THE DIGESTIVE PHYSIOLOGY AND FOOD REQUIREMENTS OF *GAMMARUS PULEX* (L) (AMPHIPODA)

BY

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ABSTRACT

Several freshwater invertebrates, including Gammarus pulex (L), were shown to possess gut cellulase activity. The nutritive physiology of G.pulex was further investigated in order to elucidate the manner in which plant detritus is utilised as food. This was approached by studying physical and chemical conditions in the gut and the effects of test diets on ingestion, assimilation and digestive enzyme secretion. The extents to which some of these diets and others supported growth and survival were also examined. The results of an investigation into the digestion of decomposing leaf litter and fine detritus by commercial enzymes are described.

Cellulase activity in the midgut glands was endogenous. The pH of the midgut gland fluid was little affected by diet and, along with that of the foregut, was well suited to carbohydrate digestion. The pH at the posterior end of the midgut was better suited to protein digestion. The tract transit time was normally independent of the rate of ingestion. Intact plant cells that were not broken open mechanically were not digested chemically in the gut. The occurrence of enzyme inhibitors in leaf litter was investigated.

The assimilation efficiency and rates of ingestion and enzyme secretion varied with different diets, but were not correlated with the nutritive values of the diets. Palatability and physical ease of ingestion controlled rates of ingestion and, probably, enzyme secretion. The assimilation efficiency seemed to depend on the surface area of the gut contents available for enzymic attack. Growth and survival were affected by the water chemistry and the initial size of the animals used, as well as by the nature of the diet. Fine particulate detritus was of little nutritive value and the nutritive value of intact elm litter depended on the amount of microbial growth which it supported. Food nutritive

value was not directly related to protein content, although the microbial flora was important in furnishing nitrogen requirements, at least during certain critical phases of growth.

An energy budget was calculated and food utilisation in G.pulex and other aquatic invertebrates discussed.

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CHAPTER 1

INTRODUCTION

a) Detritus in the Freshwater Environment.

The production of organic matter in the freshwater environment is dependent upon the existence of certain well known cycles which recirculate various essential elements (Hynes, 1970), and the productivity of the environment may be considered a function of the rate of cycling, which in turn is dependent on various environmental parameters. Such cycles involve the synthesis of complex organic materials from simple minerals by plants, and their eventual return to the simple state by the action of micro-organisms, sometimes after incorporation in to animal tissue. The terrestrial and some aquatic environments support much plant life, and this mediates the initial synthesis of the great majority of the organic matter found in them. Most estuaries, rivers and streams however support only sparse plant life and much of the plant material available as food to heterotrophs in those environments was initially synthesised by terrestrial plants (Darnell, 1961, 1964, 1967 ; Hynes, 1963, 1970 ; Fisher and Likens 1972 ; Cummins 1973). Such material makes up the bulk of the detritus that is found in those environments. This has been described by Cummins (1973) as non-living particulate organic matter and the non photosynthetic micro-organisms which are invariably associated with it.

Many authors have shown that such allochthonous plant material is frequently ingested by freshwater invertebrates (e.g. Slack, 1936 ; Hynes, 1941 ; Badcock, 1949 ; Jones, 1950 ; Hanna, 1957 ; Scott, 1958 ; Brown, 1961 ; Minshall, 1967 ; Koslucher and Minshall, 1973). Even in communities where algae predominate in the diet of the invertebrate fauna, many species still consume significant amounts of detritus (Percival and Whitehead, 1929 ; Coffman et al, 1971). The quantitative importance of the various components of the diet seems usually to depend upon their availability in the environment (Cummins, 1973) but aquatic macrophytes, even when available, do not seem to be eaten (Koslucher and Minshall, 1973). Further literature concerning the food of 71 species of aquatic insects from 7 orders has been reviewed by Cummins (1973). In only one order (the Odonata) was no detritus ingested, and in 61 of the species mentioned detritus formed a part of the diet.

The importance of detritus as a food source is also apparently reflected in the effect which its distribution on the stream bed has on the distribution of invertebrates (Egglishaw, 1964 ; Mundie et al, 1973).

The manner in which detritus contributes to the production of organic matter at higher trophic levels depends largely on its nutritive value to the animals which consume it. Much of the allochthonous plant material found in the freshwater environment is deciduous tree leaf litter. This generally contains a high proportion of cellulose, hemicellulose and lignin, but little protein (Kaushik and Hynes, 1971 ; Jensen, 1974). The nutritive value of such material may be limited, and dependent upon its chemical and physical composition and the physiological abilities of the animals which consume it. During its decomposition however, leaf litter is invaded by saprophytic fungi and bacteria which may themselves have considerable nutritive value to any animal which ingests them.

The productivity of the environment may thus be controlled to some extent by the relative importance of the saprophytic microflora and the leaf substrate in animal nutrition. If the microflora is of little importance the main factors controlling the rate of detritus cycling might presumably be its origin and the identity of the consuming species. If however the saprophytic microflora is of overriding importance in the nutrition of the consuming animals, factors which may influence the development of the saprophytic population, such as temperature and water chemistry, may have a great influence on the rate of detritus cycling.

Known correlations between water chemistry, the rate of breakdown of organic matter, the biomass of invertebrates, and the rate of production of fish in streams of differing chemical composition (Egglishaw and Morgan, 1965 ; Egglishaw, 1968, 1972) suggest the importance of the saprophytic microflora in animal nutrition. Little direct evidence is available however concerning the nutritive value of detritus to freshwater invertebrates nor, particularly, concerning their physiological abilities to utilise it. This thesis is an attempt to provide some such information.

The decomposition of leaf litter in freshwater has been shown to follow a broadly similar pattern to that described by terrestrial ecologists (Mathews and Kowalczewski, 1969 ; Kaushik and Hynes, 1971). When leaf litter falls into fresh water there is an initial loss of soluble substances, including fatty and amino acids (Nykvist, 1963). This is complete within 1-4 days and is little affected by temperature. Decomposition, as measured by weight loss, continues at a slower rate after this period. Mathews and Kowalczewski (1969) considered that leaf litter in the Thames would disappear after about one year. Growth of micro-organisms results in an uptake of nitrogen from the water, and a concomitant increase in protein content which may reach a maximum after 2-16 weeks depending on the conditions and species of leaf. After this the protein content may decline. Kaushik and Hynes (1968) showed that the increased protein content is associated with the growth of fungi, which are the organisms most responsible for the initial decomposition of leaf litter. Iversen (1973) found that the total microbial nitrogen on decomposing beech leaves comprised only 1-4% of the total leaf nitrogen, and suggested that most organic nitrogen was released and deposited on the leaves by micro-organisms. The rate of decomposition, the extent of nitrogen uptake, and the extent of the initial leaching of soluble substances may vary in different species of leaves. Kaushik and Hynes (1971) found that these processes were greater in elm and maple leaves than in oak, alder or beech leaves. Decomposing elm leaves have been used in the present work because of the marked change in protein content they may show during decomposition, and their great palatability to Gammarus.

Decomposition may be limited if there are insufficient inorganic nutrients present in the water. Kaushik and Hynes (1971) found that the greatest uptake of nitrogen by leaves in small bowls occurred when nitrogen and phosphorus were added to the water in amounts greater than those normally encountered in unpolluted waters. Litter placed in streams however, showed a similar uptake of nitrogen. Leaf litter placed in the Thames also showed an increased nitrogen content (Mathews and Kowalczewski, 1969). Egglishaw (1968) showed that the rate of break-down of rice grains in different streams was related to the calcium

concentration in the water, and in a later paper (Egglishaw, 1972) showed that the rate of breakdown of cotton strips in various streams was most closely correlated with the nitrate content of the water.

The ability of an animal to obtain nutrients from decomposing leaves may depend upon its ability to destroy their cellulose cell walls. Although the guts of some invertebrates are equipped with an armature for triturating ingested substances, it seems unlikely that more than a small percentage of the cell walls will be broken open mechanically. Utilisation of the cell contents may therefore depend on the presence of cellulose digesting enzymes in the gut of the consumer. However Waldbauer (1968) lists many terrestrial leaf eating insects which can assimilate a large proportion of the weight of the material they ingest, although they are not normally considered to possess a cellulase (Wigglesworth, 1965).

It has long been considered that the digestive enzymes secreted by most animals do not include a cellulase (e.g. Cummins, 1973). The work of several authors however (Tracey, 1951 ; Yokoe and Yasumasu, 1964 ; Crosby and Reid, 1971 ; Elyakova, 1972) has shown that enzymes which hydrolyse soluble cellulose derivatives are widespread in the Mollusca, Annelida and Crustacea, although the presence of such enzymes does not necessarily mean that cellulose itself can be digested (Halliwell, 1959). It is not known whether the apparent ubiquity of such enzymes within certain invertebrate groups extends to the Insecta, which is one of the most important groups in fresh water. Some work has recently been published concerning the occurrence of cellulase in certain freshwater invertebrates (Bjarnov, 1972). He found that Gammarus pulex could digest cellulose, and the work described here concerns the ability of that species to utilise detritus as food.

In order to investigate the distribution of cellulase in other freshwater invertebrates, a preliminary survey of its occurrence has also been carried out, the results of which are shown in Chapter 3.

The utilisation of foods by heterotrophic animals involves several different processes and thus may be studied and measured in different ways. Ingestion is followed by digestion and absorbtion (assimilation). Unassimilated food is egested and absorbed nutrients

may be immediately utilised for energy production, stored as an energy reserve, utilised to form new tissues (growth) or excreted. Food utilisation in G.pulex has been studied in this thesis by an examination of the ways in which certain diets are ingested, digested and assimilated, and of the extents to which these and other diets support growth.

In some animals complex molecules may possibly be absorbed by the gut lumen (Dadd, 1970) but the absorbtion of nutrients by crustaceans is doubtless dependent upon the secretion of extra-cellular digestive enzymes which catalyse the breakdown of complex foodstuffs to small molecules. The efficiency of the digestive system depends on the nature of the enzymes secreted and upon other chemical and physical conditions in the gut including pH, temperature, the length of time which food remains in the gut, the degree of mechanical breakdown to which it is subjected and the presence of any inhibitory substances contained in the food. The significance of these factors in the digestive physiology of G.pulex is described in Chapter 4.

As assimilation is largely the result of digestion, the assimilation efficiency may be considered as a quantitative measure of the efficiency of digestive processes. Both digestion and assimilation might be expected to be affected by the rate of ingestion, so a study of ingestion and the factors which control it, and of assimilation, is described in Chapter 5.

Assuming that the strengths of the various enzymes in the gut are important in determining the efficiency of the digestive processes, any effect that the nature of the diet had on enzyme secretion would be expected to influence the extent to which that diet was assimilated. It is known that in mammals the secretion of specific enzymes may be induced by the presence of their substrate in the diet (Bell et al, 1968) but there is little to suggest that this is the case in invertebrates. Little seems to be known of any relationship between diet and secretion in crustaceans and results of investigations into this relationship in G.pulex are described in Chapter 6.

The ultimate arbiter of the nutritive value of any food is its ability to support growth and survival, and much knowledge concerning insect food requirements has been obtained from studies of their growth with defined diets under sterile conditions. Little work of this kind has been done on crustaceans (Dadd, 1970a) with the exception of extensive studies on Artemia salina (Provasoli and Shiraishi, 1959 ; Provasoli and D'Agostino, 1969), some preliminary investigations on the isopod Oniscus asellus (Beerstecher et al, 1954 ; 1954a), and recent investigations of various decapods of commercial importance (e.g. Castell and Budson, 1974). Several studies of growth with natural diets have been carried out, e.g. Broad (1957), Regnault (1969), Roberts (1974), and as such studies indicate which foods available in the environment are of nutritive value, this approach has been adopted here (Chapter 7). The nutritive values of individual species of fungi for Gammarus pseudolimnaeus have been studied by Barlocher and Kendrick (1973) and the relative importance of bacteria and fungi in the nutrition of G.minus has been examined by Kostalos (1971). The approach adopted here is dependent on the hypothesis suggested by the literature that the increased protein content of decomposing plant material may lead to an increase in its nutritive value. Growth has been considered in respect of the origins and protein contents of the diets offered, and of any changes in protein content which may occur as a result of microbial colonisation.

As microbial growth and protein enrichment have been stimulated here by the addition of high concentrations of inorganic nutrients, experiments were also carried out (Chapter 8) which were designed to determine whether such protein enrichment does occur in plant material decomposing in the field,and to determine the extent to which changes in composition affect the fraction of the diet which is likely to be assimilated. Most detritus feeders assimilate only a small proportion of the food they ingest (e.g. Gere, 1956). This fraction presumably represents the most easily digestible part of the diet, and that which would be most rapidly invaded by micro-organisms if the diet were not ingested. Slight changes in the total chemical composition of the diet, if attributable to changes in the composition of this fraction, may

thus result in relatively large changes in the amounts of nutrients released by enzymic hydrolysis in the gut. In Chapter 8 changes in the protein and carbohydrate content of leaves decomposing in the field were studied by partial digestion with commercial enzymes.

Information concerning the digestive physiology of C.pulex has previously been published by Willer (1922), Agrawal (1965) and Bjarnov (1972), and a study of ingestion, assimilation and growth has recently been published by Nilsson (1974).

b) The Biology of Gammarus pulex

G.pulex (Class Crustacea, Sub Class Malacostraca, Order Amphipoda, Sub Order Gammaridea, Family Gammaridae) is a common inhabitant of fresh waters throughout much of Western Europe (Pinkster, 1972). It occurs throughout the British mainland except in the North of Scotland, but is absent in most of Ireland and on islands off the coast (Hynes, 1954 ; Hynes et al, 1960). It is an oviparous animal which retains its eggs in a brood pouch until they hatch. Hynes (1955) has elucidated its life history in the field. He showed that breeding occurs throughout most of the year with a resting period in the early winter. The young take from 3 to 7 months to mature depending on the temperature, and the total life span is little more than a year. The females lay 5 to 6 batches of eggs during their lifetime, with an average of 16 eggs per batch. An extensive literature exists concerning many aspects of the biology of the fresh water Gammaridae which will not all be reviewed here.

The significance of a study of the nutritional physiology of any animal depends upon the diet which it normally consumes in the field. This has not been examined here but there are several references in the literature to the food of G.pulex and other species of fresh water Gammaridae which give a fairly consistent view of their normal dietary habits.

The gut contents of G.pulex were first examined by Haempel (1908) who looked at 100 specimens and found mostly plant detritus, but also some green algae, insect larvae and worms. Willer (1922a) looked at the gut contents of groups of 25-30 animals from contrasting habitats in France and Poland. He concluded that the tissues of higher plants were the most important food and that filamentous algae may serve as an additional food source. He also found occasional diatoms and pieces of moss. Insect and entomostracan remains formed a very minor part of the gut contents. His data clearly show that anything available is likely to be eaten, for instance he found pollen grains from Pinus sylvestris in the guts of animals taken from a lake near a pine forest. Further evidence of the catholic tastes of Gammarids was provided by Dahl (1915) and Clemens (1950) who mentioned that cotton fish nets were

eaten. Percival and Whitehead (1929) found that the guts of G.pulex from rivers in the West Riding of Yorkshire contained detritus and Chironomidae. The only author who has found no vegetable material in the guts of G.pulex is Margalef (1948) who listed the food of Spanish specimens, in decreasing order of abundance, as Cyclops, adult Diptera, Ephemeroptera nymphs and other insects. Bjarnov (1972) also considered G.pulex a carnivore but gave no evidence. Badcock (1949) looked at 17 specimens from tributaries of the Welsh Dee and found that decomposing leaves and other plant tissue was the main food. Diatoms, desmids, other algae, chironomid larvae and ephemeropteran larvae were also present. Dunn (1954) examined the gut contents of 50 specimens from Lyn Tegid, Merioneth. From her histogram showing the percentage of animals containing various foods the following figures were derived: detritus 66%, diatoms 37%, higher plants 23%, other algae 13%, larval insect remains 4%, unidentified 3% and empty 30%. Hynes (1954) found that of 50 specimens from the Isle of Man, 52% contained only vegetable matter, 34% contained both animal and vegetable matter and 14% contained animal matter only. The vegetable matter was mainly vascular plant tissue with occasional algae and moss, and the animal matter was entirely arthropod. Ladle (1974) quotes unpublished work by Scorgy which showed that the food of G.pulex in the River Lambourn varied during the year according to what was available. Fine organic detritus made up the bulk of the food at all times, with a fairly constant but small proportion of autochthonous leaf tissue. A small percentage of animal material was ingested throughout the year and some algae were ingested during the autumn. A significant proportion of allochthonous leaf tissue was eaten between October and January.

From these papers it is apparent that the food of G.pulex mainly comprises the tissues of higher plants and unrecognisable organic detritus. An appreciable proportion of algae is often ingested but animal food only rarely forms a large part of the diet, although it is almost always present. The importance of higher plant tissues may be underestimated here as some authors have used the term detritus to cover any non living plant material, whereas others have used the term solely to cover unrecognisable organic debris. In papers where detritus

is mentioned and higher plant tissues are not (Haempel, 1908 ; Percival and Whitehead, 1929), the latter may still have formed a large part of the diet. The source of the plant tissue is not always stated but, at least in flowing waters, it must be largely of terrestrial origin as aquatic macrophytes are comparatively rare in lotic environments (Hynes, 1963).

Studies of the gut contents of G.pulex are generally in agreement with observations of their feeding habits, and with studies of the gut contents and feeding habits of other species. The gut contents of G.lacustris taken from lakes in Norway have been examined by Dahl (1915), and from lakes in Eastern Europe by Ermolaeva (1962) and Deksbakh and Sokolova (1965). Algae apparently formed a major part of the diet but plant detritus and animal remains were also found. Plant detritus was apparently less important in the diet of the Eastern European than in that of the Norwegian animals. Minshall (1967) found that the guts of the American species G.minus contained 94% allochthonous leaf detritus, 4% animals, 1% green algae and 1% diatoms, and Kostalos (1971) found the same species to contain mainly higher plant tissues and a small proportion of diatoms. She also noted a few fungal hyphae and spores. Hynes (1954) found that the gut contents of G.duebeni were similar to those of G.pulex.

Many authors have commented on the feeding habits of the fresh water Gammaridae. Embody (1911) and Clemens (1950) observed various American species feeding on aquatic plants, but Embody noted that the dead leaves of such plants were preferred to the live ones. Mottram (1934) found that several species of water weed were not eaten by G. pulex but two, water cress and water parsnips, were freely eaten. Only the dead leaves were consumed however. Peart (1938) found that young animals would not eat aquatic plants, apple, swede, turnip, potatoes or carrot, although older ones would thrive on such food. Young animals would eat algae, however. Several authors have observed that various species of Gammarus will consume leaf litter both in the field (Haeckel et al, 1973) and in the laboratory (Sexton, 1928 ; Mottram, 1930 ; Le Roux, 1933 ; Clemens, 1950 ; Elton, 1956 ; Bick, 1959 ; Dolling, 1962 ; Kaushik and Hynes, 1968 , 1971 ; Barlocher and

Kendrick, 1973, 1973a; Nilsson, 1974). Bick fed G.pulex fossarum with 15 species of leaves and found that the softer varieties such as hazel and alder were eaten in preference to hard leaves such as oak and beech. Dolling and Haeckel et al both noted that the hardest leaves, including pine needles, were eaten if there was no alternative.

Some of these authors have also investigated the factors which mediate the preference shown by Gammarus spp. for different types of leaves. Kaushik and Hynes (1971) fed decomposing leaves to G.lacustris limnaeus and found that the species which were preferred (elm and maple) were also the ones which showed the highest rate of weight loss and the greatest increase in protein content during decomposition. Barlocher and Kendrick (1973a) showed that the leaves which were most acceptable as food were also those which were invaded most rapidly by fungi, and that pure fungal colonies were eaten in preference to maple leaf discs with no microbial growth. The colonisation of leaves by different species of fungi increased their palatability to different extents, and the normal order of preference for different species of leaves could be reversed by inoculation with appropriate fungal species. Kostalos (1971) and Sedell (1970) have both shown that bacteria are less important than fungi in increasing the palatbility of leaf litter.

Sexton (1928) noted that Gammarus spp. preferred animal to plant food and Sexton and others (Embody, 1911 ; Clemens, 1950 ; Hynes, 1954) have noted their 'cannibal propensities'. Hynes offered G.duebeni and G.pulex several species of molluscs and platyhelminths, but only one species of mollusc was eaten. Oligochaetes were eaten but Asellus and any animals in an advanced stage of decomposition were not. Embody found that Asellus was eaten but platyhelminths were not. Clemens observed G.fasciatus eating cladocerans and copepods and cultured them with elm leaves and dog food in the laboratory. Le Roux (1933) fed G.duebeni with elm leaves and chopped earthworm.

Overall, it seems likely that Gammarus spp will consume a wide variety of plant and animal material. Certain foods are eaten more readily than others, but almost anything is consumed if there is no choice. Dead plant material is generally preferred to living and this preference seems to be mediated by the growth of fungi on the plant

material, at least in the initial phase of its decomposition. Most animal food is readily taken whether alive or dead, but probably not if it is in an advanced state of decomposition. The diet of Gammarus is probably affected more by the relative availabilities than by the relative palatabilities of its components.

Another important factor in the nutritional physiology of G.pulex which has not been considered here, but which is well known from the literature, is the morphology of the digestive system. This was described by Cussans (1904) and studied histochemically by Mabillot (1955) and Agrawal (1965). Martin (1964) described the structure of the midgut gland and, particularly, the proventriculus of Marinogammarus obtusatus, and reviewed the earlier work on amphipod gut morphology. He mentioned that the proventriculus of G.pulex is similar in all respects to that of the species he studied.

The alimentary canal is divided into foregut (stomodaeum), midgut (mesenteron) and hindgut (proctodaeum). The foregut comprises a short oesophagus and a proventriculus (stomach) which is divided into anterior cardiac and posterior pyloric portions. The proventriculus opens into the long midgut which is a straight tube joining the short hindgut between the third and fourth abdominal segments. The foregut and hindgut, unlike the midgut, are lined with cuticle. The midgut gland opens into the midgut immediately posterior to the proventriculus. It consists of four blind tubules which extend posteriorly the length of the midgut. Other diverticula are the single anterior dorsal caecum which arises from the anterior end of the midgut and passes over the roof of the proventriculus, and the paired posterior caeca which opens into the posterior end of the midgut.

The functioning of the proventriculus has been described in detail by Martin (1964). Food is passed directly into the cardiac proventriculus where it undergoes trituration by spines and teeth, and is mixed with digestive fluid which is passed forward from the midgut gland. It is then passed into the pyloric proventriculus. Digestive fluid, digestive products and small particles of solid food are passed into a filter chamber which lies ventral to the pyloric proventriculus. Here the solid particles are removed and passed into the

midgut along with other undigested food. The digestive products are passed into the midgut gland where they are absorbed. The action of the filter chamber and a pyloric valve combine to prevent any solid food entering the midgut gland. Mabillot (1955) believed that absorbtion occurred in the anterior part of each tubule and secretion was confined to the posterior part. Martin (1964) did not find this in Marinogammarus obtusatus however. Absorbtion may occur in parts of the midgut, but no enzymes are secreted.

The experiments described in Chapters 4 and 5 were carried out on the assumption, which seems to be justified from an examination of the literature, that the midgut glands are the only source of digestive enzymes. Willer (1922) described the presence of glands in the labrum and mentioned earlier work which suggested that these glands may produce an amylase. He could not verify this experimentally however and Yonge (1924), who described similar glands in Nephrops, mentioned that they are present in many decapods, and occur all over the body surface as well as in the chitinised fore and hind guts. He considered that they were not connected with digestion of food, but may be connected with the secretion of chitin. Martin (1965) discussed the possible role of these tegumental glands in the Peracarida and considered them most likely to be associated with the hardening of the cuticle after ecdysis. Agrawal (1965) could detect enzyme activity only in the midgut gland of G.pulex and similar results have been obtained for the amphipods Corophium volutator, Orchestia gammarella and Marinogammarus obtusatus (Agrawal, 1963, 1964 ; Martin, 1966). Vonk (1960) and Van Weel (1970) believed that all, or the great majority of digestive secretion takes place in the midgut gland of the Decapoda.

CHAPTER 2

GENERAL MATERIALS AND METHODS.

a) Origin and Treatment of Animals

All specimens of G.pulex used were collected from the Fincastle burn, Perthshire. This is a narrow stream 0.5-2m in width in which Gammarus is abundant, particularly amongst moss and in dead, rough pasture grass which frequently hangs into the stream. Animals were transferred into a 1.5 x 0.5 x 0.2 metre fibre glass tank in the laboratory, along with other species of invertebrates, detritus, silt and sand from the burn. The tank was provided with a slowly flowing supply of lake water. Under these conditions the animals lived for long periods with no apparent detrimental effects. The reproductive cycle seemed to occur normally in the tank as evidenced by the appearance in January of couples in precopula and ripe females, and their disappearance in the autumn. Animals to be used for experimental work were separated from the other contents of the tank in a sorting tray immediately before they were required. The stock of animals was replenished from the burn whenever necessary. Animals were not generally used for experimental work until they had been in the tank for a few days, and it is not likely that many such animals had been in the tank for more than 3-4months.

Two species of Gammarus, G.pulex and G.lacustris occur in fresh-waters in the Perthshire area (Hynes et al, 1960). In order to check that all animals used were G.pulex, samples of animals from the tank were examined and identified at irregular intervals throughout the study according to criteria described by Reid (1944), Hynes et al (1960) and Pinkster (1972). More than 300 animals were examined and identified as G.pulex. Egglishaw (1968) reported the occurrence of only G.pulex in the Fincastle burn. All experimental work was carried out in containers immersed in a water bath at a constant temperature of 10 - 11^{o}C. This was maintained by the action of a refrigerator unit and a thermostirrer (Gallenkamp) working in opposition. During one period of four months the temperature was maintained by the action of a thermo-statically controlled refrigerator unit only. During this period the temperature varied between 9 and 11.5^{o}C.

b) Diets

The natural diets used in this work are referred to as - Normal Leaves (N.L.), Enriched Leaves (E.L.), Fine Detritus (F.D.) and Enriched Fine Detritus (E.F.D.) All leaves used were fallen elm leaves (Ulmus sp) collected from under a single tree in the autumns of 1972 and 1973 and stored deep frozen until required. Before feeding to Gammarus as N.L., these leaves were kept in flowing lake water for 2-5 weeks.

F.D. was prepared by washing large samples of organic and inorganic debris from the Fincastle burn through two Endecott sieves. The mesh size of the first was 0.7 mm and of the second 0.3 mm. The material remaining in the smaller mesh sieve was stirred in a glass cylinder and the lighter organic fraction decanted after settling. Most of this material comprised cellular plant fragments, but it also contained occasional animal remains and clumps of amorphous brown material. The appearance of the plant fragments suggested that a considerable proportion of them were derived from the dead pasture grass which hangs into the burn. Some samples of F.D. also contained a few live animals. Only very occasional copepods, water mites and young Gammarus were seen. Enriched diets were prepared by keeping elm leaves or F.D. in a flask containing 2 litres of enriched filtered lake water for 2-5 weeks. The water was aerated. Nutrients added were 20 mg/l of nitrogen as $(NH_4)_2 SO_4$ and 5 mg/l of phosphorus as $KH_2 PO_4$ (Kaushik and Hynes, 1971). The effect of the enrichment procedure was evident in the case of elm leaves as considerable amounts of fungal mycelium could sometimes be seen growing from E.L. but not from N.L. No such visible effects occurred in the case of E.F.D. As a check on the effect of enrichment the protein contents of samples of N.L. and E.L. were determined. Individual leaves from batches treated on different occasions were dried and stored together. The protein and ash contents of 100 mg samples of these leaves, details of which are given in Table 33 (Chapter 7), indicated that the enrichment procedure caused an approximate doubling of protein content. The ash and protein contents of sub samples of most batches of F.D. and

E.F.D. were determined separately as it was thought likely that these may vary considerably. The results, also shown in Table 33, indicated that the enrichment procedure did not affect the protein content of E.F.D.

The artificial diets used are referred to as 100% cellulose (100C), 90% cellulose plus 10% protein (90C+10P), 60% cellulose plus 40% protein (60C+40P) and 100% protein (100P). The cellulose used was B.D.H. Column Chromedia CC 31 which is a very pure crystalline cellulose powder with a mean wet particle size of 15-40 microns. The protein used was denatured bovine albumen fraction V (Sigma Chemical Co.) or egg albumen (G.T. Gurr). The albumen was dissolved in distilled water and boiled and the precipitated protein removed by filtration. The cellulose powder was suspended in water and filtered. The precipitated albumen and the cellulose powder were mixed wet in a measuring cylinder in the desired proportions. Artificial diets were always freshly prepared before use.

c) Collection of Midgut Gland Fluid (M.G.F.)

Live specimens were dried on filter paper and the urosome and last segment of the metasome pinched off with a pair of forceps. The head, with the gut attached, could then be gently pulled away from the thorax. In some cases this was done under distilled water (wet method) and in other cases on a dry glass plate (dry method). The head and gut were immediately placed in a watch glass under liquid paraffin. This procedure was repeated with 3-30 animals depending on the volume of M.G.F. required and the size of the animals. All the guts were placed together in the same watch glass. When sufficient had been collected the midgut glands were pierced with fine pins sufficiently to release all the fluid they contained. This was removed with a 10μl micropipette and stored as a discrete globule in another watch glass containing liquid paraffin. The substance collected in this way mainly comprised the fluid derived from the lumens of the

tubules of the midgut glands, but sometimes included some pieces of gland tissue and very occasional fragments of gut contents. Small bubbles of liquid paraffin occurring in the M.G.F. globule slowly rose to its surface where they broke of their own accord, or could be broken with a fine pin.

d) Enzyme Assays

Before use in enzyme assays the M.G.F. was diluted into an insect Ringer's solution (Clarke, 1966). The M.G.F. globule was transferred in a 10μl micropipette in which its volume was estimated. The Ringer's solution contained 1.3% w/v NaCl, 0.02% KCL and 0.02% $CaCl_2$. It was used initially for the preparation of gut homogenates of various insects as well as of G.pulex, and after the work with insects was abandoned its use was continued as it seemed satisfactory. Experiments with M.G.F. were always begun within 4 hours of its collection.

The activities of carbohydrate and protein digesting enzymes have been estimated in most cases by assaying the digestive products released when diluted M.G.F., or in a few cases gut homogenate, were incubated 'in vitro' with specific substrates. Incubations were carried out in a thermostatically controlled water bath at $30^{o}C$ in 10 x 75 mm pyrex glass tubes. The pH of the mixture, the volumes of the constituents and the duration of incubation varied in different experiments and are indicated at the appropriate points in the text. All enzyme assays were accompanied by control tubes, the diluted M.G.F. in which had been boiled for five minutes. The results shown represent experimental minus control values. Digestion of carbohydrate substrates was terminated by pipetting an aliquot (usually 0.1 or 0.2 ml) into 2 ml of the de-proteinising solutions of Nelson (1944) before assaying for reducing sugars. Digestion of proteins was terminated by pipetting 0.2 or 0.4 aliquots into 1 ml of trichloracetic acid (TCA) before assaying for TCA soluble substances. A drop of toluene was added to every incubate to prevent microbial activity.

e) Other Assays

Reducing sugars were assayed by the colorimetric method of Somogyi (1952) as recommended by Gascoigne and Gascoigne (1960) and used by many authors (e.g. Lasker and Giese, 1956 ; Elyakova, 1972). Precipitates were removed from de-proteinising solutions by filtration and the colour was developed with the arsenomolybdate reagent described by Nelson (1944). Optical densities were converted into micrograms of reducing sugars from a calibration curve determined with standard glucose solutions. The small amounts of starch present in solution in some cases had no effect on the final optical density as calibration lines drawn in the presence and absence of starch were identical.

T.C.A. soluble substances were assayed by the method of Lowry et al (1951) as modified by Macdonald and Chen (1965). The protein contents of the diets used were assayed by the same technique after extraction in 2% NaOH and precipitation in 10% TCA by the method of Kaushik and Hynes (1969). A calibration curve was drawn up with bovine albumen fraction V. All filtrations were through No. 3 porosity sintered glass filters. Optical densities were measured against a distilled water blank with a 'Spekker' filter photometer using a blue/green filter for reducing sugar assays and a red filter for protein assays.

In order to test the reproducibility of reducing sugar and protein assays, replicate samples of a glucose and an albumen solution were assayed. The mean weight of reducing sugars found was 27.7 µg and the range was 25.5 to 29.0 (S.D. = 1.1, S.E.=0.25, N=19). In practice differences between assays of less than 4.4 µg (O.D. 0.44) (4 x S.D.) were not considered significant. The mean weight of protein found was 57.1 µg and the range was 49.0 to 63.0 (S.D. = 3.36, S.E. = 0.75, N = 20). Differences between assays of less than 13.3 µg (O.D. 0.35) were not considered significant.

f) Terminology

Published work concerning the digestive physiology of the Crustacea sometimes suffers from confusion in the terminology pertaining

to the various parts of the digestive system. In the present work the term midgut gland is used in favour of liver or hepatopancreas as recommended by Van Weel (1970, 1974). Tract is used to mean the tube along which solid food passes and gut is used to mean the entire digestive system, i.e. tract plus midgut gland plus other diverticula.

CHAPTER 3

THE DISTRIBUTION OF CELLULASE IN VARIOUS FRESHWATER INVERTEBRATES

a) Methods

Methods were chosen which would give measures of specific activity comparable to those determined by Elyakova (1972). Animals were collected live from various streams and lakes in Perthshire, and from a population of animals living in an artificial concrete stream adjacent to the Freshwater Fisheries Laboratory, Pitlochry. Where possible guts were dissected out and ground with 0.6 to 1 ml of insect Ringer. If particularly small however, the entire animals were homogenised, and in one case (G.pulex) M.G.F. was also used. In interpreting the results it should be remembered that specific activities determined from whole animal homogenates will be considerably lowered by the presence of much non-gut protein. Furthermore the use of whole animal homogenates may inhibit cellulase activity as Ray and Julian (1952) could not detect cellulase activity in whole animal extracts of Limnoria lignorum but could do so in gut extracts. Homogenates were filtered and the filtrate used to assay enzyme activity within five hours of preparation. Substrates used were 0.4 ml of 1% sodium carboxymethyl cellulose (CMC), which is a water soluble cellulose derivative, and a few milligrams of cellulose powder. As animal cellulases are often optimally active at pH 5 to 6 (Gascoigne and Gascoigne, 1960) all incubates were buffered at pH 5.5 with 0.2 ml (CMC) or 0.4 ml (cellulose powder) of McIlvaines Na_2 HPO_4/citric acid buffer. The incubation period was started by pipetting 0.2 ml of homogenate into each reaction mixture. The remaining 0.2 to 0.6 ml of homogenate was assayed for protein content. 0.2 ml aliquots of the reaction mixtures were immediately withdrawn for control reducing sugar analysis and a further 0.2 ml was withdrawn after 3 (CMC) or 18 (cellulose powder) hours incubation. Specific activity was calculated in every case as μg of reducing sugars/mg protein/3 hours incubation.

b) Results

The results are shown in Table 1. Diets were determined from the literature. Most authors quoted differentiate between detritus, which means brown amorphous organic material of unknown origin, and higher plant tissues. This differentiation has been retained in Table 1. The various algal phyla quoted have been lumped together as Algae. Figures based on differences between experimental and control reducing sugar analyses of less than 4.4 µg have not been included in the table but in some cases differences between 1 and 4.4 µg were found for both substrates, or were found for one substrate when the other was definitely hydrolysed. In those cases, which are marked with an asterisk, it seems probable that a slight hydrolysis occurred.

The strongest cellulase activities were detected in the crustaceans and molluscs tested. The higher activity found in M.G.F. than in gut extracts of G.pulex suggests that the enzyme originates in the midgut glands. Elyakova (1972) found specific activities in gut extracts from various crustaceans between 820 and 2,080 against a CMC substrate using a similar method to that used here, except that his assays were carried out at 40°C. As the Q_{10} of cellulase from G.pulex between 30 and 40°C is about 1.1 (Chapter 4) the specific activity at 40°C equals 945 (1.1 x 850). Cellulase activity in G.pulex is therefore similar to that encountered in other crustaceans.

A weak cellulase was found in gut homogenates from many aquatic insects but no correlation with diet is apparent. One of the groups most important in consuming freshly fallen leaf litter is the Trichoptera, in particular the Limnephilidae. The feeding habits of one of the limnephilid genera tested here for cellulase activity, Halesus spp., have been examined to some extent in the laboratory. The animals used were a mixture of roughly equal numbers of H.radiatus and H.digitatus, as indicated by the rearing of about 240 larvae and the subsequent emergence and identification of 17 adults in the laboratory. These larvae would consume a wide range of leaf material including extremely tough types such as rhododendron, as well as softer varieties such as

elm, nettle and aspen, although filter paper was not ingested. Ingestion of fresh leaf litter or green leaves occurred readily, in contrast to the reluctance of Gammarus to eat leaf litter which had not been decomposing in water for several days. The natural diet of animals in the field mainly comprises leaf material, although algal and animal matter may be ingested (Table 1). It might be expected that an animal with this type of diet could digest cellulose, and cellulase activity was found in the gut although at a lower level than that found in Gammarus. Many other analyses of cellulase activity in Halesus spp have been carried out, apart from that shown in Table 1, but in no case did the activity approach that found in Gammarus.

The highest activities found in the Trichoptera were in the predominantly carnivorous net spinning members of the Polycentropidae, Polycentropus flavomaculatus and Plectrocnemia geniculata. Other net spinners however, Hydropsyche pellucidula and Wormaldia subnigra, showed little or no cellulase activity and another carnivore, Rhyacophila obliterata, showed none.

Within the Trichoptera there was thus no obvious correlation between the activity of cellulose digesting enzymes in the gut and the amount of cellulose likely to occur in the diet, and this seems to apply generally to the species tested. Distinct activity was found in the carnivorous members of the Megaloptera and Coleoptera tested, but was slight or absent in the largely herbivorous members of the Ephemeroptera and Plecoptera. The relatively high cellulase activities found in some carnivores did not extend to the plecopteran Dinocras cephalotes. As well as the tests shown in Table 1, preliminary investigations of gut extracts of Baetis rhodani and Protonemura sp were carried out using reducing sugar detection by Fehlings solution and the iodometric method of Somogyi (1952). No activity was detected in any extract of B.rhodani but slight activity was found in some gut extracts from Protonemura sp.

KEY TO TABLE 1.

Cellulase Activity in Freshwater Invertebrates

S.A. = Specific activity

G = Gut homogenate

W.A. = Whole animal homogenate

R.S. = Reducing sugars produced

M.G.F. = Midgut gland fluid

* 4-19 (CMC) or 3-13 (cellulose powder) μg R.S. produced

References

1. See Chapter 1.
2. Ermolaeva 1962.
3. Deksbakh and Sokolova 1965.
4. Percival and Whitehead 1929.
5. Badcock, 1949.
6. Hynes, 1949.
7. Jones, 1949.
8. Jones, 1950.
9. Moon, 1938.
10. Slack, 1936.
11. Own observations.
12. Dunn, 1954.
13. Winterbourne, 1971.
14. Hynes, 1970.
15. Macan & Worthington, 1951.
16. Crisp, 1962.

TABLE 1.

Cellulase Activity in Freshwater Invertebrates

Order	Species	Origin	Diet	Extract	Protein (mg)	CMC R.S. (µg)	CMC S.A.	Cellulose R.S. (µg)	Cellulose S.A.
Amphipoda	Gammarus pulex	Fincastle Burn	Detritus, Higher Plant Tissue, some Algae and Animals (1)	G	0.22	189	859	191	145
"	"	"	"	MGF	0.06	81	1,342	65	181
"	Gammarus lacustris	Loch Kinardochy	Algae, Higher Plant Tissue, some animals (2, 3)	G	0.36	137	381	434	201
Gastropoda	Lymnaea peregra	Artificial Channel	Algae (4)	G	0.32	339	1,060	138	72
"	Ancylus fluviatilis	"	Algae, some Higher Plant Tissue (4, 5)	WA	0.25	37	148	34	23
Plecoptera	Dinocras cephalotes	Moulin Burn	Animals, some Algae and Higher Plant Tissue (6, 7)	G	0.35	0	0	0	0
"	Leuctra hippopus	Fincastle Burn	Detritus, Higher Plant Tissue, some Algae (6, 8)	WA	0.09	0	0	0	0
Ephemeroptera	Ephemerella ignita	Artificial Channel	Algae, some Animal Tissue (5)	G	0.07	0*	0	0*	0
"	Heptagenia sulphurea	"	Algae (4)	G	0.17	-	-	21	20
"	Ecdyonurus dispar	"	Algae, Detritus, Higher Plant Tissue (4, 5, 7)	G	0.13	0*	0	18	23
"	Ecdyonurus venosus	"		G	0.19	0*	0	0*	0
"	Caenis horaria	Loch Dunmore	Detritus, Algae, Higher Plant Tissue (4, 9)	WA	0.17	0*	0	35	35
Trichoptera	Halesus spp	Fincastle Burn	Higher Plant Tissues, some Algae and Animals (4, 8, 10, 11, 12)	G	0.29	66	228	32	19
"	Potamophylax sp	"		G	0.26	48	186	28	18
"	Limnephilus lunatus	Artificial Channel	Probably mainly Higher Plant Tissue, perhaps also some Algae and Animal Tissue (10, 13, 14)	G	0.33	0*	0	21	10
"	Limnephilus sp	Fincastle Burn		G	0.08	0*	0	13	27
"	Wormaldia subnigra	Artificial Channel	Fine particulate material, mainly Algae & Detritus, some animal material (14, 15)	G	0.18	0	0	0	0
"	Polycentropus flavomaculatus	Moulin Burn	Animals, occasional Plant Tissue and Algae (4, 5, 8, 12)	G	0.12	44	367	22	31
"	Plectrocnemia geniculata	"	Animals (4, 7)	G	0.08	52	650	0*	0*
"	Rhyacophila obliterata	Fincastle Burn	Animals (7)	G	0.23	0	0	0	0
"	Hydropsyche pellucidula	"	Algae, some Animals and Higher Plant Tissue (5, 10)	G	0.15	0*	0	0*	0
Megaloptera	Sialis lutaria	Loch Dunmore	Animals (12)	G	0.18	50	276	16	15
Coleoptera	Dytiscinae (Adults)	Fincastle Burn	Animals (7, 8)	G	0.08	31	385	0*	0
"	Hydroporini (Larvae)	"	Animals (7, 8)	G	0.18	32	178	18	17
Diptera	Simulium sp	"	Detritus, Algae, Higher Plant Tissue (8)	WA	0.18	0	0	0	0
"	Dicranota sp	"	Detritus (?)	WA	0.23	0	0	0	0
Hemiptera	Corixa dorsalis	Loch Dunmore	Detritus, Animals, Algae (15, 16)	G	0.06	0*	0	0*	0

c) Discussion

Bjarnov (1972) found that gut homogenates from 6 out of 10 species of Trichoptera he tested, including Rhyacophila septentrionis, Potamophylax nigricornis and Hydropsyche angustipennis, possessed a definite but slight ability to digest CMC, but only one, Neureclipsis bimaculata, digested cellulose prepared from filter paper. He also found that G.pulex could digest CMC and cellulose and Chironomus antharacinus could digest CMC. He concluded that the ability to digest CMC was widespread but never vigorous, and this conclusion is substantiated by the results presented here. Even in G.pulex, where cellulase activity was relatively high, only 4.7% of the CMC present was degraded to glucose and it will be shown later that after 6 days incubation this figure was only 8.4% (Table 6). Corresponding figures for the insects used here ranged between 0 and 1.7%. Bjarnov's finding that cellulose could not be broken down by most of the species he tested is in contrast to the slight digestion of cellulose powder mediated by many of the extracts tested here. The cellulose used by Bjarnov was prepared from filter paper but it will be shown later (Chapter 4) that cellulose powder is not broken down to a greater extent than filter paper by gut extracts from G.pulex after 6 days incubation. However, as cellulose powder is in the form of very small particles its initial rate of hydrolysis may be more rapid than that of filter paper because of the larger surface area available for enzyme action. During the relatively short incubation period used by Bjarnov (1-2 days) insufficient hydrolysis may have occurred for detection.

As a result of their review of the occurrence of cellulase in the animal kingdom, Yokoe and Yasumasu (1964) suggested that its distribution is more closely correlated with phylogenetic relationships than with feeding habits. The present results, and those of Bjarnov (1972) substantiate the lack of correlation with diet in aquatic invertebrates, but do not show clear phylogenetic correlations. It seems probable that within certain taxa, e.g. the Crustacea, Mollusca and Annelida, the occurrence of cellulase may be universal, but within the Insecta the occurrence of a weak activity may be sporadic. Bjarnov

(1972) and Nielsen (1962) concluded that extensive breakdown of cellulose by higher animals does not occur and no evidence has been presented here to show that that is not the case. The suggestion frequently made that the great majority of invertebrates do not possess a cellulase may be incorrect, but the physiological and ecological significance of the weak cellulase which is found in many animals, regardless of their feeding habits, awaits further elucidation.

CHAPTER 4

PHYSICAL AND CHEMICAL CONDITIONS IN THE GUT

a) Methods

i) The Origin of Cellulase Activity in the M.G.F.

In order to determine whether the cellulase found in the M.G.F. is secreted by the glands or is of microbial origin, the cellulase activity of crude and cell-free M.G.F. were compared, and the abilities of micro-organisms occurring in the M.G.F. to utilise cellulose substrates and produce extra cellular cellulases were investigated.

To measure cellulase activity in cell-free M.G.F. a sample of 10 µl of M.G.F. was diluted with 6 ml of Ringer's solution. A 2 ml aliquot was passed through an 'Oxoid' membrane filter (0.45 µ pore size) with a 'Turner' micro-filter syringe, and a drop of toluene added. A drop of toluene was added to a second unfiltered aliquot and the third was left as a control. Triplicate 0.2 ml samples of these solutions and of boiled controls were incubated with a few milligrams of cellulose powder and 0.2 ml of pH 5.5 buffer before assaying for reducing sugars. In a second experiment with another sample of M.G.F., cell-free and control solutions only were incubated with CMC.

To investigate any microbial population in the M.G.F., smears were made on to corn meal agar (pH 6.0), potato dextrose agar (pH 5.6) and yeast extract agar (pH 7.2). This was done by withdrawing the gut from the thorax and abdomen as previously described, resting the posterior ends of the midgut gland on the surface of the agar, piercing them with a sterile pin, and then drawing the glands across the agar. This usually left a smear of M.G.F. on the plate but portions of the gland or of the tract and its contents sometimes remained. After 7 days incubation at 25°C some of the bacterial colonies which had developed were streaked on to cellulose agar (Eggins and Pugh, 1962). After incubation of this medium at 25°C for 7 days any growth of colonies and clearing of the agar was noted. Developing colonies were inoculated into the cellulose medium made up in liquid form. This was then placed in a shaking incubator at 25-30°C for 14 days. To check whether the inoculated bacteria grew in the liquid media the populations were sampled by the pour-plate method at the beginning and end of the incubation period. An undiluted 0.1 ml inoculum and yeast extract agar culture media were used. After the incubation period each liquid culture was passed through a membrane filter

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and toluene was added to the filtrate. 1 ml aliquots of the cell-free solution, buffered at pH 5.5, were tested for enzymic activity against CMC and cellulose powder using a 24 hour incubation period.

In a further experiment M.G.F. was smeared on to potato dextrose agar only. Developing colonies were inoculated directly into the liquid culture medium. Other methodology was as described for the previous experiment except that potato dextrose agar was the culture medium used for the pour-plates.

ii) The pH of the M.G.F. and the Tract

The pH of undiluted M.G.F. collected by the dry method was determined with a cup type glass micro pH electrode, supplied by 'Activion Glass Ltd.", and a 'Pye Unicam' pH meter. Groups of about 50 animals 8 to 11 mm in length were fed the various test diets for 24 hours prior to the collection of MGF. The artificial diets, made up with egg albumen, were fed to animals isolated singly in 100 ml plastic bottles to prevent their feeding upon each other in preference to the diet offered. Those animals feeding on natural diets fed communally in large flasks as cannibalism rarely occurred when a palatable alternative was available. The pH of MGF. from animals feeding normally in the laboratory tank and that of animals starved for seven days was also measured. Animals were starved individually in 100 ml plastic bottles, the water in which was changed daily. 5-10 µl of M.G.F. was collected from groups of 4-8 animals and its pH determined to the nearest 0.05 units.

The pH of the tract was determined with Whatman - B.D.H. close range indicator papers. All the animals used were taken directly from the laboratory tank. The guts were dissected out as previously described, the midgut glands removed and the head dissected away. After removing excess moisture from the tract with filter paper it was placed on a piece of indicator paper and punctured at several points along its length. These points were the proventriculus, and anterior, medium and posterior positions along the length of the midgut. Sufficient

fluid usually ran out at each point to allow the determination of its pH to the nearest 0.25 units by comparison of the colour of the indicator paper with that of standards under a binocular microscope. The hindgut was removed during the initial dissection but in some cases the pH of its contents was determined by squeezing them out on to a piece of indicator paper.

iii) Properties of M.G.F. Enzymes

Initial experiments were carried out to determine the extent to which various cellulose substrates could be hydrolysed, and to determine the relative activities of M.G.F. carbohydrases at the two main pH valves encountered in the gut. In order to obtain a powerful cellulase preparation, a gut extract was used in the first experiment. Thirty-five guts were homogenised in 7 ml of Ringer, the solution was centrifuged, and 0.5 ml of the resulting supernatant incubated with known weights of various cellulose substrates and 1 ml of pH 5.5 buffer for 6 days before assaying aliquots for reducing sugars. Some of the substrates were crushed with a pestle and mortar before use. In the second experiment a pooled sample of M.G.F. from 20 animals was diluted with 7 ml of Ringer's solution and 0.2 ml aliquots were incubated overnight with 0.4 ml of various carbohydrate substrates and 0.2 ml of pH 5.5 or 7.0 buffer before assaying for reducing sugars. 0.6 ml of buffer was used in the case of the cellulose powder substrate.

The pH sensitivities of carbohydrases which hydrolyse cellulose powder, CMC, soluble starch maltose and cellobiose were determined by incubation of M.G.F. and substrates with buffers of different pH values. The buffers used were McIlvaines between pH 3.0 and 7.8 and boric acid, potassium chloride and sodium hydroxide mixtures between pH 7.8 and 10.0 (Gortner and Gortner, 1949). The duration of the incubation period and the volumes of the components of the incubation mixture are stated with the results in each case. In some experiments (starch and CMC substrates) the pH of the buffer solution was first determined with the pH meter and this value was considered to equal the pH of the incubation mixture. In these cases the results shown are the means of several experiments. In other experiments the exact pH of the

incubation mixture in each tube was determined by withdrawing 0.1 ml aliquots during the incubation period and determining their pH with the micro-electrode.

The pH sensitivities of proteases which hydrolyse casein, fibrin and bovine albumen substrates, and of peptidases which hydrolyse leucyl glycylglycine, hippuryl-L-phenylalanine and hippuryl-L-arginine (Sigma) substrates were determined. Hydrolysis of the peptide substrates was taken to represent amino tripeptidase, carboxypeptidase A and carboxypeptidase B activities respectively. Peptidase activity was measured by adding a 0.1 - 0.2 ml aliquot of each reaction mixture or of controls to 5 ml of a 0.2% solution of ninhydrin in pH 6.5 phosphate buffer. These mixtures were held at 100^{o}C until the increase in optical density was maximal. The pH of each reaction mixture was determined during the incubation period as previously described. In one case (bovine albumen substrate) the pH of the reaction mixtures were measured after 2 hours and again after 48 hours. In only one case had the pH altered by more than 0.1 units during this period (8.8 - 8.5). The pH of an aliquot of the mixture taken during the incubation period was therefore a good measure of the mean pH of the mixture throughout the period in most cases.

The temperature sensitivities of enzymes hydrolysing CMC, soluble starch and casein substrates were determined by incubating these substrates with aliquots of diluted M.G.F. at different temperatures for a similar period. The incubation mixtures were buffered at approximately the optimal pH of the enzyme concerned. In order to make pH and temperature sensitivity curves obtained from different preparations of M.G.F. comparable, results are expressed in all cases in terms of the percentage of maximum activity, as used by Martin (1966).

Results to be described later suggested the possibility that elm litter may contain substances which inhibit the digestive enzymes of Gammarus. In order to test this hypothesis aqueous extracts of elm leaves were made by homogenising about 2 grams wet weight of leaves with a pestle and mortar, and mixing the homogenate with 8-10 ml of Ringer's solution and a few drops of toluene. Either immediately or after storage for up to two weeks the mixture was centrifuged and the resulting viscous solution used as the extract. In order to test this extract for enzyme

inhibitors, M.G.F. was collected (15-20 µl) and diluted with 0.2 to 0.4 ml of Ringer. 0.1 ml aliquots of this solution were diluted with equal volumes of the extract to be tested or of Ringer. 0.2 ml aliquots of each solution were then incubated with various substrates and a buffer. After a suitable period aliquots were withdrawn for assay.

In order to investigate further the effect of inhibitors on cellulase activity, and to determine whether any inhibitors could be detected in fine detritus extracts, the digestion of filter paper was investigated. Leaf and fine detritus extracts were made up as described using 2.5 g wet weight of material, and mixed with gut homogenate obtained from 25-40 animals. This was used in preference to M.G.F. in an attempt to obtain a stronger enzyme preparation. In initial experiments pieces of Whatmans No. 1 filter paper were incubated with 0.5 ml of enzyme solution and 0.5 ml of pH 5.5 buffer. Aliquots (0.2 ml) for analysis were taken after 0, 3 and 6 days. As the amounts of reducing sugars produced were slight, in later experiments cellulase activity was estimated by measuring the loss in weight and tensile strength of filter paper. Pieces of filter paper between 5 and 15 mg were weighed dry before and after the experiment in order to estimate weight change. Tensile strength was measured after weighing by cutting a 1/16" strip from each piece of filter paper and securing a standard length (½") between clamps. One clamp was then fixed and weights were suspended from the other. The tensile strength was taken as the weight which broke the filter paper strip, and was measured to the nearest 10 grams.

iv) The Passage of Food along the Tract

The times taken by ingested 100C and N.L. to pass along the tract were estimated by a method based on that described by Cummins (1973). Groups of 50 animals, 8 to 11 mm in length, were fed the appropriate diet overnight. They were then transferred to another diet on which they were allowed to feed for a short period before being returned to the original diet. Rapid transfer between diets was accomplished by confining the animals within perspex tubes, the bottoms of which were covered with

fibreglass mesh. These could be rapidly lifted out of the containing beakers leaving finely divided diets behind. Leaves were added or removed by hand. The food ingested during the labelling period, if of a different colour to the main diet, appeared in the tract as a discrete mass the passage of which could be easily followed. It will be shown later that Gammarus may ingest different diets at widely differing rates. Some materials, notably leaves, may be ingested quite slowly. These are unsuitable as labelling substances as during a short period none might be ingested. However by using diets which may be rapidly ingested, i.e. cellulose powder or fine detritus, some material was almost always consumed during the labelling period.

The passage of N.L. was followed using a cellulose powder label for 5 to 10 minutes. The passage of 100C was followed using a fine detritus label, but in this case labelling lasted twenty minutes as fine detritus was ingested less rapidly than cellulose. After the labelling period several animals were removed from the containers at regular intervals and their guts quickly removed. The position in the tract of the posterior end of the label, i.e. the part which was ingested first, was readily noted through the transparent tract wall and assigned to one of ten positions previously designated along its length.

In order to observe any change in the physical appearance of the food as it passed along the tract, faeces and proventriculus contents from animals fed certain diets were examined using both conventional and phase contrast microscopy. It was assumed that any physical degradation which occurred as a result of cellulose digestion might be reflected in such a change. Groups of several animals were fed on F.D. and N.L. overnight so that any other food would be eliminated from the tract. Faeces produced up to this time were discarded and fresh faeces were then collected at 30 minute intervals and preserved in 5% formalin. When sufficient had been collected the animals were dissected and the proventriculus and its contents preserved. Samples from the proventriculus may not be entirely representative of newly ingested food as some digestion may have occurred before dissection. In an attempt to collect freshly ingested food, only animals which were apparently actively feeding were dissected.

Faeces and proventriculus contents were teased apart with fine needles and after thorough dispersion by shaking a sample was placed in a Sedgewick-Rafter counting chamber and the sizes of particles in random squares were measured with an eye piece graticule under x100 magnification. In practice the counting chamber square was racked across the field of vision by an automatic stage and the size of each particle was measured in the plane in which it crossed the graticule, i.e. the particles were measured in random planes. About 300 particles were measured in this way from each sample.

b) Results

i) The Origin of Cellulase Activity in the M.G.F.

The cellulase activity of M.G.F. treated in various ways is shown in Table 2. The removal of any micro-organisms by filtering or their inactivation by toluene did not cause a reduction in cellulase activity.

The results of the investigation of the M.G.F. microflora were not entirely conclusive. In the first experiment a total of 30 smears were prepared and bacterial colonies grew from 16 of them. No growth occurred on 9 plates and 5 plates showed growth of fungi. This last often occurred where part of the gut contents had been left on the plate. Of the 10 streaks which were made on to cellulose agar, small colonies developed in 6 cases. No clearing of the agar was visible however. When colonies from these 6 plates were transferred to the liquid cellulose medium, growth occurred in every case as indicated by populations on the pour-plates. No formal counts of bacterial populations on the pour-plates were attempted as serial dilutions had not been made, but subjective comparisons of the plates made at the beginning and end of the incubation period clearly indicated that growth had occurred in every case. The cell-free liquid culture media showed no hydrolytic activity against CMC or cellulose powder substrates, although the presence of some protein in solution was suggested in one case by the formation of a cloudy precipitate in the boiled control.

In the second experiment 10 smears were prepared and bacterial colonies grew in 5 cases. Pour-plate populations indicated that 4 of these grew in the liquid cellulose medium. Once again the cell-free media showed no hydrolytic activity against CMC or cellulose in any case.

Thus bacteria were cultured from the M.G.F. of some specimens of G.pulex which grew on solid or liquid cellulose media, but positive evidence of cellulose utilisation in the form of the clearing of cellulose agar plates was not obtained. These bacteria did not produce an extra-cellular cellulase which could be detected in the cell-

TABLE 2.

Cellulase activity in the M.G.F. (µg Reducing Sugar /0.2 ml Aliquot)

Cellulose Powder Substrate (24 hours incubation)

Replicate No.	Control	Control & Toluene	Cell-Free
1.	9.0	17.0	16.0
2.	8.0	13.0	16.0
3.	10.0	20.0	13.0
$\bar{x}$	9.0	17.0	15.0

C.M.C. Substrate (3 hour incubation)

Replicate No.			
1.	13.0	-	13.0
2.	14.0	-	13.0
3.	15.0	-	15.0
$\bar{x}$	14.0	-	14.0

- Not tested.

free medium, although cellulose powder and CMC were digested by cell-free dilutions of M.G.F. This would suggest that the enzyme or enzymes bringing about the hydrolysis of cellulose were secreted by the animal itself.

ii) The pH of the M.G.F. and Tract

The pH of M.G.F. from groups of animals fed various natural diets and from starved animals is shown in Table 3. As the variances were independent of the means, as indicated by the graphical method (Elliot, 1971), a one way analysis of variance was carried out on the untransformed data. This showed that all the results did not come from the same population ($F = 5.6$, $P = < .05$). The results of t-tests between the individual means are shown in Table 4.

It is evident that the pH of the M.G.F. was only slightly influenced by the nature of the diet. That of animals feeding normally was slightly but significantly lower than that of animals fed the other diets, but was the same as that of animals starved for seven days. Enrichment of diets had no significant effect on M.G.F. pH. Animals fed E.L. had significantly higher M.G.F. pH than those fed F.D. or E.F.D. The pH of the fluid from the midgut glands of a few normally feeding individuals was also tested by the indicator paper method. In each case a pH of 5.5 was indicated.

The pH of M.G.F. from animals fed artificial diets (figure 1) decreased with increasing dietary protein content. The regression coefficient is significantly different from 0 ($t = 2.73$, $P = < .05$) (Snedecor, 1946).

The tract was divided into two regions of distinctly different pH (Table 5). The proventriculus and the anterior part of the midgut were slightly acid, whilst the posterior part of the midgut and the hindgut were neutral. The exact point where the transition occurred varied somewhat between individuals but mainly depended on the amount of food in the tract. Where the tract was full the neutral region was usually confined to the hindgut and the posterior part of the midgut, but in individuals with empty or partially empty tracts this region often extended forward to the median area of the midgut, or occasionally to the anterior region. Neither the sex of the individuals, nor the

TABLE 3.

The pH of the M.G.F.

Replicate No.	Diet	pH.
1.	Normal	5.60
2.	"	5.50
3.	"	5.55
4.	"	5.60
5.	"	5.70
6.	"	5.80
7.	"	5.60
x		5.62
S.E.		0.04
1.	E.L.	5.90
2.	"	6.00
3.	"	6.00
4.	"	6.10
5.	"	5.80
x		5.96
S.E.		0.05
1.	N.L.	5.60
2.	"	5.75
3.	"	6.10
4.	"	6.00
5.	"	5.60
6.	"	5.85
7.	"	5.80
x		5.81
S.E.		0.07

Replicate No.	Diet	pH.
1.	E.F.D.	5.90
2.	"	5.65
3.	"	5.75
4.	"	5.60
5.	"	5.90
6.	"	5.80
7.	"	5.90
x		5.79
S.E.		0.05
1.	F.D.	5.70
2.	"	5.80
3.	"	5.80
4.	"	5.80
5.	"	5.65
6.	"	5.65
x		5.73
S.E.		0.03
1.	Starved	5.45
2.	"	5.70
3.	"	5.50
4.	"	5.60
5.	"	5.70
6.	"	5.80
x		5.63
S.E.		0.06

TABLE 4.

t values for mean M.G.F. pH values

E.L.	$t = 5.4$ S_1				
N.L.	$t = 2.39$ S_5	$t = 1.75$ N.S.			
E.F.D.	$t = 2.81$ S_5	$t = 2.48$ S_5	$t = 0.24$ N.S.		
F.D.	$t = 2.25$ S_5	$t = 3.9$ S_1	$t = 1.04$ N.S.	$t = 1.07$ N.S.	
Starved	$t = 0.15$ N.S.	$t = 4.4$ S_1	$t = 2.022$ N.S.	$t = 2.21$ S_5	$t = 1.585$ N.S.
	Normal	E.L.	N.L.	E.F.D.	F.D.

S_1 = Significant at 1% probability level.

S_5 = " " 5% " "

N.S. = Not significant.

FIGURE 1.

The effect of artificial diet composition upon M.G.F. pH.

Regression line $y = 5.91 - .0033x$.

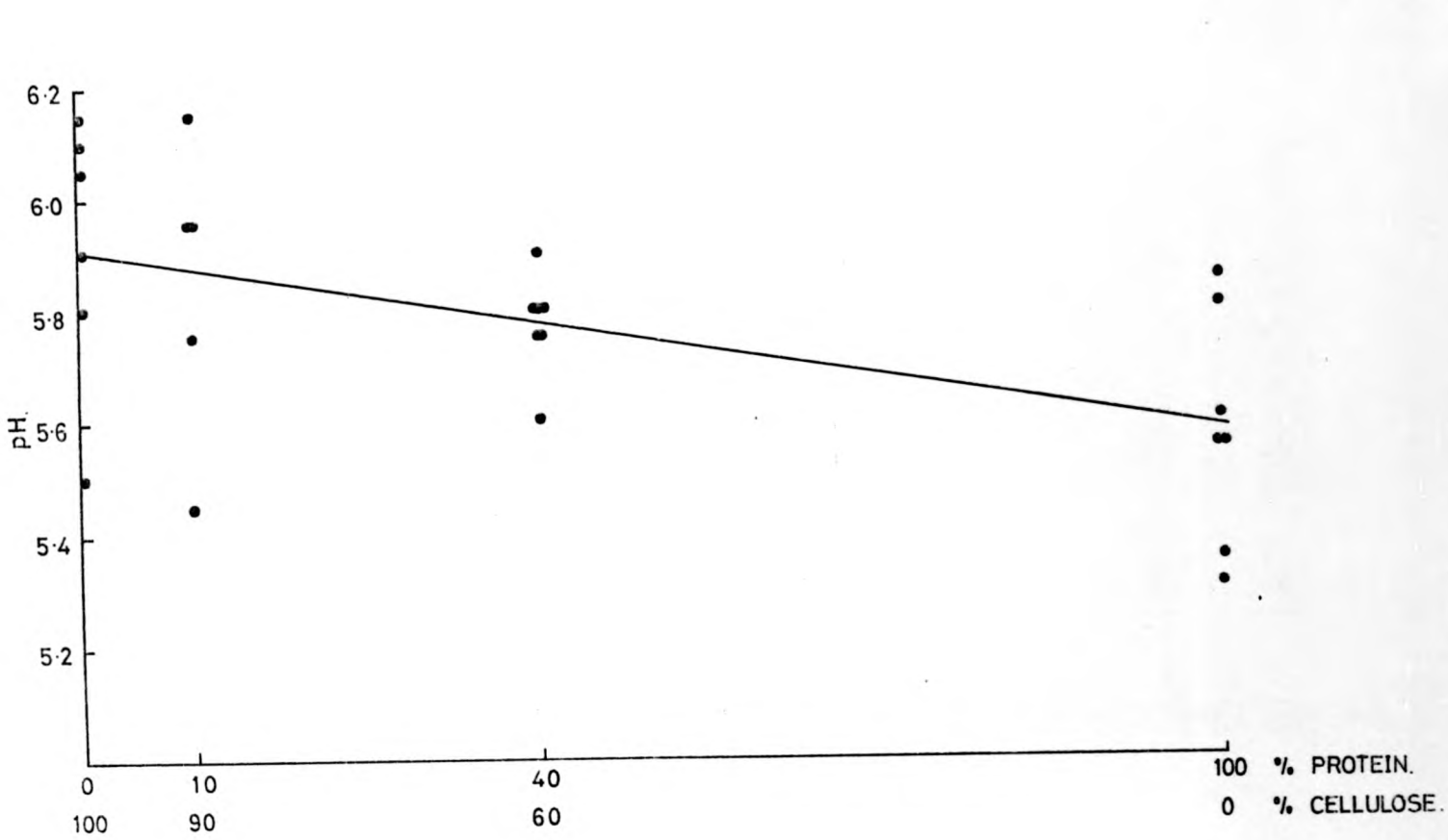

6·2
6·0
5·8
pH. 5·6
5·4
5·2
0
100
10
90
40
60
100 % PROTEIN.
0 % CELLULOSE.

TABLE 5.

The pH of the Tract

Length of Animal (mm)	Sex	Region of Tract: Proventriculus	Ant.Mid	Med.Mid	Post.Mid	Hind.
14.0	M	-	-	-	-	7.00
13.5	M	5.25	5.25	5.25	7.00	-
13.0	M	5.25	5.00	5.00	7.00	7.00
13.0	M	5.75	5.75	7.00	7.00	7.00
13.0	M	5.25	5.00	5.00	7.00	-
12.5	M	5.00	5.00	5.00	7.00	7.00
11.0	M	5.00	4.50	4.50	-	7.25
10.0	M	-	-	-	6.75	-
10.0	M	5.25	4.75	5.25	7.00	7.25
9.5	M	5.50	5.50	5.00	7.00	-
9.5	M	5.25	5.25	5.25	-	6.5
9.5	M	5.00	5.00	-	7.00	-
9.5	M	-	-	-	7.50	-
11.5	F	5.50	5.50	7.00	7.00	-
10.0*	F	5.00	5.00	5.00	7.00	7.00
9.5*	F	5.25	7.00	7.00	7.00	-
9.5*	F	5.50	5.50	5.50	6.50	-
9.0*	F	5.00	4.75	4.75	7.00	-
9.0	F	5.00	5.00	-	-	7.25
9.0	F	6.00	5.00	5.00	7.00	-
8.5	F	5.00	-	-	-	-
8.5	F	6.00	5.25	5.25	7.00	-
8.5	F	-	-	-	7.50	7.25
8.0	F	5.25	5.25	5.25	7.00	-
$\bar{x}$		5.30	5.22	5.41	7.01	7.05
N		20	19	17	19	10

* Gravid

\- No value obtained

presence of developing eggs in the brood pouch had any obvious effect on the pH of the tract.

The food generally consumed by Gammarus is probably fairly acid. The pH of a few samples of N.L. was determined by grinding them to a paste in a pestle and mortar, and then bringing them into contact with a piece of close-range indicator paper. The pH was approximately 5.0. The mixing of acid food with the M.G.F. presumably causes the slight lowering of the pH in the anterior part of the tract compared with that in the fluid. As the food passes along the tract the pH is unchanged until it enters the neutral region at the posterior end. This presumably results from secretion at the posterior end of the tract of a fluid which can move in an anterior direction if no food is passing the other way.

iii) Properties of M.G.F. Enzymes

The extent to which various cellulose substrates were broken down by gut extracts is shown in Table 6. In all cases, although readily detectable amounts of reducing sugars were produced, only a small percentage of the substrate was converted to reducing sugars. None of the solid substrates showed any change in appearance during the incubation period, but physical distruption of the substrate prior to its use caused a great increase in the extent of its hydrolysis. The figures shown are minimal estimates of the true extent of hydrolysis as they are calculated on the assumption that glucose is the sole product of hydrolysis. If other products of lower reducing power were formed their amounts would be underestimated. Although some of the substrates may have contained impurities which could have been hydrolysed by other enzymes in the gut extract, a real hydrolysis of cellulose must have occurred as cotton wool and cellulose powder are particularly pure forms of cellulose.

The activities of M.G.F. carbohydrases at the two pH values encountered in the gut are shown in Table 7. All dissacharides tested, with the exception of lactose, were hydrolysed. α glucosidases were more active than β glucosidases and soluble starch was broken

TABLE 6.

The Hydrolysis of Various Cellulose Substrates

Substrate	Weight (µg)	Total Reducing Sugars Produced (µg)	% Hydrolysis
Cotton Duck	40,100	76	0.20
Cotton Duck (Crushed)	49,200	1,755	3.60
Filter Paper	20,600	362	1.80
Filter Paper (Crushed)	19,000	1,044	5.50
Cotton Wool	42,700	111	0.30
Cellulose Powder	48,900	785	1.60
NaCMC	10,000 (1 ml 1% Sol.)	844	8.40

TABLE 7.

Carbohydrase Activity in M.G.F.

Enzyme Classification	Substrate	Concentration (% W/V)	Total µ Reducing Sugars Produced pH 5.5	pH 7.0
α Glucosidase	Maltose	1.0	1,390	740
"	Sucrose	1.0	1,560	790
β Glucosidase	Salicin	1.0	260	80
"	Cellobiose	1.0	740	110
β Galactosidase	Lactose	1.0	0	0
Polysaccharase	Starch	0.5	1,000	698
	NaCMC	1.0	149	103
	Cellulose Powder	-	182	173
	Gum Arabic	1.0	0	0
	Gum Xanthan	0.5	0	0

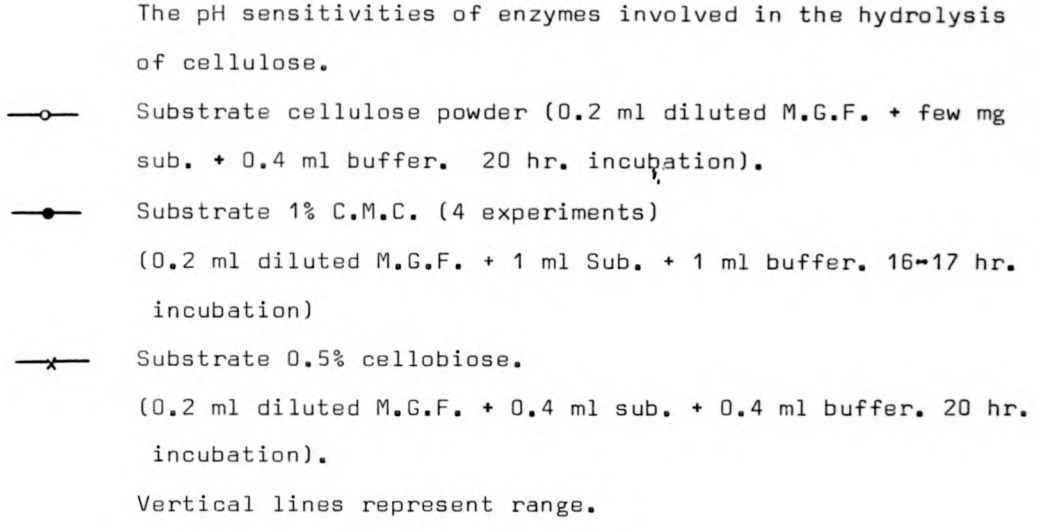
down more rapidly than C.M.C. or cellulose powder. Gum arabic, which contains hemicelluloses, and gum xanthan, a bacterial polysaccharide, were not hydrolysed to reducing sugars. Hydrolysis was more extensive at pH 5.5 than at pH 7.0 in all cases.

The pH sensitivity curves determined confirmed that carbohydrate hydrolysis was generally optimal under slightly acid conditions. Curves for the digestion of CMC and cellulose powder showed broad optima between pH 5.0 and 6.5 but that for the digestion of the dissacharide cellobiose showed a more defined optimum at pH 5.3 (figure 2). Similarly the digestion of starch (optimum 5.8) was tolerant of a wider pH range than was that of maltose (optimum 5.1) (figure 3).

The pH sensitivity curves of enzymes digesting proteins are shown in figure 4. All three curves were basically similar in that each probably had two optima, although the positioning of the optima differed. The curves for fibrin and bovine albumen were similar with optima at about pH 6.4 and 7.5, whilst that for casein indicated a more alkaline hydrolysis with optima at pH 7.5 and 8.75. Albumen was generally hydrolysed considerably less extensively than casein or fibrin. The pH sensitivities of peptidases are shown in figure 5. Optimum hydrolysis occurred around neutrality, carboxypeptidases A and B having optima at pH 7.25 and 6.7 respectively, and amino tripeptidase at pH 7.3. The second optimum on the leucyl-glycyl-glycine hydrolysis curve at pH 6.5 may have been caused by the action of leucine-amino-peptidase which also hydrolyses this substrate. Experiments with a substrate specific for this enzyme, leucine amide, indicated that optimum activity occurred at pH 6.8, but the results are not included here as the controls also showed an apparently high level of activity.

If these results are considered in conjunction with those concerning the pH of the various parts of the gut, it becomes evident that different types of food must be digested in different regions. The pH of the foregut and midgut glands is well suited to the digestion of carbohydrates but is too acid for protein digestion which must occur at the posterior end of the midgut, where the pH corresponds well with the sensitivities of protein digesting enzymes.

FIGURE 2.



The pH sensitivities of enzymes involved in the hydrolysis of cellulose.

—o— Substrate cellulose powder (0.2 ml diluted M.G.F. + few mg sub. + 0.4 ml buffer. 20 hr. incubation).

—●— Substrate 1% C.M.C. (4 experiments)
(0.2 ml diluted M.G.F. + 1 ml Sub. + 1 ml buffer. 16-17 hr. incubation)

—x— Substrate 0.5% cellobiose.
(0.2 ml diluted M.G.F. + 0.4 ml sub. + 0.4 ml buffer. 20 hr. incubation).

Vertical lines represent range.

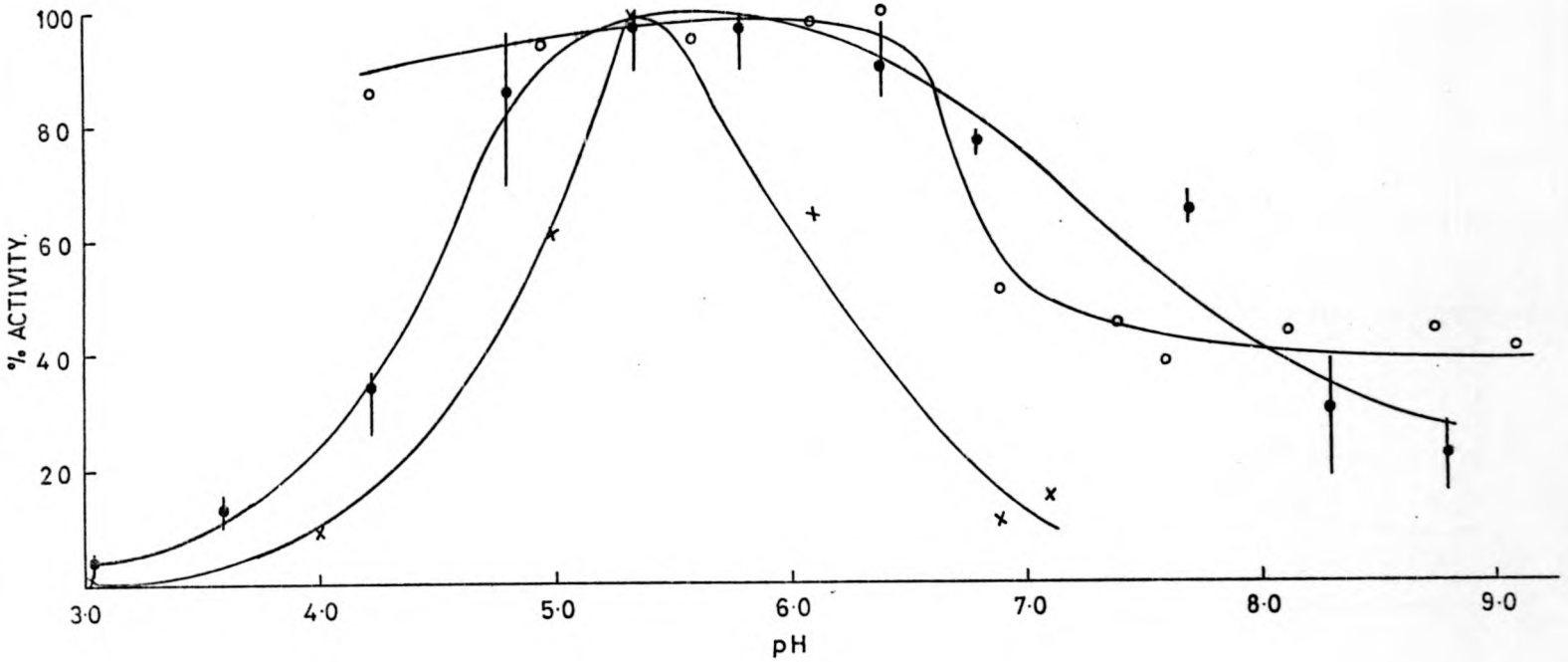


FIGURE 2.

FIGURE 3.

The pH sensitivities of enzymes involved in the hydrolysis of starch.

—●— Substrate 0.5% soluble starch. (3 experiments)
(0.2 ml diluted M.G.F. + 0.5 ml sub. + 0.2 ml buffer. 3 hrs. incubation).

—○— Substrate 0.5% maltose.
(0.2 ml diluted M.G.F. + 0.4 ml sub. + 0.4 ml buffer. 20 hrs. incubation).

Vertical lines represent range.

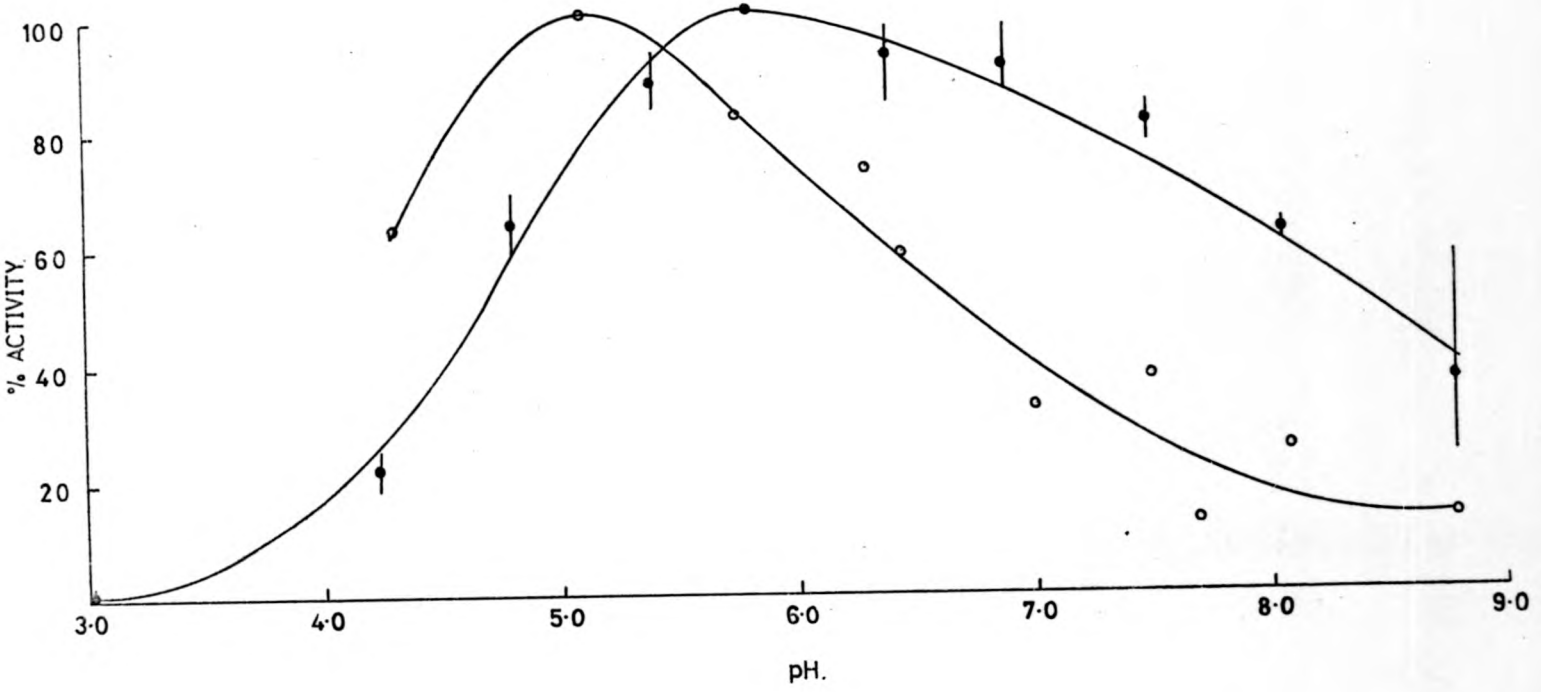


FIGURE 3

FIGURE 4.

The pH sensitivities of protein digesting enzymes.

(a) Substrate 1% Casein.
(0.2 ml diluted M.G.F. + 0.4 ml substrate + 0.2 ml buffer. 2 hrs. incubation).

(b) Substrate Fibrin.
(0.2 ml diluted M.G.F. + few mg. substrate + 0.4 ml buffer. 20 hrs. incubation).

(c) Substrate 1% Bovine Albumen
(0.2 ml diluted M.G.F. + 0.4 ml sub. + 0.2 ml buffer. 2 hours incubation).

rotein digesting

.4 ml substrate
incubation).

few mg. substrate
. incubation).
men
.4 ml sub. +
incubation).

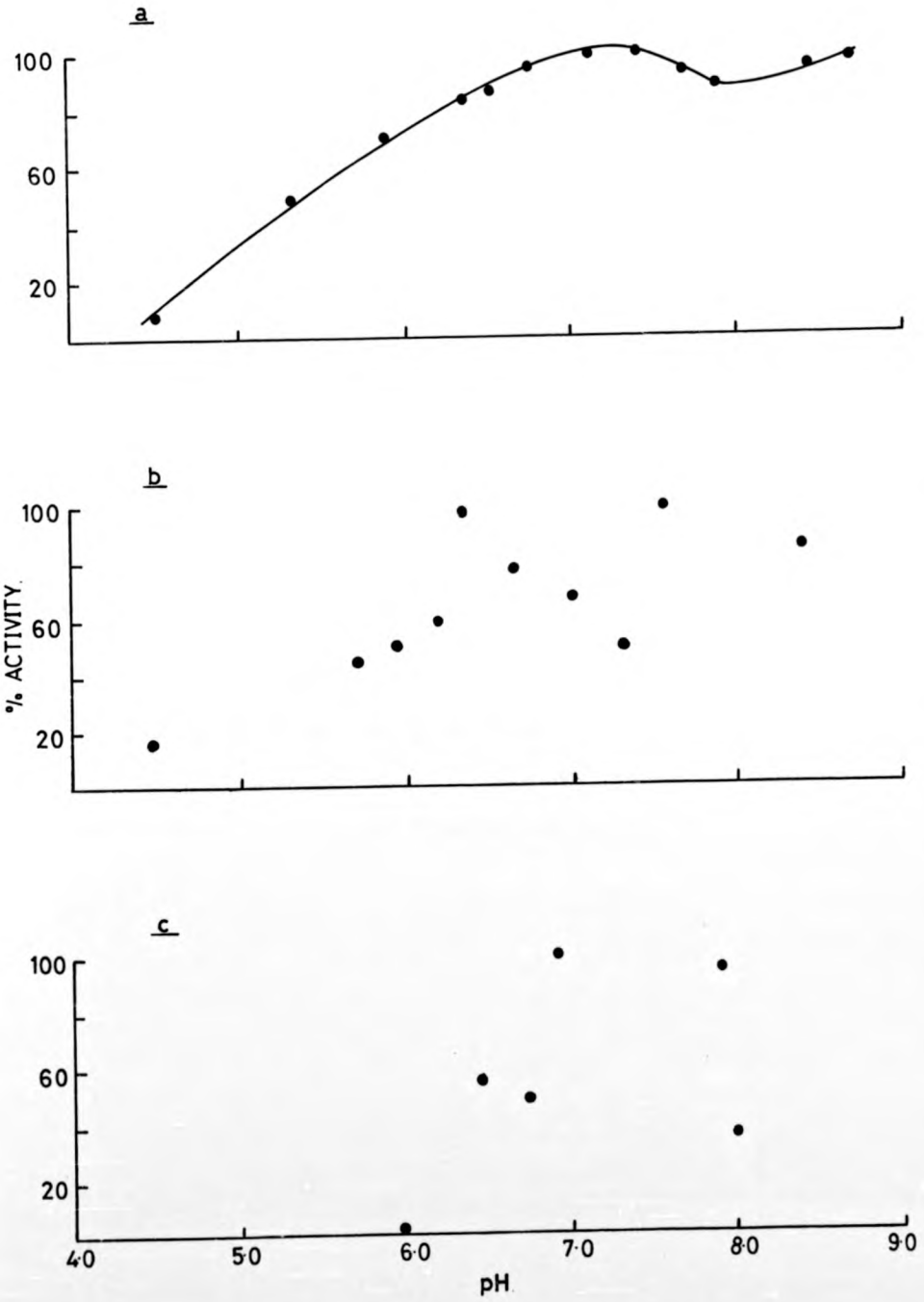


FIGURE 5.

The pH sensitivities of peptidases.

Amino Tripeptidase.

(0.2 ml diluted M.G.F. + 0.2 ml 1% leucyl-glycyl-glycine + 1 ml buffer. 20 hours incubation).

Carboxypeptidase A.

(0.2 ml diluted M.G.F. + 0.3 ml 0.1% Hippuryl-l-phenylalanine + 1 ml buffer. 20 hours incubation).

Carboxypeptidase B.

(0.2 ml diluted M.G.F. + 0.2 ml 1% Hippuryl-l-arginine + 0.5 ml buffer. 20 hours incubation).

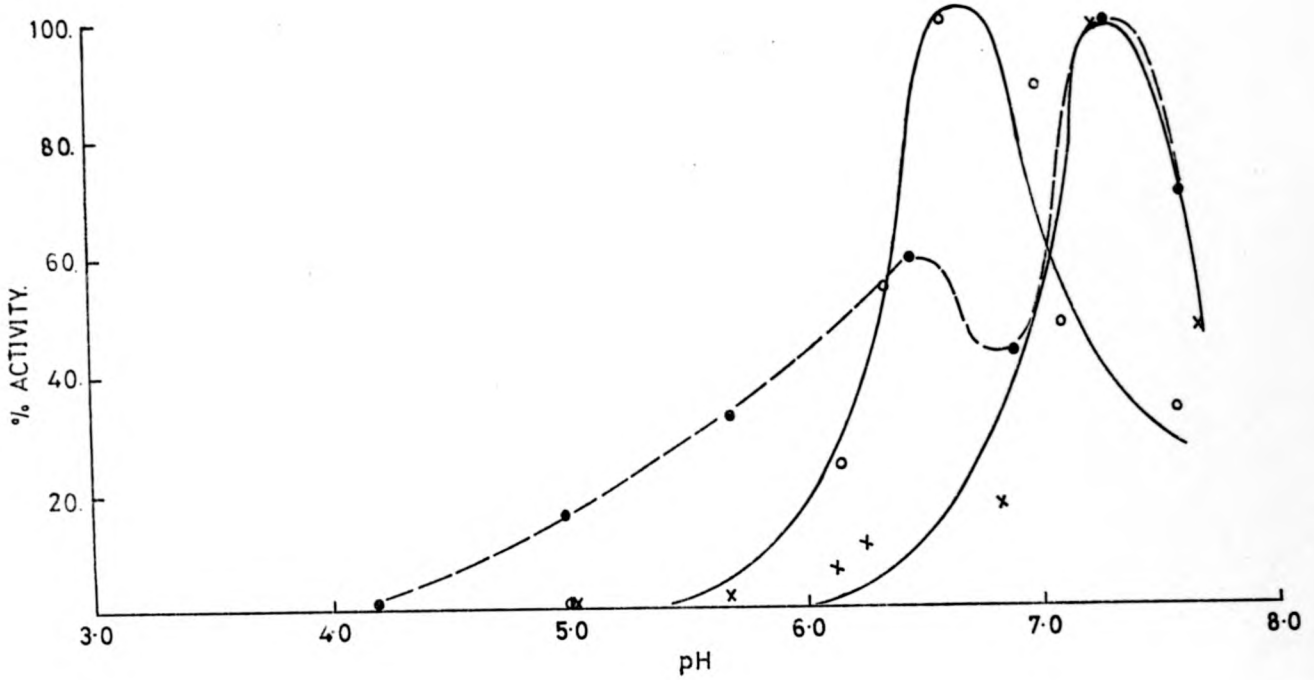


FIGURE 5.

The pH sensitivities of peptidases.

The temperature sensitivity curves of enzymes digesting CMC, soluble starch and casein are shown in figure 6. Amylase and protease had similar sensitivities with optima at about 50°C, but cellulase had a lower optimum temperature ($\leq$ 40°C) and was considerably more active at low temperatures.

Aquedus extracts of elm leaves had an inconsistent effect on the activities of the enzymes tested (Table 8). An inhibition was often observed but in some cases an apparent activation occurred. The inconsistency of the results was probably partly caused by the different treatments of the extracts prior to their use, and partly by the presence of substances in the extracts which could act as substrates for enzymes in the M.G.F. In particular this latter factor may have been the cause of the apparent activation observed in some cases.

In order to overcome some of these problems, the digestion of filter paper in leaf and fine detritus extracts was studied using controls containing active enzyme, but no substrate. The results are shown in Table 9. Changes in the amounts of reducing sugars in the controls in each case confirmed that the hydrolysis of substances in the extracts and,perhaps, adsorbtion onto materials in suspension may have been responsible for the inconsistent results in the previous experiment. At the end of the incubation period a slight softening and disintegration of the filter paper from the fine detritus extract and Ringer experiments was noticeable, but none in that from the leaf extract experiment. Although the amounts of reducing sugars produced were too low to give meaningful differences between experimental and control value in many cases, the results obtained did confirm that more reducing sugars were produced as a result of cellulose hydrolysis in the Ringer and fine detritus extract tubes. The extract used in this experiment was stored at 4°C overnight.

To confirm the apparent inhibition of cellulase by elm leaf extract, enzyme activity was assayed by measuring changes in weight and tensile strength of filter paper after nine days incubation. Two extracts were used, one of which was stored for 2 hours (1) and one for 14 days (2) at 0.5°C. Control tubes contained boiled enzyme solution. There was no apparent decrease in weight in any case but in many cases there was a

FIGURE 6.

The temperature sensitivities of various digestive enzymes.

Cellulase (0.2 ml diluted M.G.F. + 0.4 ml 1% C.M.C. + 0.2 ml pH 5.5 buffer. 3 hours incubation).

Amylase (0.2 ml diluted M.G.F. + 0.4 ml 0.5% soluble starch + 0.2 ml pH 5.5 buffer. 0.5 hours incubation).

Protease (0.2 ml diluted M.G.F. + 0.4 ml 1% casein + 0.2 ml pH 7.4 buffer. 1 hour incubation).

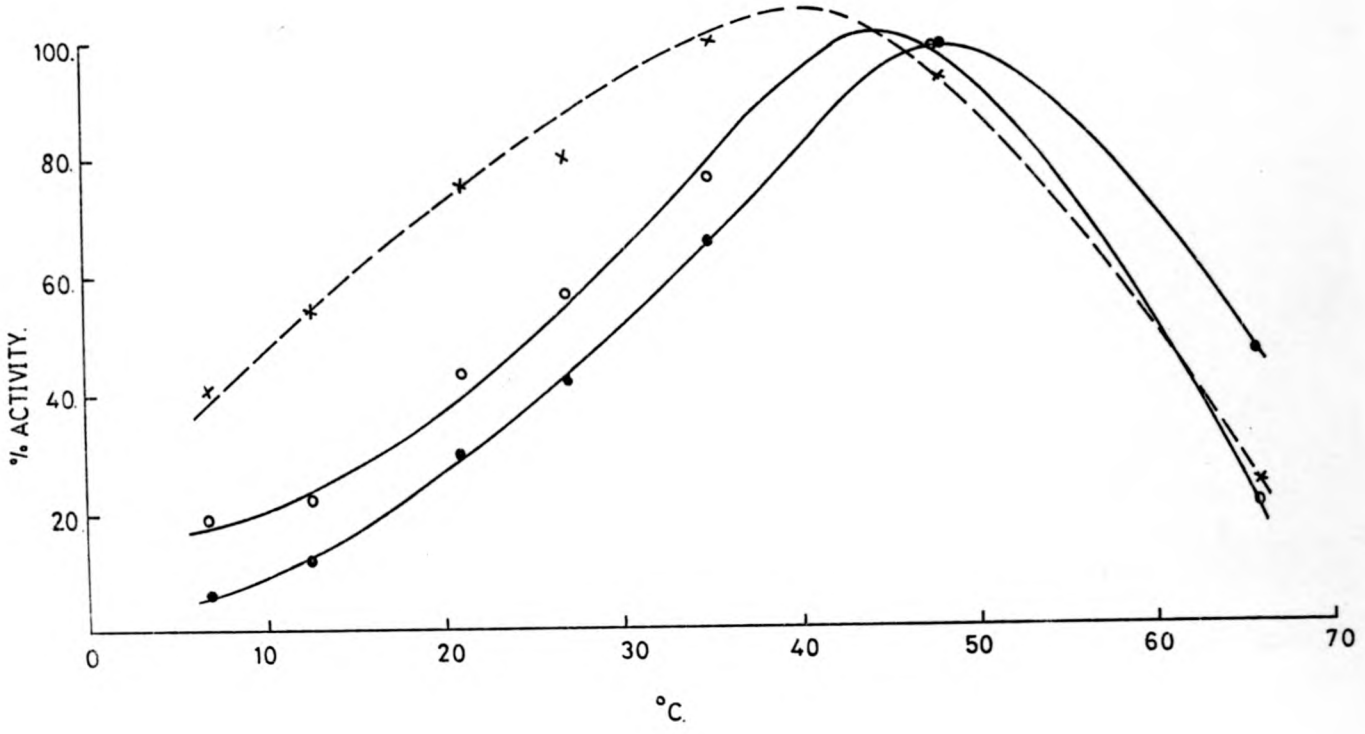


FIGURE 6.

The temperature sensitivities of various digestive enzymes.

TABLE 8.

The Effect of Elm Leaf Extracts on Enzyme Activity (O.D.)

Extract No.			1.			2.			3.			4.			5.			6.			7.			8.		
Storage Time (hr)			48			96			0			48			48			96			96			24		
Storage Temp (°C)			0 - 5			0 - 5						0 - 5			0 - 5			0 - 5			0 - 5			35		
Type of Leaf			Fresh			Fresh			Decomposing			Decomposing			Decomposing			Decomposing			Decomposing			Decomposing		
Sub-strate	pH	Incubation Time (hr)	E. (T)	C (T)	%C	E	C	%C	E (T)	C (T)	%C	E (T)	C (T)	%C	E (T)	C (T)	%C	E (T)	C (T)	%C	E	C	%C	E (T)	C (T)	%C
Casein	7.4	1.0-2.0	0.8	1.1	-27 (NS)	3.7	3.7	0	1.0	1.0	0	0.9	1.1	- 20 (NS)	0.9	1.3	-31 (S)	2.2	2.6	-15 (NS)	5.7	3.8	+50 (*)	1.8	1.6	+12.5 (NS)
Fibrin	7.4	24.0	-	-	-	3.0	4.5	-33 (*)	-	-	-	-	-	-	-	-	-	-	-	-	3.1	4.5	-31 (*)	-	-	-
Cello-biose	5.5	24.0	-	-	-	1.4	2.0	-30 (*)	-	-	-	-	-	-	-	-	-	-	-	-	1.4	2.0	-30 (*)	-	-	-
Maltose	5.5	2.5	-	-	-	1.8	3.3	-45 (*)	-	-	-	-	-	-	-	-	-	-	-	-	3.2	3.3	- 3 (O)	-	-	-
Starch	5.5	0.5 - 1.0	1.3	1.4	-7 (NS)	5.3	7.1	-25 (*)	-	-	-	1.3	1.4	-7 (NS)	-	-	-	-	-	-	7.3	7.1	+ 3 (O)	-	-	-
C.M.C.	5.5	2 - 24.0	1.1	1.6	-31 (S1)	3.8	3.6	+ 6 (O)	1.1	1.3	-15 (S5)	1.2	1.6	-25 (S1)	1.2	1.3	- 8 (NS)	1.4	1.7	-18 (S5)	3.4	3.6	- 6 (O)	1.4	1.3	+ 8 (NS)
Cell. Powder	5.5	24.0	-	-	-	5.2	3.5	+49 (*)	2.6	2.0	+30 (NS)	-	-	-	-	-	-	2.3	3.5	-34 (S1)	3.1	3.5	-11 (O)	2.4	2.4	0

Key : E : Experiment : C : Control
%C: % Change : (T) : Assays in Triplicate.

(*) Difference between assays significant)
(O) " " " not significant) Chapter 2
S1 Difference between assays significant at 1% level)
S5 " " " " " 5% ") t tests
NS " " " not significant)

TABLE 9.

Digestion of Filter Paper in Leaf and Fine Detritus Extracts (µg Reducing Sugar)/Aliquot

	Days	0	3	6
Ringer	Experiment	3.0	6.0	9.0
	Control	3.0	0.0	5.0
	R.S.H.	0.0	6.0*	4.0
Leaf Extract	Experiment	10.0	5.0	7.0
	Control	9.0	4.0	9.0
	R.S.H.	1.0	1.0	0.0
Fine Detritus Extract	Experiment	7.0	2.0	11.0
	Control	6.0	0.0	5.0
	R.S.H.	1.0	2.0	6.0*

R.S.H. = Reducing sugars produced by hydrolysis of filter paper.

* Difference between experimental and control assays significant.

TABLE 10.

Tensile Strength of Filter Paper after Hydrolysis

(Grams)

	Extract 1.	Extract 2.
Control (Prior to Incubation)	220	170
	260	180
	260	210
	220	170
	240	160
	$\bar{x}$ 240	$\bar{x}$ 180
	S.E. 9	S.E. 9

	Extract 1. Experiment	Control	% Decrease	Extract 2. Experiment	Control	% Decrease
Ringer	140	220		20	70	
	170	170		120	190	
	190	160		60	170	
	190	210		90	130	
	200	210		110	140	
	$\bar{x}$ 180	190	5(25)	$\bar{x}$ 80	140	43(56)
	S.E. 11	12		S.E. 18	20	
Leaf Extract	190	220		70	120	
	130	260		110	110	
	250	230		60	130	
	160	220		120	90	
	190	220		150	130	
	$\bar{x}$ 180	230	22(25)	$\bar{x}$ 100	120	17(44)
	S.E. 20	8		S.E. 17	8	
Fine Detritus Extract	180	200		200	120	
	220	260		110	150	
	150	240		80	220	
	190	160		100	160	
	130	180		110	130	
	$\bar{x}$ 170	210	20(29)	$\bar{x}$ 120	160	25(33)
	S.E. 16	19		S.E. 21	18	

Figures in brackets represent % difference between experimental values, and control values prior to incubation.

slight increase (0.1 - 0.6 mg), presumably caused by adsorbtion of substances from solution. In every case hydrolysis evidently caused a decrease in tensile strength (Table 10), but interpretation of the results is difficult as in some cases the tensile strengths of the controls also decreased. In some cases the individual control values were similar to those determined prior to the incubation period, but in others they were as low as those in the experimental tubes. If the control results are considered similar and equal to the value determined prior to incubation, the decreases in tensile strength in Ringer, leaf extract and fine detritus tubes are not significantly different in either case. Tensile strength measurements therefore provided no evidence for the presence of cellulase inhibitors in aqueous elm leaf extracts.

iv The Passage of Food along the Tract

The times taken by N.L. and 10OC to pass along the tract are shown in figure 7. The results of an experiment in which the animals were transferred from N.L. to 10OC and the passage of the junction between the two diets followed are also shown. It will be demonstrated in a later chapter that N.L. are ingested at a much slower rate than 10OC but it is evident from figure 7 that both diets passed through the tract in about 5-7 hours. When the animals were transferred from N.L. to 10OC they appeared to feed continuously and filled the tract completely with cellulose in about an hour. The tracts of animals feeding on both diets were not always full of food, and those of animals consuming N.L. usually contained very little. The passage of food along the tract was therefore apparently independent of the intake of further food, except where ingestion occurred at a particularly high rate when the food was forced through the tract in a short time. This conclusion is substantiated by the observation that when Gammarus was completely starved, the production of faeces continued until the tract was almost empty. A small amount of food sometimes remained in the tract for several days however.

FIGURE 7.

The passage of food along the tract.

100 C

N.L.

100 C, after feeding N.L. (See text).

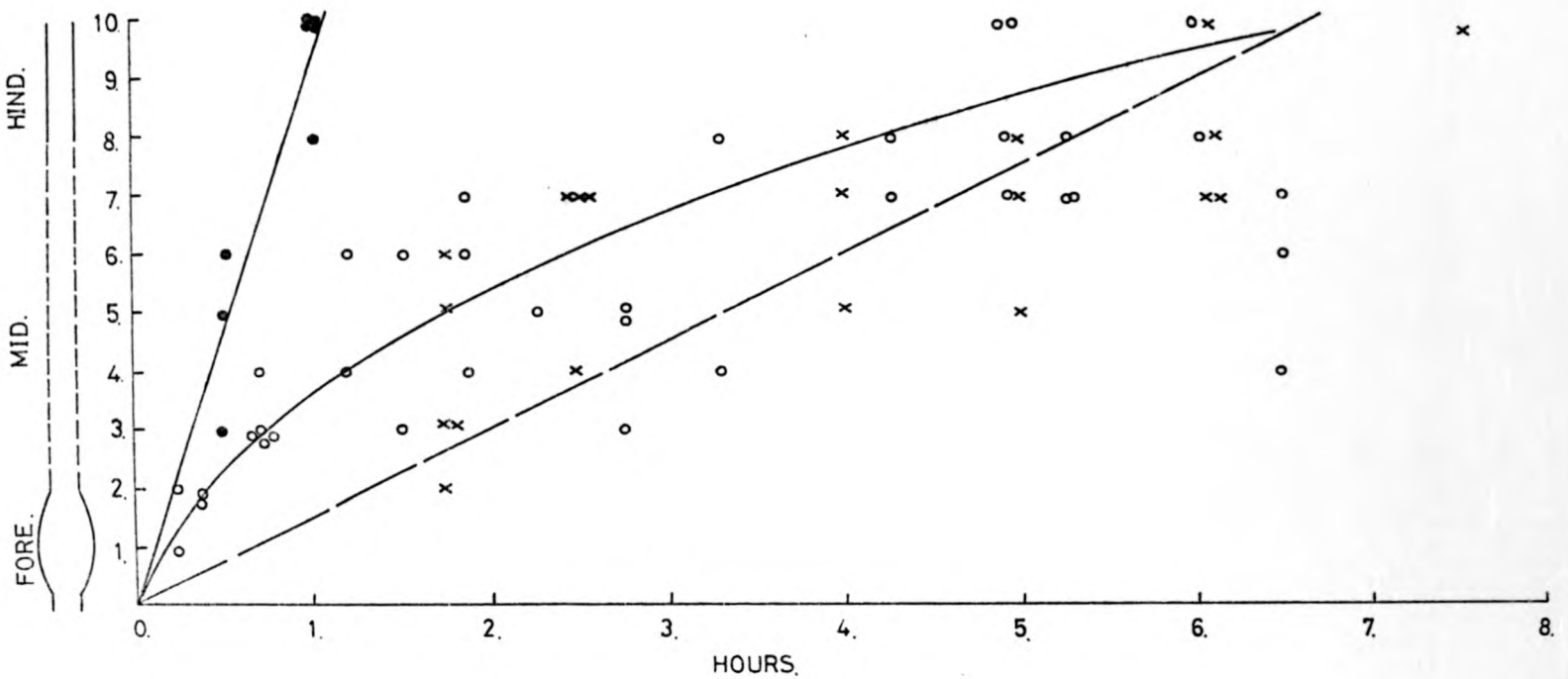


FIGURE 7.

The size distribution of particles in the proventriculus and faeces of animals fed N.L. and F.D. is shown in figure 8. The similar appearance and size distribution of freshly ingested and freshly defaecated particles suggested that little physical breakdown of either diet occurred during its passage along the tract. Very small particles of both diets were numerically dominant in both faeces and proventriculus. Particles smaller than 36μ were almost always non-cellular fragments, those from 0 - 18μ being slightly more abundant in the faeces and those from 18 - 36μ in the proventriculus. Particles larger than 54μ were usually recognisable cellular fragments. These particles, although numerically few, obviously made up a large bulk of the contents of the faeces and proventriculus. They presumably represented food that had been cut up by the mouth parts and ingested, but that had not been further triturated in the tract, whilst small particles were the result of mechanical and chemical digestion of the food. The almost equal abundance of large particles in faeces and proventriculus suggested that little further trituration occurred after the food entered the proventriculus. The approximate percentage of the volume of the faeces and proventriculus contents made up by the various sizes particles is shown in Table 11. These were calculated by assuming that each particle was a sphere, the diameter of which was the dimension measured. As the size distribution of particles between 72 and 300μ seemed to be roughly random, these figures were lumped together. The mean size of all particles measured in this range was calculated (163μ, N = 82), and the volume worked out from this figure. About 90% of both diets evidently passed along the tract as large particles and were not further broken up after ingestion.

The appearance of large cellular particles from animals fed N.L. suggested that the cell walls were not chemically broken down in the gut. The cells in the particles were evidently intact and usually contained visible cell contents, although some cells around the outsides of the particles had obviously been mechanically broken open. No differences in the appearance of these particles could be detected between samples from the faeces and proventriculus. The most common cells measured roughly 12 by 60μ and the number that were broken open depended upon the size and shape of the particle, but subjective imp-

ressions suggested that this varied between 10 and 30% in the larger particles. Overall, it seems unlikely that on average more that 30-40% of the cells in N.L. passing through the tract were broken open.

There was also little difference between the appearance of faeces and proventriculus samples from animals fed F.D. Both contained some large particles of cellular material, the cells of which were apparently intact, but a large proportion of the particles were more amorphous, having lost much of their cellular appearance. In some cases a 'skeleton' of empty cells was seen, the cell walls being only partially intact, and in other cases no trace of a cellular appearance was visible. As particles in faeces and proventriculus were similar it was concluded that their appearance was the result of microbial degredation of the food before it was ingested, rather than the result of the digestive processes of Gammarus.

FIGURE 8.

Particle size distribution in the foregut and faeces of animals fed different diets.

(a) F.D.

(b) N.L.

Foregut contents cross hatched.

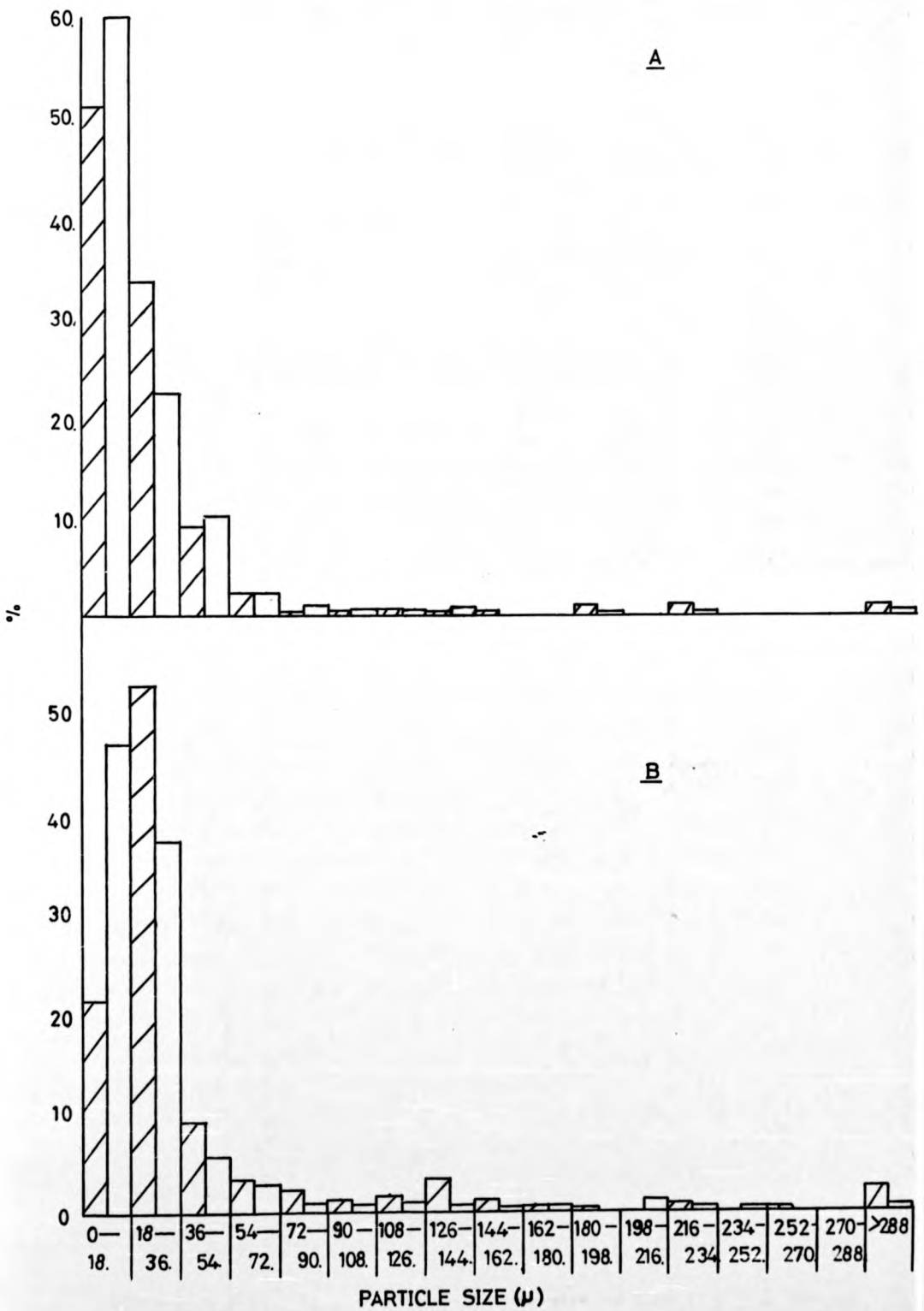

A
B
%
60.
50.
40.
30.
20.
10.
50
40
30
20
10
0
0—18.
18—36.
36—54.
54—72.
72—90.
90—108.
108—126.
126—144.
144—162.
162—180.
180—198.
198—216.
216—234.
234—252.
252—270.
270—288.
>288
PARTICLE SIZE (μ)

TABLE 11.

Size Distribution of Particles in Proventriculus and Faeces as a Percentage of the Total Volume

Size (μ)	N.L.		F.D.	
	Proventriculus	Faeces	Proventriculus	Faeces
0-18	0.03	0.10	0.20	0.20
18-36	1.80	2.30	3.50	2.00
36-54	1.40	1.60	4.40	4.30
54-72	1.40	2.30	3.00	2.80
72-300	95.30	93.70	88.90	90.70

c) Discussion

The digestive physiology of decapods has been the subject of extensive research since the latter half of the nineteenth century and considerable information concerning this group, which was reviewed by Jordan (1904), was available by the beginning of this century. Relatively little was known concerning the Peracarida at that time however, although Weber (1880) described and illustrated the structure of the midgut glands of various gammarids and noted their similarity to that of Astacus. Apart from the studies of G.pulex (Willer, 1922) and Ligia oceanica (Nicholls, 1931) little work seems to have been published concerning these groups until relatively recent years. The digestive physiology of the Crustacea has been reviewed by Mansour Bek (1954), Vonk (1960), Barrington (1962), Huggins and Munday (1968), Arvy (1969) and Van Weel (1970).

Although some of the early workers reported the presence of a cellulase in the digestive fluid of decapods, this was not confirmed until recently (e.g. Kooiman, 1964 ; Yokoe and Yasumasu, 1964 ; Elyakova, 1972). The origin of the cellulase is not known in many cases but Van Weel (1970) considered that cellulase is secreted by the midgut glands of at least some crustaceans. Its origin in G.pulex is apparently endogenous. Other workers (Wildish and Poole, 1970 ; Halcrow, 1971) have found that cellulase activity in the guts of the amphipods Orchestia gammarella and Gammarus oceanicus was slightly reduced when antibiotics were included in the diet, and concluded that the enzyme was partly endogenous and partly of bacterial origin. Wildish and Poole also succeeded in culturing bacteria from the midgut gland of Orchestia which could disintegrate filter paper. The occurrence of bacteria in.the gut of G.pulex which can grow on cellulose media is not surprising in view of the animals diet. The possibility that these bacteria may contribute to the extra-cellular cellulase found in the M.G.F. can not be excluded, although none of the bacteria examined here produced such an enzyme.

Cellulases in some other crustaceans are also believed to be endogenous (Ray and Julian, 1952 ; Hartenstein, 1964 ; Kooiman, 1964) although that in the crayfish Procambarus clarkii is known to be of

mixed origin (Yokoe, 1960), and Newcomer (1956) suggested that cellulose digestion in the isopod Porcellio may be entirely bacterial. This suggestion was substantiated by the finding of Jeuniaux (1956) that chitin digesting bacteria were present in the gut of Porcellio. Cellulases in other invertebrates may be endogenous (Lasker and Giese, 1956 ; Lewis and Whitney, 1968) or of bacterial origin (Florkin and Lozet, 1949, or of protozoan origin (Hungate, 1943). This last possibility is unlikely to be of importance in Gammarus as no large numbers of protozoa have been observed in the M.G.F. either here, or in the anatomical studies of Mabillot (1955) or Agrawal (1965).

The pH of the M.G.F. of G.pulex has previously been investigated by Willer (1922) and Agrawal (1965). Willer considered that the entire gut displayed an alkaline reaction but this conclusion was based on the yellow colour observed in the gut upon immersion in methyl orange solution. As the pK of methyl orange is 3.7 and the colour change is complete at pH 4.4 this only means that the pH was higher than the latter figure, which is in agreement with the results obtained here. The value of 6.5 found by Agrawal does seem to differ from the present results however.

The exhaustive studies of Zankai (1966) on six crustaceans, including three amphipods, led her to conclude that the pH of the M.G.F. was between 6.1 and 6.3 in every case. Agrawal (1963, 1964) also showed that the amphipods Corophium volutator and Orchestia gammarella had M.G.F. of similar pH (Table 12). Martin (1966) found a M.G.F. pH of 5.6 in Marinogammarus obtusatus but the animals had been previously starved for two days, which is known to cause increased acidity in the digestive fluid of some crustaceans (Van Weel, 1970). The only real exceptions to the 6.1 - 6.3 pH range are the fluids from the midgut glands of Hyallela azteca and Gammarus limnaeus which are considerably more acid (De Giusti et al, 1962). When G.pulex was fed 100C, which was unlikely to have any effect on the pH of the gut contents either directly, or via the effects of its hydrolytic products, the pH of the M.G.F. of most groups tested was between 5.8 and 6.15, which range is close to the 'normal' one previously discussed. When increasing amounts of protein were added to the diet the pH fell, lying between 5.3 and

TABLE 12.

The pH of the Gut of various Amphipods

Species	Author	Food	Midgut Gland	Proventric.	Mid Gut Ant.	Mid Gut Med.	Mid Gut Post.	Hind Gut
G.pulex	Present work	Various decomposing plant material.	5.60-5.90	5.30	5.20	5.40	7.00	7.10
G.pulex	Agrawal 1965	?	6.50	6.75	-	6.70	-	6.80
Corophium volutator	Agrawal 1963	Mud Particles	6.20	6.75	-	6.60	-	6.80
Orchestia gammarella	Agrawal 1964	?	6.05	6.70	-	6.45	-	6.95
Marinogammarus obtusatus	Martin 1966	Starved 2 days	5.60	6.10	-	6.20	-	-
Dicerogammarus spp.	Zankai 1966	Agar	6.10	6.30	6.30	7.10-7.40	7.60-8.10	-
Gammarus roeseli	Zankai 1966	Agar	6.20	6.40	6.40	7.10-7.80	7.60-8.00	-
Hyallela azteca	De Giusti et al 1962	Yeast, Agar, Gelatin	3.80-4.80	4.10-4.70	4.10-4.70	6.80-7.20	7.20-7.70	7.20-7.70
Gammarus limnaeus	De Giusti et al 1962	Yeast, Agar, Gelatin.	3.80-4.80	4.10-6.30	4.10-6.30	6.40-7.20	7.20-7.90	7.20-7.90

5.85 when the diet was 100% protein. The M.G.F. pH from animals fed natural diets lay between these two sets of figures. This would suggest that the 'natural'M.G.F. pH of G.pulex, like that of other amphipods, may be around 6.0, but that when feeding normally it is lowered by the effects of digestive products such as amino and fatty acids. If this were the case it might be expected that during starvation the M.G.F. pH would rise back to the 'normal' level, but, as previously mentioned, starvation itself may cause a fall in M.G.F. pH, and the range of 5.45 to 5.8 found here in starved animals may represent such a condition.

Little other work seems to have been done concerning the effects of different test diets fed over short periods on the pH of the M.G.F. Agrawal (1965) fed G.pulex with algal filaments after 50 hours of starvation and found that the pH of the entire gut was similar to that of animals feeding normally. In a previous paper (Agrawal, 1964), he treated Orchestia gammarella in the same way and obtained similar results. Zankai (1966) used various feeds such as starch, gelatin and yeast and concluded that there was no connection between the pH of the feed and that of the gut. She did suggest however that the acid conditions found in the midgut glands of the amphipods studied by De Giusti et al (1962) might be explained by the normal feeding habits of the species concerned. She pointed out that those species were omnivorous scavengers, whereas the species she studied were either herbivorous or carnivorous. The results obtained here do not fit in well with that hypothesis as M.G.F. from G.pulex, which may also be considered an omnivorous scavenger, is much less acid than that from the species studied by De Giusti et al. However, the disagreement between the results presented here and those of Agrawal (1965) might be explained by differences in the normal feeding habits of the populations of animals used. Agrawal collected his animals from various reservoirs around London in which their normal diet may have been different to that of the animals used here, perhaps mainly consisting of algae. If so, that population may have developed less acid gut conditions, mediated either by a physiological response of individuals after long periods of exposure to a less acid diet, or by a genetic response of the population. Perhaps it is

selectively more advantageous for the M.G.F. pH to be buffered near to the pH of the diet, than for the animal to secrete a buffering system sufficiently powerful to maintain a large pH differential.

Most of the published literature concerning amphipods confirms that the tract may be divided into an anterior acid region and a posterior neutral or alkaline region (Table 12). An alkaline or neutral fluid must be secreted at the posterior end of the tract and Zankai (1966) has suggested that the posterior dorsal caeca may be the source of such a fluid. This secretion is of considerable importance as it creates in the tract a region in which the pH is suitable for the activity of protein digesting enzymes. Absorbtion of digestive products may also occur here as Mabillot (1955) noted that there was a short region of absorbtive epithelium at the posterior end of the midgut.

Although the properties of crustacean digestive enzymes may be affected by the presence of other substances in crude preparations and so may alter after purification (Vonk, 1960), those determined from such preparations are presumably more relevant to 'in vivo' activity than would be those determined from purified preparations. Most studies of crustacean enzymes have utilised crude preparations and the results described here will be discussed in relation to the results of such studies and the known conditions in the gut.

As Agrawal (1965) and Bjarnov (1972) have previously extensively studied the range of gut carbohydrases occurring in G.pulex, no attempt to do so has been made here. Those authors showed that the polysaccharides starch, glycogen, xylan, chitin and inulin could be hydrolysed, but not pectin. Agrawal could not detect the hydrolysis of laminarin or CMC and Willer (1922) did not believe that G.pulex possessed a cellulase because the cell walls of Elodea could not be digested. However, Willer's experiments were carried out in a 0.5% solution of Na_2Co_3 which has a pH of about 11, under which condition little hydrolysis would be expected (figure 2). Bjarnov (1972) demonstrated the hydrolysis of CMC, filter paper and laminarin by gut extracts of G.pulex, and all of the many gut extracts and samples of M.G.F. tested for their ability to hydrolyse various cellulose substrates in the present work have given positive

results. CMC was not extensively hydrolysed (Table 6), which may explain the results of Agrawal (1965), bearing in mind the rather insensitive qualitative tests he used.

Although there is no doubt that an enzyme exists in the gut which can produce reducing sugars from cellulose substrates, the importance of this enzyme in the nutritional physiology of the animal is less clear. The results shown in Table 6 show that only small proportions of various cellulose substrates were digested under 'in vitro' conditions after six days incubation. Similarly Ray (1959) found that after two days incubation only 96 µg of reducing sugars were liberated from 20 mg of ground filter paper by the action of a cellulase preparation from the wood boring isopod Limnoria. Cowling and Brown (1969) emphasised the importance of the physical nature of the substrate and its accessibility to the enzyme, and this obviously had an effect on the results described here as more hydrolysis occurred where the substrate had been previously physically broken down. The appearance of the contents of the proventriculus and the faeces suggested that any plant cells which were not broken open during ingestion or trituration in the proventriculus would not be broken open by enzymic hydrolysis, and that in the case of certain diets a large proportion of the cells may fall into this category. Similarly Willer (1922) found many undigested algal cells in the guts of G.pulex collected in the field, and Agrawal (1961, 1964) found that Orchestia gammarella, which possesses a cellulase, could not break down algal cell walls. The ability of Gammarus to digest cellulose and the role of cellulase in its nutritional physiology will be returned to in a later discussion when further significant experimental results have been described.

The carbohydrases of most crustaceans seem to have pH optima between 5 and 7 (Van Weel 1970) and those of G.pulex follow this pattern. The optimum of 5.8 found here for the digestion of soluble starch is similar to those found for the amphipods Orchestia gammarella (5.9), Corophium volutator (5.8) and Marinogammarus obtusatus (5.6 - 6.3) (Agrawal, 1964a, 1963 ; Martin, 1966) and for the isopod Ligia oceanica (5.65) (Nichols, 1931). Nothing seems to be known of the pH sensitivities of maltose digesting enzymes from the Peracarida but Van Weel (1970)

quotes unpublished work by Yee and James which gives the optima from the decapods Procambarus clarkii and Thalamita crenata as 5.6 and 4.4 - 5.0 respectively. These figures are similar to that found here for G.pulex (5.1).

The broad pH optimum found for the hydrolysis of both CMC and cellulose powder between 5.0 and 6.4 presumably resulted from the production of cellobiose and glucose. As cellobiose was optimally hydrolysed to glucose at the lower end of this range, the true optimum for the hydrolysis of cellulose to cellobiose may lie at the upper end. This value would be similar to that of 6.5 found by Wildish and Poole (1970) for Orchestia gammarella. pH optima reported for other crustacean cellulases are 5.1 for Oniscus asellus (Hartenstein, 1964), 5.8 for Procambarus clarkii (Yokoe, 1960) and 4.0 - 4.5 for Astacus fluviatilis (Kooiman, 1964). Ray (1959) found that the optimum for Limnoria cellulase was between 4.5 and 4.8, but after a long incubation period maximum hydrolysis occurred between pH 5.2 and 7.2, suggesting greater stability at the less acid pH values. Cellulases from molluscs also usually have slightly acid pH optima (Gascoigne and Gascoigne, 1960) but Lasker and Giese (1956) found three optima for cellulase from the silverfish Ctenolopisma lineata at pH 4.5, 6.0 and 7.7. As optima for cellobiose digestion also occurred at the first two values, optimum cellulose hydrolysis may have been in the alkaline range in this case.

Protein digesting enzymes occur almost universally in the guts of holotrophic animals. That some crustaceans can digest protein has been known for many years (e.g. Weber, 1880 ; Stamati, 1888) and extensive studies in recent years have shown the wide distribution of such enzymes in the Crustacea (Degkwitz, 1957 ; De Villez and Buschlen, 1967 ; Sather, 1969 ; Koslovskaya and Vaskovsky, 1970). The occurrence of protease in the gut of G.pulex was investigated in the early work of Willer (1922) who found that proteins of animal and plant origin could be digested, and more recently Agrawal (1965) demonstrated the digestion of gelatin by midgut gland extracts.

As a result of studies of the pH sensitivities of protein digesting enzymes from many crustaceans Degkwitz (1957) and De Villez and Buschlen (1967) both concluded that most crustaceans, including

amphipods, digested protein optimally at neutral or slightly alkaline pH values, but that a few, notably barnacles, exhibited more acid optima. The amphipods Corophium volutator and Gammarus locusta were included in the study of Degkwitz, and Synurella sp in that of De Villez and Buschlen. The former species hydrolysed casein and the latter T.A.M.E. (p - toluenesulfonyl-L-arginine methyl ester) at an optimum pH around 8.0, which is similar to that found here for G.pulex. Other amphipods have been studied by Agrawal (1963a), who found that midgut gland extracts of Orchestia gammarella liquified gelatin optimally at pH 7.7 to 8.3, and by Ponyi (1966) and Ponyi and Zankai (1967) who found that Gammarus roeseli and Dicerogammarus haematobaphes balatonicus both digested haemoglobin optimally at pH 6.6 to 7.1. It is well known that the pH sensitivity of protein digestion varies with the use of different substrates, and this is evidently the case in G.pulex. This may be important in accounting for the variations in the results described above for different amphopods, particularly as Ponyi and Zankai (1967) found that the two amphipods they tested, although of different feeding habits, had similar protease pH optima when tested under identical conditions. No other authors have found double pH optima in amphipods but studies of protease activity in gut extracts of several other crustaceans have revealed the presence of such double optima (Degkwitz, 1957a ; De Villez, 1965).

The pH optima of carboxypeptidases A and B and amino tripeptidase found here, 7.25, 6.7 and 7.3 respectively, are not dissimilar to the few other values reported in the literature. Ponyi et al (1969) found that the carboxypeptidase A activities from Gammarus roeseli and Asellus aquaticus were optimal at pH 8.0 - 8.2 and 7.5 - 7.7 respectively, and De Villez (1965) found optimum carboxypeptidase A and B activities in Oronectes virilis at pH 6.0 and 7.0 respectively. Kleine and Ponyi (1967) found optimal carboxypeptidase A activity in Astacus astacus and Cambarus affinis at pH 7.6.

Optimum temperatures are particularly dependent upon assay conditions, particularly the duration of incubation, and are mainly of interest in relation to the incubation temperatures used during enzyme assays.

That used here, (30°C), is on the ascending phase of the temperature sensitivity curves of all three enzymes tested, and is too low to cause any denaturation during incubation (Blandamer and Beecher, 1966).

Although the presence of cellulase inhibitors in elm leaves seemed a possible explanation for the apparent non-digestion of the leaf cell walls, none could be definitely shown to exist in aqueous leaf extracts. However, the methods used were very crude and the presence of high concentrations of various organic compounds in the extracts, and their varying stabilities when stored, led to considerable variation in the results. Enzymes may be inhibited or activated by a wide range of substances and it would have been surprising if some of the enzymes tested had not shown different activities in Ringer's solution and leaf extracts. Although confirmation of the presence of a cellulase inhibitor in leaf extracts must await the use of purified enzyme preparations and separated fractions of leaf extracts, subjective impressions suggested that the digestion of filter paper was inhibited by at least one extract tested, and this was confirmed by the amounts of reducing sugars produced in each case. Gascoigne and Gascoigne (1960) have listed many substances which may inhibit or activate cellulases. Whilst proteins and some sugars may stimulate enzyme activity, other sugars and substituted phenols may be inhibitory. These last were particularly effective inhibitors and Ray (1959) has reported that phenol will inhibit the activity of cellulase from Limnoria. It is therefore of considerable interest that polyphenolic substances (tannins) do occur in leaf litter, although they may be rapidly broken down, and may be responsible for the initial low palatability of leaf litter to many animals (Bocock, 1964 ; Satchell, 1967).

The time which food takes to pass along the tract of crustaceans seems to vary according to the normal feeding habits of the species concerned. Some, such as the filter feeding branchiopod Streptocephalus dichotomus and the sediment feeding amphipod Hyallela azteca, have rapid turnover times, emptying their tracts every 17-18 minutes and every 30 minutes respectively (Bernice, 1971 ; Hargrave, 1970). Others such as the terrestrial isopod Oniscus asellus which eats decaying woody materials, have slower turnover times, emptying their tracts every 13-

17 hours (Hartenstein, 1964). Martin (1966) found that Marinogammarus obtusatus, which eats live and decaying plant materials (mainly seaweeds), normally retains food in its tract for about 12 hours, although some types of food were passed through in 2.5 hours, and the optimum time for maximum digestion was about 5 hours. G.pulex, with a normal retention time of 5-7 hours, is therefore not unusual in this respect. The rate of food passage along the tract was apparently not normally dependent on the rate of ingestion, although this is often assumed to be the case in arthropods, and may be the case in G.pulex when ingestion is particularly rapid. Peristaltic movements have been observed in the tracts of crustaceans by Yonge (1924) and Hartenstein (1964). Other arthropods may depend on ingestion to keep food moving along the tract as Applebaum et al (1964) found that movement of food through the tracts of larvae of the lepidopteran Prodenia litura stopped when feeding ceased and I have noticed that larvae of Halesus spp (Trichoptera) ceased production of faeces immediately their food supply was removed, and this behaviour did not vary when fed a considerable range of foods.

Overall, consideration of the results described here and of those reported in the literature suggests that the chemical and physical conditions in the gut of G.pulex are similar to those in comparable crustaceans. Although the use of crude preparations makes the properties of digestive enzymes difficult to compare, there are no reasons to believe that those of G.pulex are atypical in any way. Among the Amphipoda at least, the pH of the different regions of the gut and the pH sensitivities of the digestive enzymes are probably fairly uniform between different species. The main ways in which the chemical and physical conditions in the guts of different species are likely to vary are in the duration of food passage along the tract, and in the presence and origins of cellulase and perhaps other polysaccharide digesting enzymes. These factors may also be amongst the most important in determining the extent to which intractable diets such as leaf litter can be digested. As G.pulex secretes its own cellulase and retains food in its tract for a longer period than some amphipods, it might be expected that such diets could be efficiently utilised. The presence of apparently undigested plant material in the faeces however suggests that this is not the case. Further evidence bearing on the ability of G.pulex to utilise such diets is presented in the remainder of this thesis.

CHAPTER 5

THE EFFECT OF DIET ON INGESTION AND ASSIMILATION

a) Methods

The accurate determination of ingestion and assimilation in aquatic animals is beset by methodological problems. Movements of inorganic material across the gut wall and the loss of endogenous organic matter in the faeces make accurate estimates of assimilation difficult to obtain, and the changes in weight which diets and faeces may undergo upon standing in water lead to problems in determining ingestion and egestion rates.

Ingestion and assimilation have been measured here by gravimetric methods, which entail weighing the food eaten and the faeces produced and assuming that the difference represents assimilation, and in a few cases the results compared with the ash-ratio method of Conover (1966). This latter method does not necessitate quantitative estimation of consumption and egestion, but depends on measuring the increase in the proportion of ash in the faeces compared to that in the food. The ash-ration method therefore depends upon the assumption that no inorganic material passes in to or out of the gut lumen.

The conditions under which the test diets were offered were based on those described by Hargrave (1970). Groups of animals, from 7-11 mm in length, were confined in perspex tubes of 9.5 cm diameter, the bottoms of which were covered with 1.5 mm mesh fibreglass netting. These tubes were placed in 13 cm diameter glass troughs which contained about 400 ml of filtered water, and were supported 1 cm above the bottoms of the troughs by small petri dishes. Faeces produced immediately fell through the mesh and so could not be ingested. The diets were contained in vial caps of 2.7 cm diameter which rested on the mesh. The animals spent only a small part of their time feeding in the caps and most of the faeces produced were free to fall through the mesh. Before the experiments animals were starved for 4-5 hours in order to partially empty their guts. A short starvation period was used to avoid stimulating rapid ingestion at the beginning of the feeding period. As 90C + 10P, 60C + 40P, 100P, and both fine detritus diets were prepared wet, it was not possible to weigh accurately a known amount into the vial caps. To overcome this, a suspension of

each diet was made in water and vigorously stirred while 4 ml aliquots were sucked up into a wide bore glass tube and emptied into the vial caps. A further 5 or 6 replicate aliquots were collected on pre-weighed glass fibre filters. The mean dry weight of these samples was taken as the initial weight of the food. The weights of these replicate samples rarely varied by more than ± 5% of the mean. It was found possible to immerse the vial caps in the experimental containers without losing any of the food from them by firstly placing them in the mesh, and then filling the trough and the cap with water until a meniscus formed inside and outside the cap. When the food in the cap was settled the menisci were touched simultaneously with a broad blunt instrument, which made them run together. Water could then be gently run into the trough to the desired volume. At the end of each experiment food remaining in the vial cap was collected on glass fibre filters. The decrease in the dry weight of the food from the initial weight was taken as the weight ingested. After starvation overnight to collect the majority of food in the gut at the end of the feeding period, faeces were collected by filtration. In some cases faeces were also collected at regular intervals during the course of the feeding period. Experimental animals, food and faeces were dried to constant weight at room temperature before weighing on a Mettler four place balance. Oven dry weights were not used as these experiments were started during the 1974 3 day week when the oven was not often available. Four samples of about 80 mg of F.D. were weighed daily for a period of 5 weeks during which many of these experiments were carried out. The total weight did not vary by more than 2.3 mg (2.8%) in any case during this period, and the water content varied between 10.2 and 12.6%. The water contents of another sample of F.D., and of N.L. and of faeces derived from N.L. were 10.7, 12.2 and 9.5% respectively. All weighings concerned with any one experiment were carried out at the same time. Ash contents were determined after ignition in a muffle furnace at 500^{o}C for 5 hours. Control filters were treated similarly to allow for any weight loss upon ignition.

Hargrave (1970) has mentioned the difficulties of carrying out gravimetric experiments with elm leaves caused by their tendency to change in weight during the course of the experiment. In the present

work replicate leaf discs were cut with a cork borer and placed in the vial caps. Others were placed in beakers of water and kept at the experimental temperature until the end of the experiment when, along with the remains of leaf discs offered as food, they were dried and weighed and the difference between experimental and control weights taken as the weight consumed. In preliminary experiments replicate discs were cut from one leaf to the same diameter as the caps, and held tightly in place at the bottoms of the caps. Under these conditions however ingestion was very slow, apparently because the animals could not get under the discs. Once a hole large enough for them to get through was eaten away ingestion was more rapid and several animals were often seen feeding under each disc. Furthermore the initial weights of the discs varied considerably because they had been cut from different areas of the leaf and contained varying amounts of vascular material. In order to overcome these problems 2 leaf discs of about 1.5 cm diameter, which Gammarus could easily get under, were placed in each vial cap. The discs were cut in pairs, either from corresponding positions on either side of the central leaf vein, or from adjacent positions on the blade. In either case an attempt was made to incorporate equal amounts of vascular tissue in each disc. One of the pair was used as an experimental disc and one as a control, the controls being kept in pairs, corresponding to the pairs of discs used in each experiment. Under these conditions the ingestion rate increased four fold and there was relatively little variation between the initial weights of experimental and control discs. The differences in weight between the individuals of 12 pairs of discs cut in this way varied between 0.1 and 1.7 mg, with a mean value of 0.6 mg.

In deciding the duration of experiments a compromise had to be reached between allowing sufficient time for measurable amounts of food to be ingested and faeces produced, and not allowing sufficient time for large weight changes in the diets offered or for microbial attack of the artificial diets to occur. In practice, the experiments with artificial diets were run for two days and faeces collected at the end of the experiments, and those with natural diets were run for periods up to 8 days, the faeces being collected at shorter intervals.

Prus (1971) has shown that the ingestion and assimilation of leaf litter by the isopod Asellus aquaticus may vary with sex and season. The experiments described here were carried out in the winter and spring of 1974. Most experiments with artificial diets were completed by the end of January when couples in precopula were first seen in the population. No attempt was made to separate the sexes in these experiments, but in those with natural diets which were carried out later, the animals were first separated into three categories, males, females and gravid females.

In order to estimate the percentage of their time the animals spent feeding on the various diets, they were observed continuously for 2-4 thirty minute periods during the course of the experiment. Where possible each period was on a different day. During these periods the number of animals actually feeding in each of the replicate troughs was noted at one minute intervals. Any animal in a vial cap was assumed to be feeding. The percentage of animals feeding during the course of the observation period was assumed to be equal to the percentage of its time each individual spent feeding ('Feeding Activity') and was calculated as:-

$$\frac{\text{total animal minutes feeding observed}}{\text{duration of period in minutes x No. animals present.}} \times 100$$

From the data available the following feeding parameters were also calculated for each diet:-

1) The weight of food consumed per unit time spent feeding ('Ingestion Efficiency')

$$\mu g/\text{hr/animal} = \frac{\text{Ingestion rate } (\mu g/\text{mg B.W./hr}) \times \text{Wt.Animal} \times 100}{\text{'Feeding Activity'}}$$

2) The time required for the tract to be completely filled if the animal fed continuously ('Tract Filling Time')

$$\text{hours} = \frac{\text{Weight of Tract Contents } (\mu g)}{\text{'Ingestion Efficiency'}}$$

3) The percentage of the volume of the tract which, on average, was filled with food ('Tract Fullness')

$$\% = \frac{\text{Ingestion rate } (\mu g/\text{mg B.W./hr}) \times \text{Wt.Animal} \times \text{Tract transit time} \times 100}{\text{Wt. of tract contents when full}}$$

4) The percentage of its time an animal must spend feeding in order to keep its tract full ('Full Tract Feeding Activity').

$$\% = \frac{\text{'Feeding Activity'} \times 100}{\text{'Tract Fullness'}}$$

In calculating these parameters the effect of any reduction in volume of the diets due to their assimilation as they pass along the tract has not been taken into account.

b) Results

All the diets used were ingested quite readily as evidenced by visible feeding activity and the obvious presence in the gut of diets containing cellulose powder. Natural diets generally stimulated a higher 'Feeding Activity' than artificial mixtures (Tables 13 and 14) but there were no significant differences between any of the mean values for 'Feeding Activity' within the groups fed artificial mixtures or natural diets (Mann Whitney U test).

The ingestion rate of 100C was determined both by initially weighing out the powder dry and by using the suspension method previously described. There was no significant difference between the mean rates obtained in each case (Table 15), although there was greater variation when the suspension method was used. The variation obtained in the estimates of assimilation efficiency was also presumably caused by the initial variation in the estimate of ingestion rate. The presence of protein in cellulose powder evidently stimulated its ingestion as 90C + 10P was ingested significantly more rapidly than 100C ($t = 3.9$, $P = <.01$) (Table 16). Further increasing the protein content of the diet to 40% had little effect however (Table 17). Animals feeding on 90C + 10P and 60C + 40P were apparently not selecting cellulose or protein particles as the tracts of most animals were seen to be full of cellulose, and yet animals were frequently to be seen feeding on pieces of protein. The ingestion rate of 100P was similar when the diet was made up with bovine or egg albumen (Table 18) and was significantly less than that of 90C + 10P ($t = 9.0$, $P = \ll .01$). 60C + 40P and 90C + 10P were made up with bovine albumen.

The main source of error in experiments with artificial mixtures and fine detritus diets was caused by food leaving the vial cap and falling through the mesh without being ingested. This was mainly brought about by the locomotory and respiratory activities of the animals, and caused the greatest error where the ingestion rate was low, particularly with 100P. This error resulted in ingestion rates being over-estimated and assimilation efficiencies under-estimated. It seems likely that the best estimates for ingestion and assimilation of 100P might come from

TABLE 13.

'Feeding Activity' with Natural Diets (%)

Bowl No.	N.L. Observation No. 1.	2.	3.	4.	$\bar{x}$	E.L. Observation No. 1.	2.	3.	4.	$\bar{x}$	F.D. Observation No. 1.	2.	3.	$\bar{x}$	E.F.D. Observation No. 1.	2.	3.	$\bar{x}$
1.	33.0	10.0	0.0	0.0	10.8	18.0	4.0	0.0	0.0	5.5	0.0	0.0	0.0	0.0	3.3	0.0	2.4	1.9
2.	20.0	20.0	4.0	0.0	11.0	0.0	50.0	0.0	0.0	12.5	14.0	10.0	29.0	17.7	0.5	1.0	0.0	0.5
3.	0.0	0.0	0.0	0.0	0.0	0.0	12.0	0.0	0.0	3.0	0.0	0.4	11.9	4.1	3.3	3.7	23.6	10.2
4.	8.0	0.0	0.0	9.0	4.3	59.0	23.0	30.0	0.0	28.0	60.0	29.0	9.7	32.9	7.3	38.3	15.7	20.4
5.	61.0	55.0	30.0	12.0	39.5	30.0	68.0	28.0	0.0	31.5	11.1	16.7	0.0	9.3	6.7	4.3	1.0	4.0
6.	5.0	15.0	10.0	0.0	7.5	0.0	1.0	0.0	0.0	0.3	0.7	3.3	4.7	2.9	2.0	8.3	0.7	3.7
7.	0.0	1.0	0.0	0.0	0.3	12.0	34.0	0.0	0.0	11.5	10.7	15.3	0.0	8.7	13.0	11.1	11.1	11.7
8.	0.0	4.0	0.0	0.0	1.0	2.0	38.0	0.0	0.0	10.0	19.3	53.0	28.6	33.6	0.3	0.0	0.0	0.1
9.	11.1	11.1	17.8	11.1	12.8	8.0	29.0	25.0	63.0	31.3	0.0	0.0	0.0	0.0	10.7	14.0	6.0	10.2
10.	0.0	10.0	10.0	0.0	5.0	3.0	31.0	4.0	49.0	21.8	11.1	15.2	14.0	13.4	13.0	6.7	15.3	11.7
$\bar{x}$					9.2					15.5				12.3				7.5

TABLE 14.

'Feeding Activity' with Artificial Mixtures (%)

Bowl No.	100C Observation 1.	100C Observation 2.	100C No. $\bar{x}$	90C + 10P Observation 1.	90C + 10P Observation 2.	90C + 10P No. $\bar{x}$	60C + 40P Observation 1.	60C + 40P Observation 2.	60C + 40P No. $\bar{x}$	100P (Bovine A.) Observation 1.	100P (Bovine A.) Observation 2.	100P (Bovine A.) No. $\bar{x}$	100P (Egg A.) Observation 1.	100P (Egg A.) Observation 2.	100P (Egg A.) No. $\bar{x}$
1.	0.7	10.0	5.3	0.3	0.0	0.2	10.0	0.0	5.0	1.7	0.0	0.9	6.3	0.0	3.2
2.	6.5	0.7	3.5	8.7	0.0	4.4	0.0	0.0	0.0	0.3	0.0	0.2	7.7	0.0	3.9
3.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	0.0	0.0	0.0	0.3	0.0	0.2
4.	0.3	0.7	0.5	10.4	10.4	10.4	15.3	13.3	14.3	0.0	10.0	5.0	0.0	0.0	0.0
5.	0.7	0.0	0.4	0.0	0.0	0.0	1.3	0.0	0.7	10.0	11.7	10.9	0.0	0.0	0.0
6.	0.0	0.0	0.0	4.0	0.3	2.2	1.7	0.0	0.9	6.3	0.0	3.2	0.0	10.0	5.0
7.	10.0	0.0	5.0	0.0	0.3	0.2	5.3	3.0	4.2	10.0	0.0	5.0	0.0	0.0	0.0
8.	0.5	0.0	0.3	10.0	0.0	5.0	10.0	0.0	5.0	2.5	0.4	1.4	0.0	0.0	0.0
9.	0.0	0.0	0.0	0.0	0.0	0.0	10.0	0.7	5.4	5.0	0.0	2.5	7.0	10.0	8.5
10.	1.1	0.3	0.7	0.0	0.0	0.0	0.0	0.7	0.4	0.0	0.0	0.0	0.0	9.0	4.5
$\bar{x}$			1.6			2.2			3.6			2.9			2.5

TABLE 15.

Ingestion and Assimilation of 100C

a) Food Initially Weighed out dry

Bowl No.	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	218.3	204.1	14.2	-	43	10	3.4	9.6	-
2.	294.4	285.3	9.1	-	43	10	3.4	6.2	-
3.	243.0	234.7	8.3	-	43	10	4.1	4.7	-
4.	237.5	232.1	5.4	-	43	9	3.1	4.6	-
5.	339.9	335.3	4.6	-	43	9	3.4	3.5	-
6.	268.7	262.1	6.6	-	43	10	3.4	4.5	-
7.	465.0	461.5	3.5	-	43	7	3.1	3.8	-
8.	354.1	350.2	3.9	-	43	7	2.4	5.5	-
9.	432.6	426.6	6.0	-	43	9	3.3	4.7	-
$\bar{x}$								5.2	
S.E.$_{\bar{x}}$								0.6	

b) Food Initially Weighed out by 'Suspension' Method.

1.	311.5*	305.6	5.9	5.3	44.5	10	4.3	3.1	10.2
2.	311.5	300.3	11.2	7.4	44.5	10	4.8	5.2	33.9
3.	311.5	303.8	7.7	3.5	44.5	10	4.0	4.4	54.5
4.	311.5	296.8	14.7	8.6	44.5	9	3.7	10.0	41.5
5.	311.5	307.1	4.4	1.1	44.5	8	4.6	2.7	75.0
6.	311.5	309.1	2.4	3.0	44.5	10	3.8	1.4	0.0
7.	311.5	296.9	14.6	5.7	44.5	10	4.1	8.0	61.0
8.	311.5	310.1	1.4	2.4	44.5	10	3.9	0.8	0.0
9.	311.5	302.9	8.6	2.1	44.5	10	3.5	5.6	75.6
$\bar{x}$								4.6	39.1
S.E.$_{\bar{x}}$								1.0	10.0
$\bar{x}$)								4.9	
S.E.$_{\bar{x}}$) Overall								0.6	

- not measured. *S.D. = 0.9, N = 5.

TABLE 16.

Ingestion and Assimilation of 90C + 10P

Bowl No.	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (mg/mg/hr)	Assimilation Efficiency
1.	160.4*	149.5	10.9	5.2	47.5	10	2.5	9.0	52.3
2.	160.4	150.2	10.2	5.1	47.5	10	2.8	7.6	50.0
3.	160.4	148.3	12.1	5.8	47.5	10	2.9	8.7	52.1
4.	160.4	141.2	19.2	14.3	47.5	10	3.3	12.4	25.5
5.	160.4	155.5	4.9	3.1	47.5	10	2.2	4.6	36.7
6.	160.4	150.4	10.0	4.0	47.5	10	2.8	7.6	60.0
7.	160.4	143.7	16.7	8.2	47.5	10	3.4	10.5	50.9
8.	160.4	150.4	10.0	4.4	47.5	10	2.4	9.0	56.0
9.	160.4	152.7	7.7	5.4	47.5	9	2.5	7.1	29.9
10.	160.4	150.2	10.2	5.5	47.5	10	2.6	8.3	46.1
$\bar{x}$								8.5	46.0
S.E.$_{\bar{x}}$								0.7	3.6

* S.D. = 7.9 N = 4

TABLE 17.

Ingestion and Assimilation of 60C + 40P

Bowl No.	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	99.3*	82.4	16.9	10.7	44.5	10	3.2	12.1	36.8
2.	99.3	92.1	7.2	3.5	44.5	10	2.4	6.9	51.7
3.	99.3	90.3	9.0	4.9	44.5	10	2.9	7.0	45.8
4.	99.3	89.6	9.7	4.5	44.5	10	3.4	6.5	53.8
5.	99.3	90.7	8.6	5.1	44.5	10	3.2	6.1	41.0
6.	99.3	89.1	10.2	4.1	44.5	10	3.2	7.2	60.0
7.	99.3	88.3	11.0	5.9	44.5	10	2.8	9.0	46.6
8.	99.3	92.5	6.8	2.8	44.5	10	4.1	3.8	59.0
9.	99.3	93.0	6.3	2.3	44.5	10	2.0	7.2	63.7
10.	99.3	90.5	8.8	3.6	44.5	10	2.7	7.3	59.3
$\bar{x}$								7.3	51.8
S.E.$_{\bar{x}}$								0.7	2.8

* S.D. = 0.5, N = 5

TABLE 18.

Ingestion and Assimilation of 100P

a) Egg Albumen

Bowl No.	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	29.1*	22.4	6.7	-	41.5	10	4.6	3.5	-
2.	29.1	24.4	4.7	-	41.5	10	4.4	2.6	-
3.	29.1	26.3	2.8	-	41.5	10	3.9	1.8	-
4.	29.1	29.2	0.0	-	41.5	10	3.2	0.0	-
5.	29.1	26.0	3.1	-	41.5	8	4.6	2.0	-
6.	29.1	26.2	2.9	-	41.5	10	3.6	1.9	-
7.	29.1	23.7	5.4	-	41.5	10	4.5	2.9	-
8.	29.1	26.7	2.4	-	41.5	10	3.8	1.5	-
9.	29.1	27.2	1.9	-	41.5	10	4.4	1.0	-
$\bar{x}$								1.9	
S.E.$_{\bar{x}}$								0.4	

*S.D. = 1.4 N = 6

b) Bovine Albumen

1.	18.3*	14.8	3.5	0.5	43.5	10	4.8	1.7	85.7
2.	18.3	15.8	2.5	0.9	43.5	10	4.7	1.2	64.0
3.	18.3	12.8	5.5	2.6	43.5	10	4.4	2.8	52.7
4.	18.3	15.6	2.7	1.0	43.5	10	4.3	1.4	63.0
5.	18.3	14.4	3.9	2.1	43.5	10	4.5	2.0	46.0
6.	18.3	15.9	2.4	0.5	43.5	10	4.2	1.3	79.0
7.	18.3	14.9	3.4	1.1	43.5	8	2.9	3.4	68.0
8.	18.3	16.9	1.4	0.2	43.5	8	3.0	1.3	86.0
9.	18.3	14.5	3.8	1.3	43.5	10	3.7	2.4	65.8
$\bar{x}$								1.9	67.8
S.E.$_{\bar{x}}$								0.3	

* S.D. = 1.2 N = 5

the more extreme values reported in Table 18, i.e. 1.3 µg mg B.W./hr and 80% respectively. This latter figure may in fact approach 100% as no discrete faeces were observed in experiments with 100P. The high efficiency with which protein could be assimilated was reflected in the increasing assimilation efficiency found when the proportion of protein in the diet was increased (Tables 15, 16, 17), although there were no significant differences between the mean values obtained with 100C, 90C + 10P and 60C + 40P.

F.D. and E.F.D. were ingested at a similar rate to 100C and a similar proportion, about 40%, was assimilated (Tables 19 and 20). Enrichment had no effect on the ingestion or assimilation of fine detritus diets. There were also no differences between animals of different sex in any of the experiments with natural diets. In experiments with fine detritus diets, faeces were collected at 72 hour intervals. As the results were fairly reproducible this time interval was also used in experiments with leaf diets, the results of which are shown in Tables 21 and 22. Ingestion was very slow in both cases, presumably reflecting the mechanical difficulties of ingesting such a diet, as 'Feeding Activity' was quite high in both cases (Table 13). E.L. was ingested significantly more rapidly than N.L. ($t = 2.7$, $P < .05$). In most cases no assimilation could be detected.

Faeces from leaf diets may have gained in weight before collection, so experiments with N.L. and E.L.were repeated using an interval between faeces collection of 24 and 12 hours respectively, as these were the shortest periods in which sufficient faeces for accurate weighing were produced. In these experiments 4 leaf discs were presented as food freely in the perspex tube, rather than confined in vial caps, and 23-26 animals were used per tube. In order to determine whether shortening the interval between faeces collections might have any effect on the results of experiments with F.D., an experiment with this diet and faeces collections at 12 hour intervals was also carried out. Under these conditions (Table 23) the rate at which leaf discs were ingested increased significantly in the case of E.L. ($t = 3.76$, $P < .01$), and insignificantly in the case of N.L., when compared to results from the previous experiments (Tables 21 and 22). Positive assimilation efficiencies were obtained in the experiment with E.L. The mean value

TABLE 19.

Ingestion and Assimilation of F.D.

Bowl No.	Sex	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	GF	42.3*	21.9	20.4	12.9	145	9	3.9	4.1	36.8
2.	F	42.3	13.5	28.8	17.0	145	10	2.9	6.9	41.0
3.	F	42.3	25.5	16.8	10.4	145	9	3.0	4.4	38.1
4.	F	42.3	22.4	19.9	14.3	145	10	2.3	6.1	28.1
5.	M	42.3	17.7	24.6	12.6	145	9	3.6	5.3	48.8
6.	M	42.3	21.4	20.9	15.1	145	10	3.1	4.6	27.8
7.	M	42.3	23.5	18.8	12.2	145	10	2.6	5.0	35.1
8.	M	42.3	14.8	27.5	17.1	145	9	2.8	7.5	37.8
9.	M	42.3	19.1	23.2	13.2	145	8	3.8	5.3	43.1
10.	M	42.3	25.8	16.5	9.2	145	9	2.0	6.2	44.2
$\bar{x}$									5.5	38.0
S.E.$_{\bar{x}}$									0.4	2.1

* S.D. = 4.3 N = 6

G.F. = Gravid Female

F = Female

M = Male

TABLE 20.

Ingestion and Assimilation of E.F.D.

Bowl No.	Sex	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg mg/hr)	Assimilation Efficiency
1.	GF	33.3*	17.0	16.3	10.6	138	7	3.5	4.8	35.0
2.	GF	33.3	16.5	16.8	10.6	138	7	3.9	4.4	36.9
3.	F	33.3	13.3	20.0	12.1	66	10	3.4	9.0	39.5
4.	F	33.3	18.0	15.3	10.3	138	10	2.9	3.9	32.7
5.	F	33.3	22.3	11.0	6.0	138	10	2.2	3.7	45.5
6.	F	33.3	18.6	14.7	6.9	138	10	3.1	3.4	53.1
7.	M	33.3	13.3	20.0	10.8	138	9	3.1	5.2	46.0
8.	M	33.3	14.9	18.4	10.4	138	10	3.8	3.5	43.5
9.	M	33.3	10.5	22.8	12.6	66	10	3.4	10.1	44.7
10.	M	33.3	14.8	18.5	12.6	66	10	4.0	6.9	31.9
$\bar{x}$									5.5	40.9
S.E.$_{\bar{x}}$									0.8	2.2

* S.D. = 2.3 N = 6

TABLE 21.

Ingestion and Assimilation of N.L.

Bowl No.	Sex	Initial food (mg)	Final food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	F	14.0	7.3	6.7	8.5	163.5	10	3.0	1.4	-
2.	F	14.4	1.8	12.6	11.9	162.5	10	2.8	2.8	5.6
3.	F	14.1	12.3	1.8	3.2	162.5	10	2.9	0.4	-
4.	F	8.7	2.7	6.0	6.0	162.5	10	2.8	1.3	0
5.	M	9.4	3.9	5.5	5.8	162.5	10	3.3	1.0	-
6.	M	10.8	3.5	7.3	8.8	162.5	10	3.8	1.2	-
7.	M	16.2	10.6	5.6	7.5	162.5	10	3.5	1.0	-
8.	M	15.7	14.6	1.1	3.6	162.5	10	3.2	0.2	-
9.	M	16.0	8.7	7.7	8.6	162.5	9	4.0	1.3	-
10.	M	11.1	7.5	3.6	4.3	162.5	10	2.9	0.8	-
$\bar{x}$									1.1	
S.E.$_{\bar{x}}$									0.2	

- Positive assimilation efficiency cannot be calculated.

TABLE 22.

Ingestion and Assimilation of E.L.

Bowl No.	Sex	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assimilation Efficiency
1.	F	10.7	7.8	2.9	3.1	73	10	2.5	1.6	-
2.	F	12.5	7.2	5.3	4.6	73	10	2.1	3.4	13.2
3.	F	8.9	7.2	1.7	1.6	73	10	2.2	1.1	5.9
4.	F	9.0	3.3	5.7	6.3	73	10	2.8	2.8	-
5.	M	8.9	4.0	4.9	4.8	73	10	2.1	3.2	2.0
6.	M	9.2	5.9	3.3	3.3	73	10	2.1	2.2	0
7.	M	14.4	12.7	1.7	2.3	73	10	2.5	1.0	-
8.	M	14.1	13.2	0.9	1.5	73	10	2.3	0.5	-
9.	M	14.0	6.6	7.4	6.0	73	10	2.6	4.0	18.9
10.	M	12.3	7.1	5.2	6.6	73	10	2.4	3.0	-
$\bar{x}$									2.3	-
S.E.$_{\bar{x}}$									0.4	

- Positive assimilation efficiency cannot be calculated.

TABLE 23.

Ingestion and Assimilation of Various Diets

Bowl No.	Diet	Sex	Initial Food (mg)	Final Food (mg)	Ingested (mg)	Faeces (mg)	Duration (hr)	No. Animals	Mean Wt. (mg)	Ingestion rate (µg/mg/hr)	Assim. Eff.
1.	NL	F	32.2	21.8	10.4	9.6	113	24	2.7	1.4	7.7
2.	NL	F	44.1	31.6	12.5	13.0	113	23	3.2	1.5	-
3.	NL	M	41.6	30.7	10.9	13.5	113	23	3.3	1.3	-
4.	NL	M	41.1	25.5	15.6	16.0	113	24	3.5	1.7	-
$\bar{x}$										1.5	
S.E.$_{\bar{x}}$										0.1	
1.	EL	F	26.9	4.2	22.7	18.5	43.5	26	2.6	7.7	18.5
2.	EL	M	33.7	8.9	24.8	23.1	43.5	23	2.7	9.1	6.9
3.	EL	M	49.6	33.3	16.3	16.0	43.5	23	3.7	4.5	1.9
4.	EL	M	29.2	9.0	20.2	18.7	43.5	24	3.6	5.4	7.4
$\bar{x}$										6.7	8.7
S.E.$_{\bar{x}}$										1.1	3.5
1.	FD	GF	99.8*	84.8	15.0	4.8	73	10	3.6	5.7	68.0
2.	FD	F	99.8	88.6	11.2	6.2	73	9	2.5	6.8	44.6
3.	FD	M	99.8	74.4	25.4	13.1	73	10	2.7	12.8	48.4
4.	FD	M	99.8	87.6	12.2	10.6	73	10	3.0	5.5	13.1
$\bar{x}$										7.7	43.5
S.E.$_{\bar{x}}$										1.7	11.4

* 2 caps, each containing 44.9 mg (S.D. = 2.4, N = 6)

TABLE 24.

Assimilation Efficiencies Calculated by the Ash-Ratio Method.

Bowl No.	Diet	Organic Matter in Food (F^1)	Organic Matter in Faeces (E^1)	Assimilation Efficiency (%)
1.	E.L. [1]	0.945	0.935	16.3
2.	"	0.945	0.805	76.0
3.	"	0.945	0.800	76.7
4.	"	0.945	0.882	56.5
5.	E.L. [2]	0.945	0.807	75.6
6.	"	0.945	0.804	76.1
7.	"	0.945	0.938	11.9
8.	"	0.945	0.841	69.2
9.	"	0.945	0.833	71.0
10.	"	0.945	0.910	41.2
11.	"	0.945	0.696	86.7
12.	"	0.945	0.866	62.4
13.	"	0.945	0.933	19.0
14.	"	0.945	0.788	78.4
1.	F.D.	0.708	0.736	- 15.0
2.	"	0.708	0.759	- 30.0
3.	"	0.708	0.731	- 12.0
4.	"	0.708	0.776	- 42.9
5.	"	0.708	0.762	- 32.0
6.	"	0.708	0.755	- 27.0
7.	"	0.708	0.787	- 52.4
8.	"	0.708	0.784	- 49.7
9.	"	0.708	0.803	- 68.1
10.	"	0.708	0.750	- 23.7

1 Faeces collected at 12 hour intervals.

2 " " " 72 " "

$$\text{Assimilation Efficiency} = \frac{(F^1 - E^1)}{(1-E^1)(F^1)} \times 100$$ (Conover 1966)

was significantly lower then the lowest value found with any other diet, i.e. F.D. ($t = 7.2$, $P \ll .01$). The ingestion rate and assimilation efficiency found in this experiment with F.D. was similar to that found in the previous experiment (Table 19).

The assimilation efficiency was calculated using the ash-ratio method in experiments with E.L. and F.D. (Table 24). It is apparent that values calculated by this method are quite different to those derived from gravimetric data, suggesting a secretion of inorganic material into the gut lumen when fed E.L., and an uptake of such material from the tract contents when fed F.D. A single experiment was carried out with E.L. to determine whether the use of oven dry weights would give different results. A single leaf was cut in half, one half fed to 25 speciments of Gammarus and the other half used to determine food ash content. After two days a sample (20 mg) of the resultant faeces were collected. Ash contents were determined after drying to constant weight at 105°C. The food contained 90.75% organic matter and the faeces 83.15% giving an assimilation efficiency of 49.7%. This figure is similar to those quoted in Table 24.

Using the mean data for ingestion and egestion of E.L. and F.D. shown in Tables 23 and 19, and the mean values for ash content shown in Table 24, the percentage assimilation of organic matter in these diets may be calculated as 17.2 and 33.1% respectively.

Calculated feeding parameters are shown in Table 25. The tract passage time was taken as 7 hours in all cases (Chapter 4). In order to determine the weight of the tract contents, groups of animals of similar size feeding either normally in the laboratory tank, or on N.L., or on 10°C were dissected and the contents of full tracts dried and weighed as pooled samples. The mean weights of individual tract contents were 333 µg (normal, N = 6), 300 µg (N.L., N = 8) and 320 µg (10°C, N = 5). The mean length of all the animals was 8.7 mm (3.4 mg). As the samples included some small pieces of gut tissue which could not be completely separated from the contents, and as the high values from animals feeding in the laboratory tank were probably caused by large amounts of inorganic material in the tract, the mean weight of the full tract contents of a 3.4 mg animal was taken as 300 µg for the purposes of the calculations.

TABLE 25.

Feeding Parameters for 3.4 mg Animal

Diet	'Ingestion Efficiency' (µg/hr/Animal)	'Tract Filling Time' (Hours)	'Tract Fullness' %	'Full Tract Feeding Activity' %
100C	1041	0.3	39	4
90C + 10P	1314	0.2	67	3
60C + 40P	689	0.4	58	6
100P (Egg Alb.)	258	1.2	15	17
100P (Bovine Alb.)	223	1.3	15	19
F.D.	152	2.0	44	28
E.F.D.	249	1.2	44	17
N.L.	41	7.4	9	102
E.L.	50	5.9	18	86

The diets seem to fall into 3 categories :-

1) Those containing a high proportion of cellulose powder (100C, 90C + 10P, 60C + 40P) could be rapidly ingested, as evidenced by a high 'Ingestion Efficiency'. The tracts were generally kept quite full of these diets, and animals would only have had to spend a small proportion of their time feeding in order to keep the tracts completely full.

2) 100P, F.D. and E.F.D. could not be ingested as rapidly as diets in group 1, and more of the animals time would have had to be spent feeding in order to keep the tract full.

3) N.L. and E.L. were distinctly different from the other diets as they could only be ingested at a relatively low rate, and the animals would have had to spend all their time feeding in order to keep the tract full. The low values of 'Tract Fullness' calculated for this group were confirmed by visual observations of the gut contents of dissected animals which had been offered these diets.

The error in these parameters caused by the assimilation of food during its passage through the tract was probably only large in the case of 100P, where the majority of the food was assimilated. In this case the figures for 'Tract Filling Time', 'Tract Fullness' and 'Full Tract Feeding Activity' are meaningless as the tract would never be filled with this diet. In animals which had been feeding on 100P and were dissected, solid food was never actually observed in the gut.

c) Discussion

After the completion of this work a report appeared in the literature concerning the ingestion and assimilation of beech and alder leaves by G.pulex (Nilsson, 1974). The results he described are not entirely comparable with those described here as they were mostly given in caloric terms. However, measured gravimetrically, he found that alder leaves were assimilated with an efficiency of 30-35% at 10°C, but the assimilation of beech leaves rose from 0 to 35% in relation to the advancing state of decay of the leaves. However, in terms of energy assimilation, the figures were about 18% for beech and 8% for alder. These figures are quite similar to that of 17% found here for the assimilation of organic matter from E.L.

The main variables which Nilsson found to affect the rate of ingestion were temperature, type of leaf and the length of time and the temperature at which the leaves had been immersed prior to the experiment. The experimental temperature was constant in the present work and all leaves were immersed for at least two weeks prior to their use. Nilsson found that the rate of consumption of leaves with an immersion time of more than 1,000 degree-hours was roughly constant. The minimum immersion time used here was 3,360 degree-hours, so it is probable that the different ingestion rates found here were caused by the different origins of the diets and their treatment (enrichment) prior to the experiments. Nilsson found that alder leaves were ingested at a mean rate of 1,161.5 cal/gm/day at 10°C which, assuming a calorific value of 4,528 cal/g air dry weight (Kaushik and Hynes, 1971), is equivalent to 10.7 µg/mg B.W./hour, which is slightly higher than the value found here for E.L.(Table 23). This presumably reflects the preference found by Nilsson for alder leaves over elm leaves.

Consideration of the feeding parameters calculated from the experimental data suggests that the rate of ingestion was affected both by the physical ease with which a diet could be consumed, and the 'Feeding Activity' which the diet stimulated. Some authors, e.g. Kaushik and Hynes (1971) and Mackay and Kalff (1973) have considered

ingestion rates as measures of palatability whereas others, e.g. Barlocher and Kendrick (1973a), have also made observations on feeding behaviour. The latter authors showed that Gammarus pseudolimnaeus had a behavioural preference for fungal colonies over sterile maple leaf discs as food, but in another paper (Barlocher and Kendrick, 1973) they showed that sterile maple and elm leaf discs were eaten about ten time more rapidly, measured gravimetrically, than fungal colonies. Similarly, in the present experiments, the diet which stimulated the highest level of 'Feeding Activity', E.L., was also one of the slowest to be ingested, and the diets which were ingested most rapidly (90C + 10P, 60C + 40P) stimulated a low level of 'Feeding Activity'. It may be that those diets which are hardest to ingest elicit the highest level of 'Feeding Activity', resulting in a constant ingestion rate. Although some of the data fits in well with that hypothesis, that for 100P does not. This diet had a similar 'Ingestion Efficiency' to F.D. and E.F.D., but the level of 'Feeding Activity', and hence the ingestion rate, was much lower. It seems unlikely that 'Ingestion Efficiency' is directly related to palatability as this would infer that a diet would be unpalatable purely because it could be easily ingested. The 'Tract Fullness' results suggest that in no case was ingestion limited by a full tract in these experiments. 'Feeding Activity' is probably the best measure of palatability, and the inverse relationship observed between 'Feeding Activity' and 'Ingestion Efficiency' in these experiments was probably the result of using a readily ingested but unpalatable basis, i.e. cellulose powder, for the artificial diets.

The relationship between the feeding parameters calculated here and the situation in the field is not entirely clear. Certain parameters which depend on behavioural factors (ingestion rate, 'Feeding Activity' and 'Tract Fullness'), may be quite different in the field. The greater availability of food probably means that these factors are all higher in the field and animals taken in the field usually have full tracts. A reasonable measure of 'Feeding Activity' in the field may be given by the values of 'Full Tract Feeding Activity' for natural diets given in Table 25, which suggest that the amount of time spent feeding may depend largely on the type of food consumed. Martin (1964) observed that G.pulex, like

marine amphipods, feeds intermittently in the field, taking in small quantities at a time but feeding at frequent intervals.

The accuracy of gravimetric studies of assimilation depends partly on the validity of assumptions made about the functioning of the gut which are never entirely correct. The occurrence of processes which are outside the normal concept of assimilation, i.e. excretion or absorbtion of minerals and secretion of organic material, means that the values calculated for assimilation efficiency are only an approximation, which Waldbauer (1968) calls the approximate digestibility.

One such source of error in the present work was evidently the uptake and loss of inorganic substances from the tract contents in different experiments. Where the ash content of the diet was high there was an apparent net uptake by the animal, and where it was low there was a net loss. It seems unlikely that these results could be explained by inaccuracies present in the methods used due to the use of air dry weights and the varying loss of weight of glass fibre filters upon ashing. Inaccuracies which could have been introduced by any differences in the water content of food and faeces were slight and the one experiment carried out using oven dry weights gave a similar result. For instance, in Table 24, replicate No. 4 (E.L.), even if the diet contained no water and the faeces contained 10%, the estimate of assimilation efficiency would be increased by only 5% to 61.5%. or if the diet contained 10% water and the faeces none, the estimate would be lowered by 5.2% to 51.3%. The weight loss from filters upon ashing varied between 2.5 and 4.0 mg, so this was a more likely source of error. However, ash ratio estimates of assimilation were not changed when based only upon the ash contents of relatively large samples of faeces ($>$ 4mg), rather than upon those of all samples collected, so it seems likely that the figures obtained were real. It was notable that in certain cases little excretion of ash apparently occurred, resulting in a more realistic ash-ratio estimate of assimilation (figure 24 E.L. replicates 1, 7 and 13). In these cases the gravimetric estimates of assimilation were similar to the ash-ratio estimates (Table 23 replicate 1 and Table 22 replicates 3 and 9), and reasonably close

to the calculated estimate of organic matter assimilation from E.L. (17%). Much of the difficulty encountered in obtaining gravimetric estimates of assimilation with elm leaf diets was evidently caused by the weight of excreted ash in the faeces.

Although it is not known which ions are involved in uptake and loss from the food, it seems likely that G.pulex could compensate for a loss of the order of that found here when fed E.L. If a 3 mg animal were feeding at a rate of 7 µg mg/hr, the rate of loss of inorganic ions to the gut contents when feeding on this diet would be about 1.6 µg/hour/animal. The maximum rate at which most ions can be taken up from the environment is not known, but Shaw and Sutcliffe (1961) have shown that the maximum rate of sodium uptake is about 0.3 µM/animal/hour, which is equal to about 7 µg/hour/3mg animal.

The ash-ratio method of determining assimilation efficiency is evidently of no value in the present case, and it seems unlikely that the critical assumptions implicit in its use would be justified in many animals. It is known that millipedes take up a considerable proportion of the calcium contained in ingested leaf litter (Raw, 1967), and Prus (1971) has shown that the ash-ratio method is of no value in studying the assimilation of decaying alder leaves by Asellus aquaticus, as an uptake of minerals from the food may occur in the summer, and a loss to the faeces in the winter. Taking into account absorbtion and excretion of minerals in G.pulex, the figures of 17 and 33% for the assimilation of organic matter from E.L. and F.D. respectively, probably represent the best estimates for the assimilation of these diets available from the present data.

Most studies concerning ingestion and assimilation have emphasised the importance of these processes in trophic relationships and largely ignored their potential as a means of elucidating certain aspects of digestive physiology, although some workers with terrestrial insects (Ishaaya and Meisner, 1973 ; Lasker and Giese, 1956) have studied assimilation in this context. The results described here largely confirm those described in Chapter 4 concerning the digestion of cellulose. The assimilation of a considerable proportion of ingested 100C, 90C + 10P and 60C + 40P confirms the ability of G.pulex

to digest cellulose. Lasker and Giese (1956) found that the silverfish Ctenolepisma lineata could assimilate 71-86% of a diet of pure cellulose, compared with a figure of 39% found here. Although the results for assimilation of 100C were not consistent, they were largely confirmed by the assimilation efficiencies found for 90C + 10P and 60C + 40P, as in both cases the great majority of food ingested was probably cellulose. If it is assumed that selective feeding did not occur, the proportions of effort put in to feeding on cellulose and protein would have been similar to their proportions in the diet. If the 'Ingestion Efficiencies' for the two dietary components were similar to those computed from animals feeding on 100C and 100P (Table 25), the proportions of cellulose ingested would have been 97.7% and 87.5% for 90C + 10P and 60C + 40P respectively. The slightly increased assimilation efficiency of 90C + 10P and 60C + 40P when compared to 100C can therefore be quantitatively related to the increased amount of highly digestible protein in the diet, i.e. using the figure calculated above, when the proportion of protein passing along the tract rose from 0 to 12.5% (100C to 60C + 40P), the assimilation efficiency increased by 12.8%.

The extent to which cellulose was assimilated was much greater than the extent to which it was broken down under in vitro conditions by gut extracts, even after a long period of incubation (Chapter 4). This presumably was the result of instability of the cellulase under 'in vitro' conditions, or its excessive dilution in gut extracts. It may be that studying the assimilation of pure cellulose is not only of more physiological and ecological significance than studying 'in vitro' cellulose hydrolysis by gut extracts, but is also a more sensitive method for the detection of cellulose digestion.

E.L. was ingested significantly more rapidly than N.L. As enrichment caused an increase in the protein content of elm leaves due to the development of an extensive fungal flora, and several authors have shown that the growth of fungi on leaf litter stimulates its ingestion by aquatic herbivores (Seddell, 1970 ; Barlocher and Kendrick, 1973a ; Mackay and Kalff, 1973), it may be concluded that

it was the rich fungal flora which stimulated the ingestion of E.L. here. Kaushik and Hynes (1971) similarly showed that leaves which had been kept in water enriched with nitrogen and phosphorus were more rapidly ingested by Gammarus lacustris limnaeus than leaves which had been kept in unenriched water, and at a lower temperature. The similar rates of ingestion of F.D. and E.F.D. were to be expected as the growth of fungi on F.D. was apparently not stimulated by enrichment.

The low assimilation efficiency found for N.L. and E.L. confirms the finding of Chapter 4 that the cell walls of elm leaves were not digested, and that the fraction of leaf material assimilated was confined to broken cells and cell fragments resulting from trituration by the mouth parts and proventriculus. A major part of the assimilated material must comprise fungi and any other saprophytic micro-organisms. It is not possible to say whether assimilation efficiencies for N.L. and E.L. differed, as no value could be obtained for the former diet. Difficulties in measuring assimilation efficiencies with elm leaf diets were evidently partly caused by the faeces increasing in weight before collection, so the apparently lower assimilation efficiency of N.L. than E.L. (Table 23) may have been caused by the faeces being collected at 24 hour, rather than 12 hour intervals. However, as micro-organisms presumably made up a major part of the assimilated fraction, and E.L. carried a greater population of micro-organisms than N.L., it may be that a greater proportion of E.L. was assimilated than of N.L. Hargrave (1970) has shown that various types of microflora and some algae are assimilated more efficiently than elm leaves by Hyallela azteca, using gravimetric and radiotracer techniques. He found that the assimilation efficiency of different diets was 60-82% for bacteria, 75% for diatoms, 5-15% for blue-green algae, 45-55% for green algae, 53-92% for the epiphytic growth on Chara, 6-15% for surface sediment and its attached micro-organisms, and 8.5% for elm leaves.

Several other workers have demonstrated that leaf litter is assimilated inefficiently by aquatic and terrestrial arthtropods, values between 4 and 30% generally being reported (Gere, 1956 ; Van der Drift and Witkamp, 1960 ; Bocock, 1963 ; Hubbel, 1965 ; Mcdiffet, 1970 ; Otto, 1974). The ingestion and assimilation of leaf litter by millipedes

has been studied relatively extensively and from a review of the literature Raw (1967) concluded that they may eat large amounts of leaf litter and excrete most of it relatively unchanged chemically, but greatly fragmented. It may be that a similar conclusion would apply to many other arthropod taxa, although it must be borne in mind that values for assimilation efficiencies obtained in the laboratory may differ considerably from those determined under field conditions (Hubbel et al, 1965).

The greater extent to which fine detritus diets were assimilated than elm leaf diets suggests that some cellulose could have been digested in these cases. As described in Chapter 4, these diets contained a high proportion of particles, the cell walls of which had been broken down by microbial attack. More of the food was therefore available for enzyme attack, and the cellulose in the diet may have been physically and chemically altered by microbial action which made it more liable to decomposition by the cellulase of Gammarus. Furthermore, fine detritus diets, unlike elm leaves, do not contain a high proportion of soluble substances, some of which may inhibit the action of digestive enzymes. There is no evidence to suggest however that cells which were ingested intact in fine detritus diets were chemically broken open in the gut.

CHAPTER 6

THE EFFECT OF DIET ON M.G.F. ENZYME ACTIVITY

a) Methods and Preliminary Investigation

The rationale of this investigation was to feed groups of animals with the various test diets and then to collect known volumes of M.G.F. and test them for enzyme activity per unit volume. This measure was chosen in preference to one of specific activity as in that method an increase in the activity of an M.G.F. enzyme could be masked by an increase in the overall protein content of the fluid. Some preliminary investigations concerning the methods used were first undertaken however.

The enzymes studied were protease, amylase and cellulase, using casein, soluble starch and CMC substrates. The protease assay mixture comprised 0.4 ml 1% w/v casein solution, 0.2 ml pH 7.0 buffer and 0.2 ml diluted M.G.F. Incubation time was 60 minutes and a 0.4 ml aliquot was taken for analysis. The amylase assay mixture comprised 0.4 ml 0.5% w/v soluble starch solution, 0.2 ml pH 5.5 buffer and 0.2 ml diluted M.G.F. Incubation time was 30 minutes and a 0.2 ml aliquot was taken for analysis. The cellulase assay mixture comprised 0.4 ml 1% w/v CMC, 0.2 ml pH 5.5 buffer and 0.2 ml diluted M.G.F. Incubation time was 3 hours and 0.2 ml aliquots were taken for analysis. The assays were begun by adding aliquots of the diluted fluid to prepared tubes of substrate and buffer with a teat operated micropipette. After the required incubation period aliquots were removed with the pipette in the same sequence in which the incubations had been started.

In order to investigate the linearity of the reactions under these conditions, incubates were set up in duplicate and 0.1 ml samples were withdrawn at regular intervals for analysis. The results, which are shown in figures 9, 10 and 11, demonstrate that the hydrolysis of starch and casein proceeded linearly with time during the incubation period. The hydrolysis of CMC was not linear however. To determine directly the effect of enzyme concentration on the rate of hydrolysis observed under the prescribed conditions, a sample of M.G.F. was diluted to varying extents by serial dilution and enzyme activity in each solution was determined. The degree of hydrolysis of starch (figure 12) was directly proportional to the enzyme concentration, but the curve for

FIGURE 9.

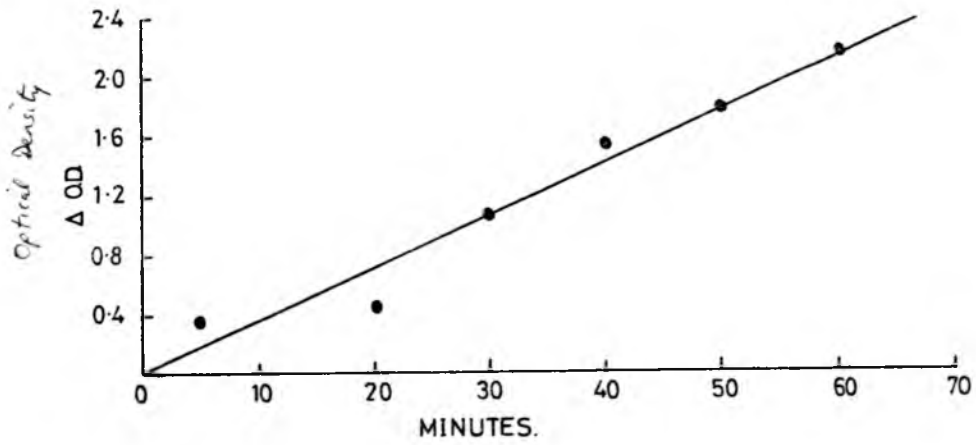
Digestion of soluble starch against time.

FIGURE 10.

Digestion of casein against time.

gainst

ime.


Optical Density
Δ OD
2·4
2·0
1·6
1·2
0·8
0·4
0
10
20
30
40
50
60
70
MINUTES.

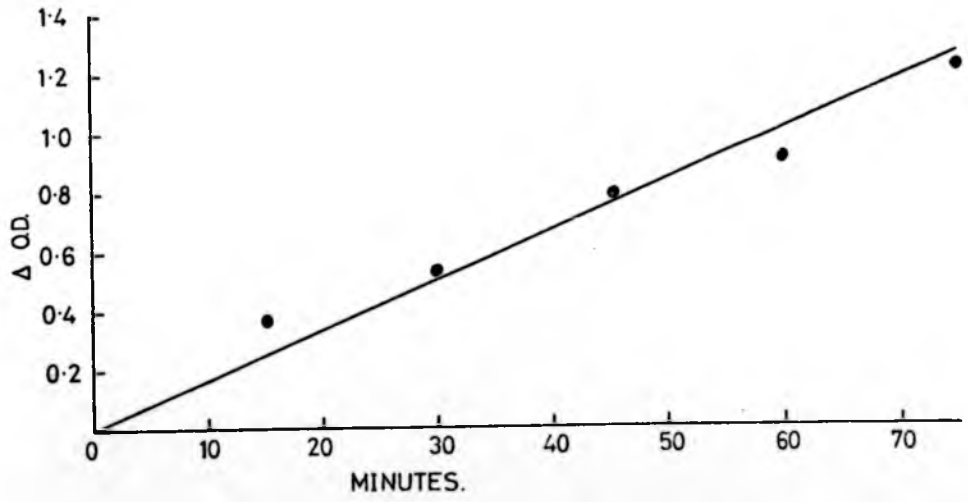
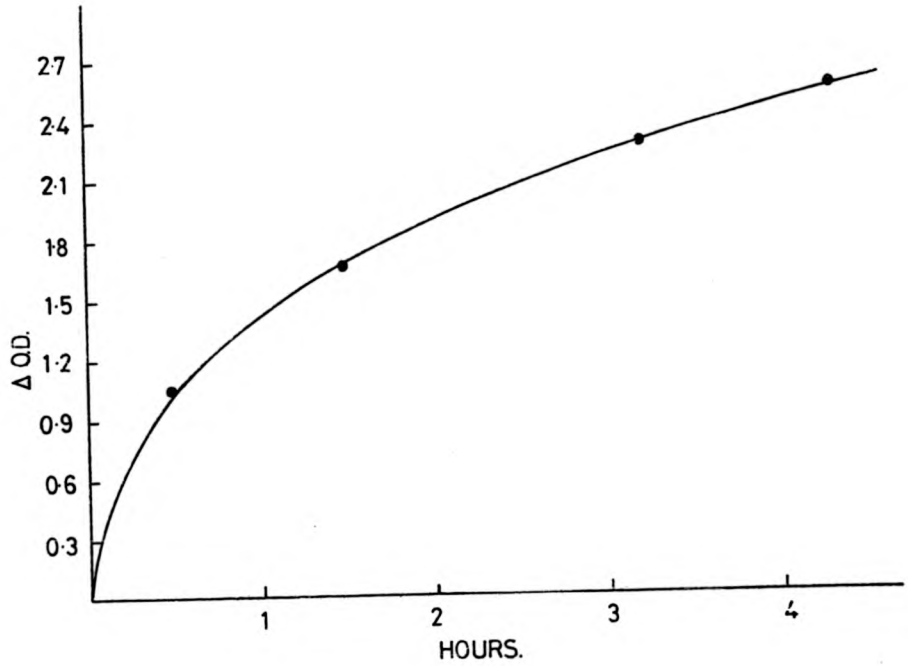

Δ O.D.
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
10
20
30
40
50
60
70
MINUTES.

FIGURE 11.

Digestion of C.M.C. against time.

FIGURE 12.

The effect of M.G.F. dilution on amylase activity.


2·7
2·4
2·1
1·8
1·5
1·2
0·9
0·6
0·3
Δ O.D.
1
2
3
4
HOURS.

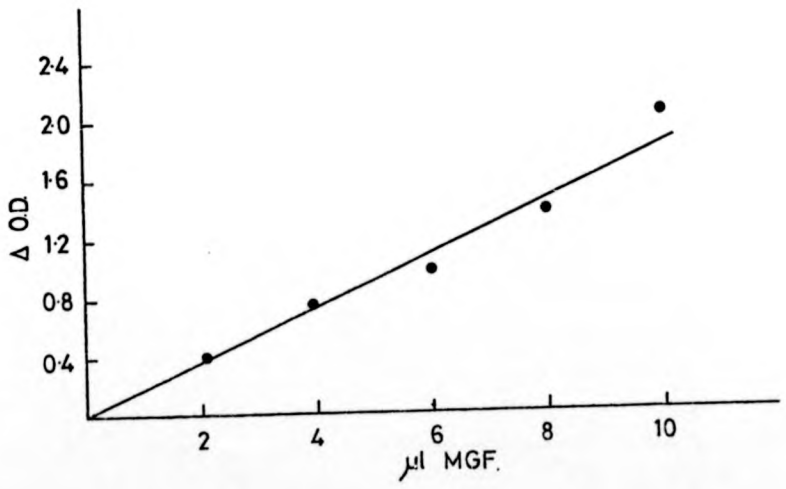
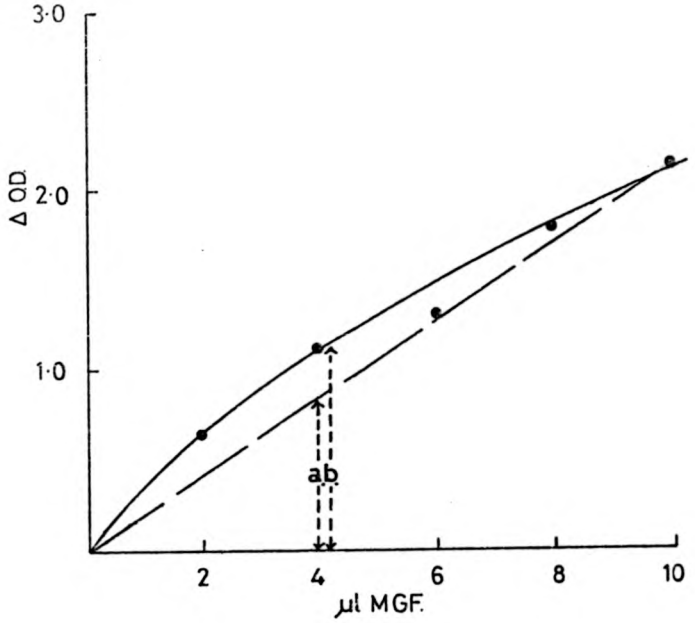

2·4
2·0
1·6
1·2
0·8
0·4
Δ O.D.
2
4
6
8
10
μl MGF.

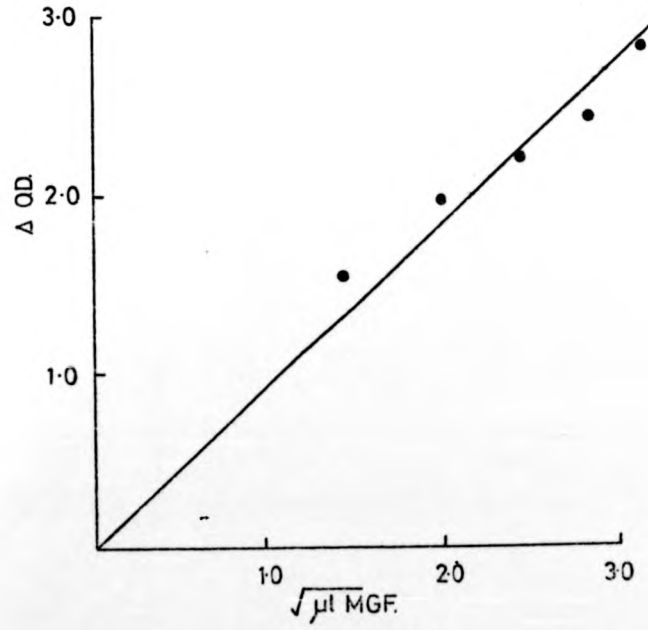
FIGURE 13.

The effect of M.G.F. dilution on protease activity.

FIGURE 14.

The effect of M.G.F. dilution on cellulase activity.


3·0
Δ OD
2·0
1·0
a b
2
4
6
8
10
µl MGF.


3·0
Δ OD
2·0
1·0
1·0
2·0
3·0
√µl MGF.

casein digestion (figure 13) showed the slight upward trend typical of the digestion of protein substrates (Dixon and Webb, 1964). The cellulase dilution curve also showed a non-linearity. Although this may be assumed to have been caused by the non-linear rate of hydrolysis during the incubation period, Gascoigne and Gascoigne (1960) considered that this type of curve was typical of cellulases and quoted values between 1.2 and 1.8 for the increase in the rate of hydrolysis upon doubling the enzyme concentration. Values found here when the M.G.F. volume was increased from 1 to 2, 2 to 4, 4 to 8 and 5 to 10μl were 1.5, 1.33, 1.25 and 1.2 respectively. If the square root of the M.G.F. volume was plotted against the extent of hydrolysis (figure 14) the relationship was approximately linear.

Taking these results into account, enzyme activities have been calculated as follows:-

Amylase activity = $O.D. \times \frac{10}{V}$

Cellulase activity = $O.D. \times \frac{10}{\sqrt{V}}$

Protease activity = $O.D. \times \frac{10}{V} \times k$

where V = the volume of M.G.F. used, k = a correction factor, calculated from figure 13. This equals the amount of hydrolysis which would occur at any given dilution (v) if the curve was a straight line (a), divided by the observed hydrolysis (b). k varied between 1.00 (V = 10μl) and 0.76 (V = 3μl). In each case the resulting figures are arbitary units of enzyme activity per 10μl of M.G.F. The enzyme activity of M.G.F. from animals fed the standard test diets was also expressed as a % of that from animals feeding normally in the laboratory tank.

To determine whether the size of the animals used affected M.G.F. enzyme activities, investigations were carried out using fluid collected by the wet method. 3-8μl of fluid were collected from groups of animals of different sizes, each group containing 3-25 animals depending on their size, and diluted into 2 ml of Ringer. In some cases the pH of the fluid was measured before dilution, so the volume of fluid recovered from the pH electrode was measured.

The relationships found between protease, cellulase and amylase activities and length are shown in figures 15, 16 and 17. The regression coefficients for protease and cellulase are not significantly different from 1.0 ($t = 1.12$, $P = 0.2 - 0.3$; $t = 0.1$, $P = 0.9$ respectively), but that for amylase is ($t = 2.39$, $P = .05 - .02$). Therefore, there was a real increase in amylase activity with increasing size. It was felt however that this increase was caused by the methods used. When guts were extracted by the wet method a certain amount of distilled water was transferred to the liquid paraffin with each gut and, as larger numbers of the smaller animals were required, the resulting dilution was progressively greater as the length of the animals decreased.

To overcome this problem, in all experiments using the test diets M.G.F. was collected by the dry method. The mean size of the groups of animals used was kept between 8.5 and 11 mm, and individual animals rarely were smaller than 8 mm or larger than 12 mm. In this way it was thought unlikely that the size of the animals used would have any effect on the results. Some of the results for animals feeding normally are derived from M.G.F. collected by the wet method. To correct these figures to equivalent volumes collected by the dry method, the 'dry' volume in each case has been calculated using a value of 1.5 $\mu\ell$ / 10 mg dry animal weight. This figure was calculated from the volumes of M.G.F. collected by the dry method from groups of animals feeding normally, and on leaf and fine detritus diets ($N = 37$, S.D. = 0.39, S.E. = 0.064). The dry weights were derived from the length/weight curve shown in figure 18. This curve was drawn from the dry weights at 55°C of 96 animals weighed in groups of 1-8.

The conditions under which the animals were fed the various test diets were those previously described in the section on the effect of diet on M.G.F. pH (Chapter 4). As Degkwitz (1957) found a decrease in midgut gland protease activity during the breeding period in several crustaceans, all experiments with standard test diets were carried out between September abd December, 1973. This period is the resting phase in the life cycle of G.pulex, no females with mature ovaries or couples in precopula being found. No attempt was made to determine the sex of animals used in these experiments. The 'normal' values were derived from several different experiments carried out between February and December, 1973.

FIGURE 15.

The relationship between animal length and protease activity.

Regression line $y = 0.99 + 0.56x$.

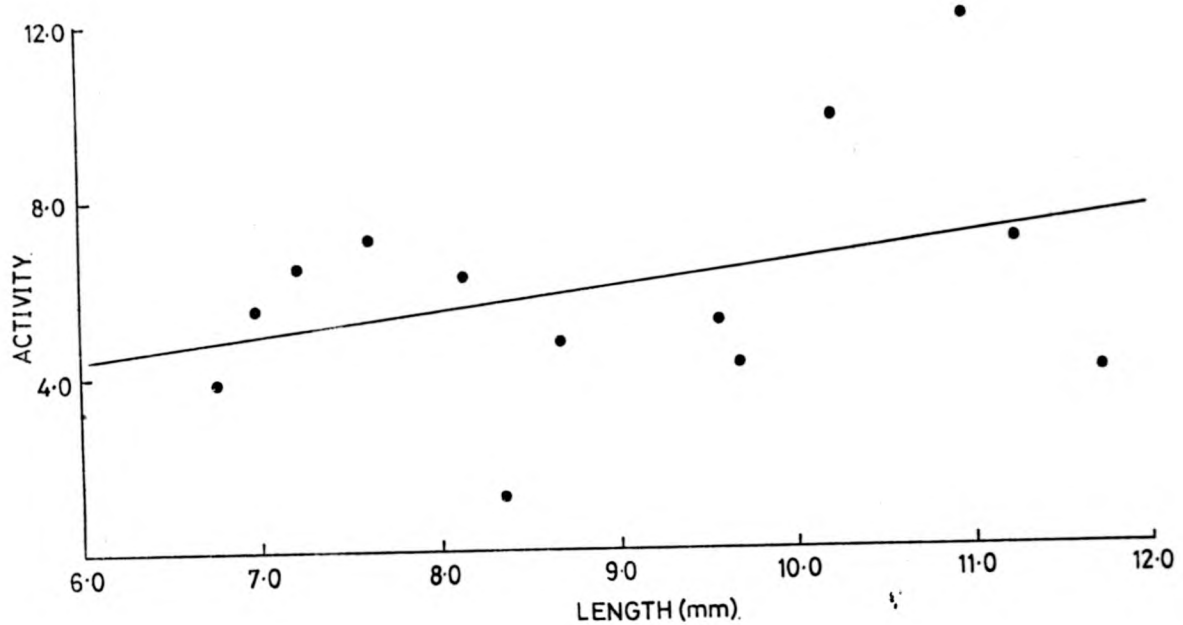


FIGURE 15

FIGURE 16.

The relationship between animal length and cellulase activity.

Regression line $y = 4.49 - 0.025x$.

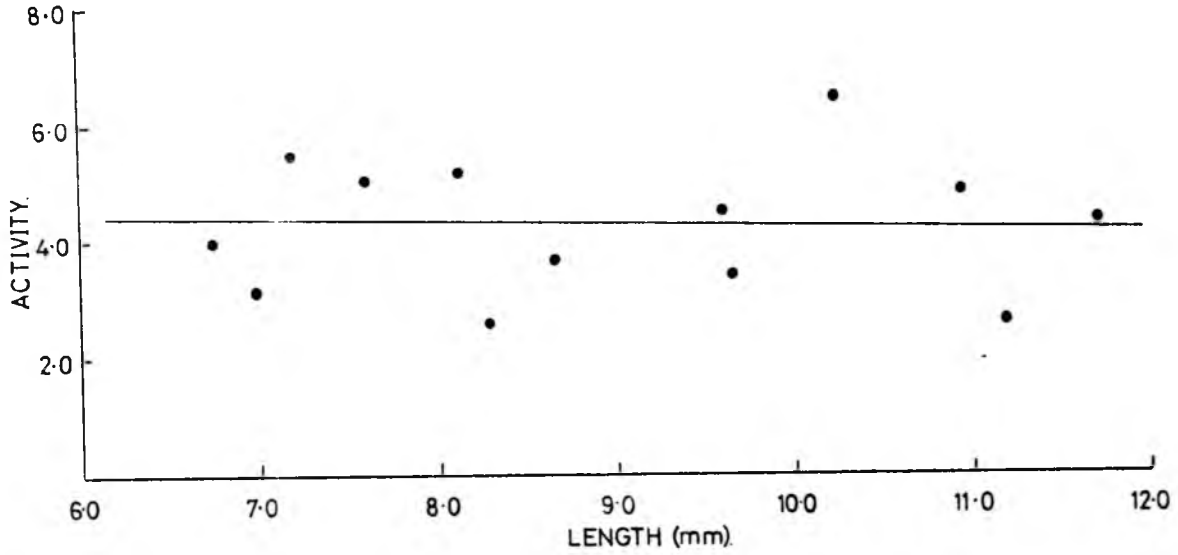


FIGURE 16.

FIGURE 17.

The relationship between animal length and amylase activity.

Regression line $y = -6.25 + 2.3x$.

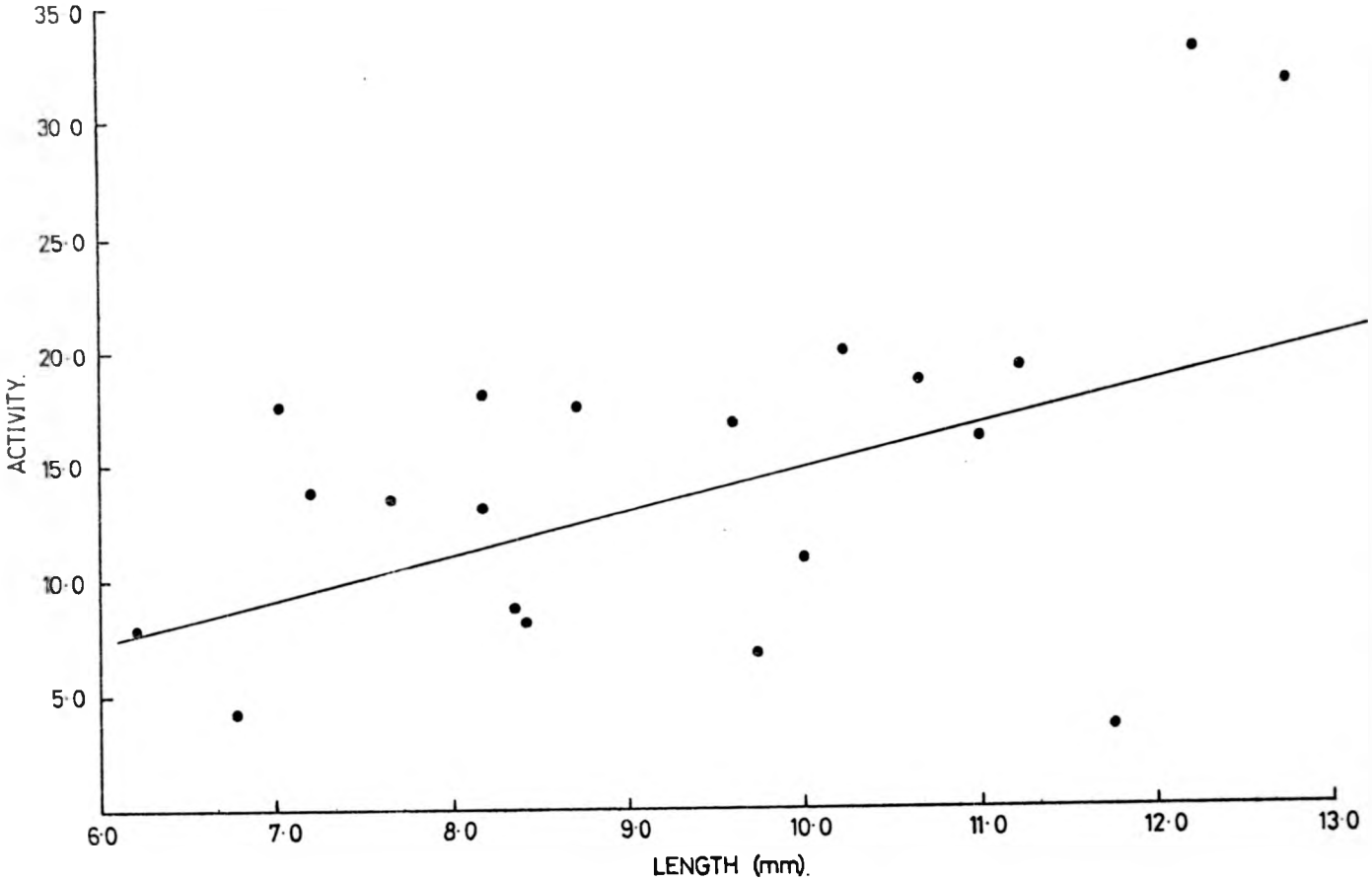
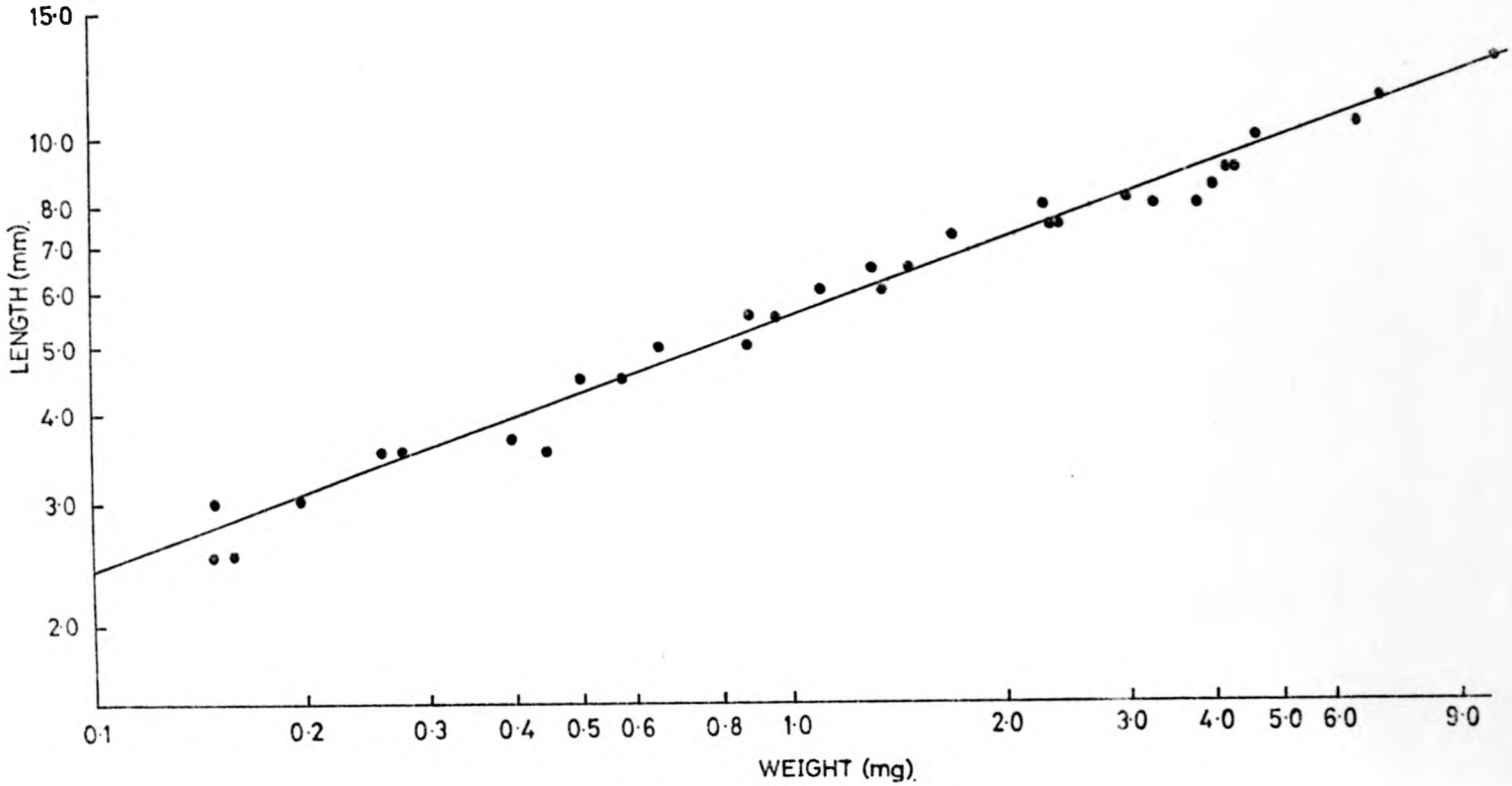

35·0
30·0
25·0
20·0
15·0
10·0
5·0
ACTIVITY.
6·0
7·0
8·0
9·0
10·0
11·0
12·0
13·0
LENGTH (mm).

FIGURE 17.

FIGURE 18.

The relationship between animal length and dry weight at 55°C.


15·0
10·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
LENGTH (mm).
0·1
0·2
0·3
0·4
0·5
0·6
0·8
1·0
2·0
3·0
4·0
5·0
6·0
9·0
WEIGHT (mg).

b) Results

The amylase, protease and cellulase activities of M.G.F. from animals feeding 'normally' are shown in Table 26 and those from starved animals and animals fed the test diets are shown in Table 27. Results of t tests between some of the mean % normal figures are shown in Tables 28 and 29. It is apparent that the activities of starch and protein digesting enzymes were affected similarly by the various treatments, although changes in amylase activity were generally of greater magnitude than those of protease activity. Generally, when 100C was consumed, activities of both enzymes fell to a minimum level. Addition of protein to this diet caused a rise in enzyme activity towards the normal level, which was reached when 40% of the diet was protein. Increasing the protein content of the diet to 100% (100P) caused no further rise in enzyme activity, in fact a slight but non significant decrease occurred in both cases. Amylase activities were similar when 100P was made up with egg albumen or bovine albumen, although the results for the latter were very varied. 90C + 10P and 60C + 40P were made up with egg albumen.

When fed on natural diets, enzyme activity rose above that found in 'normally' feeding animals in most cases. The difference in protease activity between those animals feeding normally and those consuming the various natural test diets was not significant in any case, but that in amylase activity was significant in all cases except N.L.

Enrichment of the diets caused an increase in enzyme activity. Activities of both enzymes in M.G.F. from animals fed E.L. were greater than those in M.G.F. from animals fed N.L., although the difference was only significant in the case of protease. Enzyme activities were also higher with E.F.D. than with F.D., but in neither case was the difference significant. The protease activities from animals feeding on those natural diets which stimulated the highest activities, i.e. E.L. and E.F.D., were not significantly different to those from animals feeding on 60C + 40P ($t = 1.5$ $P > .05$; $t = 1.84$ $P > .05$ respectively), although the differences in amylase activity between these groups were

TABLE 26.

Enzyme Activities in M.G.F. of Animals Feeding Normally

Mean Length	No.	Method Collection	Volume (µl) MGF	Amylase Activ.	Amylase % Norm	Protease Activ.	Protease % Norm	Cellulase Activ.	Cellulase % Norm
10.3	4	Wet	3.0	41.1	202	18.3	250	9.2	177
9.6	8	"	5.5	27.5	135	7.4	101	5.8	111
9.7	6	"	4.0	11.7	57	6.1	84	4.8	93
8.7	6	"	3.0	17.4	86	4.7	64	3.6	70
10.0	6	"	4.5	8.5	42	-	-	-	-
9.7	7	"	5.0	21.5	105	6.6	91	-	-
9.8	9	"	5.5	18.1	89	5.9	81	-	-
10.2	5	"	4.0	22.8	112	7.9	108	-	-
10.0	6	"	4.5	13.3	65	7.4	102	-	-
9.3	7	"	4.5	21.8	107	8.2	113	-	-
10.0	6	Dry	4.5	-	-	6.1	84	3.9	75
9.8	5	"	4.0	-	-	5.6	77	4.5	86
9.8	5	"	3.5	-	-	7.3	100	5.8	112
10.1	5	"	4.0	-	-	7.5	103	5.3	103
9.8	5	"	3.0	-	-	7.7	106	4.4	84
9.8	5	"	4.0	-	-	7.8	107	4.7	91
9.5	7	"	5.0	-	-	2.4	32	-	-
$\bar{x}$				20.4	100	7.3	100	5.2	100
S.E.$_{\bar{x}}$					14.4		11.2		9.6
N					10		16		10

TABLE 27.

Enzyme Activity in M.G.F. from Animals fed Various Diets and Starved.

(Mean % normal figures ± 1 S.E only. The complete data is shown in Appendix 1)

Diet.	Amylase	Protease	Cellulase
100C	52±5	79±5	89±5
90C + 10P	72±9	83±8	101±11
60C + 40P	105±6	102±5	96±5
100P (Egg Al.)	98±6	90±3	89±3
100P (Bovine Al.)	92±21	-	-
N.L.	128±7	88±4	95±3
E.L.	153±20	125±14	99±11
F.D.	146±9	111±8	100±4
E.F.D.	175±11	115±5	97±8
Starved (3 days)	-	137±10	106±2
Starved (7 days)	221±9	177±8	106±10

- = Not Tested

TABLE 28.

't' Tests between Mean % Normal Amylase Activities

a) Artificial Diets

	Normal	100C	90C+10P	60C+40P
100P	t = 0.1 N.S.	t = 5.53 S.1.	t = 2.39 S.5.	t = 0.72 N.S.
60C + 40P	t = 0.3 N.S.	t = 6.27 S.1.	t = 2.97 S.5.	
90C + 10P	t = 1.66 N.S.	t = 1.92 N.S.		
100C	t = 3.19 S.1.			

b) Natural Diets and Starved

	Normal	Starved (7d)	N.L.	E.L.	F.D.
E.F.D.	t = 4.06 S.1.	t = 3.16 S.1.	t = 3.52 S.1.	t = 0.93 N.S.	t = 1.94 N.S.
F.D.	t = 2.66 S.5.	t = 5.60 S.1.	t = 1.53 N.S.	t = 0.29 N.S.	
E.L.	t = 2.12 S.5.	t = 3.01 S.5.	t = 1.14 N.S.		
N.L.	t = 1.73 N.S.	t = 7.68 S.1.			
Starved	t = 7.10 S.1.				

N.S. = Not Significant

S.5 = P = .05

S.1 = P = .01

TABLE 29.

't' Tests between Mean % Normal Protease Activities

a) Artificial Diets

	Normal	100C	90C+10P	60C+40P
100P	t = 0.87 N.S.	t = 2.12 N.S.	t = 0.92 N.S.	t = 2.01 N.S.
60C + 40P	t = 0.17 N.S.	t = 3.37 S.1.	t = 2.14 N.S.	
90C + 10P	t = 1.3 N.S.	t = 0.44 N.S.		
100C	t = 1.75 N.S.			

b) Natural Diets and Starved

	Normal	Starved (7d)	N.L.	E.L.	F.D.
E.F.D.	t = 1.23 N.S.	t = 7.03 S.1.	t = 4.32 S.1.	t = 0.68 N.S.	t = 0.43 N.S.
F.D.	t = 0.82 N.S.	t = 6.19 S.1.	t = 2.67 S.5.	t = 0.86 N.S.	
E.L.	t = 1.38 N.S.	t = 3.23 S.1.	t = 2.49 S.5.		
N.L.	t = 1.00 N.S.	t = 10.21 S.1.			
Starved	t = 5.70 S.1.				

N.S. = Not Significant

S.5 = P = .05

S.1 = P = .01

significant ($t = 2.30$ $P < .05$; $t = 5.60$ $P < .01$ respectively).

The tracts of animals starved for three days often still contained a little food and the M.G.F. retained its normal brownish or greenish colour. After seven days starvation most tracts were completely empty and the midgut glands were full of a practically colourless fluid. In some cases however,particularly where a little food remained in the tract, the M.G.F. had a reddish appearance. Amylase and protease activities from animals starved for seven days were higher than those from all the feeding groups. Increased protease activity was evident after three days although the level reached was not significantly higher than that found in M.G.F. from animals feeding on E.L. or E.F.D.

The cellulase activities determined in these experiments (Table 27) suggest that differing diets or starvation have little effect. The null hypothesis that the activities found in all treatments were similar was tested by a one way analysis of variance. The values were not first transformed as the graphical method suggested that the variances were independent of the means. The resultant value of F, 0.71, did not reject the null hypothesis ($P > 0.2$). However, t tests between the results from three day starved and 100C, and three day starved and 100P groups, showed significant differences in both cases ($t = 2.9$ $P < .05$; $t = 4.86$ $P < .01$ respectively). In case the criteria for a one way analysis of variance were not fulfilled by the data, a Kruskal-Wallis one way analysis by ranks was also carried out, but the null hypothesis again could not be rejected ($k = 8.77$ $P = 0.3$).

In order to determine whether there was any correlation between the stimulation of enzyme secretion by various diets and the effect of those diets upon the feeding parameters described in Chapter 5, linear regression analyses between the data for certain of the feeding parameters and protease and amylase activities have been carried out (Table 30). In no case is the regression coefficient significant at the 9% level and in most cases the probability that $b = 0$ is high. However there is a fairly high probability that the activities of both enzymes were positively correlated with 'Feeding Activity' and negatively correlated with 'Ingestion Efficiency'.

TABLE 30.

Linear Regression Analyses between Feeding Parameters and Enzyme Activity

Feeding Parameters (x)	Enzymes Activities (y)	
	Amylase	Protease
'Feeding Activity'	y = 72.8 + 6.4x	y = 81.7 + 2.6x
	t = 1.93	t = 1.95
	P = 0.1 - 0.2	P = 0.05 - 0.1
Ingestion Rate	y = 122.5 - 1.2x	y = 89.6 + 1.8x
	t = 0.17	t = 0.66
	P = 0.8 - 0.9	P = 0.5 - 0.6
"Ingestion Efficiency'	y = 150 - 0.1x	y = 109.5 - 0.02x
	t = 1.97	t = 1.57
	P = 0.05 - 0.1	P = 0.1 - 0.2
'Tract Fullness'	y = 136.5 - 0.6x	y = 102.1 - 0.08x
	t = 0.67	t = 0.25
	P = 0.5 - 0.6	P = 0.8 - 0.9
'Assimilation Efficiency'	y = 146.9 - 0.74x	y = 119.7 - 0.4x
	t = 0.83	t = 1.25
	P = 0.4 - 0.5	P = 0.3 - 0.4

c) Discussion

Little is known concerning the control of digestive enzyme secretion in the crustacea. Secretion by the midgut gland may be holocrine, as in Astacus, or merocrine, as in Atya spinipes (Van Weel, 1955 ; Vonk, 1960). Secretion in the midgut gland of Marinogammarus obtusatus is probably merocrine (Martin, 1964) but Mabillot, (1955) described secretion in G.pulex as apocrine. If Astacus, Atya or Thalamita are starved for long periods and then fed, enzyme secretion is stimulated and occurs rhythmically in time (Hirsch, 1931 ; Van Weel, 1955, 1960). These results probably have little relevance to animals feeding normally, and are probably the result of the cyclic and synchronous development of new secretory cells or ripening of secretory granules, initiated by feeding after the long starvation period (Vonk, 1960). Martin (1964) could not detect a rhythm of secretion in Marinogammarus because he was unable to clear the gut of food with the result that the secretory cells were in various phases of secretion. In the present experiments the animals were not first starved and it would seem unlikely that the midgut glands as a whole were secreting rhythmically.

Where simultaneous measurements of the activities of different enzymes have been carried out e.g. Van Weel (1960), they have been found to vary synchronously. Van Weel considered that this was to be expected as all enzymes are thought to be secreted by one type of cell. Cellulase however does not seem to have been considered in this respect, although Yasumasu and Yokoe (1965)showed that the cellulase of the crayfish Procambarus clarkii was probably synthesised in the cells of the midgut gland, as it was associated with the microsome fraction of the gland. It has been suggested that the crystals found in the midgut gland of isopods may represent a phase in cellulase secretion, but there is no evidence to support this suggestion (Arvy, 1969). Mabillot (1955) and Martin (1964) did not mention the presence of any such crystals in the midgut gland of G.pulex and Marinogammarus obtusatus.

It would appear from the results presented here that cellulase is secreted independently of dietary stimuli, whereas the secretion of amylase and protease is affected by the nature of the diet, the former more than the latter. This hypothesis does not necessarily imply that the three enzymes are secreted separately into the gland lumen, but may suggest that the rates at which they are synthesised and incorporated into the secretory granules are affected to different extents by dietary stimuli. Little other information concerning the effect of diet on enzyme secretion in crustaceans is available. Halcrow (1971) measured cellulase activity in the gut of Gammarus oceanicus after feeding them on mussels, algae and deciduous tree leaves, and after starving them. The results were quite similar to those obtained here as enzyme activity did not vary significantly between the three diets, although a slight decrease occurred after starvation. His animals were starved for one month however and he used homogenates of entire guts. These results were in contrast to those of Lewis and Whitney (1968) who found that cellulase activity in the polychaete Nereis virens was very low in animals starved for six weeks or fed cockles, but was greatly stimulated by feeding algae. Hoyle (1973) measured amylase, proteinase and lipase activities in gastric juice from the lobster Homarus americanus after feeding them with diets containing varying amounts of starch. Although feeding stimulated production of varying proportions of all three enzymes, the starch content of the diet had no effect.

The increased M.G.F. amylase and protease activities observed during starvation in the present experiments were presumably the result of a build up of secreted enzymes in the lumen of the midgut gland, assuming that passage of the fluid from the gland into the tract ceases during starvation. Hartenstein (1964) showed that the passage of digestive fluid from the glands into the tract was stimulated by ingestion in the isopod Oniscus asellus, but some fluid may pass into the tract during starvation in G.pulex as the tracts of starved animals often contained fluid. This may have originated from the posterior dorsal caeca however (Chapter 4). Fingermann et al (1967) found that during starvation amylase activity increased in the fluid and tissues of the midgut gland of the crayfish Procambarus

clarkii. They believed that during starvation the gland continued to synthesise amylase, but did not release it in to the lumen. After a week however amylase activity in the tissues was so high that some began to leak into the digestive fluid. A similar explanation may apply to the present results although a rise in enzyme activity in the fluid was observed after only three days starvation. It may be that enzymes are not stored in the epithelium of the midgut gland of Gammarus, but pass into the fluid where activity builds up. This explanation suggests that the liquid basis of the fluid and the digestive enzymes are secreted separately into the lumen of the gland. Some evidence for this has been provided by Martin (1964) who carried out histochemical tests for esterases in the midgut gland of Marinogammarus. He did not detect any enzyme activity in the large vacuoles of the so called secretory cells, which are usually considered to be the origin of the digestive fluid and which have been shown to be discharged into the lumen in Atya (Van Weel, 1955), but did detect activity in the distal cytoplasm of these cells. He suggested that the large vacuoles may provide the fluid basis of the secretion. Alternatively, if the liquid basis of the fluid and the enzymes are secreted together, the build up of enzyme activity may occur because secretion into the lumen slows but synthesis and discharge of enzymes into the secretory granules continues, resulting in the slow production of a fluid of high enzyme activity.

Similarly the various levels of enzyme activity induced by different diets may be explained by differing relative rates of enzyme and fluid secretion. Some diets evidently stimulate a more rapid rate of enzyme synthesis than others, and the rates of synthesis of certain enzymes are evidently more affected by dietary stimuli than others. Fingermann et al (1967) showed that the eyestalks exerted a neuroendocrine control over RNA and amylase synthesis in the midgut gland of Procambarus and a similar control may occur in Gammarus, but the nature of the dietary components which stimulate any such control mechanism is not obvious. There seems to be no obvious correlation between any chemical parameter of the diets and the enzyme activity which they stimulated. When fed artificial mixtures the presence of protein up to a maximum of 40% stimulated increased enzyme activity,

but in those animals consuming natural diets higher activities were found, although the protein content of the food was always less than 6% (see Table 33). Any other substances which may have been present in the natural diets and stimulated enzyme activity are not likely to have been present in those artificial mixtures which stimulated the higher activities. The results of these experiments would therefore seem to suggest that the stimulus for enzyme synthesis cannot be simply explained on the basis of the chemical composition of the diet. The higher activities found when animals were fed the natural and more palatable diets may suggest that the stimuli for enzyme synthesis are closely associated with those concerned with the ingestion of food and its passage along the tract.

Studies of the control of enzyme secretion in insects have shown that different mechanisms may be involved in different groups, but mechanical stimuli are often important (Dadd, 1970). In insects taking occasional meals, secretion seems to be associated with the receipt of a suitable meal in the gut, and in adult Aedes aegyptic the amount of protease secreted depended on the size of the blood meal (Shambaugh, 1954). Earlier work also showed that enzyme secretion was stimulated by the reception of food in the gut of "frequently-feeding" insects but gave little information concerning the effect of different diets. Day and Powning (1949) found that all enzymes were secreted irrespective of the type of diet in Blatella germanica, but amylase activity was reduced following a starch meal. Dadd (1956) found that in Tenebrio molitor enzyme secretion was induced by feeding, and that the rate of discharge of enzyme from the tissues was dependent on the rate of synthesis, whereas in Dytiscus, which takes occasional meals, enzymes were stored in the tissues and discharged into the gut lumen on receipt of a meal.

Recently, considerable information has been published concerning the stimulation of digestive enzyme secretion in larvae of Spodoptera littoralis (Lepidoptera) by various dietary constituents. This insect may be reared on artificial diets and will eat pieces of foamed polystyrene (Styropor) or mixtures of cellulose powder and yeast. Applebaum et al (1964) found that adding starch to a mixture of cellulose and yeast caused an increased amylase activity in the gut, whereas adding

maltose to the mixture caused a decrease, and Ishaaya et al (1971) found that both amylase and protease activities were dependent on the protein concentration in an artificial diet. Meisner et al (1973) found that invertase, amylase and protease activities in guts of animals feeding on Styropor lamellae all increased when sucrose was added to the diet, and the extent of the increase depended on the concentration of sucrose. Ishaaya and Meisner (1973) found that enzyme activities in animals fed Styropor lamellae treated with different sugars were raised to differing extents, but that amylase, protease and invertase were all affected in broadly similar ways. Overall, in Spodoptera the secretion of digestive enzymes seems to be affected by the levels of several dietary constituents, in fact all the substances tested had some effect on enzyme activity. The true nature of the stimulant for enzyme secretion may be suggested by the findings of Ishaaya and Meisner (1973), that the extent to which the digestive enzymes were stimulated by various sugars correlated well with the extent to which those sugars stimulated the ingestion of Styropor lamellae.

It seems that there may be a link between stimuli eliciting enzyme secretion and stimuli concerned with the ingestion of food in Spodoptera, and probably in some other insects. A similar relationship may exist in Gammarus but the lack of any correlation between ingestion rate or 'Tract Fullness' and enzyme activity suggests that physical stimuli associated with the presence of food in and its passage through the tract are not connected with digestive enzyme secretion. The lack of correlation with assimilation efficiency suggests that there is also no secretory response to an indigestible diet. There seems to be a relationship however between enzyme activity and 'Ingestion Efficiency' and 'Feeding Activity'. As discussed in Chapter 5, 'Ingestion Efficiency' and 'Feeding Activity' may both be correlated with palatability, so enzyme secretion may be best correlated with food palatability. It is known that the crustacea have olfactory and gustatory receptors on their thoracic limbs, antennae and mouth parts which allow them to discriminate between different foods (Barber, 1960) and that the control of digestive secretion is probably mediated by a neuroendocrine mechanism (Finger-

mann et al, 1967). The simplest hypothesis to account for the results described here is that chemoreceptors are stimulated according to the palatability of the food, resulting in the release of hormones which stimulate digestive enzyme synthesis and secretion. The same hormone, or others released in response to the same stimuli, may control 'Feeding Activity', although the cessation of feeding may be affected by direct nervous control as Bethe (quoted in Schone, 1960) has shown that Carcinus maenas will continue to feed after its supra oesophageal ganglion has been removed, but may not stop until its stomach bursts.

CHAPTER 7

THE EFFECT OF DIET ON GROWTH

a) Methods

i) General

The experiments described here were carried out in order to determine whether the nutritive values of various kinds of plant detritus to G.pulex can be directly related to their protein contents, or whether other factors, such as digestibility, might be more important. However, it became apparent that the effect which diet had upon growth and survival was dependent both on the stage of development of the animals and the chemical characteristics of the water used. The relationships between diet, growth, survival and water chemistry were therefore first elucidated as far as possible, so that a growth parameter could be found which would be directly dependent on the nutritive value of the diet, and would allow comparisons between the various treatments.

Growth has been measured in all experiments by estimating the increase in the mean population length. This was done by measuring all individuals in the population by the method of Hynes (1954). At regular intervals each animal was compared to a set of preserved individuals of known length, and its length estimated to the nearest half millimetre. In order to test the reproducibility of this method a random sample of 100 animals was taken from the laboratory tank and preserved in 70% alcohol. On ten occasions over a period of 14 days the lengths of all individuals in the sample were estimated. The results are shown in Table 31. The mean population lengths obtained do not differ significantly as the standard errors of the two most widely separated estimates overlap.

ii) Experiments in Running Water

Preliminary investigations were carried out in a recirculating system to determine the effect of decomposition upon the nutritive value of dead plant material to both G.pulex and the limnephilid Halesus spp. The experiments were carried out in plastic bowls of 29 cm diameter and 10 cm depth, containing about 4 litres of water. Peristaltic

TABLE 31.

Reproducibility of Estimates of Mean Population Length.

Estimate No.	Size Classes (mm) 4.5	5.5	6.5	7.5	8.5	9.5	$\bar{x}$	S.E.$_{\bar{x}}$
1.	17	23	20	32	6	2	6.43	0.13
2.	13	32	16	31	5	3	6.42	0.13
3.	17	24	14	35	7	3	6.50	0.14
4.	16	26	15	34	6	3	6.47	0.13
5.	20	25	11	35	6	3	6.41	0.14
6.	16	24	15	35	8	2	6.51	0.14
7.	13	27	14	36	7	3	6.56	0.13
8.	18	20	12	41	7	2	6.55	0.13
9.	14	24	13	40	6	3	6.59	0.13
10.	15	24	15	37	6	3	6.54	0.13

pumps were used to continuously remove water from one side of the bowl and pump it back to the other side through 0.8 cm diameter tubing, thus creating a circumferential current. Suspended material was removed by a folded nylon mesh filter inserted into the intake side of the pump, which also prevented animals from passing through the pump. The filters were cleaned daily and the bowls were kept covered to exclude light and prevent the growth of algae. Two types of vegetable material were used, elm litter and dead coarse grass taken from the area of the Fincastle burn. Two bowls were set up for each type of material. In one the material was removed and replaced by fresh material every two days, and in the other the water was enriched with nitrogen and phosphorus as previously described, and the plant material was left untouched during the course of the experiments. Leaves and grass were stored dry prior to their use and soaked for one day before addition to the bowls. Elm leaves offered in enriched water did not therefore strictly correspond to E.L. as they were not incubated with nutrient enriched water prior to feeding. An excess of food was made available in each case, the amount consumed being insignificant compared to that left. To ensure that faeces were not ingested a 0.5 mm mesh nylon net, through which the faeces fell, was stretched across the bowls about one centimetre from the bottom.

As well as 120 to 140 speciments of G.pulex, 30 to 35 larvae of Halesus spp were initially placed in each bowl. G.pulex was obtained by sorting out the smallest members of the population from the Fincastle burn at that time (January, 1973), lengths varying between 2.5 and 4.5 mm. Halesus spp, which was obtained similarly, varied in length from 2.0 to 10.0 mm between the openings of the case. Measurements of head capsule width indicated that most of these animals were in their second or third instars. At the end of the experiments in which Halesus spp had grown most rapidly, lengths varied between 8 and 18 mm representing third, fourth and fifth instars. All animals were removed from the bowls, counted, and their lengths measured (Halesus spp) or estimated at roughly two weekly intervals, at which times the water was changed and nutrients renewed. The experiments were terminated after six weeks because of mechanical failure of the pumps.

iii) Experiments in Still Water

As the rates of growth and mortality found in the above experiments did not differ greatly from rates found in still, aerated water in later experiments with similar foods, the remaining experiments were carried out under the latter conditions.

In previous experiments it was suspected that young Gammarus did not ingest the fresh plant material, so a comparable experiment was performed in which the leaves were first broken into fine particles which it was thought more likely the young animals would ingest. The extent to which supplementing a diet of fresh leaf litter with protein improved its nutritive value was also studied. Freshly fallen elm leaves, collected in October, 1973, were homogenised in a Silverson laboratory emulsifier. Two distinct particulate fractions resulted. One (fine homogenate) passed through a 0.375 mm mesh readily and comprised the softer parts of the leaf. Microscopic examination showed that although it contained some intact cells, most of this fraction comprised small amorphous particles. The other fraction (coarse homogenate) would not pass through the mesh and mainly comprised the fibrous parts of the leaf and pieces of cellular epidermal tissue. Considerable material went into solution and was discarded. A large sample of each fraction was placed in a 2½ litre dark winchester bottle with two litres of enriched filtered water and 100 specimens of G.pulex, 1.5 to 3mm in length, collected as described previously. The water was aerated. A further sample of the fine homogenate was mixed with about 30% (V/V) of denatured egg albumen. This, and non-protein supplemented fine homogenate were offered fresh to Gammarus in unenriched water. These diets were placed in petri dishes which rested at the base of 17 cm diameter plastic bowls. The bottoms of the bowls had been previously cut away and replaced with 0.375 mm mesh. Two such bowls were set up for each diet, each containing 50 animals. Every two days the petri dishes were removed and the plastic bowls shaken so that all unconsumed food passed through the mesh. This was replaced by fresh food, which was stored deep frozen, and the water was changed. The animals were counted and their lengths estimated at one or two week intervals.

The remaining experiments were carried out in dark glass 2½ litre winchester bottles, the contents of which were aerated. An excess of all diets was offered, leaf diets being replenished periodically with more leaves which had previously been incubated under appropriate conditions. The water used was first filtered through Whatman GF/C glass fibre filters and was renewed every time the animals were counted and measured (approximately fortnightly intervals).

The first series of experiments, begun in September, 1973, was carried out using 100 1.5 - 3 mm animals in each case, collected as described previously. Diets offered were :-

a) Cellulose powder and Whatmans No. 1 filter paper. Cellulose powder was offered in both normal and enriched water. Decomposition proceeded for several weeks before the animals were introduced.

b) Coarse detritus. This was cellular organic debris collected from the Fincastle burn which would pass through a 0.75 mm mesh but not a 0.425 mm mesh.

c) Silt. This was very fine black amorphous material collected from pools in the Fincastle burn, which would pass through a 0.2 mm mesh.

d) N.L. and E.L. offered in normal and enriched water respectively.

The second series of experiments, begun in April, 1974, was carried out using animals which had not left the brood pouch for more than four days. Their lengths varied between about 1.5 and 2 mm. Gravid females and pairs in precopula were placed with a few leaves in a plastic bowl, the bottom of which was formed of glass fibre mesh. This was placed inside of, and held about a centimetre above the bottom of, a slightly larger bowl. Every 3 to 4 days the inner bowl was lifted out after shaking and young animals which had emerged from the brood pouches were collected from the larger bowl. Young animals which had passed through the mesh prior to their collection

could ingest the faeces of the adults, which are known to be of nutritive value to them (Hynes, 1954). Experiments were begun immediately after the animals were collected as they soon died if suitable foods were not available. The initial number in each experiment varied between 98 and 71. In some cases where young animals did not survive with the diets offered, 50 larger animals with a mean length of about 6 mm, or in one case 8.5 mm, were used. In order to determine whether the rather high and often sudden mortalities which occurred in earlier experiments were attributable to the chemical characteristics of the water supply, some experiments in this series were also carried out in water from the Fincastle burn.

The following groups of diets were offered:-

a) Cellulose powder in water from the Fincastle burn (2, 6 and 8 mm animals). Decomposition of the powder had been in progress for several weeks before the experiment began.

b) F.D. and E.F.D. offered in normal and enriched water respectively (2 and 6 mm animals.)

c) N.L. and N.L. plus protein (2 mm animals).

d) E.L. and E.L. plus protein offered in enriched water (2 mm animals).

e) Elm litter and elm litter plus protein in water from the Fincastle burn (Fincastle leaves and Fincastle leaves plus protein) (2 mm animals).

The protein added in each case was bovine albumen fraction V denatured by boiling and stored deep frozen. A few milligrams were added every 3 to 4 days.

In series 1 and 2 experiments controls were set up containing animals and the appropriate water but no food. The protein contents of most foods used were determined.

b) Results

i) Protein Contents and Water Chemistry

Chemical analyses of the laboratory water supply and of Fincastle burn water, carried out by Mr. R. Harriman, are shown in Table 32. The protein contents of the diets used in this Chapter and in Chapters 4, 5 and 6, are shown in Table 33. Significance tests between the mean protein contents of leaf diets are shown in Table 34. Absolute protein contents were low ($< 9\%$) in all cases except where the diet was supplemented with protein, and were particularly low in the case of elm leaves. The percentage of organic matter which comprised protein in fresh leaves was only 3% and this figure did not change significantly in leaves decomposed in normal water, or Fincastle burn water, or where solid protein was present. In enriched water however, the protein content doubled. All the samples of F.D. and E.F.D. tested were roughly similar, 10.6 - 14.4% of the organic matter being protein. Although the protein content of F.D. was not affected by enrichment. that of fine homogenate was increased. After about five months decomposition in Fincastle burn water, cellulose powder had a measurable protein and ash content.

ii) Experiments in Running Water

Growth and survival with fresh and decomposing grass and elm leaves are shown in figures 19 and 20. Survival was clearly better with decomposing than with fresh diets, but there was no clear difference between survival with elm and grass diets. Growth was also similar with elm and grass diets and during the first fourteen days there was no difference between growth rates with fresh and decomposing diets. The few animals which survived in the 'fresh' bowls after fourteen days grew as well as those in the 'decomposing' bowls, but there was a certain amount of material circulating in the 'fresh' bowls which originated from faeces, dead bodies and cast cuticles. This may have been the main source of food for these few animals. As in later experiments, few dead bodies were recovered from any of the bowls as these were readily eaten by the survivors. No starved controls

TABLE 32.

Chemical Analyses of the Laboratory Water Supply and Fincastle Burn Water (mg/l).

	Laboratory Supply	Fincastle Burn
pH	6.9	7.9
Alkalinity (as $CaCo_3$)	9.0	76.8
Conductivity (μMHOS)	57.0	235.0
Phosphate (as P)	0.000	0.015
Nitrate (as N)	0.17	0.68
Total P	0.010	0.031
Soluble P	0.009	0.021
Na^+	3.90	5.20
K^+	0.48	1.36
Ca^{++}	6.4	33.8
Mg^{++}	0.97	1.97
TKN	0.152	0.201

TABLE 33.

Protein Contents of Diets used here, and in Chapters 4, 5 and 6.

Diet	No. Determinations.	% O.M.	Protein Content			
			% Dry Weight			% O.M.
			$\bar{x}$	Range	S.E.$_{\bar{x}}$	
N.L.	7	92.9	2.7	1.9 - 3.3	0.25	2.9
E.L.	9	94.5	5.8	5.3 - 6.7	0.17	6.1
Fresh Elm Leaves	5	94.9	2.8	2.2 - 3.0	0.29	3.0
Fincastle Leaves	4	96.4	2.5	2.3 - 2.8	0.12	2.6
N.L. + Protein[1]	6	95.6	2.2	1.8 - 3.5	0.27	2.3
Fresh Grass	2	-	4.4	4.0 - 4.8	-	-
Decomposed Grass	2	-	5.2	5.0 - 5.4	-	-
Fine Homogenate (Fresh)	1	-	3.5	-	-	-
Fine Homogenate (Decomposing)	1	89.1	7.5	-	-	8.4
Fine Homogenate (Fresh)+ Protein	1	-	33.7	-	-	-
Coarse Homogenate (Decomposing)	3	89.0	6.2	5.9 - 6.5	0.18	7.0
Silt	4	33.3	4.3	3.6 - 4.8	0.26	12.9
Decomposing Cellulose Powder	4	99.7	0.1	0.1 - 0.2	0.02	0.1
F.D. (Ch. 4 & 6)	2	43.6	5.4	5.4 - 5.5	-	12.4
F.D. (Ch. 5)	4	59.0	7.9	7.4 - 8.5	0.22	13.4
F.D. (This Ch.)	4	71.5	8.0	7.3 - 8.8	0.33	11.2
E.F.D. (Ch. 4 & 6)	4	32.8	4.3	4.2 - 4.4	0.04	13.1
E.F.D. (Ch. 5)	4	59.0	8.5	7.6 - 10.3	0.61	14.4
E.F.D. (This Ch.)	3	71.7	7.6	7.2 - 8.3	0.33	10.6

[1] All Albumen washed off leaves before analysis.

TABLE 34.

't' values between Mean Leaf Protein Contents (% dry weight) from Table 33.

	N.L.	E.L.	Fresh L.	Finc. L
N.L. + Pr.	t = 1.36 N.S.	t = 11.28 S.1.	t = 1.51 N.S.	t = 1.02 N.S.
Finc. L.	t = 0.07 N.S.	t = 15.90 S.1.	t = 0.96 N.S.	
Fresh L.	t = 0.26 N.S.	t = 8.92 S.1.		
E.L.	t = 10.25 S.1.			

N.S. = Not Significant.

S.1. = Significant at 1% level.

FIGURE 19.

Survival in running water experiments.

—●— Decomposing elm.

—○— Decomposing grass.

—▲— Fresh elm.

—△— Fresh grass.

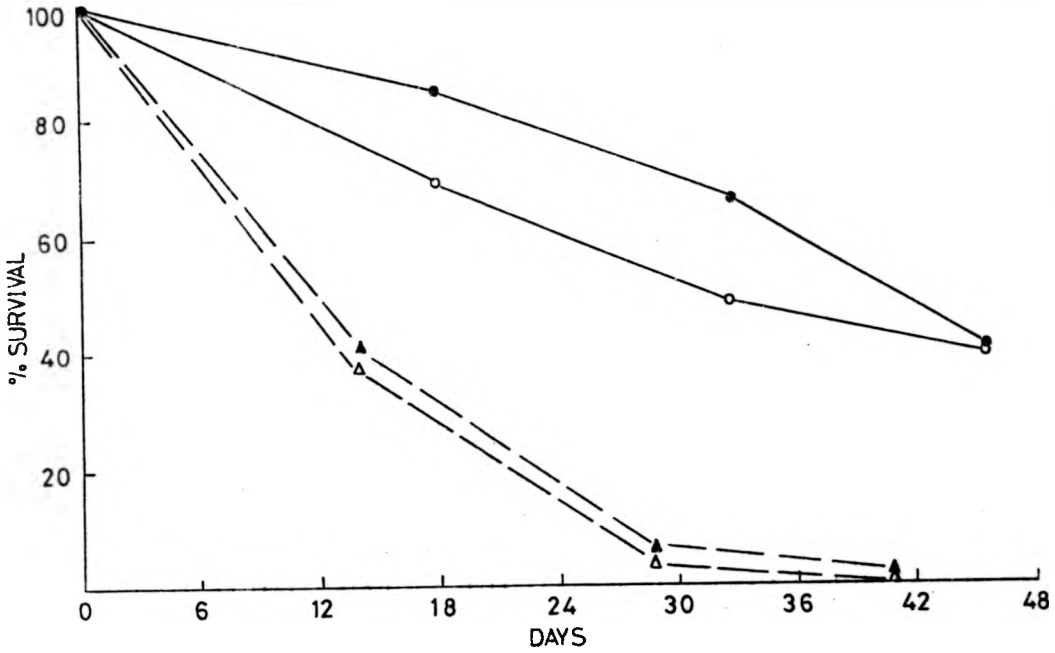


FIGURE 19.

FIGURE 20.

Growth in running water experiments.

—●— Decomposing elm.

—○— Decomposing grass.

–▲– Fresh elm.

–△– Fresh grass.

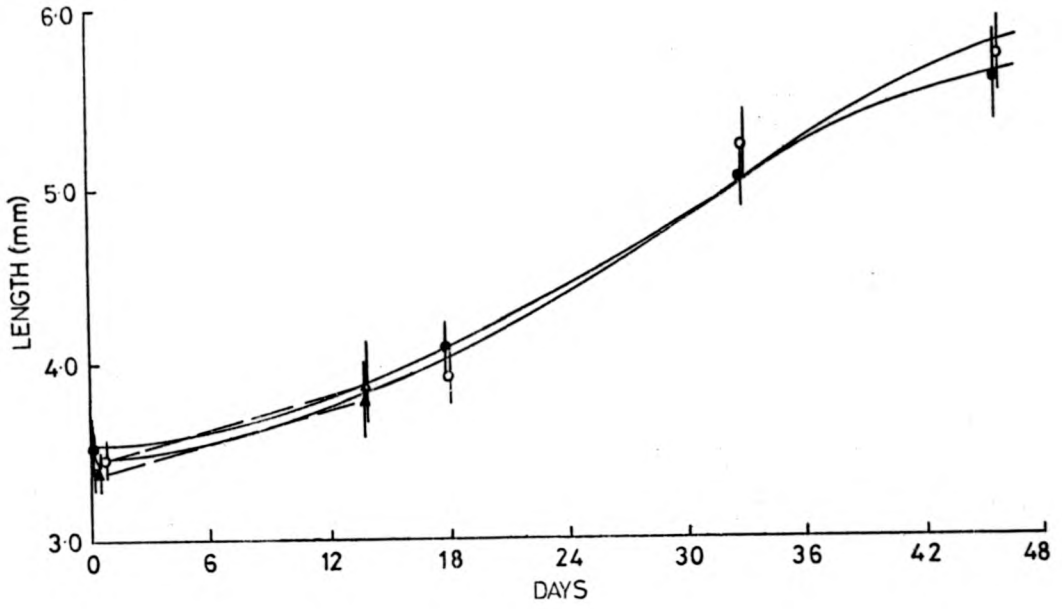


FIGURE 20.

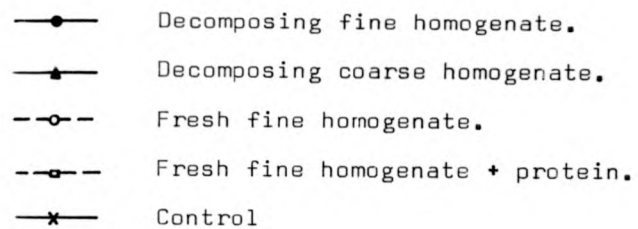
were used in this experiment but in later experiments it was found that some growth did occur in controls, presumably as a result of this habit. The results for Halesus spp, shown in Appendix 2, show that growth and survival was generally superior with decomposing foods, although fresh foods were not without nutritive value.

iii) Experiments in Still Water

Growth and survival with elm homogenates are shown in figures 21 and 22. Survival was best with decomposing fine homogenate, although rapid mortality occurred after 50 days. Decomposing coarse homogenate initially supported little better survival than the control but after 14 days there was a long period with no mortalities. Results obtained from each experiment with fresh fine homogenate and fresh fine homogenate plus protein were similar, so they have been lumped together for the purposes of figures 21 and 22. Fresh fine homogenate supported little better survival than the control, but the addition of protein improved survival slightly. Growth with elm homogenates was generally slow, but growth with decomposing fine homogenate was significantly more rapid than with fresh homogenate. Addition of protein to fresh fine homogenate caused a slight increase in growth rate. After 14 days the mean size of the animals with the protein supplemented diet was significantly larger than that of the groups without ($t = 2.06$ $P < .05$), but there was no significant difference after 21 days. In the control, which has been omitted from figure 22 for clarity but is included in a later figure (24), slight growth occurred as a result of cannibalism.

In series 1, two experiments were started with cellulose powder in enriched water, one with cellulose powder in normal water and one with filter paper in enriched water, but in no case was survival any better than in the control, all animals being dead within 19 days in every case, and many dead bodies recovered. In the control however a few animals survived up to 42 days and few dead bodies were found. This was a result of the non-selective feeding habits of the animals, which led to them eating whatever was most readily available. Where an excess of cellulose powder or filter paper was available it was readily ingested

FIGURE 21.


Survival with elm homogenates.
Decomposing fine homogenate.
Decomposing coarse homogenate.
Fresh fine homogenate.
Fresh fine homogenate + protein.
Control
Vertical lines represent range.

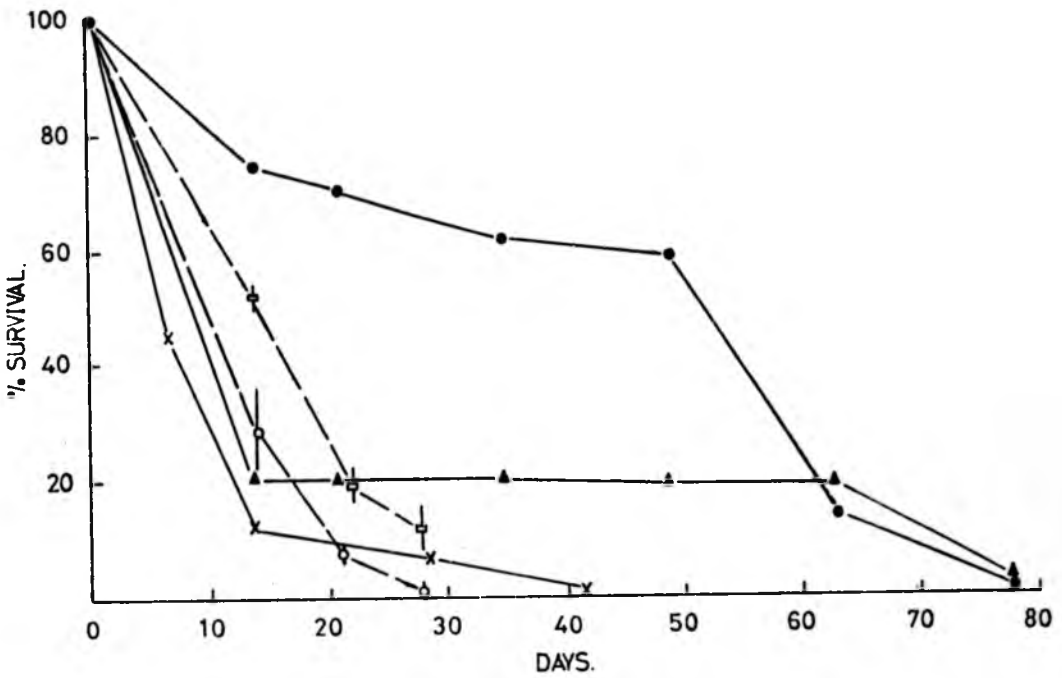


FIGURE 21.

FIGURE 22.

Growth with elm homogenates.

(1)—●— Decomposing fine homogenate.

(2)—▲— Decomposing coarse homogenate.

(3)—o— Fresh fine homogenate.

(4)—▫— Fresh fine homogenate plus protein.

Vertical lines represent ± 2 S.E.

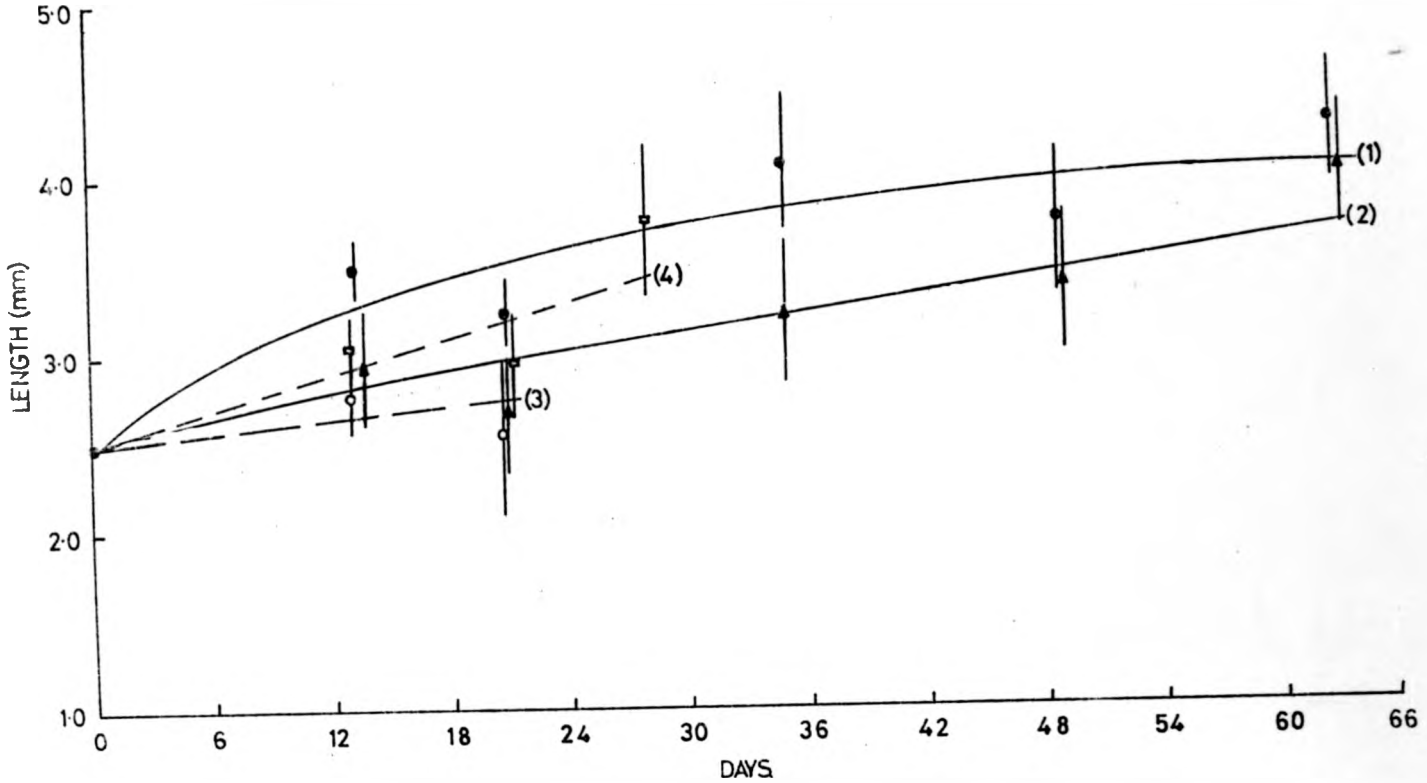
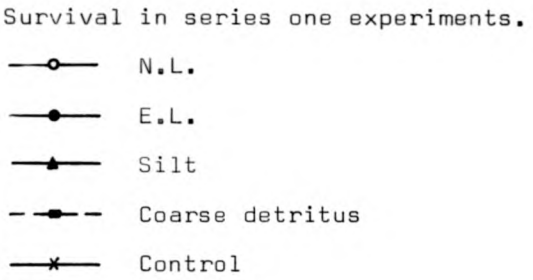


FIGURE 22.

and dead bodies were rarely consumed. Where no other food was available most dead bodies were consumed and a few animals were able to survive for a longer period because of the greater nutritive value of dead Gammarus than cellulose. 100% mortality occurred in 19 days with filter paper and 12 days in each case with cellulose powder. As the latter food was the more widely dispersed within the environment ingestion of dead bodies was less likely than with filter paper, so more rapid mortality resulted. Twelve days is therefore the best available estimate for the survival time of starved animals of this size (2.0 - 3.5 mm). In order to estimate the survival time of starved 8.5 - 10 mm animals, 50 specimens were starved individually in plastic bottles in which the water was changed daily. 50% mortality occurred after 8 days and after 17 days 94% mortality had occurred. This compares with 50% mortality after 17 days and 90% mortality after 70 days when 8.5 - 10 mm animals were starved communally in a control experiment (figure 25).

The growth and survival of animals fed other diets in the first series of experiments are shown in figures 23 and 24. Survival with coarse detritus was no better than with the control, but silt did support slightly greater survival, some animals still being alive after 116 days. Growth with silt was extremely slow however. The best growth and survival was obtained with leaf diets. Growth with E.L. was considerably more rapid than with N.L., but mortality was also greater with E.L. Growth apparently occurred in three phases. The initial growth rate levelled off after about 90 to 100 days and little growth occurred between 100 and 125 days. After this time rapid growth recommenced in both cases. Growth with E.L. was more rapid than with N.L. during both periods of rapid growth. At the end of the experiment with E.L., one female with clearly visible eggs developing in her ovaries, and one gravid female, were present. The first female with visible developing eggs was seen after 139 days when 7.5 mm in length. In the experiment with N.L. one such female, only 4.5 mm in length, was seen after 120 days, but probably died as no such females were seen in this experiment after 146 days.

FIGURE 23.


Survival in series one experiments.
N.L.
E.L.
Silt
Coarse detritus
Control

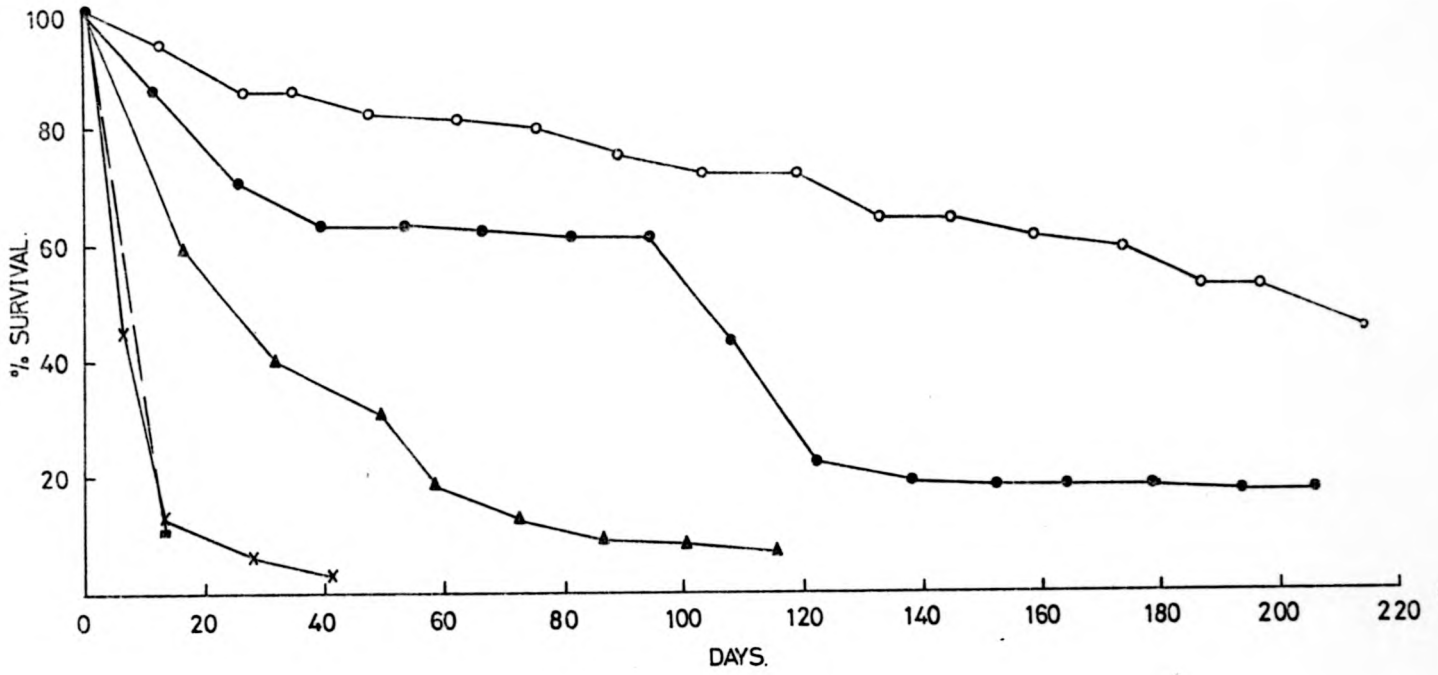
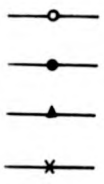

100
80
60
40
20
% SURVIVAL.
0
20
40
60
80
100
120
140
160
180
200
220
DAYS.

FIGURE 23.

FIGURE 24.

Growth in series one experiments.



Vertical lines represent ± 2 S.E.

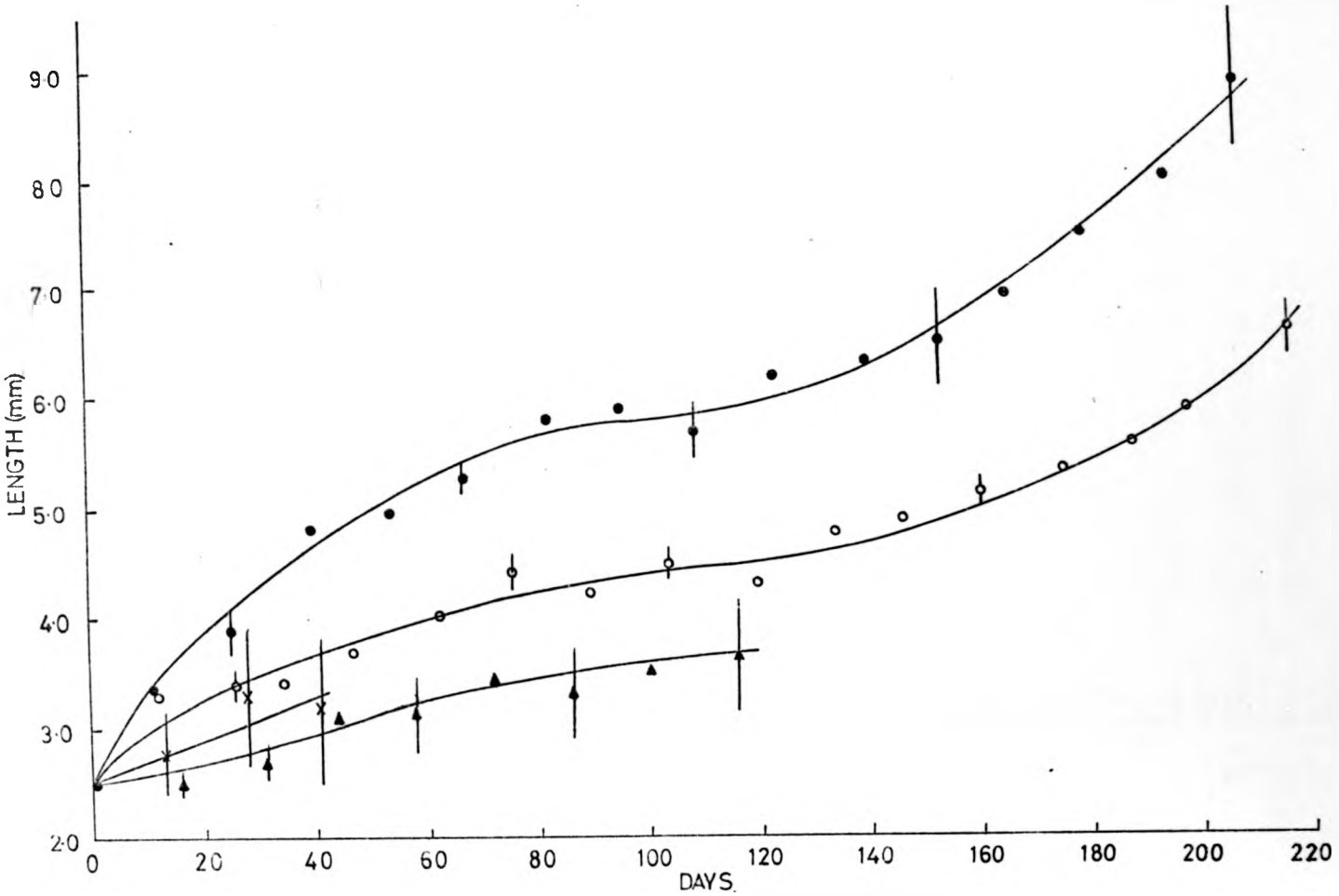

LENGTH (mm)
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
0
20
40
60
80
100
120
140
160
180
200
220
DAYS.

FIGURE 24.

Attempts to grow 2.0 mm animals (series 2) with cellulose powder in Fincastle burn water were no more successful than those described in series 1. Animals of this size were very susceptible to starvation and all animals in control and experimental containers were dead within 5 days (figure 25). Using 6 mm animals mortality was again rapid, although survival in the control bottle was considerably better, presumably for the reasons discussed previously. Some of the dead bodies and moribund survivors were examined to determine whether death could have been caused by clogging of the gills by cellulose powder. No signs of any such clogging were seen. The animals appeared quite pale and had empty and colourless tracts and midgut glands. Healthy animals in these and other experiments with cellulose powder were frequently observed to have tracts full of the powder, although the midgut glands often had the red coloration previously seen in starved animals (Chapter 6). When animals with an initial mean size of 8.5 mm were used (range 7.5 to 11.5 mm) there was a suggestion of a positive nutritive value as evidenced by slightly improved mortality and growth (figures 25 and 26). As the growth curve was apparently a straight line in this case, linear regression analyses were carried out. The regression coefficients of experimental and control curves differ significantly ($t = 5.77$ $P = < .001$) and that of the experimental curve is significantly greater than 0 ($t = 6.24$ $P = < .001$). The powder used in this experiment had been decomposing for 2½ months before the experiment began, and had changed little in appearance during that period. Twenty-five days after the beginning of the experiment however, the powder coagulated into large lumps of a light brown colour which separate observations showed were very attractive as food for Gammarus. Powder used in previous experiments never had this appearance.

F.D. and E.F.D. were of little nutritive value for 2.0 mm animals as they supported only slightly better survival than controls (figure 27). When 6.0 mm animals were used however, survival was considerably enhanced, but no growth had occurred in either case after 74 days (figure 28). Linear regression analyses of the growth curves were again carried out, but the regression coefficients did not differ significantly ($t = 1.74$ $P = > .05$).

FIGURE 25.

Survival with cellulose diets (series two).

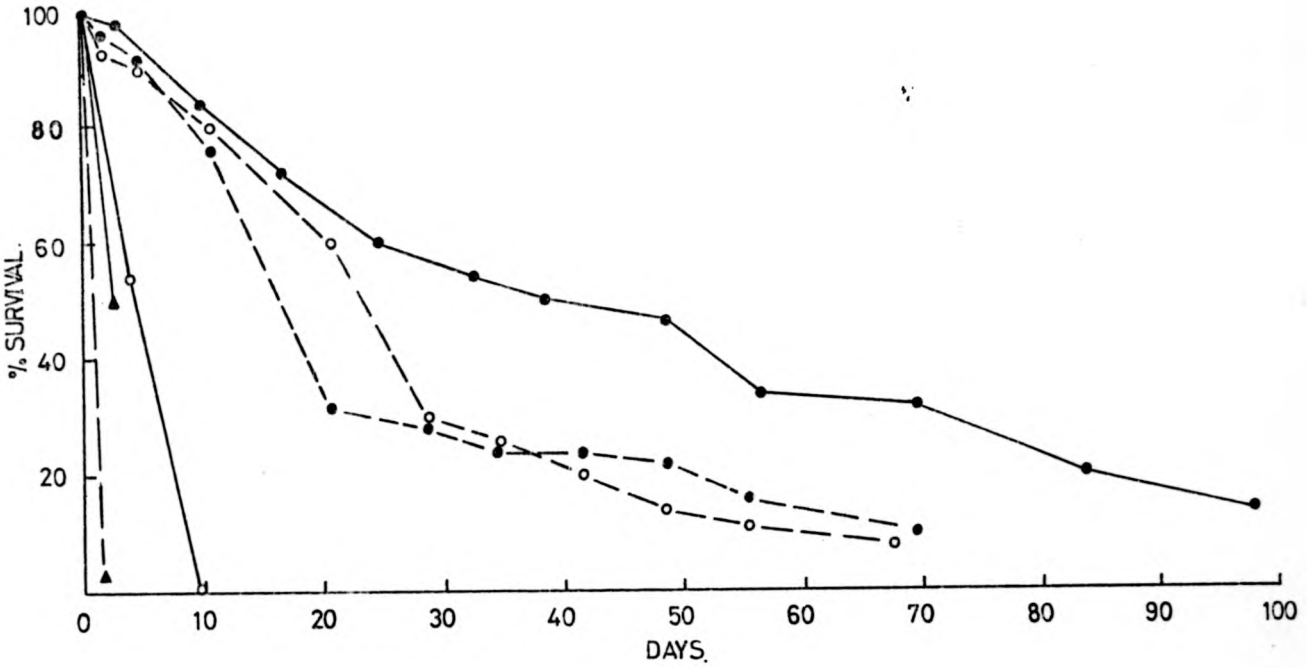


FIGURE 25.

FIGURE 26.

Growth of 8.5 mm animals with a cellulose diet.

—●— Experiment. Regression line $y = 8.34 + 0.212x$.

–○– Control. Regression line $y = 8.56 + -.010x$.

Vertical lines represent $\pm$ 2 S.E.

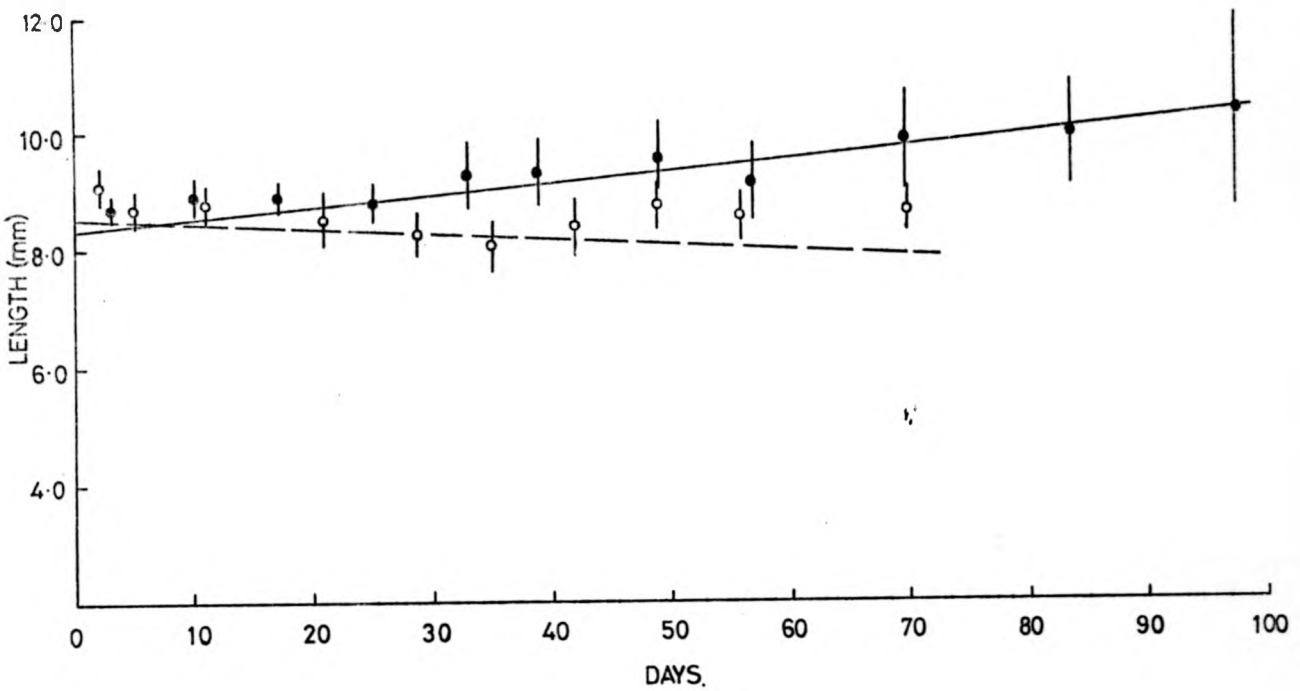


FIGURE 26.

FIGURE 27.

Survival with F.D. and E.F.D. (series two).

Animal size		Diet
2 mm	6 mm	
△	○	F.D.
▲	●	E.F.D.

Solid line - experiment.

Broken line - control.

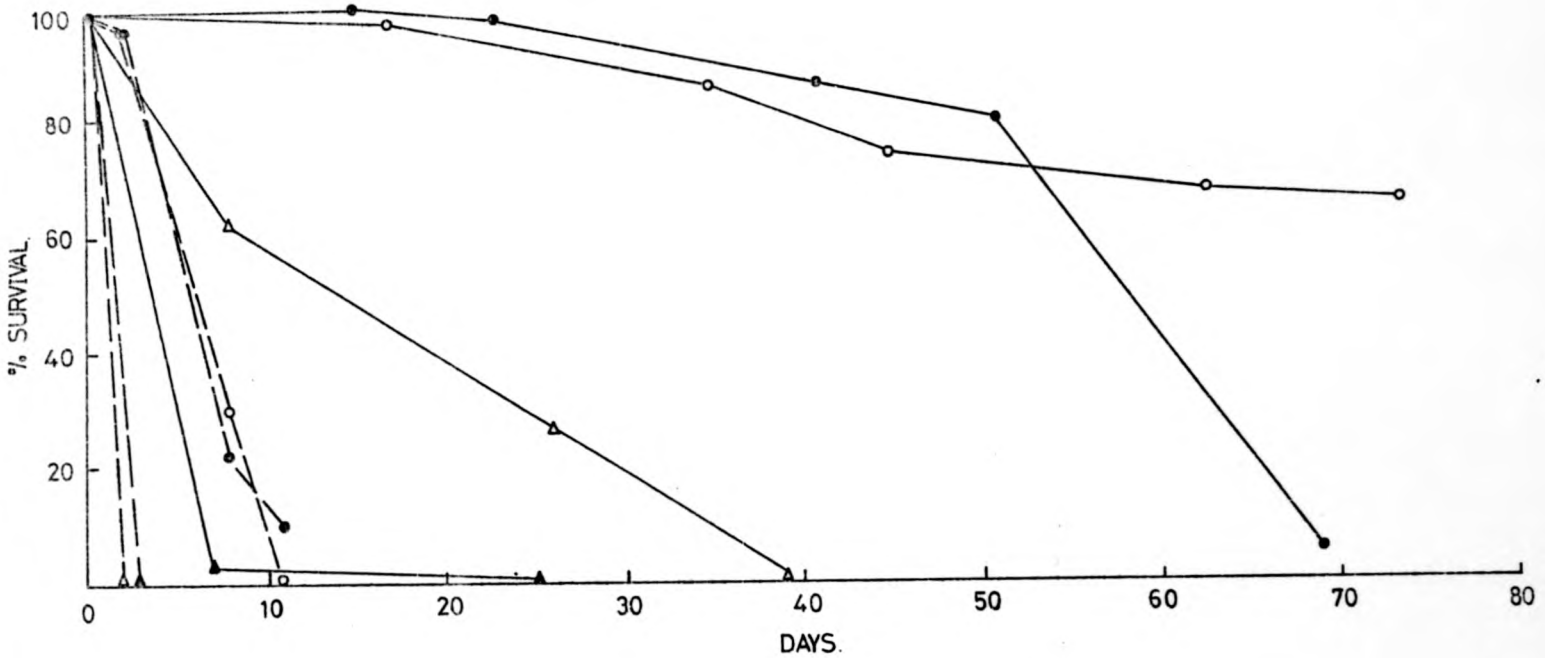
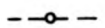


FIGURE 27.

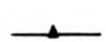
FIGURE 28.

Growth with F.D. and E.F.D. (series two).



F.D. Regression line y = 6.36 - .0054x.

E.F.D. Regression line y = 6.023 + .0002x.



F.D.

Vertical lines represent ± 2 S.E.

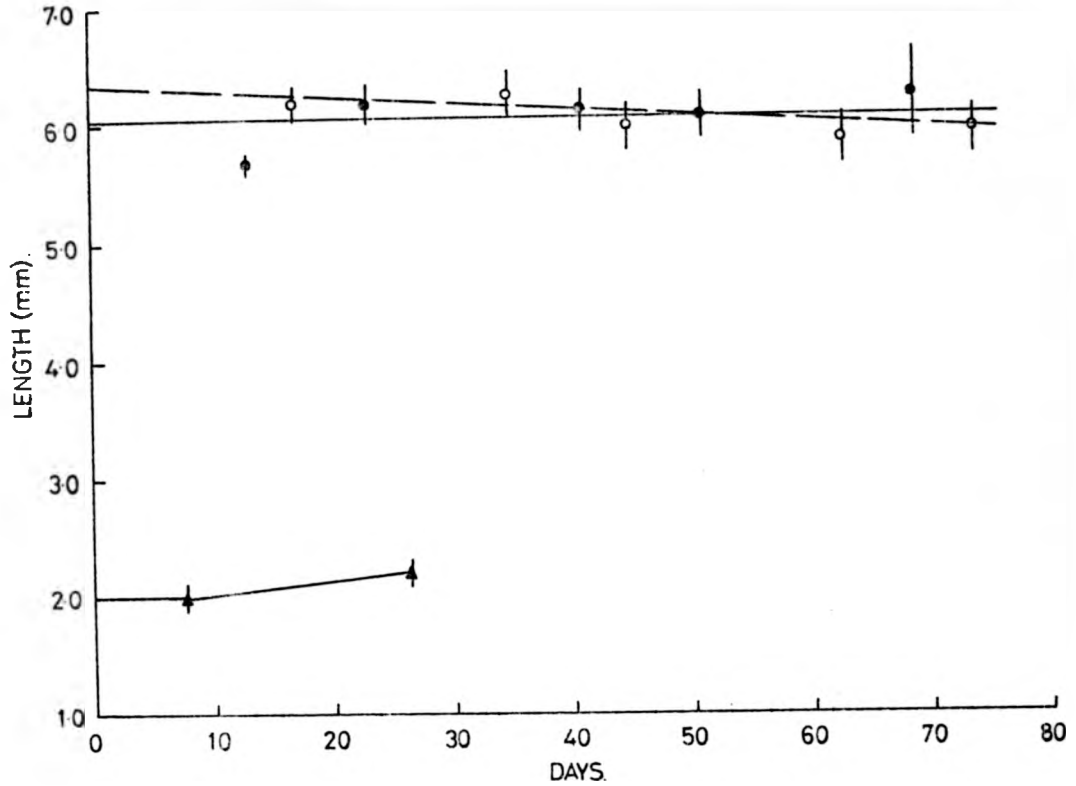


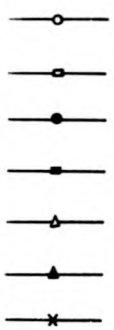
FIGURE 28.

Survival with series 2 leaf diets is shown in figure 29. In spite of several attempts, survival of 2 mm animals beyond 35 days with N.L. could not be achieved. In other experiments not shown in figure 29, 100% mortality occurred after 7 days and 10 days. Rapid mortality of 2 mm animals in normal, enriched and Fincastle burn water controls showed that animals of this size were particularly sensitive to any lack of the appropriate nutrients and it is evident that N.L. could not provide those nutrients. Initial survival was greatly enhanced by feeding N.L. plus protein and was comparable to that achieved with E.L. Addition of protein to E.L. again improved the initial survival slightly. Sudden heavy mortalities occurred in all the populations in normal and enriched water, as had occurred in earlier experiments. Mortality in Fincastle burn water however ceased after initial losses, which were again cut down by the addition of protein. A few of the animals fed Fincastle leaves plus protein also died towards the end of the experiment. The initial growth rate (figure 30) was similar in animals fed E.L., E.L. plus protein and Fincastle leaves plus protein, but was slower in animals fed N.L. plus protein and Fincastle leaves. Growth with both diets in Fincastle burn water continued steadily throughout the experiments but after about 50 days there was no difference in growth rate caused by added protein. Growth in enriched water levelled off as in series 1 experiments, but the series 2 experiments were not continued long enough to determine whether the growth rate would have increased again, as occurred in series 1 experiments.

Rates of mortality and growth evidently varied considerably in the different experiments carried out here with leaf diets. However, if mortality of animals fed these diets is plotted as a function of length (figures 31 and 32), the curves obtained are rather similar. Considered alongside the figures already described these show that the growth and mortality of animals in these experiments may be divided into several phases which were affected to different extents by diet and water chemistry.

FIGURE 29.

Survival with leaf diets (series two).



N.L.

N.L. plus protein.

E.L.

E.L. plus protein.

Fincastle leaves.

Fincastle leaves plus protein.

Control.

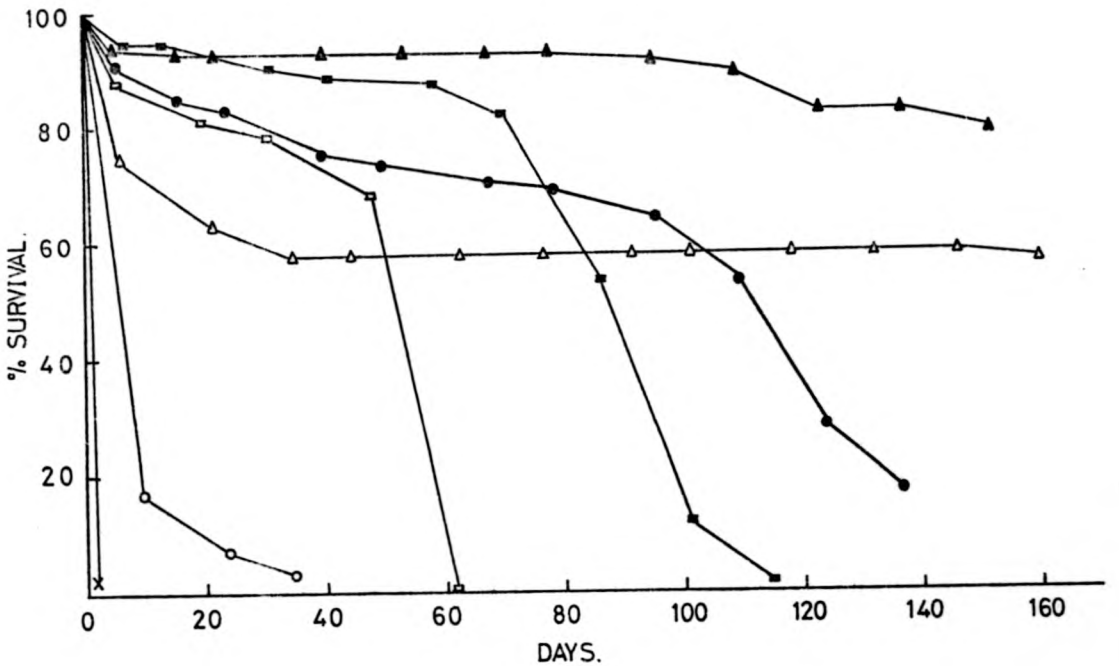
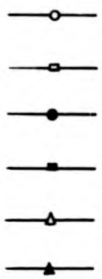


FIGURE 29.

FIGURE 30.

Growth with leaf diets (series two).



N.L.

N.L. plus protein.

E.L.

E.L. plus protein.

Fincastle leaves.

Fincastle leaves plus protein.

Vertical lines represent ± 2 S.E.

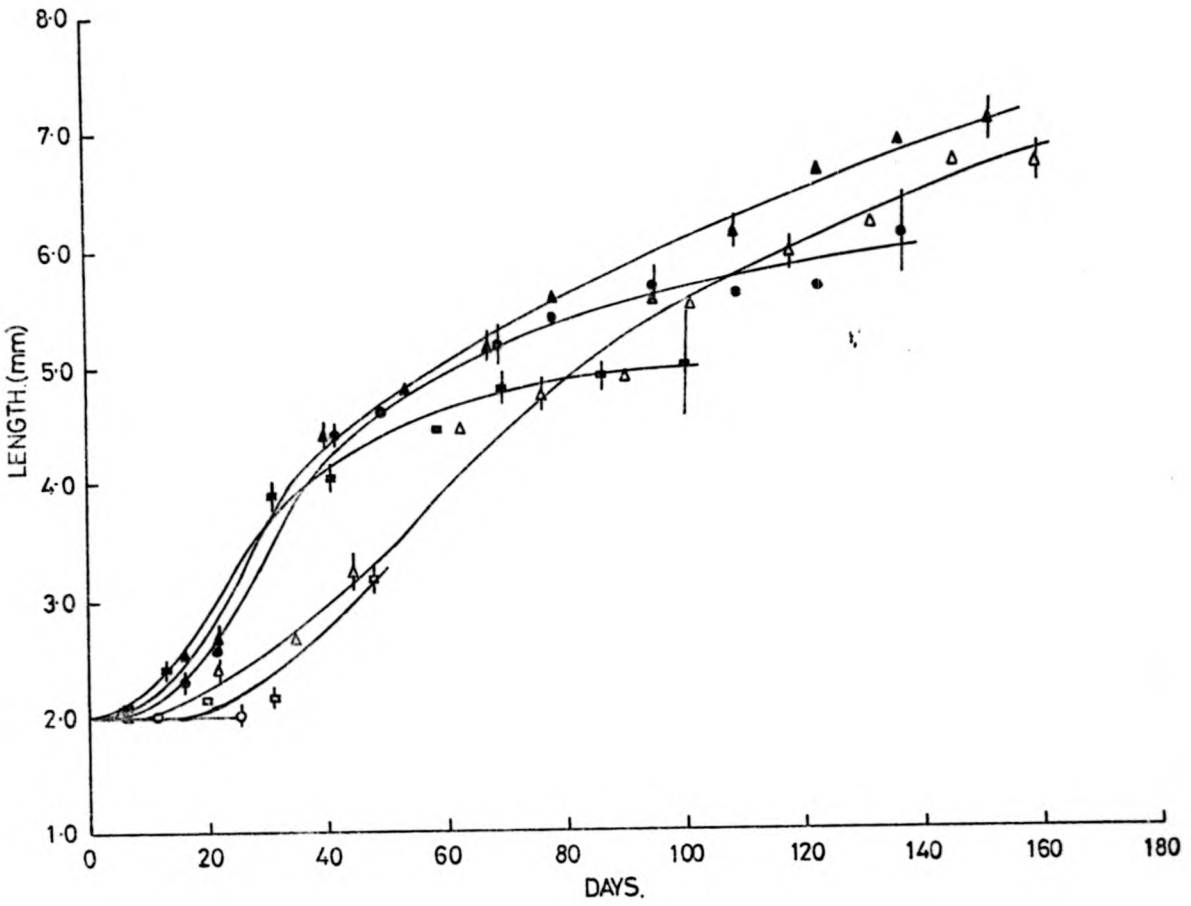

8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
LENGTH.(mm)
0
20
40
60
80
100
120
140
160
180
DAYS.

FIGURE 30.

FIGURE 31.

The relationship between survival and growth (series one leaf diets).

—o— N.L.

—●— E.L.

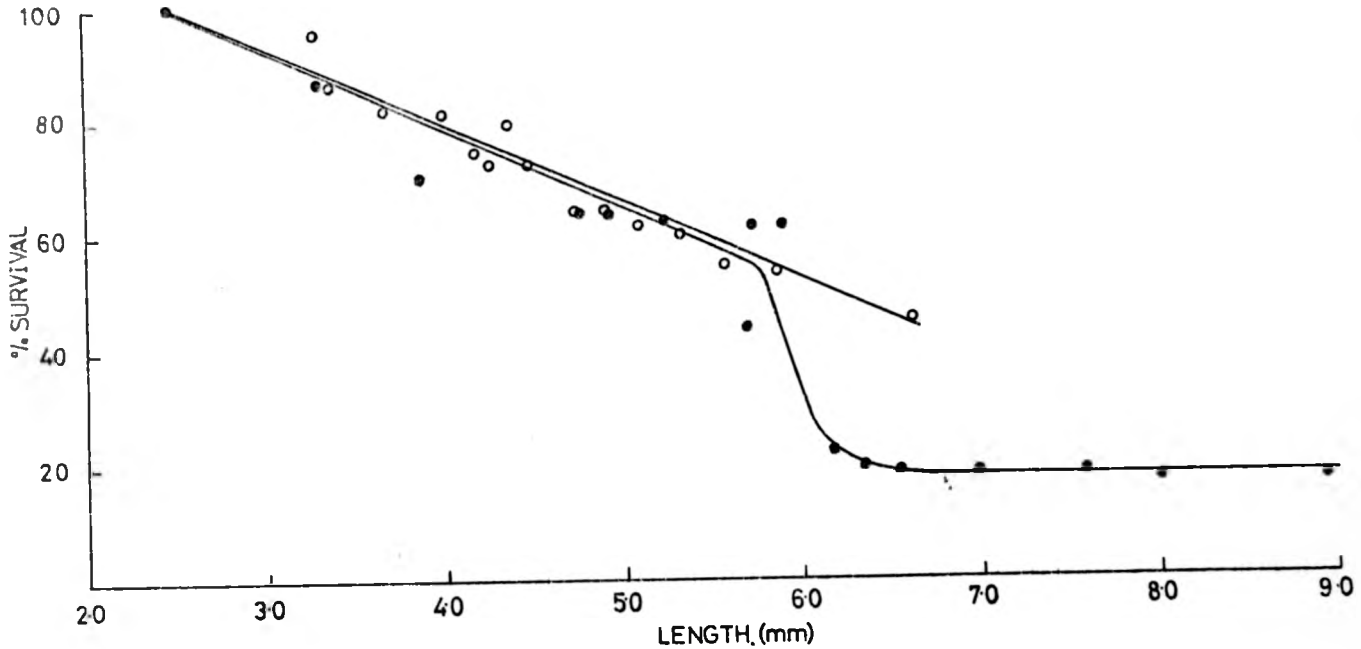
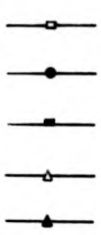


FIGURE 31.

FIGURE 32.

The relationship between survival and growth (series two leaf diets).



N.L. plus protein.

E.L.

E.L. plus protein.

Fincastle leaves.

Fincastle leaves plus protein.

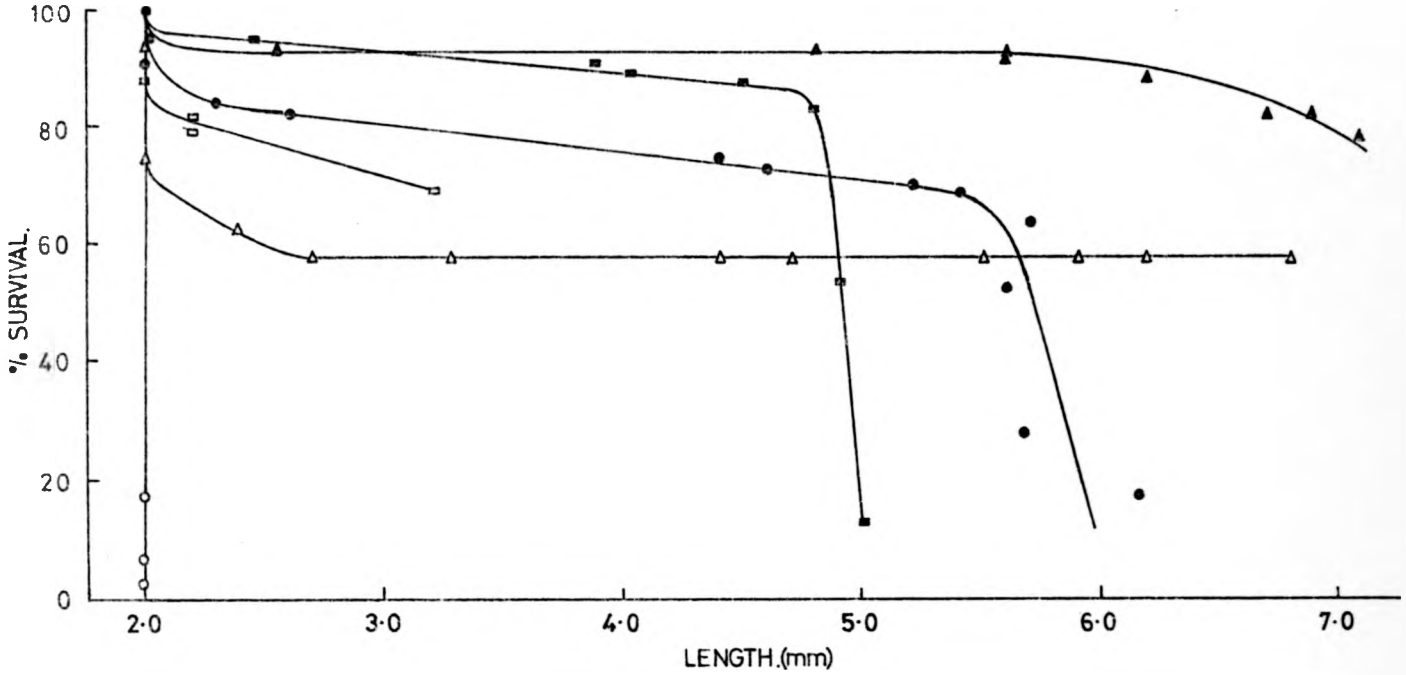


FIGURE 32.

The first phase, studied only in series 2 experiments, consisted of the period in which the animals had not yet reached about 2.5 mm. Rapid mortality occurred at this time (figure 32) and was greatest where the diet was not enriched with protein, either by direct addition or by enrichment of the leaves with microbial protein. Microbial enrichment of N.L. during this period increased survival from 0 to 82%, and addition of protein to N.L. increased survival from 0 to 77%. Simultaneous enrichment and addition of protein increased survival to 94%. Water chemistry evidently had a direct effect on survival at this time as more animals feeding on Fincastle leaves survived (58) than did those feeding on N.L. (0%). Addition of protein to Fincastle leaves increased survival from 58 to 93%. Growth during this period was slow (figure 30) and was mainly affected by the nature of the diet, although water chemistry again had some effect.

The second phase, which lasted until the animals were 4 - 6 mm in length, was characterised by a linear relationship between mortality and growth which was similar in all experiments, except those in Fincastle burn water where there was no mortality. If mortalities are calculated as a percentage of the animals surviving at the beginning of phase 2, the rates of mortality of animals in series 1 N.L. and E.L. and in series 2 E.L. and E.L. plus protein experiments were 14%, 14%, 6% and 4% of the initial number per millimetre increase in length respectively. The growth rate was dependent on the nature of the diet in all cases (figures 24 and 30) during this phase, enriched diets stimulating more rapid growth in both series 1 and 2 experiments.

The third phase occurred when the animals were 4 - 6 millimetres in length. In Fincastle burn water growth continued steadily (figure 30) at a rate which was not affected by the addition of protein to the diet, but in other experiments the growth rate slowed and there was a rapid mortality (figures 24, 30, 31 and 32). In order to determine whether the apparently different growth rates which occurred in series 2 experiments in the laboratory water supply and in Fincastle burn water could have been caused by the death of the larger animals in the laboratory supply, adjusted growth curves have been calculated from the data for

animals fed Fincastle leaves plus protein. The initial growth rate of these animals was similar to that of animals fed E.L. and E.L. plus protein (figure 30), but during phases 2 and 3 the growth rates of those animals consuming the latter diets fell behind. Taking mortality rates from experiments in the laboratory water supply after excluding phase 1 mortalities, the numbers of animals which would have been dead at points on the Fincastle leaves plus protein growth curve were calculated. New mean lengths were then calculated in each case assuming that the animals that died were the largest members of the population. For instance, after 138 days 79.7% of the animals which had survived phase 1 were dead in the E.L. experiment. A similar percentage mortality occurring among the 65 phase 1 survivors of the Fincastle leaves plus protein experiment would have resulted in 13 survivors after 138 days. The population in the Fincastle leaves plus protein experiments after 138 days comprised four 5.5 mm, twenty-nine 6.5 mm, twenty-three 7.5 mm and two 8.5 mm animals, giving a mean length of 6.9 mm. If only the 13 smallest animals had survived the population would have comprised four 5.5 mm and nine 6.5 mm animals, giving a mean length of 6.2 mm. Adjusted growth curves have been calculated in this way from the Fincastle leaves plus protein curve using mortality rates from the E.L. and E.L. plus protein experiments. Mortality rates during phase 2 were calculated on the basis of 6% and 4% mortality of phase 1 survivors per millimetre increase in length respectively, whilse those for phase 3 were calculated using figures directly read off the curves in figure 29. The few mortalities which occurred towards the end of the Fincastle leaves plus protein experiment (figure 29) have been ignored for the purposes of these calculations, the number of survivors being calculated as a percentage of the animals surviving at the end of phase 1, as in the above example. The resulting curves (figures 33 and 34) show that whilst such size specific mortality could fully account for the apparent difference in growth rate between animals fed Fincastle leaves plus protein and those fed E.L., such a mechanism could not fully account for the reduced growth rate of animals fed E.L. plus protein.

FIGURE 33.

Adjusted growth curve for E.L.

—●— E.L.

—▲— Fincastle leaves plus protein.

– –✱– – Adjusted curve.

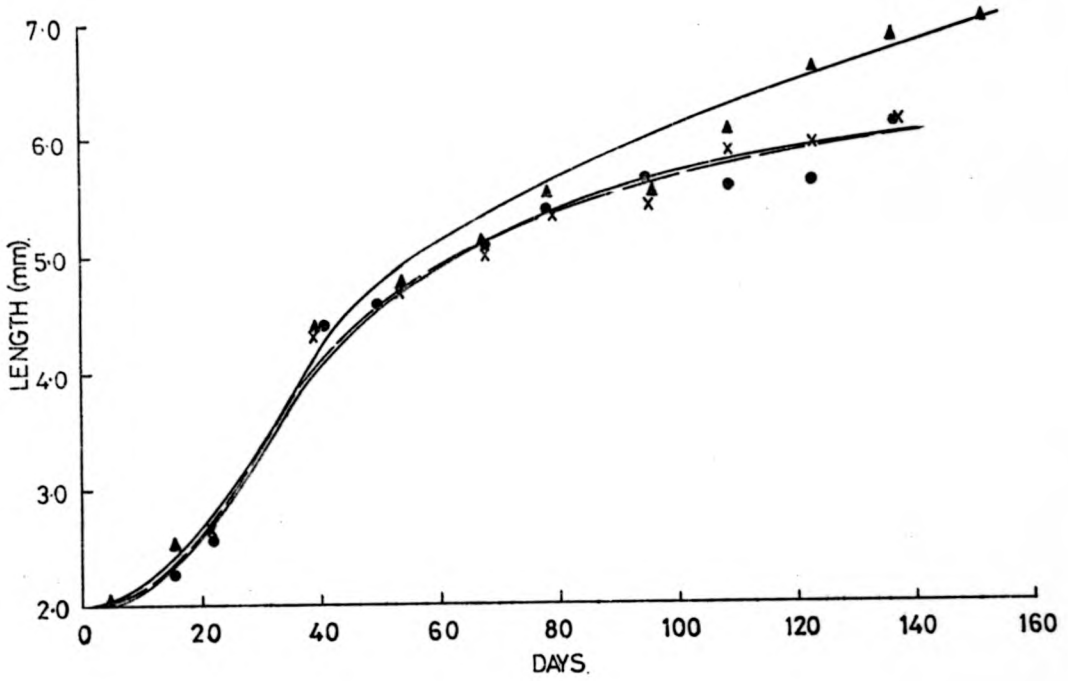


FIGURE 33.

FIGURE 34.

Adjusted growth curve for E.L. plus protein.

—■— E.L. plus protein.

—▲— Fincastle leaves plus protein.

—×— Adjusted curve.

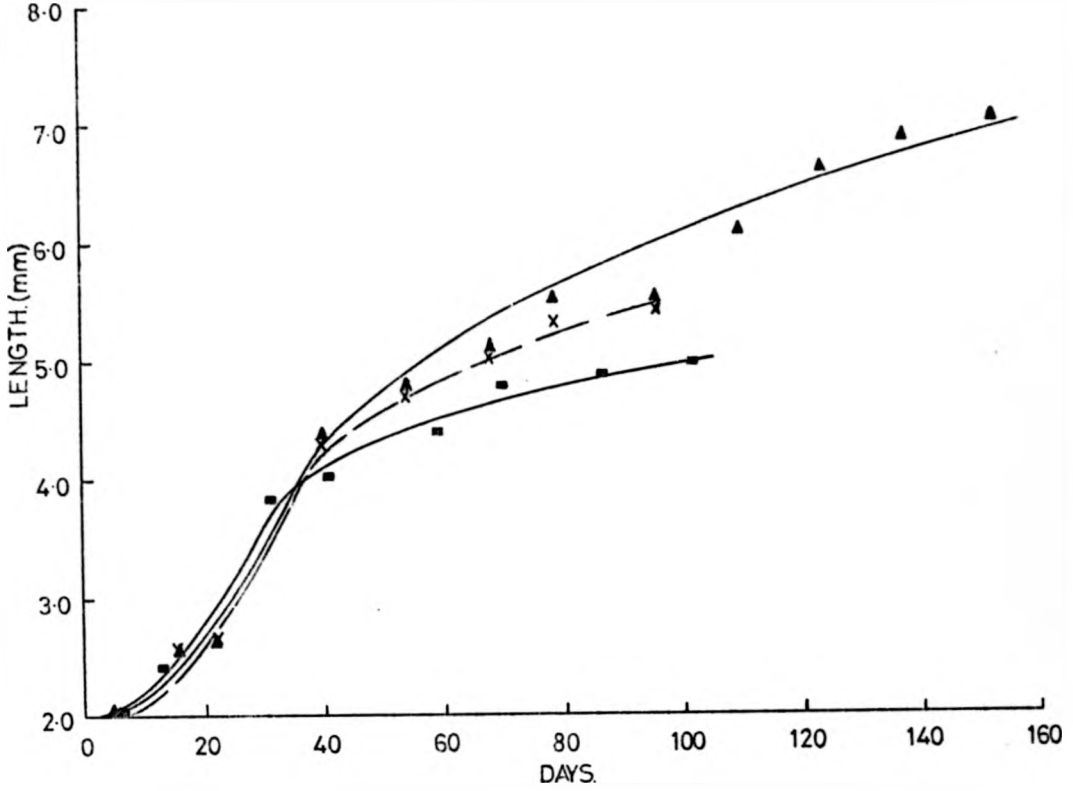


FIGURE 34.

A fourth phase was observed in series 1 experiments when the rate of growth increased considerably. During this phase the growth rate of animals fed N.L. was similar to that of those fed Fincastle leaves and Fincastle leaves plus protein (series 2) (1 mm per 40 days) but less than that of animals fed E.L. (series 1) (1 mm per 25 days).

As the results of experiments described here were thus greatly influenced by the initial size of the animals used, the periods over which the experiments were conducted and the chemistry of the water used, direct comparisons between experimental groups are best made after eliminating as far as possible variations caused by these factors. To do this it is necessary to eliminate the possible effects of high mortality rates upon apparent growth rates, and compare growth rates at a time when they were most affected by diet and least affected by water chemistry. These requirements are best fulfilled by the early part of phase 2 growth. Relative growth rates have been calculated for each experiment over a period of 21 days after the animals reached a mean size of 2.5 mm. Experiments in running water began with animals of 3.5 mm and so could not be included in such a comparison, but the relative growth rates of these animals have been calculated over the first 14 or 21 days of the experiments, and some figures from other experiments calculated over the same period for comparison. Relative growth rates for the 9.0 mm animals fed decomposing cellulose powder have also been calculated, along with comparable growth rates from series 1 experiments.

The relative growth rates, which are shown in Table 35, were calculated according to the formula given by Waldbauer (1968) :-

$$\text{R.G.R.} = \frac{\text{mean final individual weight - mean initial individual weight}}{\text{median weight} \times \text{duration of the experiment.}}$$

Weights were determined from lengths using the curve shown in figure 18. Lengths were determined from the curves shown in figures 20, 22, 24, 26 and 30.

TABLE 35.

Relative Growth Rates during certain 21 day periods

Diet	Initial Length 2.5 mm Mth.	R.G.R.	3.5 mm Mth.	R.G.R.	8-9 mm Mth.	R.G.R.
Decomposing Elm	———		Jan.	.025	———	
Fresh Elm (14 days)	———		Jan.	.022	———	
Decomposing Grass	———		Jan.	.025	———	
Fresh Grass (14 days)	———		Jan.	.025	———	
Decomposing Fine Homog.	Nov.	.042	———		———	
Decomposing Coarse Homog.	Nov.	.023	———		———	
Fresh Fine Homog.	Nov.	.016	———		———	
Fresh Fine Homog.+P	Nov.	.032	———		———	
Series 1.						
E.L.	Nov.	.049	Nov.	.030	May	.017
N.L.	Nov.	.034	Dec.	.016	———	
Silt	Oct.	.008	Dec.	.005	———	
Starved	Oct.	.014	———		———	
Series 2.						
E.L.	June	.059	June	.034	———	
E.L.+ Protein	June	.054	June	.027	———	
Fincastle Leaves	May	.044	June	.032	———	
Fincastle Leaves+P	June	.059	June	.034	———	
Cellulose Powder	———		———		June	.003

Relative Growth Rates for F.D. and E.F.D. = 0.000

c) Discussion

When the growth rates (Table 35) are considered in conjunction with the protein contents given in Table 33 it becomes evident that the nutritive value of the diets used cannot be directly related to their protein contents, and in fact those diets with the highest protein contents (fine detritus diets and decomposing fine homogenate) were not the most nutritive. The failure of coarse detritus, silt, F.D. and E.F.D. to support growth suggests that once leaf litter has become fragmented by decomposition it loses its nutritive value to Gammarus. The great difference in nutritive value between fresh and decomposing material, suggested by preliminary experiments in running water, was confirmed by the leaf homogenate experiments which also suggested that part of the effect of decomposition could be replicated by adding protein. Indeed, the relative growth rates with non-protein supplemented homogenates were in direct proportion to their protein contents. The addition of protein to Fincastle leaves similarly resulted in a considerably increased growth rate. Under certain circumstances however, the presence of excess protein apparently inhibited growth as the addition of protein to E.L. caused a decrease in growth rate. This inhibition was also evident in the latter stages of growth in series 2 experiments in both Fincastle burn and enriched water, where the presence of added protein apparently caused a slight decrease in growth rate.

In numerous studies of invertebrate food requirements, survival and growth have been considered synonymous with food nutritive value. In the aquatic environment however, the possibility of direct uptake of both organic and inorganic substances from the water can not be discounted and may complicate the normally direct relationship between growth, survival and food nutritive value. Freshwater animals must take up inorganic substances from the water in order to maintain the ionic composition of their body fluids and, although the uptake of organic substances is often discounted, many aquatic animals apparently can perform such an uptake. Stevens and Schinske (1961) found that many invertebrates could take up amino acids from solution although arthropods could not, but Lubyano and Zubchenko (1970) found that

Gammarus ballanicus could take up glucose and amino acids, as well as calcium and phosphorus, direct from solution. The survival of animals in the present experiments was evidently largely influenced by the chemistry of the water used for much of the experimental period. During the second phase of growth in the laboratory water supply, mortalities seemed to occur as a result of growth, 4 - 14% of the numbers present after phase 1 dying for each millimetre increase in length. No such mortalities occurred in water from the Fincastle burn and consideration of the chemical composition of the two water supplies (Table 32), and of the literature, suggests that the calcium content of the laboratory supply may have been critical. Schumann (1928) (quoted in Hynes, 1954) showed that Gammarus pulex could not harden its cuticle after moulting in water with a lower calcium concentration than 5.2 mg/litre, which is not far below the figure of 6.4 found here for the laboratory water supply. The calcium concentration of this supply may fluctuate considerably from this figure. Egglishaw (1972) gave a value of 5.7 for the same supply. It seems likely that at each moult in the laboratory supply a small but roughly constant number of animals died, presumably representing those most susceptible to calcium deficiency. For instance, in the first series of experiments with E.L. a female matured at 7.5 mm which, as Sexton (1924) found that 10 moults occur between hatching and maturity, means that about 1.7 moults occurred per millimetre increase in length on average. This suggests that of the original 100 animals about 8 died per moult. As the sudden mortalities of phase 3 only occurred in the laboratory water supply it seems reasonable to suggest that these were also caused by the low calcium concentration in the water, and may have been the result of increased calcium demand incurred at a critical phase of growth, or by a physiological process such as the onset of maturity. Hynes (1954), when carrying out experiments on rearing G.pulex and G.duebeni in various types of water, also noted that when the water was not suitable, animals sometimes died after growing to a length of about 4 mm. If mortalities occurred during or after moulting, the fastest growing individuals would have had the highest probability of dying. This could at least partly account for the apparent decrease in growth rate seen in animals in the laboratory water supply, but not in animals in Fincastle burn water. As is shown in figure 33, the difference between

growth rates observed with Fincastle leaves plus protein and with E.L. could have been completely accounted for if all mortalities with E.L. occurred in the largest members of the population.

Further complications incurred in long term growth experiments with Gammarus may be caused by endogenous seasonal rhythms. Sexton (1928) found that young hatched in the winter months took longer to mature than those hatched in the spring and summer irrespective of temperature. The fluctuations in growth rates observed here during series 1 experiments could have been partially explained by some seasonal rhythmicity, in spite of the constant temperature. This seems particularly likely in the case of N.L. where mortality occurred steadily throughout the experiment and so would have been unlikely to account for the fluctuations in growth rate observed. Series 1 experiments were started in the autumn of 1973 and the growth rate was at a minimum during January and February. The rapid increase in growth rate which occurred towards the end of those experiments began at the end of February and the beginning of March in the cases of E.L. and N.L. respectively.

In spite of the high mortalities observed in some of these experiments, and their possible effects upon apparent growth rates, the animals used here grew at similar rates to those studied by other authors. Hynes (1955) found that animals matured in the field in 90 to 120 days in summer conditions (10 - 15^{o}C), in about 210 days in winter conditions (5 - 10^{o}C), and in 120 days in the laboratory (15 - 20^{o}C). He also calculated from data given by Sexton (1924) that the ten moults to maturity took 180 days in the winter and 70 in the summer. Mottram (1934) considered that 100 days were needed to reach maturity. Taking the length of maturity as 7.5 mm and allowing 20 days for growth to 2.5 mm in series 1 experiments, growth to maturity with N.L., E.L. (series 1), Fincastle leaves and Fincastle leaves plus protein (series 2) would have taken about 270, 200, 190 and 165 days respectively at 10^{o}C. These figures may be overestimates as Hynes (1955) found that many females were mature at 6.5 mm.

The work of several different authors suggests that the nutritive value of plant detritus may be directly dependent on its protein content. Cummins (1973) has pointed out that the calorific content of most foods available to aquatic invertebrates is similar and that the kinds of amino acids available from any proteinaceous food source are probably fairly constant, but that the total protein content of different food sources may be more variable. If it is assumed that the content of vitamins and other trace nutrients does not limit the nutritive value of ingested materials, the protein content of such materials may be critical. The protein requirements of crustaceans are not as well known as are those of insects, but available figures suggest that they may not be fulfilled by the ingestion of plant detritus. Beerstecher et al (1954) found that the best growth of the ispod Oniscus asellus occurred when the protein content of the artificial diet used was 10%, and Andrews et al (1973) found 28-32% protein optimal for growth of shrimps. Castell and Budson (1974) showed that the lobster Homarus americanus required at least 60% casein in an artificial diet in order to ensure maximum survival and minimum weight loss.

As the C : N ratio of micro-organisms is about 10 : 1 to 12 : 1, compared with values for freshly fallen leaf litter between 20 : 1 and 50 : 1 (Burges, 1967) micro-organisms are likely to be a more concentrated source of nitrogen than plant litter. Kostalos (1971), in discussing the results of her study of the nutritive value of various types of microflora for Gammarus minus, concluded that the most probable reason for the high nutritive value of fungi was that they represented a more concentrated protein source than their detrital substrate. Furthermore Iversen (1974) found that the growth rate of larvae of the caddis-fly Sericostoma personatum was linearly related to the nitrogen content of three of the four species of decomposing leaf litter upon which it was fed.

The results of the experiments described here however show that protein content alone can not account for the nutritive value of plant detritus to G.pulex. However, the best growth was achieved with leaf diets on which the development of a luxuriant microflora had been stimulated.

Although several authors have reared Gammarus spp on elm litter alone, (e.g. Sexton, 1928 ; Hynes, 1954), the few observations that have been made concerning the nutritive value of plant detritus to amphipods tend to confirm that leaf litter is a rather inadequate food source unless enriched with a luxuriant microbial fauna or supplemented with other protein rich foods. This conclusion is substantiated by the finding of Mathews (1967) that the nitrogen production in a population of G.pulex was greater than that available from ingested leaf litter. Clemens (1950) found that there was a high mortality of Gammarus fasciatus during the first moult if they were fed elm leaves, but when fed elm leaves plus yeast survival was greatly increased. Those findings are similar to the situation found here where N.L. would not support the survival of animals smaller than 2.5 mm, but enrichment or the addition of protein greatly decreased mortality. Le Roux (1933) fed Gammarus duebeni with a mixed diet of leaf litter and earthworms as such a diet gave better growth than either component alone, growth with leaves alone being particularly slow. Clemens (1950) found the best food for adult G.fasciatus was elm leaves plus dog food. No observations seem to have been made concerning the nutritive value to amphipods of plant detritus in a more advanced state of decay than intact leaf litter, but Hargrave (1970a) found that Hyallela azteca grew and survived less well when consuming the surface sediment upon which it normally fed, than when consuming the epiphytic growth on Chara. Wilcox and Jeffries (1974) found that the decapod Crangon septemspinosa grew when fed particulate detritus derived from marsh grass, but did so more rapidly when fed various animal tissues.

A comparable situation to the feeding of amphipods upon plant detritus is the feeding of decapod larvae upon unicellular algae, which has been studied considerably. Broad (1957), Regnault (1969) and Roberts (1974) all found that algae were of little or no nutritive value to different species of decapod larvae, and all found that the diet was much improved by the addition of animal material. In many cases this may have been the result of their inability to digest the algae as Gibor (1956) found that the nutritive value of 3 species of unicellular algae to Artemia depended upon the thickness of their cell walls. The possibility

that the small percentage of animal material that almost inevitably forms part of the diet of Gammarus is necessary to furnish protein enrichment of the diet in the field cannot be overlooked.

It is evident that regarding the protein content of detritus as the sole mediator of its nutritive value for Gammarus is an over-simplification which ignores other factors which affect its suitability as a food, e.g. its digestibility, its palatability, the physical ease with which it may be ingested, its ash content, and the type of micro-flora which it supports. From the results given in this Chapter and in Chapter 5 there is no reason to believe that digestibility is an important factor in determining detrital nutritive value. F.D. and E.F.D., in spite of being more digestible than elm litter and having a higher protein content, were of much less nutritive value than leaf diets. As discussed in Chapter 5 the rate of ingestion of a diet depends both on its palatability and the physical ease with which it can be ingested. Coarse and fine detritus, silt and decomposing elm leaf homogenates, were probably all more easily ingested than, and equally palatable to, leaf diets, but were generally of less nutritive value than those diets. The ash contents of the diets can be better correlated than any of the above factors with their nutritive value, as those diets with the highest ash contents, i.e. silt, F.D. and E.F.D., were also of least nutritive value. Although dietary ash content cannot account for differences in growth rates between animals fed decomposing fine and coarse homogenates, or between N.L. and E.L., its possible importance cannot be ignored.

Taking into account the above considerations it seems an inescapable conclusion that the rate of growth of animals fed non protein supplemented diets was mainly the result of the suitability of the detritus concerned as a substrate for microbial growth, nutritive value depending both upon the extent and the type of microflora. Several authors have studied the suitability of various types of micro-organisms as food for crustaceans and most have found that their nutritive value is considerable, although Lubyano and Zubchenko (1970) considered that micro-organisms were of no nutritive value to Gammarus ballanicus but were important only in the maceration of leaf litter before its ingestion.

Of those authors who have looked at the nutritive value of bacteria, most have found them to be of limited importance. Zobell and Feltham (1938) found that bacteria, when fed to the sand crab Emerita analoga, supported growth and survival to a limited extent, but were of more nutritive value to mussels. Zhukova (1963) calculated that Pontogammarus maetoticus could get only 1.2% of its total nitrogen needs from bacteria passing through its gut, although other invertebrates could get a higher proportion. Kostalos (1971) studied the survival of Gammarus minus and found that diets of pure bacteria or elm leaves with a bacterial flora were of more nutritive value than diets of sterile leaf litter, but were of less value than diets of elm litter with a natural microbial population or a fungal microflora, or than a diet of pure fungi. Wilcox and Jeffries (1974) found a pure bacterial diet to be of less nutritive value to the shrimp Crangon septemspinosa than several animal diets. However Rodina (1963) grew Daphnia magna through several generations with a detrital diet which had a bacterial microflora, but found that sterile detritus would not support normal development and reproduction.

The increased protein content of leaf diets stimulated here by inorganic enrichment was undoubtably the result of fungal growth, and several authors have found that fungi have a high nutritive value to crustaceans. Kostalos (1971) found that leaves with a fungal flora but a reduced bacterial population, leaves with a unifungal population, or fungal colonies alone, supported better survival of Gammarus minus than leaves with a natural microbial population. Barlocher and Kendrick (1973) found that eight out of ten species of fungi supported growth of G.pseudolimnaeus and were more efficiently utilised than sterile elm or maple leaf discs. The marine wood borer Limnoria could not survive long with a diet of sterile wood, but could do so if the wood was infested by a living fungus, although some component of the wood was apparently important as survival with a pure fungus was shorter than that with infested wood (Becker, 1959). The amino acid contents of Limnoria, sterile wood and a marine fungus also suggested that fungi were important in furnishing the protein requirements of Limnoria (Lane, 1959).

It seems that the nutritive values of the diets used here were influenced by the relative importance of the bacterial and fungal microfloras, and by the species of microflora concerned. During the early stages of growth, the great importance of fungi was evidently based upon their suitability as a protein source for G.pulex. Apart from the work of Barlocher and Kendrick (1973) there seem to have been no comparative studies of the nutritive values of different fungal species to aquatic crustaceans, and that approach may be more productive than the 'protein content' approach adopted here. A study of the nutritive value of 23 fungal taxa to 3 species of Cecidomyidae (gall midges) was carried out by Nikolei (1961) who found that relatively few were satisfactory food sources.

In view of the varying nutritive values of different groups and species of micro-organisms it may be that the presence of certain trace nutrients or essential amino acids is the critical growth stimulating factor, rather than the general increase in protein availability. The considerable extent to which adding pure protein to whole elm leaves and leaf homogenates could replace the nutritional benefits of an enhanced fungal flora in the critical early stages of growth in G.pulex suggested that, at least during that stage, specific amino acid requirements may have been the more important. The importance of vitamins and other trace nutrients may be considerable however as Rodina (1963) found that the nutritive value of bacteria for Daphnia magna could be partly replaced by B vitamins.

CHAPTER 8.

DIGESTION OF PLANT DETRITUS BY COMMERCIAL ENZYME PREPARATIONS

a) Methods

Elm litter was collected during leaf fall in the autumn of 1972 and stored dry. At monthly intervals between November 1972 and May, 1973 about 10 grams of leaves were placed in nylon mesh bags (38 meshes per inch), which were in turn placed in the Fincastle burn. All the bags were removed from the stream in June, 1973. The leaves contained therein, along with fresh leaves which had been soaked in running water for 24 hours, were stored deep frozen until required as substrates in enzymic digestion experiments. Sufficient material remained in the first bag placed in the stream for only one experiment.

A single experiment was also carried out using fine detritus from the Fincastle burn. This was collected in a hand net in January, 1973 and sorted into fractions of differing particle size by washing through a set of Endecott sieves and a piece of nylon mesh. This resulted in fractions with median particle diameters of 1315μ, 525μ, 332μ, 210μ and 144μ, which were stored at 0^{o}C until required.

In order to approximate conditions in the digestive tract, a small volume of concentrated enzyme solution was incubated with each substrate for a short period. The enzymes used were cellulases of fungal origin (B.D.H. and Sigma Chemical Co.), amylase of bacterial origin (Sigma) and protease of fungal origin (Sigma). All incubates were buffered at the optimal pH quoted by the manufacturers. Preliminary experiments had suggested that the uptake of digestive products by micro-organisms might be a considerable problem which might not be entirely overcome by the addition of toluene. As well as a drop of toluene, antifungal and antibacterial antibiotics were therefore added to the incubation mixtures. These were penicillin (120μg/tube), streptomycin (120μg/tube), cycloheximine (200μg/tube) and nystatin (200μg/tube).

Fine detritus was incubated with B.D.H. cellulase only. Roughly equal amounts of detritus were placed in 10 x 77 mm pyrex glass tubes with 0.5 ml of pH 4.3 buffer and 0.5 ml of 0.66% w/v cellulase dissolved in Ringer, plus antibiotics and toluene. Eight replicate

tubes were prepared for each batch of detritus. After 14 hours incubation, 0.6 ml of solution was withdrawn and assayed for reducing sugars.

Leaf substrates were ground into a thick paste with a glass rod in a solid watchglass before incubation. The size distribution of particles in the paste was similar to that in the tract of Gammarus, although there were more large particles up to 900 µ in length. When sufficient paste had been prepared pieces between 30 and 40 mg were weighed and placed in tubes. After all reagents had been added the contents of the tubes were thoroughly mixed by holding the tubes against a rapidly revolving glass rod.

Leaf incubates with Sigma cellulase contained 125 µl of 33% w/v cellulase made up in 2:1 Ringer : buffer solution (pH 4.5). Three replicate tubes were used for each batch of leaves and after 3 hours incubation 2 10 µl samples were withdrawn for reducing sugar analysis. In amylase incubates 125 µl of 1.4% amylase in 2 : 1 Ringer : buffer (pH 7.0) was added to each tube. Two replicate tubes were prepared for each batch of leaves and 20 µl samples were withdrawn for reducing sugar analysis after 0.5, 1.5 and 3.0 hours incubation. Protease incubations were carried out similarly but the protease solution was buffered at pH 7.5 and used at two concentrations, 3.3% w/v and 12% w/v. In different experiments analyses were carried out for T.C.A. soluble substances and total amino acids. The latter were analysed by the method of Mutting and Kaiser (1963). Heating time was reduced from 10 to 3 minutes as optical density was greatest after this time with the reduced sample volume used. As amino acid assay results were not particularly reproducible a glycine standard (100 µg) was run with each batch of assays. Samples for analysis from all leaf incubates were withdrawn from the incubation mixture with a 10 µl micropipette through fine nylon mesh, which prevented the pipette from being blocked.

In most cases control tubes contained boiled enzyme solution. However, in one case (the leaf incubate with the higher protease concentration assayed for amino acids) control values obtained in this way were higher than experimental values. Analyses of the amino acid content of the enzyme solution when active, and after denaturation by

boiling or addition of T.C.A. showed that denaturation caused a 33% increase. Accordingly, true control values for amino acid assays were taken as 75% of the experimentally determined control values. In cellulase/leaf incubatas control samples were withdrawn from the experimental tubes at the beginning of the incubation period.

b) Results

The amount of reducing sugars produced from fine detritus by cellulase digestion apparently decreased with increasing particle size. As it was thought likely that the surface area of the particles may have been a critical factor, reducing sugars formed were plotted against the reciprocal of the square of the median particle diameter (figure 35). The regression coefficient was not quite significantly different from 0 at the 5% level ($t = 2.00$ $P = .05 - .1$). Assuming an average of about 5 mg dry weight of detritus per tube, in the region of 0.5% of this was converted to reducing sugars.

There was no apparent change in the extent of digestion of leaf substrates by cellulase with increasing time of leaf decomposition (figure 36). The regression coefficient was not significantly different from 0 ($t = 0.26$ $P = \gg .05$). Assuming a water content of 75%, between 1 and 4% of the dry weight of the leaf tissue was converted to reducing sugars.

Digestion of leaf substrates by amylase was complete after 0.5 hours in most tubes. The results of analyses of samples withdrawn after different periods were therefore lumped together to obtain a mean value. Although the amounts of reducing sugars produced did vary with time of leaf decomposition (figure 37) the relationship was irregular. Between 0.1 and 0.5% of the dry weight of the leaves appeared as reducing sugars.

Digestion by protease was also complete after 0.5 hours in most cases when TCA soluble substances were assayed. After this time, in the case of the lower protease concentration (Table 36), most T.C.A. soluble substances had been released from leaves decomposed for two months (about 3.4% of the leaf dry weight). In leaves decomposed for more than two months the amount of digestive products apparently declined slightly. Results from the higher protease concentration were very erratic and did not confirm this result. They suggested that up to 8% of the leaf dry weight was converted to T.C.A. soluble substances. When amino acids were assayed there was generally an increase up to 3 hours incubation. In most cases only very small amounts of amino acids were detected in the experiment with the lower protease concentration, and the results were again erratic. The results of incubations with the

lower protease concentration after 3 hours, and of those with the higher concentration after 0.5, 1.5 and 3 hours are shown in Table 37.. These results confirm those obtained with T.C.A. soluble substance assays. There was a peak in the amount of digestive products released after two months decomposition. The amounts of amino acids released represented about 0 - 1.6% of the leaf dry weight.

FIGURE 35.

The digestion of fine detritus by a commercial cellulase, as a function of particle size.

Regression line $y = 21.38 + 27.99x$.

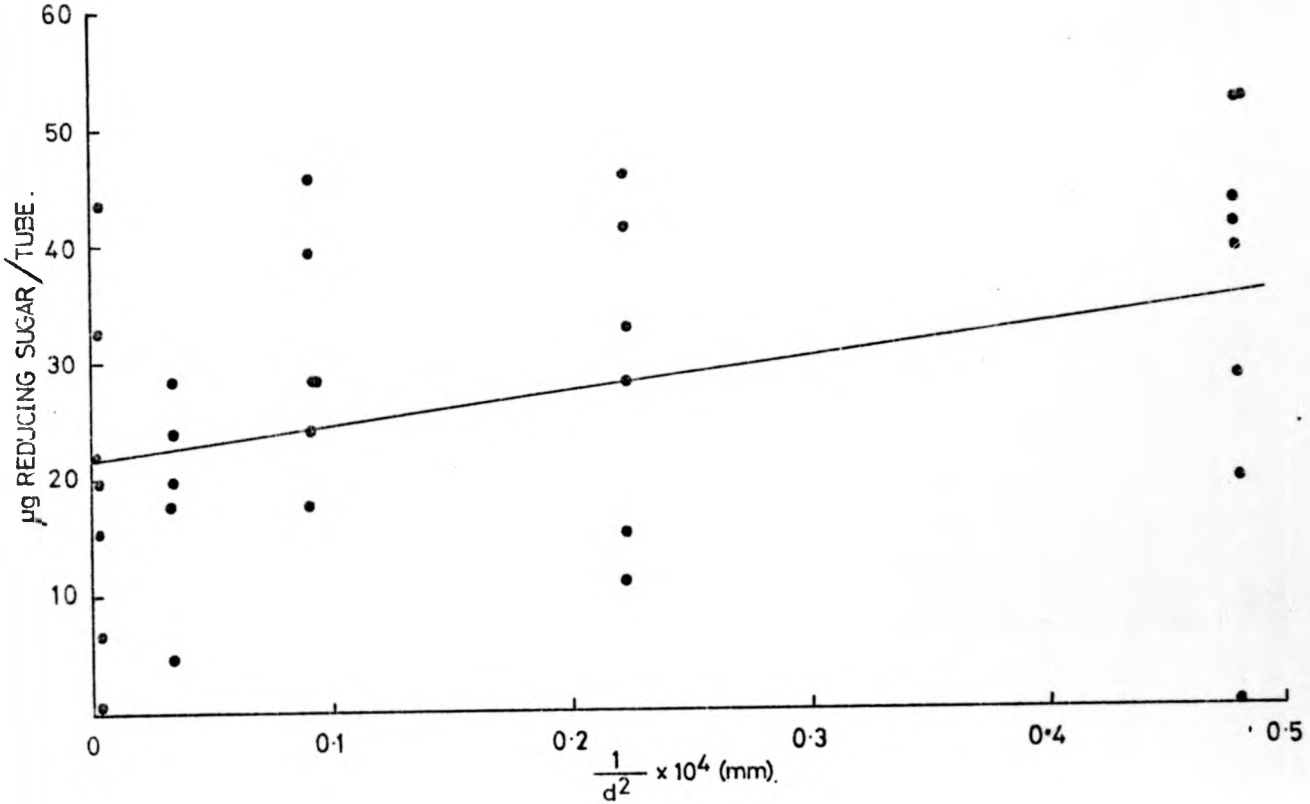

60
50
40
30
20
10
µg REDUCING SUGAR / TUBE.
0
0·1
0·2
0·3
0·4
0·5
$\frac{1}{d^2} \times 10^4$ (mm).

FIGURE 35.

FIGURE 36.

The digestion of decomposing elm leaves by a commercial cellulase.

Regression line $y = 552.5 - 6.56x$.

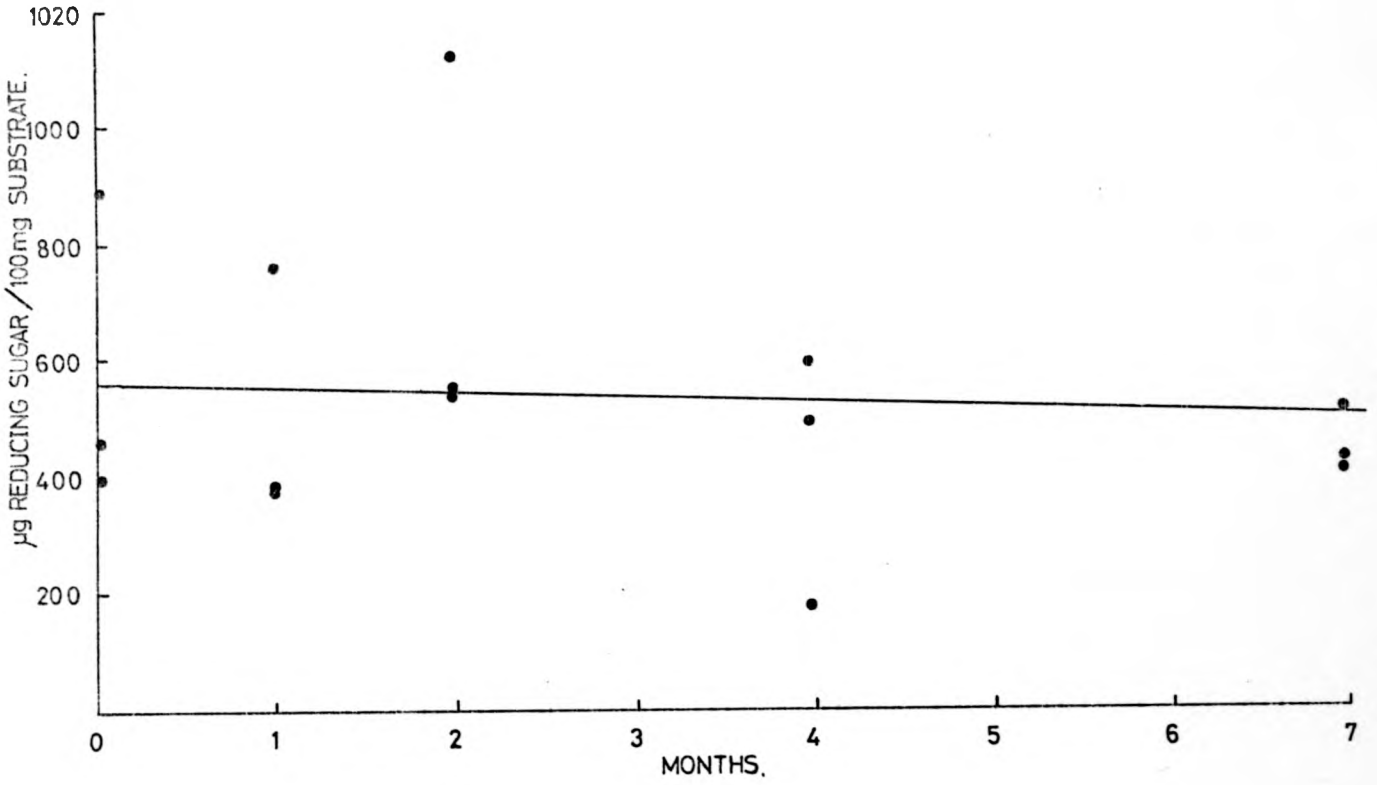

1020
1000
800
600
400
200
µg REDUCING SUGAR / 100mg SUBSTRATE.
0
1
2
3
4
5
6
7
MONTHS.

FIGURE 36.

FIGURE 37.

The digestion of decomposing leaves by a commercial amylase.

Vertical lines represent ± 1 S.E.

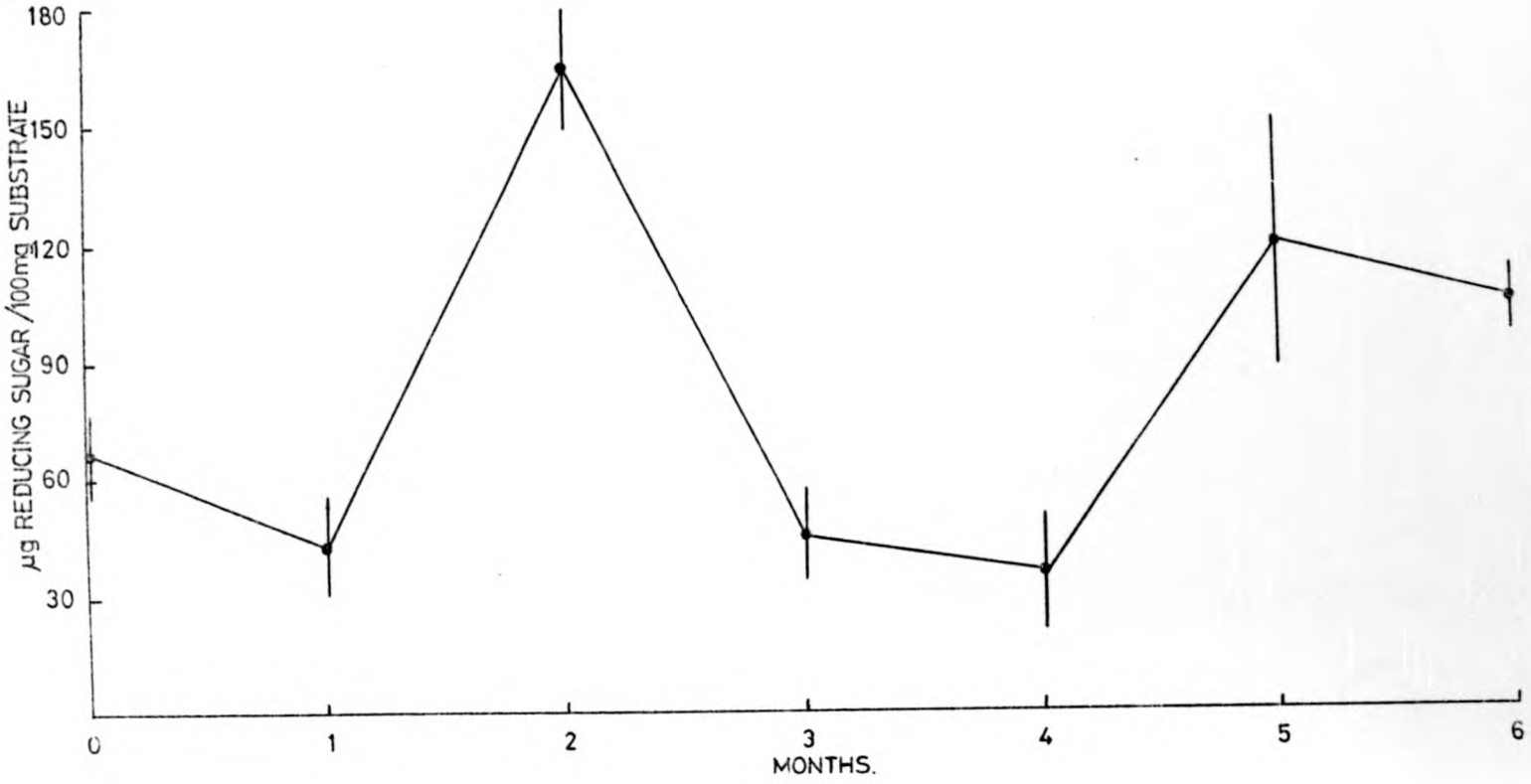

180
150
120
90
60
30
µg REDUCING SUGAR /100mg SUBSTRATE
0
1
2
3
4
5
6
MONTHS.

FIGURE 37.

TABLE 36.

Release of T.C.A. Soluble Substances by Protease Digestion

Protease conc.		3.3% W/V			12% W/V		
Replicate No.	Months Decomp.	Wt Leaf (mg)	Wt T.S. A.A.	Wt/ 100 mg Leaf	Wt Leaf (mg)	Wt T.S. A.A.	Wt/ 100 mg Leaf
1.	0	32	24	521	39	119	2110
2.	0	38	20	356	35	30	595
$\bar{x}$				439			1353
1.	1	45	29	440	40	92	1588
2.	1	44	24	379	39	78	1388
$\bar{x}$				410			1488
1.	2	36	42	810	30	60	1388
2.	2	31	41	907	34	38	766
$\bar{x}$				859			1077
1.	3	37	39	732	31	90	2016
2.	3	35	23	456	35	17	327
$\bar{x}$				594			1072
1.	4	40	17	287	44	0	0
2.	4	32	33	716	32	23	488
$\bar{x}$				502			244
1.	5	42	30	496	31	66	1478
2.	5	39	23	410	42	62	1017
$\bar{x}$				453			1248
1.	6	38	32	576	36	56	1071
2.	6	31	30	672	35	47	923
$\bar{x}$				624			997

T.S.A.A. = T.C.A. soluble substances assayed per aliquot.

TABLE 37.

Release of Amino Acids by Protease Disgestion

(μg A.A./100 mg Substrate)

Protease Conc		3.3% W/V		12% W/V	
Incubation Time		3 hrs.	0.5 hrs.	1.5 hrs.	3 hrs.
Replicate No.	Months Decomposition				
1.	0	39	118	71	402
2.	0	0	0	208	174
$\bar{x}$		20	59	140	288
1.	1	227	201	183	354
2.	1	45	98	253	283
$\bar{x}$		136	149	218	318
1.	2	297	289	351	402
2.	2	170	192	269	356
$\bar{x}$		233	240	310	379
1.	3	202	152	152	228
2.	3	144	180	57	388
$\bar{x}$		173	166	104	308
1.	4	136	108	221	389
2.	4	100	183	131	300
$\bar{x}$		118	146	176	344
1.	5	193	78	19	179
2.	5	316	0	94	242
$\bar{x}$		254	39	56	211
1.	6	283	154	154	233
2.	6	244	81	155	129
$\bar{x}$		264	118	155	181

c) Discussion

The general increase in the extent of fine detritus digestion by cellulase with decreasing particle diameter is interesting in view of results already described in Chapters 4 and 5. It was suggested in those chapters that the greater efficiency of assimilation of fine detritus than of leaves was the result of previous decomposition of fine detritus by micro-organisms, and the resultant increase in the area available for enzymic digestion. The results described in this chapter confirm that available surface area may be an important factor in determining the extent of digestion of a given diet. The relatively high efficiency with which cellulose powder was assimilated chapter 5 was also probably a result of such a surface area effect as the particle diameter of the powder was only 15 to 40 μ, which is considerably less than that of the bulk of particles in the tract when feeding on N.L. or F.D. (Table 11).

Little attention has been paid to changes in the carbohydrate content of decomposing leaves but Odum and de la Cruz (1967) showed that during decomposition the crude fibre content of Spartina initially declined, and then rose slightly. Their results were based on decreasing particle size rather than on increasing time of decomposition. The roughly constant level of cellulose digestion of elm leaves found here was obtained using pieces of intact leaf which were retained in the mesh bags. Any change in the extent of cellulose digestion would probably have been associated with fragmentation of the leaves into fine particles which would have been lost from the mesh bags. At the end of the incubation period only a small proportion of the leaf dry weight had appeared as reducing sugars. Digestion was probably incomplete however and a much longer incubation period would have been needed to hydrolyse a significant proportion of the cellulose present. The importance of long gut transit times in ensuring efficient cellulose digestion has been discussed by Tracey (1959), and the variation in gut transit times occurring in amphipods was discussed in Chapter 4. It seems likely that this factor, and the surface area factor discussed above, may be the most important determinants of the efficiency of utilisation of dietary cellulose.

Jensen (1974) states that it is difficult to detect appreciable quantities of starch in leaf litter as it is rapidly decomposed. Some reducing sugars were released as a result of amylase digestion in the present experiments but, although digestion was complete, the total amount of reducing sugars released was slight.

The results described here of protease incubations, although rather erratic in places, are generally confirmed by those described in the literature derived from normal methods of chemical analysis. They suggest that more protein would be available for absorbtion to an animal ingesting elm litter which had been decomposing for two months, than to one ingesting fresh leaf litter. Similarly Mathews and Kowalczewski (1969) found a peak in the nitrogen content of various species of leaves after 3 - 4 months decomposition in the River Thames, and Kaushik and Hynes (1971) found such a peak after 3 - 6 months using leaves decomposing in two streams.

CHAPTER 9

OVERALL DISCUSSION

a) Energy Budget

Although the data presented here were not collected for the purpose of determining an energy budget, it is of value to calculate such a budget. This may give a further insight into the utilisation of ingested materials and allow quantitative comparison with other species which have known differences in feeding habits or digestive physiology, and with data published recently for G.pulex (Nilsson, 1974). Budgets have been calculated here for E.L. and F.D. As no differences in ingestion, assimilation or growth have been determined between F.D. and E.F.D., the energy budgets for these two diets may be assumed to be similar. A complete energy budget for N.L. was not calculated because of the lack of an estimate of assimilation.

The relationship between the various parameters of the energy budget may be expressed as $I = A + E$ and $A = P + R + S$ where I = ingestion, A = assimilation, E = egestion, P = production, R = respiration and S = soluble excretory products. From the data previously presented, I, A and E are known for animals of around 2.75 mg dry weight (8 mm length). That part of P made up by growth can be determined for 2.75 mg animals from Chapter 7, but no estimates for losses due to moulting and reproduction are available. For the purposes of the present calculations these have been neglected. Calorific values have been estimated from the literature. Kaushik and Hynes (1971) gave calorific values for elm litter after 4, 6 and 8 weeks decomposition in normal and nutrient enriched water. Mean values calculated from their data give figures of 4,628 and 4,905 cal/gm for N.L. and E.L. respectively. Nilsson (1974) showed that a seasonal fluctuation in the caloric content of G.pulex occurred, and the figure used here, 5,250 cal/gm ash free dry weight, was derived from the approximate mean value he gives for males and females during May, at which time the animals in the appropriate growth experiments have reached 8 mm. This value was converted to 3,937 calories/gram, assuming a mean ash content of 25%, which was derived from data given for a number of gammarids by Cummins and Wuycheck (1971).

An estimation of growth rate at 8 mm was derived from series 1 experiments with E.L. (figure 24) by drawing a straight line through the last five points on that curve, which gave a value of 1 mm increase in length every 25 days. It is less easy to extrapolate a figure for growth with N.L. from figure 24 as the experiment terminated when the animals were 6.6 mm in length, at which time the growth rate appeared to be increasing. However, reference to figure 30 shows that in Fincastle burn water, where there was no mortality, the length/time curve for growth with Fincastle leaves was approximately straight, and of equal slope to a straight line drawn through the last five points in figure 24, representing an increase in length of 1 mm every 40 days. The growth rate for N.L. was therefore derived from this line. Growth with F.D., derived from figure 28, was taken as 0. Lengths were converted into dry weight at 55^{o}C using the curve shown in figure 18.

Ingestion figures were taken from Table 23. These were the experiments in which the food source was most freely available and the ingestion rates highest. Assimilation of calories was assumed to be equal to assimilation of organic matter, which is only true if the calorific value of food and faeces is similar. Hargrave (1971) could not determine the calorific value of faeces of Hyallela azteca because of their rapid colonisation by micro-organisms, so assumed it to be equal to that of the food. Nilsson (1974) showed that the energy contents of ingested alder and beech leaves and of the resultant faeces varied, but the faeces in his experiments had stood for two days, in which time considerable microbial colonisation and weight change may have occurred. Overall, the assumption that the caloric content of food and faeces were similar seems a reasonable approximation.

An energy budget for 8 mm animals, based on the above considerations, is shown in Table 38, along with energy budgets for other aquatic herbivores and detritivores. The respiration figures, which were derived from the other data by difference, can be compared with values given for the respiratory rate of G.pulex in the literature if the R.Q., and thus the oxycalorific value, can be estimated and soluble excretory products are assumed to be negligible. Martin (1965) has shown that assimilated carbohydrates are not normally stored as

TABLE 38.

Energy Budgets of Various Invertebrates (cal/g/day)

Species	Author	Food	Temp.	Ingestion %	Ingestion Cal.	Assimilation %	Assimilation Cal.	Growth %	Growth Cal.	Respiration %	Respiration Cal.	Net Growth Efficiency (%)	Egestion %	Egestion Cal.
G.pulex	Present work	Enriched leaves	10°C	100	789	17.2	136	6.9	55	10.3	81	40.2	82.8	653
"	"	Fine Detritus	10°C	100	589	33.0	197	0.0	0	33.0	197	0.0	67.0	393
"	Nilsson (1974)	Alder Leaves	10°C	100	1,300	38.0 (9.0)[1]	400 (117)[1]		238[2]					
								3.8	50[3]	13.8	179	42.7	91.0	900 (1183)[1]
"	"	Beech Leaves	10°C	100	188	(18.0)[1]	(34)[1]						82.0	(154)[1]
Hyallela azteca	Hargrave (1971)	Sediment	15°C	100	1,800	15.0	270	2.3	41	12.7	230	15.2	85.0	1,530
Potamophylax cingulatus	Otto (1974)	Alder Leaves	10°C	100	2,000	32.0	640	[2]24.5 [4](5)	[2]490 [4](100)	7.5	150	[4]76.6 (40)	68.0	1,360
"	"	Beech Leaves	10°C	100	1,400	14.0	196	3.3	46	10.7	150	23.5	86.0	1,204
Pteronarcys scotti	McDiffet (1970)	Mixed Leaves	10°C	100	840	15.1	127	3.6	52	7.0	75	41.0	89.4	713
Stenonema pulchellum	Trama (1972)	Navicula minima (diatom)	20°C	100	647	57.0	369	15.8	102	41.3	267	27.6	43.1	279

KEY TO TABLE 38.

1 Figure calculated using calorimetrically determined assimilation efficiency.

2 Calculated by difference from other data.

3 Observed in growth experiments with mixed diet.

4 Calculated from growth in field.

$$\text{Net Growth Efficiency} = \frac{\text{Calories used in growth}}{\text{calories assimilated}} \times 100 \quad \%$$

glycogen in G.pulex, but are converted to fats, and Huggins and Munday (1968) have suggested that carbohydrates are not the main energy substrate in crustaceans. This would suggest a low R.Q., which is confirmed by a figure of 0.72 given by Sizer (1887), quoted in Wolvekamp and Waterman (1960). This figure gives an oxycalorific value of 4,740 cal/litre of oxygen (Bell et at, 1968). Using this value, the oxygen consumption with E.L. and F.D. can be calculated as 24 and 58 mg O_2/ gram/day respectively, which may be compared with figures of about 55 given by Nilsson (1974) for 2.75 mg animals at 10^{o}C, and 45 given by Wautier and Troiani (1960). These figures are all quite comparable, particularly considering the approximations and assumptions involved, although that for E.L. seems rather low. If the figures for the E.L. energy budget are adjusted so that respiration is equivalent to the value given by Wautier and Troiani (153 cal/gm/day), and ingestion is unchanged, assimilation increased to 208 cal/gm/day, and the assimilation efficiency becomes 26.4%. Although these figures seem reasonable, caution must be used in making such calculations as the implicit assumption that soluble excretory products are negligible is doubtful. Such an assumption is often made (e.g. Nilsson, 1974 ; Otto, 1974) but Hargrave (1971) has shown that losses of soluble excretory products may be considerable. The apparent difference found here between calories used in respiration with E.L. and with F.D. may perhaps be explained on this basis.

Only part of the energy budget for N.L. can be calculated, but if it is assumed that the figure for respiration is similar to that obtained with E.L., assimilation can be calculated by difference. On this basis 167 cal/gm/day were ingested, 115 assimilated, 34 used in growth and 52 egested. This however implies a 69% assimilation efficiency, which was certainly not the case. The probable reason for this is that ingestion rates in Chapter 5 were too low. It was noted in some experiments with N.L. that consumption was very slow at first, often no obvious feeding occurring during the first 24 hours. After longer periods feeding became more rapid. With E.L., rapid feeding started immediately. In growth experiments where the animals consumed N.L. for over 200 days, the ingestion rate may have been similar to that of E.L., a suggestion which was corroborated by the rapid disappearance of the leaves offered

as food. If the ingestion rate for E.L. is used to calculate an energy budget for N.L., an assimilation efficiency of 14.6%, a gross growth efficiency of 4.3% and a net growth efficiency of 29.7% result. If the respiration figures of Wautier and Troiani are again substituted these figures become 23.8%, 4.3% and 18.2% respectively.

Nilsson (1974) did not attempt to present a balanced energy budget for G.pulex, presumably because the growth rates he calculated from assimilation and respiration data were much higher than those he observed under pseudo-field conditions. He considered that the cause of this was the energy lost in moults and reproduction under field conditions, which was not measured in short term ingestion and assimilation experiments. However, it also appears that his estimates of assimilation were too high. He found that the assimilation efficiency when fed alder leaves was much higher when measured gravimetrically than when measured calorimetrically, yet apparently used gravimetric data for estimating the rate of caloric assimilation. If the calorimetrically derived assimilation efficiency is used in the calculations (in brackets in Table 38) the estimate of assimilation is reduced almost four fold, which would account for most of the difference between observed and calculated growth rates. Nilsson carried out growth experiments with a mixed alder and beech leaf diet under pseudo-field conditions and obtained a very similar growth rate to that found here with E.L. He found that alder leaves were consumed much more readily than beech leaves and, indeed, insufficient calories could apparently have been assimilated from beech leaves to account for the observed growth rate. Most of the material consumed in his growth experiments was therefore presumably alder leaves. The figures for net and gross growth efficiency in Table 38 were calculated assuming that all growth was supported by alder leaves using his assimilation figures derived from calorimetric data. Taking into account these considerations, the figures of Nilsson can be used to calculate an energy budget which, although not completely balanced, is similar to that described here with a diet of E.L. It should be considered however, that the figures presented here are for a non-existent animal which is of neither sex, as values for growth, ingestion and assimilation are means derived from experiments involving both sexes.

Females are generally mature when 8 mm in length, although males may not be, but where the energy for egg production comes from is not known. As females grow more slowly than males (Sexton, 1928 ; Nilsson, 1974), but ingestion and assimilation seem similar (Chapter 5), it seems likely that in females some energy may be diverted from somatic into gonadal growth. This is in contrast to the situation in Asellus aquaticus in which females have a higher assimilation efficiency (Prus, 1971).

Comparison with the other animals in Table 37 which ingest leaf litter would suggest that G.pulex is quite typical in the way in which it utilises the energy in its food. As ingestion rates and assimilation and growth efficiencies often alter during growth, usually decreasing, (Waldbauer, 1968 ; Mcdiffet, 1970 ; Nilsson, 1974 ; Otto, 1974), the figures given in Table 38 are computed as far as possible from figures for animals of similar size at similar temperatures. Those quoted from Mcdiffet (1970), Trama (1972), Nilsson (1974) and Otto (1974) are not necessarily the mean figures quoted by the authors concerned for the entire life cycles of the animals, but have been selected or calculated from their data as being most comparable with the results described here in terms of temperature and animal size. The data may therefore not be shown entirely accurately as it was often determined by inspection of, and in some cases extrapolation from, graphs presented in those papers. The resulting energy budgets are quite similar. Assimilation efficiencies of leaf litter are mostly between 9 and 17%, with only one exception, which is the higher value of 32% for assimilation of alder leaves by Potamophylax cingulatus. Gross growth efficiencies are also very similar, lying between 3.3 and 6.9%. Again Potamophylax is an exception to this with a gross growth efficiency calculated from A, I and R figures of 24.5%. This figure is calculated from graphs given by Otto, the highest figure quoted by the author being 20%. Otto found that growth rates in the field were much lower than his calculated values for most of the year, and a figure for growth derived from his field data (in brackets in Table 38) gives a value of gross growth efficiency similar to others in the Table. Net growth efficiencies are also similar with values around 40% for alder and elm leaves, again with the exception of Potamophylax when calculated growth data is used. The amphipod Hyallela azteca utilised sediment in a generally similar way, although net and gross growth efficiencies are rather low. Hyallela

is known to pass food rapidly through its gut, to be incapable of digesting cellulose, and to lose a high proportion of the material it assimilates as soluble excretory products (Hargrave, 1970, 1971.). Perhaps to an even greater extent than the leaf litter consumers, it relies on passing large amounts of material through its tract, which it uses rather inefficiently (see also later discussion).

The utilisation of a diatom as food by Stenonema pulchellum is markedly more efficient than the utilisation of detritus by the other species in Table 38. Ingestion is a little lower than in the other examples, particularly considering that the figures are calculated for a rather small animal (6 mm length, 1.29 mg dry weight) and at a rather high temperature. Assimilation efficiency is high, as is gross growth efficiency, although the latter is still at the lower end of the 15-35% scale proposed by Welch (1968) as the normal range.

Several authors have studied energy budgets in terrestrial consumers of leaf litter, e.g. Gere, 1956, who found assimilation efficiencies of about 6-17% and gross growth efficiencies of 2-3%, and Bocock (1963) who found assimilation efficiencies of 6-10.5% and gross growth efficiencies of 0.29 - 0.45%. Terrestrial consumers are therefore no more efficient, and perhaps less so, in converting ingested leaf litter into body tissue.

Most of these studies were carried out using decomposing leaf litter but no conclusions can be drawn from them regarding the relative roles of ingested leaf material and micro-organisms in the energy budget. Barlocher and Kendrick (1973) have however studied growth and food consumption in Gammarus pseudolimnaeus when offered ten species of fungi and leaf litter with no microbial biomass as food. They found that leaves without any microbial biomass were rapidly ingested and would support growth, as would the fungi. From data they give for consumption and growth, gross growth efficiencies can be calculated. These ranged from 4.6 to 23.8% for the various species of fungi, and were 0.75% for elm leaves and 0.41% for sugar maple leaves. Ingestion rates of leaves, measured at 17^{o}C, were much higher than those found here (equivalent to about 2,200 cal/gm/day) but were similar to those reported by Nilsson (1974) at 15^{o}C. Those of fungi (equivalent to about 50-150 cal/gm/day) were much lower than those reported here. The result-

ant growth rates with fungi and leaves were similar, the former ranging from 8 to 33 cal/gm/day and the latter being 14.3 and 7.7 cal/gm/day for elm and maple leaves respectively. It seems reasonable to conclude that when G.pseudolimnaeus ingests leaf litter with a normal fungal population, although the proportion of fungal hyphae consumed may be small, their contribution to the energy assimilated and eventually converted into new tissues may be considerable.

b) Digestion and Nutrition in Freshwater Invertebrates

The ingestion and digestion of a given diet by G.pulex, and the nutritive value of that diet, are evidently mainly controlled by different parameters. Ingestion is dependent upon the availability of the food in the environment, the mechanical ease of its ingestion and its palatability (Chapter 5), but the extent of digestion (assimilation) probably depends on the availability of surface area for enzyme activity (Chapters 4, 5, 8). The pH conditions in the gut of Gammarus suggest that it is better adapted to carbohydrate than to protein digestion (Chapter 4), and protein availability is probably a critical factor in its nutrition. However, the nutritive value of a given type of detritus is little affected by the rapidity with which it is ingested or the extent to which it is assimilated, but is dependent upon the type and extent of microflora which it supports.

Of the natural diets used here, F.D. was most representative of the majority of the food normally passing through the tract. Intact leaf litter is probably only available in large amounts for a few months of the year, and the great majority of the detritus available to Gammarus and many other freshwater invertebrates during most of the year is undoubtably the more or less amorphous material formed by the breakdown of plant debris (Chapter 1 ; Hynes, 1970). Although F.D. and E.F.D. were rapidly ingested by Gammarus, and reasonably efficiently digested, they were of little nutritive value. It would seem therefore that the fraction of the diet comprising plant detritus contributes relatively little to its total nutritive value, except perhaps where a large proportion of the detritus comprises intact leaf litter enriched with a luxuriant microflora, e.g. whole elm leaves after two months decomposition in the field. (Chapters 7 and 8). The small proportion of the diet which usually comprises algal and animal material may thus be of disproportionate nutritive importance. The nutritive value of algae is probably fairly limited as most have cellulose cell walls (Prescott, 1968), but Moon (1939) believed that the occurrence of algal filaments in the detrital diet of Caenis horaria stimulated its growth. Animal material undoubtably has a high nutritive value, which may be enhanced by the generally more complete amino acid spectrum of animal than of plant proteins (Bell et al, 1968). Winterbourne (1971) considered that

the change from a plant to an animal diet shown by the caddis larva Banksiola crotchi during development was necessary to obtain a rapid increase in body weight before pupation. Another possible nutrient source is the type of organic aggregate described by Lush and Hynes (1973) which could constitute a significant proportion of the amorphous detritus in the diet of Gammarus. The nutritive value of such particles is unknown, but comparable ones formed in sea water supported some growth of Artemia (Baylor and Sutcliffe, 1963).

Much of this thesis has been concerned with the role of cellulase in the digestion of detritus. The production of reducing sugars from several cellulose substrates and the decrease in tensile strength of cellulose strips after incubation with M.G.F. or gut extracts showed that these enzymes were present in the gut of Gammarus. Furthermore, the considerable extent to which cellulose powder was assimilated suggested that cellulose might be an important source of carbohydrate. However, the lack of any change in the appearance of plant cells during their passage along the tract, and the small extent to which F.D., E.F.D. and,particularly, N.L. and E.L. were assimilated, suggested that the majority of the cellulose in the diet was not digested.

There are several possible reasons for the lack of extensive cellulose digestion. The most important is probably the tract transit time of 5-7 hours. Tracey (1959) emphasised the slow action of cellulases and the resultant need for gut turnover times measured in days rather than hours if cellulose is to be efficiently digested. Another possible reason for the non-digestion of plant cell walls may have been the lack of either a component of the cellulase complex, or of another necessary enzyme. Nielsen (1962) considers that the main barrier to cellulose digestion in invertebrates is in the lack of enzymes which mediate the initial conversion of native cellulose to a soluble substrate. In fungal enzyme preparations the component which mediates this initial conversion (C_1) acts synergistically with the hydrolytic enzymes, although having no hydrolytic activity itself (Selby, 1969). However, the hydrolysis of various forms of native cellulose which was mediated by gut extracts from G.pulex (Chapter 4) showed that this component was not missing. A more likely factor may have been the absence of other enzymes which are necessary for degrading plant cell walls (Tracey, 1968). Plant

cells are held together by pectin, and Toyama (1969) has shown that before the cell walls of potato tubers can be broken down, their intercellular structure must first be destroyed by the addition of pectinase. Bjarnov (1972), using sensitive chromatographic methods, could not detect the presence of pectinase in the gut of G.pulex. Although other factors such as the presence of enzyme inhibitors in leaf litter may also reduce the efficiency of cellulose digestion, it seems unlikely that the presence of cellulase in the gut of an animal which normally consumes large amounts of cellulose is without physiological significance. The available evidence suggests that although intact cells are not digested, cellulose could provide a significant portion of the energy requirements of G.pulex via the digestion of smaller particles. Large numbers of non-cellular particles, produced by trituration of N.L. and F.D., were found in the tract (Chapter 4) and cellulose particles of a similar size (15-40μ) were assimilated reasonably efficiently (Chapter 5). The digestion and assimilation of such non-cellular particles, in which cellulase probably plays a major role, may account for a large proportion of the total nutrients assimilated by G.pulex. In view of the relative insensitivity of cellulase to low temperatures (Chapter 4), cellulase activity may be particularly important during the winter when Gammarus remains highly active and continues to grow slowly. From the point of view of fulfilling dietary carbohydrate requirements, the efficient utilisation of all dietary cellulose may not be necessary as dietary carbohydrate is available in large amounts.

Tracey (1959) suggested five possible relationships between animals and the cellulases found in their guts :-

1. The animal produces a cellulase and the products of digestion represent a significant part of its food (some protozoa, insects and shellfish).
2. The animal produces cellulase but uses it to gain access to other foods and benefits mainly from the use of cellulase as a tool (plant pathogenic nematodes).

3. The animal produces cellulase but the products of its action do not appear to form a significant part of its food (some protozoa, earthworms).

4. The animal does not produce cellulase and does not benefit significantly from breakdown of cellulose, but its gut harbours a population of micro-organisms, some of which produce cellulase. Any benefit from their activity is only significant indirectly, if at all, e.g. by virtue of vitamin synthesis (most vertebrates, herbivorous echinoderms? many invertebrates?).

5. The animal does not produce cellulase but benefits from breakdown of cellulose by gut micro-organisms. This situation often is indicated by major structural adaptations of the gut and sometimes by an altered metabolism of the host (low blood sugar, ability to use volatile fatty acids in bulk etc. ruminants, macropod marsupials, many other vertebrate herbivores, some insects and some herbivorous molluscs?).

Evidence presented here suggests that G.pulex falls into group 1. The possibility that cellulase is used as a tool to gain access to cell contents, and thereby falls into group 2, was not confirmed here in respect of the digestion of higher plant cells, and was evidently not the case in respect of the digestion of algae as Willer (1922a) found many intact green algae in the gut of G.pulex. Some diatoms were apparently digested, but their cell walls are porous and comprise silicon and pectin (Prescott, 1968). The possibility that Gammarus belongs in group 3 cannot be rejected however as much of the cellulose passing through the gut was not digested. The results described in Chapter 4 suggest that Gammarus does not belong in groups 4 or 5, although the occurrence of micro-organisms in the gut which produce a cellulase is probable in view of the nature of the normal diet.

It is evident that the animals which make most efficient use of diets with a high cellulose content do so with the aid of an enteric microflora or microfauna. Ruminant mammals assimilate 45 to 80% of their diet (Tilley and Terry, 1963) including up to 50% of the cellulose (Consolazio and Iacono, 1963), and termites, which have a protozoan enteric fauna, can digest more than 50% of ingested plant material (Jensen, 1974). Many molluscs assimilate leaf litter with an efficiency of 50-70%, after digestion by endogenous and exogenous enzymes and, unlike crustaceans (Chapter 7), they are able to grow when fed a purely algal diet (Mason, 1974). The efficiency of digestion by enteric micro-organisms may be associated with the satisfaction of protein and trace nutrient requirements. Rumen bacteria are able to synthesis protein from other nitrogen compounds, e.g. urea and ammonia (Bell et al, 1968) and many enteric micro-organisms synthesise vitamins (e.g. Beerstecher et at, 1954a). Therefore animals with an enteric microflora need be less concerned with the intake of large amounts of food in order to satisfy protein and trace nutrient requirements. As a result, food may be retained in the gut for long periods in order to efficiently utilise all dietary compounds.

In Gammarus, when consuming intact leaf litter, and probably in many other freshwater invertebrates, the situation may be thought of similarly. The saprophytic microflora associated with the leaves replaces the gut microflora however, and the same food must be re-ingested several times by the same or other individuals before it is efficiently utilised.

The nutritional physiology of Gammarus is analagous to that of the marine wood boring isopod Limnoria (Ray and Julian, 1952 ; Becker, 1959 ; Lane, 1959 ; Ray, 1959). This animal secretes its own cellulase and lives solely on a diet of wood and any fungi which may be ingested with it. 36-53% of the wood passing through the gut is assimilated, including about half the cellulose. However, Limnoria bores into wood only after it has been colonised by marine fungi, and relies upon the fungi to furnish its protein requirements.

Also comparable is the prosobranch Hydrobia ulvae. Practically all molluscs tested, including many prosobranchs, possess a wide range of gut polysaccharases including cellulase (Yokoe and Yasumasu, 1964 ; Crosby and Reid, 1971 ; Mason, 1974) as it can therefore reasonably be assumed does Hydrobia. This mollusc ingested deposits of silt from which it utilised very little of the carbon and little of the total weight, but a high percentage of the nitrogen (Newell, 1965). The nitrogen content of the diet arose from a population of non-photosynthetic micro-organisms and, on standing, the faeces of Hydrobia rapidly regained the nitrogen content of uningested silt at the expense of a small drop in carbon content. If the faeces were then ingested the cycle was repeated.

Gammarus, Limnoria and Hydrobia are similar in that they ingest a diet containing a high proportion of refractory materials. Some of these materials can be digested and each animal presumably assimilates sufficient to satisfy its energy requirements. The limiting factor in the nutrition of these animals is probably concerned with fulfilling nitrogen requirements. In each case this is achieved by assimilating nitrogen which has been synthesised from the environment into microbial protein. The digestive processes are presumably adapted to provide the correct nutrient balance. For instance, if the tract transit time in Gammarus were longer, dietary cellulose would probably be more efficiently digested, but the total amount of fungal nitrogen passing through the gut would be less, perhaps resulting in a protein deficiency. If the tract transit time was shorter, more proteins and trace nutrients may be available, but energy requirements could be less easily satisfied because of inefficient digestion of the more refractory part of the diet.

Although the majority of detritivorous freshwater invertebrates are insects, the basic picture of food utilisation in Gammarus is probably applicable, at least in part, to many of them. Although it was found in Chapter 3 that many aquatic invertebrates have weak cellulase activity in their guts, it seems unlikely that many of them are able to effect an extensive breakdown of ingested plant detritus. Members of the Trichoptera are among the first to consume freshly fallen leaf

litter. The occurrence of a cellulase in several species tested (Chapter 3 ; Bjarnov, 1972), and the normally fairly long gut retention time of 8 to 24 hours (Cummins, 1973) might suggest that they are particularly well adapted to deal with such a diet. It is not known whether the cellulase activity in the guts of the caddis larvae examined in Chapter 3 or by Bjarnov was endogenous or exogenous, but if any of these animals are capable of efficiently utilising fresh leaf litter it may be expected from considerations already discussed that they might do so with the aid of an enteric microflora, and that the gut may be morphologically adapted to allow a long period of microbial digestion. Caddis larvae, with their very capacious midguts, are likely candidates for this mode of food utilisation. Results obtained here (appendix 2) showed that some growth occurred when members of one genus, Halesus, were fed on fresh leaves, but comments made by other authors concerning the appearance of the gut contents of the Trichoptera suggest that plant detritus is not extensively digested. Jones (1949) mentioned that in the Trichoptera identifiable material occurred in the whole of the midgut. Winterbourn (1971a) examined gut contents of Lepidostoma unicolor, which fed solely on decaying leaves, and noted that leaf fragments in the foregut were normally coated with a clearly visible microflora, but the surfaces of fragments in the hindgut were clear. He concluded that the leaf microflora formed a high proportion of the material assimilated.

The ability of Halesus spp to utilise fresh leaf litter may lie in the large amount of food which they pass through their gut. Cummins (1973) has commented on the exceptionally rapid ingestion rates of some Trichoptera. These may reach 160% of the body weight per day compared to the normal range of 4 to 20%, which is similar to that found here for Gammarus. In some preliminary experiments carried out here with Halesus spp it was found that fresh aspen and nettle leaves (green), and fresh elm litter, were ingested at mean rates of approximately 70, 91 and 208% body weight per day respectively at 10^{o}C. Even if only a small proportion of this material was utilised, the animals nutritive requirements may have been satisfied.

In recent years, much ecological research has been concerned with the dynamics of energy flow in freshwater communities (e.g. Fisher and Likens, 1972). It has long been recognised that attempts to categorise invertebrates into trophic levels are oversimplifications (e.g. Darnell, 1961), but it is not easy to take this into account in describing energy flow through the community. Coffman et al (1971) calculated a 'tissue support structure' based on the percentage of animal, algal and detrital material in the guts of animals examined. From these figures they calculated the percentage of invertebrate biomass which was being supported by the intake of algal, detrital or animal calories. The accuracy of such an approach depends on the ways in which the various food items were utilised by the animals concerned. Coffman et al stated that assimilation efficiencies are dependent upon species enzyme systems, and suggested that species would tend to ingest what they could assimilate. Although this must be at least partly true, the results presented here suggest that such assimilated materials may support no growth in some cases, and production may be supported to a large extent by a disproportionately small fraction of ingested materials. Coffman et al calculated community energy budgets based on an assumed overall assimilation efficiency of 40% and a net growth efficiency of 30%. Such figures may well be good estimates of the mean parameters involved, but if information concerning the assimilation and, particularly, growth efficiencies with different diets were available, far more accurate 'tissue support structures' and community energy budgets could have been calculated.

The approach adopted in this thesis has uncovered many problems but resolved few. Those such as the uptake and loss of ash from the diet and the direct effects of water chemistry upon growth require to be elucidated before data concerning food utilisation can be fully interpreted, and further work on digestion and nutrition is needed. Further study of tract transit time and the factors which control it, and of the properties of cellulase and other polysaccharases, may shed further light on the significance of these enzymes in the animals nutritive physiology. Greater knowledge of these factors, and of the exact

physical and chemical composition of the diets ingested would probably enable much of the variation observed in assimilation efficiency to be accounted for. As emphasised by Cummins (1973), further information concerning the control of ingestion would be valuable, but the control of enzyme secretion, although one of the more interesting aspects of digestive physiology, is probably of little significance in the overall picture of food utilisation. Evidence presented here (Chapter 6) suggested that enzyme secretion would be stimulated to similar levels by the ingestion of most foodstuffs available in the field.

The greatest advance towards complete understanding of food utilisation in Gammarus could probably be achieved by further studies of food requirements. Although more information concerning the nutritive value of different types of micro-organisms is needed, a more valuable approach would necessitate the development of a completely artificial diet. If this was fed under sterile conditions, specific nutritional requirements could be determined and compared with chemical analyses of the foodstuffs available.

SUMMARY

Summary

1) Twenty-six species of freshwater invertebrates were tested for cellulase activity. Weak activity was found in several insects and stronger activity in some molluscs and crustaceans. There was no correlation between the occurrence of cellulase and the abundance of cellulose in the diet.

2) The digestive physiology and food requirements of Gammarus pulex were studied using natural and artificial test diets. The former were fine detritus, elm litter and elm litter on which microbial growth had been stimulated by incubation in nutrient-enriched water, and the latter were mixtures of cellulose powder and protein.

3) Cellulase activity in the midgut gland fluid was found to be endogenous. The pH of the fluid varied between 5.6 and 6.0 depending on the diet consumed. The pH of the various regions of the tract and the pH sensitivities of the digestive enzymes suggested that carbohydrate digestion occurs in the foregut, midgut glands and the anterior midgut, but protein digestion is limited to the posterior part of the midgut. The possible occurrence of enzyme inhibitors in elm litter was investigated.

4) Food normally passed through the tract in 5 to 7 hours irrespective of the rate of ingestion. Plant cell walls that were not ruptured mechanically during their passage along the tract were not digested chemically.

5) The rate of ingestion was controlled both by the palatability of the diet, and by the physical ease with which it could be ingested.

6) Study of assimilation was complicated by movements of inorganic substances across the gut wall. About 40% of the cellulose and more than 80% of the protein in artificial diets was assimilated, compared with about 18% of enriched leaves and 33% of fine detritus.

7) The activities of amylase and protease in the midgut gland fluid were higher when the animals were fed natural diets than when fed artificial diets, but were highest when the animals were starved. The activities of these enzymes were best correlated with the palatabilities of the diets. The nature of the diet had little effect on cellulase activity.

8) Decomposing plant litter supported better growth and survival than did fresh plant litter. Fine detritus and other fine particulate diets were of little or no nutritive value. Growth was more rapid with enriched than with normal elm litter, but the addition of protein to normal litter only partly replicated the effect of enrichment.

9) The composition of the water used had a direct effect upon survival and apparent growth rates. The nutritive values of the diets were best compared from growth rates observed when the animals were 2.5 to 4.5 mm long. No direct correlation existed between food nutritive value and protein content. The nutritive value of detritus probably depends largely upon the type and extent of microflora which it supports.

10) The digestion of fine detritus and decomposing elm litter by commercial enzyme preparations was studied. It was concluded that the surface area of substrate available for enzymic attack is the most important factor controlling assimilation efficiency.

11) An approximate energy budget was calculated and compared with energy budgets for other freshwater invertebrates. The role of cellulase in the nutritional physiology of G.pulex, and food utilisation in this and other invertebrates were discussed.

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APPENDIX 1.

APPENDIX 1.

ENZYME ACTIVITY IN M.G.F. FROM ANIMALS FED VARIOUS DIETS AND STARVED.

Diet	No.	Mean Length (mm)	Volume M.G.F. (µl)	Amylase Activity	Amylase % N	Protease Activity	Protease % N	Cellulase Activity	Cellulase % N
100C	5	9.2	4.0	13.4	66	6.2	85	5.5	106
"	4	9.5	3.0	13.7	67	6.8	93	5.3	101
"	5	10.3	3.5	11.4	56	4.4	61	3.7	71
"	5	9.8	4.0	5.3	26	4.4	61	3.8	73
"	5	9.9	4.5	11.0	54	5.6	76	5.0	95
"	5	9.4	5.0	8.0	39	6.9	95	4.8	93
"	5	10.8	6.0	8.8	43	5.6	76	3.8	72
"	5	9.9	4.5	12.4	61	6.1	84	5.4	103
$\bar{x}$					52		79		89
S.E.$_{\bar{x}}$					5		5		5
90C + 10P	5	10.2	3.0	15.1	74	7.6	104	6.5	125
"	4	9.4	3.0	22.7	111	7.8	107	7.6	145
"	5	9.9	2.0	9.7	48	4.8	66	4.8	93
"	4	10.8	4.0	15.3	75	5.2	71	4.3	83
"	5	9.4	6.5	14.0	69	4.9	67	3.9	75
"	5	9.8	3.5	10.7	53	6.0	82	4.6	88
$\bar{x}$					72		83		101
S.E.$_{\bar{x}}$					9		8		11

Diet	No.	Mean Length (mm)	Volume M.G.F. (µℓ)	Amylase Activity	Amylase % N	Protease Activity	Protease % N	Cellulase Activity	Cellulase % N
60C + 40P	5	10.0	6.5	21.8	107	6.5	89	4.8	92
"	5	10.1	8.0	22.1	108	6.7	91	4.0	77
"	6	9.7	7.0	16.3	80	6.1	83	4.2	80
"	5	9.8	3.5	17.6	89	8.2	113	5.7	110
"	6	9.8	5.0	25.6	126	8.1	111	5.4	103
"	6	9.5	6.5	21.2	104	8.2	113	5.2	100
"	5	10.3	4.0	24.8	122	8.4	115	5.6	108
$\bar{x}$					105		102		96
S.E.$_{\bar{x}}$					6		5		5
100P (Egg Albumen)	5	9.6	5.0	20.7	101	6.2	85	4.8	92
"	5	9.4	3.5	17.8	88	6.6	90	3.9	75
"	5	9.3	4.5	22.6	111	7.1	97	5.0	96
"	6	9.3	7.0	20.1	99	6.7	92	4.9	94
"	5	10.4	4.5	25.0	123	7.3	100	4.6	88
"	6	10.0	4.0	14.4	71	6.0	82	4.8	92
"	6	10.0	6.0	19.6	96	6.2	85	4.3	82
$\bar{x}$					98		90		89
S.E.$_{\bar{x}}$					6		3		3
100P (Bovine Alb)	5	8.5	5.0	5.9	29	-	-	-	-
"	5	8.8	4.0	26.4	130	-	-	-	-
"	5	9.1	4.5	31.3	154	-	-	-	-
"	5	9.1	4.0	11.7	58	-	-	-	-

Diet	No.	Mean Length (mm)	Volume M.G.F. (μℓ)	Amylase Activity	Amylase % N	Protease Activity	Protease % N	Cellulase Activity	Cellulase % N
100P (Bovine Alb)	5	8.4	4.0	10.3	51	-	-	-	-
"	4	9.4	2.5	25.8	127	-	-	-	-
$\bar{x}$					92				
S.E.$_{\bar{x}}$					21				
N.L.	5	9.4	3.0	19.4	95	6.0	83	4.8	91
"	5	9.5	3.5	23.1	114	5.6	77	5.0	96
"	6	9.3	3.5	30.7	151	7.0	96	5.1	97
"	5	9.8	6.5	30.3	149	7.7	106	5.6	107
"	4	9.3	3.5	27.7	136	6.2	85	4.4	84
"	4	9.9	4.0	25.7	126	5.5	75	4.7	89
"	5	9.0	3.0	25.6	126	6.7	92	5.2	99
$\bar{x}$					128		88		95
S.E.$_{\bar{x}}$					7		4		3
E.L.	6	10.6	4.0	37.9	186	10.4	142	4.5	86
"	5	9.4	3.5	37.4	184	9.3	127	5.2	101
"	4	9.3	2.5	32.2	158	10.0	136	3.8	73
"	6	10.5	2.0	52.0	255	14.7	201	8.8	169
"	6	9.4	3.5	31.0	152	8.6	117	5.4	104
"	7	9.1	2.5	15.0	74	4.2	57	4.1	79
"	6	9.7	4.5	21.3	104	8.5	116	4.7	91
"	6	8.6	4.5	22.3	110	7.5	103	4.7	89
$\bar{x}$					153		125		99
S.E.$_{\bar{x}}$					20		14		11

Diet	No.	Mean Length (mm)	Volume M.G.F. (µl)	Amylase Activity	Amylase % N	Protease Activity	Protease % N	Cellulase Activity	Cellulase % N
F.D.	7	9.6	6.0	22.6	111	6.2	85	4.8	92
"	8	8.9	3.0	33.1	162	9.1	124	5.7	110
"	6	9.0	4.5	31.4	154	8.7	119	5.3	101
"	7	9.2	4.0	29.6	146	7.5	103	4.7	90
"	6	9.3	4.0	32.1	158	9.0	123	5.4	104
$\bar{x}$					146		111		100
S.E.$_{\bar{x}}$					9		8		4
E.F.D.	5	10.0	3.5	28.7	141	8.7	119	3.5	66
"	6	10.0	4.5	31.6	160	7.0	96	4.9	95
"	5	9.8	4.0	39.0	192	9.0	123	4.8	91
"	6	9.9	3.5	33.7	166	7.9	108	5.1	97
"	5	9.9	4.0	42.6	209	8.9	121	7.3	140
"	7	9.7	4.0	34.3	167	7.8	106	4.9	94
"	7	8.9	3.5	38.2	188	9.6	131	4.9	94
$\bar{x}$					175		115		97
S.E.$_{\bar{x}}$					11		5		8
Starved (3 days)	5	9.3	5.0	-	-	10.5	144	5.6	108
"	6	9.5	4.0	-	-	9.2	126	5.2	100
"	5	10.3	4.5	-	-	10.2	140	6.0	114
"	6	10.2	3.5	-	-	12.5	171	-	-
"	6	9.9	5.0	-	-	10.5	143	5.5	106

Diet	No.	Mean Length (mm)	Volume M.G.F. (μl)	Amylase Activity	Amylase % N	Protease Activity	Protease % N	Cellulase Activity	Cellulase % N
Starved (3 days)	6	10.0	3.5	-	-	7.3	100	5.4	103
$\bar{x}$							137		106
S.E.$_{\bar{x}}$							10		2
Starved (7 days)	6	10.1	3.5	47.6	234	12.4	169	4.5	86
"	6	10.0	4.0	39.6	195	11.8	161	4.4	85
"	6	9.5	3.5	40.4	199	12.2	166	7.2	139
"	6	10.5	4.5	43.2	212	12.2	166	5.0	96
"	6	10.9	4.0	50.3	247	15.1	207	5.2	100
"	6	10.4	3.0	49.1	241	14.3	195	6.9	132
$\bar{x}$					221		177		106
S.E.$_{\bar{x}}$					9		8		10

- = Not Tested

APPENDIX 2.

APPENDIX 2.

Survival and Growth of *Halesus* spp in running water experiments.

A Survival

B Growth

—●— Decomposing Elm

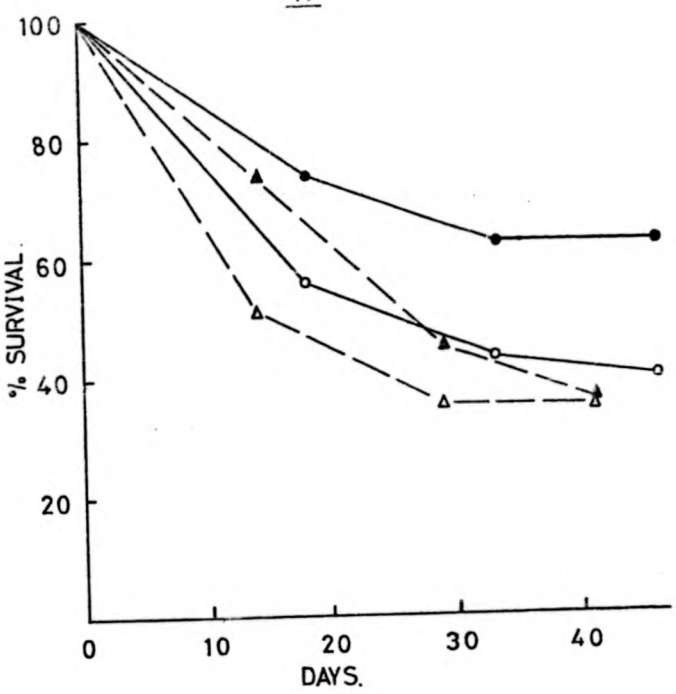
—○— Decomposing Grass

–●– Fresh Elm

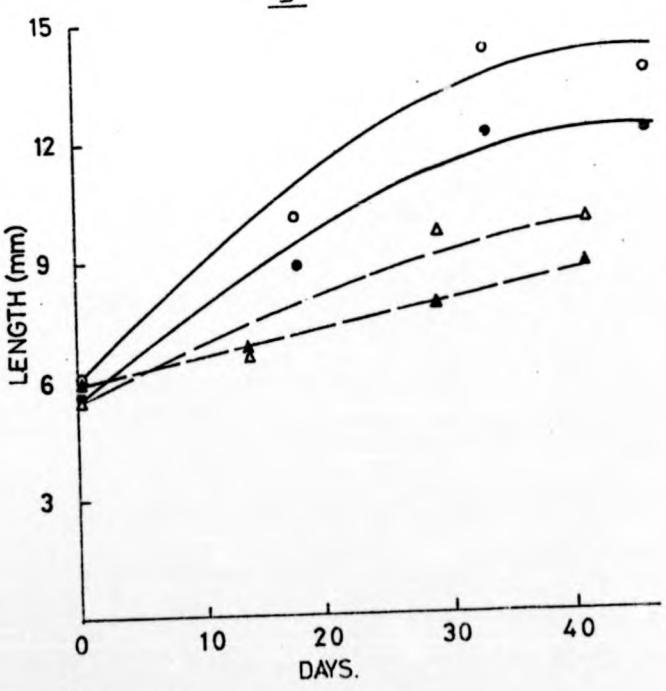
–△– Fresh Grass

us spp

A


100
80
60
40
20
% SURVIVAL.
0
10
20
30
40
DAYS.

B


15
12
9
6
3
LENGTH (mm)
0
10
20
30
40
DAYS.