day 10: Inoculated leaf strips had disintegrated; control strips were intact.

Organism: Tricladium splendens (c)

Day	pH		PG assay		PTE & PME assay	
	Control	Culture	Control	Culture	Control	Culture
1	6.9	6.9	X	X	X	X
3	6.8	6.9	X	1mm	X	2mm
6	6.6	7.0	X	2mm	X	4.0mm
8	6.6	7.4	X	2mm	X	5.0mm

Day 9: Inoculated leaf strips had disintegrated; control strips were intact.

Organism: Articulospora tetracladia (d)

Day	pH		PG assay		PTE & PME assay	
	Control	Culture	Control	Culture	Control	Culture
1	6.9	6.9	X	X	X	X
3	6.8	6.9	X	X	X	X
6	6.6	7.1	X	1mm	X	1mm
8	6.7	7.0	X	1mm	X	3mm
10	6.8	7.2	X	2mm	X	9mm

TABLE 17 (a), (b), (c) & (d): pH values & enzyme assays (cup-plate) on inoculated & control alder-leaf strips.

From Figures 47 and 48 it can be seen that within 3-4 days of inoculation there was significant loss of tensile strength (80%) in the leaf strips with each of the four fungal species tested. Suberkropp & Klug (in press), in a similar pure culture study with Tetracladium marchalianum, found loss of tensile strength in leaf discs of hickory (Carya glabra) after 6 days. The time taken in the present experiment for leaf strips to become skeletonized by fungal activity was in the range of 9-12 days. For hickory leaf discs, Suberkropp & Klug measured 10-12 days. It is possible that the differences between species are due to differences in chemical content of the individual leaves; or they may be due to real differences in enzymatic activity. The purification and characterization of pectinases showed that each species of aquatic Hyphomycete can elaborate a characteristic assay of pectinases which differ in their properties from one another.

As enzyme activity is marked by a fairly narrow range of pH, it is noteworthy that in the control strips the general trend was for pH values to fall overall, probably due to leaching of acidic substances. In the inoculated leaf supernatant there was an overall rise in pH, within the values observed for the stream, but in line with observations made in pure substrate studies Expt. III. The rise in pH is probably due to the extracellular products of fungal metabolism.

At the pH of the stream it is apparent from the data, that PTE and PME enzymes are more active than PG's. In the case of T. elegans and M. angulata which were dominant species in the field study, PG expression appears to decline with time and it is possible that the levels of PG observed were constitutively produced and may not have been active. In the case of T. splendens and A. tetracladia which have been demonstrated in Expts. II and III to produce more active PG's, the level of activity of these enzymes increased with time, though they did not match PTE activity. Although in pure culture on Napp substrates the PG's of these species showed very low activity at pH7, Cooper (1974)

found, with very similar PG's, that on a cell wall substrate, their pH optimum shifted from pH5 to pH6. PTE's, he found, with the addition of Ca^{2t} ions had a pH optimum lowered from 9 — 8. If this situation holds for the aquatic Hyphomycetes tested, the stream pH would be close to the optimum for PTE activity, but would not preclude PG activity. It appears that T. elegans and M. angulata with higher levels of PTE activity are metabolizing chiefly on these enzymes, whereas A. tetracladia and T. splendens with lower PTE activity and increasing PG activity are possibly metabolising on both. It was noted in Expt. II that T. elegans and M. angulata though they elaborated PG's did not grow at the optimum pH of these enzymes. In their pure culture experiments with T. marchalianum, Suberkropp and Klug (in press) detected both PG (pH optimum C.5) and PTE (pH optimum C.8) in the supernatant fluid of inoculated hickory leaf discs. They, too, found the PTE to be more active than PG's in leaf litter incubated in a stream at low temperatures (2°-4°C) and pH 7.2-8.0.

For individual species, only the pectin lyases of T. elegans and M. angulata have been investigated. On the evidence of leaf maceration experiments, there is a difference in activity with time between the two species. T. elegans appears to produce an initial high level of enzyme matched by swift loss of tensile strength in the leaf strips and this level is maintained with time. As this is an exo-enzyme, acting more slowly than endo-enzymes, with low Km for V max and a lower V. max, comparatively, than that of M. angulata, the slower degradation rate overall in T. elegans may be due to the rate of enzyme activity. In M. angulata, PTE activity starts at a low level but increases greatly within the period of leaf degradation for both species. Similar differences are observable in the activity of PTE's in T. splendens and A. tetracladia.

Although the differences observed in these pure culture experiments are not great, they be extended in stream conditions in which

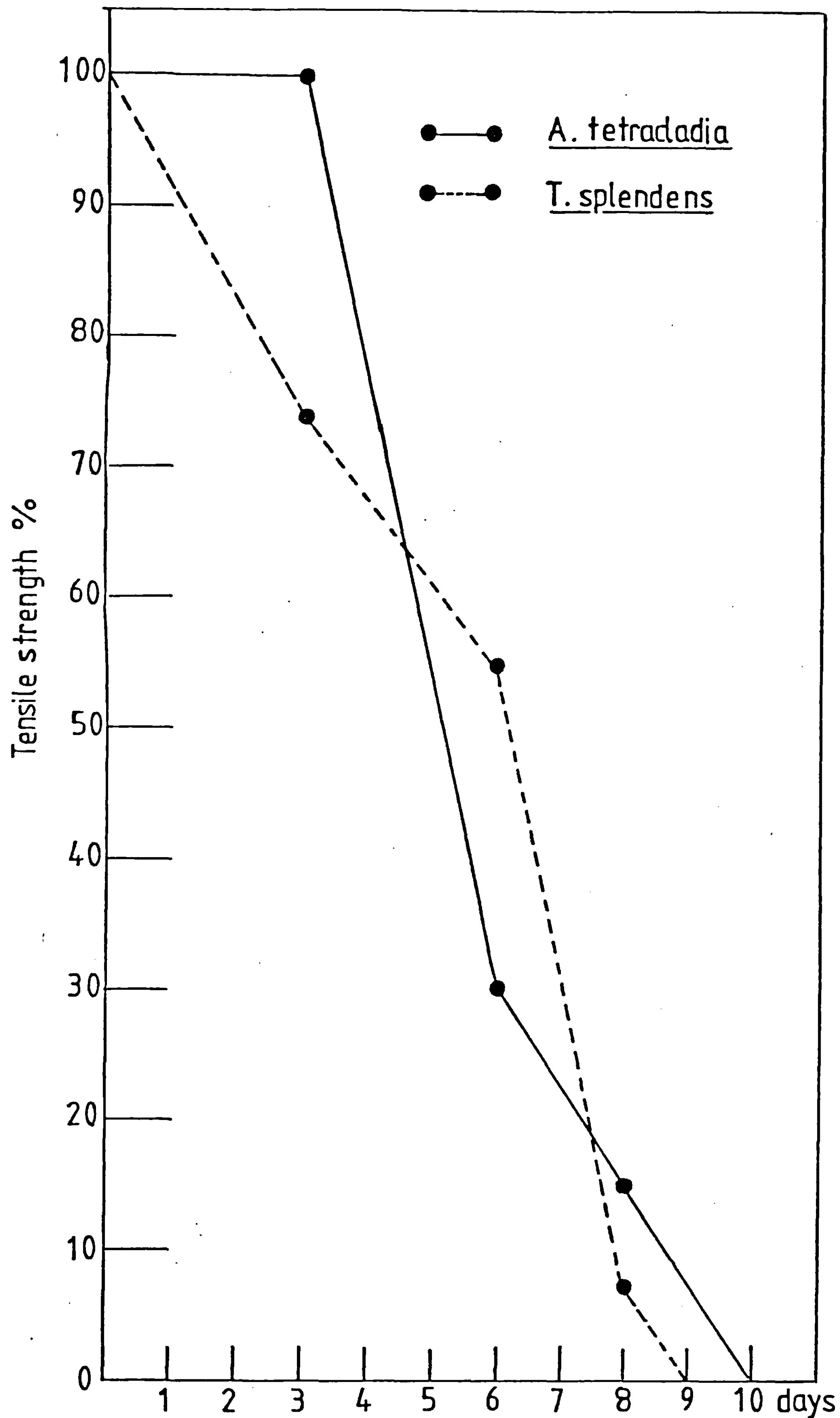


FIGURE 47: Graphs showing loss of tensile strength with time in alder leaves inoculated with Articulospora tetracladia or Tricladium splendens. Values represent the average tensile strength of 3 leaf strips expressed as a percentage of uninoculated control strips.

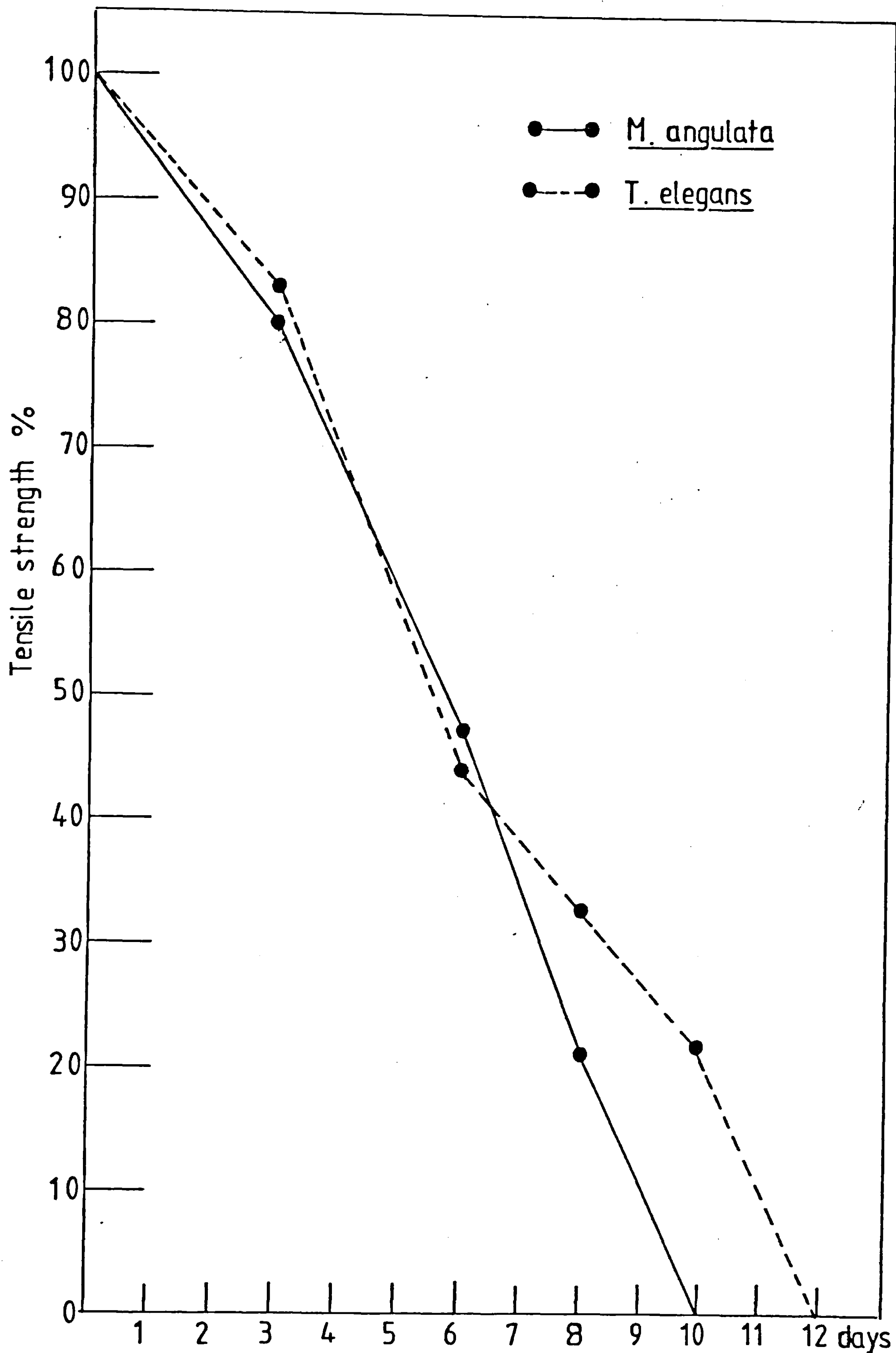


FIGURE 48: Graphs showing loss of tensile strength with time in alder leaves inoculated with *Mycocentrospora angualata* or *Tetrachaetum elegans*. Values represent the average tensile strength of 3 leaf strips expressed as a percentage of inoculated control strips.

less stable temperature regimes could be a modifying factor. It is noteworthy, in this respect, that in the results of the field study, although T. elegans and M. angulata are both the dominant early colonizers of alder and oak leaves in the stream, M. angulata reaches peak in every case before T. elegans and drops away as T. elegans reaches its peak. Patterns of colonization may well depend in large measure on the enzymatic properties and capablities of individual species of aquatic Hyphomycetes modified by stream conditions. Under laboratory conditions, however, there appears to be little difference in the pectolytic capacity of all four species to macerate leaf tissue and the differences in the frequency observed in the field study must be due either to the techniques employed for detecting active species, or to other physiological differences including total enzymatic capability.

It is shown by the experiments on pectinase production by the aquatic Hyphomycetes tested, that they elaborate a system of pectic enzymes. This phenomenon is reported for pathogenic fungi (Bateman 1963). The fact that these enzymes have differing pH optima, would give the organism the ability to degrade the pectic polymers in plant cell walls over a wide range of environmental pH. From the results of the experiments done on inoculated leaf strips, it would appear that in the case of aquatic Hyphomycetes the pH of the stream water determines which of the pectic enzymes are most active. Although PG's were detectable in the culture filtrate of all four organisms tested, they may have been constitutively produced in the initial stages of infection, serving only to induce the production of lyases. Zucker and Hankin (1970) compared the production of endo-pectate lyase in a pathogen and a saprophyte. The pathogen produced a small amount of the enzyme constitutively, whereas the saprophyte showed only inducible synthesis. The authors considered that the constitutive enzyme serves only for the initial attack of the substrate. The products formed

induce the synthesis of a new enzyme which then takes over cleavage of the substrate.

The production of a PME at the same pH and on the same non-methylated substrate (Napp) as PTE induction is a puzzle. PME's are found in conjunction with endo-galacturonase production in the course of fruit maturation and are found to act in conjunction with pectate lyase on highly esterified pectin (Lee and Macmillan 1970). In theory a PME would lower pectin lyase activity and this was found to be the case when both enzymes were reacted on a pectin substrate at pH9 and the results compared with those of lyase alone. The PME reduced lyase activity by 50%. The two enzymes were found together in the culture filtrates of inoculated leaf strips of both T. elegans and M. angulata.

GENERAL DISCUSSION

The significance of extracellular production of pectinases by a saprophyte, lies in the fact that the organism possesses the potential to break down the pectic substances in the middle lamella and primary cell walls of plant material which will result, firstly, in the exposure of other cell-wall polysaccharides and, secondly, in the maceration of the plant tissue. Polysaccharidases capable of degrading all the components of plant cell walls have been found in both pathogenic and saprophytic fungi. They are produced sequentially in the order in which wall polymers become available to enzymic exploitation. The first enzymes secreted in this sequence are pectinases (Jones et al 1972) and the evidence both from this study and that of Suberkropp & Klug (in press) is that pectinases elaborated by aquatic Hyphomycetes are active in leaf maceration both at laboratory temperatures ($15^{\circ}C$ is within the temperature range of temperate streams - 0-$20^{\circ}C$) and at stream temperatures in the winter (0-$4^{\circ}C$).

The effect of maceration is to soften leaf tissue and eventually to break apart the cells of the epidermis and mesophyll so that the CPOM ($>$ 1mm) is reduced to FPOM ($<$ 1mm) (Suberkropp & Klug, in press) These studies have not explored the extent to which other polysaccharidases are elaborated by aquatic Hyphomycetes and which may become active as pectic degradation proceeds. Evidence that hemicelluloses and celluloses are processed slowly and uniformly with time and that their percentage remains constant in relation to other leaf components is provided by Suberkropp et al (1976). This suggests that a complex sequence of polysaccharidases is acting on the degradable components of the leaf tissue. Further evidence of a complex of polysaccharidases at work is given by Suberkropp & Klug (1978) who found that the FPOM released from leaf tissue through maceration by *T. marchalianum* contained a lower percentage of the structural constituents, cellulose

and hemicellulose, than the intact tissue.

Without concrete evidence of the full range of the enzymic capabilities of the microbes associated with a unit-community of a resource like leaves, it would be unwise to make generalizations about the nature of their competitive or synergistic inter-relationships. This study has shown that the seven species of aquatic Hyphomycetes tested were all capable of elaborating pectinases. The four species from which these enzymes were purified and characterized proved to have a formidable array, each different from one another, and characteristic of the individual species. When each species was inoculated onto leaf strips, they were all found to skeletonize the strips in about the same time. Yet in the field study, only one species, T. elegans, was dominant almost throughout on both oak and alder leaves, having initially outcompeted another isolate, M. angulata. The other two isolates investigated, A. tetracladia and T. splendens were infrequently or rarely encountered. It is possible that the initial colonization pattern of T. elegans and M. angulata is based on the differences in action pattern between the two PTE's of both. Exo-enzymes act more slowly than endo-enzymes and the exo-PTE of T. elegans may result in a slower growth-rate than other species and make it a more persistent colonizer. Triska (1970) found T. elegans to be an early colonizer of freshly-fallen leaves is his study of Linesville Creek. But early colonizers are generally replaced by succesive dominant species, e.g. F. curvula in Suberkropp & Klug's study (1976). On both oak and alder leaves in this study, T. elegans persisted as the dominant species. It is possible that it may be less palatable to grazing aquatic invertebrates than other species. Bärlocher & Kendrick (1973b) found that Gammarus pseudolimnaeus had marked species preferences in its fungal diet.

If fungal food preferences of aquatic invertebrates is not the governing factor in determining the pattern of dominance in unit-

communities of the fungal flora on leaves discussed under the Field Study, then physiological explanations suggest themselves. The initial colonization pattern may be based on competitive interactions which would include the affinity or specificity of the organism for the resource. Between oak and alder leaves there is a marked similarity in the colonizing species - 14 in common out of 16. But the resident group of species in the stream are likely to be those which are capable of colonizing both leaf species, as the riparian vegetation along the catchment area is mainly oak and alder. Gönczöl's study (1975) showed a marked difference in the fungal flora on Fagus sylvatica and Carpinus betula, at one site and on Alnus glutinosa at a lower site on the same stream.

T. splendens may be rare on leaves in our study because its preferred substrate is wood. Jones (in press) has shown that T. splendens can elaborate cellulases and that it is capable of causing soft rot in wood. It was found on wood by Willoughby & Archer (1973) and by Saunders and Anderson (1979) on wood-blocks. It was one of the 6 species common to all sizes of wood blocks. So was A. tetracladia which was also on Willoughby's list. Jones (in press) detected cellulolytic activity in this species but found it unable to degrade wood, though it was subject to the same experimental conditions as T. splendens.

This suggests that there may be ecotypic variations in species from different streams and that dominance is gained over other species by the fine adjustment of the dominant species to particular environmental conditions. There is some evidence from this study to support the idea that T. elegans in the R. Bourne may be an ecotypic variant. Firstly, this isolate was unable to grow at pH5 and though T. elegans is not found in the species lists for the acidic Dartmoor streams, investigated by Iqbal & Webster (1977), it was found in Narrator Brook - pH 4.2 (Sanders & Anderson 1979). Secondly, in a preliminary experiment described above, of the five ionic environments investigated,

T. elegans grew significantly better in water from the stream than in other ionic media. The only factor that could be identified as contributing to this was the concentration of calcium ions in the stream, together, possibly, with low phosphate levels. The pH of the stream does have a significant effect on the species present and seems to work through the enzymes elaborated by a given species. From Experiment II one would expect T. splendens, A. tetracladia and V. elodeae all to be able to support acidotrophic conditions since they have a robust system of PG's and can grow at c.pH5. They are indeed fairly common species in the Dartmoor stream, despite the fact that these streams have few trees along the banks. The are found too, in the species list for Narrator Brook.

There are, in general, fewer species of aquatic Hyphomycetes in oligotrophic than in eutrophic waters. This, in turn, affects the numbers of aquatic invertebrates found in streams of differing nutrient status. Egglishaw (1964) found that there was a close correlation between benthic invertebrates and the distribution of plant detritus in Shelligan Burn and noted that the stream had many more animals than another that was chemically poorer. Egglishaw (1968) went on to suggest that the calcium concentration in stream water was an important factor governing the rate of breakdown of plant material, and that the higher the concentration of calcium, the faster the rate of plant degradation. He suggested that calcium concentration affected the rate of microbial metabolism. This study offers an explanation in support of Egglishaw's proposition.

In streams with a pH of 6.5 or higher, the microbial population degrading plant material would be operating on PGTE (bacteria) and PTE (fungi) enzymes. The former have an absolute dependence on Ca^{2+} (Garibaldi & Bateman 1971); the latter are stimulated by Ca^{2+} if they have alkaline pH optima. Cooper (1974) found that in the case of two

PTE's of fungal origin with pH optima c.9, a rise in concentration of Ca^{2+} from 0.001M to 0.01M resulted in a 19-fold enhancement of enzyme activity. The stimulatory effect of Ca^{2+} ions on the growth of T. elegans at pH7 has been noted above. Other studies reviewed by Macan (1974a) support Egglishaw's observations.

However Minshall & Minshall (1978) in a study of two sites on the River Duddon reviewed in Chapter 1, dispute the proposition that it is the calcium content of the water which accounts for the abundance and distribution of benthic invertebrates in a stream. From their experiments they concluded that the concentrations of any of the nutrients present in the water might be as important as that of calcium, and that in the case of the distribution of Gammarus pulex, the controlling factor was probably potassium concentration. They also concluded that it was not the quality of the food provided to the animals that affected their survival and distribution, but a direct chemical effect of the water on the animals. This seems a sweeping statement in the face of slender evidence from one species of invertebrate. Ecological communities are based on a number of inter-related factors and though these can, to some extent, be examined individually by laboratory experiments, the essence of the natural situation is the dynamic relationship between the plant and animal communities within a given physical and chemical environment. Water quality must have a direct effect upon members of the animal population, just as it does upon the microbial population; and no doubt the concentrations of all dissolved ions are to some extent important in the composition and balance of flora and fauna. But it cannot be concluded from the study of Minshall & Minshall that calcium ion concentration does not effect the metabolism of microbes on plant litter. An explanation has been offered above for its stimulatory effect which would lead to the quicker breakdown of plant material in streams of high calcium concentration and thus, through the food chain provide resources to support greater numbers of aquatic

invertebrates than could calcium-poor streams. This proposition might be tested by a laboratory experiment similar to that conducted to test leaf-tissue maceration. At pH7, species known to elaborate PTE's could be tested at varying Ca^{2+} concentrations for their capacity to macerate leaf tissue.

The field of microbial interactions in leaf degradation in aquatic ecosystems needs further investigation. Competitive interactions may determine the initial composition of dominant fungal colonizers, but thereafter their relationship may be synergistic, in the sense that the individual enzymic complement of each enables it to fill a niche which does not overlap with those of other species. Bärlocher & Kendrick (1974) found that when they inoculated five species of aquatic Hyphomycetes onto leaf discs, decomposition proceeded faster than it did for any species individually. It has been suggested above, that antagonistic phenomena between species may influence the pattern of dominance, and Webster (1970) has demonstrated the way in which these may increase during the decomposition of herbivore faeces. Antagonism may result in the overall decline in the number of species observed of oak and alder leaves as decomposition proceeds from a peak of colonization. It is noteworthy in this respect, that when levels of T. elegans, the dominant species, declined, other 'subject' species in the association increased in percentage frequency.

The relationship between fungi and bacteria which can metabolise the same pectic substrate may be synergistic or competitive. This study merely records that both are present on the same resource, and that their colonization pattern illustrates their dependence on the same substrate.

It is easier to dissect an ecological community into its parts than it is to investigate the dynamics of the natural situation. The erection of 'functional' groups based on nutritional capabilities may have a limited validity. It is possible, for example, that in stream

conditions T. elegans may not be exploiting its pectolytic capacity due to catabolic repression. It may be acting as a 'sugar fungus' living off the excess proceeds of the pectolytic bacterial metabolism.

Further investigation of the enzymic capabilities of aquatic Hyphomycetes is required in the context of the unit-community, along with the exploration of possible ecotypes. Interactions between species of fungi, including groups other than aquatic Hyphomycetes, and between fungi and bacteria need looking into. From there it would be possible to investigate the role of fungal enzymes in the digestive systems of aquatic invertebrates. Martin (1979), for example, has reported that cellulases active in the gut of termites are of fungal origin. Further physiological investigations would help to elucidate the phenomenon of successional patterns of colonization by aquatic Hyphomycetes, which represent dynamic changes in species associations.

ACKNOWLEDGEMENTS

I am very grateful to the Natural Environmental Research Council for the award of a Special Competition Postgraduate Research Scholarship.

Without the understanding co-operation of my family, I could not have carried out this research and I thank my husband, Anthony, and my children, Amy and Daniel, for all their kindness and support on the domestic front.

Professor R. K. S. Wood F.R.S. and his associates at Imperial College, particularly Dr. F. Cervone, provided excellent advice on techniques for separating, purifying and characterizing pectinases. I thank them warmly.

Professor J. Webster and his associates at Exeter University were most helpful on matters concerning aquatic Hyphomycetes.

Professor C. T. Ingold very kindly lent me reprints and Professor E. B. G. Jones and Professor P. Albersheim generously sent me material in press.

Professor R. H. Vaughn of the University of California, Davis, sent a gift of unsaturated digalacturonic acid which was greatly appreciated.

Mr. J. G. Brennan of the National College of Food Technology gave much time to helping me use the Instron tensiometer. I am most grateful to him.

Dr. J. H. Evans and Dr. Janet Kerr of this department were generous with advice when it was needed.

Dr. P. M. Dey of R. H. C. Biochemistry Department was very helpful in discussing work on enzymes.

The technical experts at Pharmacia and LKB deserve all my praise.

I record my thanks to the technical staff of this department; to Ms. Carole Spurdon in particular, who gave so generously of her time

and skills; and to Mr. Bruce Ellis and Mr. Stephen Janes for making and adapting equipment.

Finally, and most of all, I thank my supervisor, Mr. Peter A. Dixon. His optimism, enthusiasm, encouragement and sound advice were unfailing and have been very greatly appreciated. I thank him, too, for reading the manuscript of this thesis.

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APPENDIX

Appendix Table 1 Scum Samples Species Present

	10/10	7/11	22/11	13/12	13/1	9/2
Alatospora acuminata			/	/	/	/
Angullospora longissima	/	/	/	/	/	/
Articulospora inflata					/	/
A. tetracladia	/	/	/	/	/	/
Clavariopsis aquatica	/	/	/	/	/	/
Clavatospora longibrachiata					/	/
C. stellata			/	/	/	/
Culicidospora aquatica			/		/	/
Flagellospora curvula	/	/	/	/	/	/
Lemonniera aquatica			/	/	/	
Lunulospora curvula		/	/			
Mycocentrospora acerina	/					
M. angulata	/	/	/	/	/	/
Tetrachaetum elegans	/	/	/	/	/	

T. marchalianum						/
Tetracladium setigerum		/				
Tricladium chaetocladium			/	/	/	/
T. patulum			/			
T. splendens	/		/	/	/	/
Triscelophorus monosporus	/		/		/	
Varicosporium elodeae			/		/	/
No. of species	9	8	16	11	16	14

Total spp = 21

Appendix Table 1 contd.

% Frequency. Filter Counts.

Species	5/12	19/12	3/1	17/1	30/1	13/2	27/2	13/3	4/4	18/4	3/5	15/5
Alatospora acuminata	1	5	15	10	10	7	16	25	17.5	22	22	5
Articulospora tetracladia	6	1	4	6	6	3	1		1.5	2		
Clavariopsis aquatica	10	22	10	21	15	7	47	31	22	29	7	40
Clavataspora longibrachiata			18	12	23	5	5	8	6	7	14	4
C. stellata	4					2		2				
Culicidospora aquatica			2	1	3	2			1.5	2	7	2.5
Filiform*	19	45	37	29	25	42	16	14	14.5	14	22	18
Heliscus lugdunensis											7	
Lemonneira aquatica	7	6	5	1			1			2		
Mycocentrospora acerina											7	
Tetrachaetum elegans	30	19	5	3	2	5		1	1.5	2		4
Tetracladium marchalianum			0.5							2		1
T. setigerum								2	1.5			1
Tricladium angulatum	1						1	1				2.5
T. anomalum							2					
T. chaetocladium			2	3	10	19	7	7	22	9	7	12
T. splendens	10	2	4.5	5	6	5	4	9	12	7		9
Triscelophorus sp.	1		2	3			1			2		
Varicosporium elodeae	11		3	6		3					7	1
No. of species*	13	9	15	14	11	13	13	12	12	14	11	14
Spores/litre	142	430	792	500	211	200	337	350	136	112	47	166
					F						F	

*Filiform includes:-
Flagellospora curvula
Anguillospora longissima
Mycocentrospora angulata
The latter two species predominate: = 2spp. in the total.

Appendix Table 2.
Percentage frequency of individual species found in filtered stream water. (500mls)

F = Floods

Total spp. = 21

Appendix Table 3. Spore Counts from Submerged Leaves. % Frequency.

OAK

Started 21/11/77	5/12	19/12	3/1	17/1	30/1	13/2	27/2	13/3	4/4	18/4	15/5	25 weeks
Alatospora acuminata			18	5	15	31	47	32	6	15	4	
Articulospora tetracladia		4		5	11	1	3.5		16	5		
Clavariopsis aquatica	SAMPLE LOST	12			3	0.3	2.5		3	16	4	
Clavatospora longibraciata			1	1	6							
C. stellata				1								
Culicidospora aquatica					4.5	0.3						
Filiform		44	15	12	3	0.3	1		35	3		
Flagellospora curvula						3		2.5			8	
Lemonniera aquatica		16	7	13	9	5	7	11.5				
L. terrestris					1	1	11					
Tetrachaetum elegans		24	58	56	21	51	37	25	26	48	32	

Tricladium chaetocladium									10	2	4
T. eccentricum											24
T. splendens				1	12	2			3	5	
Triscelophorus sp.				3	3	6	2	29		2	20
Varicosporium elodeae				2	1.5						
No. of species		5	6	11	13	10	7	5	7	9	8
Spores/g dry wt leaf		1470	17700	58000	35200	150800	75350	19300	5400	97900	3700

Total = 16

Appendix Table 3 contd. OAK.

ALDER I

Appendix Table 3 contd.

Spore counts from submerged leaves. % Frequency.

Started 21/11/77	5/12	19/12	3/1	17/1	30/1	10 weeks
Alatospora acuminata	SAMPLE LOST		9			
Articulospora tetracladia		8	5		25	
Clavariopsis aquatica			2			
Clavatospora longibrachiata			3		12.5	
Culiciospora aquatica			2			
Filiform		38	8	10		
Lemonniera aquatica		15	11	20	25	
L. terrestris			1	5	12.5	
Tetrachaetum elegans		31	59	65	12.5	
Tricladium splendens					12.5	
Varicosporium elodeae		8				
No. of species		5	9	4	6	Total = 11
Spores/g dry wt of leaf		2,900	10,500	5,300	1,070	

Appendix Table 3 contd.

Spore counts from submerged leaves. % Frequency

ALDER II

Started 5/12/77	19/12	3/1	17/1	30/1	13/2	27/2
Alatospora acuminata		6		14	7	13.5
Articulospora tetracladia		5	6		8	4.5
Clavariopsis aquatica		2				
Clavatospora longibrachiata		1			2	
Culicitospora aquatica				2		
Filiform	60	3	10	6	8	
Flagellospora curvula					5	
Lemonniera aquatica	6	21	29	10	7	13.5
L. terrestris		5	10	29	18	
Tetrachaetum elegans	28	52	42	29	39	57
Tricladium chaetocladium				2		
T. splendens	3			8		4.5
Triselophorus sp.		3	1		3	
Varicosporium elodeae	3	2			3	7
No. of species	5	10	6	8	10	6
Spores/g dry wt leaf	1950	23300	8550	4670	8130	37000

12 weeks

Total = 14

ALDER III

Started 19/12/77	3/1	17/1	30/1	13/2	27/2	13/3	12 weeks
Alatospora acuminata			2	25	21	15	
Articulospora tetracladia			2	1			
Clavatospora longibrachiata			1	1			
Culicidospora aquatica			1	3			
Filiform	94	2	7	5			
Lemonniera aquatica	2	9	14	16	8	8	
L. terrestris		9	18	8	3	7	
Tetrachaetum elegans	4	78	51	32	65	68	
Tetracladium marchalianum			1				
Tricladium angulatum				5			
T. chaetocladium			1				
T. splendens		1	2	3			
Triscelophorus sp.				1	3	2	
Varicosporium elodeae		1					
No. of species	3	6	11	11	5	5	Total = 14
Spores/g dry wt leaf	143750	102500	40300	17500	31500	10900	

APPENDIX TABLE 4

Mean dry-weight values (3 replicates) with standard errors for Experiment III

Articulospora tetracladia

Day	D.Wt(pH7)mg	(pH5)
4	4.83 ± 0.34	
5	6.93 ± 0.21	13.43 ± 0.27
6	15.10 ± 0.49	
7	19.40 ± 0.32	33.07 ± 1.5
8	20.40 ± 0.12	
9	37.70 ± 0.38	53.63 ± 0.39

Articulospora tetracladia repeat

Day	D.Wt(pH7)mg	pH5
4	3.43 ± 0.24	
5	7.67 ± 0.34	12.77 ± 0.54
6	8.70 ± 0.20	
7	20.23 ± 0.18	35.90 ± 1.8
8	22.87 ± 0.15	
9	36.10 ± 0.30	48.00 ± 0.38

Tricladium splendens

Day	D. wt mg (pH5)
3	3.92 ± 0.12
4	4.28 ± 0.15
5	9.76 ± 0.21
8	28.72 ± 0.30

Varicosporium elodeae

Day	D. wt mg (pH5)
4	22.40 ± 0.93
5	57.87 ± 0.84
6	68.20 ± 1.05
7	77.90 ± 0.81

Tetrachaetum elegans

Day	D. wt mg (pH7)
5	4.87 ± 0.13
7	7.63 ± 0.27
9	27.70 ± 0.71
11	75.43 ± 0.78

Tetracladium setigerum

Day	D. wt mg (pH7)	(pH5)
4	17.0 ± 0.35	
5	20.70 ± 0.11	64.50 ± 0.81
6	20.83 ± 0.06	67.07 ± 1.07
7	17.37 ± 0.06	
9	16.40 ± 0.27	
12	16.30 ± 1.15	

Lemonniera aquatica

Day	D. wt mg (pH7)	(pH5)
7	3.87 ± 0.10	
8		51.13 ± 1.06
9	7.37 ± 0.38	67.50 ± 0.51
10	8.60 ± 0.40	
11	12.50 ± 0.20	
12	52.03 ± 1.18	

Mycocentrospora angulata

Day	D. wt mg (pH7)	(pH5)
7	4.80 ± 0.26	
8	8.07 ± 0.26	
9	15.47 ± 0.49	18.27 ± 0.73
10	24.70 ± 0.12	
11	21.10 ± 0.12	

Appendix Table 4 contd.

APPENDIX TABLE 5

Raw data for dry weights (mg) of mycelium of Tetrachaetum elegans from Experiment IV

Treatments - 3 replicates of each. Carbon source: 1% (w/v) Napp. pH7-8.

A - Flask medium + distilled water (Control)

B - Flask medium + filtered stream water.

C - Yeast extract + 50% d. water; 50% mineral salts soln.

D - Flask medium + 0.5mM $CaCl_2$

E - Flask medium + 1.0mM NaCl

Ag - As A + 0.025% glucose

Bg - As B + 0.025% glucose

Cg - As C + 0.025% glucose

Dg - As D + 0.025% glucose

Eg - As E + 0.025% glucose

	A	B	C	D	E
	39.4	79.1	29.4	68.5	33.8
	48.9	74.3	33.4	68.4	39.2
	48.8	76.6	31.4	69.4	31.1
Σ	137.1	230.0	94.2	206.3	104.1
	$\bar{x}$ = 45.7	$\bar{x}$ = 76.6	$\bar{x}$ = 31.4	$\bar{x}$ = 68.8	$\bar{x}$ = 34.7

	Ag	Bg	Cg	Dg	Eg
	43.3	66.2	10.7	51.6	60.8
	58.7	64.0	12.9	53.2	53.6
	51.7	67.7	16.1	51.6	51.8
Σ	153.7	197.9	39.7	156.4	166.2
	$\bar{x}$ = 51.2	$\bar{x}$ = 65.9	$\bar{x}$ 13.2	$\bar{x}$ 52.1	$\bar{x}$ 55.4