ABSTRACT

SOME EFFECTS ON OAK-FEEDING INSECTS OF SEASONAL CHANGES IN THE NATURE OF THEIR FOOD

The present work has been restricted to an investigation of the relationship between the leaves of the common oak Quercus robur L. and the larvae of the order Lepidoptera which feed on them. At least 210 species of Lepidoptera have been recorded as larvae feeding on oak leaves in Britain and there is evidence that insect growth and survival on young oak leaves may be higher than on the leaves of other species. More species and higher numbers of Lepidoptera larvae are found on oak leaves in April and May than in any other months of the year and there is evidence, from the high mortality of young winter moth Operophtera brumata L. larvae due to premature hatching, that there may be strong selection pressure for early larval feeding in this species. It is unlikely that predation or parasitism are responsible for this selection pressure and it was suspected that the concentration of larval feeding in the spring might be due to changes in the nature of the oak leaves as the season progresses. This thesis describes some investigations into the seasonal variation of the toughness, nutrient content and tannin content of oak leaves and into the possible effects which these factors might have on caterpillar growth and subsequent insect survival.

Fieldwork was largely carried out in Wytham Wood, near Oxford. The caterpillar populations on the leaves of one oak tree were sampled

during the spring of 1964. Although the number of caterpillars in May and June was greater than 200/sq.m., very few larvae could be found on the leaves after mid-June.

The winter moth was selected for laboratory study. It was found that winter moth larvae which had been fed on oak leaves, collected in late May and early June, reached very much lower peak and pupal weights than larvae which had been fed on leaves collected in mid-May. Adult emergence from the pupae resulting from larvae fed on the early leaves was about 50%, whereas no adults emerged from pupae resulting from larvae fed on the later leaves. This shows that a difference of only 12 days in leaf age can significantly affect larval growth, pupal weights and adult emergence.

The toughness of oak leaves collected in June, measured with a specially constructed device, was found to be considerably greater than that of leaves collected in May. The effect of leaf toughness on the fresh weight increase of winter moth larvae was investigated by rearing larvae on two agar diets, each containing a finely ground powder derived from May and June oak leaves, respectively. It was found that the larvae fed on the June leaf powder diet increased in weight at almost the same rate as the larvae reared on the May leaf powder diet. This result, in conjunction with that from the previous experiment, suggests that leaf toughness may be important in determining the ability of winter moth larvae to grow satisfactorily on oak leaves. It is considered unlikely, however, that increase of leaf toughness can adequately account for the evident selection pressure favouring early larval feeding.

Abstract (continued)

If leaf toughness was the only barrier preventing late feeding, stronger larval mandibles would probably have been evolved, as has happened in some species which do feed on older leaves.

Analyses were carried out on the leaves of one oak tree to determine the seasonal variation of sugar content. It was found that variation in the concentration of reducing sugars (glucose and fructose) in the leaves followed no obvious trend during the growing season, though sucrose concentration increased rapidly during early June and remained at a level of about 6% (dry weight of leaf) during the summer months. The sugar content of oak leaves is therefore lower during April and May than during other months of the year. For this and other reasons, such as the apparent lack of an amylase enzyme in the winter moth, it was considered that the growth of winter moth larvae is normally not limited by the level of carbohydrates in oak leaves.

Analyses were carried out on the leaves of six oak trees to determine the seasonal variation of total nitrogen content. It was found that the 'protein' content of the leaves (calculated as total nitrogen x 6.25) is high in buds and young leaves (about 30% dry weight of leaf), but drops rapdily to about half this value by the end of May and then remains steady at about 12% dry weight of leaf at least until September. There is therefore a correlation between early larval feeding periods and a high nitrogen content of the leaves, which may suggest that total nitrogen, or the concentration of certain nitrogenous compounds, may be important in determining the optimum feeding times for Lepidoptera larvae.

Abstract (continued)

Oak leaves have been known for centuries to contain tannins. Tannins are known to have biological effects, including inhibition of fungal growth and virus infection, which are believed to be due to the ability of tannin to form complexes with proteins. It was considered possible that the presence of tannins in oak leaves might have an adverse effect on the growth and survival of caterpillars, either by directly inhibiting digestive enzymes or by reducing the availability of leaf protein to insect digestion.

The phenolic compounds of oak leaves were investigated during the growing season by means of two-way paper chromatography. The most obvious seasonal change was found to be the appearance of condensed tannin, and its probable precursors (+)-catechin and (+)-gallocatechin, in the leaves at the end of May and the apparent increase in concentration of condensed tannin in the leaves during subsequent months. Tannins were isolated from oak leaves by an ether-precipitation method. The crude tannin material was found to contain at least one hydrolysable component, in addition to the condensed tannin. The crude material was eventually separated into the two major components (condensed tannin and hydrolysable tannin) by Sephadex column chromatography, followed by purification on thick paper.

Quantitative analysis of the leaves of one oak tree showed that the concentration of tannins inthe leaves increases from about 0.5% dry weight in April (when only hydrolysable tannin is present) to about 5% dry weight in September (when both hydrolysable and condensed tannins

Abstract (continued)

are present). The analyses were carried out by a gravimetric adaptation of the ether-precipitation method, previously used for isolating tannins. However, a new spectrophotometric method for the rapid determination of condensed tannin in leaf extracts was subsequently devised.

Effects of oak leaf tannins on the fresh weight increase and pupal weight of winter moth larvae were investigated by rearing larvae on artificial diets, to which crude oak leaf tannins were added. Although the experiments were marred by mortality, due to fungus, virus and microsporidian infections, the results nevertheless clearly showed that larval fresh weight increase and pupal weight are reduced by the presence of tannin in the diet. It is considered likely that reduction in larval peak weight and pupal weight is an indication of a potential adverse effect on subsequent adult weight and fecundity, though this was not proved, owing to the high mortality.

Several in vitro experiments were carried out to determine the ability of oak leaf tannins to form complexes with casein (the protein incorporated in the artificial diet experiments) and leaf protein (from nettle leaves). It was found that both protein preparations were completely complexed at pH 5.0 by their own weights of oak leaf tannin and probably by lower quantities of tannin. Oak leaf tannin was found to inhibit almost completely the hydrolysis of starch by α-amylase at pH 6.0. Moreover, digestion of casein by mammalian trypsin was almost completely inhibitied at pH 7.6.

There is evidence that tannin-protein complexes are dissociated

Abstract (continued)

to some extent under alkaline conditions. The effect of hydrolysable and condensed oak leaf tannins on the hydrolysis of casein and nettle leaf protein was therefore determined separately at pH 9.2, found by two methods to be the pH of the winter moth mid-gut. It was found that inhibition of hydrolysis at this pH was much less pronounced than at pH 7.6, indicating some dissociation of the complexes in the mid-gut. Hydrolysable tannin was found to have little effect on the hydrolysis of either protein, but a concentration of condensed tannin equivalent to only 30% of the concentration of the casein reduced the hydrolysis of this protein by about 50%. Condensed tannin is therefore much more effective as an inhibitor of protein hydrolysis than is hydrolysable tannin. This is of interest in view of the different seasonal occurrence of these two tannins, already mentioned. It is possible that one of the functions of the high mid-gut pH found in many phytophagous larvae, may be to increase the availability of protein by partial dissociation of protein-tannin complexes.

It was found by autoradiography that ^{14}Carbon labelled Chlorella vulgaris protein can be incorporated into the mid-gut tissues of the winter moth in the presence of oak leaf condensed tannin, though the protein, on account of its denatured condition, was not initially present as a tannin-protein complex. Both condensed and hydrolysable tannins were labelled with ^{14}Carbon by enclosing oak leaves in situ in an atmosphere containing labelled carbon dioxide. These labelled tannins were fed to winter moth larvae to determine by autoradiography the fate

Abstract (continued)

of tannins in the larval mid-gut. This experiment is still in progress.

It was concluded from the experiments described in this thesis that oak leaf tannins might have an adverse effect on larval growth and survival in the field, by reducing the availability of leaf nitrogen for larval digestion. It was estimated that the concentration of protein in oak leaves which is available for insect growth might be about five times higher inApril than it is in September. It is not known to what extent this effect may be responsible for the evolution of early feeding periods by the larvae of many Lepidoptera species. The increase in the tannin content of oak leaves does not seem to be sufficiently rapid to account for the evident strength of the selection pressure favouring early feeding by winter moth larvae and it is possible that other factors, such as the availability of micronutrients, might be important. However, it is also possible that even small concentrations of tannins in oak leaves might have much greater effects on insect survival under natural conditions than have been indicated by the laboratory experiments.

SOME EFFECTS ON OAK-FEEDING INSECTS OF SEASONAL CHANGES IN THE NATURE OF THEIR FOOD

A thesis submitted to the Board of the
Faculty of Biological Sciences, Oxford University,
for the degree of Doctor of Philosophy.

by P. P. Feeny



November 21st, 1966.

FRONTISPIECE

FRONTISPIECE

Part of the study area in Wytham Wood, August 1966.

Winter moth caterpillar feeding on oak leaves in Wytham Wood, May 1966.





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INTRODUCTION

1. Oak leaves as a source of food for Lepidoptera

The present work has been restricted to an investigation of the relationship between the leaves of the Pedunculate or Common Oak *Quercus robur* L. and the larvae of the order Lepidoptera which feed on them. For reasons of time and since their ecology is comparatively poorly known, insects of other orders have not been considered, though many are known to feed on oak leaves.

The pedunculate oak (subsequently referred to in this thesis simply as the oak) is widespread in Europe as a dominant species in the natural deciduous forest climax vegetation. Before the advent of large scale deforestation by man, much of central Europe from the Urals westward to Britain was covered by deciduous forest, in which oak was a prominent species (Jones, 1959).

Probably more species of Lepidoptera have been recorded feeding on the leaves of oak trees than on the leaves of any other species of plant in Europe. A search through four standard reference works (South, 1939; Allan, 1949; Scorer, 1913; Ford, 1949) on the Lepidoptera of Great Britain revealed that 210 species have been recorded as larvae feeding on oak leaves in this country, comprising 125 'Macro-Lepidoptera' and 85 'Micro-Lepidoptera' (Appendix 1, p. 150). The actual figures are likely to be much higher than these, since only a few food-plants are listed for the more widely polyphagous insect species.

In Britain, the two most abundant species of Lepidoptera feeding on oak are the winter moth Operophtera brumata L. (Frontispiece) and the green tortrix Tortrix viridana L. Over a seven year period, in Wytham Wood, Berkshire, these two species were found to outnumber all the other hundred oak-feeding species put together (Varley and Gradwell, 1958b). In western Russia, however, very high population densities are reached by two further species Phigalia pilosaria Schiff. (= pedaria Fab.) and Apocheima (= Biston) hispidaria Schiff. (Golosova, 1965), which are present in comparatively low densities in Britain.

It is evident from Figure 1 (p. 2α), obtained from the data in Appendix 1 (p. 150), that the largest number of species feeding on oak leaves are found as larvae in the spring. If the total insect biomass, rather than number of species, is considered, the concentration of feeding in the spring is even more marked. While Lepidoptera larvae are relatively scarce on oak leaves after mid-June, very high densities are frequently reached in May, when the populations of winter moth and green tortrix alone are often at a density of over 100 larvae per sq. m. of oak canopy area (Varley and Gradwell, 1958a). The infestations are occasionally on such a scale as to cause complete defoliation of oak trees in late May and early June, as occurred in Wytham and elsewhere in southern England in 1965 (Figure 2a, p. 2(b)). The trees respond to the defoliation by putting out a secondary set of leaves, the so-called 'lammas' shoots (Figure 2b, p. 2(b)), so that the photosynthetic area is largely re-established by late June and only close

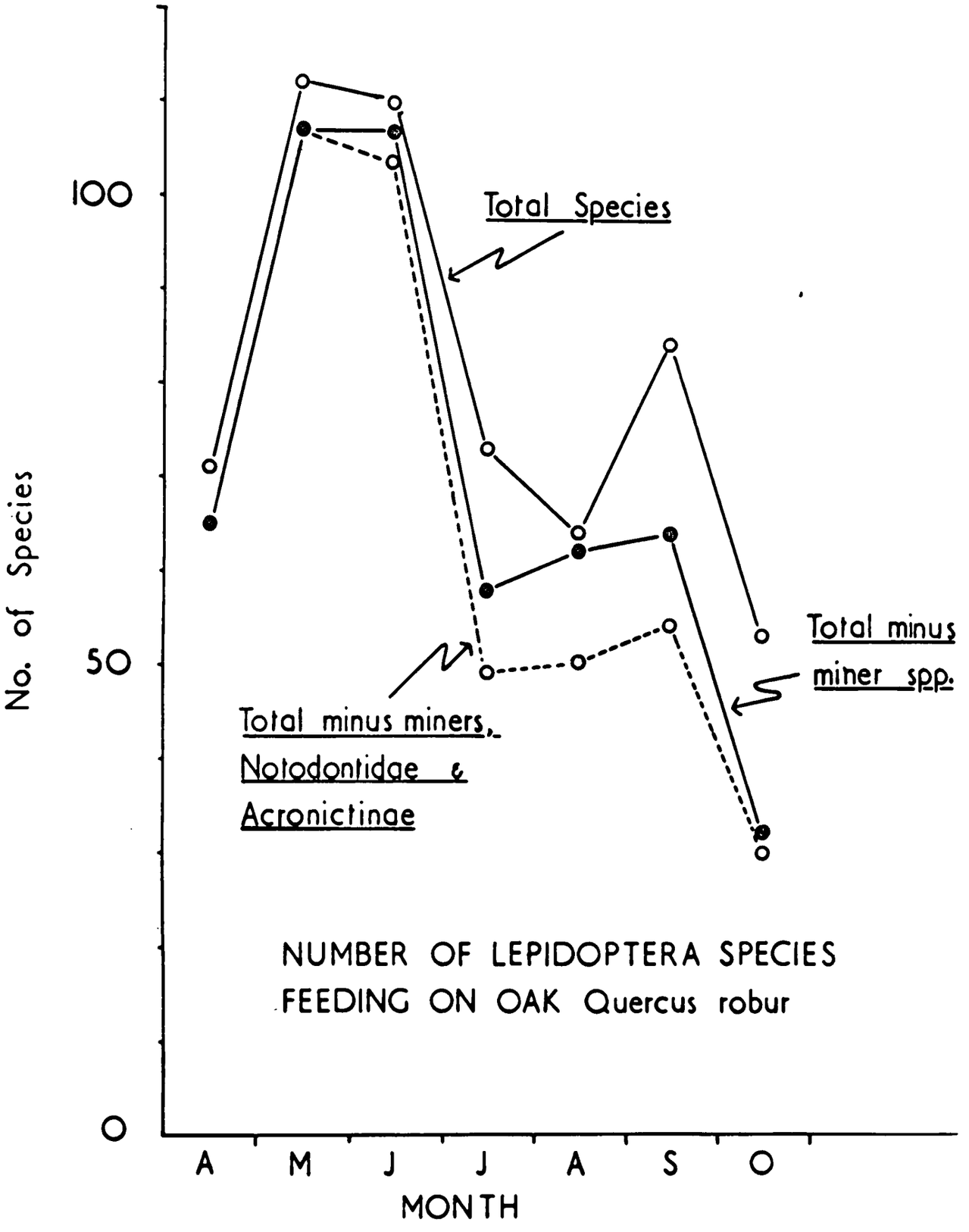


FIGURE 1 Number of Lepidoptera species recorded as larvae on oak leaves in each month from April until October.

FIGURES 2a and 2b

FIGURE 2a Oak tree defoliated by winter moth larvae in Wytham Wood, June 1965. It can be seen that leaves of the Sycamore Acer pseudo-platanus L. were also attacked.

FIGURE 2b Lammas shoots on the branch of an oak tree, following defoliation by Lepidoptera larvae, July 1965. Traces of the earlier defoliation can still be seen.





examination reveals traces of the earlier defoliation. The trees suffer relatively little insect damage during the remainder of the year.

The unusually large number of Lepidoptera species feeding on oak may be related to its history as one of the commonest species of tree in Europe for a considerable period of time. However, there is some evidence that young oak leaves may be more nutritious for the larvae than the leaves of other trees. Winter moth larvae develop into heavier pupae when fed on young oak leaves than when fed on the leaves of hazel Corylus avellana L. or blackthorn Prunus spinosa L. available at the same time of year, though pupae obtained from larvae fed on hawthorn Crataegus monogyna Jacq. were not significantly different in weight from those obtained on oak leaves (Gradwell G.R., personal communication). A comparison of the growth of Lymantria dispar L. larvae on the leaves of several different tree species has also shown oak to be the most satisfactory food-plant (Kansu, 1962).

2. Population fluctuations of oak-feeding Lepidoptera

Since 1949, Varley and Gradwell (1958a, 1958b, 1960, 1962a, 1963a, 1963b) have been investigating in detail the population dynamics of oak-feeding Lepidoptera in Wytham Wood, Berkshire, with particular reference to fluctuations in the populations of the winter moth. They have found that, although large fluctuations occur in the populations of all species, these variations tend to be synchronous for many species and the relative densities of the dif erent species do not vary greatly.

By far the heaviest mortality of winter moth occurs between the

time in November and December when the females lay their eggs on the twigs and the following April and May, when the larvae are feeding on the young oak leaves. This mortality - called the 'winter disappearance' by Varley and Gradwell - includes the 'key factor' (Morris, 1959), largely responsible for the annual variations in population density, since it is the only mortality factor to fluctuate in synchrony with the total annual mortality and to a similar extent. The winter disappearance is related to weather, which influences the relative timing of bud burst and egg hatch. If the majority of the trees open their buds later than the peak egg hatch, a high mortality of caterpillars results - about 90% on average (Gradwell G.R., personal communication) - since the young larvae are unable to penetrate the closed buds. In any one year, oaks in Wytham vary by as much as two weeks in the date of bud burst, the order of bud opening remaining the same from year to year, so that the early trees tend to carry higher caterpillar populations than the late-opening trees, which may escape defoliation even in years of very high caterpillar density. The number of larvae surviving on early trees may reach five times the number on the late-openers, leading to a higher number of pupae in the soil beneath the trees in June. However, pupal predation in the soil, largely by beetles and small mammals, operates in a density dependent manner, the predators showing a 'functional response' (Holling, 1959) to pupal density, so that the balance is partially restored by higher pupal predation under the trees of high caterpillar density.

Mortality due to weather accounts for the synchrony of the large

annual variations in the populations of the different caterpillar species, since it generally acts in a density independent manner ('catastrophic factor' - Howard and Fiske, 1912). The winter disappearance actually fluctuates to a greater extent than the total annual mortality, but is partially compensated for by pupal predation, which operates in a density dependent manner from year to year, as well as from one tree to another. Weather, however, cannot regulate insect densities and does not account for the more or less constant relative abundance of different species - i.e. it cannot explain why one species is always common and another always rare. Varley and Gradwell suggest that the relative abundance of the different species is governed by the differing efficiencies of the specific parasites, by which most of the Lepidoptera species are attacked. Thus, the tachinid fly Cyzenis albicans Fall., a parasite of winter moth larvae, appears to be relatively inefficient, with a small 'area of discovery' (Nicholson and Bailey, 1935), allowing the winter moth to maintain a comparatively high density relative to other species, which appear to suffer from more efficient specific parasites.

Since parasites in general show a 'numerical response' (Holling, 1959) to increases in host densityand thus operate in a 'delayed density dependent' manner (Varley, 1947), inefficient parasites are unable to prevent outbreaks of the more abundant host species to a level at which the trees suffer complete defoliation. This is especially true of parasites such as C. albicans which are univoltine, like their hosts. Varley and Gradwell consider that parasitism may be sufficient to con-

trol at a low level the less common Lepidoptera species, which seem to suffer from more efficient specific parasites, but that parasitism of the more abundant species (which has never exceeded 40% in the last fifteen years) is unable to prevent increases in host density to outbreak level.

There is a tendency for years of greatest oak defoliation to be followed by decreases in the numbers of many Lepidoptera species, while years of light damage are frequently followed by population increases. Mass starvation of larvae has never been recorded in Wytham. Larvae from defoliated trees tend to be carried on silk threads to other plant species (see Figure 2a, p. 2(b)), where they complete their growth. It is likely that competition for food, if it exists, manifests itself in reduced fecundity, perhaps resulting from low pupal weights attained on alternative food-plants.

Insects at high density are especially vulnerable to the spread of disease, which may also contribute to the decrease in numbers following a defoliation year. Golosova (1965) reports the almost total annihilation of very large populations of P. pilosaria and A. hispidaria on oak trees in western Russia within ten days by nuclear polyhedrosis virus. This virus was detected by the author among a high density of winter moth larvae in Wistman's Wood, Devon, in 1965 (see p. 20), though its effect on the caterpillar population there is unknown. In Wytham, virus does not seem to have been a major mortality factor, though Microsporidian infection regularly leads to some mortality (Varley and Gradwell, 1962a).

Predation by birds appears to be unimportant in regulating numbers

of Lepidoptera feeding on oak leaves (Varley and Gradwell, 1962a). In Wytham, populations of the chief bird predators, the great tit Parus major L. and the blue tit Parus caeruleus L., fluctuate in parallel with those of the oak-feeding caterpillars, though there was no significant correlation between clutch size and availability of larvae (Perrins, 1965). Perrins considered that the fluctuations of both caterpillars and tits were caused by a third factor, probably the weather, which might favour high bird populations in years of early bud opening on the oaks. Royama (1966), in a detailed study of the food taken by the parents to great tit nestlings, found that oak-feeding larvae were taken in quantity only for a very limited period. Moreover, a study of the predation by tits during the outbreak of winter moth in Wytham in 1948 revealed that only 2% - 5% of the caterpillars were taken (Varley and Gradwell, 1962a; Betts, 1955).

Clark (1964), in a recent study of the predation by birds on Cardiaspina albitextura Taylor (Psyllidae) on leaves of Eucalyptus spp. in Australia found that at low insect densities birds took an increasing number of both nymphs and adults as the density increased. However, the response of the birds was 'functional' and not 'numerical' (Holling, 1959), depending partly on factors other than psyllid density. When high psyllid density was strongly favoured by other environmental factors, the birds were powerless to prevent an increase to outbreak level. It seems that a similar situation may prevail in Wytham, as the tits do appear to show a functional or opportunist response to the caterpillar densities both on oak and on other trees (Royama, 1966). Morris

and co-workers (1963) have concluded that, even though both a numerical and a functional response are shown, at least by some bird species, to increasing density of Spruce budworm Choristoneura fumiferana (Clem.) larvae in Canada, the birds have no appreciable influence on the insect populations at high density.

It does not seem that numbers of tits are limited by population density of oak-feeding caterpillars during May. Winter mortality is likely to be at least as important, and Perrins (1965) found that tit numbers are greatly influenced by the size of the beech mast crop, which fluctuates in parallel with the crops of many other trees.

Summarising, therefore, we can say that some Lepidoptera species on oak are apparently efficiently controlled at a low density by their specific parasites. Oscillations in density, which would be expected on the Nicholson-Bailey model (Nicholson and Bailey, 1935), are damped by density-dependent factors, the most important of which appears to be pupal predation, resulting in relatively steady caterpillar densities. Other species, such as the winter moth, have less efficient specific parasites, which are unable to prevent the larvae from occasionally reaching such a level that complete defoliation of oaks occurs. The timing of these outbreaks is governed by the weather, which influences the crucial relative timing of bud burst and egg hatch and therefore superimposes large density independent variations on the caterpillar population levels, again partially compensated for by pupal predation. Predation by birds is unlikely to have a significant effect on controlling caterpillar populations. It does seem probable that several

caterpillar species occasionally reach the limit of their food supply, and though the larvae do not necessarily starve to death, since they turn to alternative food, subsequent populations are probably reduced owing to lowered fecundity and increased susceptibility to disease. In a sense, therefore, it can be said that there is competition for food in defoliation years.

3. Effect on the oak trees of insect damage

This thesis will largely be concerned with the effects of changes in oak leaves on the growth and survival of caterpillars. However, in view of the evolutionary implications of the insect-plant relationship, it is also relevant to look briefly at the problem the other way round and ask what effect the persistant annual attack of caterpillars has on the growth and survival of the trees.

Varley and Gradwell (1962b) estimated the amount of spring and summer growth in the annual rings of five oak trees over an eight year period by means of core samples from the tree trunks, and compared the resulting data with caterpillar density over the same period. They found that spring growth is apparently unaffected by the larvae. This one would expect, since the spring growth is largely laid down from reserves before the main caterpillar damage occurs, during the 5th instar in late May. However, there was a significant correlation between the percent mean summer growth of timber in any year and the percent mean caterpillar density in that year. Assuming a linear relationship between these two quantities, Varley and Gradwell estimate that in the absence

of caterpillars the mean summer growth of the trees, as represented in the annual growth rings, might increase by as much as 60%, and therefore the total annual timber increment by 40%. It is probable, therefore, that the caterpillars have a substantial adverse effect on tree growth.

Unfortunately, there do not appear to be data available to compare the acorn crop of oak trees with caterpillar density, which might make it possible to assess the effect of larval damage on the reproduction of the trees. This could provide some idea of a possible selection pressure giving rise to a defensive mechanism in the leaves. However, as Varley pointed out (1962b, discussion), acorn production would be expected to suffer from high caterpillar density, since developing fruits are eaten by the larvae, in addition to leaves.

Young (1965) reports that insect larvae are apparently able to kill within a short period oak trees which have previously been weakened by drought.

It is clear, therefore, that oak trees are adversely affected by caterpillar attack and it would not be surprising to find that some kind of defensive mechanism against insects has been evolved by natural selection.

4. Lack of insect attack on mature oak leaves - reason for this research

From the summary of the work so far discussed in this Introduction, it can be seen that the factors governing the population fluctuations of oak-feeding insects in the spring are now fairly well known. How-

ever, one of the most striking features of the Lepidoptera/oak leaf relationship - namely the overwhelming concentration of insect attack in the spring - is at present unexplained. The enormous mortality of around 90% suffered by first instar winter moth larvae, apparently to ensure that the remaining 10% enter the opening buds at the first possible opportunity, suggests that there is very strong selection pressure for early feeding. This selection pressure is most likely to act on the larvae towards the end of their feeding period. If such a selective force did not exist, it is difficult to understand why later egg hatch has not been selected for, to enable a far higher proportion of first instar larvae to survive. Moreover, it has been shown that the periodic defoliation of oaks leads to high larval mortality, probably through competition for food, resulting in lowered fecundity. It seems extraordinary that evolution has not favoured extension of insect feeding periods to reduce this competition by making use of the apparent abundance of relatively untouched food after early June. This also suggests that there is some very good reason why the insects are forced to feed early.

The earlier discussion of the factors regulating populations of phytophagous insects has shown that parasites and bird predators are unable to control at a low level many of the caterpillar species. It is almost certain, therefore, that parasitism and predation are insufficient by themselves to account for the suspected selection pressure forcing early feeding. It is more likely that some change or combination of changes in the nature of the leaves is responsible for the

relative lack of insect attack after mid-June.

Apart from the reasons mentioned above, there is further circumstantial evidence to suggest that mature oak leaves may be less suitable for insect growth than the young leaves. Lepidoptera larvae of species which do feed on oak leaves after the spring, such as Diurnea fagella Fab. and Biston betularia L., tend to grow relatively slowly, taking months to reach full size, compared with the three weeks, which is normal for the early species. Moreover, many of the later species (see Appendix 1, p. 150) are apparently unable to complete their growth on mature oak leaves and either drop to complete their growth on other plants or hibernate as larvae before completing their growth the following spring, either on young oak leaves or on other plant species.

The lunar double-stripe moth Minucia lunaris Schiff., although very rare in Britain, beautifully illustrates the difference in edibility of oak leaves of different ages. Although the larvae are found on oak in July and August, they will only survive on fresh tender oak leaves put out from the stumps of recently felled trees ('stool oak'). The larvae cannot survive when fed only on mature oak leaves (Kettlewell H.B.D., personal communication).

Lastly, it should be mentioned that many of the late-feeding species on oak are leaf miners, which restrict their feeding activities to the spongy parenchymatous leaf tissue, and ignore the pallisade layer and epidermis (see Figure 1, p. 2(a)). This suggests that the pallisade cells of mature leaves might in some way be less nutritious

than the cells of the spongy parenchyma.

In view of the strong circumstantial evidence, built up as a result of the long-term ecological study of oak-feeding caterpillars, that larval feeding periods might be influenced by changes in the nature of the leaves, Professor G.C. Varley suggested in 1963 that I should undertake the present research project. He was of the opinion that leaf tannins might in some way inhibit larval growth and reduce survival. Brown, Love and Handley (1962) had shown that condensed tannin was present in July oak leaves but absent in May. Furthermore, tannins are apparently located mainly in the vacuoles of the pallisade cells of the leaf (Esau, 1953 and Gradwell G.R., personal communication), which might account for the feeding behaviour of the leaf miner species.

This thesis presents the results of my investigations into this problem, involving not only a study of the possible effects of tannins on larval growth and survival, but also an investigation of leaf nutrient value and texture.

CHAPTER 1: FIELDWORK

1. Selection of trees

The area of oak trees, approximately 50 x 80 yards in extent, in which Varley and Gradwell have carried out their long-term study of insect population dynamics, is situated among mixed deciduous woodland in Wytham Great Wood, Berkshire, approximately three miles from the Oxford City boundary (Frontispiece). It was thought desirable to carry out the present research on trees in or immediately adjacent to this study area, the ecology of which was already well known. A single tree (referred to subsequently as tree M), immediately adjacent to the area mentioned above, was therefore selected for study. A census of Lepidoptera larvae was carried out on this tree in 1964 and leaf collections for chemical analysis were taken from it in all three years 1964-6.

In 1966, leaf collections for chemical analysis were extended to the five other trees studied by Varley and Gradwell, whose numbering for the trees (1, 5, 8, 9 and 15) is retained in this work. On May 6th, 1966, the longest leaf in each of twenty terminal leaf clusters from the lower shade branches of each of the six study trees were measured to gain an idea of the relative bud-opening dates (Appendix 2, p. 161). The means, with standard errors of the means, were as follows:

Tree:	M	1	5	8	9	15
Mean Leaf Length (cm.)	5.5	2.4	3.3	3.5	2.9	2.2
S.E.M.	± 0.2	± 0.1	± 0.2	± 0.1	± 0.1	± 0.1

Representative leaf clusters from each tree are shown in Figure 3a (p.15(a)). The measurements show that tree M is the earliest and tree 15 the latest of the six trees.

In addition to the six trees mentioned above, leaves were occasionally collected from elsewhere in Wytham Woods for laboratory experiments.

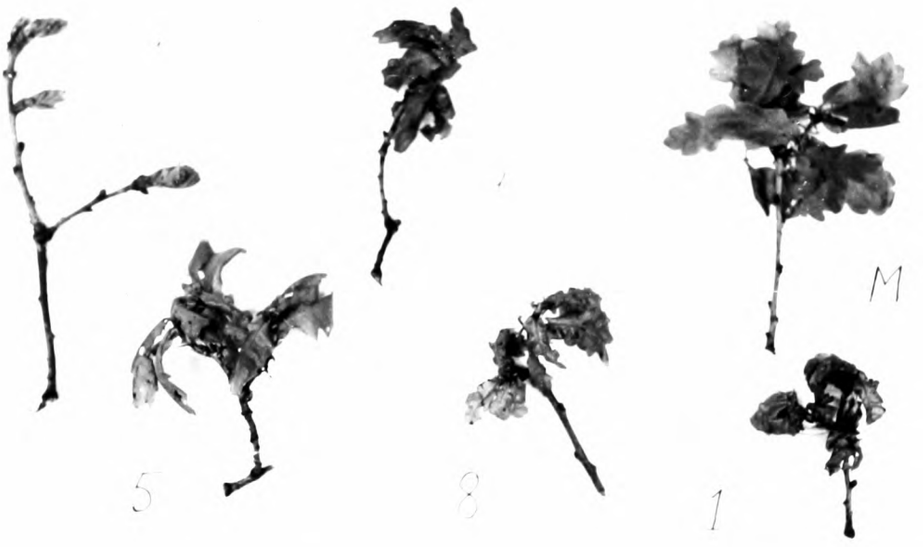
2. Insect census on tree M.

A census of the caterpillars feeding on tree M was taken in the spring of 1964, in order to obtain an idea of the population density on the tree and to gain familiarity with the dominant species. On four dates from May 12th to June 1st, 25 leaf clusters were collected, as far as possible at random, from both the upper and lower branches of the tree and the number of insects of each species on each sample counted. The results are given in detail in Appendix 3 (p.162) and in abbreviated graphical form in Figure 4 (p.15(b)). The number of larvae dropping from the tree to pupate was estimated by the method used by Varley and Gradwell. Two metal trays, each $\frac{1}{2}$ sq. m. in area, were placed beneath the tree canopy within 10 ft. of the trunk. The trays contained sufficient water to drown all the larvae falling into them and were protected from bird predation by wire netting (Figure 3b, p.15(a)). The number of larvae which had fallen into the two trays was counted at eight 3 - 5 day intervals between May 22nd and June 16th. These results are also given in Appendix 3 (p.162) and, in graphical form, in Figure 4 (p.15(b)). It can be seen from Figure 4 that the peak fall of fully fed winter

FIGURES 3a and 3b

FIGURE 3a Oak twigs from the lower branches of the six study trees on May 6th., 1966, to indicate the relative order of bud burst.

FIGURE 3b Expanded polystyrene refrigeration container, used for transport of leaf samples before analysis, and metal tray ($\frac{1}{2}$ sq. m. in area) used for sampling Lepidoptera larvae dropping to pupate in the soil.



OAK TWIGS MAY 6th 1966



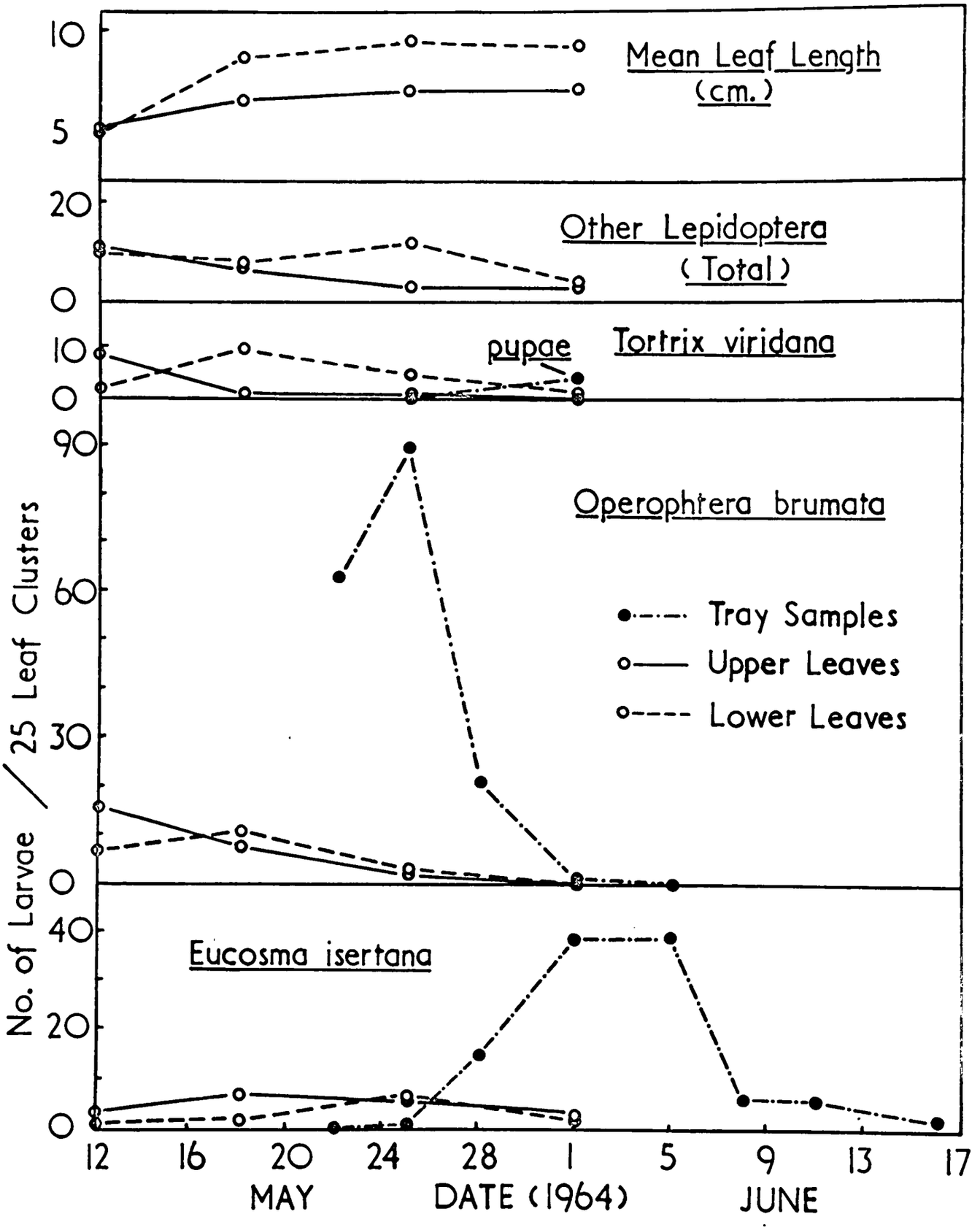


FIGURE 4 Census of Lepidoptera larvae on tree M, 1964.

moth larvae took place around May 25th, followed several days later by Eucosma corticana Hubn. (= isertana Fab.). The number of larvae counted on the 50 leaf clusters fell from 59 on May 12th to 13 on June 1st. Altogether, 175 winter moth larvae fell into the 1 sq. m. of tray area, so that the winter moth density on the tree was of the order of 200 per sq. m. ground area, since a number had probably already fallen prior to the first tray sample. Corresponding figures obtained by Varley and Gradwell (unpubl.) for winter moth density in 1964 on the other five trees were 310 per sq. m. on tree 5 (highest density tree) and 20 per sq. m. on tree 15 (lowest density tree). Surprisingly, 108 larvae of E. corticana fell into the two trays beneath tree M, indicating that this species was present at about half the density of the winter moth. From the insect densities obtained, it seems that tree M has a fairly high caterpillar population compared with other trees in the vicinity. This is consistent with its relatively early date of bud opening (see p. 15).

The lower branches of tree M and three other trees in its vicinity were thoroughly beaten for larvae on three later dates in 1964 and produced the following results:

July 1st.	nil.
July 24th.	2 Bena prasinana L. larvae
September 30th.	nil.

Although leaf miner species are not detected by beating, the contrast with the insect density of several hundred larvae per sq. m. earlier in the year is, nevertheless, striking.

3. Collection of leaf samples for analysis

Buds and leaves from tree M and subsequently from the other trees were always collected as intact leaf clusters to reduce the risk of chemical change during return to the laboratory, where the individual leaves or buds were cut off with scissors immediately before homogenisation in the appropriate solvent. To further minimise the risk of chemical change, the leaf clusters were collected into polythene bags and at once placed in a specially constructed portable refrigeration box, made of expanded polystyrene and containing crushed ice, for transport back to Oxford (Figure 3b, p. 15(a)).

Representative leaf samples for analysis were ensured by spreading the collection of leaf clusters over several branches in the appropriate part of the tree. All the leaf clusters were then mixed together after return to the laboratory before samples of leaves were cut away and weighed prior to analysis. Most analyses were carried out on leaves or buds from the south side of the tree crown ('upper sun leaves') and from the northern branches at the base of the tree ('lower shade leaves'), since it was considered that these two regions would provide the two extremes of any chemical variations among the leaves of a single tree. Bud or leaf samples were occasionally taken from other parts of the tree and these are specified in the relevant analytical results.

Leaves were always collected between 1400 hrs. and 1630 hrs. B.S.T. to reduce any variation in results due to time of day. Samples for sugar analysis were collected as far as possible on days of high sun-

shine hours, again to reduce variation due to differing rates of photosynthesis in different weather conditions.

The leaves were always processed in the laboratory during the evening immediately following the afternoon collection. Samples of leaves from each randomised collection of leaf clusters were dried at 80°C to constant weight in an oven to obtain the moisture content of the leaves, in order to convert analyses of leaf components into dry weight percentages.

4. Selection of larvae for laboratory experiments

The winter moth was selected for laboratory study, since it is the species for which the necessity for early feeding has most clearly been demonstrated (see Introduction). It is also the most abundant species on oak, its life history is well known and its eggs can be conveniently collected from the field for laboratory experiments by trapping the wingless females as they climb the trunks of the trees after mating in November and December. It was expected that conclusions reached from experiments with the winter moth would in all probability apply to many of the other early-feeding species.

The life cycle of the winter moth is as follows. After emergence in November and December and pairing on the oak trunks at night (Figure 6b, p.20(a)), the wingless female climbs to lay its eggs (about 150) in crevices in the bark of twigs high up in the crown of the trees. These eggs hatch the following April at about the time of bud burst (see Introduction) after which the caterpillars (Frontispiece) feed on the young oak leaves, passing through five instars before they spin

down to the ground on silk threads as fully fed larvae in late May. On reaching the ground, they pupate within a short distance of the soil surface, where they remain until the adults emerge to repeat the cycle the following winter. There is thus only one generation each year.

5. Wistman's Wood

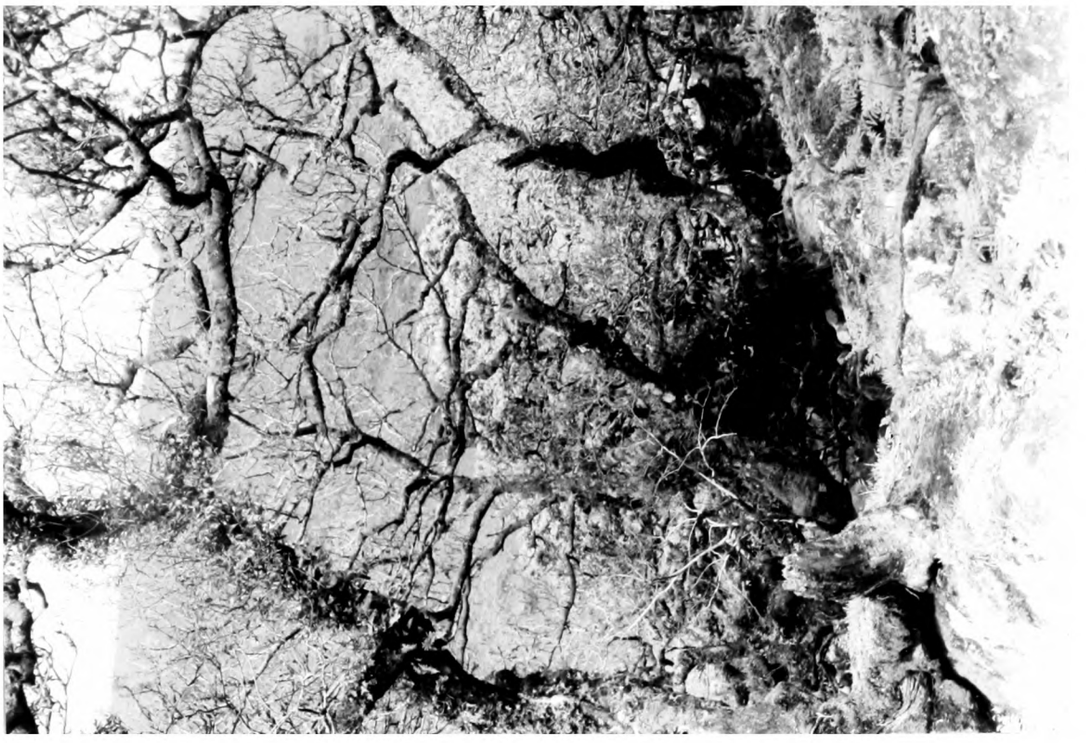
Towards the middle of Dartmoor, Devon, there is a small and very isolated oak wood known as Wistman's Wood. This is thought to be a natural relict of the formerly much more extensive Dartmoor Forest and has a number of curious features, perhaps partly attributable to its exposed isolation and elevation (1500 ft. a.m.s.l.). Although many of the trees are apparently old, they rarely reach a height of more than 20 ft. They are stunted and carry an extensive epiphytic flora of ferns and mosses, which also cover the boulder-strewn ground beneath the trees (Figure 5a, p. 19(a)). In the present context the Wood is remarkable in that it apparently carries a very high density of winter moth, which has led to extensive defoliation for at least the last three years (Varley G.C., personal communication). Also, probably because of the temperature effect of altitude , bud-burst and larval egg hatch occur annually about two to three weeks later than in Wytham.

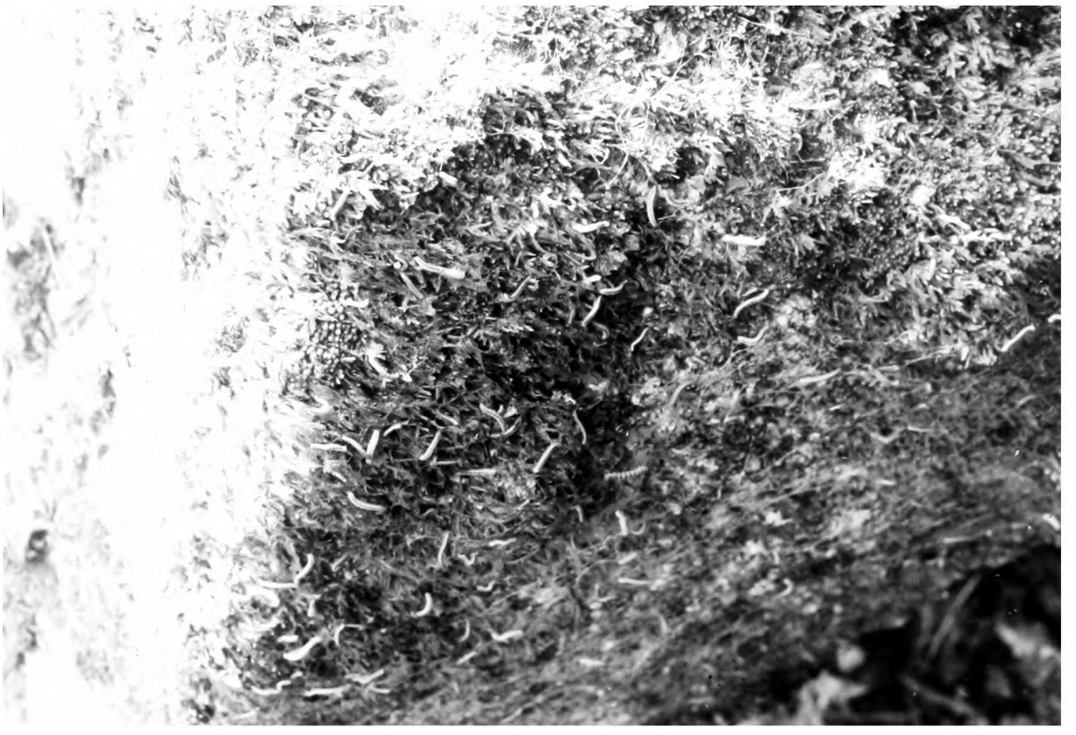
A visit was made to Wistman's Wood on June 17th-18th, 1965, with the object of collecting larvae for laboratory experiments, since stocks of Wytham insects were no longer available. As expected, the wood was largely defoliated, though some trees had escaped extensive damage (Figure 5a, p. 19(a)), probably owing to their relatively late

FIGURES 5a and 5b

FIGURE 5b Winter moth larvae on moss-covered boulders in Wistman's Wood, Devon, June 1965.

FIGURE 5a Part of Wistman's Wood, Devon, showing oak trees defoliated by winter moth larvae. In the background can be seen an oak tree which has escaped defoliation, probably owing to late bud burst. In the foreground can be seen boulders, one of which is shown in Figure 5b.





bud burst (see p. 4). Both the trees and the boulders beneath them were found to be carrying very large numbers of larvae, largely winter moth with some Operinia dilutata Schiff. and a very few Erannis spp. (Figure 5b, p.19(a)). Having stripped the trees of foliage, the larvae - many still in their fourth instar - were evidently attempting to complete their growth on the mosses covering the boulders. Several hundred larvae were collected and brought to Oxford. Of these, a number were used in an experiment to determine the effect of leaf texture on growth (p. 30). In the course of this experiment, several larvae died. On treating gut smears of four of these larvae with Gemsia stain (Smith and Xeros, 1953), Mr. J.F. Longworth, of the Insect Pathology Unit, Oxford, detected nuclear polyhedrosis virus in very high density in the smears from two of the larvae (Figure 6a, p.20(a)). The virus was considered to be the cause of death.

This is the first recorded instance of nuclear polyhedrosis affecting wild winter moth larvae in Britain, though it has been reported in Canada and elsewhere (Longworth J.F., personal communication).

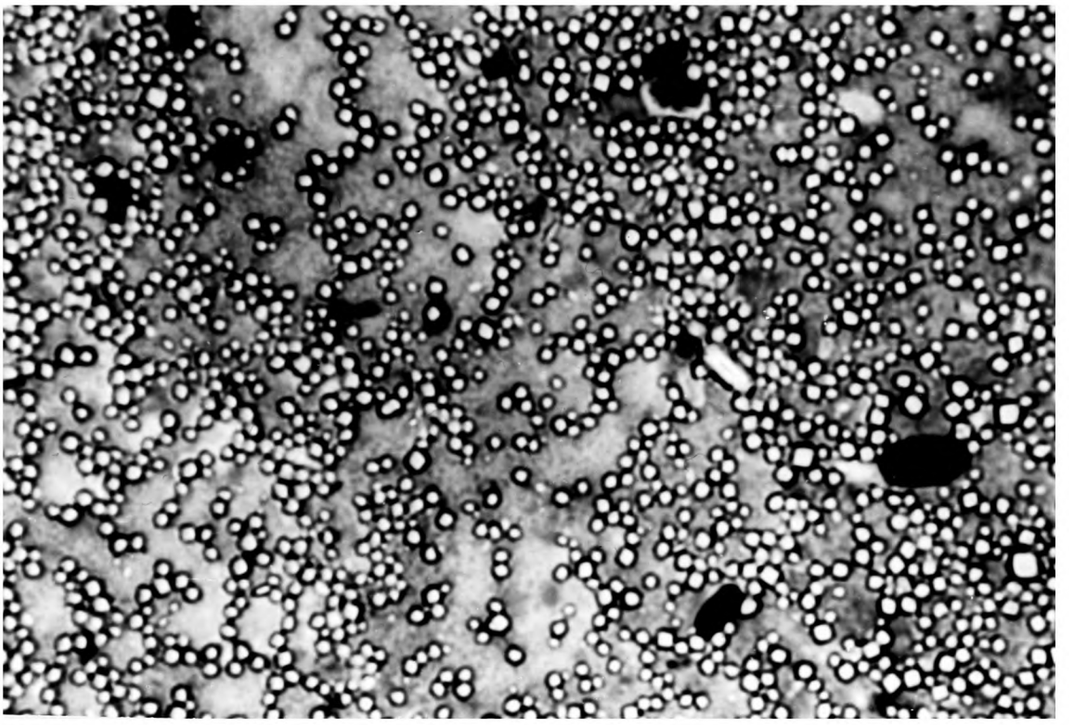
6. Comparison of growth and survival of winter moth larvae on oak leaves of different ages

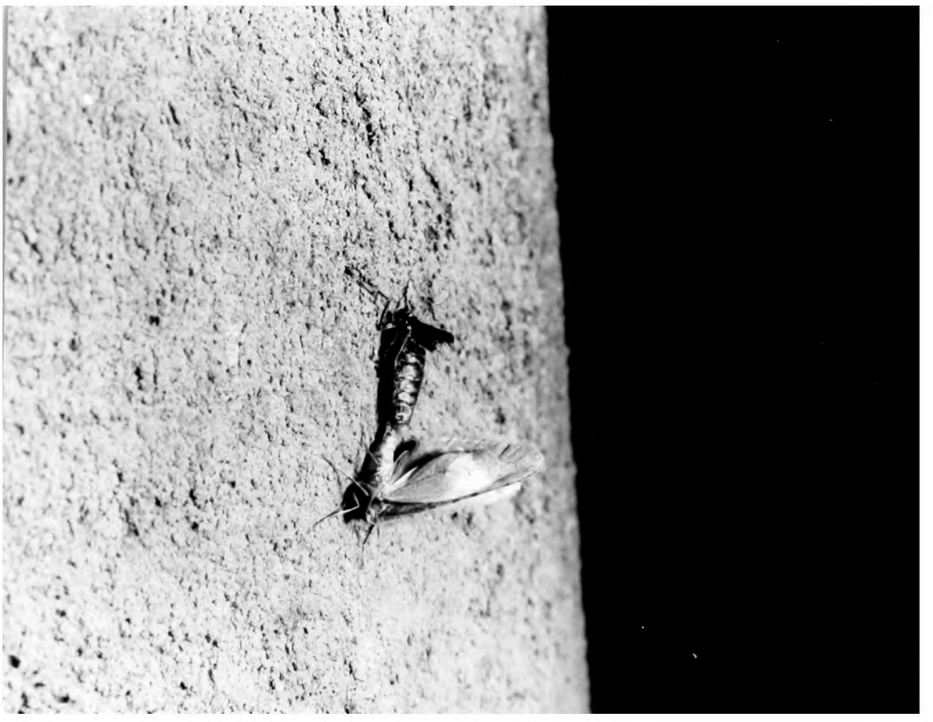
To determine whether winter moth larvae are able to grow satisfactorily on leaves slightly older than those they would normally encounter during their growth period in the field, a comparison of growth rates and pupal weights obtained on two leaf diets of different

FIGURES 6a and 6b

FIGURE 6a Virus inclusion bodies revealed in the gut smear from a winter moth larva, collected from Wistman's Wood, Devon, 1965. (Gemsia stain; magnification 2,000).

FIGURE 6b Winter moth pair in copulation on the trunk of a tree in Wytham Wood, December, 1965. Wingless female uppermost. Photograph taken with electronic flash at about midnight.





age was carried out in the spring of 1964. Lower shade oak leaves from tree M were collected on May 16th and at once transferred to the deep-freeze. A further collection from the same part of tree M was made on May 28th and these leaves were stored in the deep-freeze for one night to equalise any effects of deep-freeze storage between the two leaf collections. On May 29th, leaves from each sample were thawed at room temperature and placed in two sets of 25 small plastic containers with perforated snap-on polythene lids (Figure 35, p. 85(a)), labelled L 1 - 50. One May 16th leaf was placed in each of the containers L 1 - 25 and one May 28th leaf in each of the containers L 26 - 50.

Meanwhile, the hatch of about one hundred winter moth eggs, collected from females the previous winter, had been delayed by cooling in a refrigerator at 4°C and the resulting larvae reared to the fourth instar on the youngest oak leaves which could be found in Wytham. The May 16th leaves corresponded approximately to the leaves on which these fourth instar larvae would have been feeding, had they been allowed to hatch under natural conditions in Wytham.

On May 29th, 25 pairs of larvae were withdrawn from the rearing container and assigned at random to the two leaf diets. When the two sets of larvae were complete, each individual was weighed, after which the two sets of containers were intermingled and stored in the laboratory at room temperature and humidity.

At intervals of a few days, the larvae were reweighed to assess their growth rates. On each occasion, the leaves in the containers

were renewed. May 16th leaves were replaced by leaves from the original deep-frozen sample. May 28th leaves were replaced by lower shade leaves collected from tree M on the day before each weighing and stored overnight in the deep-freeze.

The weights recorded for the individual larvae until June 26th, when all survivors had pupated, are given in Appendix 4 (p. 164). The mean larval weights, calculated from this data, are plotted against date in Figure 7 (p. 22(a)), where the 5% confidence limits are also indicated for the mean peak weights and mean final pupal weights.

The mean growth curves can be divided into three phases. After a rapid growth phase during the fourth and especially the fifth instar, larval weights decline after reaching peak weight owing to loss of water during the non-feeding pre-pupal period. The curves finally level out as pupation is completed.

It is at once apparent from Figure 7 (p. 22(a)) that both larval peak weights and final pupal weights were markedly lower on the late leaf diet than those attained on the May 16th leaves, which the larvae would normally encounter in Wytham. The difference between the mean pupal weights was tested by the Student's t-test (Bailey, 1959) and found to be significant ($P < 0.001$).

Of the 21 pupae resulting from larvae fed on the May 16th leaves, ten eventually hatched into adult moths whereas, of the ten pupae attained on the later leaves (May 28th onwards), no adults emerged. It can be concluded, therefore, that the reduced pupal weights reached on late leaves indicate a reduced survival. Gradwell (unpubl.) has found that low pupal weight of wild winter moth females tends to be

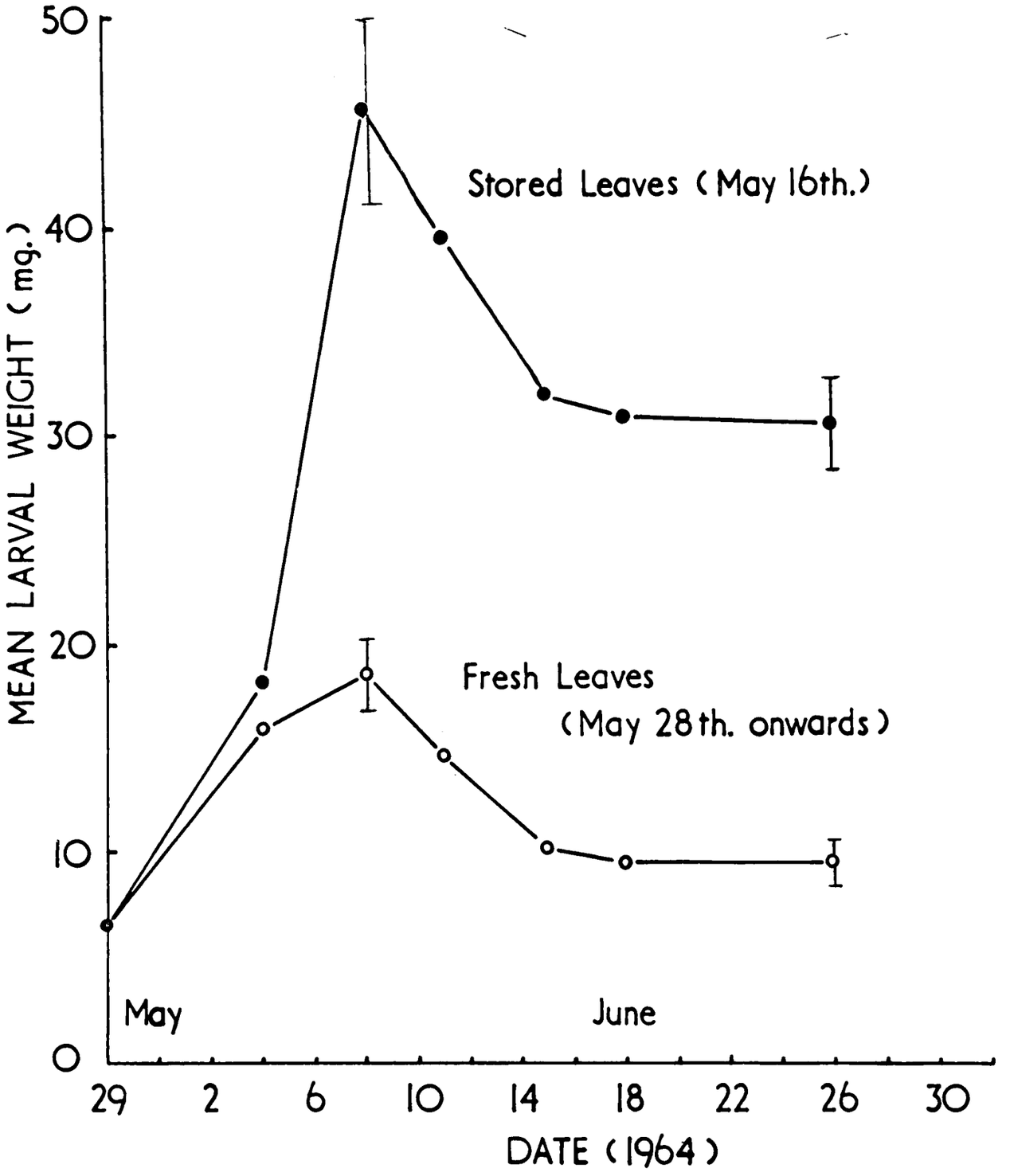


FIGURE 7. Mean larval and pupal weights of winter moth samples, fed on oak leaves of different ages. Initial steep rise in fresh weight represents 4th. and 5th. instar larval feeding. This is followed by decline in weight during pre-pupal phase, until fairly constant pupal weight is reached.

associated with reduced adult weight, which is in turn correlated with lowered egg production. It seems, therefore, that subnormal pupal weights are an indication of an adverse effect on the subsequent generation of insects, either as a result of reduced pupal survival or, in the case of pupae which succeed in hatching, as a result of reduced fecundity.

Inspection of Figure 7 (p. 22(a)) reveals a second point of interest, namely that reduced pupal weight follows a reduced growth rate and peak weight of the larvae. This is not surprising, since a proportion of the peak body weight must always be lost by metabolism and evaoporation, as well as by emptying of the gut contents, during the non-feeding pre-pupal phase. Although the proportion of loss in weight may vary to some extent with the peak weight, it is nevertheless likely that a low peak weight is usually followed by a low final pupal weight. It would clearly be desirable to confirm this correlation on the basis of dry weight or percentage tissue nitrogen. This would involve very large samples of larvae, from which individuals could be withdrawn on each weighing date for dry weight or nitrogen estimation. In view of the short duration of the larval feeding period and the amount and variety of the work to be carried out during it, such large scale experiments were considered impracticable for the investigations described in this thesis, in which inferences have been tentatively drawn from peak and pupal fresh weights.

Summarising, the experiment shows that some change or combination of changes occurs in oak leaves over a period of only two to three

weeks in late May and early June which has a markedly adverse effect on the growth rates, pupal weights and survival of fourth and fifth instar winter moth larvae feeding on the leaves. This result apparently confirms the inference from field observations (see Introduction, p. 10) that winter moth caterpillars are likely to suffer a high mortality unless they begin feeding at the earliest possible opportunity. The conclusion from the experiment rests on one assumption, namely that growth of larvae on leaves which have been deep-frozen is similar to growth on fresh leaves of comparable age. This assumption was considered to be reasonable since the only major effect of deep-freeze storage of leaves is the rupture of the cell membranes due to expansion of the cytoplasm and vacuoles on freezing. This is unlikely to affect the gross chemical composition of the leaves (though some enzymic changes probably occur on thawing) or to significantly alter their texture, which is largely governed by the cellulose framework.

The remaining chapters of this thesis will be devoted to an account of investigations into the possible reasons for poor growth and survival of winter moth larvae on mature oak leaves.

CHAPTER 2: EFFECT OF LEAF TOUGHNESS ON GROWTH OF WINTER MOTH LARVAE

1. Introduction

The most obvious change occurring in young oak leaves, apart from their rapidly darkening colour during May, is their increase in toughness. From being thin and fragile structures in early May (Frontispiece), oak leaves rapidly become thicker and more difficult to tear apart. By the end of May, they are already relatively rigid as they approach their full size. The increasing toughness of the leaves is presumably due largely to the deposition of cellulose, hemicelluloses, pectins and other materials which consolidate the cell walls. It is probably also connected with the decreasing water content of the leaves, rendering them increasingly less succulent. The cellulose content of oak leaves was not measured during the present study, but the variation in water content during the year was found from the leaf dry weights, obtained as described in Chapter 1 (p. 18). The data for tree M during 1965 are shown in Figure 8 (p.25(a)), and reveal a sharp drop in the water content of the leaves during late May and early June.

It was considered that the increase in leaf toughness might have a significant effect on the ability of insect larvae to feed satisfactorily and that such an effect might account for the results obtained in Chapter 1. Tanton (1962) has shown that mustard beetles

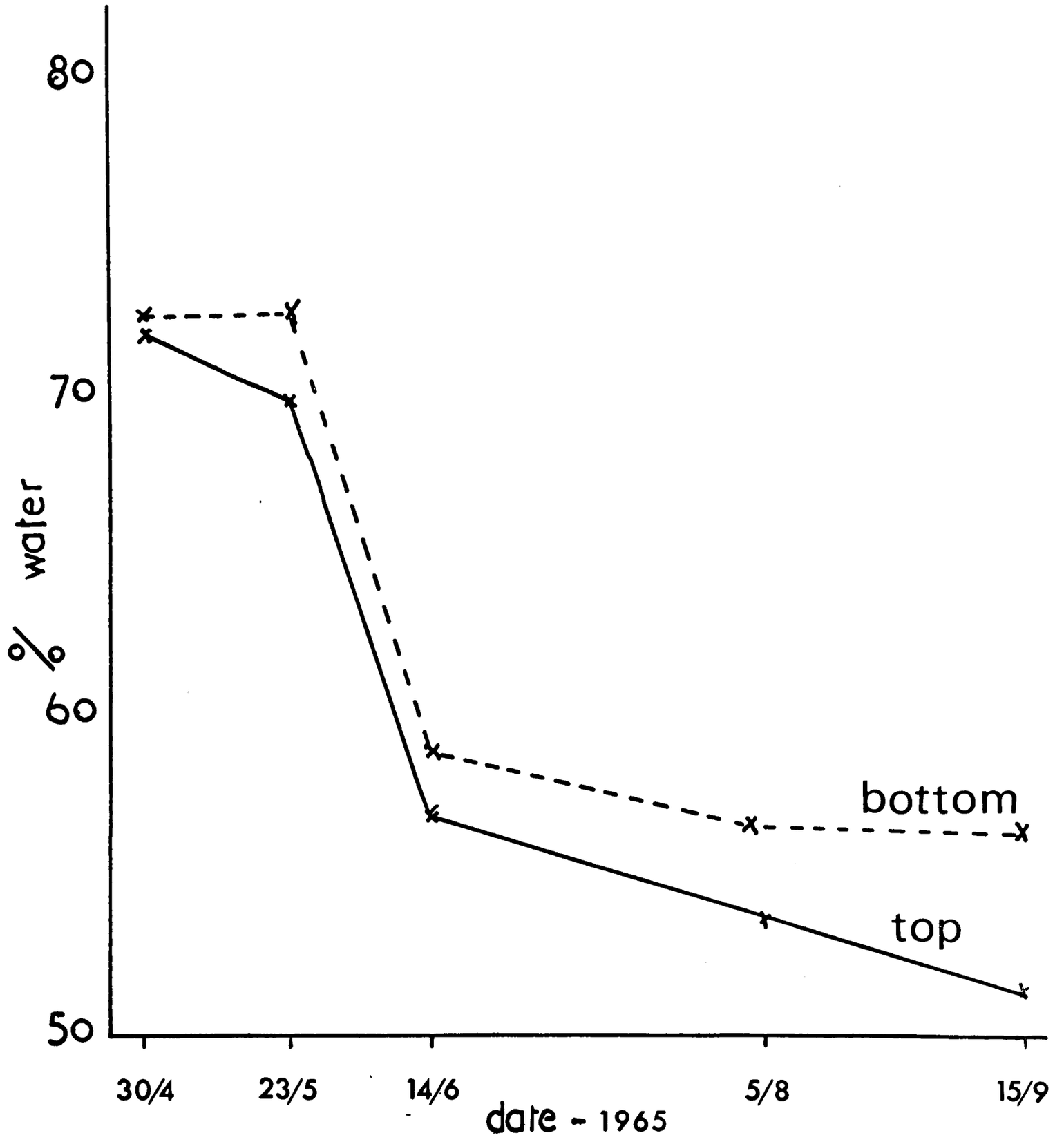


FIGURE 8 Water content of top and bottom leaf samples from tree M during 1965.

Phaedon cochleariae Fab., reared on the leaves of kale, turnip and other vegetables, suffer greater larval and pupal mortality as the texture of the leaves is increased. It has also been shown (Williams, 1954) that leaf toughness has a considerable influence on the choice of food plant by young locusts and grasshoppers.

2. Estimation of leaf texture - method

A method must clearly be devised to quantify the apparent changes in leaf toughness. In the present context, moreover, the method must be capable of measuring quantities which are related to the ability of an insect to bite off and ingest pieces of leaf tissue. The situation is further complicated by the heterogeneous structure of leaves, in which much of the tensile strength is restricted to the leaf ribs. Simple estimation of the tension required to pull a leaf apart, analogous to industrial methods for measuring the tensile strength of fabrics, plastic sheeting, etc., is therefore likely to give misleading results.

Williams (1954) describes a 'penetrometer' which he used to estimate leaf texture in connection with experiments on the feeding habits of Acrididae. His method, with minor modifications, has since been used by Tanton (1962). The principle of the method is simply to measure the force required (estimated as weight of sand) to cause a sharp metal needle to pierce the tightly stretched leaf. Although this method gives results which are apparently satisfactory, it is considered by the present author that the piercing action of a sharp needle may not be the best available method for simulating the biting

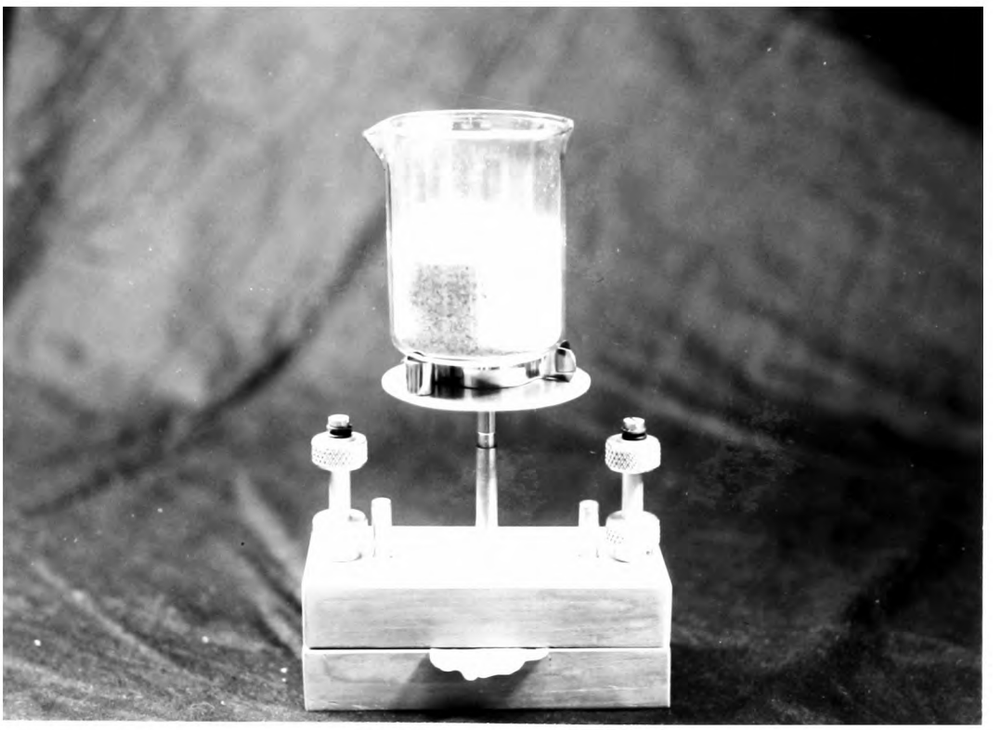
action of an insect. The action of the mandibles is likely to involve a combination of shearing and tearing and the force required to shear or tear a leaf is not necessarily related to that required to pierce it with a sharp needle. A method was therefore sought which might provide a more relevant estimate of leaf texture.

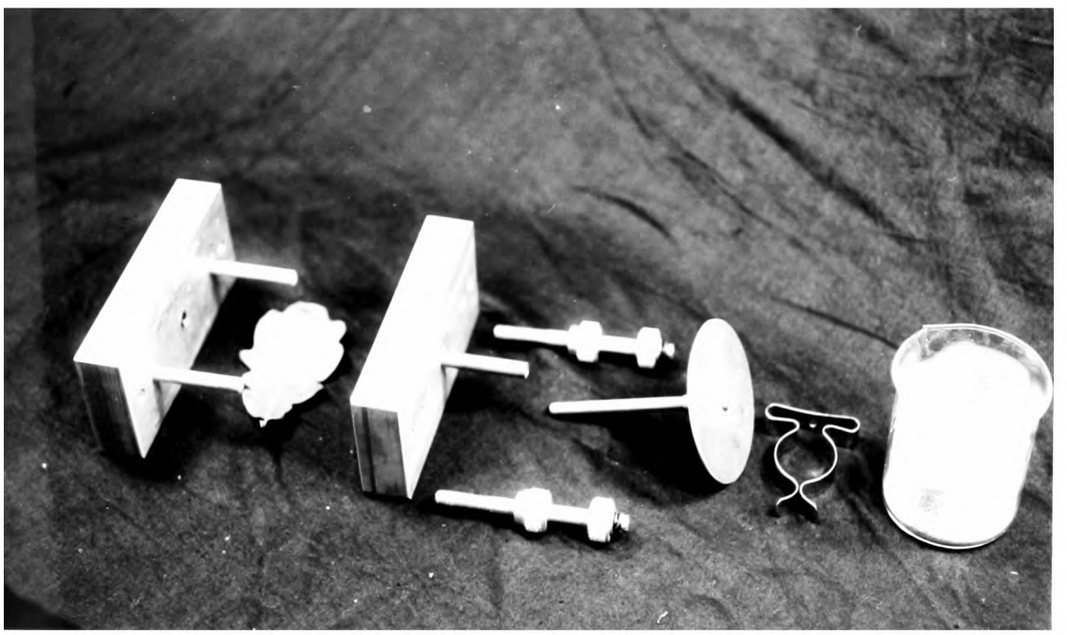
After consultation with members of the Department of Engineering, Oxford, it was decided that the best method of simulating the action of the mandibles of a caterpillar would be to punch out a disc of tissue from the leaf with a metal rod, entirely flat on its lower surface. The lower surface of the rod would be slightly rounded along its circumference to avoid immediate tearing of the leaf by a sharp metal edge. The action of the rod would thus be to break the leaf tissue initially by a shearing action, after which the disc of tissue would be detached from the remainder of the leaf by a combination of shearing and tearing forces, as is considered to happen when leaves are eaten by caterpillars. The texture of the leaves would then be estimated by measuring the force required to detach the leaf disc.

A suitable device was accordingly designed and the construction carried out by Mr. F. Filipowski in the Zoology Department workshop. The complete assembly, five inches in length, is shown in Figure 9a (p.27(a)) and its component parts in Figure 9b (p.27(a)). The leaf is placed between the upper and lower halves of an aluminium block, which are then fastened together by two slip screws, ensuring that the tension applied to successive leaves is reproducible. A cylindrical

FIGURE 9a Device for measuring toughness of oak leaves.

FIGURE 9b Component parts of device for measuring toughness of oak leaves.





hole runs through both halves of the block and is lined with a brass sleeve. Precise alignment of the holes in the two halves of the block is assured by two guide rods, screwed firmly into the lower half of the block, down which the upper half of the block slides into position by a corresponding pair of holes. The brass sleeve lining the central hole in the upper half of the block is extended upwards for 1½ inches. Into this is inserted the steel punching rod, fractionally narrower than the lining sleeve, through which it runs easily. For reasons mentioned above, the lower edge of the rod is very slightly rounded. To its upper end is attached a circular metal plate, on which is placed a beaker. This is gradually filled with sand at a constant rate from a wide bore chromatography column. As soon as the lower end of the punching rod punches a disc of tissue from the leaf, the rod drops suddenly into the hole in the lower block. As soon as the rod drops, the flow of sand is stopped and the beaker with its contents weighed on a Mettler balance. The punching rod and the inside of the sleeves are cleaned carefully with a pipe cleaner dipped in ether before repeating the procedure on the next leaf.

Leaves were placed between the two halves of the block in such a way that the disc of leaf punched out was well clear of the major leaf ribs. This could be checked when the leaf had been screwed into position by looking through the central hole against the light, before the central rod was inserted.

3. Difference in leaf toughness between May and June oak leaves

The device described in the previous section became available

in time for the 1966 season only and it was unfortunately not possible to make a detailed comparison of leaf toughness between different trees or between different parts of the same tree. However, the device was used to assess quantitatively the change in toughness of similar leaves from a single tree over the period in late May and early June when most of the caterpillars pupate.

Samples were taken from the lower sun leaves of tree 9 on May 19th and June 10th, 1966. These dates correspond approximately with those of the two leaf samples fed to winter moth larvae in the experiment already described in Chapter 1 (p.20). On each occasion, a large number of leaf clusters were collected and transferred to the laboratory in the polystyrene refrigeration box, containing a layer of crushed ice. The leaf clusters of each sample were thoroughly mixed before individual clusters were withdrawn at random. Although it is realised that some variation of toughness can be expected between the leaves of a single cluster, to make comparison between the two samples more precise, toughness estimations were made only on the longest leaf in each of the clusters withdrawn. From casual observation, it appeared that variation of toughness between the leaves of a single cluster was trivial in comparison with the variation between May and June leaves, though it is now realised that this should have been confirmed by measurement.

In all, twenty leaves from each sample were measured for toughness. The results are shown in Appendix 5 (p.167) and in Figure 10, where the toughness values are plotted graphically against leaf

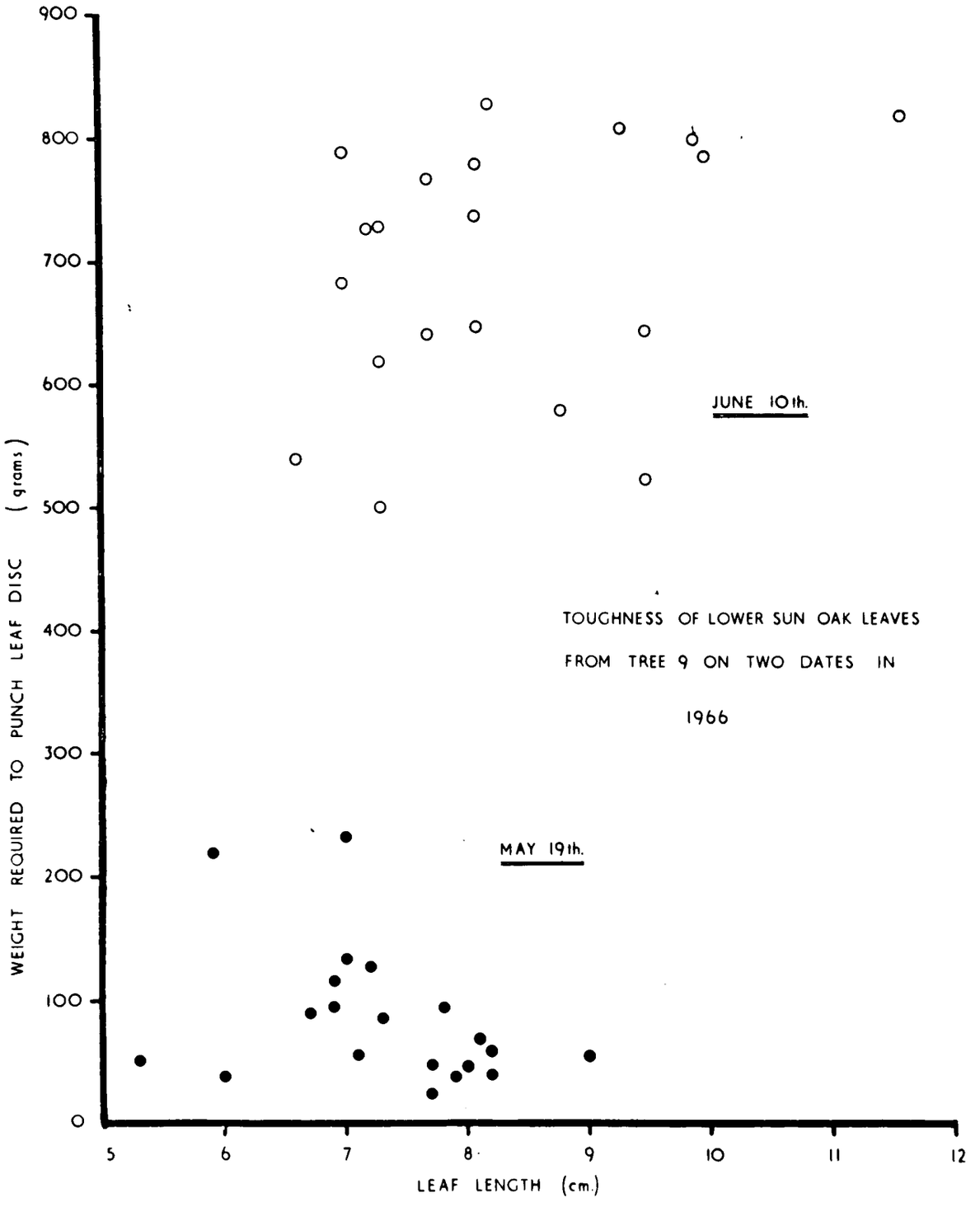

900
800
700
600
500
400
300
200
100
0
WEIGHT REQUIRED TO PUNCH LEAF DISC (grams)
5
6
7
8
9
10
11
12
LEAF LENGTH (cm.)
JUNE 10th.
MAY 19th.
TOUGHNESS OF LOWER SUN OAK LEAVES
FROM TREE 9 ON TWO DATES IN
1966

FIGURE 10

length. Although there is evidently considerable variation between the toughness values obtained within each sample, it can be seen that there is a substantial difference between the two samples, with no overlap of results. In fact, the force required to punch out a leaf disc in the manner described is approximately seven times greater on June 10th than it is on May 19th. - a difference of only 22 days. This result clearly shows the rapid increase in the toughness of oak leaves during late May and early June which might well be responsible for the results obtained in Chapter 1.

4. Effect of leaf texture on growth of winter moth larvae - methods

The method adopted for investigating the effect of leaf texture on the growth of winter moth larvae was that used by Moreau (1965). By powdering dried leaves of different ages and incorporating them in plain agar diets, he was able to eliminate the effect of leaf toughness in his study of the growth of Arctia caja L. larvae on vine leaves of different ages. By adopting this method in the present work, it was hoped to discover the effects, if any, of eliminating leaf toughness differences on the growth, pupal weights and survival of winter moth larvae fed on oak leaf diets of different ages. Any effects of leaf toughness would be revealed by comparison of the results with those obtained on intact leaves of similar ages (Chapter 1).

Lower sun oak leaves were collected from tree M on May 13th and June 1st, 1965. The leaves were transferred rapidly from the polystyrene refrigeration container to an oven, already at 100°C, to

minimise enzymic changes in the leaves. The dried leaves were coarsely powdered with a mortar and pestle and then ground to a very fine powder in a ball mill.

The two leaf powders were incorporated into agar diets, which contained the following ingredients:

Distilled water	22 mls.
Leaf powder	14 gms.
Aureomycin	0.03 gm.
Methyl p-hydroxybenzoate	0.15 gm.
Vitamin solution	1.0 ml.
Agar (plain Japanese)	2.5 g., dissolved in 62 mls. distilled water

The aureomycin and methyl p-hydroxybenzoate were included as antibiotics. The vitamin solution, the composition of which is given on p. 212, was included to compensate for the probable destruction of the leaf vitamins during the drying process. The first five ingredients were thoroughly mixed in an M.S.E. 'Atomix' blender, whereupon the agar solution was added at about 70°C and the whole mixture blended until homogeneous. The diets were poured into autoclaved glass jars and allowed to set to a fairly rigid gel. A number of small plastic pots, about 1½ inches in diameter and 1 inch deep, with snap-on polythene lids were obtained from P.K. Dutt and Co. Forty of these were thoroughly washed and rinsed in 80% ethanol, to effect partial sterilisation (plastic containers have the advantage of being very cheap, but they cannot be autoclaved). The pots were labelled M 1 - 20 and J 1 - 20. About 2 grams of the May

13th and the June 1st leaf diets, respectively, were then placed in each pot of the two series.

On June 5th, 1965, forty 5th instar winter moth larvae, which had been collected the previous morning from Wistman's Wood on Dartmoor (p. 19), were weighed and placed individually at random in the pots. A disc of What an No. 1 filter paper was placed over each pot to regulate humidity and the lids, perforated with small holes, then snapped on. The pots of the two series were then intermingled and placed upside down on a wire mesh tray on a laboratory bench, out of direct sunlight. The pots were inverted so that frass pellets from the larvae would drop on to the filter paper disc instead of fouling the diet. The arrangement of a pot with its constitutent parts is shown in Figure 25, (p.85(a)).

At intervals of a few days, the larvae were re-weighed. On account of heavy mortality, the experiment was discontinued after June 17th, when the samples had become too small for meaningful analysis and when most of the surviving larvae had pupated.

5. Mortality

It was found that 8 out of the 40 larvae had been parasitised prior to the experiment, and developed into pupae of Phobocampe crassiuscula (Grav.). Of the remainder, only 14 survived until the end of the experiment. The mortality was puzzling, since there was no obvious cause of death, such as extensive growth of fungus on the diets. Apparently healthy larvae were transformed within a day or so into fluid-filled carcasses. Smears of the 'gut contents' of six of these corpses were examined by Mr. J.F. Longworth and at least two

were found to contain a high density of nuclear polyhedrosis virus inclusion bodies (see p.20 and Figure 6a, p.20(a)). The virus was considered to be responsible for at least part of the mortality encountered in this experiment.

6. Effect of leaf texture on growth of winter moth larvae - results

The larval weights obtained on the six weighing dates, together with their means and relevant statistical data, are given in Appendix 6 (p.168). The mean larval weights are plotted against date in Figure 11 (p.33(a)). For simplicity, the 5% confidence limits are only indicated in Figure 11 for the second re-weighing, though it can be seen from the data in Appendix 6 that the confidence limits of the two curves overlap throughout the experiment. Only those larvae were considered for analysis which were known to be unparasitised and which showed a weight increase at the first re-weighing. Prepupae and unhealthy larvae were eliminated by this means, and are indicated by brackets in Appendix 6.

It can be seen from Figure 11 that at no point do the mean larval weights become significantly different, indicating that growth on the two diets is similar. It is possible that the use of larger samples and younger larvae would have shown up a difference in the growth achieved, but the small samples available in this experiment are sufficient to show that there is no very great difference in the amount of growth achieved, such as was found when larvae were fed on whole leaves of a similar difference in age (Figure 7, p.22(a)).

Because such a small number of larvae survived to the pupal stage,

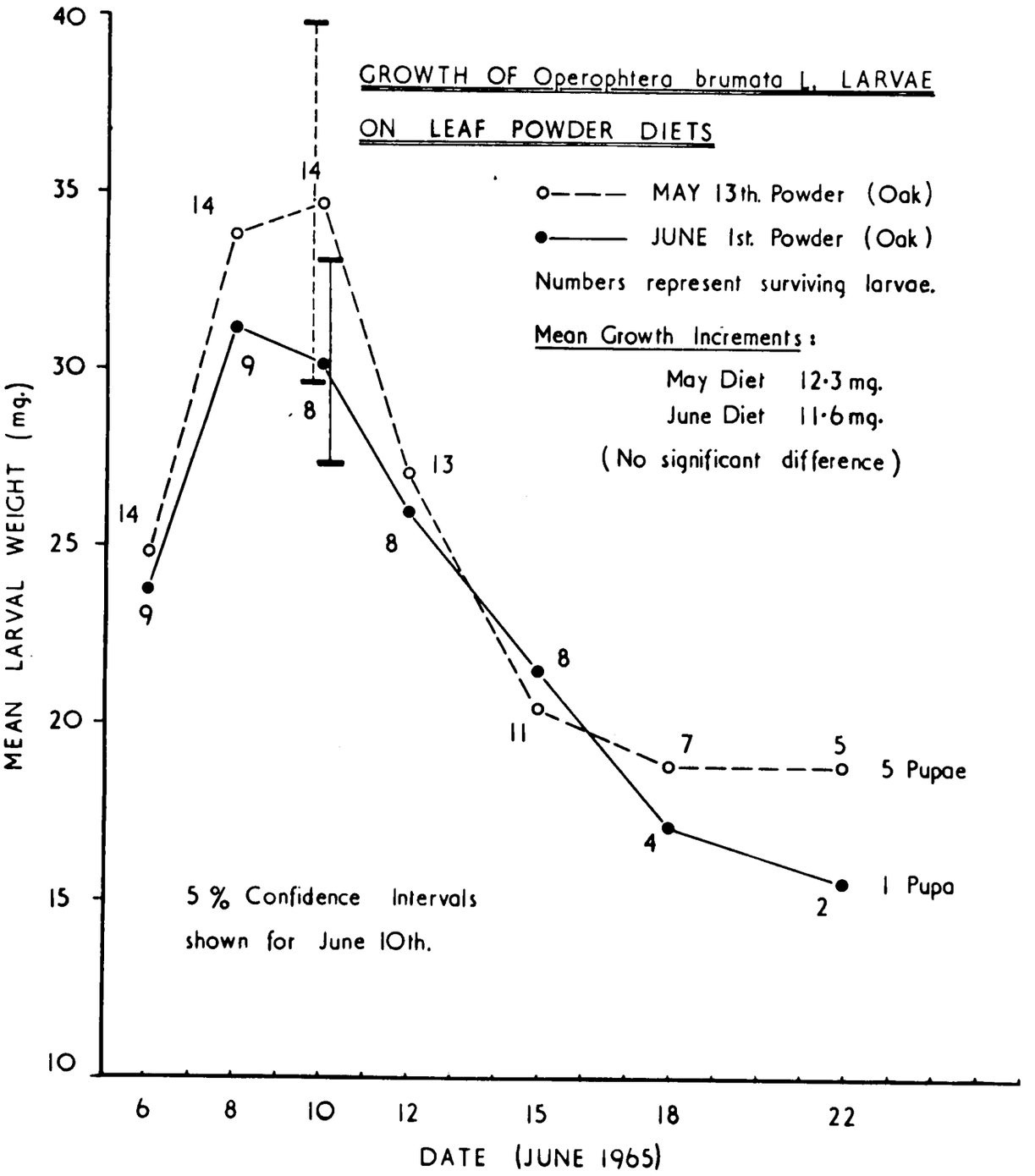

GROWTH OF Operophtera brumata L. LARVAE
ON LEAF POWDER DIETS
MAY 13th. Powder (Oak)
JUNE 1st. Powder (Oak)
Numbers represent surviving larvae.
Mean Growth Increments :
May Diet 12·3 mg.
June Diet 11·6 mg.
(No significant difference)
5 % Confidence Intervals shown for June 10th.
5 Pupae
1 Pupa
MEAN LARVAL WEIGHT (mg.)
DATE (JUNE 1965)
40
35
30
25
20
15
10
6
8
10
12
15
18
22

FIGURE 11

it was not realistic to make a direct comparison of pupal weights. The most satisfactory method of comparing the suitability of the two diets for insect growth was therefore considered to be by comparison of the mean growth increments from initial to peak weight. Although much less satisfactory than direct comparison of pupal weights and adult emergence and fecundity, it was considered, nevertheless, that comparison of peak weights would reflect any large potential differences in these quantities, since peak weight is apparently related to pupal weight and pupal weight, in turn, to survival and fecundity (see Chapter 1, p. 23). An advantage of comparing the initial parts of the growth curves in the present experiment is that the results are less likely to have been influenced by the virus infection.

The growth increments of the larvae fed on the two diets are shown in Table 1 (next page), excluding the data for unhealthy larvae or prepupae, as explained earlier. The mean growth increments and their standard errors are also shown.

The mean growth increments obtained on the two diets were compared by the Student's t-test for small samples (Bailey, 1959). It was found that they were not significantly different (Appendix 7, p. 170).

Table 1. Growth increments (initial to peak weight) of winter moth larvae fed on two oak leaf powder diets, together with means and standard errors of the means.

	May 13th leaf diet	June 1st leaf diet
Growth Increments (mg.)	27.5	3.3
	9.6	23.3
	5.9	23.8
	13.2	9.1
	22.7	11.2
	10.2	7.3
	14.2	20.8
	2.6	5.4
	21.6	0.3
	13.6	
	6.8	
	6.5	
	2.3	
	14.8	
Mean Growth Increment and S.E.M.	12.3 ± 2.0	11.6 ± 3.0

7. Conclusions

In spite of the reduction in sample size owing to larval mortality during the experiment, the results show that larval growth on June 1st leaf powder is at least comparable to that on the May 13th powder. Even with the reduced samples, a difference in growth of the order of that observed for larvae growing on whole leaves of similar ages (Chapter 1, p. 20) would have been evident. It seems very likely, therefore, that the marked difference of larval growth found between the two samples fed on whole leaves is largely attributable to the difference in toughness of the leaves, which has been shown in

this chapter to be substantial, though it must be borne in mind that the leaf diets were augmented with equal quantities of vitamins. Major nutrients, such as sugars and proteins, are unlikely to be rendered less available to insects by the drying and powdering process and, to anticipate a later chapter, the level of tannins in the leaves does not appear to change markedly within the crucial period of about three weeks under consideration. The evidence suggests that, whatever the 'ultimate' factors governing early feeding on oak leaves, the 'proximate' factor which prevents larvae from feeding successfully in June is the rapidly increasing toughness of the leaves.

The results described in this chapter are presented with some diffidence, since the sample sizes were small and fifth instar larvae had to be used. It would clearly be desirable to repeat the growth experiment using much larger samples of younger larvae. Such an experiment was planned for 1966, but had to be cancelled owing to circumstances beyond the author's control. It is considered, however, that the results described are sufficient to indicate the probable importance of leaf toughness as a factor determing the successful growth of caterpillars.

CHAPTER 3: SEASONAL VARIATIONS OF SUGARS AND PROTEIN IN OAK LEAVES

1. Introduction

It has been seen in the previous chapter that the inability of winter mothlarvae to feed on mature oak leaves could be due largely to leaf toughness. This is not, however, a completely satisfactory explanation for the lack of extensive insect attack on mature oak leaves in the field. Many insect larvae, even in their early instars, have mandibles which would be amply strong enough to cut through mature oak leaves. Moreover, several Lepidoptera species feed as larvae on old oak leaves in the normal course of their life histories, in some cases only in their early instars (Appendix 1, p. 150). In view of the 90% mortality of young winter moth larvae, mentioned earlier (p. 11), it seems remarkable that selection has not favoured the development of stronger mandibles by many more caterpillar species, enabling them gradually to extend their feeding periods. That this has not occurred in the majority of species suggests that there is some other factor or combination of factors which causes oak leaves to become less suitable for larval growth as the season progresses.

Nutritional value seemed the most obvious property of oak leaves which might have an influence on larval feeding seasons. Assuming that caterpillars feed as rapidly as possible, growth rate could be

determined by the concentration in the ingested leaf material of the essential ingredients in shortest supply relative to the insect's requirements, i.e. the limiting food factors. An excellent example of this phenomenon concerns the aphids, where excess sugars and much soluble nitrogen, though apparently valuable food materials, are excreted as honeydew. It is thought that the insects must ingest more nitrogen and carbohydrate than they need in order to obtain enough of the essential nitrogen compounds in shortest supply or perhaps of certain vitamins, present in low concentration in the phloem (Auclair, 1963).

Insects need to obtain from their food a number of vitamins and other micronutrients (e.g. House, 1962) and it is possible that larval growth rates may be restricted in the field by deficiencies of these chemicals in leaves. It was, however, clearly beyond the scope of this work to undertake a detailed investigation of the fluctuations of leaf micronutrients during the year, together with breeding experiments to discover their likely effect on growth rates. Attention was therefore restricted to the major leaf nutrients, protein and carbohydrate, which are likely to be important in determining maximum growth rates, owing to the rapid formation of new tissue and its associated requirements for energy.

2. Carbohydrates

Insects have rarely been shown to digest cellulose, the chief structural carbohydrate of leaves, and the few examples known, such as the termites, achieve this breakdown with the aid of a specialised

protozoal gut fauna. Digestion of cellulose has never been reported in phytophagous Lepidoptera larvae (Wigglesworth, 1953) and it seems, therefore, that this source of carbohydrate is ignored. This in itself is of interest, since it implies that carbohydrate is not generally limiting for phytophagous insects. If it were, one would have expected heavy selection for the production of a cellulase enzyme, as has occurred in the snails, or in the development of a symbiotic cellulase containing gut fauna, as in the termites.

Ability to digest starch, the other chief polymeric carbohydrate source in leaves, is widespread among insects, including phytophagous larvae (Wigglesworth, 1953). However, attempts to detect the presence of an amylase in the salivary glands or the mid-gut of winter moth larvae were all unsuccessful (Appendix 8, p.172), although some 300 larvae were involved in the tests. It must be concluded that the enzyme is either completely absent as has been shown for at least two other phytophagous Lepidoptera species (Wigglesworth, 1953), or present in such small amounts that its activity is very restricted. It was concluded that starch is unlikely to influence the larval feeding of the winter moth and starch analyses of oak leaves were not undertaken.

3. Sugars

Since the winter moth does not appear to make use of leaf polysaccharides, it must be assumed that its needs for energy are adequately met by the free sugars and, perhaps, nitrogenous compounds present in leaves. The results obtained from work on aphids suggest

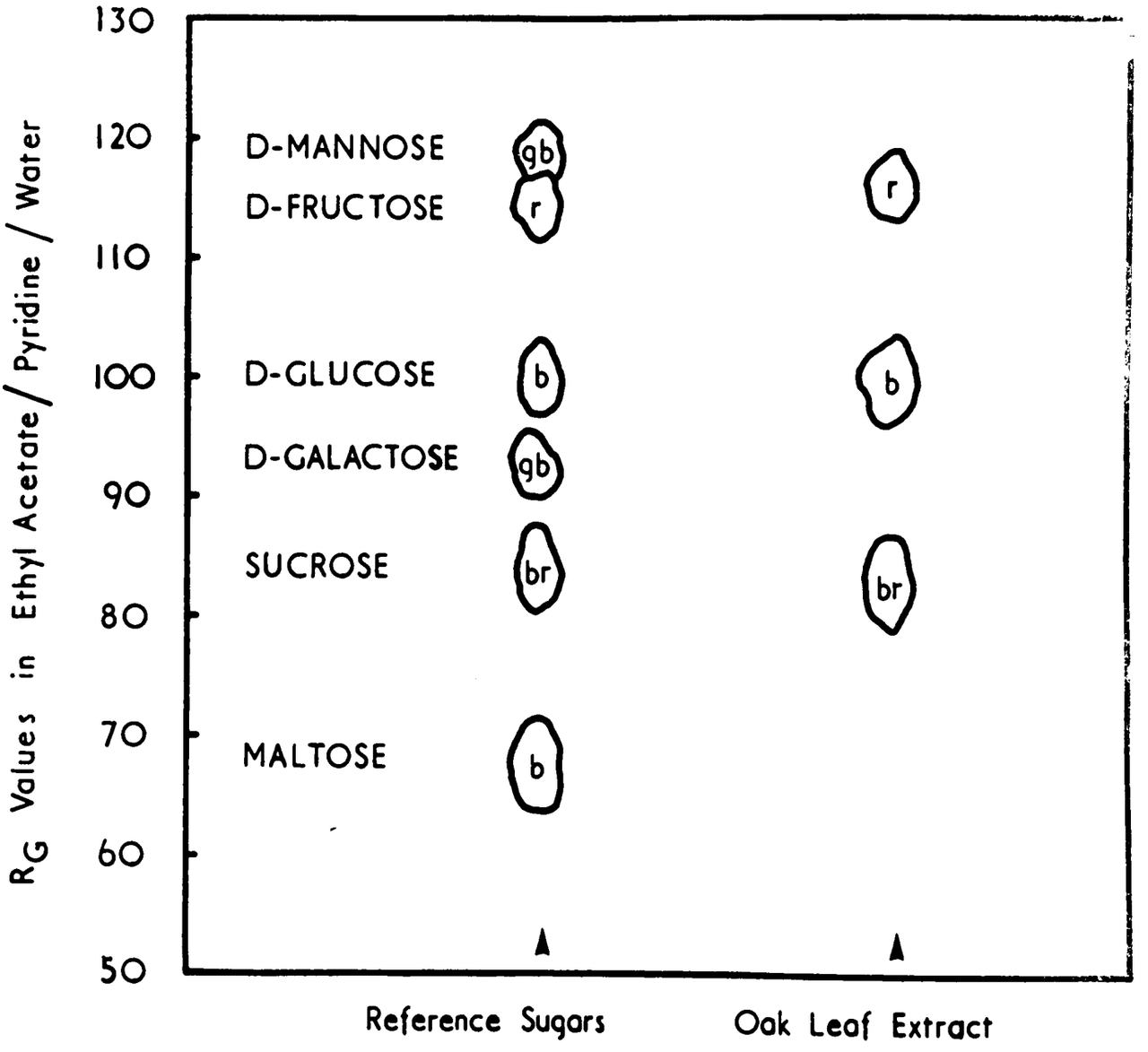
that it is nitrogenous compounds, rather than sugar, which might be limiting growth, but it was nevertheless decided to ascertain qualitatively and quantitatively the sugars present in oak leaves at different times during the year, to determine whether there was any correlation between sugar content and feeding period which might be ecologically significant.

Most,if not all, phytophagous Lepidoptera larvae so far studied secrete the enzyme invertase (Wigglesworth, 1953), so that both sucrose and monosaccharides are likely to be important items of diet.

a) Qualitative analysis

To discover which sugars are present in oak leaves, an ethanolic extract of July oak leaves was examined by one-way paper chromatography, as described in Appendix 9 (p. 175). The solvent used for chromatography was ethyl acetate/pyridine/water (12:5:4). Solutions of six pure reference sugars (D-Glucose, D-Fructose, D-Galactose, D-Mannose, Sucrose and Maltose) were run in parallel, for comparison with the oak leaf sugars. Chromatograms were developed with aniline/diphenylamine (Figure 12, p. 40(a)) or silver nitrate reagents to show up sugars. The colours of the developed spots, together with their R_G values (R_f values expressed as % R_f of D-Glucose), are given in Table 2, (p. 40(b)).

It can be seen from Table 2 and Figure 12 (p. 40(a)) that only three sugars were present in appreciable amount in the leaf extract. These were, as expected, sucrose, D-Glucose and D-Fructose. Judged by spot area on the original chromatograms, sucrose was present in



PAPER CHROMATOGRAM of ETHANOLIC EXTRACT of JUNE OAK LEAVES Quercus robur L. . Aniline / Diphenylamine Spray. b = blue , gb = grey-blue , br = brown , r = red .

FIGURE 12

TABLE 2 Chromatography of oak leaf sugars in ethyl acetate/pyridine/water on Whatman 3MM paper (see Appendix 9, p. 175)

	CONTROL SUGARS			OAK LEAF SUGARS		
	R_G	An/D[1]	$AgNO_3$[2]	R_G	An/D[1]	$AgNO_3$[2]
Maltose	68	blue				
Sucrose	84	brown	-	83	brown	-
Galactose	93	grey-blue				
Glucose	100	blue	brown	100	blue	brown
Fructose	115	red-brown	brown	116	red-brown	brown
Mannose	117	grey-blue				

[1] Colour of spot after development with aniline / diphenylamine reagent.

[2] Colour of spot after development with silver nitrate reagent.

the greatest concentration.

b) Quantitative Analysis

The quantitative determination of the sugar content of oak leaves was confined to tree M, with the intention of extending analysis to other trees if any significant trends were detected. Samples from both upper sun leaves and lower shade leaves were collected during the 1964 season, as described in Chapter 1 (p. 17), and ethanolic extracts prepared.

Some difficulty was encountered in adapting a suitable analytical method for sugar analyses of the extracts (Appendix 10, p.177) but the ferricyanide oxidation method of Hagedorn and Jensen (1923) was eventually found to be suitable. In conjunction with invertase digestion, this method was used to estimate sucrose independently of the reducing sugars, largely glucose and fructose. The method gave comparable results to those obtained by the α-naphthol procedure of Devor (1950). An account of the investigations leading to a suitable method of sugar analysis, together with the results obtained for the tree M samples, is given in Appendix 10 (p.177). The results are expressed graphically in Figure 13 (p.41(a)) and Figure 14 (p.41(b)).

It is apparent from Figure 13 (p.41(a)) that the content of reducing sugar in the leaves follows no obvious seasonal trend, in spite of considerable fluctuation during May and June. Monosaccharide content remains between 1.5% and 2.5% fresh weight throughout the season in both upper and lower leaves. Sucrose content, however, shown in Figure 14 (p. 41(b)), shows a marked increase in early June, reaching

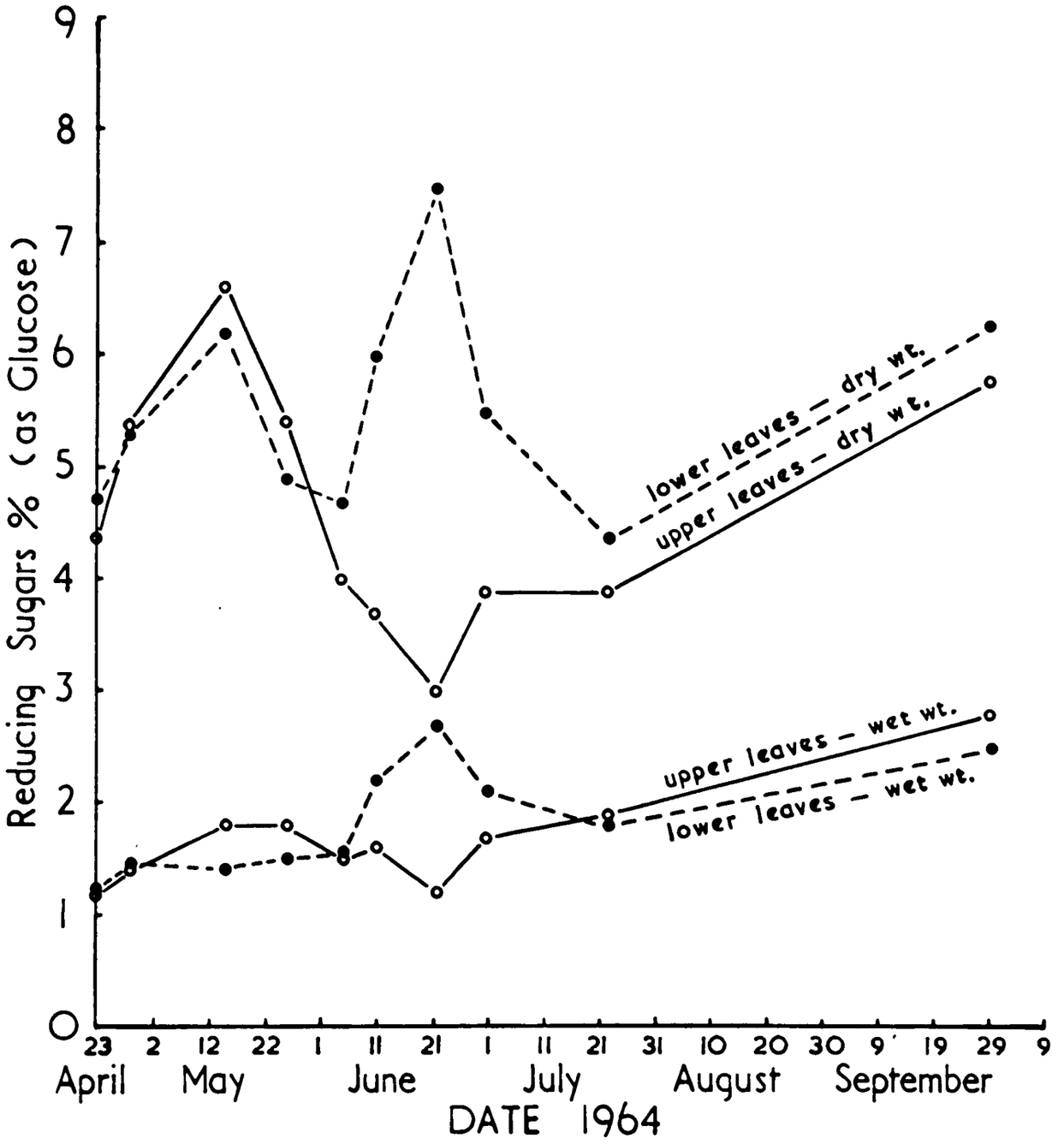


FIGURE 13 Content of reducing sugars in the upper and lower leaves of tree M during 1964.

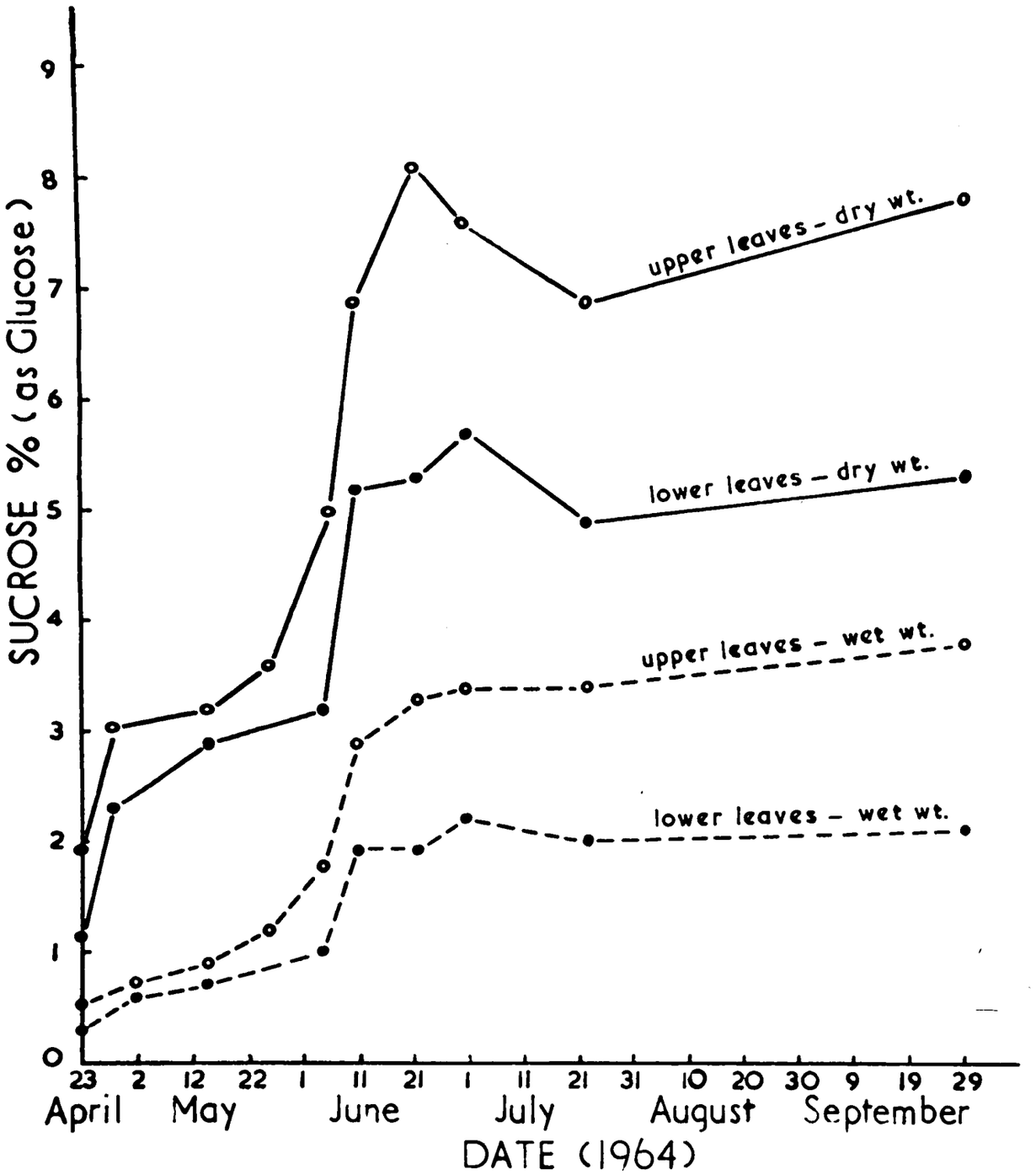


FIGURE 14 Content of sucrose in the upper and lower leaves of tree M during 1964.

3% fresh weight in the upper sun leaves and about 2% fresh weight in the lower shade leaves. These levels are maintained at least until late September. This situation is what one might expect since early in the year the photosynthetic rate is relatively low and, on account of the rapid rate of new tissue formation, metabolism of sucrose would be expected to be high. It has been known for some time that the percentage of sucrose in leaves tends to remain low during rapid growth and to increase as the leaves approach full size (e.g. Barnell, 1936).

It is clear from these results that the majority of oak-feeding Lepidoptera larvae feed during the period when the sugar content of the leaves is at its lowest and not, as would be expected if sugars were nutritionally limiting, when sugar content is at or near its maximum seasonal value. This tends to confirm the previous suspicion that carbohydrate content is not limiting the growth of these insects and that it is not likely to be a significant factor in determining early larval feeding periods.

On May 15th, 1964, analyses were also made of the sugar content of hazel and hawthorn leaves from trees in the immediate neighbourhood of oak tree M. These trees were apparently receiving about the same amount of light as the lower leaves of tree M. It is of interest that, although the sugar content of the hawthorn leaves was very similar to that of the oak leaves, the content of both sucrose and the monosaccharides was found to be about twice as high in hazel leaves (Appendix 10, p.177). Gradwell, G.R. (personal communication) has

found that winter moth larvae grown on hazel reach lower pupal weights than those grown on oak leaves. If the figure obtained for the sugar content of the hazel sample is representative of the situation throughout the year and for hazel trees in general, it would imply that the reduction in pupal weights is not due to a lack of carbohydrate.

4. Seasonal variation of oak leaf nitrogen content

Most of the nitrogen of plants (usually 80% to 90% in leaves) is present in the form of amino acids, either existing free or combined in proteins. The total nitrogen content of a plant is then a rough measure of its amino acid and protein content (Long, 1961).

All phytophagous larvae so far studied are apparently able to digest leaf protein (Wigglesworth, 1953) so that a determination of total nitrogen is probably a good estimate of the maximum amount of nitrogen available for insect growth. Leaf protein content is frequently determined as %N x 6.25 (Long, 1961) and this procedure was followed in the present work.

On five dates during the 1965 season, buds or leaves from tree M were collected for analysis to see whether the total nitrogen content varied during the year in a manner likely to affect insect larvae. Upper sun and lower shade leaves were analysed separately. The leaves or buds (descaled) were dried, ground to a fine powder in a ball mill and analysed for total nitrogen by the micro-Kjeldahl method as described in Appendix 11 (p.190). Analyses were carried out in triplicate, the mean values being shown in Table 3 (p. 44). In this

table the equivalent protein values, calculated by multiplying total nitrogen percentage by 6.25, are also shown.

TABLE 3. Nitrogen content of oak leaves from tree M during 1965

Sample	Date	Total Nitrogen (% Dry Weight)	Protein Equivalent (% Dry Weight)
Upper Sun Buds	April 30th	4.46	27.8
" " Leaves	May 23rd	2.58	16.1
" " "	June 14th	2.10	13.2
" " "	August 5th	2.21	13.8
" " "	September 15th	2.08	13.0
Lower Shade Buds	April 30th	4.80	30.0
" " Leaves	May 23rd	2.63	16.4
" " "	June 14th	2.27	14.2
" " "	August 5th	2.29	14.3
" " "	September 15th	2.21	13.8

These results are presented graphically in Figure 15 (p.44(a)). Although there is little difference in nitrogen content between upper and lower leaves, there is a marked decrease in percentage nitrogen from nearly 5% dry weight (30% protein equivalent) in April buds to about half this value in late May and thereafter. A high nitrogen content in young growing leaf tissue is, of course, expected and has been shown for many plants (e.g. Long, 1961). Its coincidence in oak leaves with the main period of larval feeding is striking and supports the view that nitrogen content might be one of the most important factors governing early feeding.

5. Comparison between nitrogen content of several trees.

To compare the nitrogen content of different trees, in order to verify the decrease found in tree M in 1965, leaf samples were

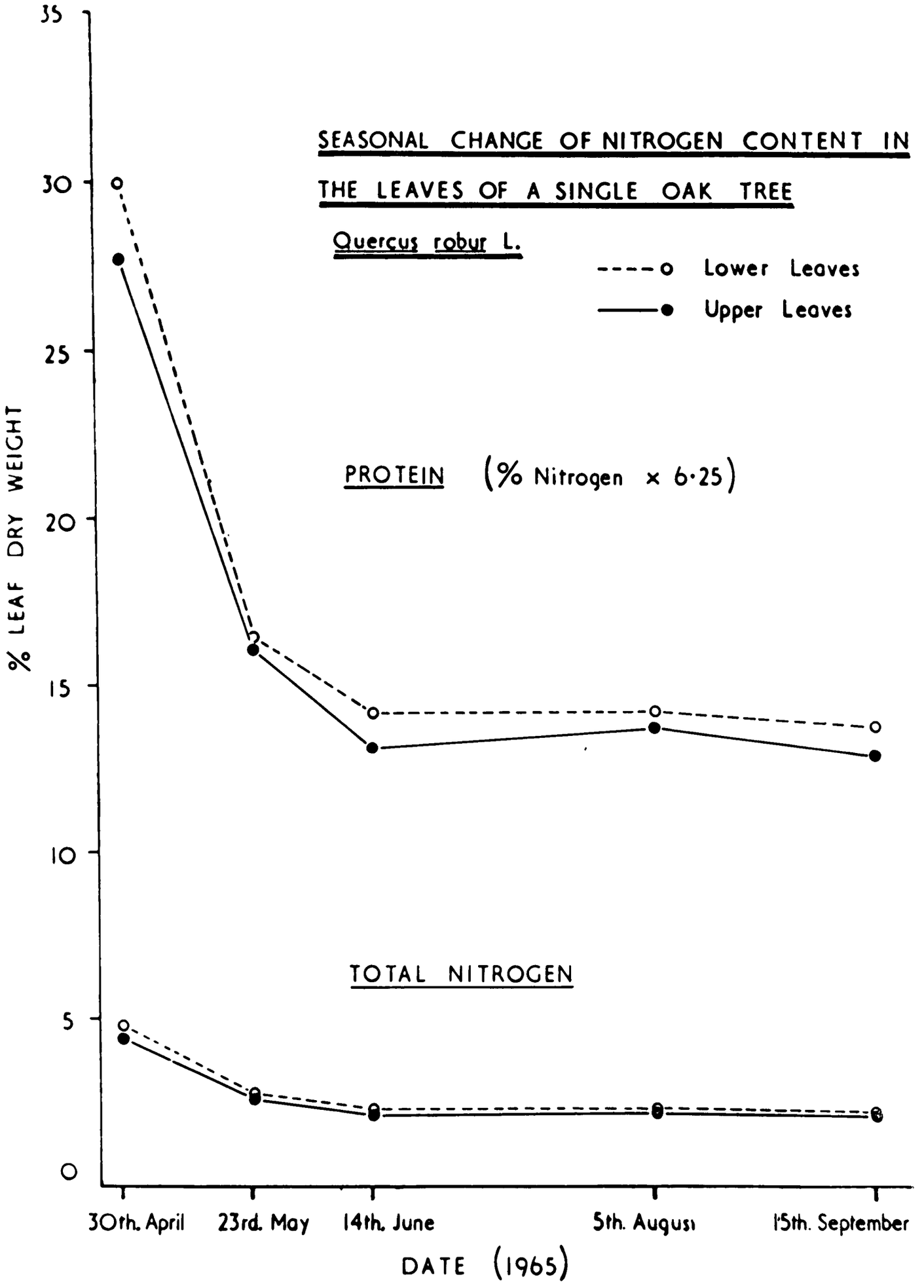

SEASONAL CHANGE OF NITROGEN CONTENT IN
THE LEAVES OF A SINGLE OAK TREE
Quercus robur L.
Lower Leaves
Upper Leaves
PROTEIN (% Nitrogen × 6·25)
TOTAL NITROGEN
% LEAF DRY WEIGHT
35
30
25
20
15
10
5
0
30th. April
23rd. May
14th. June
5th. August
15th. September
DATE (1965)

FIGURE 15

collected from six oak trees (1, 5, 8, 9, 15 and M) on two dates (May 6th and July 1st) during 1966. Samples were also obtained from tree 9 on the two intermediate dates, May 19th and June 10th. Since the nitrogen content of upper and lower oak leaves had been shown from the 1965 data to be very similar, only lower shade leaves were collected in 1966. As before, leaves were collected by the method described in Chapter 1 (p. 17).

The leaf samples were kindly analysed by Miss Jenkins in the Department of Forestry, Oxford, using the same method as that employed by the author in 1965. The results are included in Appendix 11 (p. 190) and are expressed graphically in Figure 16 (p.45@).

It is at once apparent from Figure 16 that in all six trees the nitrogen content of the leaves was much higher on May 6th than it was on July 1st, and in most cases the figures are very similar to those obtained for tree M the previous year. In particular, the drop in nitrogen content of leaves from tree 9 is almost identical to that found in 1965 for tree M. The previous finding that nitrogen content of oak leaves drops to about fifty percent of its initial value during May is thus broadly confirmed from results from several trees.

It is of interest that the leaves of trees 8 and M contained noticeably less nitrogen on May 6th than the other four trees. These two trees, however, are the first to flush of the six trees (Chapter 1, p. 14) and their leaf nitrogen content had presumably started to drop in advance of the other four trees. The nitrogen content found for the leaves of tree 1 on July 1st is substantially higher than the

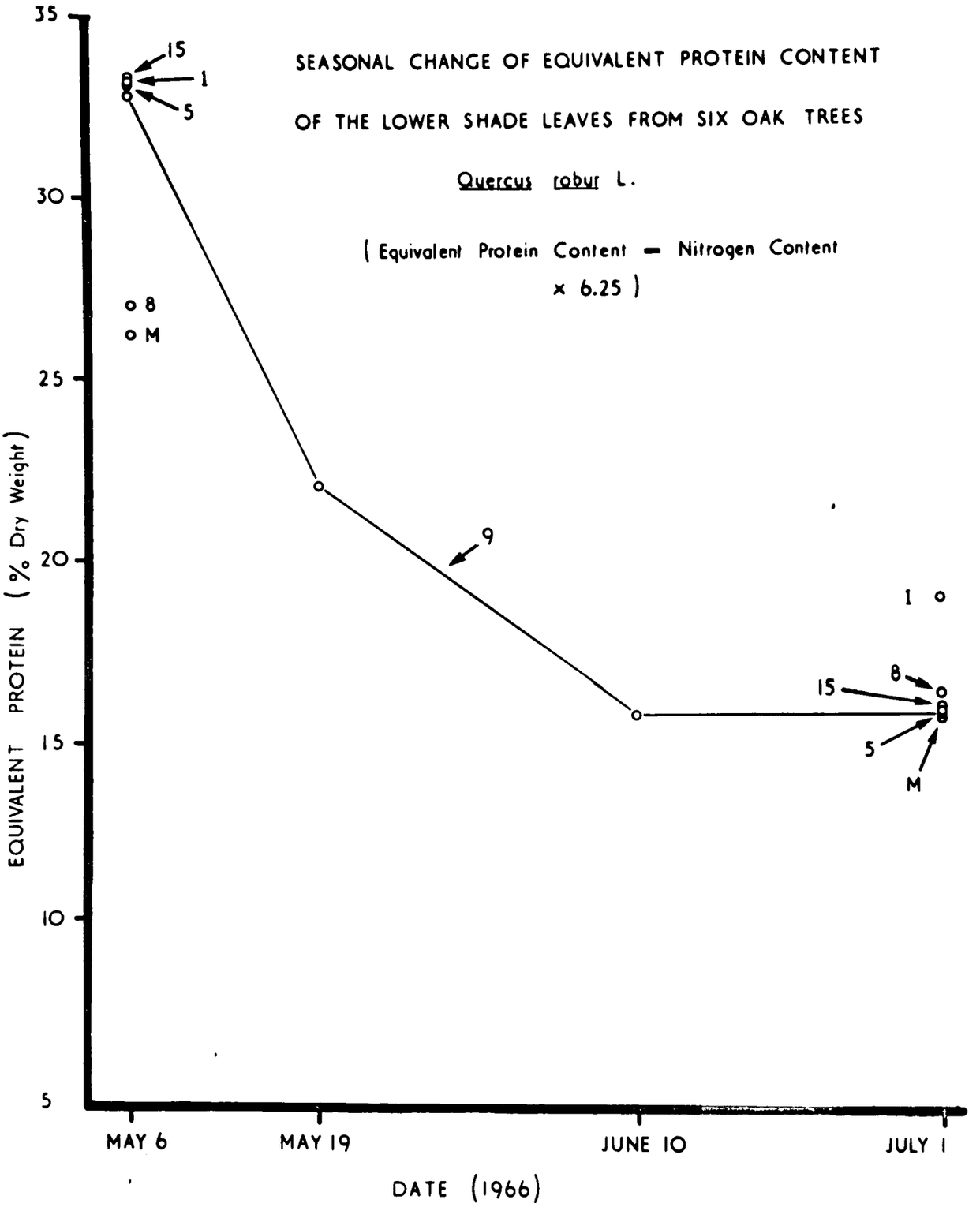

SEASONAL CHANGE OF EQUIVALENT PROTEIN CONTENT
OF THE LOWER SHADE LEAVES FROM SIX OAK TREES
Quercus robur L.
(Equivalent Protein Content = Nitrogen Content
× 6.25)
EQUIVALENT PROTEIN (% Dry Weight)
35
30
25
20
15
10
5
15
1
5
8
M
9
1
8
15
5
M
MAY 6
MAY 19
JUNE 10
JULY 1
DATE (1966)

FIGURE 16

figure obtained from the leaves of the other five trees. No explanation can at present be given for this, unless some lammas shoots were accidentally included in the original leaf sample. These would be expected to have a higher nitrogen content than contemporary mature leaves.

6. Conclusions

The results described in this chapter show that the feeding period of the majority of oak-feeding caterpillars is correlated with a high nitrogen and low sugar content in the leaves. This suggests that larval feeding periods may be influenced by the content of nitrogenous compounds in oak leaves. Evidence will be presented in later chapters to suggest that it is not the nitrogen content *per se* but rather the protein/tannin ratio in theleaves which may be important in determining the availability of nitrogen for larval growth.

It is possible that a seasonal difference in the content of certain micro-nutrients in oak leaves may be important in determining the optimum feeding time for caterpillars. It has been shown (Ellis, Carlisle and Osborne, 1965) that sexual maturation in the desert locust *Schistocerca gregaria* Forskål is delayed when the insects are fed on senescent leaves of *Brassica* spp. The senescent leaves were found to be short of gibberellins, and a dietary supplement of gibberillin A_3 restored the rate of maturation of the locusts to that found in animals feeding on green leaves. The present author is well aware that a similar phenomenon may be affecting caterpillar growth periods on oak leaves, but time was unfortunately insufficient to include research into this aspect of the problem.

CHAPTER 4: PHENOLIC COMPONENTS OF OAK LEAVES

1. Introduction

It is now well established that polyphenols in the leaves of plants can have adverse effects on the growth and survival of other organisms. It is known, for example, that leaf tannins can inhibit the growth of fungi (e.g. Williams, 1963) and the transmission of viruses (e.g. Cadman, 1960), probably by tanning the fungal pectolytic enzymes and virus nucleoprotein (White, 1956; Cadman, 1960). Moreover, Handley (1961) has shown that protein may be rendered resistant to microbial decomposition by the formation of relatively indigestible complexes with tannins. It is also of interest that although there is no marked change in the crude-protein content of oak fodder from July to September leaves, the digestibility of the crude-protein by sheep shows a marked fall, in parallel with the probable increase in leaf polyphenols during this period (Vukavíc and Maksimovíc, 1956; Handley, 1961). There is also evidence that simple phenols in leaves such as chlorogenic acid and catechins may be biologically active in a similar way, though their effect may be to some extent dependent on prior oxidation to quinones, which may be followed by polymerisation (e.g. Byrde, Fielding and Williams, 1960; Johnson and Schall, 1952).

Oak leaves, bark and galls have been known for centuries to develop high concentrations of tannins, which have been used in the manufacture of leather and inks. Although no definite evidence could

be found in the literature to show that tannins have an inhibitory effect on the growth and survival of insects, it nevertheless seemed that an investigation into the effects of oak leaf tannins on oak-feeding caterpillars, as suggested to the author by Professor G.C. Varley, might be rewarding. There was casual evidence to suggest that the tannin content of oak leaves increased during the year and it will be remembered (Introduction, p. 13; Brown, Love and Handley, 1962) that a qualitative change in oak leaf tannins had also been shown to occur during the summer.

Study of the phenolic components of leaves, apart from certain groups of compounds such as the flavanols, has so far been limited and much of the present day knowledge is restricted to the leaves of economically important plants such as tea (e.g. Roberts, 1962; Bradfield, Penney and Wright, 1947). The only thorough investigation of oak leaf phenolic compounds apparently undertaken so far is that of Hathway (1958), who discovered the presence of (+)-catechin, (+)-gallocatechin, ellagic and gallic acids, leucodelphinidin, quercetin, and unidentified flavonol pigments and leuco-anthocyanins in mature August sun leaves.

In view of the inadequate knowledge about phenolic compounds in oak leaves, it was decided to carry out suitable leaf analyses throughout the year and so determine any seasonal variation of these compounds. This, it was hoped, would indicate which phenols, if any, were negatively correlated with larval feeding periods.

2. Qualitative analysis of oak leaf phenols

a) Methods

To reduce the possibility of confusion due to phenotypic or genotypic differences between individual trees and to differences between leaves from different parts of the same tree, analyses were initially restricted to the upper sun leaves of tree M. It was subsequently found, however, that there were no obvious qualitative differences in the phenolic constitutents of oak leaves from different parts of the same tree or from several different trees in Wytham Wood.

Leaf samples were collected monthly during 1965 by the method described in Chapter 1 (p. 17) and extracted with 70% acetone/water. This solvent was chosen since it results in more efficient extraction of tannins than would be achieved by using, say, ethanol or water (Brown, B.R., personal communication). Similar aliquots from each extract were examined by two-way paper chromatography, using 2% acetic acid and n-butanol/acetic acid/water (60:15:25) as solvents (Appendix 12, p. 193). These solvents have been widely used in phenol chromatography (e.g. Smith, 1960) and were found to be satisfactory for the present investigation. For identification of some of the phenols, reference compounds were also run simultaneously on separate chromatograms. These compounds included gallic acid, ellagic acid, (+)-catechin (Koch-Light Laboratories, Ltd.), quercetin (Koch-Light) and (+)-gallocatechin (kindly supplied by Dr. D.E. Hathway).

All chromatograms were examined in ultraviolet light before and

after exposure to ammonia vapour so that certain components could be readily recognised by their fluorescence. The chromatograms were then sprayed with suitable reagents to show up phenolic compounds as coloured spots. The most useful developing sprays were ferric chloride/potassium ferricyanide reagent, which shows up all phenols as blue spots or streaks, and vanillin, which reveals as pink spots those phenols, such as the catechins, which contain resorcinol residues. Other developing sprays, such as diazotised p-nitraniline and tetrazotised benzidine, were occasionally used, but did not provide much additional information (Appendix 12, p. 193).

b) Phenolic components in oak leaf extracts

Representations of chromatograms obtained for the phenolic constitutents of April oak buds and June oak leaves from tree M are shown in Figure 17 (p.50(a)). These show all the spots and streaks revealed by the ferric chloride/potassium ferricyanide spray. Spots which were, in addition, vanillin-positive are indicated by black shading. The phenolic compounds revealed in the chromatograms were all assigned letters of the alphabet for convenient reference. These are indicated in Table 4 (p.50(b)), in which are listed the R_f values for all the phenolic components detected by the author in oak leaf extracts, together with their behaviour under ultraviolet light and with the various spray reagents. The R_f values listed are the average values obtained from a large number of chromatograms.

It can be seen that more than twenty spots or streaks attributable to phenols were detected in oak leaf extracts. The ferric chloride/

FIGURE 17

TWO WAY CHROMATOGRAMS SHOWING PHENOLIC COMPONENTS OF OAK LEAVES REVEALED BY $FeCl_3 / K_3Fe(CN)_6$ SPRAY. ● = Vanillin positive. ◌ = Stored leaves only.

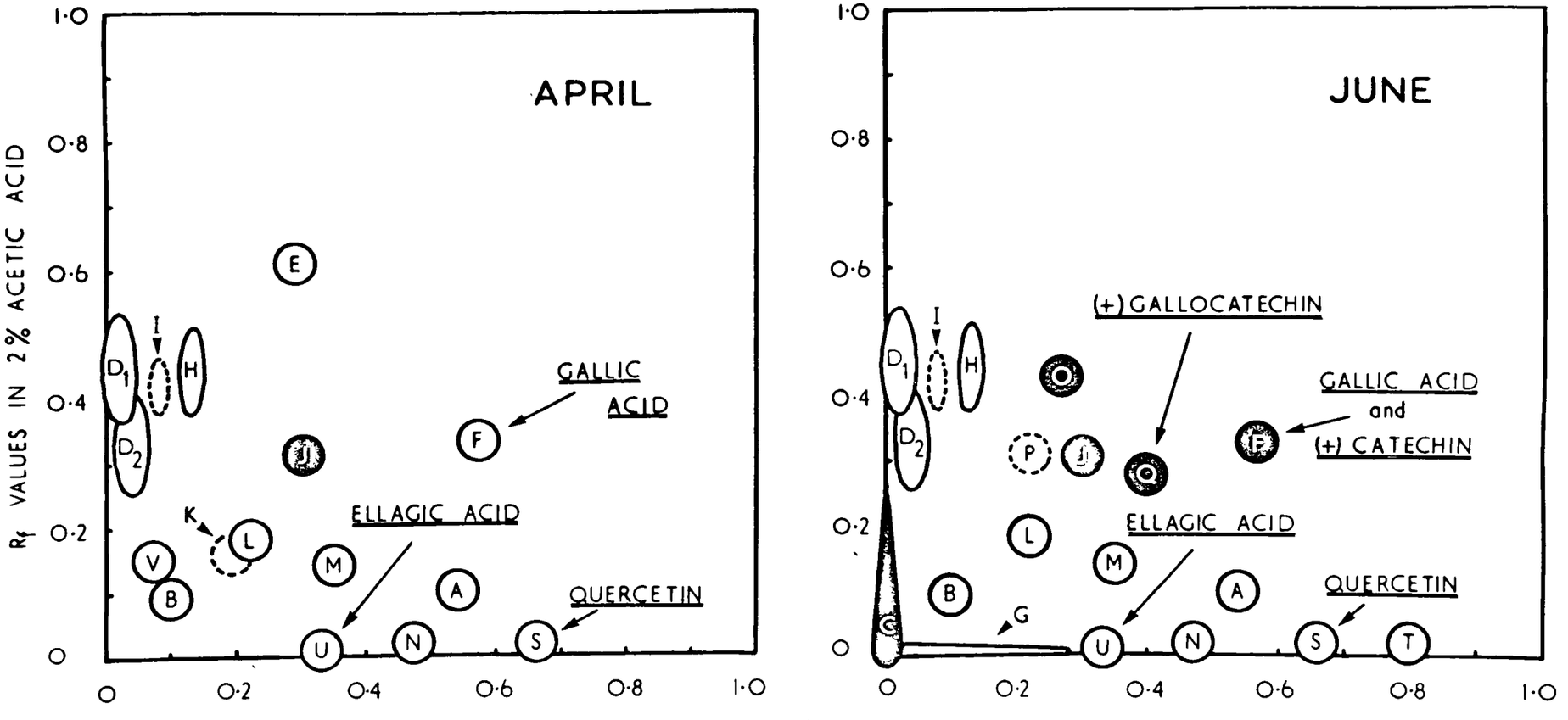


TABLE 4 R_f values and chromatographic behaviour of phenolic compounds in oak leaf extracts.

Symbol	Identity (if known)	UV	UV/NH_3	Van.[1]	D-pN[2]	Tet.B[3]	R_fHAc[4]	R_fBAW	Comments
A		abs.	YG	-	-	-	0.10	0.54	
B	hydrol tan.	abs.	abs.	-	-	-	0.09	0.10	sometimes streal
C	condd. tan.	abs.	abs.	R	Y	YBr	0-0.25	0	streak
D_1	hydrol. tan.	abs.	abs.	-	Br	YBr	0.45	0.02	streak
D_2	hydrol tan.	abs.	abs.	-	Br	YBr	0.33	0.04	streak
E		-	-	-			0.61	0.29	
F_1	gallic acid	-	-	-			0.33	0.57	
F_2	(+)-catechin	-	-	R	Y	R	0.33	0.57	
G	?hydrol. tan.	-	-	-			0	0-0.30	v.feeble
H	hydrol. tan.	abs.	abs.	-	-	-	0.44	0.13	streak
I[6]							0.42	0.08	streak
J		-	-	R			0.31	0.30	
K[6]							0.16	0.19	
L		-	-	-			0.18	0.22	
M		-	abs.	-			0.14	0.35	
N		-	-	-			0.02	0.47	
O		-	-	R	Y	R	0.43	0.27	
P[6]							0.31	0.22	
Q	(+)gallocatech.	-	-	R			0.28	0.40	
S	quercetin	Y	Y	-			0.02	0.80	

contd.

TABLE 4 (contd.)

Symbol	Identity	UV	UV/NH_3	Van.[1]	D-pN[2]	Tet.B[3]	R_fHAc[4]	R_fBAW	Comments
T		Y	Y	-			0.02	0.80	
U	ellagic acid	bV	Y	-			0.01	0.33	
V		-	B	-			0.15	0.07	
W		-	-	-			0.08	0.17	

R = red; Y = yellow; YG = yellow-green; Br = brown; YBr = yellow-brown; bV = blue-violet; B = blue; [6] = in extracts from stored leaves only; [1] = vanillin reagent; [2] = diazotised p-nitraniline reagent; [3] = tetrazotised benzidine reagent; [4] = 2% acetic acid; [5] = butanol/acetic acid/water.

potassium ferricyanide reagent detects quantities of less than a microgram of many phenols (Smith, 1960), so that it is unlikely that any major phenolic components of oak leaves (> 0.01 - 0.05% fresh weight) were overlooked.

c) Effects of storage of leaves and extracts on their phenolic constituents

Since it was rarely possible to carry out chromatographic analysis of leaf extracts immediately after preparation, it was important to determine the effects, if any, of storage on the phenolic components. Accordingly, aliquots from an acetone/water extract of fresh September oak leaves were run on chromatograms in the usual way, a) immediately after extraction and b) after storage for four months under nitrogen at room temperature in the dark. The pattern of spots on the two sets of chromatograms was identical and there was no obvious difference in the relative spot intensities. This shows that extracts can be stored for considerable periods under the conditions used, without risk of significant change in the phenolic constitutents. As a further precaution, a trace of potassium cyanide was added to the acetone/water solvent before each extraction, since cyanide has been shown to completely inhibit the copper-containing polyphenol oxidase enzyme of tea leaves (Roberts, 1962) and it is likely that a similar enzyme in oak leaves would have similar properties.

The effects of storing leaves in the deep-freeze before extraction of phenolic constitutents were also investigated, since it was convenient to accumulate large reserve stocks of leaves for subsequent

extraction of tannins. Fresh acetone/water extracts of April buds and June oak leaves were therefore compared chromatographically with extracts from the same samples of leaves after deep-freeze storage at -20°C for a year. Almost no difference was detectable between the two sets of chromatograms, except that substances I and K were present in the stored April bud extract and substances I and P in the extract from stored June leaves (Table 4, p. 50(b) and Figure 17, p. 50(a)). I, P and K have not been found in extracts of fresh leaves. The only other change evident on storage of leaves before extraction was the disappearance of substances S (quercetin) and T from both April and June samples.

As might be expected, therefore, changes in leaf phenols, though slight, do occur during storage of leaves in the deep-freeze. However, deep-freeze storage was occasionally used for leaves destined for tannin extraction, since the tannins did not seem to be affected by this procedure.

d) Identity of phenolic components

The R_f values of the reference phenols, previously found in oak leaves by Hathway (1958), and their behaviour under ultraviolet light and with the developing reagents were found to correspond exactly with those of certain spots on the chromatograms of oak leaf extracts. Gallic acid and (+)-catechin have very similar R_f values in the two solvents used, corresponding to substance F. It had previously been realised that substance F represented two components, since, although under the conditions used, the spot is fairly intense in April, it

is vanillin negative (F_1), becoming vanillin-positive only in late May (F_2). It seems reasonable to suppose, therefore, that F_1 represents gallic acid, probably present in the leaves throughout the year, while F_2 represents the vanillin-positive (+)-catechin, which appears in the leaves from May onwards.

Ellagic acid and quercetin correspond both in their R_f values and their characteristic behaviour in ultraviolet light (Table 4, p.50(b)) with substances U and S respectively. Also present as a trace impurity in the commercial sample of quercetin was a substance with identical R_f values and behaviour in ultraviolet light with substance T. Its nature is unknown, though it could perhaps be a glycoside of quercetin. The fifth reference phenol, (+)-gallocatechin, was indistinguishable chromatographically from compound Q.

Because of their tendency to run as streaks on chromatograms, B, C, D (under some conditions resolved into D_1 and D_2), G and H were tentatively described as tannins. Condensed tannins tend to streak in acetic acid solvents and many hydrolysable tannins in n-butanol/acetic acid/water (Brown, Love and Handley, 1962; Brown, B.R., personal communication), so that C, being vanillin-positive, clearly represented the oak leaf condensed tannin, previously reported by Brown (loc. cit.) (Figure 17, p.50(a)). The streak G in n-butanol/acetic acid/water presumably represents the hydrolysable tannin referred to by Brown (loc. cit.) but it seems to be a minor constitutent of oak leaf tannin, being barely visible on the chromatograms. Components D, B and H, all vanillin-negative, were designated 'anomalous'

hydrolysable tannins, since they streaked in acetic acid.

Since the terms 'condensed' and 'hydrolysable' will frequently be used in this thesis to distinguish the two major kinds of plant tannin, a brief summary of the structural type and properties of condensed and hydrolysable tannins is given in Appendix 13 (p.196). The term 'tannin' itself is widely used in the literature to describe the heterogeneous group of phenols which have the property of forming complexes with proteins, largely by hydrogen-bonding. In this thesis, the term is restricted to polymeric phenols of high molecular weight which have this property.

To confirm the identity of the suspected oak leaf tannins, July oak leaves were extracted by the ether precipitation procedure developed by Brown and Love (1961) for the isolation of high molecular weight polyphenols from leaf extracts (Appendix 14, p.201). The resulting pale brown, crude tannin powder, dissolved in 70% acetone was run on chromatograms using the usual two solvents. C, D and H were revealed on the developed chromatograms as heavy dark blue streaks and B and G as very faint pale blue streaks. C was the only vanillin-positive component. All other substances found in the original leaf extracts (Figure 17, p.50(a)) were absent and presumably represent low molecular weight phenols.

e) Absorption by hide powder

One method of determing whether or not a compound can be described as a tannin consists in shaking an aqueous solution of the

compound with specially prepared animal hide powder, available commercially. If the compound has a tanning action, it will be completely adsorbed from solution by an excess of the powder (Brown and Love, 1960). To determine which compounds of those detected in oak leaf extracts were able to tan protein, an experiment was carried out (Appendix 15, p.203) in which an aqueous extract of fresh September oak leaves was shaken with hide powder. It was found that all the phenolic constituents suspected of being tannins - C, D, B, H and G - were adsorbed from the solution, so confirming their suspected identity as tannins. The simple phenols O and J also appear to combine with protein, since they, too, were missing from the supernatant.

f) Seasonal variation in the phenolic constituents of oak leaves

The variation in the occurrence of the oak leaf phenols from April to September is shown in Table 5 (p.55(a)). An approximate idea of the relative amounts of each component, judged subjectively by the intensity of the spots given with the ferric chloride/potassium ferricyanide reagent, is indicated by plus signs. Owing to the different colour intensity given with this reagent by similar amounts of different phenolic components, such estimates can only be used to compare very approximately the relative amounts of the different phenols.

It is apparent from Table 5 (and also from Figure 17, p.50(a)) that the pattern of the phenolic constituents of oak leaves remains fairly constant during the growing season, though some components increase and others decrease in concentration. The most notable change

TABLE 5 Seasonal occurrence of phenolic compounds in oak leaf extracts.

Symbol	Identity, if known	April[1]	May	June	July	August	September
A		++	+	+	+	+	+
B	hydrol. tannin	+	++	+++	+++	+++	+++
C	condensed tannin		+	++	+++	+++	+++
D_1	hydrol tannin	+++	+++	+++	+++	+++	+++
D_2	hydrol. tannin	+++	+++	+++	+++	+++	+++
E		+	+				
F_1	gallic acid+	++	++	?	?	?	?
F_2	(+)-catechin		+	+++	+++	+++	+++
G	hydrol. tannin		+	++	+	+	+
H	hydrol. tannin	+++	+++	+++	+++	+++	+++
J		+	+	++	++	++	++
L		++	++	++	++	++	+
M		+++	+++	+++	+++	+++	+++
N		+++	++	+	+	+	+
O				+	+	+	+
Q	(+)gallocatechin			+	+	+	+
S	quercetin	+	+	++	++	++	++
T			+	+	+	+	+
U	ellagic acid	+	+	+	+	+	+
V		+++	+				
W			+				

+++ = strong; ++ = medium; + = weak; + = after May shielded by (+) catechin. Latter judged by intensity of vanillin reaction. [1] = descaled buds.

55(a)

concerns the vanillin-positive compounds, C, F_2, J, O and Q. With the exception of J, present in low concentration, these compounds are absent or at a very low level in April and only begin to appear in the leaves during May, reaching a high level, in the case of the condensed tannin C, only in July. Thus condensed tannin and its probable precursors, (+)-catechin and (+)-gallocatechin (see Appendix 13, p. 196), are absent from oak leaves during the early summer, when larval feeding is at its peak. This broadly confirms the finding of Brown, Love and Handley (1962) that condensed tannin is present in oak leaves in July, but absent in May. The present work shows that condensed tannin is present in May leaves, but at a relatively low level. It is of interest that the suspected hydrolysable tannins, especially D_1 and D_2 (Figure 17, p. 50(a)) are present at a high level even in the oak buds. G, the hydrolysable tannin apparently referred to by Brown, Love and Handley (loc. cit.), is, however, never more than very weakly shown on chromatograms.

In the present work, attention has been focussed on the tannins C and D (by far the strongest spots on the chromatograms) and, to a lesser extent H and B, since these were considered to be the four most likely phenolic components of oak leaves to have an effect on caterpillar growth due to their possible effect on the digestibility of leaf protein. The remaining components showed little seasonal change, apart from the disappearance of E and V after May, and their tanning effect is likely to be slight (p. 55). No further attention was therefore paid to them.

3. Properties of oak leaf tannins

To save time by avoiding duplication of research, much of the investigation of the properties of oak leaf tannins was shared with Mr. H. Bostock, working in the Dyson Perrins Laboratory of Organic Chemistry, Oxford. The research carried out by Bostock was written up separately and submitted for an Oxford Chemistry Part II thesis, a copy of which is available in the Dyson Perrins Laboratory. This will be referred to as Bostock (1966).

a) Extraction of tannin

Oak tannin was isolated in bulk from 70% acetone/water leaf extracts for chemical investigation by the ether precipitation method of Brown and Love (1961) (Appendix 14, p.201). During the removal of salt by dialysis, some of the tannin came out of solution as a dark brown precipitate. Before freeze-drying, this water-insoluble fraction (P) was centrifuged down and subsequently freeze-dried from aqueous suspension, separately from the soluble fraction (S). After freeze-drying, both fractions were obtained as light brown powders, soluble in acetone.

b) Elemental analysis

Several analyses of September oak leaf tannins, extracted by Bostock using the Brown and Love method, were carried out by Messrs. Weiler and Strauss Microanalytical Laboratories, Oxford. The following values were obtained:

Sample	% C	% H	% N	% Ash
Oak leaf tannin (both fractions)	48.8	4.8		
Oak leaf tannin - fraction (S)*	48.7	4.7	nil	nil
Oak leaf tannin (S), dried for 1 day	49.4	4.7		
" " " " 3 days	49.7	4.6		
" " " " 9 days	49.8	4.4		
Oak leaf tannin - fraction (P)*	51.8	5.4	nil	nil

Drying was carried out in a drying pistol, containing phosphorous pentoxide, at 30° - 35°C. The samples marked by an asterisk were analysed in duplicate and the average values are shown (Bostock, 1966).

The figures are similar to those obtained by Brown and Love (loc. cit.) for the condensed tannin of heather leaves Calluna vulgaris (L.) Hull (% C 49.6 and 49.3; % H 5.0 and 5.0; % ash 1.0 and 1.5), suggesting that the crude oak leaf tannin may be largely composed of condensed tannin in the late summer.

It is of interest that the hydrogen content of fraction (P) is somewhat higher than that of fraction (S), though the carbon contents are similar. No explanation can at present be given for this, though it is probable, from its insolubility in water, that fraction (P) contains tannin molecules of higher molecular weight than those in fraction (S). It is also possible that (P) and (S) differ to some extent in the nature and proportions of the functional groups attached to the phenolic nuclei.

c) Chromatography

Two-way paper chromatography of the soluble tannin fraction (S) in the usual solvents showed that it was made up of the components C (the vanillin-positive condensed tannin), D (usually resolved into D_1 and D_2) and H. Other spots visible on the chromatograms, though much weaker, were B, I_1 and I_2 (in the same position as the substance I, found in extracts of leaves stored in the deep-freeze) and E' (in a similar position to substance E, present inextracts of spring oak leaves). Two-way cellulose thin-layer chromatography of the crude oak tannin by Bostock (1966) showed the same pattern, but revealed in addition a component Y, hidden by C on the paper chromatograms owing to the poorer resolution. The thin layer chromatograms also showed that D_1 was resolvable into two components, D_a and D_b, and D_2 into three components, D_c, D_d and D_e (the latter being relatively weak). The positions of the components and their R_f values, obtained by Bostock (1966) on cellulose thin layer plates, are shown in Figure 18 (p. 59(a)) and Table 6 (p. 59(b)) respectively. Comparison of Tables 4 (p. 50(b)) and 6 (p. 59(b)) shows that although the R_f values of the tannin components were very similar on paper and on cellulose thin-layer plates, the R_f values in n-butanol/acetic acid/water (subsequently referred to as BAW) were approximately twice as high on thin layer as on paper.

Chromatograms of the water-insoluble tannin fraction (P) differed from those of (S) only in the presence of the streak G along the BAW axis and in the greater concentration at the origin of the reaction

TWO WAY CELLULOSE THIN LAYER CHROMATOGRAM of OAK LEAF TANNINS (after Bostock, unpubl.)

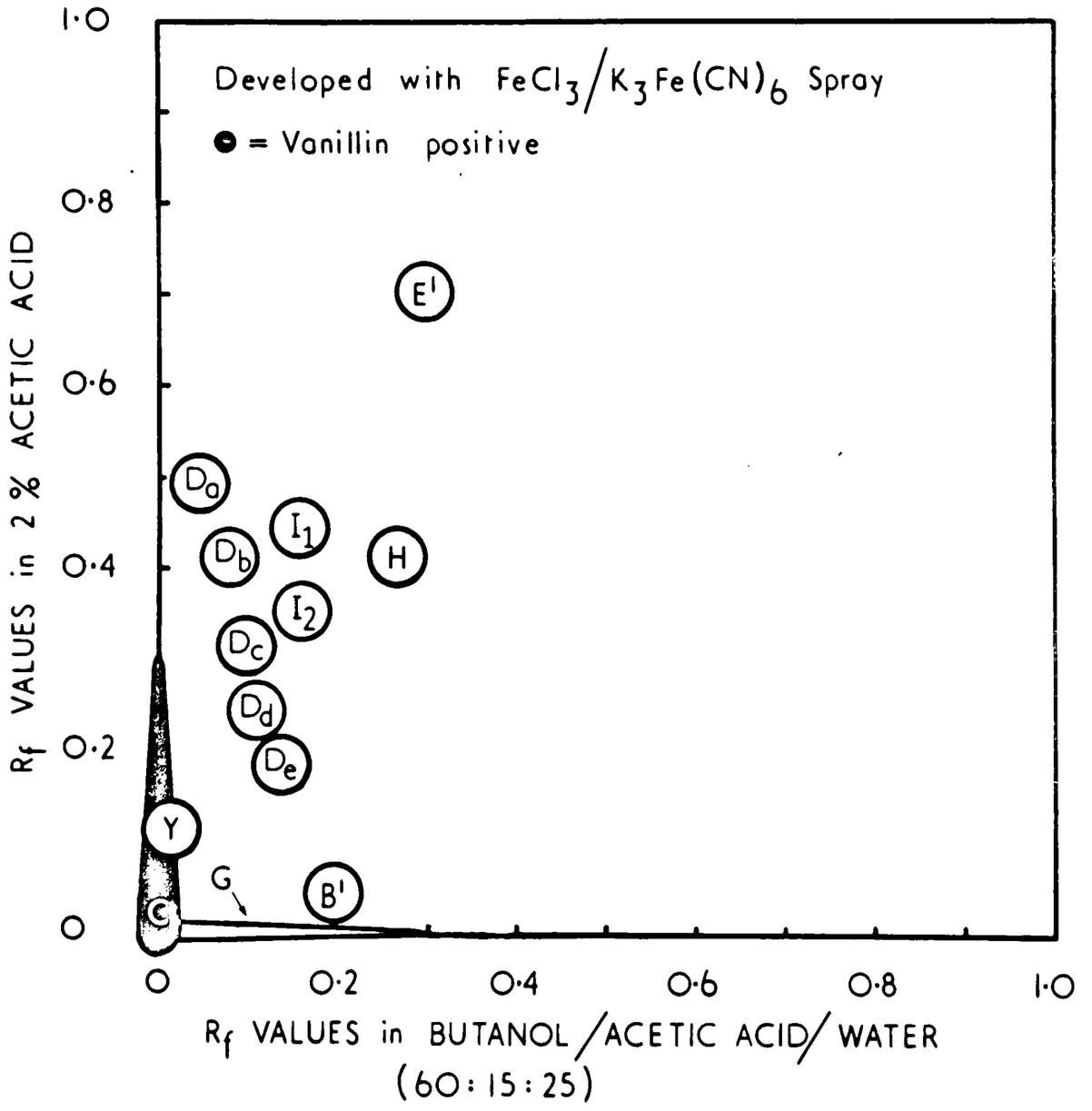


FIGURE 18

TABLE 6 R_f values of oak tannins, obtained by Bostock on cellulose thin-layer. (HAc = 2% acetic acid; BAW = butanol/ acetic acid/ water).

Symbol	Identity, if known	UV	UV/NH_3	R_fHAc	R_fBAW
B		-	-	0.04	0.20
C	Condensed tannin	abs.	abs.	0-0.3	0.00
D_a		abs.	abs.	0.49	0.05
D_b		abs.	abs.	0.41	0.08
D_c		abs.	abs.	0.31	0.10
D_d		-	-	0.24	0.11
D_e		-	-	0.18	0.14
E'		abs.	abs.	0.70	0.30
H		abs.	abs.	0.41	0.27
I_1		-	-	0.44	0.16
I_2		-	-	0.35	0.16
Y		-	-	0.11	0.02
G		-	-	0.00	0-0.30

abs. = absorption of ultraviolet light.

of C with vanillin, again suggesting that (P) contains higher molecular weight components than (S).

d) Hide powder

All the components of the crude oak tannin fraction (S) were adsorbed from aqueous solution by hide powder. The complexes were stable to water, but were dissociated, at least partially, by 70% acetone.

e) Gelatin

An aqueous solution of crude oak tannin fraction (S) gave a whitish precipitate on addition of a 1% aqueous solution of gelatin, containing 10% (w/v) sodium chloride, at pH 4.0. This reaction is given by all tannins (Long, 1961).

f) Hydrolysis

On boiling samples of both (S) and (P) fraction of crude July oak leaf tannin with concentrated hydrochloric acid, a deep red colour was produced, extractable into n-butanol. One-way paper chromatography of the hydrolysate in Forestal solvent with the pure reference compound confirmed the identity of the red material as cyanidin chloride (Appendix 16, p. 205). This was further confirmed by comparison of the visible spectrum of the hydrolysate with that of a solution of pure cyanidin chloride (Appendix 16). Cyanidin, previously reported by Brown, Love and Handley (1962), in hydrolysates of oak leaf tannins, is a characteristic hydrolysis product of condensed flavanoid tannins. Crude oak tannin from April buds was found by the present author to give no red colour on boiling with concentrated

hydrochloric acid and the reaction with tannin from May leaves was extremely weak, so confirming the virtual absence of condensed tannin from oak leaves in the early spring.

Prolonged hydrolysis of July oak leaf tannin with dilute sulphuric acid at 90°C in a sealed tube again gave rise to a cherry red solution. Two-way paper chromatography in 2% acetic acid and BA revealed the presence of ellagic and gallic acids (identified by their R_f values and behaviour in ultraviolet light - see Table 4, p. 52(b)) in addition to cyanidin. One-way paper chromatography in iso-propanol/water (4:1), together with the pure sugar, revealed the presence of D-Glucose. The presence of a sugar and of the simple phenols gallic and ellagic acid in the hydrolysate confirmed for the first time the occurrence of at least one hydrolysable tannin in oak leaves, the presence of which had previously been suspected solely on the basis of chromatographic behaviour (Brown, Love and Handley, 1962). Further details of the methods used for hydrolysis of the tannins are given in Appendix 16 (p. 205).

g) Molecular weight

Aqueous solutions of fraction (S) from July oak tannin were run by Mr. C. Teal on the Beckmann analytical ultracentrifuge in the Department of Biochemistry, Oxford, with the kind permission of Dr. I.O. Walker, in order to gain an indication of the average molecular weight of the crude tannin. The approximate figure obtained was 5,100 (Appendix 17, p. 209).

4. Separation of oak tannins

For further chemical investigation and subsequently to determine the relative biological effects of the condensed and hydrolysable tannins, a method was sought for separating in quantity the components of the crude oak tannin mixture.

a) Paper chromatography

By running samples of the tannin fractions (S) and (P) on two-dimensional paper chromatograms in acetic acid and BAW, cutting out the spots and homogenising them in solvents such as ethanol, aqueous acetone, and dimethyl formamide, it was possible to obtain very small quantities of the major hydrolysable tannins, D and H. However, the tannins, and in particular, the condensed tannin C, proved to be difficult to extract from the paper, owing to adsorption on to the cellulose. Although up to 60% of D could be extracted, yields of C were negligible. Attempts to overcome these losses by increasing the applied sample resulted in very poor resolution, even with only 5 mg. of applied sample. The method is clearly unsuitable for separating tannins in any quantity and was therefore abandoned.

b) Countercurrent separation

Attempts by Bostock (1966) to separate oak tannins by countercurrent methods met with only limited success. Using various two-phase systems of n-butanol, acetic acid and water, he was able to separate the streak G from other components, which, however, were not separated from each other. Partition of fraction (S) between ethyl

acetate and water resulted in separation of H and B into the organic phase but the other components remained in the aqueous layer and could not be resolved. Since the major objective was the separation of the two primary tannin components C and D, which did not seem possible by countercurrent methods, this technique, too, was abandoned.

c) Column chromatography

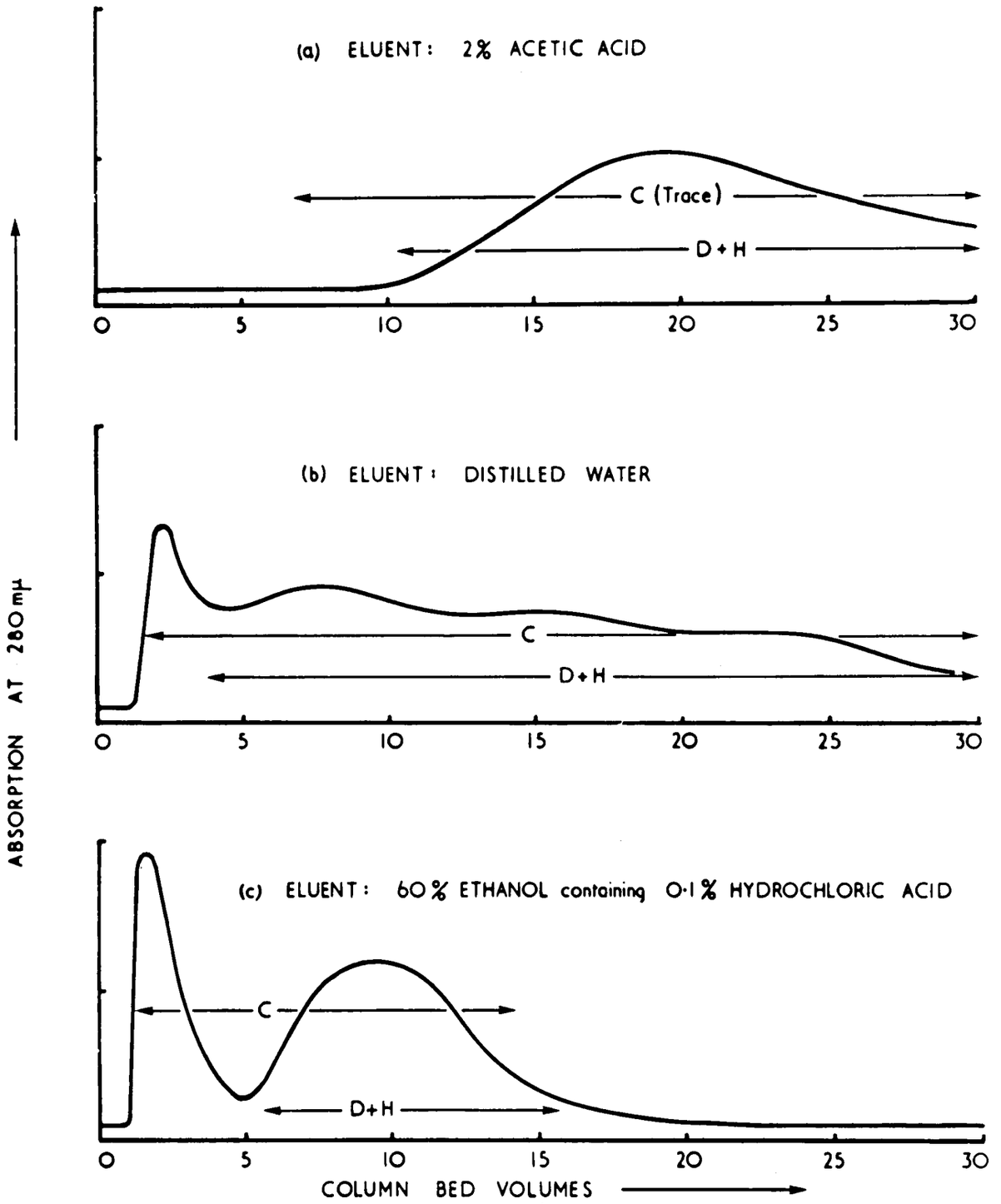
For the next attempt to separate oak tannin in gram quantities, methods of column chromatography were investigated. Cellulose and perlon powder have already been shown to be unsuitable as stationary phases for the isolation of the condensed tannin of common heather owing to irreversible adsorption (Love and Brown, 1959; Brown and Love, 1960). The condensed tannin of oak is similar to that of heather chromatographically so that the same difficulty would presumably arise. Elution from a silica gel column was attempted with fraction (S), but found to be subject to the same difficulty.

Since condensed tannins are normally of much higher molecular weight than hydrolysable tannins, it was decided to investigate the ability of Sephadex gels (Pharmacia, Uppsala, Sweden) to effect a separation. The stationary phase of a Sephadex column consists of beads of a three-dimensional cross-linked dextran network, which swell in water to form a gel. The pores within the dextran beads are of well-controlled size and function as a molecular seive. Molecules too large to fit the pores are not held back by the dextran network, but smaller molecules are retarded on passing down the column, owing to 'partition' between the eluent and the pores of the gel.

The average molecular weight of oak tannin, as reported above, had been found to be approximately 5,100. This suggested that the condensed tannin was probably of higher molecular weight than this figure and the hydrolysable tannin presumably considerably lower. Sephadex G 25 (fine) was therefore selected for the initial experiments, since this gel has a 'molecular exclusion limit' of 5,000 in water (Pharmacia technical handbook).

In view of the separation of tannins C and D in 2% acetic acid on paper, this solvent was used as eluent in the first Sephadex experiment. 0.1 gm. July oak tannin (S) was applied to the top of a 15 x 1 cm. G 25 Sephadex column and eluted with 2% acetic acid. The top 2 cm. of the column at once turned dark brown and this band did not move further, presumably owing to irreversible adsorption and/or transformation. The eluate was passed through the flow cell of a Beckmann ultraviolet spectrophotometer, set at 280mμ, before delivery into the tubes of a fraction collector. No adsorption was noted until 10 bed volumes of eluate had passed through the column. A long sprawling peak then began to appear on the spectrophotometer chart, taking some 30 bed volumes to pass through completely (Figure 19 (a), p.64(a)). Chromatography of fractions corresponding to this peak revealed the presence of D and H, together with a trace of C in all fractions. Thus, although the method could be used to obtain reasonably pure D and H, it was clearly of no value for obtaining pure C.

A second column was eluted with distilled water in place of acetic acid. As before, a dark brown band formed at the top of the



SEPARATION OF OAK LEAF TANNINS BY SEPHADEX (G 25 Fine)

COLUMN CHROMATOGRAPHY.

FIGURE 19

column and remained stationary. After one column volume, a peak appeared on the chart, closely followed by a long sprawling second peak (Figure 19 (b), p.64(a)) which took several hours to emerge, involving more than thirty column volumes of eluent. Chromatography of the first fraction of the first peak revealed a small amount of C only. Fractions from the second peak were found to contain C, D and H. It seemed therefore that small amounts of C could be obtained by this method but the resolution was very poor, owing to extensive 'trailing' of C into the fractions containing D and H. As the method was clearly unsuitable in its present form for separating the major components, C, D and H, no attempt was made at this stage to locate the whereabouts of the minor tannin components (B and G), by concentrating the individual fractions.

A subsequent elution with distilled water, using a smaller tannin sample (0.05 gm. July tannin (S)) and a longer column (60 cm.), in an attempt to improve resolution, produced three broad overlapping peaks, again taking over 30 column volumes to elute. In this case, chromatograms from all fractions revealed material at the origin and fractions corresponding to the first two peaks also contained phenolic material, which streaked along the whole acetic acid axis. Degradation of the tannin seemed to be occurring. A dark brown band again formed at the top of the column. The colour was only weakly extracted from the gel on shaking with acetone.

Elution from Sephadex columns using aqueous methanol was attempted

by Bostock (1966) but similar unsatisfactory results were obtained.

In January 1966, the successful use of 60% ethanol (containing 0.1% hydrochloric acid) as an eluent for tannins on Sephadex was reported by Somers (1966). He was able to separate condensed flavanoid pigments from wine into one fraction which was totally excluded from the G 25 gel and another fraction, showing weak adsorption effects but which could be eluted within $1\frac{1}{2}$ bed volumes of eluate. In the hope that irreversible adsorption of oak tannins might also be eliminated by using this eluent, another column was run, in conjunction with Bostock, following the method of Somers. A dark brown band, shown by paper chromatography to contain the condensed tannin C only, was excluded from the gel and eluted in 0.5 bed volumes. The column remained pale brown, probably from partial adsorption of C, since traces of phenolic material immobile on paper continued to be eluted. Apart from these, however, none of the other oak tannins was eluted within 2 bed volumes, by which time C had ceased to be eluted (Figure 19 (c), p. 64(a)). On continued elution, lasting several hours, D, H and B' came off the column within the next ten bed volumes. The method was repeated by Bostock (1966) on a larger scale and it was again found that while C was totally excluded from the gel, the other tannins were very strongly but reversibly held, so that they were eluted by large volumes of eluate: B' in $3\frac{1}{2}$, and D_1, D_2, and H in 4 bed volumes.

The similar strong retentions by Sephadex of the D spots and H suggest that they all have similar sizes and structures, which allow

them to penetrate the pores of the dextran gel, where they fit closely and are presumably held by multiple hydrogen bonds. Somers (1966) gives a value of about 2,000 for the molecular exclusion limit of G 25 Sephadex in 60% ethanol, so that molecular weights somewhat lower than this would be expected for D and H and a much higher one for C. Using the Beckman analytical ultracentrifuge in the Department of Biochemistry, Oxford, the average values obtained for the molecular weights of the oak tannin fractions separated by Bostock were found to be 6,750 for pure C (condensed tannin) and 1,680 for D (Appendix 17, p.209 and Bostock, 1966). These values are therefore in good agreement with the interpretation of the behaviour of oak tannins on Sephadex columns.

Although condensed tannin, C, could be obtained chromatographically pure from a single separation on Sephadex, D and H were still contaminated with C and B' and not separated from each other. Bostock (1966) discovered that D and H could largely be freed from B' and remaining traces of C by running them down a second Sephadex G 25 column, using 50% acetic acid as eluent. Under these conditions, C appears to be irreversibly adsorbed by the Sephadex. Final purification of D and H was achieved by two-way paper chromatography in acetic acid and BAW, using Whatman 3 MM paper.

5. Properties of the major components of oak leaf tannin

Tannin C was obtained as a reddish brown amorphous powder, partly soluble in water but completely soluble in 20% acetone/water. Its ultraviolet spectrum showed a distinct maximum at 277 m/u. Its

molecular weight (average) was found to be 6,750, using the ultracentrifuge. Aqueous solutions gave copious precipitates with gelatin solution (see p.60) and were freed from C by hide powder adsorption.

The flavanoid nature of C, indicated by its positive reaction with vanillin reagent, was confirmed by liberation of cyanidin with concentrated hydrochloric acid (Appendix 16, p.205). Some gallic acid was produced by dilute acid hydrolysis (Bostock, 1966) and could either have come from contamination of C with a gallotannin, or from galloyl groups linked to the hydroxyl groups of the flavanoid nuclei. The former is unlikely, since sugars were not detected in the hydrolysate.

All the information obtained concerning C is consistent with the view that it is a condensed flavanoid tannin, built up from monomers such as (+)-catechin and (+)-gallocatechin, which are present in the leaves when the tannin begins to appear in late May (Figure 17, p.50a). Structural details have not yet been completely worked out for any condensed tannin, but investigations of Calluna condensed tannin, which has similar chromatographic behaviour to C, suggest that its structure may be based on ether linkages between the flavan -3, 4-diol units (Brown,1964; see Appendix 13, p.196).

Degradation of D_2 and D_3, the major components of the suspected hydrolysable tannins of oak leaves, was carried out by Bostock (1966), who discovered a rich source of these components, uncontaminated with condensed tannin, in oak galls caused by the gall wasp Adleria

(= Cynips) kollari Hartig. Hydrolysis of these two tannins gave ellagic acid and glucose, confirming their identity as hydrolysable tannins. They could be further classified as ellagitannins, though the occurrence of the unidentified phenolic compounds N1, N2, P' and S1 - S3, none of which could be hydrolised further, indicates a more complex constitution than those of ellagitannins so far described (Figure 20, p.69(a)). From retention volumes on Sephadex, N1, N2, and P' are thought to be sizeable fragments of D_2 and D_3. It is interesting that N1 and N2 also appear along with D_2 and D_3 in extracts of oak galls and are perhaps biosynthetic precursors.

Whether obtained from oak leaves or galls, D was a dull white amorphous powder, completely soluble in water. Its ultraviolet spectrum was featureless, showing gradually increasing absorption in the ultraviolet region, with no maxima. Solutions gave precipitates with gelatin and D was adsorbed by hide powder. H, of which a small amount was prepared by Bostock, appeared to have similar properties to D.

6. Quantitative estimation of tannins

a) Review of published methods

To determine the ecological importance of oak tannins, it is clearly necessary to have at least a rough estimate of the amount of tannin present at various times of the year. The literature was therefore searched for a suitable quantitative method.

Two methods have been used for the past fifty years or so by industrial concerns involved in the manufacture of leather, tea,

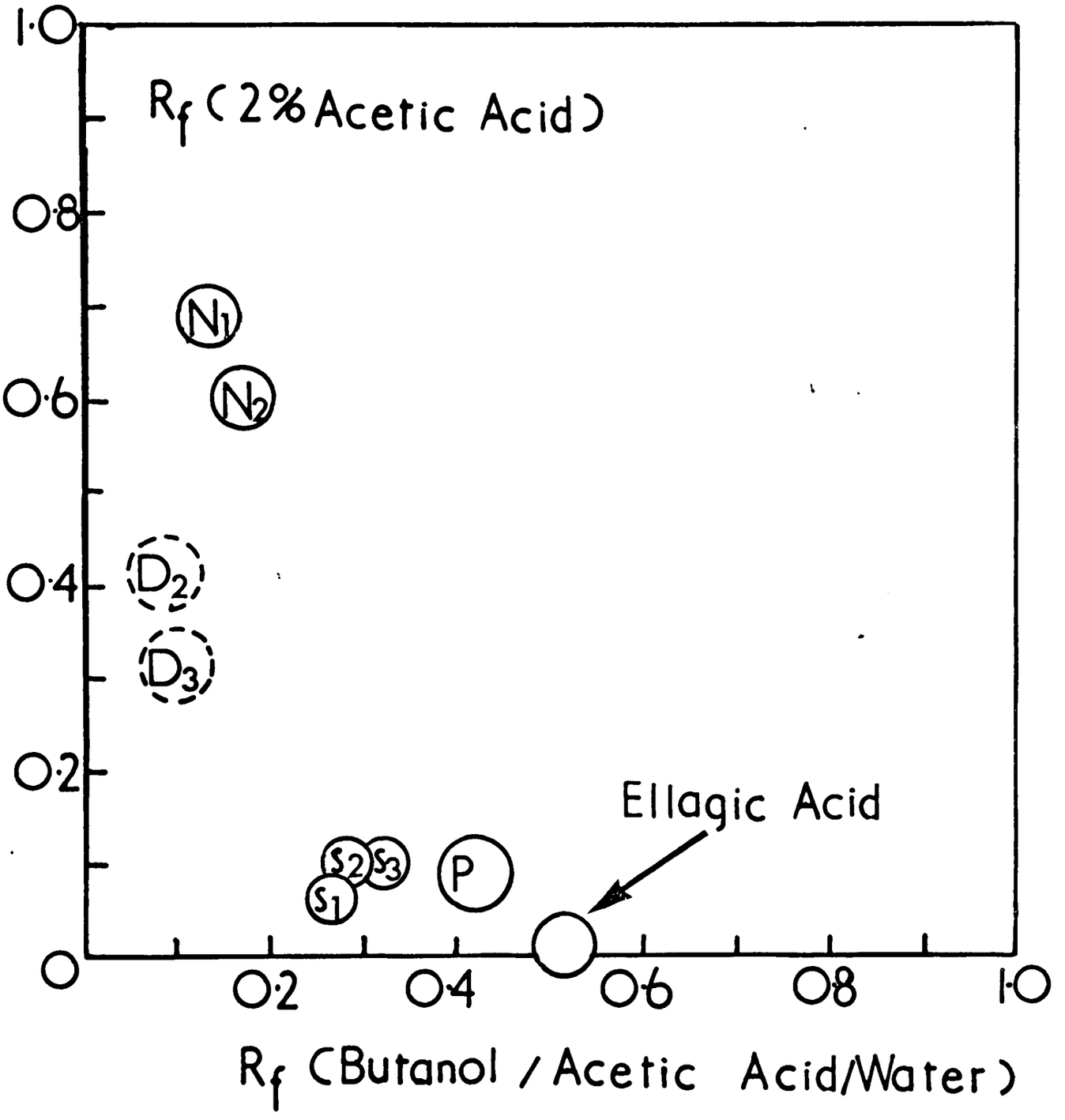


FIGURE 20 Two-way thin layer chromatogram of hydrolysis products of D_2 and D_3 from oak galls (after Bostock, unpubl.)

tobacco and other economically important commodities. Leather manufacturers have tended to use the hide powder method of tannin analysis (McCandlish, 1942). The principles of this method is quite simple. The amount of tanning material in an aqueous extract is estimated by finding the increase in weight of a sample of hide powder after standing for a period of time in a known volume of the extract. In practice, the method is very tedious, since standardisation of the method involves precise pre-chroming of the hide powder, which must be of a recognised standard grade, and adjustment of the moisture content of the chromed powder to within 1% before adsorption, by squeezing in muslin cloth. Extracts have to be laboriously prepared in a standardised way. Apart from the considerable labour involved, this method suffers from two further disadvantages. Firstly, it is unspecific, since a wide variety of phenolic components, in addition to the high molecular weight tannins, are adsorbed to varying extents by hide powder, giving rise to an arbitrary result. Although this is undoubtedly of use in industry, when the fully standardised method is adopted, it is of little use for precise determination of individual polyphenolic components. Secondly, aqueous extracts must be used for the hide powder method, since only under these conditions are the protein-tannin complexes stable. However, precisely because of this reason, water will not completely extract tannins from leaf tissue, since some of the tannin is bound to the leaf protein. It is therefore necessary to use acetone or some other solvent which will split hydrogen bonds in order to extract the maximum amount of tannin from plant tissue.

Removal of acetone by vacuum distillation of aqueous acetone extracts, prior to analysis of the resulting aqueous solution, leads to further problems, since some of the tannin was found by the present author to be precipitated in the process, along with other material. For these reasons, the hide powder method was considered unsuitable for tannin analysis of oak leaves.

A method widely used in the polyphenol analysis of tea and tobacco involves the titration of aqueous extracts with potassium permanganate before and after tannins have been removed by gelatin precipitation (U.S. Dept. Agr. Div. Chem. Bull., 1892). In this procedure (known as the 'Löwenthal' method) the tannins are estimated by their reducing properties, the difference in the two titres providing an estimate of the tannins present, in terms of standard tannic acid solutions. This method has also been shown to be quite unspecific and arbitrary (Roberts, 1962) and also suffers from the disadvantage that only aqueous extracts can be analysed. It too was discarded as unsuitable for the present work.

Modern methods for quantitative analysis of small amounts of plant chemicals mostly make use of paper and column chromatography. Spots may be cut from chromatograms, with or without prior development with a colour producing reagent, and the resulting solutions after elution estimated spectrophotometrically, again with or without treatment with a colour producing reagent. Such methods have been successfully applied to the analysis of simple phenols in plant material (e.g. Pridham, 1957; Swain and Goldstein, 1963) and, of course, are

independent of the solvent used for the original extraction. Unfortunately, no solvent has been found which will elute more than insignificant amounts of oak leaf condensed tannin, C, from homogenised chromatogram spots. Continued strip elution of excised spots, as opposed to homogenisation, also fails to remove the condensed tannin, which appears to be irreversibly bound to the cellulose. Methods involving elution of chromatographic spots are therefore of no value for the estimation of oak condensed tannin. Although much of the hydrolysable tannins can be recovered from chromatograms by homogenisation of excised spots in aqueous acetone or dimethyl formamide, the elution is not quantitative. However, it should be possible to elute these components quantitatively from the intact excised spots by strip elution, since they move appreciably more easily in 2% acetic acid than condensed tannin and do not leave immobile material at the origin. The eluted spots would have to be developed with a colour producing reagent, since the hydrolysable tannins show no maxima in their visible and ultraviolet absorption spectra (p. 69).

Several modifications of the paper chromatographic method have been successfully employed for quantitative phenol analysis. Bradfield and Flood (1952) found a quantitative relationship between the amount of phenol present and the direct light absorption reading obtained by placing the excised spots in the sample cell of a spectrophotometer, with a blank control. Such a method would be impracticable for the oak tannins, since they form streaks of uneven colouration with developing reagents, rather than concise spots, and, in view of the

concentration of condensed tannin at the origin, the method is very unlikely to be quantitative. Scanning of excised spots with a photo-electric scanner, as used by Reid (1956 and 1959) for tobacco leaf extracts, is likely to suffer from the same disadvantages. Pridham's (1957) azo-dye method has been attempted by the present author, but it was found that the orange dyes obtained from oak tannins with diazotised p-nitraniline could not be eluted with methanolic potash.

For many phenols, it has been found that there is a linear relationship between the logarithm of the phenol concentration in the initial spot and the weight of the spot cut from the developed chromatogram (Hathway, 1960). However, a preliminary experiment with oak tannin (Appendix 18, p. 210) showed that the variation in the weights of excised spots was such that, to obtain even 5% accuracy in tannin analysis, the necessary quantity of tannin in the initial spot was prohibitive. Such a chromatogram was in fact run, but the condensed tannin 'caked' at the origin and the chromatogram was so overloaded that resolution was almost negligible.

For reasons mentioned previously, column chromatography is impracticable for estimation of tannins, which tend to be irreversibly adsorbed on to cellulose, perlon, silica gel and even Sephadex. Although the latter was used for the qualitative preparation of individual tannins, following the method of Somers (1966), yields were never quantitative, owing to some losses on the column.

Further methods of quantitative analysis, also considered

unsuitable for the present work, are included in the review by Hathway (1960) and Roberts (1962).

b) Gravimetric method

As no suitable method of tannin analysis could be found in the literature and since some estimate of oak leaf tannins had become an urgent necessity, it was decided to investigate whether the Brown and Love (1961) method of extraction could be adapted as a gravimetric method for quantitative tannin analysis. An experiment was accordingly carried out, using a sample of 500 ml. 70% acetone extract of fresh September oak leaves (50 gm. wet wt.), to ascertain under what conditions, if any, the method could be made quantitative.

Three 50 ml. aliquots from the acetone layer obtained after salting out (Appendix 14, p.201), were treated according to the scheme in Figure 21 (p.74(a)). To the first aliquot, 2 volumes of ethanol and 5 volumes of ether were added. To the second aliquot, 4 volumes of ethanol and 5 of ether were added and to the third, 2 volumes of ethanol and 10 of ether (Table 7, p.74(b)). The precipitates of tannin and salt were centrifuged down (2,500 rpm. for 10 minutes), dialysed, freeze-dried and weighed separately. The ethereal supernatants in each case were concentrated by vacuum distillation to 25 mls., of which 0.01 ml. aliquots were applied to two-way chromatograms, run in the usual way in acetic acid and BAW, to discover whether any tannin remained unprecipitated. The results (Table 7) show that some tannin remained in the ethereal layers from aliquots 1 and 2, but that complete precipitation had been achieved from aliquot 3, for which 2 volumes of

FIGURE 21

TANNIN ANALYSIS BY SALTING OUT

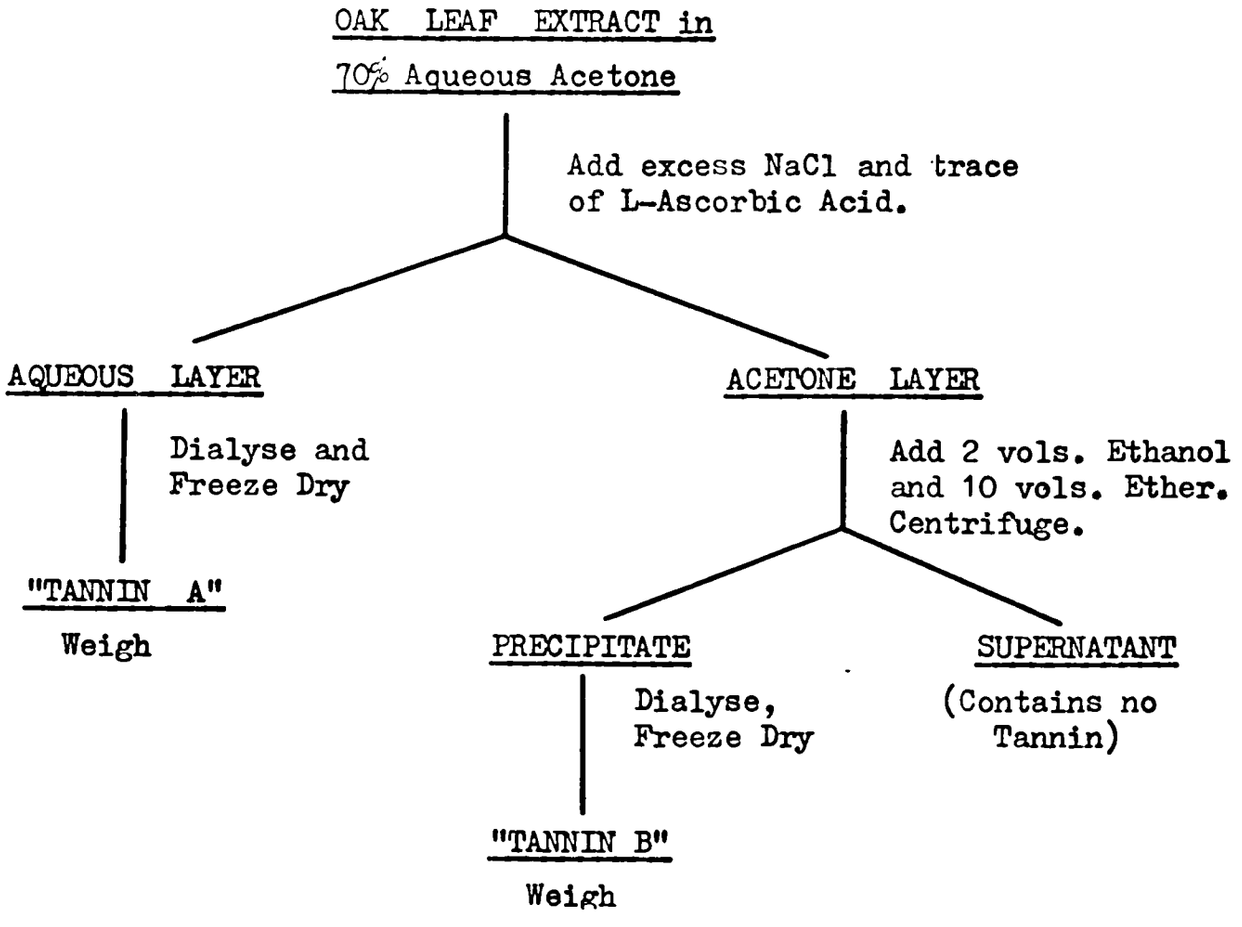


TABLE 7 Effect of alteration of the ratios of ethanol and diethyl ether on the yield of 'Tannin B' from the Brown & Love (1961) extraction procedure

Volume of 70% acetone/ water oak leaf extract[1] (ml.)	Volume of added ethanol (ml.)	Volume of added ether (ml.)	Yield of 'Tannin B' (gm.)
50	100	750	0.163
50	200	1,250	0.134
50	100	1,500	0.165

[1] Aliquots taken from ~~from~~ extract of 50gm. (fresh weight) September oak leaves in 500ml. 70% acetone/ water.

ethanol and 10 of ether had been used. However, as judged by the weight of tannin obtained, addition of ten volumes of ether is only marginally more effective than addition of five volumes (aliquot 1).

The Brown and Love method can therefore be made quantitative, although the method is somewhat tedious and involves considerable volumes of ether. The tannin fraction obtained from the acetone layer was designated 'Tannin B'. It was chromatographically free from the non-tannin phenolic material present in the original extract and consisted largely of condensed tannin, C, and the hydrolysable tannins, D and H, as discussed earlier in this chapter.

After the salting out of the 500 ml. of September extract, it was noticed that the lower, aqueous, layer was a rich golden brown colour. Suspecting that this layer might contain some tannin, it was vacuum distilled to remove traces of acetone, dialysed and freeze-dried, when it yielded a light brown powder (0.211 gm.). Heating a sample of this powder with concentrated hydrochloric acid/butanol yielded a deep red colour, identified as cyanidin by its spectrum (λ max at 555 mμ) and R_f in Forestal solvent (Appendix 16, p.205). It was therefore concluded that the material contained some condensed tannin and was labelled 'Tannin A'. Two-way chromatography of an ethanolic solution of tannin A showed only a long streak along the acetic acid axis of R_f 0 - 0.9 when sprayed with ferric chloride/potassium ferricyanide, with a concentration near the origin, which was weakly vanillin-positive. Of tannins D_1, D_2, H, etc., there was no sign. Elemental analysis by Messrs. Weiler and Strauss of tannin A

revealed the presence of a small amount of nitrogen and a large quantity of inorganic material, as shown below. The percentages of carbon and hydrogen, however, were not very different from those of tannin B, Calluna tannin, etc. (see p. 58).

Elemental analysis of Tannin A

% C	% H	% N	% Ash
51.65	4.81	1.45	6.76
51.76	4.48		

Tannin A was found to be adsorbed from aqueous solution by hide powder. Although Tannin A must contain at least some non-tannin material, the above observations indicate that it consists very largely of tannin, partly condensed tannin and partly of material of unknown nature. It was therefore decided to include tannin A in routine leaf analyses, but to tabulate the results separately.

c) Seasonal variation of oak tannins

Since the gravimetric method of tannin analysis takes approximately 6 days per sample, with only one freeze-drying apparatus available, attention had to be confined to one oak tree initially, though it was hoped that further trees might be investigated subsequently if a more convenient method of analysis could be developed.

Samples of the upper sun leaves from tree M in Wytham were collected through the 1965 season according to the procedure already described and 50 gm. homogenised in 500 ml. 70% acetone, containing 0.5 gm. L-Ascorbic acid, to reduce the risk of oxidation. Tannins A

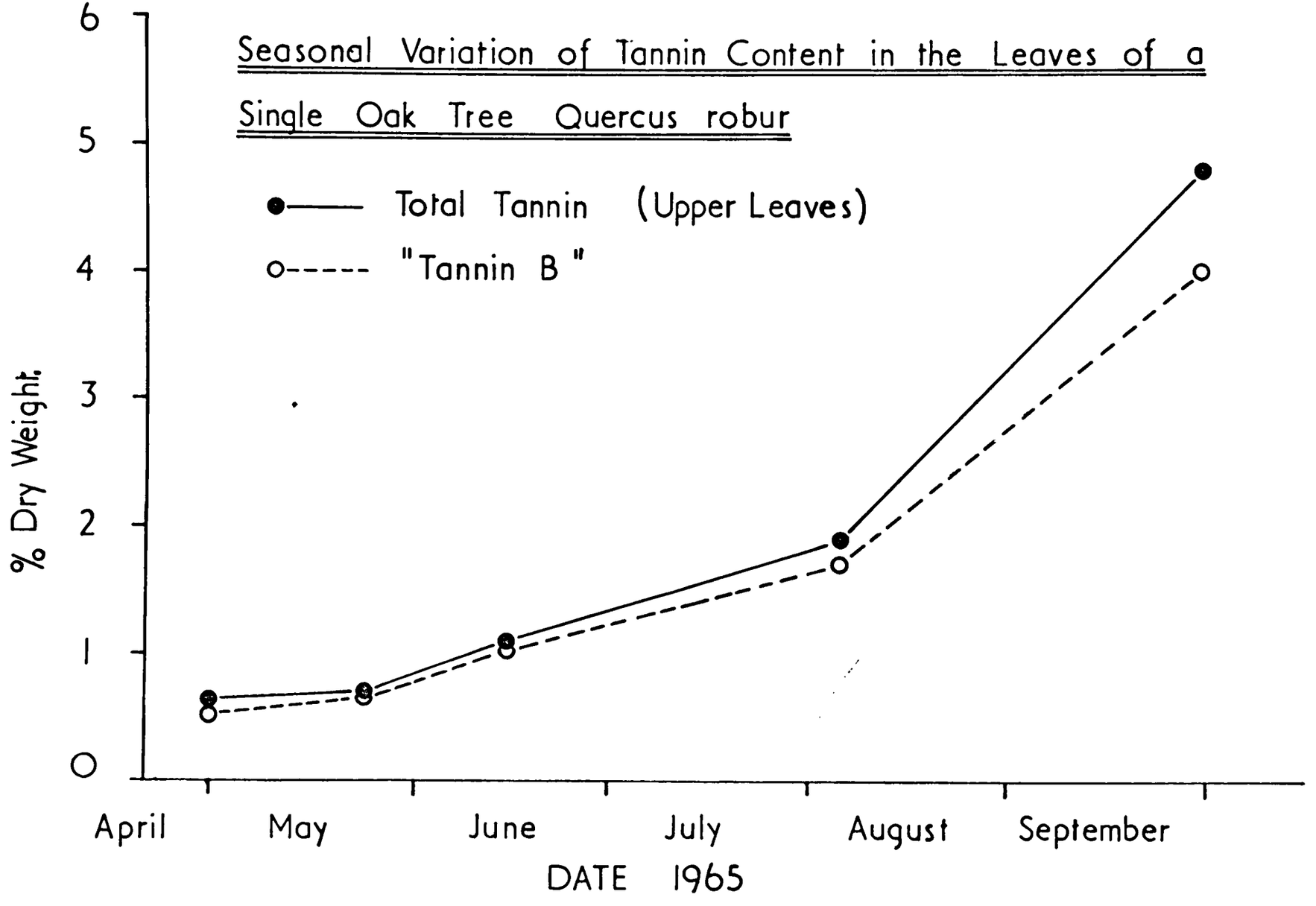
and B were estimated by the gravimetric method. The results are shown in Figure 22 (p.77(a)) and Table 8 (p.77(b)). It can be seen that the tannin content does, as suspected, increase during the season, though rather slowly at first. The most rapid increase occurs in the late summer, when the tannin accounts for as much as 5.5% of the dry weight of the leaves in September. The condensed tannin, C, and hydrolysable tannins (chiefly D and H), making up the 'Tannin B' fraction, account for about 80% of the tannin throughout the season.

d) A new spectrophotometric method for estimating condensed tannin

Chromatographic methods for estimating condensed tannin had previously been abandoned on account of the difficulty encountered in attempting to elute the tannin from paper. However, in view of the considerable time involved in analysing samples by the gravimetric method, much thought was still given to possible alternatives, preferably making possible the independent analysis of condensed and hydrolysable tannins. It seemed possible that elution from chromatograms would not be necessary if the entire spot could be dissolved, cellulose and all, and the tannin estimated in the resulting solution without interference from the cellulose.

The most obvious solvent was concentrated sulphuric acid, which converts the cellulose to sugar and subsequently to furans, before charring occurs due to dehydration. However, since condensed tannin yields some cyanidin on treatment with acid, and since the red colour produced might be found to be proportional to the amount of tannin present, it seemed worth while attempting to find conditions of acid

FIGURE 22



77(a)

TABLE 8 Tannin content of upper sun oak leaves from tree M during 1965 (estimated by gravimetric adaptation of Brown & Love (1961) extraction method.

Date of Leaf Sample	Fresh Weight of Leaf Sample (gm.)	Dry Weight of Leaf Sample (gm.) [1]	Yield of 'Tannin A'(gm.)	Yield of 'Tannin B'(gm.)	Yield of Total Tannin (gm.)	% Tannin (dry wt. of leaf)	% Tannin (fresh wt. of leaf)
30/4	50	14.1	0.019	0.074	0.093	0.66	0.186
23/5	50	15.1	0.009	0.096	0.105	0.70	0.21
14/6	50	21.6	0.010	0.230	0.240	1.10	0.48
5/8	50	23.2	0.036	0.365	0.401	1.86	0.80
30/9	50	24.3	0.211	0.990	1.201	5.50	2.40

[1] Determined on a separate sample of 50gm. leaves from the same collection.

concentration and temperature under which the cellulose and tannin could be completely dissolved without discolouration of the solution due to charring.

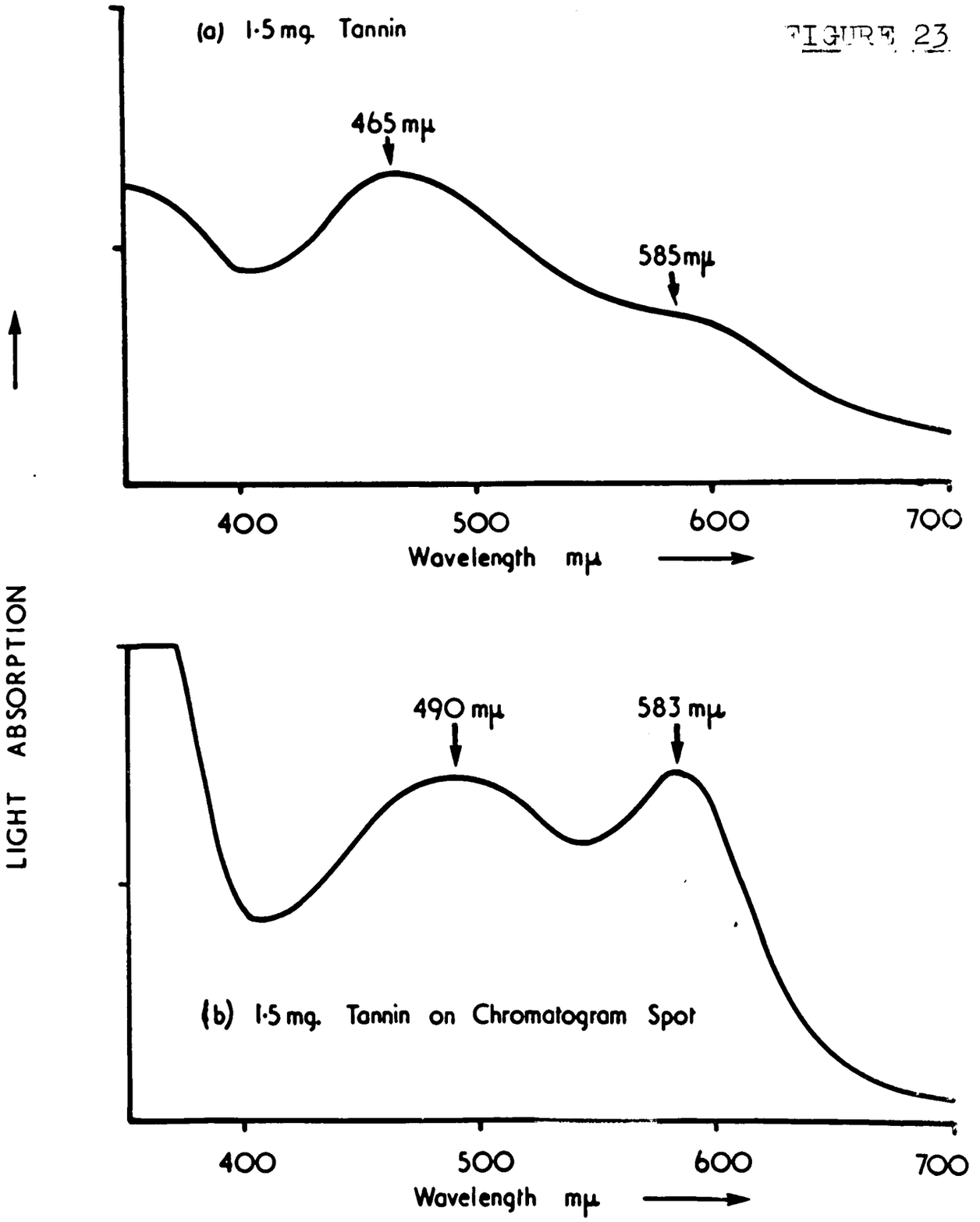
Treatment of blank spots cut from chromatogram paper with different acid concentrations for different periods of time at various temperatures showed that such conditions could be achieved using 66% acid at 60°C for 4 minutes. Under these conditions, the cellulose is completely dissolved and if the tubes are transferred to a water bath at room temperature, the solutions remain colourless and free from charring for several hours.

60°C was found to be the best compromise temperature for developing the pink colour of the tannin spots. At 50°C, the reaction is very much slower and the colour does not reach the same intensity, whereas at 70°C, charring of the solutions, resulting in the development of a brown solution, is rapid. Using 2 ml. of acid (the minimum volume required for a standard spectrophotometer cell) per excised chromatogram spot for 4 minutes in a 60°C water bath, the cellulose controls dissolved completely to give colourless solutions, while the tannin spots reached almost maximum colour development. After 4 minutes at 60°C, control, blank spots showed no absorption peak in the visible range. After 8 minutes, a very slight peak at 455 mμ appeared, which was pronounced after 10 minutes at this temperature. Cooling of the solutions to room temperature after the 4 minutes reaction time retarded formation of coloured cellulose breakdown products for several hours.

The cerise solution obtained by dissolving a cellulose spot containing 0.15 ml. of 1% September tannin (S) solution in 2 ml. 66% sulphuric acid at 60°C for 4 minutes showed adsorption peak at 465 mμ and a much lower shoulder at 585 mμ (Figure 23, p.). This contrasts with the spectrum of the supernatant obtained after hydrolysing 0.1 gm. of the same tannin (solid), not on cellulose, under the same conditions, when the normal cyanidin peaks at 585 mμ and 490 mμ, of equal height, were observed to be present (Figure 23). The shift of the 490 mμ peak to 465 mμ and the reduction of the 585 mμ peak to an inflexion suggest that the presence of cellulose degradation products has a modifying influence on the final colour. Since, however, the spectra of several tannin/cellulose hydrolysates were found to be entirely reproducible, the discrepancy need not affect the usefulness of the method.

To discover whether the colour developed from excised tannins spots is quantitatively related to the amount of tannin present, an experiment was carried out as follows. Samples of increasing size (0.02 to 0.20 ml.) from a 1% aqueous solution of September tannin (S) were applied to Whatman No. 1 chromatography paper. The samples correspond to a range of 200 μg to 2 mg. tannin (Table 9, p.). After drying, the spots were cut out with scissors, together with two control blank spots, and placed in tubes in a water bath at 60°C. 2 ml. of 66% sulphuric acid were added to each tube and after 4 minutes the tubes were removed and cooled in an ice-water bath. The colour intensities were measured in a Unicam spectrophotometer at 490 mμ

FIGURE 23



Spectra of solutions obtained by dissolving Oak Leaf Condensed Tannin (a) Alone and (b) on excised Whatman No. 1 Chromatogram Spot in 2 mls. 66 % Sulphuric Acid at 60°C for 4 Minutes.

TABLE 9 Optical density at 490mμ of excised chromatogram spots (containing oak leaf tannin), after solution in 66% H_2SO_4 at 60°C.

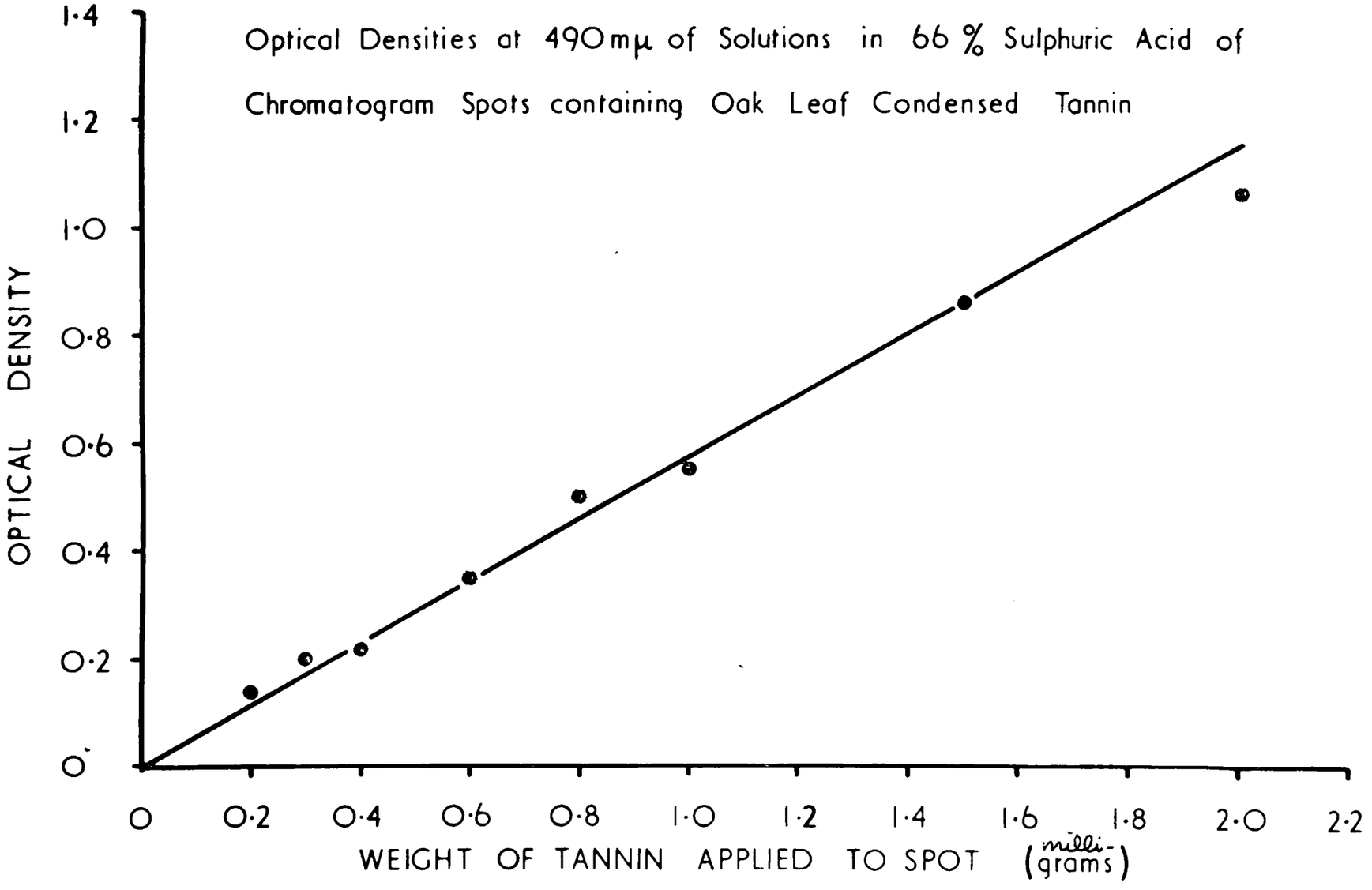
Volume of tannin solution[1] applied to chromatogram spot (ml.)	Weight of applied tannin (μg)	Optical density at 490mμ[2]
0.02	200	0.137
0.03	300	0.197
0.04	400	0.216
0.06	600	0.347
0.08	800	0.500
0.10	1,000	0.55
0.15	1,500	0.86
0.20	2,000	1.06
0 (control)	0	0

[1] 1% solution of September oak leaf tannin (fraction (S)) in water.

[2] Optical densities read in Unicam manual spectrophotometer.

against 66% sulphuric acid (control) and are recorded in Table 9. A graph of the readings (Figure 24, p.) takes the form of a straight line, showing that the colour obeys Beer's Law, at least up to a tannin concentration of 2 mg., so that the method can be used for the rapid estimation of small amounts of condensed tannin. In conjunction with the elution method suggested for the hydrolysable tannins (p. 72), therefore, a scheme is available for the rapid analysis of all major tannin components from a single chromatogram of the acetone extract of oak leaves. The only disadvantage of the method is its lack of sensitivity. Application of 400 µg of tannin would be required for an accurate reading, corresponding to about 0.2 ml. of an extract of 5 gm. August leaves in 10 ml. solvent. However, this is at least as sensitive as other methods for estimating condensed tannin and, in view of the labour and difficulties involved in other methods of tannin analysis, this new method offers very definite advantages.

FIGURE 24


Optical Densities at 490mμ of Solutions in 66 % Sulphuric Acid of Chromatogram Spots containing Oak Leaf Condensed Tannin
OPTICAL DENSITY
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
2·2
WEIGHT OF TANNIN APPLIED TO SPOT (milli-grams)

CHAPTER 5: EFFECTS OF OAK LEAF TANNINS ON LARVAL WEIGHT INCREASE AND PUPAL WEIGHT OF THE WINTER MOTH

1. Introduction

In order to investigate the possible effects of oak leaf tannins on the growth of caterpillars under controlled conditions, it was necessary to carry out experiments in the laboratory. Results from such laboratory experiments might then throw light on the effects of tannins in the field. The use of laboratory experiments to examine ecological phenomena is prone to considerable errors of deduction. Conclusions from laboratory experiments, however striking, might be invalid under natural conditions, where the effect in question may be outweighed by some of the many other, often unsuspected, factors, which cannot be taken into account during the laboratory experiments. However, there is frequently no alternative to laboratory investigation as an initial step in evaluating the effects of individual ecological variables. Provided that the results are not indiscriminately assumed to apply in the field, such experiments can be of considerable value in revealing factors which might be of ecological significance.

For the projected growth experiments involving tannins, an artificial diet was sought which might be suitable for the culture of oak-feeding Lepidoptera larvae in the laboratory. Ideally, the chemical composition of the diet should be completely defined so that

the maximum amount of information could be gained from the experiments. Diets based on leaf powders were clearly unsuitable, since the unknown and non-reproducible composition of the powders would influence the results, rendering them almost valueless. At the time when these experiments were contemplated (1964), no precisely defined chemical diets for the rearing of caterpillars were in use in this country, as far as is known, though diets approaching complete chemical definition had been used in America for rearing the larvae of certain agricultural pests, such as the cotton bollworm Heliothis zea Boddie (Berger, 1963). However, at the suggestion of Professor G.C. Varley, the formula for a possible diet was obtained from Miss A. McMorran, of the Canadian Forestry Department research centre at Sault Ste. Marie, Ontario. This agar-based artificial diet is a slight modification of the medium used by Berger (loc. cit.) and others for rearing the cotton bollworm. Since it had been used successfully for rearing caterpillars of the spruce budworm Choristoneura fumiferana (Clam.) and other Lepidoptera species in Canada (McMorran, A., personal communication), it was decided to see if it could be applied to the present problem.

The formula of the diet was subsequently published (McMorran, 1965). Since it contains wheat embryo and nutrient agar as additives, it is not strictly chemically defined. However, it seemed to be the most suitable formula available at the time.

2. Preliminary test of artificial diet

The first batch of artificial diet was prepared following exactly the procedure of McMorran (loc. cit.). The composition of the diet and details of its preparation are given in Appendix 19 (p.212).

It was mentioned in Chapter 1 (p. 18) that the winter moth had been selected for laboratory experiments. This species is widely polyphagous and is unlikely to require specific stimulant chemicals to bring about feeding, as have been found, for example, in the case of the silkworm Bombyx mori (L.) (Watanabe, 1958). However, in case the basal artificial diet proved unacceptable to the larvae, two further diets, in which oak leaf extracts were included, were tested simultaneously to save time. It was essential to discover a satisfactory diet quickly, since winter moth larvae are available for only 6 to 8 weeks each season. The second test diet, therefore, consisted of 100 gm. artificial diet to which was added 0.5 gm. of a powder obtained by vacuum-distilling a petroleum ether (40° - 60°C) extract of fresh oak buds. The third test diet consisted of 100 gm. artificial diet, to which had been added 0.2 gm. of a white solid, obtained by steam distillation of an aqueous suspension of deep-frozen May oak leaves. This solid, visible as a white film on the surface of the distillate, was dried after extraction into diethyl ether. It smelled very strongly of fresh oak leaves and was suspected to be a terpene.

Owing to its volatility and characteristic scent, which would seem to be the major component of the scent of young oak leaves, it was considered to be a likely feeding stimulant for oak-feeding insects, if

such compounds are indeed required.

Eggs from winter moth females, collected in Wytham the previous winter by Mr. G.R. Gradwell, were hatched in the laboratory in April, 1964. 15 of these freshly hatched first instar larvae were fed separately on each of the three test diets. Each group of 15 was split into 5 batches of three larvae, fed on pieces of diet placed in Petri dishes, covered with perforated polythene.

A number of the larvae died within the first 48 hours, after becoming trapped and drowned in the film of water on the surface of the moist pieces of diet. However, after 5 days it was apparent that the remainder were feeding successfully on all three test diets. After 10 days, 8 larvae were apparently thriving on both the standard diet and the diet containing 'oak scent' material and 7 were still surviving on the diet containing the petroleum ether extract. In all cases, moulting to 2nd instar had been successfully accomplished.

The preliminary experiment therefore showed that the basal artificial diet could apparently be used successfully for feeding winter moth larvae, though 1st instar larvae,on account of their small size, were vulnerable to drowning. As suspected, no additive compounds were necessary to enable the caterpillars to feed and further chemical investigation of the suspected terpene was therefore abandoned as irrelevant.

In order not to waste a complete breeding season, a more thorough test of the artificial diet was not carried out at this stage, but was included in the first of the experiments on the effect of tannins on

larval growth.

3. Effects of oak leaf tannin on larval weight increase and pupal weight of the winter moth - 1964 season

a) Method

In an experiment, commencing on May 13th, 1964, the effects of oak leaf tannin on the growth of winter moth larvae were investigated by rearing 25 4th and early 5th instar larvae on each of 5 different diets and comparing their gains in fresh weight and final pupal weights.

The diets were basal artificial diet (control), basal artificial diets to which had been added 10%, 1.0% and 0.1% September oak leaf tannin (fractions (P) and (S) unseparated) and, lastly, a diet of fresh young May oak leaves. The last of these diets was included in the experiment so that growth of the larvae on oak leaves could be compared with that on the artificial diet.

The larvae were reared individually at room temperature in plastic pots, identical to those already mentioned in Chapter 2 (p.31) (Figure 25, p.85(a)). All larvae were weighed at intervals of a few days until pupation had been completed.

Details of experimental technique are given in Appendix 20 (p.216), together with the larval and pupal weights recorded on each weighing date.

b) Mortality

As mentioned in Appendix 20 (p.216), the rearing pots were disinfected with alcohol and exposed to ultraviolet light before use



FIGURE 25. Polystyrene pots for rearing winter moth larvae on artificial diets. In the upper part of the photograph are illustrated two rearing pots, complete with diet and larva. The component parts of each rearing container are shown in the lower half of the photograph (polystyrene pot, 1½" in diameter, filter paper disc and polythene 'snap-on' lid).

and the diet was prepared in autoclave containers. However, although most of the larvae pupated, larval mortality was substantial, especially on the 10% tannin diet. Mortality did not seem, though, to be solely connected with the presence of the tannin in the diets, since many larvae also died when fed on the basal synthetic diet (Figure 26, p.86(a)). It is possible that the mortality may have been due to the difficulty which larvae encounter in adjusting to the texture of the diets, after initial rearing on oak leaves. The diet containing 10% tannin, in particular, tended to dry out more quickly than the other three artificial diets, and, in spite of humidity control by addition of water to the filter discs (Appendix 20), this drying effect, perhaps in conjunction with the high tannin content, may have been responsible for the high mortality on this diet. It is also possible that some of the mortality was due to pathogenic causes, though there was no evidence for this.

c) Results

The mean weights of the larvae on each diet at each weighing, together with other relevant statistical data, are given in Table 10, (p.86(b)). The means are plotted against date in Figure 27 (p.86(c)). Larvae with initial weights below 5 mg. or above 24.9 mg. were excluded from calculation of means. These few larvae would bias the results out of proportion to their number, since they tended to fare excessively badly or well, respectively (Appendix 20).

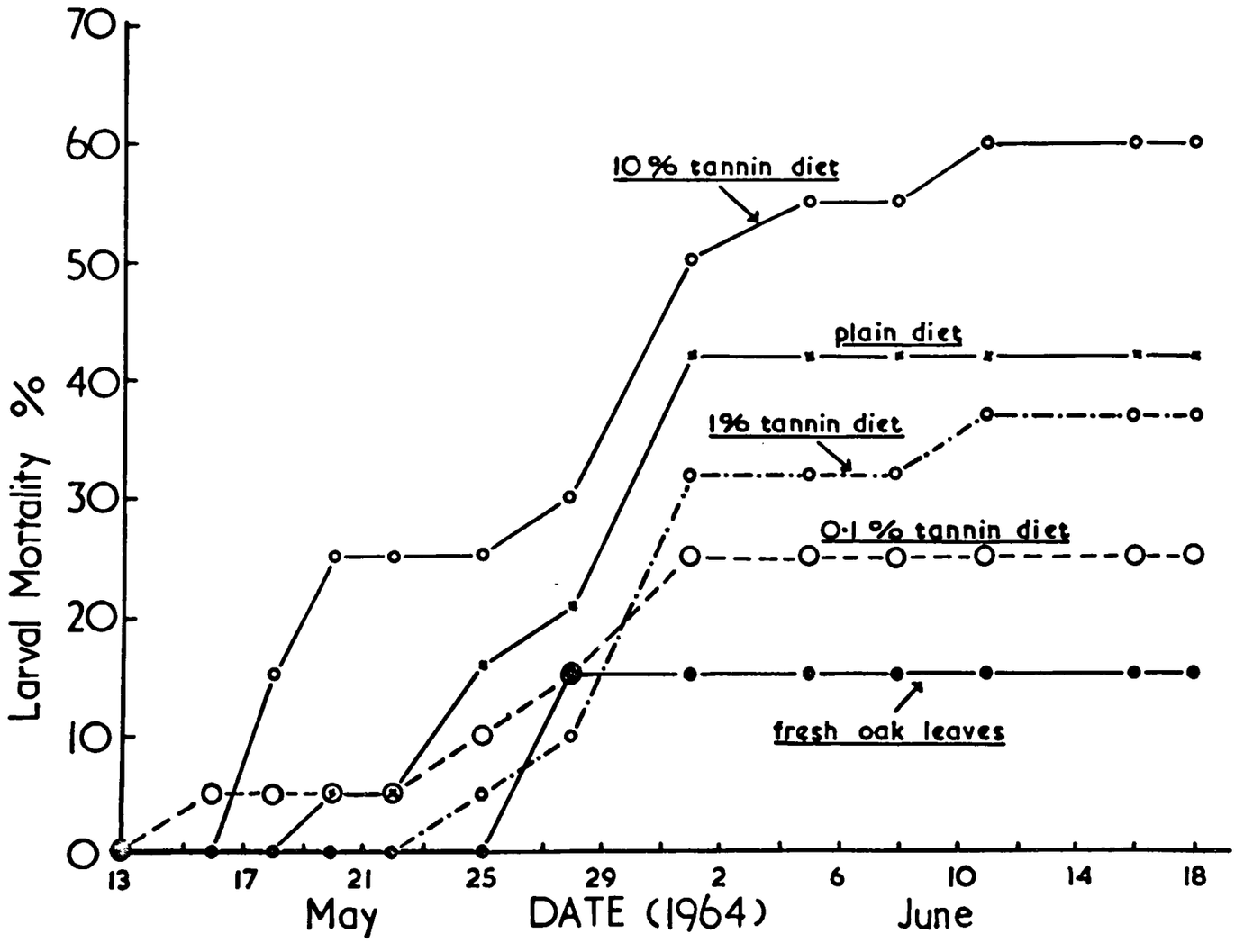


FIGURE 26 Mortality of winter moth larvae on the five diets during the 1964 experiment.

TABLE 10 Winter moth larval fresh weights and pupal weights attained on 5 different diets (1964) - means, standard errors of the means and sample sizes.

Date:	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6
A	13.2 ±1.3 n=18	22.3 ±2.3 n=18	57.5 ±2.7 n=18	53.0 ±2.3 n=18	43.3 ±1.0 n=18	37.2 ±0.8 n=18	33.2 ±1.0 n=16	31.4 ±1.1 n=15	30.8 ±1.2 n=15
B	14.0 ±1.4 n=13	25.7 ±4.2 n=13	36.9 ±3.8 n=13	43.3 ±3.6 n=13	43.9 ±4.0 n=13	38.1 ±3.4 n=13	31.3 ±2.1 n=12	30.8 ±2.1 n=7	30.3 ±2.1 n=7
E	14.8 ±1.3 n=17	32.1 ±4.2 n=17	38.8 ±4.9 n=17	39.5 ±3.1 n=17	39.7 ±2.0 n=17	34.0 ±2.2 n=17	28.6 ±1.5 n=17	27.3 ±1.7 n=14	26.5 ±1.8 n=14
D	15.4 ±1.2 n=18	27.0 ±3.1 n=18	37.4 ±3.7 n=18	38.8 ±2.5 n=18	38.4 ±2.0 n=18	31.7 ±1.2 n=18	26.2 ±0.9 n=18	24.3 ±1.0 n=13	22.4 ±1.1 n=12
C	12.8 ±1.4 n=16	15.4 ±1.8 n=16	19.5 ±2.7 n=14	27.8 ±3.2 n=12	39.1 ±3.1 n=12	35.3 ±3.4 n=12	27.1 ±2.4 n=11	22.3 ±1.7 n=9	21.2 ±1.6 n=9

Diets: A = Fresh May oak leaves; B = Basal artificial diet; C,D,E, = Artificial diets containing 10%, 1%, and 0.1% respectively of July oak leaf tannin.

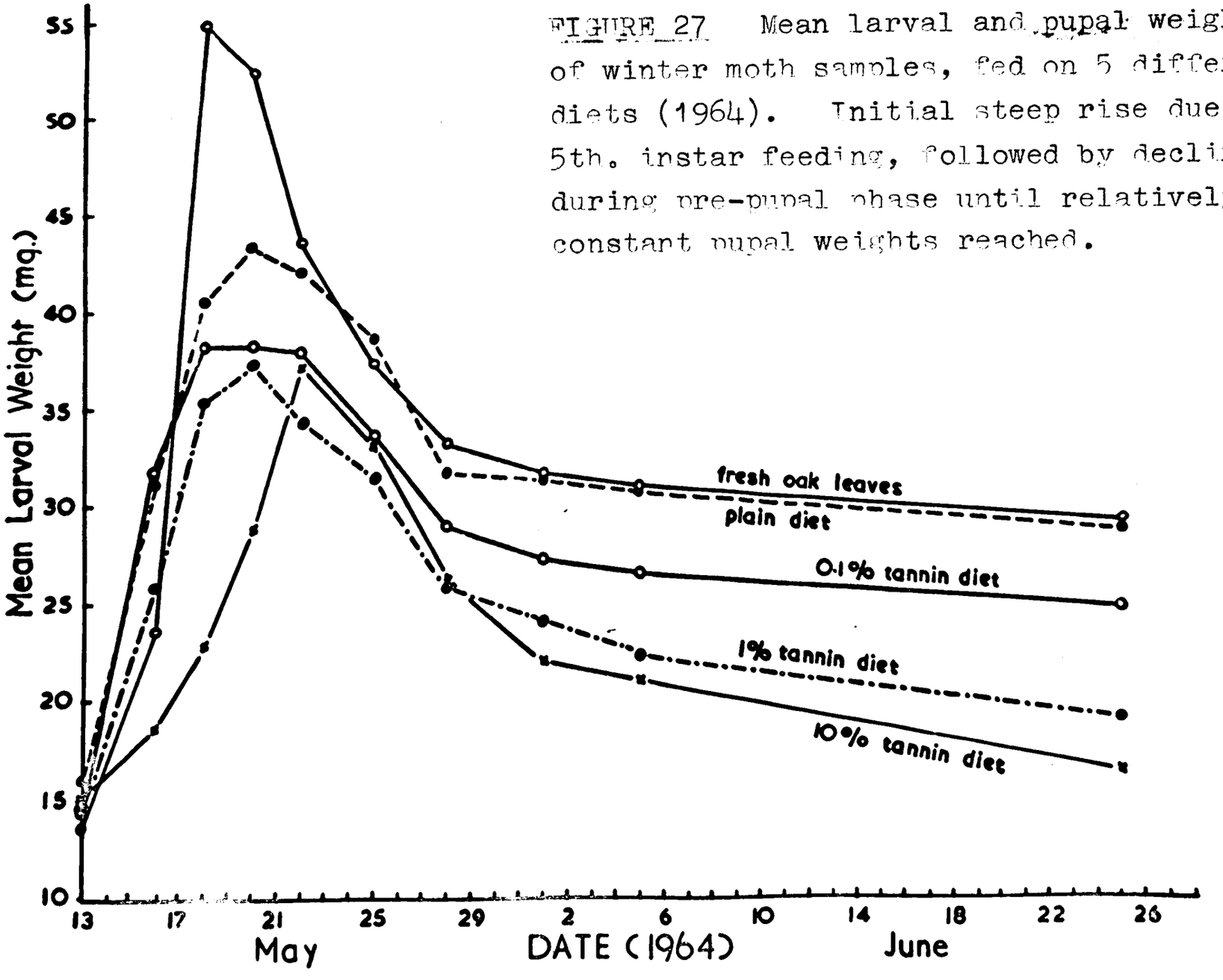


FIGURE 27 Mean larval and pupal weights of winter moth samples, fed on 5 different diets (1964). Initial steep rise due to 5th. instar feeding, followed by decline during pre-pupal phase until relatively constant pupal weights reached.

d) Comparison of pupal weights attained on artificial diet and fresh oak leaves

It is apparent from Figure 27 (p.86(c)) that the mean pupal weight attained on the basal artificial medium was very similar to that attained on May oak leaves, of a similar age to those which the larvae would have encountered in Wytham. These means (June 5th data) were compared by the Student's t-test for small samples, after establishing similarity of variance (Bailey, 1959) and no significant difference was found (Appendix 20, p.216). This suggests that the basal artificial diet is an adequate medium for experiments on the growth of winter moth larvae. No data are available for subsequent adult emergence and fecundity on the two diets, though it will be remembered (p.23) that high pupal weight is apparently correlated with high emergence and fecundity.

The high mean peak weight attained by larvae feeding on oak leaves, relative to those attained on the artificial diets, (Figure 27, p.86(c)) is due to the fact that the majority of the larvae feeding on the leaves reached their peak weights simultaneously. Individual larvae reared on the basal diet reached similar peak weights, but these were spread over a longer period (Appendix 20, p.216), resulting in a lower mean peak weight. The difference is probably due to the delay before rapid growth, caused by the change from oak leaves (on which all larvae were initially reared) to the artificial diet.

e) Comparison of pupal weights attained on control and tannin diets

It can be seen from Table 10 (p. 86(b)) and Figure 27 (p. 86(c)) that pupal weights attained on the tannin diets became progressively lower as the tannin content of the diet increased. The pupal weight means for June 5th were compared by a method involving the analysis of variance (Bailey, 1959). It was found (Appendix 20) that the mean pupal weights on the diets containing 10% and 1% tannin were significantly different from that on the control diet ($P < 0.01$ in both cases), but that the mean pupal weight on the 0.1% tannin diet, although lower than that on the control diet, was not significantly different from it.

A regression analysis was also carried out on the results, to investigate the relation between tannin content of the diet and the resulting pupal weights (Appendix 20, p. 216). A graph of pupal weight plotted against tannin content of diet takes the form of a curve, but a plot of logarithm pupal weight against logarithm tannin concentration conforms more closely to a straight line. The regression of logarithm pupal weight on logarithm tannin concentration was therefore analysed and the calculated regression line found to be significantly different from the horizontal ($P < 0.05$) (Appendix 20, p. 216 and Figure 28, p. 88(a)).

f) Conclusions from the 1964 experiment

The growth experiment carried out in 1964 showed, firstly, that winter moth larvae could be reared in the laboratory, at least to the

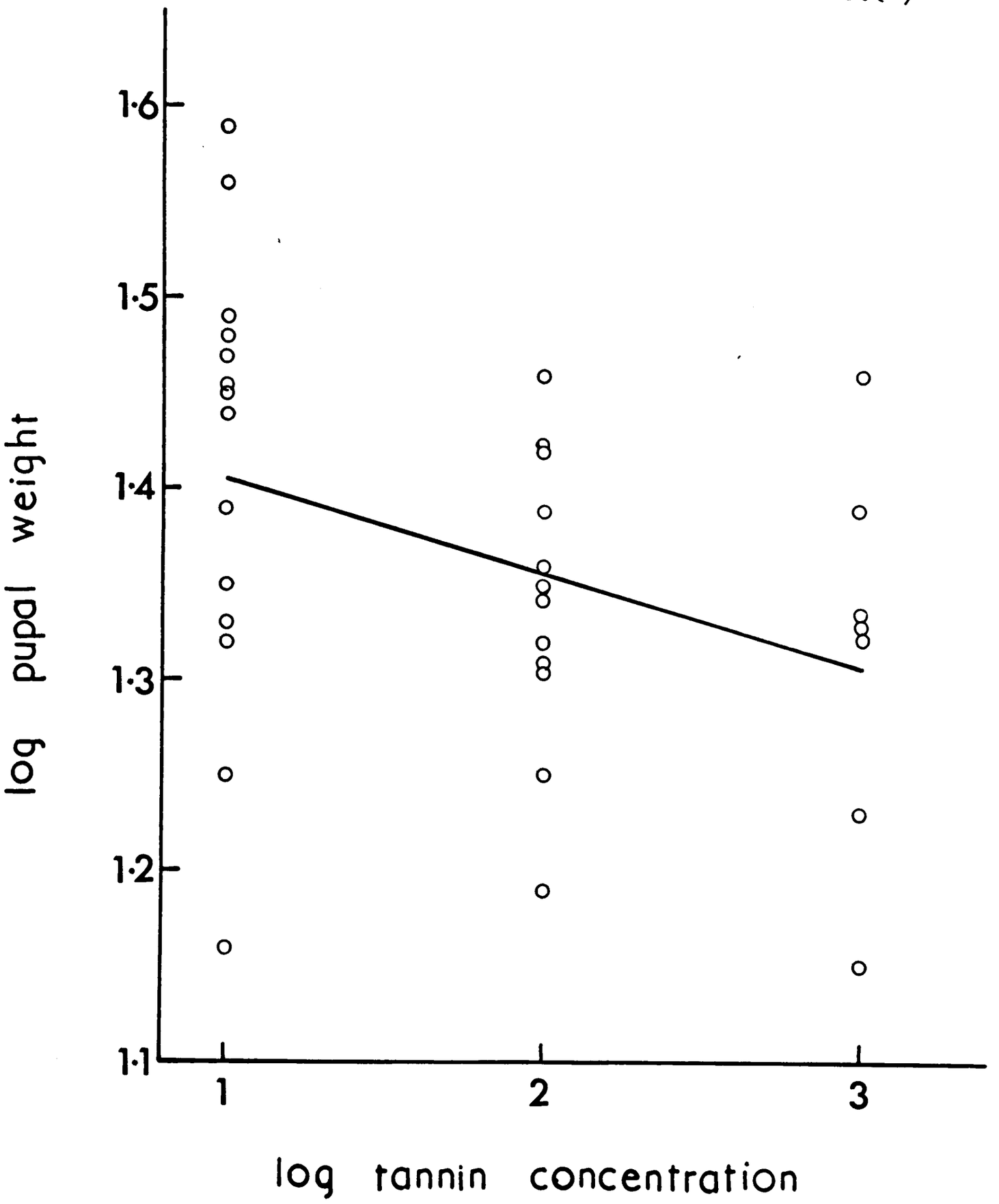


FIGURE 28 Calculated regression line from regression analysis of $\log_{10}$ winter moth pupal weight on $\log_{10}$ tannin content of diet.

pupal stage, on an artificial diet and that the pupal weights attained were similar to those of larvae reared in the laboratory on fresh oak leaves. As far as is known, this was the first occasion on which Lepidoptera larvae have been grown on an almost completely chemically defined diet in this country. However, several problems were encountered while rearing larvae on the diet, the chief of which is dessication in the presence of a high concentration of tannin.

Secondly, it was shown that oak leaf tannin significantly reduces the pupal weights of the larvae, even when present in the diet at a concentration of only 1%. This supports the hypothesis that tannins may have some inhibitory effect on caterpillar growth in the field. It will be recalled that the tannin content of oak leaves reaches 5.5% (fresh weight) in September (p.77@). There is evidence (p. 22) to suggest that low pupal weights result in lower pupal survival and lower fecundity of emerging females (Chapter 1, p. 22), though this was not checked for the 1964 experiment described above. It was, however, evident that pupae from the 10% tannin diet were unhealthy and tending to dessicate rapidly. It was hoped to check the effect of tannin on adult emergence in a similar experiment in 1965.

4. Test of completely defined diet

It will be recalled that the artificial diet used in the 1964 experiments, although approaching complete chemical definition, contained nutrient agar and wheat embryo, both of which contain a wide

variety of nitrogenous and other components. It was desirable for the present work to feed the larvae on a diet in which casein was the only source of nitrogen, so that any effects on growth of tannin-protein interactions would be more clearly revealed. Omission of ingredients from the diet in 1964 had been an unacceptable risk in view of the untried nature of the diet. However, in view of its apparent success, it was decided in 1965 to leave out wheat embryo and to substitute plain Japanese agar (British Drug Houses Ltd.) for Oxoid nutrient agar. The diet was accordingly prepared with these modifications, otherwise using the same quantities of ingredients as recommended for the Canadian diet used the previous year (Appendix 19, p. 212).

To determine whether this modified diet was acceptable to winter moth larvae, it was offered to 5 early 5th instar larvae on April 23rd, 1965. These larvae were withdrawn from a sample which had been hatched early (by keeping eggs at room temperature since February) and subsequently reared on young hawthorn leaves. After 4 days, the larvae were re-weighed. Their initial and final weights were as follows:

Larva	Weight on April 23rd (mg.)	Weight on April 27th (mg.)
1	14.0	26.0
2	20.3	37.0
3	18.5	49.0
4	18.3	37.0
5	17.5	40.0

The larvae were apparently feeding satisfactorily on the modified diet

and, although there was insufficient time to carry out a large-scale trial before the 1965 experiment with phenols, it was decided to use the modified diet for this experiment.

5. Effects of oak leaf tannins, gallic acid and (+)-catechin on the fresh weight increase of winter moth larvae - 1965 season.

a) Method

It was decided to repeat growth experiments with winter moth larvae in 1965, using the modified, completely defined diet, to confirm the inhibitory effects of oak leaf tannins and also to discover the effects on growth of two other phenols, known to occur in oak leaves, (gallic acid and (+)-catechin (Figure 17, p.50(a)). It was expected that (+)-catechin, being a probable precursor of condensed tannin (Appendix 13, p.196), with a similar arrangement of functional groups, might have a weak inhibitory effect, whereas the gallic acid would not be expected to have any effect on growth. It was also planned to compare the effects on larval growth of tannin from both May and July leaves.

25 4th instar winter moth larvae, reared in the laboratory on fresh young oak leaves, were transferred to each of 5 different diets, commencing on May 11th, 1965. The diets were basal artificial diet (modified as mentioned above) as control and four artificial diets to which had been added 1% May tannin, 1% July tannin, 1% (+)-catechin (Koch-Light) and 1% gallic acid respectively. The experimental procedure was similar to that adopted in 1964 and is described in Appendix

21 (p.225). As in 1964, precautions were taken to disinfect as far as possible the rearing pots, diets, larvae and any instruments used in weighing the larvae. The larvae were weighed at intervals of a few days.

b) Regulation of humidity

In view of the tendency of the diets to dessicate during the 1964 experiment, possibly contributing to larval mortality, larvae for the 1965 experiment were reared under conditions of constant humidity. This was achieved by placing the rearing pots on perforated plastic shelves in a specially constructed, transparent plastic box (30"x 30" x 30"), which could be sealed, except for some small holes near its base and top to allow slow circulation of air. On the floor of thecontainer, beneath the lowest shelf, was placed a shallow dish containing a saturated solution of ammonium chloride and potassium nitrate, in contact with excess of the solid salts, to maintain the humidity at a constant level of approximately 70% - the published figure for an entirely sealed container at 20°C using this solution is 72.6% (Hodgman, 1950). A drum of Whatman 3MM paper, 6 inches high, was placed in the solution to increase the rate of evaporation and consequent attainment of equilibrium.

c) Results

The larval weights for each diet on each weighing date are shown in Appendix 21 (p.225). Owing to high mortality, the experiment was discontinued after June 4th. The mean weights of the larvae on

the five diets are shown in Table 11 (p.93(a)). Only larvae which survived until at least May 24th were included in the calculation of the means. Unhealthy larvae, excluded by the above criterion, are indicated by brackets in the table of means. The larval weight means are plotted against date for each diet in Figure 29 (p.93(b)).

d) Mortality

It is clear from the results that mortality was considerably higher than in the 1964 experiment, although careful precautions had been taken (Appendix 21, p.225). The most likely cause of this mortality was fungal growth, which was much more prevalent than in 1964, probably because of the constant high humidity. In many cases, the larvae could be seen to be trapped by the fungal hyphae. Fungal growth was frequently observed to start from faecal pellets which had failed to drop from the diet and occasionally remained adhering to the rear of the larva, whence the hyphae seemed to enter the hind-gut rapidly, eventually killing the insect. Samples of the fungus were sent to the Commonwealth Mycological Institute, Kew, for identification. Two species were found: Penicillium cyclopium Westling and Penicillium brevicompactum Dierckx.

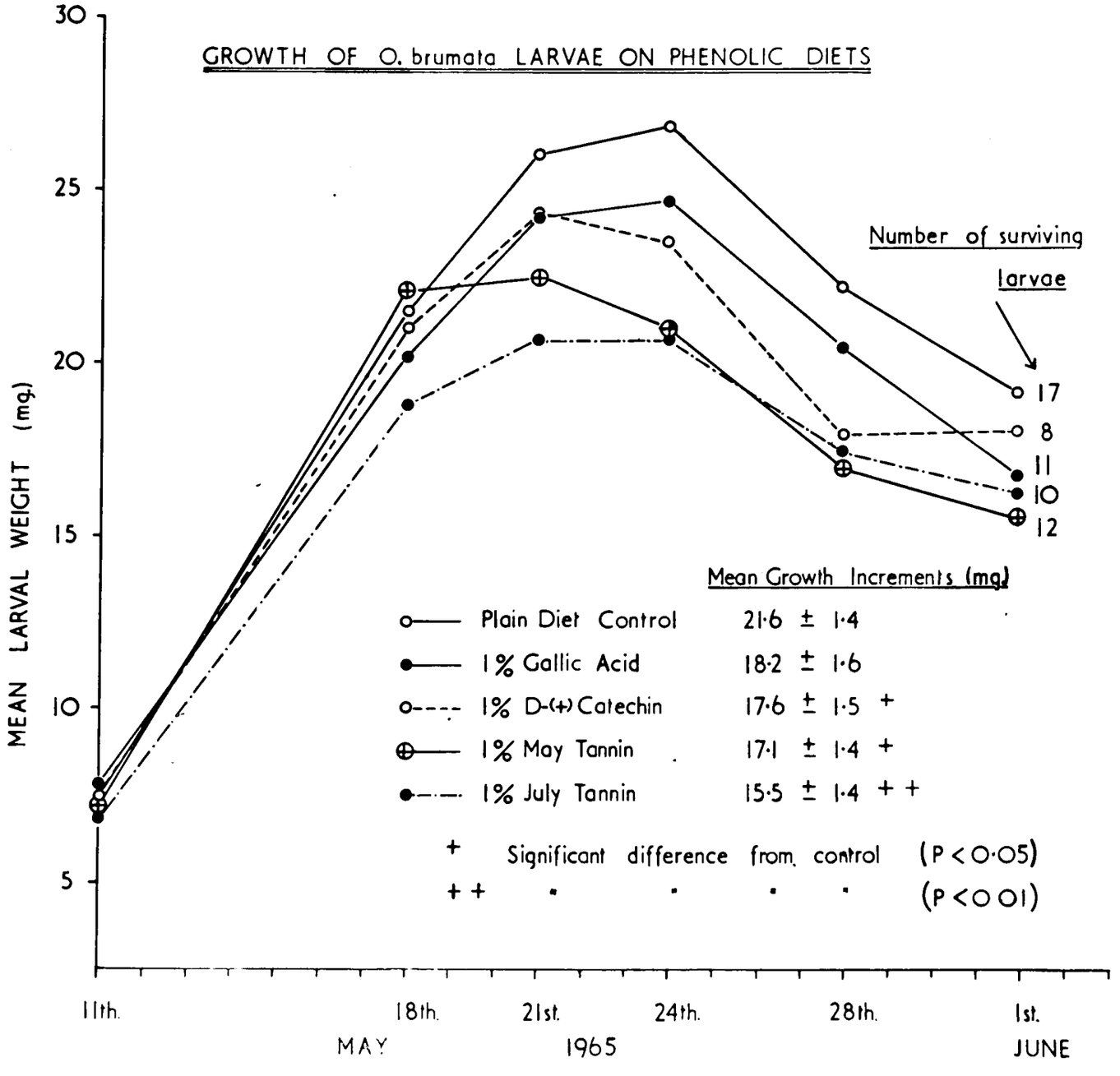
It was at first thought that death was due to pathogens other than fungi followed by secondary effects of fungal mycelium. However, direct observation (see above) suggested that fungus was, in fact, the primary cause of mortality. Staining of 20 larvae with Gemsia stain (Smith and Xeros, 1953) revealed no sign of virus infection and numbers of Microsporidia spp. did not appear to be abnormal, in comparison

TABLE 11 Fresh weights of winter moth larvae fed on 5 different diets (1965) - means, standard errors of means and sample sizes.

Date:	11/5	18/5	21/5	24/5	28/5	1/6
P	7.5 ±0.5 n=23	21.5 ±2.0 n=23	26.0 ±2.3 n=23	26.8 ±1.9 n=23	22.2 ±1.1 n=22	19.1 ±1.3 n=17
J	6.8 ±0.3 n=23	18.8 ±1.4 n=23	20.7 ±1.3 n=23	20.7 ±1.3 n=23	17.4 ±1.2 n=21	16.2 ±1.6 n=10
M	7.2 ±0.5 n=22	22.1 ±2.3 n=22	22.5 ±1.9 n=22	21.0 ±1.8 n=22	16.9 ±1.2 n=19	15.5 ±1.3 n=12
G	7.8 ±0.5 n=17	20.2 ±1.7 n=17	24.2 ±2.0 n=17	24.7 ±1.9 n=17	20.4 ±1.6 n=15	16.7 ±1.8 n=11
C	7.5 ±0.4 n=18	21.0 ±1.5 n=18	24.3 ±1.4 n=18	23.5 ±1.3 n=18	17.9 ±1.7 n=17	18.1 ±2.2 n=8

Diets: P = Basal artificial diet; J,M,G,and C = artificial diets containing 1% July oak tannin, May oak tannin, gallic acid and (+) - catechin respectively.

FIGURE
29



93(b)

with those normally present in healthy wild larvae.

It is considered probable that the high mortality encountered in this experiment was due to the introduction to the medium of fungal spores present in the guts and on the surface of the caterpillars. Such spores would have escaped destruction during disinfection of the pots and diets, and the surface treatment of the larvae was unlikely to have been 100% effective; more thorough treatment would probably have damaged the insects. The experiment clearly demonstrated the problems involved in carrying out laboratory experiments with stocks of wild larvae and the consequent necessity, not previously realised by the author, of commencing an experiment with very large samples, so that complete analysis of results is possible, in spite of very heavy mortality.

e) Comparison of the larval fresh weight increases on the five diets

Fungal growth became evident only after the experiment had been in progress for several days and is unlikely to have affected the larvae significantly until after about May 24th. Therefore, although pupal weights were not available for analysis, it was considered reasonable to draw conclusions concerning larval growth from the early part of the experiment.

Comparison of Figure 29 (p.93(b)) with Figure 27 (p.86(c)) at once shows that increase in fresh weight was very much slower in the 1965 experiment than in the previous year, and much lower peak weights were attained. This may have been due to the conditions of constant 70% humidity imposed in 1965, but is more likely to have been due to the

omission of wheat embryo and nutrient agar from the diet. Although a diet containing casein is acceptable to the larvae, it is possible that the omitted ingredients contained an essential micronutrient (see p. 46).

Examination of Figure 29 also reveals that, although weight increase on all diets was poor in comparison with the previous year, that on the tannin diets appeared to be worse than on the control diet, with growth on the other diets taking an intermediate position. Since pupal weights were not available for comparison, statistical analysis was carried out on the weight increments of the larvae, being calculated as the difference between the peak fresh weight reached by each larva and its initial fresh weight. As for the calculation of the sample means, only those larvae surviving until at least May 24th were included in the analysis (see Appendix 21, p. 225).

The weight increments available for analysis are shown in Table 12 (p. 95a), together with their means and other relevant statistical data. The mean weight increments of the larvae on each diet were compared by the method involving analysis of variance used the previous year (Bailey, 1959 and Appendix 21, p. 225). It was found that the mean weight increments obtained on the July tannin, May tannin and catechin diets were all significantly lower than that obtained on the control diet ($P < 0.005$, $P < 0.05$ and $P < 0.05$, respectively), though there was no significant difference between the mean growth increments attained on the gallic acid and control diets (Appendix 21).

TABLE 12 Fresh weight increments (initial to peak weight), attained by winter moth larvae on five diets, together with means (1965).

Diet[1]:	J	P	M	G	C
	11.9	16.7	19.4	24.3	18.8
Fresh	20.0	24.4	13.7	11.5	13.6
weight	12.8	14.8	8.3	15.7	15.2
increments	11.1	32.2	23.0	15.9	24.1
(mg.)	11.0	29.1	32.4	27.3	20.6
	14.2	19.0	10.9	17.6	12.7
	13.0	22.4	39.4	14.6	22.1
	11.3	16.2	18.2	22.5	13.5
	17.4	26.4	27.7	18.7	19.8
	10.3	39.4	24.7	23.6	16.7
	32.3	28.0	11.1	6.6	18.0
	19.2	12.3	9.6	17.2	13.1
	15.3	23.7	11.3	16.7	17.8
	15.2	17.8	8.8	14.2	20.1
	16.8	11.6	12.7	8.8	24.5
	16.9	7.6	15.2	28.7	12.5
	14.9	22.2	13.9	25.5	15.1
	13.9	35.1	15.7		13.5
	12.3	13.9	16.2		
	14.9	24.6	16.9		
	19.3	20.2	12.9		
	21.8	16.4	14.0		
	10.1	22.5			
Sample size(n)	23	23	22	17	18
Mean ($\bar{x}$)	15.5	21.6	17.1	18.2	17.6

[1] J = 1% July tannin; M = 1% May tannin; P = Plain diet; G = 1% Gallic acid; C = (+)-catechin.

f) Conclusions from the 1965 experiment

Although conditions of constant humidity (70%) achieved the desired result of preventing dessication of diets, they unfortunately encouraged the widespread growth of fungal mycelia, which could not be avoided by disinfection. Since larvae cannot be completely sterilised and antifungal agents in the diet are not 100% effective, it is probably preferable to rear larvae under conditions of much lower humidity and to replace their diet at more frequent intervals with fresh medium.

Growth of winter mothlarvae on the modified and completely defined diet was found to be substantially slower than on the original medium, containing wheat embryo and nutrient agar. It is likely that these ingredients contain some micronutrient, essential for normal larval growth, though it is possible that the slow growth was due to the conditions of high humidity and the consequent spongy texture of the diets.

Notwithstanding the slower growth and high mortality, it was possible to compare larval weight increments on the 5 diets, since the fungus did not become widespread during the first ten days of the experiment. Statistical analysis revealed that weight increments on a diet containing 1% July oak tannin were significantly lower than those attained on the control diet, thus confirming the 1964 finding that oak leaf tannin can have an inhibitory effect on larval growth. It is also of interest that May tannin and (+)-catechin appear to have

an inhibitory effect, though less pronounced than that of July oak leaf tannin. It will be remembered that whereas July tannin consists of both hydrolysable and condensed tannins (Table 5, p.55(a)), May tannin consists almost entirely of hydrolysable components. Although the data here presented are limited, the apparently weaker inhibition of growth by hydrolysable tannin, in comparison with that by condensed tannin, is of great interest in connection with protein digestibility experiments to be described in the next chapter (p.123). The weak inhibition by (+)-catechin and lack of inhibition by gallic acid were expected, as mentioned in section 5 (a) above, though the data are insufficient to draw any firm conclusions.

6. Further modification of the artificial diet for rearing larvae

a) Composition of diet

Although the synthetic diet used during the 1964 and 1965 seasons had been found to be largely successful for rearing winter moth larvae, it was evident that the larvae took some time to become accustomed to the spongy texture of the diet and, in the case of the smaller larvae, high mortality probably resulted as a result of this factor. Before the 1966 season, therefore, it was decided to modify the diet in such a way as to substantially harden the texture to that of a rigid jelly, which might be more suitable for larval feeding. Mr. D.R. Lees (personal communication) had been experimenting in the same laboratory with a diet developed from an American formula (Vanderzant and Reiser, 1956a and 1956b) at the Agricultural Research Council Unit of Insect Pathology at Cambridge (David and Gardiner, 1965).

Although this diet was not completely defined chemically, since it contained cabbage powder to facilitate rearing of Pieris brassicae L. larvae, it was found to have a suitably rigid texture. A modified version of this diet was therefore prepared in the hope that it would be suitable for the forthcoming experiment.

The formulae for the Cambridge diet and for the modification used for the 1966 winter moth experiments are as follows:

	Ingredient	Cambridge Diet	Modified Diet
(a)	Distilled Water	110 ml.	110 ml.
	Potassium Hydroxide (4 Molar soln.)	1.8 ml.	1.8 ml.
	Casein (Light White Soluble)	12.6 gm.	21.9 gm.
(b)	Sucrose	12.6 gm.	12.6 gm.
	Wheat Embryo	10.8 gm.	1.5 gm.
	Cabbage (dried powder)	5.4 gm.	-
	Salt Mixture	3.6 gm.	3.6 gm.
	Cellulose Powder	1.8 gm.	7.2 gm.
(c)	Choline Chloride	3.6 ml. (10% soln.)	0.3 gm.
	Methyl p-hydroxybenzoate	3.6 ml. (15% soln. in 95% ethanol)	0.4 gm.
	Formaldehyde soln.	1.5 ml. (10%)	0.4 ml. (40%)
	Vitamin solution	0.8 ml.	3.0 ml.
(d)	Distilled Water	200 ml.	200 ml.
	Agar (fine Japanese powder)	9.0 gm.	9.0 gm.
(e)	Ascorbic Acid	1.5 gm.	1.5 gm.
	Aureomycin	0.8 gm.	0.8. gm.

The modified diet is a combination of the Cambridge and Canadian diets. The concentrations of vitamins and other micronutrients are those found successful in the Canadian diet, while the major components were added in the proportions recommended for the Cambridge diet, in order to obtain the improved texture. The percentage composition of the components in the Cambridge and Canadian diets are compared in Appendix 19 (p.212), where it can be seen that the differences are small, though evidently sufficient to affect the final texture. The distinguishing features of the new modified diet are as follows:

Casein and wheat embryo. Although it is desirable to define the source of nitrogen in the diet accurately by restricting this to casein, omission of wheat embryo entirely is likely to have contributed to the slow rates of growth found in the 1965 experiment perhaps in conjunction with the spongy texture of the diet, already discussed. It is possible that the insects in that experiment suffered from lack of a steroid source, which would have been provided by the wheat germ. In the modified diet, therefore, wheat embryo was not completely omitted, but reduced to a level considered high enough to safeguard against any lack of essential micronutrients, while still remaining unimportant as a nitrogen source by comparison with the casein. The reduction in the amount of wheat embryo was exactly compensated for by increasing the content of casein.

Cabbage powder and cellulose. Cabbage powder was omitted from the modified diet and the content of cellulose powder increased accordingly.

Micronutrients and antibiotic agents. With the exception of aureomycin and ascorbic acid, these components were added in the proportions of the original Canadian diet. The original Wesson salt mixture and vitamin concentrations were used, though these differ slightly from those used for the Cambridge diet (Appendix 19).

Gibberellic Acid. Gibberellic acid has been recorded as an essential item of diet for the successful growth of young locusts (p. 46) and it is possible that it forms an essential source of steroids for phytophagous insects. Indeed it is likely that relatively poor growth of winter moth larvae in the 1965 experiment was partially due to a lack of gibberellic acid, hitherto provided in artificial diets by the wheat embryo. Since the wheat embryo content in the modified diet had been severely reduced for reasons previously mentioned, it was decided to add a small quantity of gibberellic acid to the diet as a precaution. The material used was the 'purriss' grade gibberellic acid provided by Koch-Light Ltd.

b) Preparation of diet

In the original mixing procedure, following the Canadian procedure, all the ingredients were mixed together in an 'Atomix' blender before addition of the agar solution. The temperature of the agar solution had, therefore, to be kept below 70°C to avoid degradation of some of the vitamin components. In consequence, only a very short time was available for thorough mixing of the agar and pouring of the diet into containers before gelation set in. A different technique

was therefore developed for future use. The ingredients (a) and (b) (see above) were mixed together in the 'Atomix' blender as usual, after which the agar solution could be added at 95°C without danger of affecting the ingredients. After mixing, the temperature was found to fall to about 65°C (instead of about 30°C), so that plenty of time was available for further mixing and manipulation of the diet before gelation. After blending of the agar, the ingredients (c) and (e) were added and blended thoroughly before the medium was poured out into the sterilised containers. This technique has the considerable advantage that many experimental materials to be incorporated in the diet can be added and thoroughly mixed without fear of gelation occurring in the blender. The technique, in combination with the modified diet, was used for the 1966 experiment and has also now been adopted successfully by Mr. D.R. Lees (personal communication), in conjunction with the Cambridge diet.

7. Effects of oak leaf tannin on larval fresh weight gain - 1966 season

a) Introduction

For the 1964 and 1965 diet experiments, the phenolic additives (tannin, gallic acid and (+)-catechin) were incorporated in the diets after addition of agar. The extent to which the casein was tanned, even after ingestion of the gel by the insect, was therefore uncertain. In 1966, therefore, it was decided to pre-tan the protein before incorporation in the diet, so making the experimental conditions closer to those in a masticated oak leaf.

Ideally, it would have been desirable to substitute leaf protein for casein in the 1966 growth experiment. However, a source of pure leaf protein in bulk was still unavailable (Appendix 24, p.238), so that casein again had to be used. Although this is an animal protein, it is evidently assimilated with ease by winter moth larvae, so that experiments with casein-tannin complexes are likely to shed light on the behaviour of leaf protein-tannin complexes.

In order to make the tanning process as realistic as possible, it was carried out at the pH likely to obtain in the field when oak leaf tissue is masticated by larvae. Several homogenates of both May and July oak leaves in CO_2-free distilled water were found to have a pH of approximately 5.0.

b) Method

Casein was thoroughly pre-tanned with an excess of September oak leaf tannin (fraction (S) only),isolated by the method of Brown and Love (1961). The aqueous solutions of casein and tannin were brought to pH 5.0 with citric acid immediately after mixing and stirred for 5 minutes, being a minimum estimate for the time available for tanning in the field, when the leaf tissue is being masticated by a larva. The precipitate of tannin-protein complex was then centrifuged and washed before incorporation in the experimental diet. Further details of procedure are given in Appendix 22,(p.230).

Two 100 gm. batches of the newly modified artificial diet were prepared. The first of these contained 7.0 gm. casein, pre-tanned

with 3.5 gm. oak leaf tannin and the second (control) diet contained the same amount of casein, though the content of cellulose powder was increased by 3.5 gm. to compensate for the addition of tannin to the experimental diet. The two diets were otherwise identical.

The diets differed from the new modified formula in only two other respects: potassium hydroxide and formaldehyde were omitted, since it was considered that these compounds might affect tannins in a manner not likely to be found in leaf tissue.

An experiment to determine the effect of feeding winter moth larvae on pre-tanned casein was commenced on May 15th, 1966. 78 3rd and 4th instar larvae were individually reared on each of the two diets in plastic containers identical to those used in the two previous seasons. All pots, diets, instruments, etc. were disinfected as far as possible by autoclaving, ultraviolet light or 80% ethanol. Larvae were surface disinfected with a dilute solution of sodium hypochlorite. In view of the high incidence of fungus found under the conditions of high humidity used in 1965, these conditions were not used in 1966. The larvae were reared under normal laboratory conditions of humidity and temperature, out of direct sunlight.

The larvae were weighed at intervals of a few days. The diets and containers were completely renewed on June 1st. It was found that the diets showed no tendency to dessicate, as was found in 1964 with the original diet, and, furthermore, faecal pellets were very rarely found to stick to the diet. These improvements probably resulted from the use of the new modified diet, with its more rigid

texture.

Further details of experimental procedure are given in Appendix 22 (p.230).

c) Results

The weights recorded for the larvae on both diets are given in Appendix 22 (p.230). The experiment was discontinued after June 6th, since mortality of larvae had reached such a level that remaining larvae were insufficient for meaningful comparison. The mean sample weights for each date on each diet are shown in Table 13 (p.104a) together with relevant statistical data. In calculating the means and associated quantities, only those larvae surviving until at least May 27th (i.e. for 12 days) were included in the samples. Among the 25 larvae excluded by this criterion were 5 which had been infected by parasites before collection in the field and subsequently became pupae of Angitia exareolata (Ratz.). These larvae will be completely ignored in the discussion of results.

The means for each date, together with their 5% confidence intervals, are plotted against date in Figure 30 (p.104(b)).

It is evident that larval growth was substantially more successful on the control diet than in the comparable experiment in 1965 and peak weights (Table 39, p.233(a)) were similar to those attained during the 1964 experiment. This can be attributed to the improvement in the diet and in the conditions under which the larvae were reared in 1966.

TABLE I3 Comparison of fresh weight increase of winter moth larvae on tannin and control diets (I966). Means, standard errors and confidence limits of the means.

Date:	I5/5	I9/5	23/5	27/5	3I/5	6/6
Control diet						
Σx	45I.2	869.5	I565.8	2056.4	I623.0	495.2
n	67	67	67	67	65	24
$\bar{x}$	6.73	I2.98	23.37	30.69	24.97	20.63
s	3.77	6.8I	I3.79	I4.I8	9.04	5.67
Standard error of mean	±0.46	±0.83	±I.68	±I.73	±I.I2	±I.I6
5% confidence limits	±0.90	±I.63	±3.29	±3.39	±2.20	±2.27
Tannin diet						
Σx	447.4	787.6	I068.5	II04.8	738.9	I69.6
n	64	64	64	64	46	I2
$\bar{x}$	6.99	I2.3I	I6.70	I7.26	I6.06	I4.I3
s	3.70	5.92	9.59	I0.26	7.58	5.44
Standard error of mean	±0.46	±0.74	±I.20	±I.28	±I.I2	±I.57
5% confidence	±0.90	±I.45	±2.35	±2.5I	±2.20	±3.08

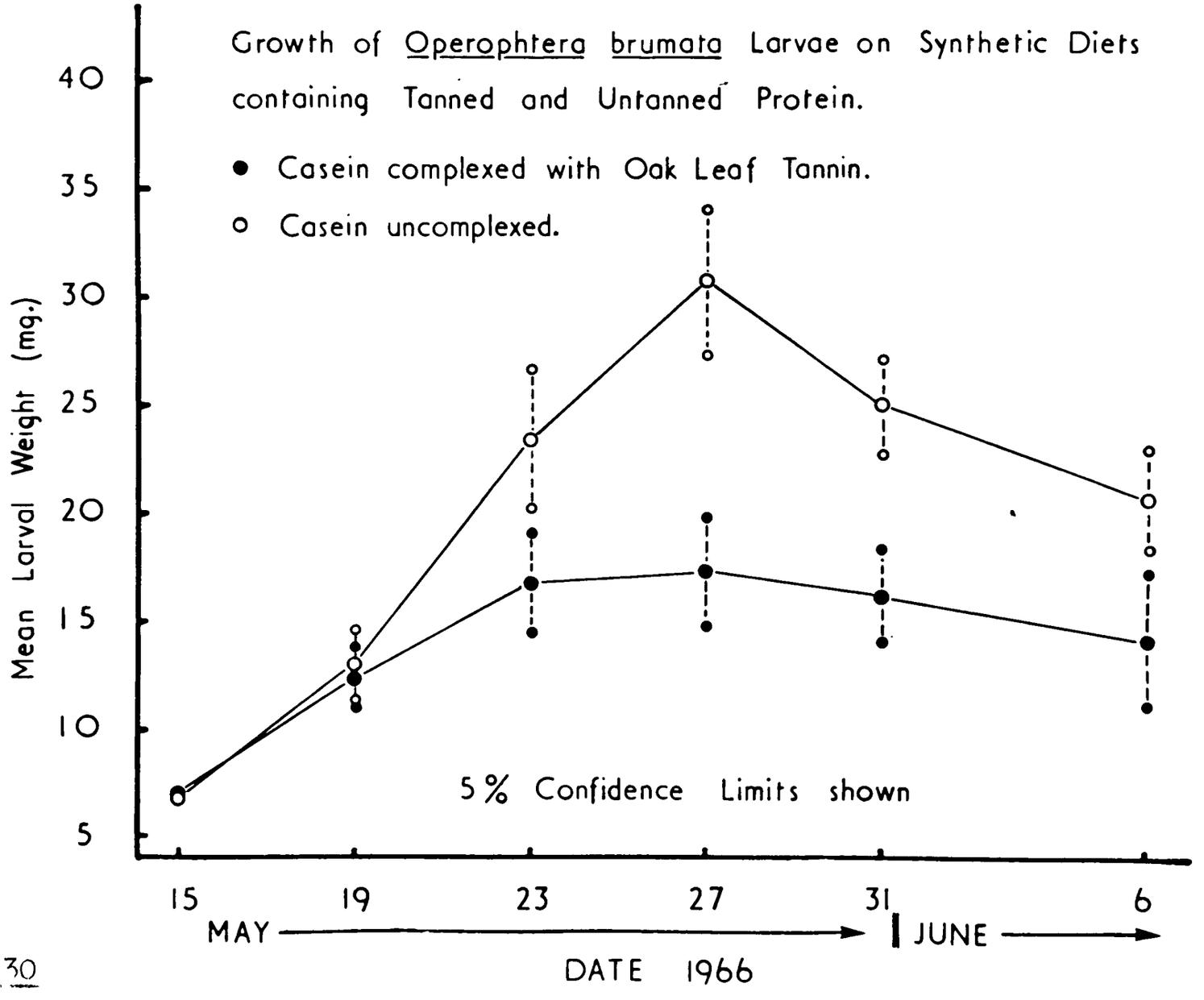

Growth of Operophtera brumata Larvae on Synthetic Diets containing Tanned and Untanned Protein.
● Casein complexed with Oak Leaf Tannin.
○ Casein uncomplexed.
Mean Larval Weight (mg.)
40
35
30
25
20
15
10
5
5% Confidence Limits shown
15
19
23
27
31
6
MAY
JUNE
DATE 1966

FIGURE 30

d) Mortality

In spite of all precautions, high mortality prevented the intended comparison of pupal weights, adult emergence and fecundity on the two diets. Mortality is shown for the two diets in Figure 31 (p.105a) as a percentage of the initial number of larvae (excluding the 5 parasitised larvae). It is seen that mortality remained low until the experiment had been in progress for 12 days and then rose sharply on the tannin diet, followed about three days later by a sharp rise in mortality on the control plain diet.

The mortality was clearly not due to fungal attack, since there was very little evidence of fungus on the diets. 6 recently dead larvae were dissected and their gut smears examined after staining with Gemsia (Smith and Xeros, 1953). There was no sign of virus, but the smears showed a very high density of the spores and other stages in the life cycles of at least two species of Microsporidia. In four of the specimens examined, the spores were oval shaped (Figure 32, p. 105(b)) while in the remaining two specimens, they were smaller and circular. Unlike fungus, the Microsporidia could not have developed in the larvae after death, since they are parasitic and not saprophytic. The slides were shown to Mr. J.F. Longworth, of the Insect Pathology Unit, Oxford, who considered that the Microsporidians were present in such high density that they were almost certainly the cause of death.

Microsporidians are normally present in wild larvae without, apparently, causing much ill effect. However, it is possible that they may multiply rapidly to a harmful density if conditions become un-

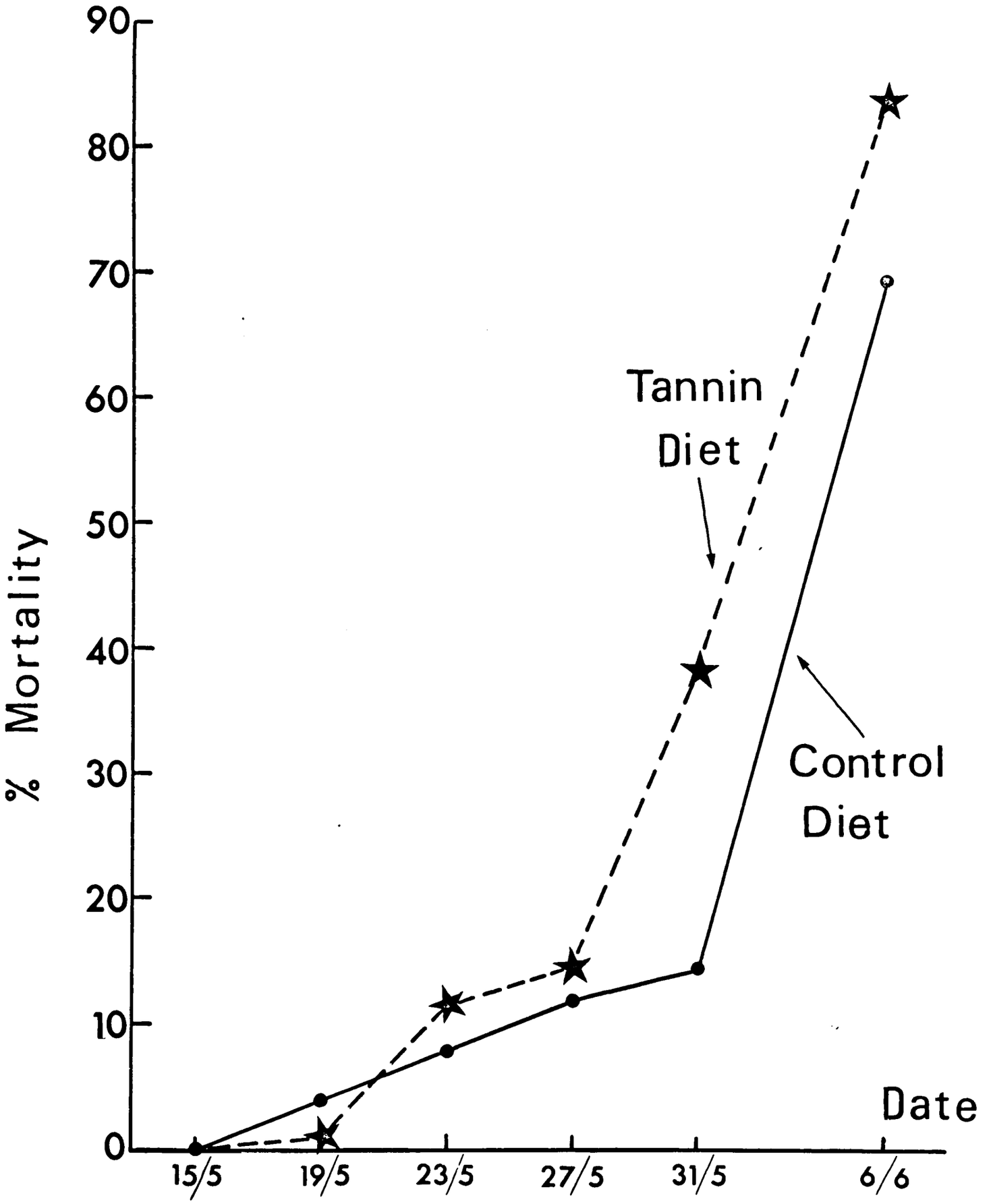


FIGURE 31 Mortality of winter moth larvae on the two diets, 1966.

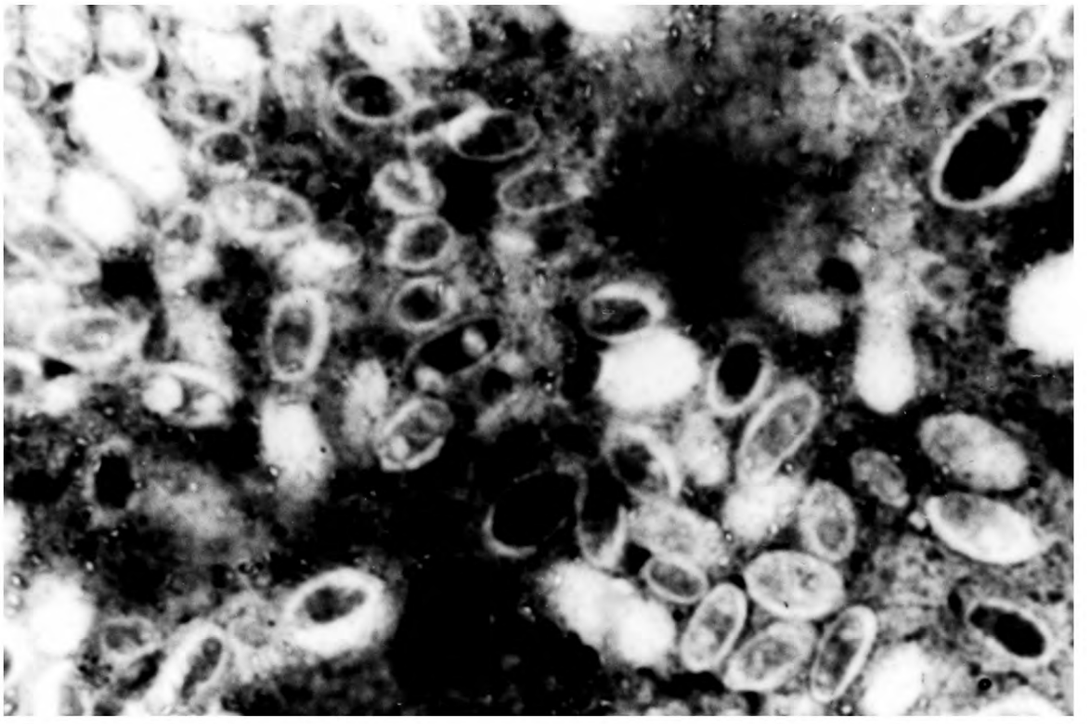


FIGURE 32. Microsporidian spores revealed in the gut smear from a winter moth larva (Gemsia stain; magnification x 2,000)

favourable for the larvae. Such conditions may have occurred in the present experiment after the weighing on May 27th, when the tray of larval containers was accidentally exposed to direct sunlight for nearly one hour. This resulted in considerable condensation on the walls of the pots and possibly on the larvae themselves. Although the filter discs were at once renewed, conditions of high humidity persisted for several days, prior to the sudden rise in mortality. The most likely cause of mortality seems, therefore, to have been a sudden increase in the numbers of Microsporidians, present initially at a low density in the wild larvae, in response to unfavourable conditions brought about by accident. Such mortality would be expected to affect the larvae on the tannin diet before those on the control diet, since the larvae on the control diet were, on average, of greater weight (Figure 30, p.104(b)) and therefore, perhaps, less susceptible to the infection.

e) Comparison of larval weight increments on control and tannin diets

Since, once again, pupal weights were not available for comparison, it was decided to compare the larval weight increments (initial to peak weight) on the two diets, as was done in 1965. There is every reason to suppose that the larvae were unaffected by the Microsporidian infection until after May 27th (see above), so that comparison of weight changes on the two diets before this date should reveal any effects due to the presence of tannin.

The weight increments for each larva, apart from those excluded

from analysis (p.104) were calculated as the difference between the maximum recorded weight up to and including May 27th and the initial weight. They are listed in the final columns of Tables 39 and 40 (p.233a). The mean weight increments attained on the two diets, together with the sample sizes, were as follows:

	Control Diet	Experimental Diet
Mean Larval Weight Increment, with S.E.M. (mg.)	25.2 ± 1.5	11.7 ± 1.0
Sample size	67	64

The mean weight increments were compared statistically by the method for large samples ($n > 30$), based on the normal distribution (Bailey, 1959). The difference between them was found to be statistically significant ($P < 0.001$) (Appendix 22, p.230). The experiment therefore showed that winter moth larvae fed on casein which had been pre-tanned with oak leaf tannin increase in weight more slowly and reach lower peak weights than larvae fed on a control diet in which no tannin is present. For reasons mentioned earlier (e.g. Chapter 1, p. 23), it is thought that an adverse effect on pupal survival and adult weight and fecundity can be tentatively inferred from low peak weights, though it was not possible to confirm this correlation in the present experiment, owing to the most unfortunate high mortality.

8. Conclusions from laboratory experiments with winter moth larvae

The use of a previously described artificial diet has been found to be essentially suitable for the rearing of winter moth larvae, though various modifications had to be devised to suit experimental requirements. The rearing of insect larvae from wild stock is very liable to high mortality, owing to pathogenic factors, and such mortality was encountered with the winter moth. In the light of this, however, there seems no reason why the new modified diet should not be entirely successful, if additional precautions are taken.

In spite of the mortality of larvae, it is possible to demonstrate clearly that oak leaf tannin has an inhibitory effect on larval weight increase under laboratory conditions and it is thought that this inhibition could have resulted in lower survival of the larvae into the subsequent generation. Both pupal weights and larval weight increments were significantly reduced by the presence of the tannin, the phenomenon being more marked if the tannin was first complexed with the protein, as might be expected to occur in the field on ingestion of leaf material. There was also some evidence that (+)-catechin may have an inhibitory effect on larval weight increase.

As mentioned at the beginning of the chapter, the explanation of ecological phenomena on the basis of laboratory results can be misleading. There is every reason to suppose, however, that oak leaf tannin has a similar effect under natural conditions as it does in the laboratory, since the leaf proteins are likely to be tannin in the same manner as casein. It is thought that the very restricted release

of mineral nitrogen from the litter of oak and other trees under some soil conditions may be due to the formation of such stable leaf protein-tannin complexes when the leaves die (Handley, W.R.C., personal communication).

CHAPTER 6: INTERACTIONS OF OAK LEAF TANNINS WITH PROTEINS

1. Introduction

This chapter describes a number of experiments designed to investigate possible reasons for the inhibitory effect of oak leaf tannin on larval growth, discussed in the previous chapter.

Tannins, by definition (p. 54), are polymeric phenols which form complexes with proteins, probably to a large extent by hydrogen bonding. These complexes can be very stable and are used, for example, to protect animal hide from decomposition by tanning it in the production of leather. Such complexes have previously been suspected to be of biological importance. Examples are the inactivation of virus particles by the formation of tannin-- nucleoprotein complexes (Cadman, 1960) and the inhibition of microbial breakdown of protein when the protein is complexed with tannin (Handley, 1961). It was considered possible, therefore, that inhibition of insect growth by oak leaf tannins may be due to a decrease in the digestibility of the dietary protein in the presence of the tannin.

There would also seem to be a second possible method of inhibition, whereby the tannin, although released to some extent from ingested tannin-protein complexes, directly inhibits digestive enzymes by forming complexes with these. Williams (1963) has shown that the pectolytic enzymes of certain fungi can be directly inhibited in this way.

In this chapter, the behaviour and digestibility of protein-oak tannin complexes will be examined, in addition to possible direct in-

hibition of certain enzymes by oak leaf tannin.

2. Quantitative investigation of oak leaf tannin— casein complexes.

a) Introduction

An experiment was undertaken to determine how much tannin is adsorbed to form an insoluble complex by a given amount of casein at pH 5.0 (previously found to be the pH of homogenised oak leaf tissue, p. 102). It was hoped to discover whether the casein-tannin complex is stoichiometric, or whether the amount of tannin adsorbed increases with the concentration of tannin present and the time of contact, as is found in the conversion of animal hide into leather. It was also hoped to ascertain the protein-tannin ratio in the complex, so that this could be compared with the protein-tannin ratios found in oak leaves during the year.

b) Method

Aliquots of a casein solution in distilled water were added to solutions of oak leaf tannin (also in distilled water) of three different concentrations in glass centrifuge tubes. The concentrations of tannin were such that the protein:tannin ratios were 1:0.5, 1:1 and 1:1.5. After addition of buffer at pH 5.0, tanning was allowed to proceed at 20°C. The experiment was performed with a triplicate set of tubes, along with relevant controls (see Appendix 23, p. 235). One tube from each triplicate set was centrifuged after 2 hours and the complex washed and then weighed after drying at 85°C for several days to constant weight. Tanning inthe other two tubes of each set was allowed to proceed for fifteen hours before the tubes were

similarly treated. The weights of the complexes were determined by difference, since the weights of the clean tubes had previously been determined.

Details of the experimental procedure, together with the weights of the complexes produced, are given in Appendix 23 (p.235).

c) Results

The weights of the complexes are plotted against the weights of tannin in the original aliquots in Figure 33 (p.112@), where the theoretical curve for complete precipitation of both components is also indicated. It can be seen that after 15 hours' contact between the tannin and casein solutions the curve follows exactly the theoretical line until the points corresponding to a protein : tannin ratio of 1 : 1. At higher initial tannin concentrations, the curve drops below the theoretical line, indicating that not all the tannin has been complexed. Evidently, all the casein is precipitated by half its weight of tannin after 15 hours, but the resulting complex can also completely adsorb a further equal amount of tannin within the same period. As expected, therefore, the complex is non-stoichiometric and it is probable that the experimental curve would continue to rise at a diminishing rate for still higher initial tannin concentrations, as proportionately smaller amounts of tannin are adsorbed on to the complex.

It is also clear from Figure 33 that the amount of tannin depends on the time of contact between casein and tannin. For all three initial concentrations, the weight of complex is consistently lower

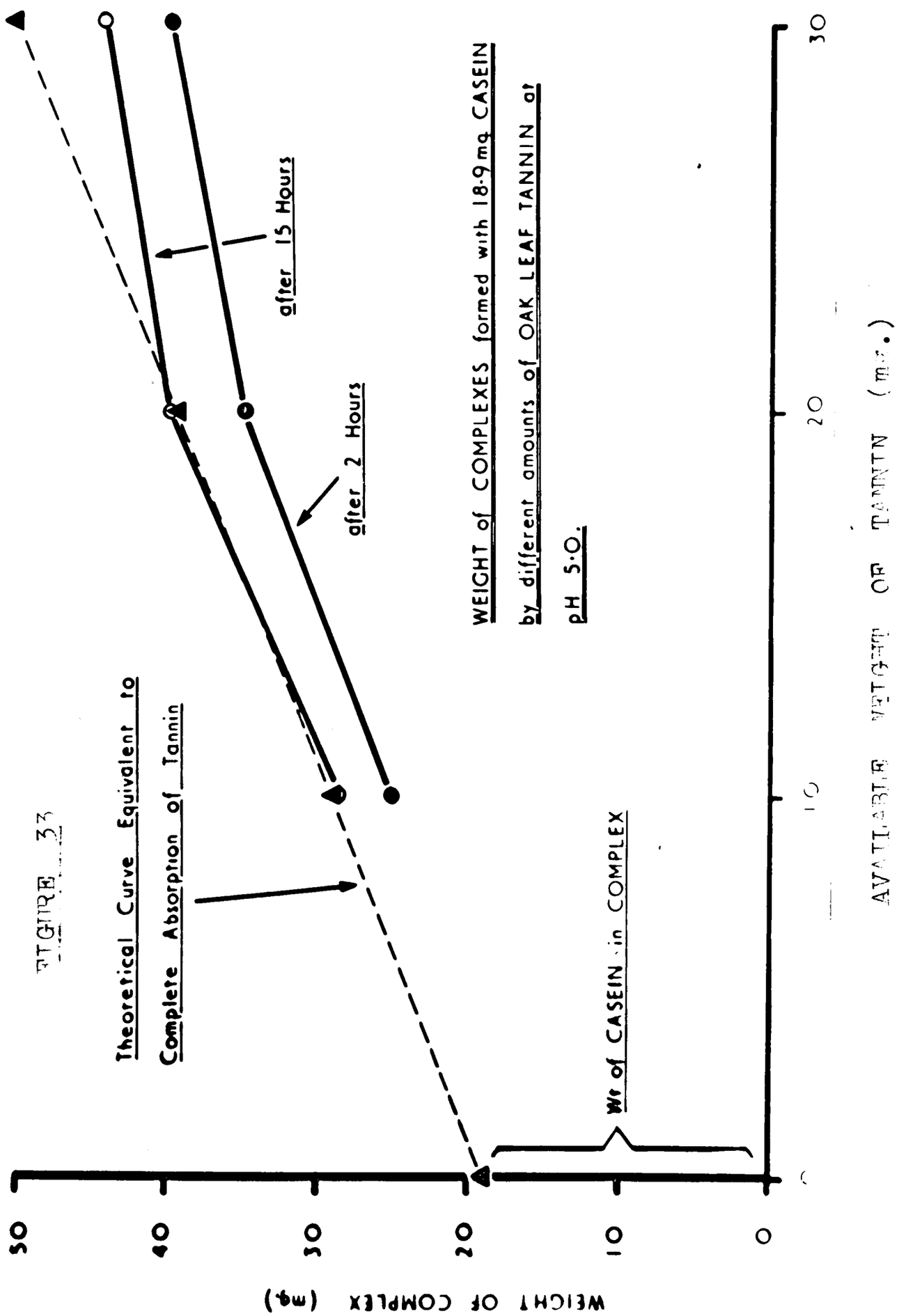

WEIGHT OF COMPLEX (mg)
50
40
30
20
10
0
0
10
20
30
AVAILABLE WEIGHT OF TANNIN (mg.)
Theoretical Curve Equivalent to Complete Absorption of Tannin
after 15 Hours
after 2 Hours
WEIGHT of COMPLEXES formed with 18·9 mg CASEIN by different amounts of OAK LEAF TANNIN at pH 5·0.
Wt of CASEIN in COMPLEX

FIGURE 33

after 2 hours than after 15 hours of contact. This is of interest in connection with the probable formation of tannin-leaf protein complexes in leaves. Presumably the time between maceration of leaf tissue by a caterpillar and its subsequent digestion is long enough for adsorption of only a proportion of the maximum amount of tannin which could be adsorbed in a leaf protein - tannin complex. It seems, however, that the casein can be completely precipitated by only a fraction of the total tannin with which it can combine. In this experiment, 18.9 mg. of casein were completely precipitated by 10 mg. of oak leaf tannin. If leaf protein behaves in a similar manner, the tannin present in September oak leaves (5% dry weight, p.77(a)) would be sufficient to precipitate almost all the leaf protein (12% dry weight, p.44 (a)). Moreover, the figure obtained for leaf protein content was almost certainly an overestimate, since it was determined from total leaf nitrogen analysis (Chapter 3).

3. Quantitative investigation of oak leaf tannin - nettle leaf protein complexes

a) Introduction

Although it is widely believed that tannins form complexes with most, if not all, proteins, it was clearly desirable to confirm that the results obtained using casein were also valid for leaf protein. An experiment similar to that described in the previous section was therefore carried out, using a leaf protein preparation in place of casein.

In view of the nature of the present problem, it would, ideally,

have been preferable to carry out experiments with oak leaf protein. Several attempts to prepare oak leaf protein in bulk were made, but it was found extremely difficult to obtain a reasonably pure preparation and the yields were so low as to make such a preparation impracticable (Appendix 24, p. 238). The difficulty was probably due to the presence of tannin in the leaves, which would render much of the leaf protein insoluble. Handley (personal communication) has used nettle leaves for the preparation of leaf protein, since this species, Urtica dioica, appears to contain very little, if any, polyphenolic material in its leaves. A substantial quantity of this nettle leaf protein preparation was kindly made available by Dr. Handley. His method of extraction of this protein from nettle leaves is given in Appendix 24 (p.238).

The nettle protein preparation, in the form of an aqueous suspension at pH 4.0, was deep brown in colour, probably owing to complex formation between the protein and a pigment degradation product. Several attempts to purify the protein by column chromatography or electrophoresis at various pH values were made, but were all unsuccessful (Appendix 24, p.238). However, Handley (personal communication) has found that the presence of this contaminating material does not prevent the protein from forming complexes with tannins in the usual way, nor does it prevent the protein from being digested quite rapidly by trypsin. It seems, therefore, that the properties of the protein are not substantially altered by the presence of the pigment material (which would in any case probably be present under natural conditions in oak

leaves) and it was decided to proceed with the experiment without further purification of the nettle protein.

b) Method

The experiment was carried out in duplicate, using a similar procedure to that described in the previous section (p. 111). Aliquots of leaf protein solution in distilled water were mixed at pH 5.0 with increasing amounts of September oak leaf tannin (also in distilled water), corresponding to tannin : protein ratios from 1 : 5 to 8 : 1, and allowed to stand at 20°C for ten minutes. The complexes were then centrifuged and washed before their weights were determined gravimetrically. The experimental technique is described in detail in Appendix 25 (p.245), where the recorded complex weights are also given.

c) Results

The complex weights are plotted against increasing initial tannin concentration in Figure 34 (p.115@), where the theoretical curve equivalent to complete precipitation of both components is also indicated.

It is seen that the general shape of the experimental curve is similar to that found for casein - tannin complexes in the previous experiment. However, the curve never reaches the theoretical line, probably because of the considerably shorter time allowed for tanning (10 minutes instead of 15 hours) to make the results more relevant to the field situation. In spite of the short time available for tanning,

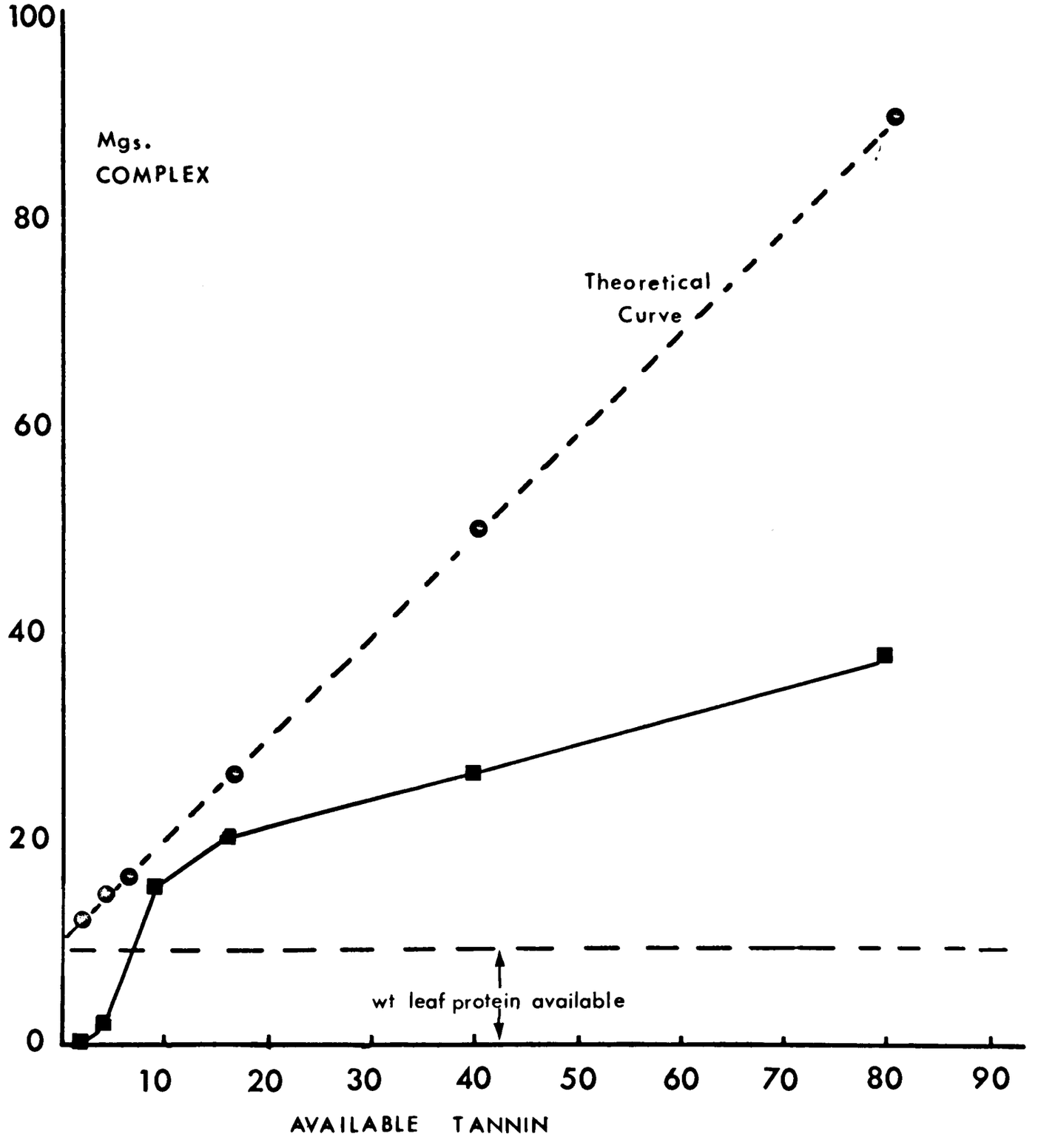


FIGURE 34 Weight of complexes formed with 9.6 mg. nettle leaf protein by different amounts of oak leaf tannin at pH 5.0.

precipitation approaches completion when the tannin : leaf protein ratio is approximately 1 : 1. It is likely that all the protein is precipitated at this tannin : protein ratio and at all higher ratios. It is of interest that, below the equivalence point of tannin and protein, the curve drops sharply. This suggests that protein which has been only partially tanned is able to remain in solution, possibly because there is insufficient tannin available to cross link adjacent colloidal particles of complex. This must not be taken to mean that tannin concentrations below the equivalence point with leaf protein are biologically ineffective, since, although the complex might be largely soluble under these conditions, it may still be less digestible by caterpillars than the pure protein. It will be remembered that inhibitory effects of tannin, discussed in the last chapter, were noted as a result of experiments involving tannin : protein ratios of as low as 1 : 12.

A further feature of the graph in Figure 34 (p.115(a)) is the continuing increase in the weight of complex formed up to tannin : protein ratios as high as 8 : 1, showing that the protein, although completely complexed by its own weight of tannin, is capable of involving at least four times its weight of tannin in the complex. This conclusion must, however, be treated with suspicion, since the two final points on the graph might have been inflated by a higher proportion of sedimented tannin (precipitated, but not bound to protein) than indic ted by the control (see Appendix 25, p.245).

d) Conclusions

The experiment showed that the interaction of nettle leaf protein, likely to be essentially similar to oak leaf protein, with September oak leaf tannin follows a similar pattern to that of casein and thus justifies the use of casein as a model protein in the artificial diet experiments described in the previous chapter. After as little as ten minutes contact at 20°C, most and probably all of the protein was complexed by its own weight of tannin. It is probable that all the protein was bound to tannin at concentrations of tannin lower than this, though still remaining in solution. Evidently, the tanning is far from stoichiometric and presumably takes place by a progressive aggregation of colloidal protein particles as the tannin concentration increases, eventually resulting in flocculation and settling out. Precipitation as a criterion of complex formation is likely, therefore, to lead to underestimates of the proportion of protein interacting with tannin at pH 5.0.

As a result of the experiments so far described in this chapter, it can be concluded that leaf protein and casein are both likely to be complexed with any oak leaf tannin present when they are ingested at a pH of about 5.0 by a caterpillar. It was now necessary to examine the digestibility of such complexes, to determine the relationship between the proportion of tannin in the complex and the digestibility of the protein. Experiments involving the digestion of protein-tannin complexes by trypsin will be discussed later in the chapter.

4. Inhibition of amylase by oak leaf tannin

a) Introduction

Possible enzyme inhibition by oak leaf tannin was first investigated by examining the effect of the presence of tannin on the degradation of starch by amylase. Though an amylase had not been shown to be present in winter moth larvae (Appendix 8, p. 172), this enzyme was selected initially because its activity is simply and rapidly determined using the external iodine indicator technique and because it was expected that any inhibitory effects of tannin on enzymes would be quickly revealed.

b) Materials

Enzyme: The enzyme solution was prepared by dissolving 0.5 gm. bacterial α-amylase (British Drug Houses Ltd.) in 500 ml. distilled water.

Substrate: 1 gm. soluble starch was dissolved in 100 ml. 0.1 Molar phosphate buffer (NaH_2PO_4/Na_2HPO_4) at pH 6.0 - the optimum pH for the enzyme (Fischer and Stein, 1960). 0.02 gm. sodium chloride was added to provide the chloride ions necessary for activation of the enzyme (Bernfeld, 1955).

Tannin: A 1% aqueous solution of oak leaf tannin (fraction (S) only, see p. 57), was prepared, using tannin which had been extracted from July oak leaves by the method of Brown and Love (1961).

Indicator: The external indicator solution contained 1 gm. iodine and 3 gm. potassium iodide, dissolved in 500 ml. distilled water.

c) Method

All solutions were equilibrated in a water bath at 25°C. 5 ml. of the starch solution were then added to each of two tubes, also in the water bath. To the first, experimental, tube was then added 0.5 ml. of the tannin solution. 0.5 ml. of distilled water was added to the second (control) tube. 0.5 ml. of the enzyme solution was then added to each tube. The tubes were shaken at intervals, and drops extracted every minute with a clean glass rod to test for the presence of starch, using rows of indicator drops on a white tile For convenience, the enzyme was added to the control tube thirty seconds before the experimental tube.

The experiment was repeated, using identical materials and quantities, at 20°C.

d) Results

The results of the two experiments are shown in Table 14 (p.119(a)). Although the starch in the control tubes was digested within 10 minutes in both cases, starch was still present in the experimental tubes after 24 hours, though the indicator colour had now become purple rather than blue, suggesting that some degradation to dextrans had occurred.

e) Conclusions

The results of the experiment show that starch is incompletely degraded by α-amylase in the presence of oak leaf tannin at pH 6.0, when the concentrations of starch, enzyme and tannin are in the ratio

TABLE 14

INHIBITION OF AMYLASE BY OAK TANNIN

Temperature	Starch (1% in 0.1M Phosphate Buffer, pH 6; 0.0002% NaCl)	α-Amylase 0.2%	Water	Oak Tannin 1.0%	Digestion Time *
25° C:	5 ml. (50 mg.)	0.5 ml. (1 mg.)	0.5 ml.	-	6 mins.
	5 ml. (50 mg.)	0.5 ml. (1 mg.)	-	0.5 ml. (5 mg.)	More than 24 hours
20° C:	5 ml. (50 mg.)	0.5 ml. (1 mg.)	0.5 ml.	-	10 mins.
	5 ml. (50 mg.)	0.5 ml. (1 mg.)	-	0.5 ml. (5 mg.)	More than 24 hours

* Digestion of substrate followed by external iodine indicator.

100 : 1 : 10. It is not known whether this inhibition is due to complex formation between the tannin and the enzyme or to the formation of a tannin - starch complex, resistant to the action of free amylase, or to both of these phenomena. The experiment does, however, confirm in a convincing manner the potential ability of oak leaf tannin from Q. robur to prevent the action of an enzyme.

Boudet and Gadal (1965a and 1965b) have studied the inhibition of β-amylase by tannins from the leaves of the Sessile Oak Quercus petraea Lieb. (= sessilis Ehrh.). The tannins from this species seem to be very similar to those from Q. robur, as obtained by the present author, consisting of both condensed tannin and hydrolysable components, the latter yielding ellagic and gallic acids on hydrolysis. Boudet and Gadal (loc. cit.) found that 1 mg. of β-amylase is totally inhibited by 0.05 mg. of crude oak leaf tannin at pH 5.0, though the inhibition was found to depend on the time of contact between the enzyme and tannin before the reaction. They found that the amount of inhibition was correlated with the amount of precipitate formed on mixing the tannin and enzyme solutions and conclude that inhibition is due to non-specific blocking of active sites by adsorption of tannin on to the enzyme. The inhibition seems to be irreversible, since on re-suspending precipitated enzyme-tannin complex at pH 5.0, the enzyme regained only 5% of its initial activity. However, on mixing enzyme and tannin solutions at higher pH values, inhibition of β-amylase decreased to zero above pH 6.9, suggesting that stable amylase - tannin complexes are not formed under alkaline conditions.

5. The effect of oak leaf tannin on the hydrolysis of casein by trypsin at pH 7.6

a) Introduction

It was decided to investigate initially the effect of oak leaf tannin on the activity of mammalian trypsin, firstly at its normal operating pH of 7.0 - 8.0 and subsequently at the mid-gut pH of the winter moth - found by two different methods to be about pH 9.2 (Appendix 26, p.248). It was hoped later to follow up these experiments with a protease extract from winter moth larvae, using leaf protein in place of casein to simulate the natural conditions as closely as possible.

The object of the present experiment was to determine whether casein was any less readily hydrolysable by trypsin at pH 7.6 when complexed to tannin than in the uncomplexed state.

b) Method

5 aliquots each of 10 mg. casein ('Light White Soluble') were complexed with an excess of July oak leaf tannin (fraction (S) only) solution at pH 5.0. The complexes were centrifuged down and washed before being re-suspended in 1 ml. distilled water. 5 further tubes were prepared, each containing 1 ml. casein solution (10 mg.) uncomplexed with tannin. 2 ml. phosphate buffer at pH 7.6 were then added to all ten tubes. 1 ml. of trypsin solution (0.25 mg./ml.) was added to 3 tubes in each series and the tubes were then incubated for 3 hours at 26°C. Any proteolytic activity was stopped after the 3 hours by

addition of 3 ml. of 10% (v/v) trichloracetic acid ('TCA') to all tubes. 1 ml. trypsin solution was then added to the two control tubes in each of the series of five tubes. After standing for 1 hour, by which time all unhydrolysed protein would have been precipitated by the TCA, the tubes were centrifuged and the supernatants analysed for soluble nitrogen by the micro-Kjeldahl method.

Further details of the experimental technique, including a description of the preparation of the casein - tannin complex, are given in Appendix 27 (p.250).

c) Results

The Kjeldahl titres, as ml. of 0.005 N sulphuric acid, for the nine supernatants (one was accidentally lost during distillation) are shown in Table 15 (p.122(a)). After subtraction of the reagent control readings, the mean titres were used to calculate the amount of nitrogen released from the 10 mg. casein in the two cases. It was found (Table 15) that 1.196 mg. of nitrogen was released from the free casein whereas only 0.014 mg. of nitrogen was released from the same amount of casein when complexed with oak leaf tannin. Since 'Light White Soluble' casein contains 14.5% nitrogen (British Drug Houses Ltd., technical handbook), the percentage of protein hydrolysed in the two cases was as follows:

Free casein	82.8%
Complexed casein	0.96%

TABLE 15 Effect of oak leaf tannin on hydrolysis of casein by trypsin at pH7.6

Tube	Casein (mg./1ml.)	Buffer[2] (ml.)	Trypsin (ml.)	T.C.A.(10%) (ml.)	Kjeldahl titre[3] (ml.)	Corrected titre[4]	Mean corrected titre	Nitrogen (mg.)
1	10	2	1[1]	3	0.93			
2	10	2	1[1]	3	0.94			
3	10	2	1	3	17.97	17.03		
4	10	2	1	3	17.98	17.04		
5	10	2	1	3	18.12	17.18	17.08	1.196
6	10^5	2	1[1]	3	0.70			
7	10^5	2	1[1]	3	0.72			
8	10^5	2	1	3	lost			
9	10^5	2	1	3	0.89	0.18		
10	10^5	2	1	3	0.93	0.22	0.20	0.014

[1] Trypsin added after T.C.A.

[2] 0.1 molar phosphate buffer (pH7.6)

[3] ml. 0.005N H_2SO_4

[4] Kjeldahl titre corrected for reagent controls

[5] Complexes with oak leaf tannin.

1931(a)

d) Conclusions

It can be concluded from this experiment that casein can be almost completely protected from hydrolysis by trypsin at pH 7.6 when it has been previously complexed with July oak leaf tannin. Since leaf protein is likely to form similar complexes, as shown earlier in this chapter, this result lends support to the hypothesis that tannins may reduce the availability of protein nitrogen to hydrolysis by caterpillars. However, there is evidence (e.g. Boudet and Gadal, 1965b and Handley, W.R.C., personal communication) to show that tannin-protein complexes may be at least partially dissociated at high pH values. It was therefore necessary to repeat the above experiment at pH 9.2 to render the results more relevant to the present ecological problem.

6. The effect of oak leaf tannin on the hydrolysis of proteins by trypsin at pH 9.2

a) Having demonstrated that the activity of mammalian trypsin is substantially inhibited by oak leaf tannin within its normal operating pH range, an experiment was now carried out to discover whether inhibition also occurred at pH 9.2 - the pH of the winter moth mid-gut (Appendix 26). Before this experiment was commenced, the successful separation of crude oak tannin into its condensed and hydrolysable components had been achieved, using Sephadex columns (Chapter 4, p. 63). It was therefore possible to compare the inhibitory effects of the two tannin components separately. Attempts to further purify the nettle

protein, however, had been unsuccessful (Appendix 24, p.238). Since the rate of hydrolysis of the protein in this material was expected to be much slower than that of casein, it was decided to keep casein as the main experimental protein, while testing sufficient samples of nettle leaf protein to determine whether or not any conclusions reached were applicable to the hydrolysis of leaf protein.

b) Method

The experiment was carried out in duplicate and was designed to simulate natural conditions as far as possible. Aliquots of casein (10 mg.) were allowed to interact with different amounts of either condensed tannin (pure 'C', see p. 50(a)) or hydrolysable tannin (pure 'D' and 'H'), ranging from 0 to 20 mg., for 30 minutes at pH 5.0. The pH was then raised to 9.2, when trypsin was added and the mixture incubated for three hours. As in the previous experiment, protein hydrolysis was estimated by assessing the soluble nitrogen released, using the Kjeldahl method.

The enzymic hydrolysis of aliquots of leaf protein, free or complexed with excess hydrolysable or condensed tannin, was investigated simultaneously, using the same conditions. Aliquots of leaf protein were also analysed by the Kjeldahl method to ascertain the total nitrogen content.

The experimental technique is described in detail in Appendix 28 (p. 253), where the final Kjeldahl titres for all 61 samples are given.

c) Results

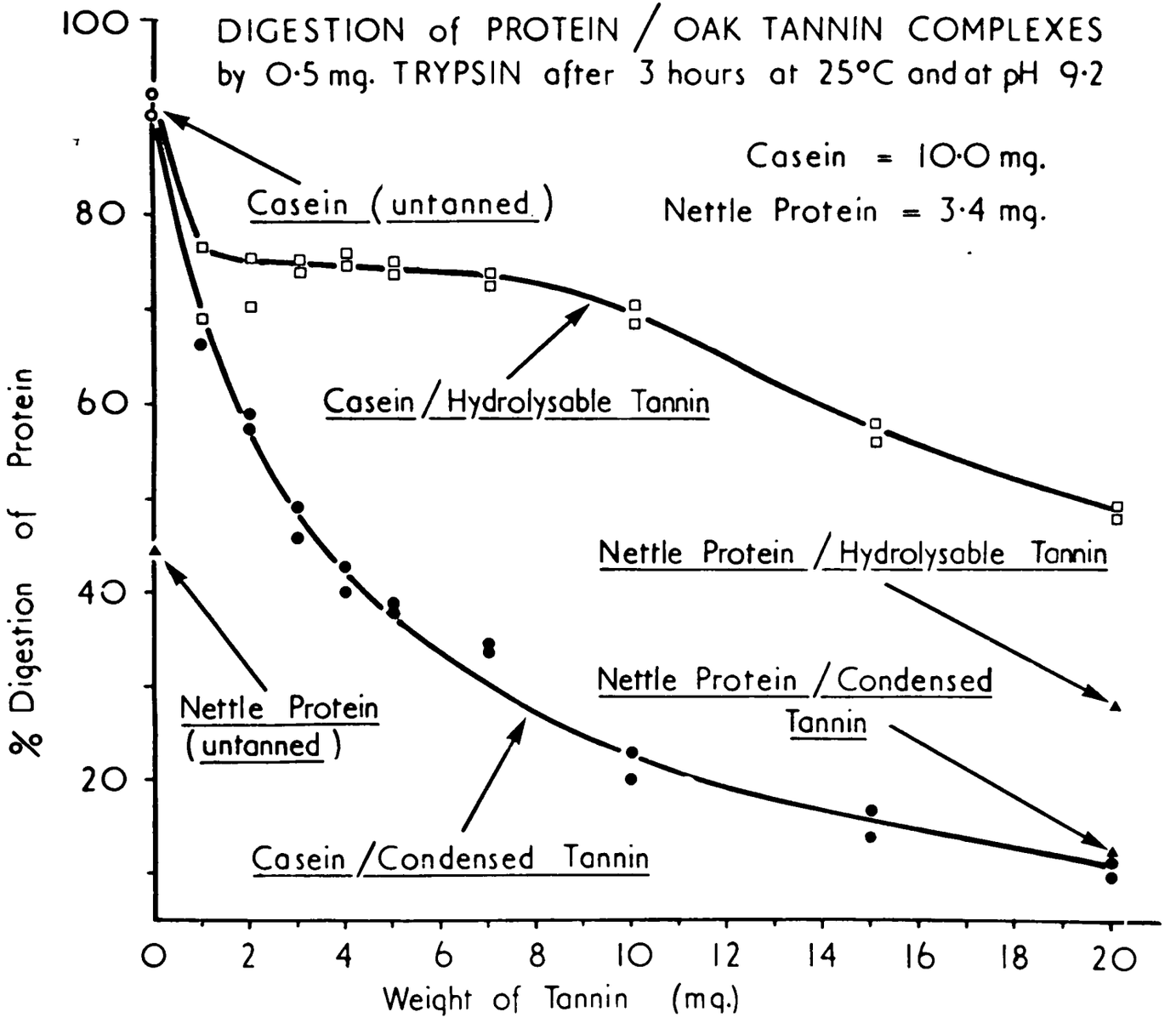
From the results of the Kjeldahl analyses, the percentage of the casein and leaf protein hydrolysed was calculated for each sample (Appendix 28). These figures are plotted against tannin concentration in Figure 35 (p. 125a).

It is seen from Figure 35 that whereas the casein control was 92% hydrolysed, complexes of casein with condensed tannin became less readily hydrolysable as the amount of added tannin increased. The results follow a reasonably smooth curve, from which it is seen that inhibition for a given weight of tannin was greatest with small amounts of tannin and gradually lessened as the amount of tannin increased. As little as 1 mg. of condensed tannin, when complexed with 10 mg. of casein, caused a reduction of 20% in hydrolysis. Casein complexed with its own weight of tannin was almost 80% less hydrolysed than the casein control.

Hydrolysable tannin was clearly a much less effective inhibitor of trypsin action under the experimental conditions. Even with 20 mg. of the tanninpresent, protein hydrolysis was still more than half that of the uncomplexed control. The curve shows an initial sharp drop, followed by a plateau and, subsequently, a gradual decline. No explanation can be given for the shape of the curve at present.

Results from the leaf protein hydrolysis are similar to those obtained for casein, though inhibition by tannins appears to be proportionately less marked. Thus the complex of 3.4 mg. of nettle protein with 20 mg. condensed tannin suffered about 25% hydrolysis

FIGURE 35


DIGESTION of PROTEIN / OAK TANNIN COMPLEXES
by 0·5 mg. TRYPSIN after 3 hours at 25°C and at pH 9·2
Casein = 10·0 mg.
Nettle Protein = 3·4 mg.
Casein (untanned)
Casein / Hydrolysable Tannin
Nettle Protein / Hydrolysable Tannin
Nettle Protein / Condensed Tannin
Nettle Protein (untanned)
Casein / Condensed Tannin
% Digestion of Protein
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
Weight of Tannin (mg.)

compared with the uncomplexed protein, whereas casein complexed with the same proportion of tannin would have suffered scarcely any hydrolysis. As with the casein complexes, hydrolysable tannin is less effective than the condensed tannin as an inhibitor of leaf protein hydrolysis at pH 9.2.

d) Conclusions

The experiment shows that both hydrolysable and condensed oak leaf tannins, when complexed with casein and leaf protein, reduce the susceptibility of the protein to hydrolysis by trypsin at pH 9.2. The effect is not so marked as that found in the previous experiment at pH 7.6, but, in the case of condensed tannin, it is nevertheless substantial. Evidently some dissociation of the tannin-protein complexes occurs at the higher pH, but dissociation is by no means complete. This suggests that the presence of tannins in leaves may significantly reduce the availability of protein nitrogen to caterpillars, in which the mid-gut pH is 9.2. It is of interest to note that hydrolysable tannin, which apparently has only a feeble effect on protein hydrolysis, is present in oak leaves throughout the season (p. 55(a)), whereas the condensed tannin, with a much stronger inhibitory action, appears in the leaves only from late May onwards.

7. Discussion

The experiments described in this chapter have shown that oak leaf tannins form complexes with both casein and a nettle leaf protein preparation, only a small proportion of tannin being required to effect

complete precipitation of casein at pH 5.0. These complexes appear to be almost completely resistant to hydrolysis by trypsin at its normal operating pH of 7.6, but can be hydrolysed to some extent at pH 9.2. At this higher pH, hydrolysable tannin is evidently considerably less effective as an inhibitor than condensed tannin, possibly because its complexes with protein are more liable to dissociation under alkaline conditions.

These results are in agreement with those of other authors. Loomis and Battarle (1966) have found that complexes of polyvinylpyrrolidone ('PVP') with condensed tannin dissociate at pH values above 7-8, whereas complexes of PVP with hydrolysable tannin are largely dissociated above pH 5.0. Moreover, Basaraba and Starkey (1966) found that inhibition of microbial hydrolysis of the proteins gelatin and gliadin by chestnut Castania sativa and wattle Acacia molissima bark tannins was much more pronounced at pH 4.0 than at pH 7.0 and was also greater at a tannin : protein ratio of 4 : 1 than at a ratio of 1 : 4. Goldstein and Swain (1965) showed that precipitation of β-glucosidase by tannic acid is virtually complete at pH values up to 7.5, but at higher pH values inhibition depends markedly on the buffer used. Condensed tannin from wattle was found to give more stable complexes than tannic acid and reactivation of enzyme - tannin complexes by removing tannin with PVP, detergents, etc. rarely restored more than 50% of the enzyme activity. Goldstein and Swain (loc. cit.) conclude that the condensed tannin - enzyme complexes may be held together by other, more stable, means in addition to hydrogen bonds.

From the results described in this chapter, a number of interesting inferences may tentatively be drawn with respect to the ecological situation under study.

1) The time taken for ingested food to pass through the mid-gut of a winter moth larva has not yet been determined, but it seems likely that three hours is longer than the period normally available for digestion. If this is the case, and if the experiment involving casein digestion at pH 9.2 can be considered to represent the situation in the larval mid-gut, then it can be said that the amount of nitrogen available to an insect from casein, complexed with its own weight of condensed tannin, is only about one quarter of the amount available from uncomplexed casein. If the period available for digestion is in reality shorter than three hours, then the amount of nitrogen released from the complex would be expected to be even further reduced. Such a reduction in the level of available nitrogen would seem to be amply sufficient to account for the reduction in growth of winter moth larvae fed on tannin diets, as discussed in the previous chapter.

2) If it is assumed that the tannins used in the laboratory experiments are chemically identical to the tannins originally present in oak leaves and, again, if the conditions obtaining in the laboratory experiments can be considered to represent the field situation, then it can be said that oak leaf tannins, and especially condensed tannin, are likely to reduce the availability of protein nitrogen to insects feeding on the leaves. It is impossible at present to estimate the extent of

this reduction quantitatively, since so many factors are unknown. The effect of the tannins would depend, for example, on the concentrations of tannins in the leaves, the nature of the proteins present, the time available for complex formation after ingestion of the leaves, the extent to which some of the tannin may form complexes with other, non-protein, material, the protease level in the insect gut and the period available for digestion. However, a rough estimate of the possible effect of the tannins can be obtained from the results so far described. If the behaviour of casein at pH 9.2 is taken to represent that of oak-leaf protein in the field, then, in three hours digestion in the presence of a third of its weight of condensed tannin, the amount of nitrogen released is approximately half that released from uncomplexed casein (Figure 35, p.125(a)). In September oak leaves, the ratio of tannin to protein is approximately 1 : 3 (Chapter 3 and 4), so that the amount of available protein would be of the order of 7 grams/100 grams of leaf (dry weight), compared with approximately 30 grams/100 grams of leaf (dry weight) in very young oak leaves, in which no condensed tannin is present. If it is assumed that a caterpillar must normally ingest, say, 10 gm. (dry weight equivalent) of leaf material in order to complete its growth during April and May, then the data imply that at least 40 gm. might have to be ingested to achieve similar growth on September leaves. Such a difference, although very approximate and based on a number of assumptions, at least gives an idea of the possible effect of leaf tannins in further reducing the level of available nitrogen as the season progresses, and may account

to some extent for the concentration of larval feeding on oak leaves in the early part of the year. It may well be possible for larvae to obtain some nitrogen from leaf protein throughout the summer and autumn, but growth would probably be much slower.

3) In view of the substantial increase in digestibility of protein-tannin complexes at pH 9.2 in comparison with their digestibility at lower pH values, it seems possible that one of the functions of the high gut pH in phytophagous insects is to increase the amount of nitrogen available from leaf protein-tannin complexes. pH values higher than 9.2 are possibly unacceptable physiologically, although dissociation of the tannin-protein complexes might be expected to proceed further. The value of 9.2 could therefore be a compromise value to obtain the maximum amount of nitrogen, compatible with the physiological limitations.

CHAPTER 7: PRELIMINARY INVESTIGATION INTO THE FATE OF PROTEIN AND TANNIN IN THE MID-GUT OF THE WINTER MOTH

1. Introduction

This chapter describes some preliminary experiments to study the fate of oak leaf tannin and of protein in the presence of tannin, after reaching the gut of the winter moth. It was hoped to ascertain to what extent protein is assimilated in the presence of the tannin and whether any of the tannin is adsorbed into the tissues of the caterpillars.

The experiments are largely incomplete and only tentative conclusions can be drawn from them. They were undertaken partly to assess the feasibility of using autoradiography to investigate this kind of problem.

2. Assimilation by winter moth larvae of protein in the presence of oak leaf tannin

a) Introduction

It was decided to investigate, during the 1966 season, the ability of winter moth larvae to digest and assimilate protein in the presence of oak leaf tannin. Ideally, it was hoped to prepare a tannin-protein complex, using ^{14}Carbon-labelled leaf protein, and discover by autoradiography the proportion of radioactive carbon adsorbed into the insect tissues in comparison with a control, in which the protein was

uncomplexed. However, a source of soluble radioactive protein was unavailable commercially and time did not permit the preparation of labelled protein by enclosing plants in an atmosphere containing labelled carbon dioxide. It was necessary, therefore, to use the only ^{14}Carbon-labelled protein available commercially, namely ^{14}C - protein from Chlorella vulgaris, available from the Radiochemical Centre, Amersham. This protein is unfortunately supplied in a denatured form, insoluble in water, so that preparation of tannin-protein complexes was impossible. It was considered worth while, however, to feed this preparation to winter moth larvae, in the presence of tannin, to ascertain whether digestion can occur under these conditions.

b) Materials

0.05 mg. of labelled Chlorella protein, containing 0.1 millicurie of activity, was obtained from the Radiochemical Centre, Amersham.

c) Method

420 mg. of casein ('Light White Soluble') was dissolved in 11 ml. of 0.1 Molar phosphate buffer (Na_2HPO_4/NaH_2PO_4) at pH 8.0. The Chlorella protein (0.05 mg.) , suspended in 2 ml. distilled water, was then added and finely ground into suspension in the casein solution by means of a hand homogeniser with a teflon plug. With washings, the final column of the protein preparation was 20 ml.

6 ml. aliquots of the protein preparation were pipetted into each of three glass centrifuge tubes, which therefore contained approximately

140 mg. mixed protein, with an activity of about 30 microcuries. To the first tube was added 200 mg. oak leaf condensed tannin, purified by the Sephadex method of Somers (Chapter 4), dissolved in 5 ml. distilled water. To the second tube was added 200 mg. of hydrolysable tannin (isolated from oak galls and purified on chromatograms by Bostock, 1966), also dissolved in 5 ml. distilled water. To the third (control) tube, were added 5 ml. distilled water. The three tubes were left overnight after shaking at intervals for one hour to ensure thorough mixing of the tannin and protein components. The pH of the contents of each tube was then adjusted to 4.0 with citric acid, using a pH meter, and the resulting flocculent precipitates centrifuged down (4,000 r.p.m. for 5 minutes) and the supernatants decanted. The supernatants in both of the tubes containing tannin were coloured, showing that excess tannin was present.

50 gm. of artificial diet was prepared, using the new modified formula described in Chapter 5 (p. 97), except for casein, wheat embryo and formaldehyde, which were omitted. 4 gm. of this freshly prepared diet, while still liquid, was poured into each of the three centrifuge tubes containing precipitated protein or tannin-protein complexes. 150 mg. of cellulose powder was added to the control tube, in place of tannin. The contents of the tubes were thoroughly mixed, before the diets were dispensed into plastic pots (6 for each diet), identical to those used for the experiments described in Chapter 5.

On May 30th, 1966, 6 4th instar winter moth larvae were placed on each diet (one per pot) and allowed to feed as usual. On June 7th, all

the larvae, now in their mid 5th instar, were dissected and their guts transferred to formol/saline fixative for 20 hours. It was noticeable that the guts of larvae fed on the two tannin diets were brown after fixation, whereas the guts of the six control insects were translucent white, indicating that the tannin had not been removed during fixation.

The frass from each series of six diets was collected, dried and stored in case opportunity permitted chromatography and determination of radioactivity to be carried out, to ascertain the fate of the tannin and labelled protein.

The guts from one larva each from the condensed tannin and control diets were embedded in paraffin and sectioned at 6/u - 15/u. The serial sections from the tannin and control diet series were transferred to microscope slides and all stained in Haematal '8' and counterstained in Biebrich Scarlet (Baker, 1962). The odd numbered slides from both the tannin and control diet material were not treated further. The even numbered slides from both the tannin and control diet material were coated with Kodak AR 10 stripping film, using the technique of Kukita and Fitzpatrick (1955), also used by Brunet and Small (1959). The coated slides were placed in a sealed box in a refrigerator at 4°C, to allow radioactive disintegration to be recorded on the photographic emulsion.

After four days, alternative even numbered slides from each series were withdrawn inthe darkroom and the photographic emulsion developed and fixed (Brunet and Small, loc. cit.). The slides were

then examined under oil immersion to locate the radioactive carbon by the positions of the black dots (silver grains) in the emulsion overlying the sections.

d) Results

A section of part of the mid-gut of the larva fed on untanned protein is shown in Figure 36a (p.134a). This section was on one of the control slides and therefore not coated with the photographic emulsion. Typical columnar epithelial and goblet cells can be seen. In Figure 36b (p.134a) is shown a similar section from the same region of the same animal which had been exposed to emulsion, as described above. It can immediately be seen, from the pattern of silver grains shown as black dots above the section, that incorporation of radioactive carbon from the Chlorella protein was widespread throughout the mid-gut tissue.

In Figure 37a (p.134b) is shown, at the same magnification (x 500) and slide thickness (6 μ) as the exposed control above, a section from the same mid-gut region of the larva fed onthe diet containing condensed tannin, also exposed to the emulsion for four days. It is at once apparent that the label has been widely incorporated in the mid-gut tissue, showing that the Chlorella protein had been at least partly digested. Although no quantitative comparison was attempted, by counting silver grains in the two cases, it is evident that incorporation of labelled protein or its constitutent amino acids had taken place on the same scale in both the larva fed on the tannin diet and that fed on the control diet.

FIGURES 36a and 36b

FIGURE 36a Section of part of the mid-gut of a winter moth larva, fed on a diet containing ^{14}Carbon-labelled Chlorella protein. Section stained in Biebrich Scarlet/Haematal '8'; not exposed to photographic emulsion. (magnification ×500; 10μ).

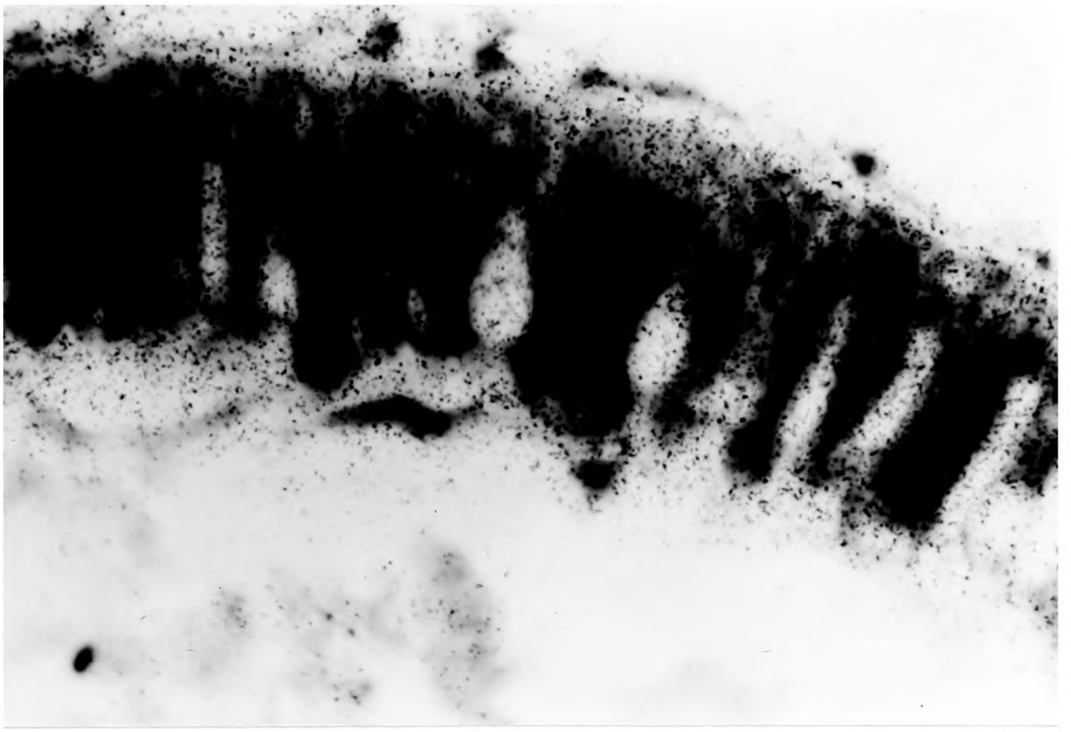
FIGURE 36b Section of part of the mid-gut of a winter moth larva, fed on a diet containing ^{14}Carbon-labelled Chlorella protein. Section stained in Biebrich Scarlet/Haematal '8'; exposed to photographic emulsion for 4 days. (magnification ×500; 6μ).





FIGURE 37a Section of part of the mid-gut of a winter moth larva, fed on a diet containing ^{14}Carbon-labelled *Chlorella* protein and unlabelled oak leaf condensed tannin. Section stained in Biebrich Scarlet/Haematal '8'; exposed to photographic emulsion for 4 days. (magnification $\times 500$). (6μ).

FIGURE 37b Enclosure of oak leaves in a polythene bag, containing $^{14}CO_2$, to prepare ^{14}Carbon-labelled oak leaf tannins. 'Interplant', Wytham Wood, July 1965.





No attempt has yet been made to examine sections of the other larvae fed on the three diets.

e) Conclusions

This experiment shows that protein in the presence of oak leaf condensed tannin, complexed with casein, can be digested by winter moth larvae. However, it is not possible to say whether or not protein can be digested when in the form of a protein-tannin complex, since such a complex was not formed with the radioactive protein in the present experiment, owing to its insoluble denatured condition. It is clearly desirable to repeat the experiment, now that the procedure has found to be satisfactory, using soluble radioactive leaf protein. Such protein is unfortunately unavailable commercially and would have to be prepared from plants, following a procedure similar to that described for preparing radioactive tannin later in this chapter.

3. Fate of oak leaf tannin after ingestion by winter moth larvae

a) Introduction

It is of interest to discover by direct observation what happens to oak leaf tannins when they are ingested in the form of protein-tannin complexes by caterpillars. The evidence presented in the last chapter suggests that protein-tannin complexes may be dissociated, at least partially, at pH 9.2 in the mid-gut of the winter moth. If this is so, is the tannin then simply passed out in the frass or is it in some way assimilated by the insect?

Hollande (1923) reported the occurrence of 'tannoid crystals' in the lumen of goblet cells in the mid-gut of Tortrix viridana larvae

which had fed on oak leaves. These 'crystals' could not be found in sections from larvae which had been fed on leaves from several other species of tree, such as ash Fraxinus excelsior L. The 'crystals' were thought, for a number of reasons, to be complexes of tannin with secretory products of the goblet cells. Among these reasons were their yellow-brown colour, their solubility in 75% ethanol and their reducing properties (e.g. reduction of ammoniacal silver nitrate solution). Hollande (loc. cit.) considered that the 'crystals' were not attributable to uric acid but his evidence for their tannin nature was entirely histochemical and his conclusions were necessarily largely arrived at by a process of elimination.

b) Histology

A number of winter moth larvae, fed on oak leaves or on artificial diets containing tannin, were dissected during 1965 and 1966. Their guts were fixed in formol/saline, Zenker or mercuric/acetic fixatives. Sections were cut from these gut preparations, after embedding in paraffin, and stained with Masson tricolor, haemalum/light green or ferric chloride. No trace could be found of 'crystals' in the goblet cells of the mid-gut, such as were found by Hollande in larvae of T. viridana. However, in view of the present author's lack of histological experience, such 'crystals' may have been overlooked.

c) Examination of the gut lining of winter moth larvae

During the 1965 season, three winter moth larvae, two of which had been reared on oak leaves and one on an artificial diet containing crude oak leaf tannin, were dissected. The mid-gut wall was carefully

removed from the peritrophic membrane, containing the gut contents. In all three cases, the gut wall and the peritrophic membrane, still containing food material, were stained with 5% ferric chloride solution to locate phenolic materials. Whereas the peritrophic membrane and its contents in each case turned deep blue, no trace of blue colour could be found on the gut lining, even after careful examination under a microscope. This suggested that the tannin was largely, if not entirely, retained inside the peritrophic membrane in the gut of the winter moth. However, it is possible that tannin, if absorbed into some of the goblet cells, did not give a coloured product with the ferric chloride or that any coloured product was too small in amount or too finely dispersed to be easily visible.

d) Preparation of radioactive tannin from oak leaves

The simplest and most elegant method available to discover once and for all the fate of ingested tannin in the winter moth gut seemed to the author to involve autoradiography. If winter moth larvae could be fed on a diet containing radioactive tannin, then it should, at least in theory, be a simple matter to locate the whereabouts of the ingested tannin.

Accordingly, since radioactive tannin is unavailable commercially, a field experiment was carried out in late June and early July, 1965, to determine whether oak leaf tannin could be labelled with ^{14}Carbon by enclosing a branch of an oak tree in an atmosphere containing radioactive carbon dioxide.

The technique used for this experiment was suggested to the

author by Dr. Daphne Osborne, of the Department of Agriculture, Oxford, and is described in Appendix 29 (p. 259). By kind permission of the Wytham Scientific Committee, it was possible to carry out the experiment on an oak tree in Wytham Wood. One of the south-facing branches of this tree was enclosed securely in a large polythene bag (capacity 130 litres) in the early morning of June 30th. At 0900 hrs., the same morning, $^{14}CO_2$ (about 1 millicurie) was released into the bag by adding 30% perchloric acid to radioactive barium carbonate, kindly supplied by the Department of Agriculture, Oxford. The gas released amounted to about 0.02% of the bag capacity. The bag is shown in position on the tree in Figure 37b (p.134(b)).

After 12 hours, including 7 hours of bright sunshine, the bag was ripped open and excess radioactive gas allowed to escape overnight. The next morning, the bag was removed and the leaves left <u>in situ</u> for one week to allow metabolism to continue, in the hope that some labelled tannin would be produced.

After a week, the leaves (360 gm. fresh weight) were harvested and extracted with 70% acetone/water. 0.475 gm. crude tannin was isolated from the extract by the method of Brown and Love (1961), followed by dialysis and freeze-drying. The mixed tannins were separated by the method of Somers (1966). The condensed tannin (streak 'C', Figure 17, p.50(a)) was subsequently separated into its water-soluble and water-insoluble fractions. The hydrolysable tannin (streaks D_1 and D_2, Figure 17) was purified by paper chromatography on Whatman 3MM paper. The activity of the three tannin fractions was

found using a Nuclear Chicago series 724 liquid scintillation system (Appendix 29, p.259). The yields and activities of the fractions were as follows:

Tannin Fraction	Yield	Activity
Condensed Tannin - water soluble (streak 'C', see Figure 17, p. 50(a))	22.0 mg.	86.9 millimicrocuries
Condensed Tannin - water insoluble (streak 'C')	95.7 mg.	344.5 millimicrocuries
Hydrolysable Tannin (streak 'D' only)	40.7.mg.	6.9 millimicrocuries

The results showed that some incorporation of the ^{14}Carbon label into oak leaf tannins had in fact been achieved. The total activity of the radioactive tannin amounted to about 0.44 microcurie. This represented only about 4.4×10^{-5}% of the activity (1 millicurie) present in the initial barium carbonate, but is nevertheless greater than the activity of labelled materials frequently used for autoradiography in, for example, developmental biology. The samples obtained were therefore considered suitable for an autoradiographic invesitgation of the fate of tannin after ingestion by larvae.

e) Investigation of the fate of radioactive tannin in the mid-gut of winter moth larvae

360 mg. casein ('Light White Soluble') were dissolved in 36 ml. 0.1 Molar phosphate buffer (Na_2HPO_4/NaH_2PO_4) at pH 8.0 and the solution divided into three aliquots of 12 ml. in separate centrifuge tubes. To the first tube was then added 20 mg. radioactive condensed tannin

(water soluble fraction), as obtained above, dissolved in 5 ml. distilled water. To the second tube was added 10 mg. of the radioactive hydrolysable tannin, also dissolved in 5 ml. distilled water. 5 ml. of distilled water only was added to the third (control) tube.

The three tubes were left overnight in a refrigerator, to allow thorough mixing of the components. The pH was then adjusted to 4.0 with citric acid solution and the precipitates centrifuged and washed with distilled water.

300 gm. of artificial diet were prepared, using the modified formula, described on page 97 , except that casein, wheat embryo and formaldehyde were omitted. 2 gm. of the diet, while still hot, were transferred in a warm pipette to each of the centrifuge tubes containing casein or casein-tannin complex. The contents of the three tubes were then thoroughly mixed with a mechanical homogeniser, which had been previously warmed by running in distilled water at 100°C. The three diets were then allowed to gel in a refrigerator.

Each of the three diets was divided into three portions and each portion was placed in a plastic pot of the usual design (Figure 25, p.85(a)), making nine pots in all. On June 17th, 1966, one 4th instar winter moth larva, reared from a batch of eggs in the laboratory, was placed in each of the nine pots and allowed to feed for 7 days.

On June 24th, all nine larvae, now in their 5th instar and apparently all healthy, were dissected and their guts fixed in formol/saline fixative for 20 hours.

The gut of one of the larvae fed on the diet containing radioactive

condensed tannin was embedded in paraffin and serial sections cut at 6/u - 15/u. The serial sections,mounted on 30 slides, were then stained in Haematal '8' and counterstained with Biebrich Scarlet (Baker, 1962). The even-numbered slides were coated with Kodak stripping film, using the technique described earlier in this chapter, and placed in a sealed container in the refrigerator on October 13th, 1966, to allow radioactive disintegration to be registered on the photographic emulsion. The odd-numbered slides were not further treated.

After 4 days, six of the exposed slides, corresponding to different regions of the insect gut, were withdrawn and developed to show up any grains of metallic silver produced in the emulsion during decay of the radioactive carbon atoms. However, very few black dots could be seen in the emulsion and these might have been due to background radioactivity. It is possible that the labelled tannin had not been incorporated into the tissue surrounding the gut, but some trace of the labelled tannin should have been revealed in the gut lumen. It is clear that the activity of the labelled tannin is so weak that exposure to the emulsion must be prolonged for many weeks, or even months, in order to obtain a conclusive result. The remaining nine slides will therefore be developed, it is hoped, in December, 1966.

4. Conclusions

Some preliminary experiments, described in this chapter, have been undertaken to discover the fate, after ingestion by winter moth larvae, of protein in the presence of tannin and also of tannin complexed

with protein. Although the experiments were incomplete and suffered from various shortcomings, they have been described since they illustrate the potential use of autoradiography in studying this kind of problem. It was shown that Chlorella protein can be assimilated by larvae in the presence of a tannin-casein complex. It was further shown that labelled tannin can be prepared by surrounding oak leaves with labelled carbon dioxide, although experiments with the resulting tannins have as yet revealed no definitie conclusions, owing to insufficiently long exposure of the photographic emulsion to the sections.

There is, therefore, no conclusive evidence to either support or reject the possibility that tannins are absorbed into the cells of the mid-gut of the winter moth, as was found by Hollande (1923) for T. viridana. However, such limited histological investigation as was possible by the present author suggests that tannin is not so absorbed.

CHAPTER 8: DISCUSSION

It was mentioned in the Introduction to this thesis that the pronounced concentration of the larval feeding periods of many species of oak-feeding Lepidoptera in the spring was considered to be due to the characteristics of oak leaves during the growing season. Several such changes have now been studied and it is possible to comment further on their ecological significance.

It was shown (Chapter 2) that leaf toughness may play an important part in preventing winter moth larvae, the commonest early feeding species in Wytham, from feeding satisfactorily and reaching normal pupal weights on mature oak leaves. However, it was considered that toughness is a proximate factor influencing larval growth and, although it could adversely affect the growth of species which at present feed early in the year, natural selection would be likely to have favoured the development of stronger jaws, if toughness was the only factor acting to prevent later feeding.

Consideration of the life histories of the oak-feeding species listed in Appendix 1 (p. 150) suggests that some factor or factors other than toughness may be important in determining the ability of Lepidoptera larvae to grow satisfactorily on oak leaves. Although the data in Appendix 1 are by no means complete, often resting only on one or two observations, it can nevertheless be seen that larvae which feed early tend to complete their growth rapidly, whereas those species which

commence their feeding in the late summer tend to grow very slowly and many species drop from the trees to complete their growth on low-growing plants or hibernate as comparatively small larvae, before completing their growth the following spring on fresh young oak leaves. Although it is possible that toughness might be responsible for the slow growth by larvae on mature oak leaves, it is considered by the author that changes in the nutrient value of the leaves are more likely to be important. As already mentioned, natural selection would be expected to favour the development of strong jaws by late feeding Lepidoptera, as an adaptation to their diet. Even young larvae of Hepialus humuli L. (not an oak-feeding species) are capable of biting through the tough plastic of the rearing pots described earlier in this thesis (Figure 25, p.25(a)) (Lees, D.R., personal communication). Although a survey of the jaw sizes of the oak-feeding species has not been carried out, it is known that at least some late feeding species, such as Biston betularia, have strong jaws.

Determination of the nitrogen content of oak leaves (Chapter 3) suggested that variation in leaf nutrients might provide a more fundamental explanation for early larval feeding than variation in leaf toughness. The early larval feeding periods are correlated with a high level of nitrogen in the leaves and it is possible that larval growth may be controlled either by the concentration of nitrogen or by the presence of particular nitrogenous compounds (as is apparently the case with some aphids; see p. 38). However, this again is likely to be only a partial explanation of the ecological situation, since there is

still a substantial concentration of nitrogen in oak leaves during the summer and early autumn.

It is known that plant tannins may render protein more resistant to breakdown (e.g. Handley, 1961) and it seemed that the availability of leaf protein nitrogen to insect larvae might be influenced by the tannin content of oak leaves, which was suspected to increase during the growing season. It was found (Chapter 4) that oak leaves contain both hydrolysable and condensed tannins, which were both eventually isolated in a chromatographically pure condition. The tannin concentration in the leaves was found to increase during the season, but not as sharply in the early summer as was previously suspected (though the data represent analyses from one tree during only one season). The increase in tannin concentration is thought to be due largely to the formation of condensed tannin. The hydrolysable tannin, judged from chromatograms, seems to remain at a relatively constant level during the growing season and the rate of incorporation of radioactive carbon (Chapter 7) into the hydrolysable tannin of July leaves was found to be about twenty times slower than into the condensed tannin.

Winter moth larvae, grown on artificial diets containing oak leaf tannins, were found to gain fresh weight more slowly and to reach lower peak and pupal weights than larvae which had been fed on tannin-free control diets (Chapter 5). This reduction in growth was considered to have a potentially adverse effect on the survival of the pupae and, perhaps, the weights and fecundity of the emerging adults, though this was not proved.

It was considered that inhibition of larval growth by oak leaf tannins was likely to be due to interference by the tannins with the digestion of protein by the insects. In vitro experiments (Chapter 6) confirmed that oak leaf tannins, and especially the condensed tannin, can inhibit the hydrolysis of casein and nettle leaf protein by mammalian trypsin; the inhibition is, however, less pronounced at pH 9.2 than at pH 7.6. This might suggest that digestion of oak leaf protein by insect proteases could be partially inhibited by oak leaf tannins and, moreover, that one of the functions of the high mid-gut pH found in many phytophagous larvae might be to overcome this inhibition to some extent by partially dissociating protein-tannin complexes.

It was found that condensed tannin has a much greater inhibitory effect on the hydrolysis of casein and leaf protein than does the hydrolysable tannin. This is of interest in view of the presence of hydrolysable tannin in young oak leaves (as well as in older leaves) and suggests that this tannin may have an insignificant effect on larval growth and survival in comparison with that of the condensed tannin, which reaches a high level only in older leaves.

The in vitro experiments showed that the proportions of protein and tannin had a marked influence on the amount of casein hydrolysed by trypsin. If a similar state of affairs is assumed to obtain in the field, it is likely that the protein : tannin ratio in leaves could well be of considerable significance in determining the proportion of the total protein nitrogen which is available for insect growth. The 'protein' (total N x 6.25) : tannin concentration ratio for the upper

sun leaves of tree M during 1965 is shown in Figure 38 (p.147(a)). In calculating this ratio from the data obtained from tree M in 1965 (Tables 3, p. 44 and 8, p. 77(b)), it has been assumed that the protein content of the leaves on September 30th was the same as that on September 15th. In view of the steady concentration of protein found in the leaves during the late summer, this assumption is probably justified. It is possible that nitrogen content had begun to drop by the end of September, as the leaves became senescent; this, however, would tend to decrease, rather than increase, the protein : tannin ratio.

Although the ratios obtained above can only be rough approximations, since the protein values are derived from total nitrogen determinations and since the tannin analyses depend on only one sample from each date, the graph in Figure 38 nevertheless indicates the substantial drop in the protein : tannin ratio which occurs in oak leaves during the growing season and therefore, perhaps, the progressive reduction in the digestibility of leaf protein by caterpillars.

It is not at present possible to draw with conviction a graph showing the level of digestible protein in oak leaves during the growing season, since it would be based on a very large number of assumptions. Suffice it to say that the experiments described in Chapter 6 suggest that this level may be at least 4-5 times lower in September than in April. It is possible that the effect is even greater, since the properties of the tannin might have been somewhat altered (for example, by combining with carbohydrate material) during

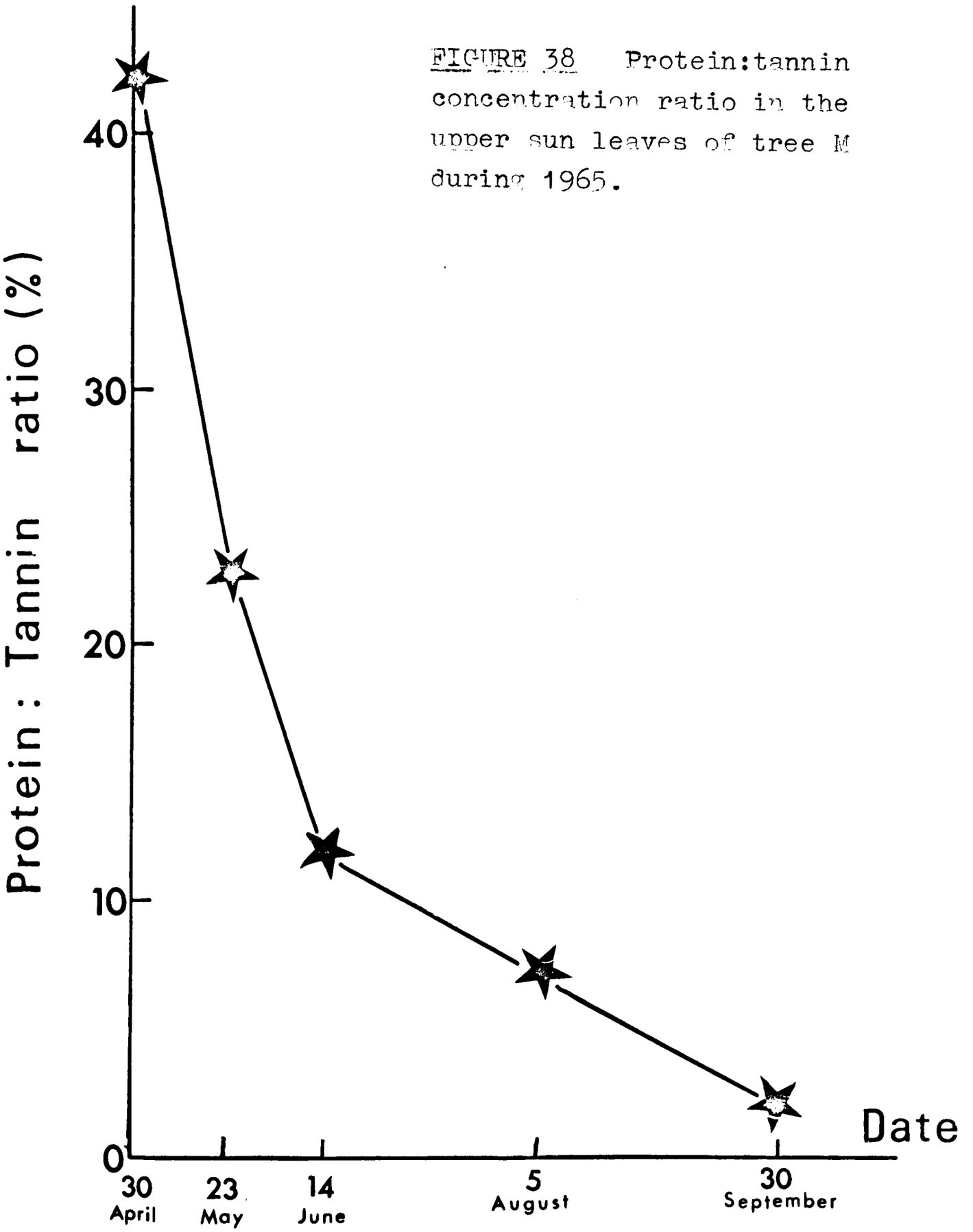


FIGURE 38 Protein:tannin concentration ratio in the upper sun leaves of tree M during 1965.

isolation from the leaves, in spite of all precautions. It is also probable that analyses of tannin content underestimated the actual tannin content, since the extraction procedure is unlikely to have been 100% efficient. Furthermore, caterpillars of the winter moth were observed by the author to bite off pieces of leaf from around the edge of previously damaged tissue. During exposure to the air between rupture of the cells and ingestion of the tissue by the larvae, it is very likely that quinone groups might be formed by oxidation of some of the hydroxyl groups on the tannin molecules, possibly resulting in the formation of covalent bonds between the tannin and leaf protein. This would almost certainly increase the proportion of leaf protein rendered unavailable to digestion by the caterpillars.

Among the exceptions to the generalisation that late-feeding larvae tend to be slow-growing are the leaf miners, many of which apparently grow fairly rapidly, even on September and October leaves (Appendix 1, p.150). Although it could be argued that leaf mining is an adaptation, evolved by natural selection, whereby the larvae avoid feeding on the tougher parts of the leaves, it is also possible that the habit is an adaptation to feeding on the spongy parenchymatous tissue while ignoring the pallisade cells, in which the tannins are believed to be largely concentrated.

Summarising, the evidence presented in this thesis supports the hypothesis that one of the effects of leaf tannins, and condensed tannin in particular, may be to inhibit the growth, and probably the survival, of caterpillars. The production of tannins by the tree,

whatever other functions it might serve, perhaps acts to reduce attack by Lepidoptera larvae.

It seems, however, that the effect of leaf tannins on larval growth does not at present adequately account for the apparent magnitude of the selection pressure for early larval feeding, resulting in 90% mortality of 1st instar larvae (see Introduction). It seems that the concentration of tannins in oak leaves does not increase sufficiently rapidly in late May and June to give rise to a pronounced effect in the early stages of its production. It is not at present possible, though, to assess the long-term effect of tannins on the larvae, since the relevant data are scanty and since many of the deductions from them verge on speculation. It is possible that tannins have a much greater (or lesser) effect than can be deduced from the present data. It is also quite possible that components other than tannins in oak leaves may be influencing the optimum feeding periods for Lepidoptera larvae. Variation in the content of micronutrients including, perhaps, gibberellins (see p. 46) might be significant. It was not possible in the present work to investigate the influence of micronutrients on larval growth but, until this has been carried out, together with much further investigation of the effects of tannins, conclusions about the ecological situation cannot be made with any certainty.

Appendix 1: Larval Feeding Periods of Lepidoptera Species on Oak Trees in Britain

Data obtained from Stovin (1944), South (1961), Allan (1949), Scorer (1913), Ford (1949), Kettlewell,H.B.D. (personal communication) and Gradwell, G.R. (personal communication). Scientific names are as in South (1961) and Ford (1949).

(+ = larval feeding recorded. h = larval hibernation)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family NOTODONTIDAE													
Stauropus fagi							+	+	+				
Drymonia dodonaea							+	+	+				Night feeder
Chaonia ruficornis						+	+	+					Prefers isolated trees
Notodonta trepida						+	+	+					
Lophopteryx capucina							+	+	+	+			
Phalera bucephala								+	+				
Family THYATIRIDAE													
Tethea duplaris								+	+	+			Night feeder
Asphalia diluta					+	+							Night feeder
Polyploca ridens					+	+	+						Night feeder
Tethea fluctuosa								+	+				Night feeder
Family DREPANIDAE													
Drepana binaria						+			?	+			

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family LYMANTRIIDAE													
Orgyia antiqua					+	+	+	+					
Orgyia recens	h	h	h	+	+		+	h	h	h	h	h	
Dasychira pudibunda							+	+	+				
Euppoctis similis	h	h	h	+	+	+		+	h	h	h	h	
Lymantria dispar				+	+								
Lymantria monacha				+	+	+	+						
Euproctis chrysorrhea	h	h	h	+	+	+		+	+	h	h	h	
Family NOCTUIDAE													
Graphiphora augur	+	+	+	+	+		+	+	+	+	+	+	Feeds on low plants in autumn.
Mesogona acetosellae	?	?	?	?	?	?	?	?	?	?	?	?	
Mamestra brassicae							+	+	+	+			
Polia nebulosa	+	+	+	+	+			+	+	+	+	+	Feeds on low plants in autumn
Hadena thalassina								+	+				
Hadena contigua								+	+				
Orthosia gothica				+	+	+							
Orthosia miniosa					+	+							
Orthosia cruda				+	+	+							
Orthosia stabilis				+	+	+	+						
Orthosia incerta					+	+	+						
Orthosia munda				+	+	+							
Brachionycha sphinx					+	+							
Lithophane socia					+	+	+						
Lithophane ornitopus				+	+	+							
Xylena exsoleta				+	+								
Griposia aprilina			+	+	+	+							

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family NOCTUIDAE (continued)													
Dryobotodes eremita			+	+	+	+							
Eupsilia transversa					+	+							Cannibal
Jodea croceago					+	+							
Dasycampa rubiginea					+	+							
Agrochola macilenta				+	+	+							
Anchoscelis helvola				+	+								
Anchoscelis litura				+	+	+							Night feeder
Conistra erythrocephala				+	+								Completes growth on low plants.
Conistra vaccinii					+	+							Ditto
Conistra ligula				+	+	+							Ditto
Apatele psi								+	+				
Moma alpium							+	+					
Apatele leporina							+	+	+				
Apatele aceris								+	+				
Apatele tridens								+	+	+			
Apatele auricoma						+	+		+				
Apatele alni							+	+	+				
Amphipyra pyramidea				+	+	+							
Dicycla oo				+	+	+							
Cosmia pyralina				+	+								
Cosmia trapezina				+	+	+							Cannibal
Bena fagana								+	+				
Pseudoips prasinana	h	h	h	+	+	+		+	+	+	h	h	Hibernates when small
Nycteola revayana						+	+						
Catocala sponsa					+	+							
Catocala promissa				+	+	+							Night feeder
Minucia lunaris							+	+					On stool oak
Colocasia coryli					+	+		+					Night feeder
Catephia alchymista								+					

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family NOCTUIDAE (continued)													
Paracolax derivalis	h	h	h	+	+	+		+	+	+	h	h	Hibernates when small; apparently eats dead oak leaves.
Herminia barbalis	h	h	h	+	+			+	+	+	h	h	Ditto
Zanclognatha nemoralis								+	+				
Enargia paleacea					+	+							
Amathes triangulum	+	+	+	+	+			+	+	+	+	+	Night feeder
Lampra fimbriata	+	+	+	+	+				+	+	+	+	Night feeder; feeds on low plants in autumn.
Family LASIOCAMPIDAE													
Malacosoma neustria				+	+	+							Gregarious
Trichiura crataegi				+	+	+							
Poecilocampa populi				+	+	+							Night feeder
Family ARCTIIDAE													
Nola strigula	h	h	h	+	+	+		+	+	+	h	h	
Celama confusalis							+	+					
Miltochrista miniata	+	+	+	+	+			+	+	+	+	+	Apparently eats dead oak leaves.

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family GEOMETRIDAE													
Alsophila aescularia				+	+	+							
Comibaena putulata	h	h	h	+	+	+	h	h	h	h	h	h	
Hemithea aestivaria	h	h	h	+	+	+		h	h	h	h	h	
Jodis lactearia								+	+				
Cosymbia porata						+	+		+	+			
Cosymbia punctaria						+	+		+				
Cosymbia pendularia						+	+	+	+				
Electrophaes corylata							+	+	+				
Chlorociysta siterata						+	+	+					
Chlorociysta miata						+	+	+	+				
Eupithecia irriguata					+	+							
Eupithecia abbreviata						+	+						
Eupithecia dodoneata						+	+						Eats young leaves only
Eupithecia exiguata									+	+			
Oporinia autumnata					+	+							
Oporinia dilutata				+	+	+							
Operophtera brumata				+	+	+							
Operophtera fagata					+	+							
Anagoga pulveraria						+	+	+					
Campaea margaritata	+	+	+	+	+				+	+	+	+	In winter can eat closed buds and bark of twigs.
Ennomos quercinaria				+	+	+	+						
Deuteronomos erosaria					+	+	+						
Selenia lunaria							+	+	+				
Selenia tetralunaria						+	+		+	+			
Gonodontis bidentata							+	+	+	+			Grows very slowly
Colotois pennaria				+	+	+							
Plagodis dolabraria							+	+	+				
Erranis leucophaearia				+	+								
Erannis aurantiaria				+	+	+							

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family GEOMETRIDAE (continued)													
Erannis marginaria				+	+								
Erannis defoliaria			+	+	+	+							
Phigalia pilosaria				+	+	+							
Apocheima hispidaria					+	+							
Biston strataria					+	+	+						
Biston betularia							+	+	+				
Boarmia roberaria	h	h	h	+	+			+	+	+	h	h	
Pseudoboarmia punctinalis	+	+	+	+	+	+	+	+	+	+	+	+	
Ectropis consonaria						+	+						Night feeder
Ectropis extersaria							+	+					
Theria rupicapraria				+	+								
Alcis repandata	+	+	+	+			+	+	+	+	+	+	Feeds on low plants after August
Deileptenia ribeata	+	+	+	+	+	+		+	+	+	+	+	
Epione repandaria				+	+	+							
Lycia hirtaria					+	+	+						Night feeder
Selenia bilunaria					+	+		+	+				
Ectropis biundulata					+	+		+	+				
Family LIMACODIDAE													
Apoda avellana							+	+	+				
Heterogenea asella								+	+	+			
Family LYCAENIDAE													
Thecla quercus				+	+	+							
Family NYMPHALIDAE													
Apatura iris	+	+	+	+	+	+		+	+	+	+	+	Night feeder

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family PHYCITIDAE													
Phycita spissicella	+	+	+	+	+				+	+	+	+	
Niphopteryx similella							+	+					
Cryptoblabes bistriga									+	+			
Acrobasis consociella	+	+	+	+	+	+			+	+	+	+	
Acrobasis sodalella					+	+							
Acrobasis tumidella					+	+							
Acrobasis tumidana	+	+	+	+	+	+			+	+	+	+	
Family PSYCHIDAE													
Sterrhopteryx hirsutella	+	+	+	+	+			+	+	+	+	+	Larval stage lasts for 2 years.
Family TORTRICIDAE													
Batodes angustiorana					+								
Capua gretiana	+	+	+	+	+			+	+	+	+	+	
Cacoecia podana					+	+							
Cacoecia crataegana					+	+							
Cacoecia xylosteana					+								
Cacoecia sorbiana (= hebenstritella)					+	+							
Cacoecia lecheana					+								
Pandemis corylana					+	+							
Pandemis ribeana (= cerasana)	+	+	+	+	+				+	+	+	+	
Tortrix loeflingiana					+								
Tortrix viridana					+								
Tortrix semialbana					+	+							
Tortrix musculana	h	h	h					+	+	h	h	h	
Tortricodes tortricella	h				+	+	h	h	h	h	h	h	
Peronea fissurana					+			+					
Peronea literana					+	+							

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Family EUCOSMIDAE													
Ancylis nutterbacheriana	h	h	h				+	+	+	h	h	h	
Gypsonoma dealbana					+	+							Overwinters as larva
Eucosma corticana (=isertana)					+	+							
Lobesia permixtana							+	+					
Argyroploce pomedaxana					+	+							
Argyroploce profundana						+							
Pammene nitidana							+	+	+				
Pammene germarana	?	?	?	?	?	?	?	?	?	?	?	?	
Pammene splendidulana						+	+						
Pammene fimbriana						+	+	+					Feeds on oak galls
Pammene argyrana						+	+	+					Ditto
Pammene gallicolana									+	+			Ditto
Pammene juliana								+	+	+			Feeds on acorns
Family GELECHIIDAE													
Stenolechia gemmella						+							Feeds on oak shoots
Telphusia humeralis					+	+							
Telphusia triparella								+	+				
Telphusia luculella									+				
Chelaria gibosella					+	+							
Family COSMOPTERYGIDAE													
Mompha stephensi	?	?	?	?	?	?	?	?	?	?	?	?	Feeds on living oak

Appendix 1 (continued)

Species	J'	F	M	A	M	J	J	A	S	O	N	D	Comments
Family OECOPHORIDAE													
Dasycera oliviella	+	+	+	+								+	Feeds on decayed wood
Schiffermuelleria grandis	+	+	+									+	Ditto
Borkhausenia panzerella	+	+	+	+			+	+	+	+	+	+	Feeds on dead oak leaves
Chimabache (= Diurnea) fagella						+	+	+	+	+			
Chimabache phryganella						+	+						
Carcina quercana					+	+							
Family HELIOZELIDAE													
Heliozela sericiella						+	+						
Heliozela stanneela						+	+						
Family HYPONOMEUTIDAE													
Argyresthia glaucinella			+	+									Feeds on oak bark
Family COLEOPHORIDAE													
Coleophora lutipennella	+	+	+	+	+				+	+	+	+	Bores holes into parenchyma
Coleophora flavipennella	+	+	+	+	+				+	+	+	+	Ditto
Coleophora ardeaepennella	+	+	+	+	+	+			+	+	+	+	Ditto
Coleophora palliatella	+	+	+	+	+				+	+	+	+	Ditto
Coleophora currucipennella	+	+	+	+	+				+	+	+	+	Ditto
Family GRACILARIIDAE													
Lithocolletis roboris							+		+	+			Leaf miner
Lithocolletis cramerella							+		+	+			Ditto
Lithocolletis heegeriella							+		+	+			Ditto
Lithocolletis messaniella			+	+			+		+	+			Ditto
Lithocolletis quercifoliella							+		+	+			Ditto

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Lithocolletis distentella							+		+	+			Leaf miner
Lithocolletis lautella							+		+	+			Ditto
Lithocolletis amyotella							+		+	+			Ditto
Lithocolletis hortella							+		+	+			Ditto
Acroceroops brongniardella						+	+						Ditto
Gracillaria sulphurella						+	+						Ditto
Gracillaria alchimiella							+		+				Ditto
Family PLUTELLIDAE													
Ypsolophus lucella					+	+							
Ypsolphus alpella						+							
Ypsolophus sylvella						+							
Ypsolophus costella					+	+							
Ypsolophus radiatella					+	+							
Family LYONETIIDAE													
Tischeria complanella									+	+			
Tischeria dodonaea									+	+			
Bucculatrix ulmella							+		+	+			Leaf miner
Family LAMPRONIIDAE													
Lampronia oehlmanniella	+	+	+					+	+	+	+	+	Mines when young; then feeds on dead leaves.
Family NEPTICULIDAE													
Nepticula atricapitella							+		+	+			Leaf miner
Nepticula ruficaptitella							+		+	+			Ditto
Nepticula basiguthtella							+		+	+			Ditto

Appendix 1 (continued)

Species	J	F	M	A	M	J	J	A	S	O	N	D	Comments
Nepticula quinquella										+	+		Leaf miner; can be found in fallen leaves.
Nepticula subbimaculella									+	+			Leaf miner
Nepticula albifasciella								+					Ditto
Family ERIOCRANIIDAE													
Mnemonica subpurpurella						+							Leaf miner
Family COSSIDAE													

Larvae of the species Cossus cossus and Zenzera pyrina feed on the solid wood of various species of tree, including oak, and take 2-4 years to complete their growth.

Family SESSIIDAE

Several species belonging to this family, such as Aegeria vespiformis, feed as larvae under the bark of many trees, including oak, and, like larvae of Cossidae spp., take several years to attain complete growth.

Appendix 2: Leaf Length of Six Oak Trees on May 6th, 1966

On May 6th, 1966, the longest leaf in each of 20 leaf clusters from each of the six study oak trees was measured. In all cases, leaf clusters were collected at random from the lower branches on the south-facing sides of the trees. The leaf lengths, together with the means were as follows:-

Tree:	M	1	5	8	9	15
Leaf lengths: (cm.)	6.0	2.5	3.7	3.8	2.5	2.2
	7.1	2.4	3.4	3.8	3.6	2.4
	4.9	2.3	2.8	2.7	3.4	2.5
	3.4	2.8	3.8	3.9	3.3	2.5
	4.6	1.7	2.0	4.1	2.5	1.6
	7.2	1.7	3.0	3.7	2.3	2.5
	4.8	4.5	5.2	3.8	2.8	2.0
	6.3	2.6	4.3	3.6	3.2	1.8
	5.2	3.0	2.4	3.4	2.9	1.9
	5.5	2.2	3.0	2.6	2.6	2.1
	6.4	1.6	4.8	3.5	2.9	1.7
	6.5	2.5	2.9	3.5	3.7	2.0
	5.6	2.6	2.5	4.2	2.7	1.8
	5.7	2.2	4.2	2.4	2.6	2.0
	6.2	3.1	3.4	2.6	2.2	2.7
	4.9	2.3	2.7	3.8	2.6	2.3
	4.9	2.5	3.0	3.5	3.5	2.4
	6.0	1.9	3.5	3.3	2.8	1.8
	4.3	1.8	3.1	4.3	2.5	2.8
	4.3	1.9	3.0	3.8	3.3	2.0
Mean Leaf Length:	5.5	2.4	3.3	3.5	2.9	2.2
S.E.M.	± 0.2	± 0.1	± 0.2	± 0.1	± 0.1	± 0.1

Appendix 3: Census of Lepidoptera larvae on tree M in spring 1964

On four dates between May 12th and June 1st, 1964, 25 leaf clusters were collected, as far as possible at random, from both the upper and the lower branches of tree M and the number of larvae of each species on each sample counted. Mr. G.R. Gradwell kindly assisted with identification of the larvae. The results of the census were as follows:

Species	May 12th		May 18th		May 25th		June 1st	
	U	L	U	L	U	L	U	L
Orthosia cruda	1							
Cosmia trapezina						2		1
Eupithecia spp.[1]			1					1
Oporinia dilutata	1							
Operophtera brumata	16	7	8	11	2	3		
Erannis leucophaearia	1	1	2		1	1		
Erannis aurantiaria				1				
Erannis marginaria	1					2		
Erannis defoliaria	1							
Phigalia pilosaria				1				
Cacoecia sorbiana (= hebenstritella)				1			1	
Cacoecia lecheana	1	3	1	3	1p			
Pandemis corylana								1
Pandemis cerasana		1				1		
Tortrix loeflingiana	2	2	2	1		1	1	
Tortrix viridana	9	2	1	10	1	5	2p	1, 2p
Eucosma corticana (= isertana)	3	1	7	2	6	7	3	2
Ypsolophus spp.[2]	3	3	1	1	2	5	1	1

(U = Upper leaf sample; L = Lower leaf sample; p = pupa; [1] mostly E. abbreviata; [2] comprising the species Y. costella, Y. sylvella and Y. alpella)

Appendix 3(continued)

Census of Lepidoptera larvae dropping from tree M in spring 1964

Two metal trays, each ½ sq. m. in area, were placed beneath the canopy of tree M, within 10 ft. of the trunks. The trays contained sufficient water to drown all larvae falling into them. The number of larvae which had dropped into the trays was counted at intervals between May 22nd and June 16th, 1964. The results were as follows:

Species	Number of larvae falling into the two trays							
	22/5	25/5	28/5	1/6	5/6	8/6	11/6	16/6
Orthosia cruda			1		1			
Conistra vaccinii		1						
Cosmia trapezina		1	2	2				
Eupithecia spp.[1]				2	2 7	1		1
Operophtera brumata	63	90	21	1				
Erannis leucophaearia		2	1	2				
Erannis aurantiaria	1	1	5	1				
Erannis marginaria			1					
Erannis defoliaria		2	1					
Phycita spissicella							1	
Cacoecia xylosteana								1
Pandemis corylana			1	1				
Tortrix loeflingiana		1	3					
Tortrix viridana	1	3	2p					
Eucosma corticana (= isertana)		1	15	39	39	6	6	2
Carcina quercana								1
Lithocolletic spp.	3	3						
Ypsolophus spp.[2]			2	6	2	1	2	

(p = pupa; [1]mostly E. abbreviata; [2]comprising the species Y. costella, Y. sylvella and Y. alpella).

Appendix 4: Comparison of weight increase and survival of Winter Moth Larvae on Oak Leaves of Different Ages (Chapter 1)

Table 16. Fresh weights (mg.) of larvae and pupae fed on May 16th oak leaves, together with mean weights.

(* = pupa; d = dead; + = Adult emerged; - = Adult failed to emerge)

Larva	29/5	4/6	8/6	11/6	15/6	18/6	26/6	Adult Emergence
L 1	4.7	13.8	45.6	34.4	29.0*	29.0*	28.9*	+
L 2	4.2	19.3	53.2	39.6	31.3*	32.3*	32.1*	+
L 3	6.6	11.7	45.9	47.8	34.3	32.3*	30.4*	-
L 4	9.0	31.6[1]						
L 5	5.9	22.7	56.5	41.7	38.9*	38.9*	38.7*	-
L 6	4.7	12.9	45.1	37.6	28.1	25.3*	d	
L 7	3.9	12.0	40.4	43.1	30.0	27.9*	27.7*	+
L 8	5.8	14.8	52.1	47.0	34.1	33.5*	32.5*	-
L 9	7.0	20.1	46.5	35.8	32.8*	32.3*	31.9*	-
L 10	3.1	18.8	47.9	50.9	38.5*	31.6*	25.3*	-
L 11	7.8	19.0	31.2	31.5	23.6	22.7*	22.5*	+
L 12	3.7	14.3	38.5	45.0	33.0	31.3*	30.4*	-
L 13	9.5	21.0	58.5	40.2	37.0*	36.4*	36.0*	-
L 14	5.0	13.3	45.2	38.0	31.3*	29.9*	29.6*	-
L 15	5.7	24.1	69.5	50.4	42.2*	41.8*	41.5*	+
L 16	10.5	13.8	32.3	29.2	23.8	22.4*	22.1*	+
L 17	7.2	17.1	42.3	31.9	28.2*	28.1*	27.9*	+
L 18	6.4	16.3	50.7	34.8	32.8*	32.2*	31.6*	-
L 19	11.8	24.6[1]						
L 20	3.1	10.7	32.6	44.2	32.1	29.7	d	
L 21	7.8	17.5	38.7	40.7	31.9*	31.2*	30.8*	-
L 22	6.6	18.2	56.3	39.3	36.1*	35.7*	35.6*	+
L 23	11.9	29.0	26.8	25.2*	23.7*	23.5*	23.0*	-
L 24	7.1	23.6	54.5	45.6	35.6*	34.9*	34.4*	+
L 25	4.6	14.3	41.5	36.2	28.6*	27.5*	26.8*	+
Mean	6.6	18.2	45.7	39.6	32.0	30.9	30.5	

[1] Removed for histological examination.

Appendix 4 (continued)

Table 17. Fresh weights (mg.) of larvae and pupae fed on oak leaves from May 28th onwards, together with means.

(* = pupa; d = dead; - = adult failed to emerge)

Larva	29/5	4/6	8/6	11/6	15/6	18/6	26/6	Adult Emergence
L 26	7.9	13.7	19.3	24.5	12.3	8.6	d	
L 27	5.2	20.5	24.9	9.8	8.1*	8.3*	7.9*	-
L 28	7.0	3.6	d					
L 29	6.0	25.2	17.8	10.8	10.1*	10.0*	9.7*	-
L 30	7.5	25.1	16.5	12.0	11.1*	11.1*	11.0*	-
L 31	4.7	11.7	20.1	12.1	7.6	6.4	d	
L 32	6.7	13.4	19.5	10.1	7.8*	5.9*	d	
L 33	4.3	11.1	17.9	21.6	9.0	8.1*	7.8*	-
L 34	7.2	10.8	18.1	22.7	13.9	13.5	d	
L 35	2.8	5.1	d					
L 36	6.6	17.4	25.2	13.9	10.4*	10.2*	9.9*	-
L 37	4.8	25.3	19.3	9.6	d			
L 38	9.4	22.2	15.4	10.6	9.8*	9.7*	9.7*	-
L 39	4.2	10.3	12.7	14.0	d			
L 40	6.0	14.0	20.4	10.6	9.4*	6.7*	d	
L 41	8.5	27.8	16.2	14.0	13.3*	13.3*	13.1*	-
L 42	11.7	31.2[1]						
L 43	4.7	17.8	20.1	18.9	9.6	8.7*	8.5*	-
L 44	3.6	11.1	18.8	19.4	10.5	9.4*	8.6*	-
L 45	3.4	8.9	16.0	16.2	12.0	7.3	d	
L 46	12.3	22.6[1]						
L 47	5.2	12.1	21.2	15.6	8.1	7.3	d	
L 48	9.3	14.6	18.3	18.7	15.1	12.7	d	
L 49	4.3	6.6	11.6	13.3	7.1	6.6	d	
L 50	8.9	16.1	20.5	11.0	8.7*	8.8*	8.8*	-
Means:	6.5	15.9	18.5	14.7	10.2	9.5	9.5	

[1] Removed for histological examination.

Appendix 4 (continued)

Table 18. Statistical data for comparison of larval growth on oak leaves of two different ages

Diet:	May 16th leaves	May 28th et seq. leaves	May 16th leaves	May 28th et seq. leaves
Date:	June 8th	June 8th	June 26th	June 26th
$\sum x$	1051.8	389.8	639.7	95.0
n	23	21	21	10
$\bar{x}$	45.7	18.6	30.5	9.5
$\sum x^2$	50,341.1	7,451.0	20,005.5	925.7
s^2	101.9	10.8	26.0	2.58
s	10.1	3.3	5.1	1.6
5% Confidence limits	±4.1	± 1.4	± 2.2	± 1.0

The data for June 26th (mean pupal weights on the two diets) were compared by the Student's t-test (Bailey, 1959). The value of t, corresponding to 29 degrees of freedom, was found to be 12.63. The difference between the pupal weights is therefore significant ($P < 0.001$).

Appendix 5: Toughness values[1] and lengths of lower sun oak leaves from tree 9 on two dates in 1966 (Chapter 2)

May 19th Leaves		June 10th Leaves	
Length (cm.)	Toughness[1]	Length (cm.)	Toughness[1]
9.0	52.9	9.3	808.5
8.1	68.3	7.3	728.6
6.9	90.2	8.2	827.7
8.2	57.8	8.1	779.8
5.9	219.4	7.0	682.5
7.1	54.7	6.6	539.6
6.0	39.1	9.9	798.6
7.8	92.1	9.5	523.2
6.9	114.1	7.7	766.6
7.9	36.9	11.6	815.9
7.7	23.6	10.0	784.8
7.0	231.0	7.2	727.9
7.7	46.6	8.1	737.0
5.3	51.6	7.0	789.5
7.2	126.2	7.7	641.2
8.0	45.8	8.8	580.1
8.2	39.4	7.3	618.5
7.3	84.7	7.3	500.3
7.0	132.4	8.1	647.6
6.7	89.4	9.5	643.5

[1] Estimated as the weight of sand required to punch a leaf disc of standard area from the leaf, using the device described in Chapter 2 (p. 26).

Appendix 6: Effect of leaf toughness on weight increase of winter moth larvae (Chapter 2, p. 30)

Table 19. Fresh weights (mg.) (together with derived statistical data) of winter moth larvae and pupae fed on diet containing May 13th oak leaf powder.

	1965					
Larva	5/6	7/6	9/6	11/6	14/6	17/6
(M 1)	35.2	30.9	par.			
M 2	14.7	31.4	39.2	42.2	21.6	17.9
M 3	28.5	36.9	38.1	24.4	18.7	16.8*
(M 4)	30.5	30.9	32.0	par.		
M 5	34.2	40.1	33.3	22.5	21.3	d
M 6	20.3	30.5	33.5	24.3	19.7	16.7*
(M 7)	39.2	34.9	27.8	par.		
M 8	20.5	33.3	43.2	31.7	24.1	19.2*
M 9	37.5	47.7	42.8	24.2	17.7	d
M 10	27.8	40.2	42.0	27.5	19.3	d
(M 11)	15.6	15.5	8.0	d		
M 12	10.7	13.3	11.8	d		
M 13	22.4	35.9	44.0	33.9	d	
M 14	20.1	28.1	33.7	26.0	d	
M 15	31.2	38.0	31.6	27.6	22.5	20.4*
(M 16)	29.3	14.5	par.			
(M 17)	23.4	22.8	20.0	17.8	d	
M 18	39.7	46.2	40.7	27.7	25.0	23.7*
M 19	22.9	25.2	19.3*	19.3*	18.8*	17.4*
M 20	17.2	26.5	32.0	20.8	16.2	d
n	14	14	14	13	11	7
$\sum x$	347.7	473.3	485.2	352.1	224.1	132.1
$\bar{x}$	24.8	33.8	34.7	27.1	20.4	18.9
s^2	74.2	81.3	86.8	36.9	7.4	6.3
S.E.M.	± 2.3	± 2.4	± 2.5	± 1.7	±0.8	± 1.0
5% Confidence Limits	± 4.6	± 4.8	± 5.0	± 3.4	± 1.6	± 1.9

Appendix 6 (continued)

Table 20. Fresh weights (mg.) (together with derived statistical data) of winter moth larvae and pupae fed on diet containing June 1st oak leaf powder.

	1965					
Larva	5/6	7/6	9/6	11/6	14/6	17/6
J 1	36.5	39.8	38.9	25.3	23.6	d
(J 2)	34.2	par.				
(J 3)	40.3	27.4	17.7	14.6	d	
(J 4)	31.7	30.9	23.0	par.		
(J 5)	33.2	18.9	10.5	d		
J 6	11.4	27.8	34.7	25.3	14.3	10.8
(J 7)	37.2	23.0	19.7	17.9*	17.5*	16.8*
(J 8)	35.1	20.3	18.0	16.4*	16.8*	16.8*
J 9	13.3	30.4	34.5	37.1	24.0	17.0
J 10	37.9	47.0	34.4	29.2	28.7	d
J 11	16.1	22.9	26.9	27.3	20.2	13.6
J 12	27.8	35.1	23.4	17.8	15.1	d
J 13	10.8	12.7	14.7	26.3	31.6	26.9
(J 14)	27.1	23.7	21.5	par.		
J 15	31.7	37.1	33.8	20.0	14.4	d[1]
(J 16)	36.3	27.1	24.4	22.4	19.8	17.7
(J 17)	37.0	25.3	17.7	16.6	d	
(J 18)	30.0	par.				
(J 19)	22.2	22.0	d			
J 20	27.7	28.0	d			
n	9	9	8	8	8	4
$\sum x$	213.2	280.8	241.3	208.3	171.9	68.3
$\bar{x}$	23.7	31.2	30.2	26.0	21.5	17.1
s^2	118.2	100.9	63.2	34.2	44.2	49.3
S.E.M.	± 3.6	± 3.2	± 2.8	± 2.1	± 2.4	± 3.5
5% Confidence Limits	± 7.2	± 6.3	± 5.6	± 4.1	± 4.7	± 7.0

par. = pupa of P. crassiuscula; d = dead; * = pupa; ( ) = larvae excluded from analysis owing to parasitism or failure to show a weight increase at the first re-weighing; [1] = death due to accident.

Appendix 7: Comparison of mean fresh weight increments (mg.) for winter moth larvae fed on two diets containing oak leaf powders

(Chapter 2, p. 26)

The individual fresh weight increments of the larvae on the May 13th and June 1st leaf diets were obtained from the data given in Appendix 6 (Tables 19 and 20) by subtracting the initial from the peak weights. Those larvae were excluded from analysis which were known to be parasitised or which failed to show a weight increase at the first re-weighing.

The fresh weight increments are presented in Table 1 (p. 35). Their means and other relevant statistical data are as follows.

	May 13th leaf diet	June 1st leaf diet
Mean Weight increment, $\bar{x}$ (mg. fresh weight)	12.3	11.6
Sample Size, n	14	9
Sum of increments, $\sum x$	171.5	104.5
Sum of squares, $\sum x^2$	2,849.53	1,843.65

An F test (Bailey, 1959) was first carried out to establish similarity of variance between the two means. For the May sample:

$$\text{Variance, } s^2 = \frac{1}{n-1}\left[\sum x^2 - \frac{1}{n}(\sum x)^2\right] = 57.59$$

For the June sample:

$$\text{Variance, } s^2 = 78.79$$

$$\text{Therefore, } F = \frac{78.79}{57.59} = 1.368$$

The F value for 5% significance with 8 and 13 degrees of freedom, given in the standard Biometrika Tables (Pearson and Hartley, 1962)

Appendix I (continued)

is 2.20. No significant difference can therefore be detected between the sample variances. It is therefore permissible to proceed with the Student's t-test for small samples (Bailey, 1959) as follows:

$$\text{Pooled variance } s^2 = \frac{1}{n_1 + n_2 - 2}\left[\sum_1 x^2 - \frac{(\sum_1 x)^2}{n_1} + \sum_2 x^2 - \frac{(\sum_2 x)^2}{n_2}\right]$$

$$= 65.66$$

$$\text{therefore } s = 8.11$$

$$\text{therefore } t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} = 0.202$$

The t value for 10% significance with 21 degrees of freedom, given in the standard table in Bailey (1959) is 1.721. From the available data, therefore, no significant difference can be detected between the fresh weight increments of the larvae on the two diets.

Appendix 8: Experiments to determine whether winter moth larvae possess an amylase enzyme

1. Salivary glands

The freshly dissected salivary glands from three batches of 80 early 5th instar winter moth larvae were homogenised separately in 5 ml. aliquots of distilled water (previously boiled to remove carbon dioxide and then cooled to 4°C in a refrigerator), using a chilled mortar and pestle. The salivary glands from each larva were placed, immediately after dissection, in a mortar maintained at a low temperature in a shallow bath, containing crushed ice. The homogenates were stored for one week in a deep-freeze (- 20°C) in stoppered vials. One of the homogenates was diluted with glycerol (to 10% v/v glycerol) as a preservative.

0.3 ml. samples of each of the above homogenates were transferred to separate test-tubes, to which were then added 2 ml. of 0.1 Molar phosphate buffer (Na_2HPO_4/NaH_2PO_4) at pH 7.0 and 1 ml. of soluble starch solution (1% aqueous). Control tubes, in which the homogenates had first been boiled briefly to inactivate any enzyme present, were prepared simultaneously. All six tubes were then covered with 'Parafilm' and incubated at 30°C in a water bath for 24 hours. 10 drops of a solution of iodine (0.2%) and potassium iodide (0.6%) in water were then added to each tube. All six solutions immediately turned deep blue. There was no apparent difference in the shade or intensity of the colour between the experimental and control tubes, indicating that there had been no appreciable amylase activity.

Appendix 8 (continued)

It was concluded that amylase activity is either absent or very weak in the salivary glands of the winter moth, since at least some activity would be expected at pH 7.0 from any enzyme present.

2. Mid-gut

The freshly dissected mid-guts of 75 5th instar winter moth larvae, which had been starved for 24 hours, were homogenised in glycine/sodium hydroxide buffer at pH 9.2, using a chilled mortar and pestle as described above. This pH value was selected as it had been found to be that prevailing in the winter moth mid-gut (Appendix 26, p. 248). After storage for one week in the deep-freeze (- 20°C), aliquots of 0.3 ml. of the homogenate were transferred to small test-tubes and incubated, alongside boiled controls, for 24 hours at 30°C, after addition of 0.5 ml. soluble starch solution (1% aqueous).

5 drops of the iodine/potassium iodide solution (as above) were then added to each tube. The solutions all turned deep blue, with no apparent difference in colour shade or intensity between experimental and control tubes.

A second experiment was carried out with five 5th instar winter moth larvae which had not been starved. A homogenate of the five fresh mid-guts in 0.5 ml. of distilled water was incubated with 0.5 ml. of 1% starch solution at 30°C, along with a boiled control. On addition of the iodine solution, following incubation, there was again no sign of any starch degradation.

A third experiment was carried out on a much smaller scale. A

Appendix 8 (continued)

5th instar winter moth larva was dissected immediately after removal from its diet of hawthorn leaves. The larva was dissected on a chilled microscope slide and the mid-gut homogenised with two drops of distilled water. A series of droplets of this suspension was at once arranged in a row along the centre of a separate microscope slide, which had previously been coated with candle wax. Along one side of this row of droplets was arranged a series of droplets of 1% starch solution. On the other side was arranged a row of droplets of the iodine/potassium iodide solution. Incubation was started by gently blowing the starch droplets into the corresponding droplets of the central row, containing the gut suspension. At intervals after this, iodine droplets were rolled (by gentle blowing) into the central reaction drop, to follow the course of the hydrolysis. This technique has been used successfully by Professor G.C. Varley (personal communication) to demonstrate to undergraduate classes the activity of amylase in salivary glands of cockroaches. In the experiments with cockroaches, the starch has usually disappeared within a few minutes after the mixing of the droplets. However, six such experiments with six individual winter moth larvae showed no trace of starch degradation after an hour.

It was concluded that amylase activity is absent or very weak in the mid-gut of winter moth larvae.

Appendix 9: Qualitative Analysis of Oak Leaf Sugars by Paper Chromatography

Lower sun oak leaves from tree M were collected on July 7th, 1964, and 50 gm. (fresh weight) was homogenised in a litre of 96% ethanol in the laboratory, soon after collection. 500 ml. of this extract were evaporated to dryness under reduced pressure and then taken up into 20 ml. of distilled water. The resulting solution was centrifuged (4,000 r.p.m. for 20 minutes) and then de-ionised by shaking for one hour with 10 gm. of a mixed-bed ion exchange resin, made by mixing one part 'Amberlite IRC 50' with two parts 'Amberlite IRA 401' (British Drug Houses Ltd.), as recommended by Smith (1960).

One-way chromatograms were run on Whatman 3MM paper in ethyl acetate/pyridine/water (12 : 5: 4), using applications of 0.01 to 0.10 ml. of the concentrated de-ionised extract. 0.05 ml. applications of 0.5% aqueous solutions of D-Glucose, D-Fructose, D-Galactose and D-Mannose, as well as of 0.7% solutions of sucrose and maltose, were run in parallel as standards, together with an aqueous solution containing all these sugars at the same concentrations. The lower edges of the chromatograms were serrated to allow the descending solvent to drip off, so extending the R_f values of the sugars and thus improving resolution.

After the chromatograms had been run for 15 hours, they were dried at room temperature and developed with either of the following two reagents (Smith, 1960).

a) Aniline/diphenylamine reagent

Aniline, 1% (1 ml.), plus diphenylamine (1%) in acetone	10 vol.
Phosphoric acid, 85%	1 vol.

After spraying, with the reagent, chromatograms are heated at 95° - 100°C for a few minutes, when most sugars yield green, blue or brown colours.

b) Silver nitrate reagent

1) Silver nitrate, saturated solution in water 0.1 vol.

Acetone 20 vol.

2) Sodium hydroxide, 0.5% in ethanol

The chromatograms are first sprayed with the silver reagent and the acetone then blown off with a current of air. When dry, the chromatograms are sprayed with the alkali and again the solvent is blown off. Within ten minutes at room temperature, reducing sugars react to give dark brown or black spots.

The R_G values of the oak leaf sugars and standards are given in Table 2 (p. 40(b)), and in Figure 12 (p. 40(a)). R_G values are calculated as R_f values relative to R_f of D-Glucose = 100. As discussed in Chapter 3 (p. 40), the only sugars detected in the oak leaf extract were D-Glucose, D-Fructose and sucrose.

Appendix 10: Quantitative Estimation of the Sugars in Fresh Oak Leaves

1. Extraction of sugars from leaf material

Leaves were collected at intervals during the 1964 season from the upper sun and lower shade branches of tree M, as described in Chapter 1 (p. 17). After being cut from the twigs in the laboratory, the weighed samples of leaves were transferred to boiling 96% ethanol for 20 minutes and then immediately homogenised in the same solvent, using an M.S.E. 'Atomix' blender. The homogenates were filtered through a Buchner funnel and the residues washed several times with fresh hot solvent. The combined filtrates were stored under nitrogen in sealed stoppered bottles at 4°C in the dark to await analysis. A little benzoic acid was added to each extract to inhibit any possible microbial activity during storage. Benzoic acid has no effect on the sugar analysis (see p.185(a)).

As mentioned in Chapter 1, further leaf samples from each collection were dried to constant weight at about 80°C, to determine the moisture content of the leaves.

2. Analytical methods

A wide variety of analytical methods for sugars has been described (e.g. Peach and Tracey, 1955; Somogyi, 1952; Morris, 1948; Horwitz, 1955). However, many of these involve reagents of low stability or are inconveniently complex. Two methods were selected for possible use in the present research, both of which are convenient and apparently suitable for fairly rapid analysis of many samples. These

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methods were the ∝-naphthol/sulphuric acid method (Devor, 1950) and the ferricyanide method originally described by Hagedorn and Jensen (1923). No information could be obtained as to the application of these methods to ethanolic leaf extracts. A number of preliminary experiments were therefore carried out, which resulted in minor modifications to both methods.

3. ∝-naphthol method

This method is essentially Devor's (1950) modification of the Molisch test for carbohydrates. Free or combined carbohydrates yield furfural derivatives on dehydration by concentrated sulphuric acid; these derivations can be condensed with various reagents (such as phenols, amines and thiol compounds) to give coloured compounds. Hexoses yield ω-hydroxymethylfufural and pentoses yield furfural. In the Devor method, these furfurals are condensed with ∝-naphthol, yielding a red coloured compound, solutions of which obey Beer's law.

Reagent:

0.4% ∝-naphthol in concentrated sulphuric acid. This solution is made up at least 8 hours before use and can be stored for several days in the dark.

Procedure:

To 1 ml. of the solution containing the carbohydrate, 2 ml. of reagent are added and mixed well. The solution is then heated at 100°C for 10 minutes and the colour intensity read at 555 mμ.

It was intended to estimate total sugar by this method, and to estimate reducing sugars by the ferricyanide method, yielding the

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concentration of sucrose by difference.

4. Application of α-naphthol method to ethanolic leaf extracts

Examination in a Beckan spectrophotometer of the coloured product resulting from the tr atment by the above method of 1 ml. of a solution containing 50 µg D-Glucose revealed a well-defined absorphtion peak at 565 mµ, with a subsidiary peak at 485 mµ.

The intensities of the colours obtained from a series of glucose samples, from 10-100 µg/ml., were determined at 565 mµ, using a Unicam manual spectrophotometer. The colour intensities of the same solutions were also determined in an Eel colorimeter, using a green filter. In both cases, the readings were found to bear a linear relationship to glucose concentration over the whole range of samples. For work on leaf extracts, therefore, it was decided touse the Eel colorimeter, since it is much more convenient when many samples are to be analysed, while it apparently gives comparable accuracy to the spectrophotometer. It has a further advantage over the spectrophotometer: the absorption peaks of the coloured furfural products given by fructose and glucose differ slightly (Devot, 1950), so that measurement at 565 mµ with a spectrophotometer of extracts containing both these sugars might lead to spurious results. Both absorption peaks are, however, included in the wave length band of the Eel colorimeter, using a green filter.

Several minor disadvantages were encountered with the α-naphthol method. Small air bubbles tended to appear in the samples after pouring into the colorimeter cell, greatly increasing the reading. Owing to the high viscosity of the acid solution, these bubbles usually took 10

Appendix 10 (continued)

minutes to clear before each reading could be made. Concentrated sulphuric acid is inconvenient to work with, in comparison with other reagents, and thorough mixing with sugar samples is more tedious. Water loss during the heating at 100°C in a water bath was prevented by placing glass marbles on the mouth of each tube.

5. Alcohol effect

Preliminary attempts to apply the α-naphthol method directly to aliquots of the 96% ethanol leaf extracts, diluted 50 times with distilled water, showed that alcohol interferes with the determination. 20 ml. aliquots of the 26th May Lower Leaf and 5th June Lower Leaf extracts in 96% ethanol were diluted fifty times to 1 litre with distilled water, and 1 ml. samples then analysed. Control samples, from 96% industrial ethanol (used for the leaf extractions) and 96% absolute ethanol, both diluted 50 times with water, were also analysed. Further 20 ml. aliquots of the two above-mentioned leaf extracts were vacuum-distilled to dryness at 60°C to remove ethanol and then taken up into distilled water by shaking the residue into suspension in an ultrasonorator and then centrifuging to remove insoluble material, before dilution to 1 litre. 1 ml. samples of these solutions were also analysed. The optical densities of the treated samples, read on an Eel colorimeter with a green filter, are shown below. The readings represent the average of two determinations in each case.

Sample	Optical Density (Eel Colorimeter)
26/May/Lower; diluted 50 times; undistilled;	3.05
" " distilled;	2.01

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Sample	Optical Density (Eel Colorimeter)
5/June/Lower; diluted 50 times; undistilled;	2.21
" " distilled;	1.22
96% ethanol (absolute); diluted 50 times; undistilled;	0.93
96% ethanol (industrial); diluted 50 times; undstilled;	0.92

The results show that the presence of ethanol in the sample has a considerable, but reproducible, effect on the final reading. However, distillation of the sample prior to analysis successfully removes this effect. Pure ethanol gives rise to the same effect as industrial alcohol, so that the effect is not due to impurities in the industrial alcohol.

6. Cellulose effect

Spurious results were obtained after filtering small samples before analysis and it was suspected that filtration through cellulose might be increasing the readings, owing to contamination of the filtrates by small amounts of carbohydrate material from the paper. This could be important if it was required to 'clear' extract samples from polymeric material, prior to analysis, by a precipit tion method.

To investigate this effect, 2 ml. aliquots of a standard sucrose solution (30 µg/ml.) were filtered through Whatman No. 42 paper discs and the papers washed through with 4 ml. of water. Control aliquots were similarly diluted but not filtered. 1 ml. samples from each aliquot were then analysed by the α-naphthol method and the optical

Appendix 10 (continued)

densities, read on the Eel colorimeter using a green filter, were as follows:

Sucrose solution, filtered	1.79 (average of 3 values)
Sucrose solution, unfiltered	0.85 (" "

Filtration of small samples therefore produces a considerable error in the reading. This means either that precipitation methods for 'clearing' solutions from polymeric material before analysis must be carried out with much larger aliquots, where the error from one filter paper is likely to be negligible, or that residues must be removed by centrifuging or glass wool filtration.

7. Ferricyanide method

Oxidation of reducing sugars by ferricyanide solution is the basis of the Hagedorn and Jensen titration method, commonly used for estimation of blood sugars (Hagedorn and Jensen, 1923). The colorimetric adaptation of this method (Folin, 1928; Fuller and Northcote, 1956) appeared suitable for estimating leaf sugars.

Reagents

a) 0.004 Molar potassium ferricyanide in 0.1 Normal sodium hydroxide.

b) 1 gm. ferric sulphate, 2 gm. sodium dodecyl sulphate and 50 ml. of syrupy phosphoric acid, all dissolved in 2.5 litres of distilled water.

Procedure

Add 1 ml. of solution (a) to 1 ml. of the sample

(Appendix 10 continued)

Heat for ten minutes in a boiling water bath

Cool and add 1 ml. of solution (b)

Mix well and read optical density of Prussian blue colour at 660 mμ

(Absorption shows a plateau in this region of the spectrum.)

8. Alcohol effect

A similar experiment to that with the α-naphthol method revealed that alcohol also interferes with the readings from the ferricyanide method. However, this interference could be successfully eliminated by vacuum distilling samples before analysis.

9. 'Clearing' with lead

The ferricyanide method detects all reducing materials in the sample, so that it is desirable, before analysis of reducing sugars, to remove polyphenols and other reducing substances of high molecular weight. The reducing substances in the extract will then consist very largely of sugars (Horwitz, 1955).

The method used to free extracts from polymeric material in the present work was based on that given by Horwitz (loc. cit.). After distillation of each 20 ml. extract aliquot to remove the alcohol (see above), the residue in the flask was shaken into solution or suspension in about 100 ml. distilled water, using an ultrasonorator. 5 ml. of saturated lead acetate solution was then added from a burette and mixed thoroughly with the distilled extract solution. 7 ml. of saturated aqueous sodium oxalate solution was then added from a second burette.

This quantity was found to be just more than sufficient to precipitate all the lead as the insoluble oxalate. The precipitate, carrying down polyphenols, chlorophyll, etc., was filtered off on a porous silica filter, using suction. The filtrate and washings were then made up to one litre, equivalent to 50-fold dilution of the original leaf extract. The final solutions were clear, colourless and free from lead.

To discover the effect of removing polymeric material from the extracts, an experiment was carried out with the 26/May/Lower leaf extracts, subsequently referred to as 26/5/L. Two 20 ml. aliquots of this extract were vacuum distilled to remove alcohol and then cleared with lead, as described above, before dilution to 1 litre with distilled water. Two 20 ml. aliquots from the extract were distilled and diluted to 1 litre without clearing. Two further 20 ml. aliquots were distilled after addition of 0.02 gm. sucrose and two more after addition of 0.02 gm. glucose. Of these last four aliquots, one containing extra sucrose and one containing extra glucose were cleared with lead and the remaining two aliquots retained as uncleared controls. All four were then diluted to 1 litre with water.

From each of two standard sugar solutions (1 gm./litre of sucrose and glucose, respectively, in 96% industrial ethanol) two 20 ml. aliquots were vacuum distilled. One of each pair was then cleared with lead and the other left uncleared, before all four aliquots were diluted to 1 litre with water. As a control, a 100 ml. sample of distilled water was treated with lead acetate and sodium oxalate in the

Appendix 10 (continued)

usual way and then diluted to 1 litre. Lastly, an alcoholic solution of benzoic acid (1 gm./litre) was also distilled and cleared with lead in the usual way before dilution to 1 litre with water.

All the above samples were then analysed by both the α-naphthol and ferricyanide methods, using 1 ml. aliquots in duplicate from each litre sample, along with 1 ml. aliquots of standard glucose solutions. The optical densities were read on an Eel colorimeter; a red filter was used for the samples treated by the ferricyanide method and a green filter for those treated by the α-naphthol method. Before estimation of the optical densities of the samples treated by the ferricyanide method, all solutions were diluted 10 times with distilled water. The results obtained from all the analyses are shown in Table 21 (p.185a).

The results show that clearing with lead reduces both sets of readings substantially but in a reproducible manner. It is clear that polymeric material in the extracts is estimated as sugar by both methods and must therefore be removed. Secondly, the experiment shows that addition of sucrose or glucose to the samples before distillation results in approximately the expected increase in the final readings. This is confirmed by the readings from the pure sugar solutions, showing that the analytical procedure used, including distillation and clearing with lead, produces reasonably accurate results by both methods.

The two final controls confirm that neither the clearing procedure nor the presence of benzoic acid (used as a preservative in the extracts) significantly affects the results.

A further finding from the experiment is that the ferricyanide

TABLE 21 Effect of clearing with lead on sugar analysis of leaf extracts by the α-naphthol and ferricyanide methods

Sample	O.D.[1] (α-naphthol)	Total Carbohydrate[2]	O.D.[1] (ferricyanide)[5]	Reducing Material[2]	Difference[2,3]
Glucose standard (10mg/l.)	0.9	10	0.97	10	
Glucose standard (20mg/l.)	2.0	20	2.01	20	
Glucose standard (40mg/l.)	3.85	40	3.80	40	
26/5/L., uncleared[4]	1.77	18.3	1.96	20.2	- 1.9
26/5/L., cleared[4]	1.10	11.5	0.81	8.2	3.3
26/5/L + 0.02g sucrose, cleared	2.97	30.8	0.81	8.2	22.6
" " , uncleared	3.69	38.3	1.97	20.2	18.1
" + 0.02g glucose, cleared	3.09	32.0	2.70	28.0	4.0
" " , uncleared	3.70	38.5	3.93	40.8	- 2.3
Sucrose (1g/l.ethanol), cleared	1.83	19	0.05	0	19
" " , uncleared	1.82	19	0.05	0	19
Glucose ("), cleared	1.85	19.3	1.92	19.9	- 0.6
" ("), uncleared	1.90	19.7	1.95	20.0	- 0.3
Distilled water, cleared	0.01	0	0.02	0	0
Benzoic acid solution, cleared	0.01	0	0.00	0	0

[1] Eel colorimeter; ~~red~~ green filter for α-naphthol, red filter for ferricyanide; [2] mg. glucose equivalent / litre. [3] Difference between estimations of total carbohydrate and reducing material. [4] Average of two determinations.

185(a).

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method is about ten times more sensitive than the α-naphthol method since, although similar readings were obtained, the ferricyanide samples had all been diluted ten times to bring the readings on to the colorimeter scale.

10 . Sucrose estimation by invertase

Since the α-naphthol method had been found to be less sensitive and much less convenient than the ferricyanide method, the use of invertase in conjunction with the ferricyanide method seemed an attractive method of estimating sucrose. Both reducing sugars and sucrose could then be determined by the same method.

An experiment was therefore carried out to determine the effect of invertase action. A yeast invertase preparation (British Drug Houses Ltd.) was diluted 2,000 times with 0.1 Molar acetic acid/sodium acetate buffer at pH 4.5. Alcohol was removed from two 20 ml. aliquots from the 26/5/L leaf extract by vacuum distillation, as described earlier. The residues were taken up in water, cleared with lead and made up in the usual way to 1 litre with distilled water. A 20 ml. aliquot from a standard sucrose solution (1gm./litre alcohol) was treated similarly. To one 2 ml. aliquot from each sample were added 2 ml. of the diluted invertase preparation. To a further 2 ml. aliquot from each sample, 2 ml. of distilled water were added. As a final control, 2 ml. of the enzyme preparation were added to 2 ml. of distilled water. The whole procedure was carried out in duplicate. The tubes containing the samples were then mechanically shaken in a water bath at 20°C for 40 minutes.

Appendix 10 (continued)

After the incubation 2 ml. of ferricyanide reagent and 2 ml. phosphate reagent were added to each tube, in the manner described on p.182. The solutions were then diluted 7.5 times (2 ml. to 15 ml. with water), thus bringing the total dilution to the same value used in the last experiment (30 times). The results (Table 22, p.187(a)) show that sucrose is effectively hydrolysed under the conditions used, though the readings (19 mg.) were slightly lower than the expected 20 mg. The invertase itself has no effect on the readings. Invertase action on the cleared leaf extract increases the sugar reading by 3.0 mg./l. glucose equivalent, which agrees reasonably well with the sucrose estimate of 3.3 mg./l.obtained in the last experiment by difference between the readings from the α-naphthol and ferricyanide methods (Table 21).

11. Sugar analysis of oak leaf extracts

In view of the results from the last experiment, it was decided to adopt the ferricyanide method for analysis of the extracts from the leaves of tree M, using the invertase to estimate sucrose. The final procedure, based on the results of the preliminary experiments described above, was as follows:

1) Each alcoholic leaf extract was thoroughly shaken to disperse any sediment. A 20 ml. aliquot was then pipetted into a suitable flask and distilled under reduced pressure in a rotatary evaporator at 60°C, until all the alcohol had been removed. About 100 ml. of distilled water were then added to the flask and the gummy residue completely shaken into suspension or solution by dipping the flask into the bath of an

Table 22: Sucrose estimation by the action of invertase

Sample	Optical Density[1]	Reducing Material[2]
Glucose standard (10 mg./l.)	1.12	10
Glucose standard (20 mg./l.)	2.20	20
Glucose standard (40 mg./l.)	3.86	40
26/5/L, cleared, no enzyme	0.92	8.5
26/5/L, cleared, with enzyme	1.26	11.5
Sucrose (1 gm./l. ethanol), cleared, with enzyme	2.10	19
Invertase alone	0.01	0
Sucrose (1 gm./l. ethanol), cleared, no enzyme	0.00	0

[1] Read on Eel colorimeter with red filter, after treatment of samples by ferricyanide method. Samples diluted 10 times before estimation of optical density. All readings represent average of duplicate determinations.

[2] Expressed as mg. of glucose equivalent/litre; values were determined from a graph prepared from the readings from the glucose standards.

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ultrasonorator.

2) To the above suspension, now transferred to a beaker, were added from a bubette 5 ml. of saturated lead acetate solution and, after thorough mixing, 7 ml. of saturated sodium oxalate solution. The resulting precipitate was removed by filtration through a sintered glass funnel and, with washings, made up to 1 litre with distilled water.

3) Four 1 ml. aliquots from the extract preparation were pipetted into separate test-tubes, which had been thoroughly cleaned before the analysis by washing in a sodium dichromate/concentrated sulphuric acid bath. To two of the tubes were then added 1 ml. of the diluted invertase preparation (British Drug Houses preparation, diluted 2,000 times in 0.1 Molar sodium acetate/acetic acid buffer at pH 4.5). 1 ml. of distilled water was added to each of the other two (control) tubes. All four tubes were covered with 'Parafilm' and incubated for 45 minutes in a water bath at 20°C, using a mechanical shaker.

4) After incubation, 2 ml. of ferricyanide reagent and 2 ml. of ferric sulphate reagent were added, following the procedure described on p. 182. After thorough mixing, the optical densities of the coloured solutions, along with those from suitable glucose controls, were read at 680 mμ on a Unicam manual spectrophotometer.

The procedure was carried out in duplicate for each leaf extract. The optical density readings were converted to sugar concentrations (expressed in terms of glucose) present in the distilled and diluted extract using a graph of optical density against glucose concentration, prepared from the control readings. Two sets of glucose controls were

Appendix 10 (continued)

used for each analysis; one was treated with invertase and the other with distilled water, before incubation alongside the experimental tubes, to simulate the experimental procedure.

The sucrose concentration was determined, in terms of glucose, by difference between the sugar concentrations of the digested and undigested aliquots. Since 20 ml. of the original alcoholic extract had in each case been diluted, after distillation, to 1 litre, multiplication of the sugar concentrations by 50 yielded the sugar concentrations (glucose equivalent) present in the original leaf extracts. Using the volumes of the original extracts, together with the fresh and dry weights of the leaf samples, values were then calculated for the percentages of sucrose and reducing sugars in the leaves.

The results of the analyses, representing in each case the average value of the duplicate determinations, are shown in Table 23 (p.189(a)), where the concentrations of monosaccharides and sucrose are expressed as percentages glucose equivalent of both fresh weight and dry weight of leaf. The results are also expressed graphically in Figures 13 (p.41(a)) and 14 (p.41(b)).

TABLE 23 Sugar content of leaves, determined by the ferricyanide method, in conjunction with invertase hydrolysis

Date (1964)	Source of Sample[1]	Monosaccharides[2]		Sucrose[2]	
		% Fresh wt.	% Dry wt.	% Fresh wt.	% Dry wt.
23 April	Upper shade buds	0.9	3.5	0.6	2.2
23 April	Upper sun buds	1.5	5.2	0.5	1.7
29 April	Upper shade buds	1.3	6.1	0.7	3.4
29 April	Upper sun buds	1.4	4.6	0.8	2.7
15 May	Upper sun leaves	1.8	6.6	0.9	3.2
26 May	"	1.8	5.4	1.2	3.6
5 June	"	1.5	4.0	1.8	5.0
11 June	"	1.6	3.7	2.9	6.9
22 June	"	1.2	3.0	3.3	8.1
1 July	"	1.7	3.9	3.4	7.6
23 July	"	1.9	3.9	3.4	6.9
30 September	"	2.8	5.8	3.8	7.8
23 April	Lower shade buds	1.4	5.5	0.3	1.2
23 April	Lower sun buds	1.1	4.0	0.3	1.1
29 April	Lower sha de buds	1.4	5.3	0.6	2.3
15 May	Lower shade leaves	1.4	6.2	0.7	2.9
26 May	"	1.5	4.9	0.5	1.7
5 June	"	1.5	4.7	1.0	3.2
11 June	"	2.2	6.0	1.9	5.2

TABLE 23 (continued)

Date (1964)	Source of Sample[1]	Monosaccharides[2]		Sucrose[2]	
		% Fresh wt.	% Dry wt.	% Fresh wt.	% Dry wt
22 June	Lower shade leaves	2.7	7.5	1.9	5.3
1 July	"	2.1	5.5	2.2	5.7
23 July	"	1.8	4.4	2.0	4.9
30 September	"	2.5	6.3	2.1	5.3
23 April	Bud scales	1.9	4.8	0.2	0.4
16 May	Hazel leaves	3.2	10.8	2.3	7.8
16 May	Hawthorn leaves	2.0	5.9	0.7	2.0

[1] All samples from oak tree 'M', except where specified.

[2] Determined as glucose equivalent.

Appendix 11: Nitrogen analysis of oak leaves

1. Method

The method used to determine nitrogen was that originally developed by Kjeldahl (1883) and since extensively modified (Bradstreet, 1965). The procedure followed in the present work was that in current use in the Department of Forestry, Oxford (Handley, personal communication). All analyses were carried out in the Department of Forestry, either by the author or with the generous help of Miss Jenkins.

The procedure used is briefly as follows:

1) The leaf material is first dried in an oven to constant weight. The dried leaves are then coarsely ground with a mortar and pestle and subsequently ground to a very fine powder in a ball mill. A representative sample of this fine powder (about 2 gm.) is then re-dried to constant weight in an oven at 105°C (about 4 days).

2) Samples of about 50 mg. are removed from the thoroughly dry powder and accurately weighed into Kjeldahl digestion flasks. 2.0 ml. of concentrated sulphuric acid is then carefully added to the flask, together with a quarter of a standard catalyst tablet (British Drug Houses, Ltd.; each tablet contains 1 gm. sodium sulphate and the equivalent of 0.1 gm. mercury). The contents of the flask are now heated gently over a low flame to allow digestion to commence. As the reaction dies down, the heat is increased and continued for one hour after the digest has become colourless. The digestion takes about 2-5 hours in all.

Appendix 11 (continued)

3) The digest is carefully poured into a Markham Still apparatus, together with washings. 11 ml. of a solution containing 320 gm. sodium hydroxide and 50.28 gm. sodium thiosulphate in 914 ml. water is now introduced into the still. Steam is blown through the now alkaline reaction mixture and about 30 ml. of distillate, containing all the ammonia derived from the nitrogen in the original leaf material, collected in 20 ml. of boric acid solution, containing indicator. The boric acid and indicator mixture is previously prepared as follows:

Bromo-cresol green : To 0.5 gm., add a little water and 7.5 ml. of 0.1 Normal sodium hydroxide and 10 ml. ethanol. Stir. When the indicator has dissolved, dilute to 500 ml. with water.

Methyl red: 0.1% in ethanol

Boric acid: 0.25% solution in de-ionised water. To every litre of boric acid solution, add 5.0 methyl red and 7.5 ml. of bromo-cresol green.

4) The distillate, in boric acid solution, is titrated against 0.01 N or 0.005 N sulphuric or hydrochloric acid. From this titre, after allowing for the control titres, the amount of ammonia released from the acid digest by addition of alkali can be determined, and therefore the amount of nitrogen present in the original weighed sample of dried leaf powder.

Analyses were invariably carried out in duplicate or triplicate, along with reagent blanks, containing distilled water in place of the leaf material.

Appendix 11 (continued)

2. Nitrogen content of leaves from tree M during 1965.

The results obtained by Kjeldahl analysis, as described above, of leaf samples from tree M in 1965 are shown in Table 24 (p.192(a)). The mean values for % total nitrogen (dry weight) from the triplicate determinations are converted to equivalent protein content (% dry weight) by multiplying by a factor of 6.25. The results are also expressed graphically in Figure 15 (p.44(a)).

3. Nitrogen content of leaves from six oak trees during 1966

The results obtained by Kjeldahl analysis, as described above, of leaf samples from trees M, 1, 5, 8, 9 and 15 in 1966 are shown in Table 25 (p.192(c)). The analyses were carried out in duplicate. The results are expressed graphically in Figure 16 (p.45(a)).

TABLE 24 Total nitrogen content of oak leaves from tree M during 1965 estimated by Kjeldahl analysis.

Leaf sample (date)	Weight of dried leaf powder (mg.)	Kjeldahl titre (ml. 0.01N H_2SO_4)	Corrected titre (ml.)	Mean % N (dry wt.)	Mean % "protein"[3] (dry wt.)
Blank	-	0.24	-		
Blank	-	0.23	-		
Blank	-	0.22	-		
Blank	-	0.25	-	Mean blank titre = 0.23	
Blank	-	0.24	-		
Blank	-	0.21	-		
Blank	-	0.23	-		
$30/5^{I}$	46.1	14.90	14.67		
	49.5	16.26	16.03	4.46	27.8
	48.8	15.97	15.74		
$23/5^{I}$	45.3	8.60	8.37		
	48.5	9.16	8.93	2.58	16.1
	48.5	9.20	8.97		
$14/6^{I}$	51.4	8.06	7.83		
	50.9	7.90	7.67	2.10	13.2
	48.0	7.70	7.47		
$5/8^{I}$	46.8	7.58	7.35		
	57.1	9.16	8.93	2.21	13.8
	47.6	7.82	7.59		
$15/9^{I}$	51.1	7.81	7.58	2.08	13.0
	51.0	7.80	7.57		
$30/4^{2}$	45.7	16.04	15.81	4.80	30.0
	45.3	15.64	15.41		
$23/5^{2}$	48.0	9.26	9.03		
	49.2	9.35	9.12	2.63	16.4
	53.3	10.34	10.11		

contd.

TABLE 24 (contd.)

Leaf sample (date)	Weight of dried leaf powder (mg.)	Kjeldahl titre (ml.0.0IN H_2SO_4)	Corrected titre (ml.)	Mean % N (dry wt.)	Mean % "protein"[3] (dry wt.)
I4/6[2]	5I.8	8.74	8.5I	2.27	I4.2
	53.6	8.84	8.6I		
	5I.8	8.56	8.33		
5/8[2]	46.8	7.92	7.69	2.29	I4.3
	56.0	9.I2	8.89		
	49.0	8.43	8.20		
I5/9[2]	48.9	8.0I	7.78	2.2I	I3.8
	48.6	7.90	7.67		
	49.I	7.90	7.67		

I = upper sun leaves

2 = lower shade leaves

3 = calculated as total nitrogen × 6.25.

TABLE 25 Nitrogen content of lower leaf samples from 6 oak trees in I966, dtermined by the Kjeldahl method.

Date of sample	Tree	Wt. of dried leaf sample	Kjeldahl titre (ml.0.005N H_2SO_4)[1]	Mean% nitrogen	Mean % "protein"[2]
May 6th	I	I7.2	I2.98		
"	"	I7.75	I3.53	5.32	33.2
"	5	2I.25	I5.57.		
"	"	I5.95	II.57	5.I0	3I.9
"	8	I6.52	I0.I4		
"	"	I8.2	II.26	4.32	27.0
"	9	I9.73	I4.75		
"	"	I7.47	I3.04	5.24	32.8
"	I5	22.05	I6.66		
"	"	I8.2	I3.87	5.3I	33.2
"	M	I9.67	II.75		
"	"	I8.35	II.0I	4.20	26.2
May I9th	9	27.75	I3.85		
"	"	25.0	I2.43	3.49	22.0
June I0th	9	25.6	9.20		
"	"	26.0	9.39	2.52	I5.7
July Ist	I	I7.98	7.74		
"	"	26.43	II.42	3.02	I8.9
"	5	30.6	I0.84		
"	"	3I.I5	II.08	2.49	I5.6
"	8	28.8	I0.72		
"	"	29.55	I0.95	2.6I	I6.3
"	9	29.7	I0.66		
"	"	3I.35	II.I[illegible]	2.5I	I5.7
"	I5	27.5	9.94		
"	"	28.65	I0.30	2.52	I5.8
"	M	29.95	I0.66		
"	"	30.42	I0.93	2.50	I5.6

[1] = corrected for reagent blank titres.
[2] = Calculated as total nitrogen × 6.25

Appendix 12: Qualitative analysis of the phenolic compounds in oak leaf extracts

1. Preparation of extracts

Leaves were collected by the method described in Chapter 1 and weighed samples homogenised in 70% acetone/water, containing a little potassium cyanide, using an 'Atomix' blender. For routine analysis, 50 gm. fresh weight of leaves were extracted into 500 ml. of solvent. The extracts were stored under nitrogen in sealed stoppered bottle, in the dark at 4°C, to await analysis.

2. Chromatography of extracts

0.01 to 0.05 ml. samples from the stored extracts were run on two-way Whatman No. 1 chromatograms, first in 2% acetic acid (about 2 hours) and then, after blowing off the solvent, in n-butanol/acetic acid/water (60 : 15 : 25) (10-12 hours). Standard Shandon 'Chromotanks' were used and the 10" x 10" papers supported in polythene frames, five at a time. The ascending solvent technique was invariably used and the polythene frame, containing the chromatograms, was placed in a shallow tray containing the solvent. Chromatograms were normally run at a temperature of 16°-18°C.

After the second solvent had been blown off in a current of air, the chromatograms were examined in ultraviolet light, to detect any fluorescent materials. The chromatograms were also examined under ultraviolet light in the presence of ammonia, since further phenolic compounds may be revealed by this procedure (Smith, 1960).

Appendix 12 (continued)

After the positions of fluorescent spots had been marked, the chromatograms were developed by spraying with one of the following reagents (Smith, 1960), toshow up the phenolic materials and to determine their R_f values and relative intensities.

Ferric chloride/potassium ferricyanide reagent:

1% aqueous of each component. Shows up all phenols as blue spots or streaks, revealing as little as 1 mg. of many. Before drying, chromatograms are dipped in dilute hydrochloric acid to prevent deterioration on storage. This was found to be the most useful developing reagent for the present work.

Vanillin Reagent:

10% w/v vanillin in ethanol : 12 Normal hydrochloric acid, 2 : 1.

Transient pink spots are given with phenols containing a resorcinol nucleus.

This reagent is less sensitive than the ferric chloride/ferricyanide reagent, but was found to be useful for detecting condensed tannin and its probable precursors, (+)-catechin and (+)-gallocatechin, which all contain resorcinol units in their structures (Appendix 13).

Diazotised p-nitraniline reagent

Aqueous sodium nitrite solution (5% w/v, 0.2 vol.) is added to a solution of p-nitraniline (1.5 gm.) and concentrated hydrochloric acid (45 ml.) in water (950 ml.) (10 vol.). Sodium carbonate (anhydrous) in water (10% w/v, 10 vols.) is then added to the mixture. The chromatogram is dried for 5 minutes at 100°C before being sprayed with the reagent. Red, yellow, grey, brown and purple azo-dyes are produced with

Appendix 12 (continued)

different phenols.

The use of this reagent was eventually abandoned, since, although the tannins and a number of other phenols gave yellow dyes (Table 4, p.50(b)), no information was provided which could not already be obtained with the first two reagents described above.

Tetrazotised benzidine reagent

Benzidine, 0.5%, in dilute hydrochloric acid	1 vol.
Sodium nitrite, 10%, in water	1 vol.

Equal volumes are mixed to form a clear, bright yellow solution.

This reagent, when sprayed on to the chromatograms, revealed a number of components as yellow-brown or red spots (Table 4, p. 50(b)).

Appendix 13: Nature and classification of plant tannins

The plant tannins represent a heterogeneous group of high molecular weight polyphenols, found in plants, which have the ability to combine with the protein of animal hide, probably by hdrogen bonding. This is an arbitrary criterion, since tanning ability depends on a number of factors, including molecular weight and the number and positions of the functional groups. If the criterion is strictly adhered to, a number of low molecular weight phenols must be included as tannins. It is customary, however, to restrict the term to materials of high molecular weight.

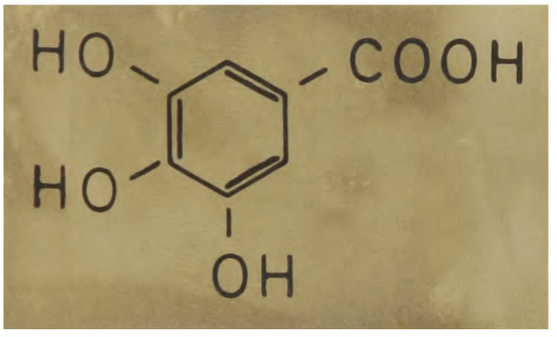
In spite of the diversity of the plant tannins, a primary classification is possible by considering the effect on them of warm dilute mineral acids. Hydrolysable tannins, under these conditions, usually yield a sugar and a phenolic acid, whereas condensed tannins are not hydrolysed, but are further co densed to form 'phlobaphenes' - red-brown solids of very high molecular weight. Hydrolysable tannins, usually of relatively low molecular weight, are therefore characterised by ester links. Condensed tannins, however, include a variety of structures, none of which has yet been completely elucidated (Brown, 1964).

Hydrolysable tannins

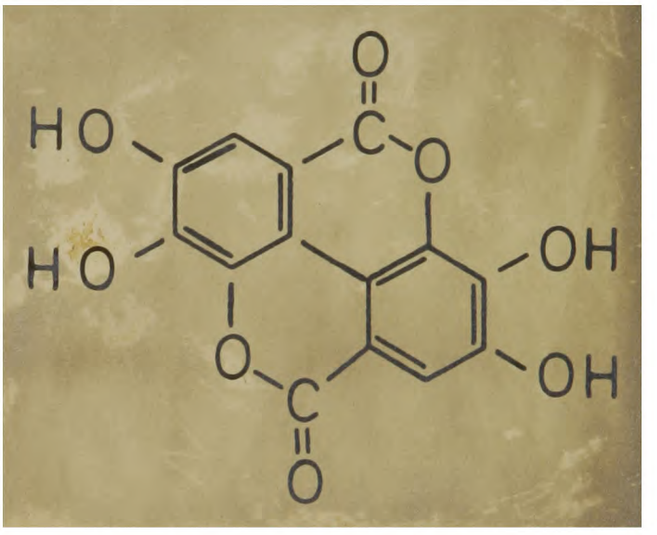
The structures of a number of hydrolysable tannins have now been discovered, largely through the work of Haworth and co-workers (e.g. Haworth, 1963). They consist of several molecules of gallic acid (I), or acids clearly derivable from gallic acid (such as ellagic acid, II),

Appendix 13 (continued)

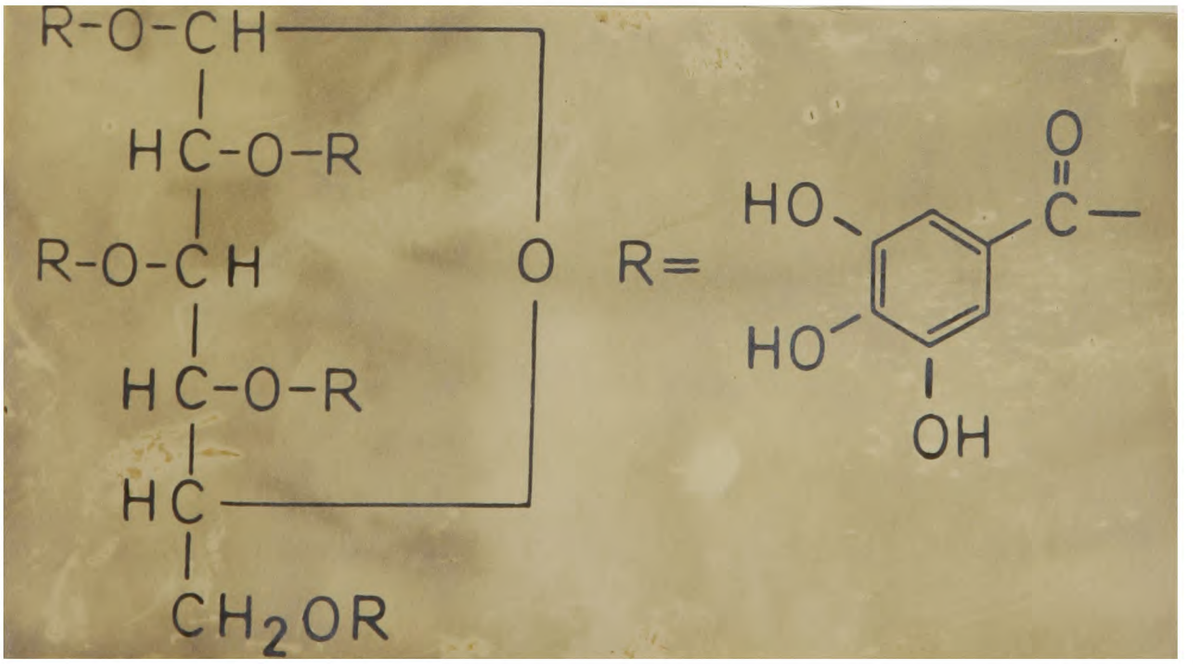
united by ester links to a central sugar residue, which is commonly glucose. They can be subdivided into the gallotannins (in which the phenolic acid is gallic acid) and ellagitannins (in which ellagic acid is the phenolic unit). The structures can frequently be complex (Haworth, loc. cit.), but some are relatively simple. The structure of one such simple gallotannin, that of Turkish Tannin from the twigs of Quercus infectoria, is shown below (III)



I Gallic Acid



II Ellagic Acid



III. Turkish Tannin: Galloyl groups (R) linked by ester linkages to a central glucose unit.

Appendix 13 (continued)

As might be expected, gallotannins yield gallic acid on hydrolysis, whereas ellagitannins yield ellagic acid.

Condensed Tannins

Condensed tannins consist of mono-, di- and tri-hydric phenolic nuclei, linked by any or all of the following:

1) Ether linkages, via oxygen atoms.

2) Carbon - carbon links; either via aliphatic chains or by direct linkage between aromatic nuclei.

3) Heterocylic rings, involving oxygen and carbon atoms.

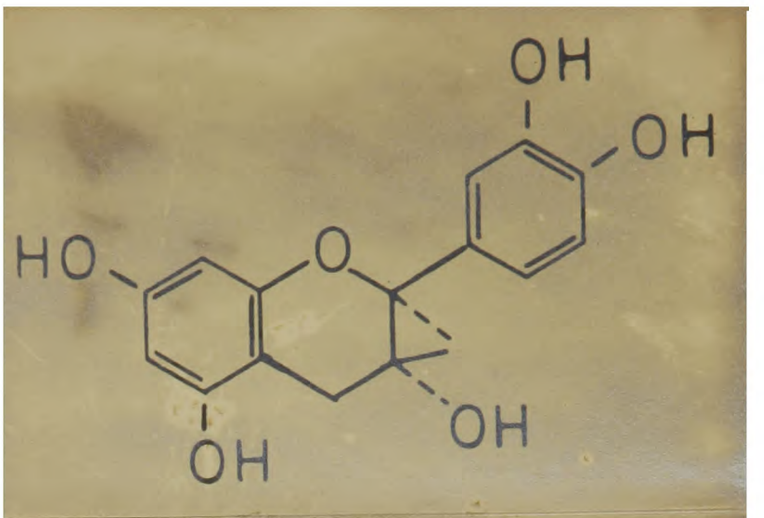
Such links are not easily broken by hydrolysis, but under sufficiently vigorous conditions, the polymers can be degraded to yield phenolic fragments. These, however, give no information as to the positions of the linkages in the polymer.

Condensed tannins may be subdivided into the polyhydroxystilbene tannins and the flavanoid tannins. Both are generally of high molecular weight. Flavanoid tannins appear to be the most widespread in leaves. They are widely believed to be derived from simple flavanol phenols, such as (+) catechin (IV) and (+)-gallocatechin (V), present in the leaves, though the precise manner in which these residues are linked together in the polymer is not yet known. Brown (1964) considers that the flavan-3, 4-diol units in the condensed tannin of heather Calluna vulgaris L. (which seems to have similar properties to oak leaf condensed tannin) may be linked by ketal and/or hemi-ketal links (VI and VII).

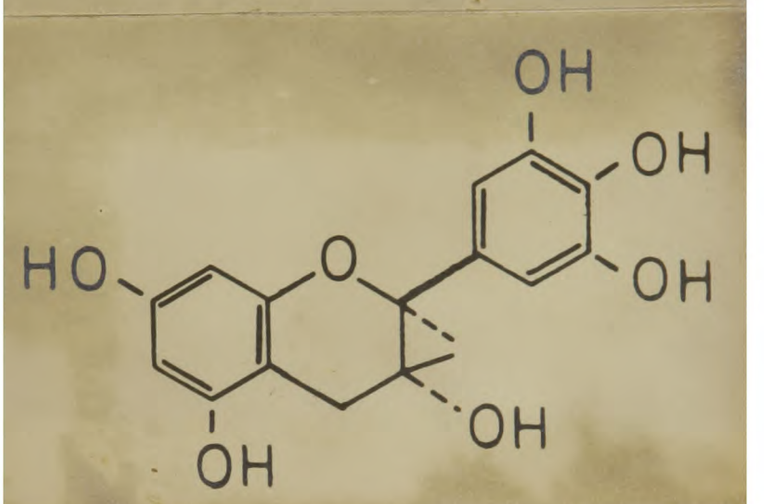
Oak leaf condensed tannin is thought to be a flavanoid tannin,

Appendix 13 (continued)

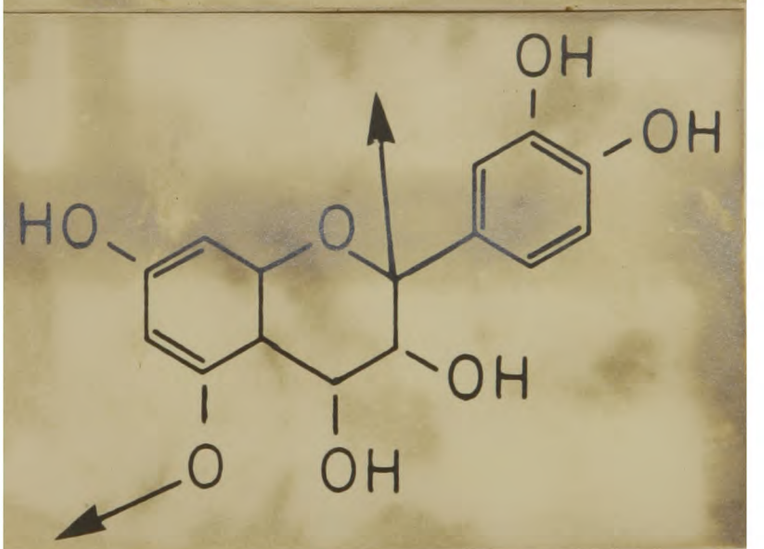
IV.(+)-catechin:



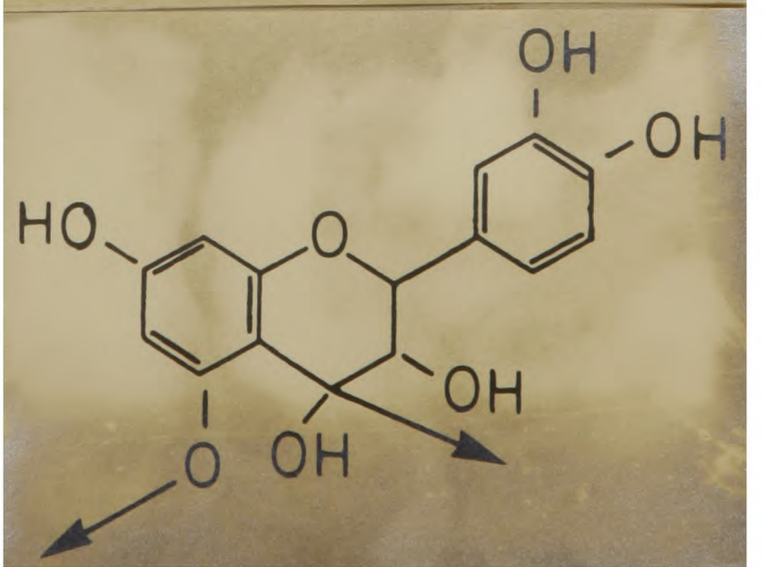
V. (+)-gallocatechin:



VI. Ketal linkage:



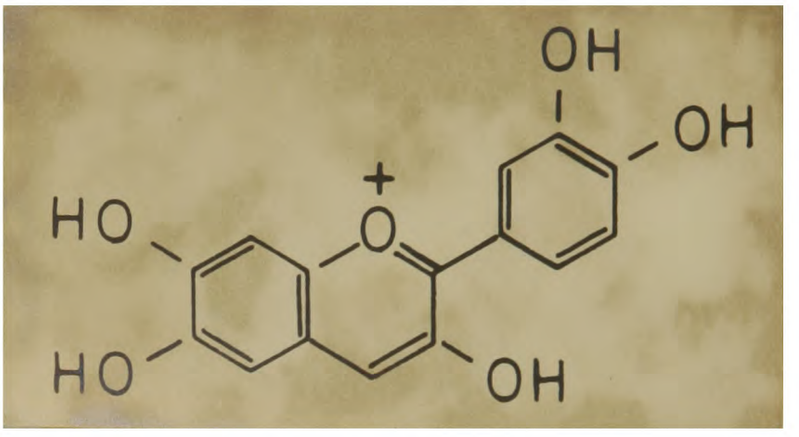
VII. Hemi-ketal linkage:



Appendix 13 (continued)

chiefly because it yields some cyanidin (VIII), in addition to phlobaphene, on heating with concentrated acid. The presence of (+)-catechin and (+)-gallocatechin in oak leaves during the period of condensed tannin synthesis suggests that one or both of these flavanol phenols may be incorporated into the polymer.

VIII. Cyanidin cation:



Appendix 14: Isolation of oak leaf tannins

Tannins were extracted from oak leaves by the ether-precipitation method devised by Brown and Love (1961) for the extraction of tannin from heather.

Leaves were homogenised in 70% acetone/water, containing a little ascorbic acid to minimise oxidation, in an M.S.E. 'Atomix' blender. The extract suspension, often amounting to several litres, was then stored under nitrogen for 1-7 days in large sweet jars, sealed with 'Lassotape', placed in the dark at room temperature. Portions of the extract were then filtered through a Büchner funnel, using suction, to remove cellular debris, and the clear green filtrates transferred to large conical flasks.

To each flask was then added excess solid sodium chloride, which caused the filtrate to separate into two layers. The upper layer (predominantly acetone) was separated and transferred to another flask. It was diluted with twice its volume of ethanol before 5-10 volumes of anhydrous diethyl ether were added to precipitate a mixture of tannins and sodium chloride. The precipitate was then centrifuged (2,500 r.p.m. for 10 minutes) and washed with fresh ether. It was then dried in a current of nitrogen, while still in the centrifuge bottles, and taken up into solution and suspension in a small volume of distilled water.

The solution/suspension of the tannin and salt mixture was transferred to 'Visking' cellophane dialysis tubing (1" diameter) and suspended in sealed sweet jars, filled with distilled water containing a little ascorbic acid. The water in the jars was changed at daily

Appendix 14 (continued)

intervals, until, usually after 3 days, it became free of salt (no precipitate with silver nitrate solution) and low molecular weight phenols (no colour with ferric chloride solution). The contents of the dialysis tubing, now consisting only of tannin in water, were poured into suitable flasks and freeze-dried (about 24 hours) to give a brown fluffy powder of crude oak leaf tannin.

During the extraction procedure, and especially during dialysis, some of the tannin separated out from solution. This fraction (P) was usually freeze-dried separately from the water-soluble fraction (S), after separation of the two fractions in a centrifuge.

Appendix 15: Adsorption of oak leaf tannins by hide powder

Tannins are adsorbed from aqueous solution by powdered animal hide to form hydrogen-bonded complexes with the hide protein. To determine which components in extracts of oak leaves possessed tanning ability, the following experiment was carried out.

50 gm. (fresh weight) of recently collected September oak leaves were homogenised in 500 ml. of 70% acetone/water, in order to extract the maximum quantity of tannin. The extract was centrifuged at 2,500 r.p.m. for 5 minutes, yielding a clear, dark green supernatant.

To remove the acetone from the extract, in order to obtain an aqueous solution for the hide powder test, the supernatant was distilled under reduced pressure under nitrogen in a rotary evaporator at 30°-35°C. A fine colloidal precipitate appeared as the acetone was removed. This precipitate remained in suspension when the volume of the extract was made up to 300 ml. with distilled water, after complete removal of the acetone, and was not retained by Whatman No. 2 filter paper, nor was it sedimented after one hour in a centrifuge at 4,500 r.p.m. The precipitate was, however, finally removed by sedimentation in a M.S.E. 'Superspeed' centrifuge at 40,000 r.p.m. for one hour. The precipitate was subsequently found, by chromatography of a solution in acetone, to contain chlorophyll and some condensed tannin, as well as other material of unknown nature.

1) 0.01 ml. of the clear pale green supernatant was run on a two-way paper chromatogram in 2% acetic acid and BAW, revealing the pattern of phenolic spots, previously found for extracts of September

Appendix 15 (continued)

leaves (Table 5, p. 55(a)), on development with the ferric chloride/ potassium ferricyanide reagent.

2) 5 ml. of the clear supernatant were shaken with 0.5 gm. of wetted hide powder (British Drug Houses, Ltd., standard grade) for 10 minutes, and 0.01 ml. of the resulting supernatant was then run on a chromatogram at the same time as the above untreated control. Most of the phenolic spots appeared on the chromatogram, after development with the ferric chloride/potassium ferricyanide reagent, but C, D_1, D_2, H, B and G were entirely absent, although present on the control chromatogram. It was concluded, therefore, that these compounds have the ability to combine with animal hide and can therefore be described as tannins. It is of interest that substance O and J were also missing from the hide powder supernatant. This suggests that these compounds also can combine with hide protein, though they are not referred to as tannins in the present work, since they are known to be of low molecular weight (for example, they pass through dialysis tubing and are not isolated by the Brown and Love method for polymeric phenols of high molecular weight).

Appendix 16: Hydrolysis of oak leaf tannins

1. Hydrolysis of crude July oak leaf tannin

Crude oak tannin, isolated from July oak leaves by the method of Brown and Love (Appendix 14) was treated with both concentrated and dilute acid to confirm the presence of condensed tannin and to determine whether or not a hydrolysable tannin was present.

a) Concentrated hydrochloric acid

0.2 gm. of both the (S) and (P) fractions of the crude July tannin were boiled briefly in separate test-tubes with 2 ml. of concentrated hydrochloric acid and 1 ml. n-butanol. In both tubes, a cherry red colour developed and was largely extracted into the butanol layer. Samples of 0.01 ml. of the butanol layers from the two hydrolysates were run one-way on a paper chromatogram, together with pure cyanidin chloride as a reference compound; the solvent used was the 'Forestal' solvent (water/acetic atid/hydrochloric acid, 10 : 30 : 3 v/v) (Love and Brown, 1959). The R_f value of the cerise spot obtained from both the hydrolysates was found to be 0.40 - identical to that of the cyanidin reference compound. The absorption spectra of the butanol layers from the two hydrolysates were obtained in a Beckman spectrophotometer, using a pure butanol reference blank. The spectra, with absorption peaks at 560 and 465 mμ, were indistinguishable from the spectrum of a butanolic solution of pure cyanidin chloride. It was concluded that cyanidin chloride was present in the tannin hydrolysates, confirming the presence of a flavanoid condensed tannin (see Appendix 13).

Appendix 16 (continued)

Similar results were later obtained by boiling pure oak-leaf condensed tannin with concentrated hydrochloric acid, confirming the identity of streak C as the flavanoid condensed tannin in oak leaves.

b) Dilute sulphuric acid

0.05 gm. of the crude July oak leaf tannin (both (S) and (P) fractions combined) was heated at 90°C in a sealed glass test-tube with 3 ml. of dilute (1%) sulphuric acid for 24 hours. The resulting pink hydrolysate was decanted from insoluble residue and 0.01 ml. aliquots run on two-way paper chromatograms in 2% acetic acid and BAW solvents. Samples of pure cyanidin chloride, gallic and ellagic acids were run as reference compounds on separate chromatograms. All the chromatograms were examined under ultraviolet light, with and without ammonia vapour, and then developed with ferric chloride/potassium ferricyanide or vanillin reagents. The R_f values and properties of the resulting spots are shown in Table 26 (p.206(a)).

It can be seen from the data in Table 26 that both gallic and ellagic acids were present in the hydrolysate; this strongly suggests that the crude July oak leaf tannin contains at least one hydrolysable tannin (see Appendix 13, p. 196). Apart from unhydrolysed D_1, D_2 and C, together with some cyanidin chloride, three other phenolic components were detected in the hydrolysate. These, labelled N', Z and A', are of unknown identity.

As mentioned in Appendix 13 (p.196), a hydrolysable tannin would be

TABLE 26 R_f values and chromatographic behaviour of hydrolysis products obtained by heating crude July oak leaf tannin with dilute sulphuric acid

(All components gave a positive reaction with the ferric chloride/ferricyanide reagent)

Compound	Colour of spot	u.v.	u.v./NH_3	Vanillin	R_f in 2% Acetic acid	R_f in BAW
'Cy'	cerise	-	-	-	0.02	0.48
Cyanidin chloride[1]	cerise	-	-	-	0.02	0.50
U	-	bV	Y	-	0.02	0.36
Ellagic acid[1]	-	bV	Y	-	0.02	0.33
F_1	-	-	-	-	0.37	0.58
Gallic acid[1]	-	-	-	-	0.44	0.59
C	brown	abs.	abs.	pink	0 - 0.30	0
D_1	-	-	abs.	-	0.45	0.02
D_2	-	-	abs.	-	0.36	0.06
N'	-	-	-	-	0.62	0.10
A'	-	-	-	pink	0.12	0.54
Z	-	-	-	-	0.02	0.55

[1]Pure reference compounds, run on separate chromatograms;

bV = bright violet fluorescence; Y = yellow fluorescence; abs. = absorbs ultraviolet light;

Appendix 16 (continued)

expected to yield a sugar in addition to a phenolic acid on hydrolysis. 0.01 ml. samples of the hydrolysate were therefore run one-way on paper chromatograms in iso-propanol/water (4 : 1) and the dried chromatograms were sprayed with aniline/diphenylamine, aniline hydrogen phthalate or phthalic acid reagents to discover whether any sugars were present. Pure D-Glucose, the most likely sugar to be present, was run on the chromatograms in parallel, as a reference compound. One spot was revealed on the chromatograms of the hydrolysate. Its R_f value and colour with the reagents is given below, together with that of the glucose control:

	Spot from Hydrolysate	Spot from pure glucose
R_f in iso-propanol/water	0.45	0.45
Aniline/diphenylamine reagent	blue	blue
Aniline hydrogen phthalate	red, with greenish-yellow fluorescence in u.v.	red, with greenish-yellow fluorescence in u.v.
Phthalic acid reagent	brown with orange fluorescence in u.v.	brown, with orange fluorescence in u.v.

It is clear from the identical properties of the spots on the chromatograms from the hydrolysate and from the reference sugar that glucose is present, as suspected, in the hydrolysate obtained by heating July oak tannin with dilute sulphuric acid. This confirms, for the first

Appendix 16 (continued)

time, the presence of a hydrolysable tannin in oak leaves. The available data cannot be used to further classify the tannin as an ellagitannin or as a gallotannin, since interconversions between these two phenolic acids may have occurred under the conditions used. However, D_1 and D_2 were subsequently hydrolysed by Bostock (1966), after separation from other tannin components on a Sephadex column, and were considered by him to be ellagitannins, since ellagic acid was the most obvious hydrolysis product.

The aniline/diphenylamine reagent has already been described (Appendix 9, p.175). The remaining two reagents used to develop the chromatograms in the above experiment are prepared as follows:

Aniline hydrogen phthalate (Dawson, et al., 1959)

0.93 gm. aniline and 1.66 gm. phthalic acid, dissolved in 100 ml. n-butanol, saturated with water. After application of the reagent, chromatograms are heated at 105°C for 5 minutes.

Phthalic acid reagent (Dawson, et al., 1959)

3% phthalic acid in water. Chromatograms are heated at 150°C for 10 minutes, after application of the reagent.

Appendix 17: Determination of Molecular Weights of Oak Leaf Tannins

An approximate value for the average molecular weight of crude July oak leaf tannin (fraction (S), unseparated into condensed and hydrolysable components) was obtained by Mr. C.J. Teal, using the Beckman 'Spinco' Model E analytical ultracentrifuge in the Department of Biochemistry, Oxford. The determination was carried out on a sample of 0.01 gm. July oak leaf tannin (fraction (S)) by the short column method of Yphantis (1960), using Schlieren optics. An approximate value of 5,100 was calculated for the average molecular weight.

The molecular weights of pure condensed and hydrolysable oak tannins were subsequently determined by the same method (Bostock, 1966). The values found were:

Condensed tannin:	6,750
Hydrolysable tannin, D:	1,680

Appendix 18: Quantitative estimation of oak leaf tannins by weight determination of chromatogram spots.

Hathway (1960) has found that, for many phenols, there is a linear relationship between the logarithm of the concentration of the phenol in the initial spot, applied to a chromatogram, and the weight of the chromatographed, developed and excised spot. A preliminary experiment was therefore carried out to determine whether this method could be applied to the quantitative estimation of oak leaf tannins.

To obtain an estimate of the accuracy of the method, 30 spots (A), of approximately 3 sq. cm. area, were cut from both Whatman 3MM and Whatman No. 1 chromatography paper (not treated in any way) with a sharp scalpel. In each case, control spots (B) were cut simultaneously from a second sheet of chromatography paper (also untreated), of the appropriate grade, placed beneath the sheets from which the spots A were cut.

The 60 pairs of spots (30 from each grade of chromatography paper) were numbered lightly in pencil and placed in a dessicator, containing anhydrous calcium chloride, for 24 hours before weighing. The weights of all the spots are shown in Table 27 (p.210(a)), where it can be seen that the difference in the weights of the A and B spots of each pair (which should theoretically be zero) frequently reached 5% of the spot weight, on both grades of paper. To obtain an estimate of tannin concentration, accurate to 10%, from one chromatogram, the weighed spot or streak must contain an amount of tannin equal to at least ten times the

TABLE 27 Comparison of the Weights (mg.) of parts of excised spots from Whatman no. 1 and Whatman 3 MM chromatography papers.

	Whatman 3 MM Paper			Whatman No. 1 Paper		
No.	A (mg)	B	Diff. %	A	B	Diff. %
1	187.7	179.5	- 4.3	38.1	38.4	+ 0.8
2	146.6	143.1	- 2.5	57.2	58.3	+ 1.9
3	103.3	102.6	- 0.7	66.5	67.4	+ 1.3
4	131.1	128.4	- 2.1	72.4	74.8	+ 3.2
5	128.6	126.1	- 2.0	56.9	57.7	+ 1.4
6	124.0	12o.0	- 3.3	57.5	59.8	+ 3.8
7	118.6	119.2	+ 0.5	54.4	55.2	+ 1.4
8	139.8	139.4	- 0.3	41.2	43.1	+ 4.4
9	110.9	113.2	+ 2.0	47.3	48.0	+ 1.5
10	140.6	141.4	+ 0.6	44.9	45.0	+ 0.2
11	124.4	128.0	+ 2.9	39.0	39.4	+ 1.0
12	98.9	1oo.5	+ 1.6	4311	45.3	+ 4.9
13.	89.7	93.3	+ 4.0	42.8	43.0	+ 0.5
14	117.7	12o.8	+ 2.6	38.2	37.7	- 1.3
15	135.8	134.2	- 1.2	39.3	39.3	0
16	157.6	161.2	+ 2.2	48.4	48.9	+ 1.0
17	123.8	124.3	+ 0.4	44.4	44.1	- 0.7
18	104.3	102.7	- 1.6	55.3	54.7	- 1.1
19	129.4	131.7	+ 1.8	43.4	44.0	+ 1.4
20	138.3	140.7	+1.8	44.0	43.6	- 0.9
21	197.4	199.1	+ 0.9	40.0	39.8	1 0.5
22	120.6	120.3	- 0.3	32.4	33.7	+ 3.9
23	136.6	139.6	+ 2.2	38.7	40.1	+ 3.5
24	101.3	102.8	+ 1.5	50.3	52.1	+ 3.5
25	135.4	135.6	+ 0.1	42.4	43.2	+ 1.8
26	135.0	138.4	+ 2.5	48.5	48.7	+ 0.4
27	164.1	170.3	+ 3.6	44.4	44.3	- 0.2
28	122.8	125.8	+ 2.4	44.0	42.9	- 2.6
29	106.3	105.4	- 0.7	48.6	49.2	+ 1.2
3o	129.7	126.6	- 2.4	41.3	40.3	- 2.5

Appendix 18 (continued)

maximum experimental error. This is equivalent to approximately 0.01 gm. tannin in the initial spot for the Whatman No. 1 paper and 0.025 gm. tannin for the Whatman 3MM paper.

Attempts to run chromatograms, using these quantities of tannin, were completely unsuccessful. Owing to the gross overloading, the tannin 'caked' at the origin and much remained immobile. Development of the chromatograms revealed that resolution of the spots and streaks was almost negligible. The method was therefore abandoned. The lack of success with this technique was considered to be due to the relatively low quantities of tannin (in comparison with many simple phenols) which can be run on chromatograms before these become overloaded.

Appendix 19: Composition and preparation of artificial diets

1. Composition and preparation of Canadian diet

The formula for this diet is that described by McMorran (1965 and personal communication). The quantities given below are those used to obtain 100 gm. of diet. The ingredients (in the listed order) are added to the mixing container of an 'Atomix' blender and the blender run for about 20 seconds after each addition:

Water (distilled)	22.0 ml.
Casein (vitamin-free)	3.5 gm.
4 Molar potassium hydroxide	0.5 ml.
Cellulose powder (e.g. 'Alphacel')	0.5 gm.
Wesson salt mixture	1.0 gm.
Sucrose	3.5 gm.
Wheat embryo	3.0 gm.
Choline chloride	0.1 gm.
Vitamin solution	1.0 ml.
Ascorbic acid	0.4 gm.
Antimicrobial agents:	
Formaldehyde (37% aqueous)	0.05 ml.
Methyl p-hydroxybenzoate	0.15 gm.
Aureomycin	0.03 gm.

Finally, nutrient agar (2.5 gm., dissolved in 62 ml. hot distilled water) is added while still hot and the entire mixture is then blended for several minutes before dispensing into storage jars or rearing pots.

The vitamin solution is made up as follows:

Water (distilled)	100 ml.
Nicotinic acid	100 mg.
Calcium pantothenate	100 mg.
Riboflavine	50 mg.

Appendix 19 (continued)

Thiamine hydrochloride	25 mg.
Pyridoxine hydrochloride	25 mg.
Folic acid	25 mg.
D-Biotin	2 mg.
Cyanocobalamine (B_{12})	0.2 mg.

This stock vitamin solution can be kept in the refrigerator for at least a month.

2. Comparison of Compositions of Canadian, Cambridge and new Modified Diets

For reasons mentioned in Chapter 5, a modified artificial diet was devised by combining features of the Canadian (McMorran, 1965) and Cambridge (David and Gardiner, 1965) formulae. The compositions of all three diets are compared on a percentage weight basis as follows:

	gm. per 100 gm. of diet		
Ingredient	Canadian Diet	Cambridge Diet	Modified Diet
Distilled Water	85.5	83.8	84.2
Potassium Hydroxide (4 Molar soln.)	0.50	0.47	0.48
Casein	3.50	3.32	5.87
Sucrose	3.50	3.32	3.37
Wheat Embryo	3.00	2.85	0.40
Cabbage Powder	-	1.43	-
Salt Mixture	1.00	0.95	0.96
Cellulose Powder	0.50	0.50	1.92
Choline Chloride	0.10	0.10	0.08
Vitamin Mixture	0.00327	0.00414	0.00262
Ascorbic Acid	0.40	0.40	0.40
Formaldehyde	0.02	0.04	0.04

Appendix 19 (continued)

Ingredient	gm. per 100 gm. of diet		
	Canadian Diet	Cambridge Diet	Modified Diet
Methyl p-hydroxybenzoate	0.15	0.15	0.11
Agar	2.50	2.37	2.41
Aureomycin	0.03	0.20	0.21
Ethanol	-	0.92	-
Gibberellic Acid	-	-	0.003

The salt mixture incorporated in the Canadian and modified diets is Wesson's modification of the salt mixture developed by Osborne and Mendel (1932). The salt mixture used in the Cambridge diet is that of Beckman et al. (1953). The compositions of the salt mixtures are compared as follows:

Component	Wesson Mixture (% by weight)	Beckman Mixture (% by weight)
Calcium Carbonate	21.000	30.000
Copper Sulphate (5 H_2O)	0.039	0.030
Ferric Phosphate (4 H_2O)	1.470	-
Ferric Citrate (6 H_2O)	-	2.750
Manganous Sulphate (anhydr.)	0.020	-
Manganous Sulphate (4 H_2O)	-	0.500
Magnesium Sulphate (anhydr.)	9.000	-
Magnesium Sulphate (7 H_2O)	-	10.200
Potassium Aluminium Sulphate	0.009	-
Potassium Chloride	12.000	-
Potassium Dihydrogen Phosphate	31.000	-
Dipotassium Hydrogen Phosphate	-	32.250
Potassium Iodide	0.005	0.080
Sodium Chloride	10.500	16.750
Sodium Fluoride	0.057	-

Appendix 19 (continued)

Component	Wesson Mixture (% by weight)	Beckman Mixture (% by weight)
Tricalcium Phosphate	14.900	-
Calcium Hydrogen Phosphate ($2\ H_2O$)	-	7.500
Zinc Chloride	-	0.025

The percentage compositions (by weight) of the added combined vitamin samples are the same for both the Cambridge and the Canadian formulae and are shown as follows:

Vitamin	% by weight of total added vitamins
Nicotinic Acid	30.6%
Calcium Pantothenate (B_5)	30.6%
Riboflavin (B_2)	15.3%
Thiamine Hydrochloride (B_1)	7.65%
Pyridoxine Hydrochloride	7.65%
Folic Acid	7.65%
D-Biotin	0.61%
Cyanocobalamine (B_{12})	0.06%

Appendix 20: Comparison of fresh weight increase and pupal weights of winter moth larvae fed on different diets - 1964 season; experimental technique and results

1. Experimental procedure

7.5 gm. of oak leaf tannin (fractions (P) and (S) combined) were isolated from September oak leaves, which had been stored over winter in a deep-freeze (- 20°C), by the method of Brown and Love (1961). The tannin was obtained as a brown powder, free from salt (Appendix 14).

500 gm. of the Canadian artificial diet were prepared (Appendix 19). Samples of 45.0, 49.5 and 50 gm. of the thoroughly blended diet were poured, while still warm, into separate 100 ml. beakers, placed in turn on a Mettler balance. The beakers were warm, to prevent gelation of the diet. Samples of the oak leaf tannin, weighing 5.0, 0.5 and 0.05 gm., were added, respectively, to the beakers containing the three portions of diet and the three mixtures then thoroughly mixed with a mechanical homogeniser. The three diets, containing 10%, 1.0% and 0.1% of tannin, respectively, were allowed to set. The remainder of the basal plain diet was also allowed to set in a separate container.

Precautions against contamination of the diets by micro-organisms were taken as far as possible. All glassware and most instruments were autoclaved before use.

125 small plastic pots (1" deep and 1.5" in diameter), identical to those described in Chapter 2, were thoroughly cleaned and disinfected with 80% ethanol, after ten holes had been drilled in all the lids. These holes, all of the same size, were small enough to prevent larvae

Appendix 20 (continued)

escaping, yet large enough to ensure ventilation. The pots were divided into 5 groups of 25 and labelled A to E, to identify the group, and 1 to 25, to identify each pot within a letter group. On May 13th, a freshly picked oak leaf was placed in each of the pots of group A. About 0.5 gm. of basal plain diet was placed in each of the pots of the B series and about 0.5 gm. of the 10%, 1.0% and 0.01% tannin diets in each of the pots of the groups C, D and E respectively. The pots with lids upturned beside them, were then further disinfected by exposure to ultraviolet light in a sterilised plastic chamber for one hour, to remove any surface contamination of the diets.

From a stock of 150 winter moth larvae, reared from eggs inthe laboratory on a diet of young oak leaves, 125 were removed and placed individually at random in the experimental pots. The larvae were mostly in their 4th instar, though a few had moulted into the 5th instar. 4th instar larvae were used in the experiment, since there would be no danger of mortality due to drowning in the surface moisture of the diets and since regular handling for weighing would have been hazardous for the delicate earlier instars. Furthermore, rapid and accurate weighing of winter moth larvae only becomes possible when the 4th instar is reached, corresponding initially to a weight of about 4 mg. It was considered that little would be lost by commencing experiments with 4th instar larvae, since about three-quarters of the weight of a fully-grown winter moth caterpillar (45 mg. out of about 60 mg.) is acquired during the fifth (last) instar alone.

Before being placed on its diet, each larva was weighed and then

Appendix 20 (continued)

lightly brushed with 80% ethanol, using a paint brush, to reduce contamination by micro-organisms to a minimum. A disc of Whatman No. 1 filter paper, previously disinfected by exposre to ultra-violet light, was then placed over each pot and the lids were then snapped into position. The pots were inverted, to allow larval frass to drop on to the filter papers. The filter papers served the additional function of helping to standardise the humidity inthe pots. A pot, lid and filter paper disc, together with two complete pots containing diets and larvae, is shown in Figure 25 (p.85(a)).

The five series of pots were intermingled, to ensure similar conditions of temperature, light and humidity, and placed on a wire mesh tray, raised from the supporting bench to allow free circulation of air into the inverted pots. The tray was placed inthe laboratory at room temperature, away from direct sunlight, which tends to cause heavy condensation on the sides of the pots.

At intervals of two or three days, all larvae were individually weighed and disinfected with 80% ethanol before being replaced on their diets. At each weighing, filter papers were removed and replaced by fresh, disinfected, papers. Also at each weighing, the oak leaves in the group A pots were replaced by fresh oak leaves, collected from Wytham shortly beforehand.

In spite of the precautions, fungus appeared in some of the pots after about 10 days. In view of this, all larvae were transferred to a completely fresh set of pots, containing fresh diet (or a fresh oak

Appendix 20 (continued)

leaf). It was also evident that the diets tended to dry out gradually, apparently altering their texture. This effect was chiefly noted on the 10% tannin diet. To minimise the dessication, two drops of water were placed on all the fresh filter paper discs after each weighing. Any diet which seemed to be drying out was replaced with fresh diet.

2. Results

The weights of the larvae in each series are shown for each weighing date in Tables 28 to 32 (pages 219a to 219e). Most larvae had pupated by the end of May, though weighings of pupae were continued until June 25th.

The mean larval and pupal weights on all five diets are given for each weighing date in Table 10 (p. 86(b)), together with the number of larvae included in each sample. To render the results more homogeneous, larvae with initial weights of less than 5 mg. or more than 24.9 mg. were excluded from calculation of means and from subsequent statistical analysis. These few larvae would bias the results out of proportion to their number, since they tended to fare excessively badly or excessively well, respectively.

3. Comparison of pupal weights attained by winter moth larvae fed on basal artificial diet and those attained on fresh oak leaves

The mean pupal weight attained by larvae fed on the basal artificial diet and that attained by larvae fed on fresh oak leaves were compared as follows, using the data for June 5th (Table 10, p. 86(b)).

TABLE 28 SERIES A Weight Increase of Winter Moth Larvae on Fresh Oak Leaves (1964)

LARVAL WEIGHT (mg.)

Date (1964):	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6	25/6
A 1	21.2	23.5	61.6	66.8	49.9	42.9	34.6*	31.5*	29.9*	25.4*
A 2	16.7	22.0	58.5	54.7	43.7	38.3	34.1*	33.8*	33.6*	33.2*
A 3	11.9	14.3	39.2	45.0	42.0	31.5	27.6*	27.2*	26.9*	26.0*
A 4	9.9	14.2	58.3	59.6	44.3*	40.3*	34.6*	32.7*	31.3*	26.9*
(A 5)	33.2	54.6	41.4	40.0*	38.1*	37.5*	37.4*	37.3*	37.2*	36.7*
A 6	5.3	16.4	56.9	61.5	52.4*	37.2*	37.3*	d		
A 7	6.4	15.6	58.9	50.7	39.3*	34.3*	30.7*	30.3*	30.2*	29.6*
A 8	7.6	20.5	72.3	60.8	46.6*	42.6*	36.9*	32.2*	30.8*	26.7*
A 9	9.3	19.3	61.5	54.1	40.9*	34.4*	29.2*	29.0*	28.7*	28.3*
A 10	6.5	18.2	60.4	50.8	42.1*	34.4*	31.3*	31.4*	31.4*	30.8*
A 11	7.7	16.2	64.5	63.1	49.1*	37.3*	31.5*	27.3*	24.9*	19.0*
(A 12)	4.0	18.9	22.1	55.8	58.9	43.1	36.8*	33.5*	33.5*	32.7*
A 13	11.1	11.6	23.4	26.1	38.8	34.1	29.3	24.3*	22.9*	19.9*
A 14	18.0	25.9	63.1	57.5	46.1*	40.2*	36.2*	35.6*	35.3*	33.4*
A 15	12.2	16.3	51.5	49.3	36.4*	31.9*	28.6*	28.3*	28.1*	27.5*
A 16	16.9	25.4	66.1	56.8	43.5*	37.3*	32.8*	32.3*	31.8*	30.4*
A 17	18.5	44.4	50,8	39.0	37.1*	34.5*	34.4*	34.0*	34.1*	33.6*
A 18	15.7	19.1	58.5	54.8	41.6*	37.4*	d			
A 19	21.3	44.6	70.7	55.0	46.3*	43.0*	42.2*	41.8*	41.8*	41.1*
A 20	21.0	33.8	59.4	47.9	40.1	37.8	d			

* Pupa ( ) Larvae excluded from analysis d = dead

219(a)

TABLE 29 SERIES B Weight Increase of Winter Moth Larvae on Plain Diet Control.

LARVAL WEIGHT (mg.)

Date (1964):	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6	25/6
B 1	15.7	17.8	29.1	37.4	38.7	29.5	26.7*	26.3*	25.9*	25.8*
B 2	20.4	51.3	63.5	53.7	41.2	37.7*	35.3*	33.9*	32.8*	30.5*
(B 3)	4.1	3.1	2.6	d						
(B 4)	37.9	89.5	78.4	52.4*	48.1*	43.5*	43.5*	42.8*	43.1*	42.4 *
B 5	19.3	60.5	46.3	34.0	31.8*	27.8*	27.5*	27.2*	27.2*	26.3*
B 6	16.0	37.7	57.2	55.4	42.6	33.7	31.6*	31.2*	30.9*	30.3*
(B 7)	26.9	57.8	60.1	49.4	41.9	27.2*	dl			
B 8	7.3	18.9	30.2	45.9	46.6	33.3	d			
B 9	9.3	19.0	38.1	50.4	57.3	45.8	42.4	d		
B 10	5.2	8.6	17.9	19.0	30.6	44.9	29.6	24.1*	23.2*	22.2*
(B 11)	9.3	dl								
B 12	11.0	23.2	39.4	53.9	44.2	31.3	26.8	d		
B 13	14.6	14.2	22.0	24.2	30.2	53.8	36.1	32.4*	31.9*	23.8*
(B 14)	3.9	3.8	11.0	13.0	20.4	46.5	36.9	24.7	22.1*	18.7*
B 15	11.0	26.9	27.2	60.6	85.4	68.3	44.4	40.6*	40.2*	39.0*
B 16	16.9	15.0	27.7	36.7	42.8	28.1	24.7	d		
B 17	14.6	17.6	49.2	55.8	45.5	36.3	31.0	d		
(B18)	26.1	47.5	61.3	49.1	36.5*	32.8*	30.3*	29.0*	28.1*	25.7*
(B 19)	25.7	57.3	80.0	58.2	41.8*	36.6*	34.9*	34.8*	34.5*	34.3*
B 20	20.2	23.8	32.4	36.0	33.9	24.3	19.1	d		

d = dead * Pupa 1 Death due to experimental accident. ( ) Larvae excluded from calculation of Means (see text).

219(b).

TABLE 30 SERIES C Weight Increase of Winter Moth Larvae on 10% Oak Tannin Diet

LARVAL WEIGHT (mg.)

Date (1964):	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6	25/6
c										
C 1	7.7	17.5	24.0	31.6	43.4	42.8	24.1	d		
C 2	21.2	28.0	37.0	40.5	54.5	41.4	31.6	29.1*	28.8*	28.4*
C 3	19.5	25.1	25.3	24.3	26.8	19.1	d			
(C 4)	42.9	53.2	36.9	30.8*	30.7*	29.4	d			
C 5	17.7	12.7	d							
C 6	20.4	21.3	17.1	27.9	43.5	32.1	26.0	21.7*	21.4*	20.3*
C 7	6.2	17.4	19.9	27.7	36.9	54.2	39.5	26.3	24.5*	13.0*
C 8	5.9	3.4	d							
(C 9)	26.2	36.5	31.5	33.8	27.8	22.5	21.2	d		
C 10	15.4	17.5	18.2	23.3	40.9	32.7	23.9	20.9*	17.1*	8.9*
(C 11)	2.6	11.9	d							
C 12	11.0	10.7	10.3	14.2	32.4	32.1	17.6	15.3*	14.1*	12.6*
C 13	11.0	12.9	21.4	30.8	39.1	35.6	24.4	21.7*	21.4*	20.9*
C 14	12.1	14.7	25.9	36.7	42.2	25.3	22.4	d		
C 15	10.5	9.3	8.5	d						
C 16	7.6	13.1	14.5	21.4	43.5	54.5	40.8	27.3	22.0	9.1
C 17	12.1	9.9	9.0	8.0	13.4	18.6	17.1	14.6	20.5	d
(C 18)	26.1	26.0	34.7	36.6	31.6	24.9	21.9*	21.0*	20.4*	19.2*
C 19	6.6	5.4	3.8	d						
C 20	20.2	27.9	37.8	47.7	42.2	34.8	30.8*	24.1*	21.4*	13.4*

219(c).

* Pupa ( ) Larvae excluded from sample means (see text) d = dead

TABLE 31 SERIES D Weight Increase of Winter Moth Larvae on 1% Oak Tannin Diet

LARVAL WEIGHT (mg.)

Date (1964):	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6	25/6
D 1	15.6	35.1	38.0	37.3	32.1	27.4	23.8*	23.1*	22.9*	21.5*
D 2	21.5	48.5	47.9	43.5	34.7	32.6	26.3*	d		
D 3	9.4	20.1	25.5	45.5	50.1	36.9	31.9	d		
D 4	18.5	45.6	57.5	45.1	34.6	31.4*	30.1*	29.6*	29.1*	27.5*
D 5	20.1	34.8	40.3	51.8	45.1	33.5	26.0*	22.5*	20.3*	15.0*
D 6	19.5	10.0	53.5	47.8	35.1*	30.5*	26.2*	d		
D 7	18.5	32.9	32.0	33.8	28.8	23.1	18.6*	17.9*	17.9*	17.1*
D 8	8.7	14.1	16.4	26.5	44.4	36.2	25.4*	23.1*	22.5*	21.2*
(D 9)	3.9	6.8	9.9	14.7	13.4	d				
D 10	8.0	18.6	26.4	35.2	39.7	37.1	28.3	17.0	d	
D 11	14.1	14.1	19.3	25.6	30.3	29.8	23.8	18.1*	15.4*	13.5*
D 12	5.3	19.5	9.7	10.5	21.6	22.1	23.1	20.3	d	
D 13	9.0	18.3	30.0	31.0	40.7	41.1	31.7	25.6*	20.6*	8.9*
D 14	16.8	17.9	29.5	35.4	49.6	34.7=	29.6	26.3*	22.5*	17.1*
D 15	16.4	23.3	40.3	43.7	43.4	31.5	22.8*	d		
D 16	16.0	25.1	43.7	51.6	42.4	31.1	27.7*	22.4*	20.8*	17.0*
D 17	18.4	26.2	36.6	45.2	51.1	35.1	29.3*	27.1*	26.3*	25.1*
D 18	19.8	44.8	54.2	37.6	27.9	25.6*	24.9*	24.4*	24.3*	22.9*
(D 19)										
D.20	22.4	46.8	71.7	50.8	39.8	30.0	27.7*	27.1*	26.5*	25.2*

219(d).

* Pupa ( ) Larvae excluded from sample means

TABLE 32 SERIES E Weight Increase of Winter Moth Larvae on 0.1% Oak Tannin Diet

Date (1964):	13/5	16/5	18/5	20/5	22/5	25/5	28/5	1/6	5/6	25/6
E 1	18.9	22.1	21.8	19.3	27.2	18.7	15.8*	14.5*	14.4*	13.7*
E 2	16.9	32.4	33.8	31.6	33.2	24.8	22.4	21.9*	20.9*	20.3*
E 3	22.5	63.2	54.3	44.3	42.4*	39.7*	39.4*	38.1*	39.2*	35.7*
E 4	8.3	16.5	30.7	39.9	31.7	22.2	19.1*	d		
E 5	19.6	58.9	48.1	35.3	32.2*	29.8*	30.0*	29.9*	29.6*	29.4*
E 6	19.5	54.6	60.2	44.3	35.5*	31.8*	29.6*	28.4*	27.5*	25.2*
E 7	20.2	58.0	61.0	43.4	35.8*	31.5*	3113*	31.3*	30.7*	30.4*
E 8	15.2	30.0	48.2	43.9	33.7*	29.[illegible]*	25.7*	23.5*	22.6*	20.3*
E 9	6.6	30.0	28.6	47.2	59.6	59.6	36.3	30.6*	30.2*	28.9*
E 10	6.3	18.8	20.4	27.2	42.3	40.7	30.8	d		
E 11	11.9	25.9	42.3	51.5	43.4	35.5	31.2*	28.7*	28.1*	25.7*
(E 12)										
(E 13)	1.9	3.4	3.3	7.9	14.9	d				
E 14	13.8	14.2	16.3	21.5	45.5	36.3	27.5	24.9*	24.4*	22.8*
E 15	16.6	17.7	26.0	43.8	52.5	38.2	32.6*	28.7*	28.9*	27.2*
E 16	12.0	17.0	42.7	56.8	39.8	31.5	21.3	d		
E 17	6.4	11.3	12.2	14.7	33.0	35.4	27.9	23.5*	21.4*	19.3*
E 18	[illegible]0.3	43.0	65.2	57.5	43.6*	39.5*	36.6*	36.6*	36.1*	35.5*
E 19	16.2	32.3	47.9	50.1	43.7*	33.7*	28.9	20.9*	17.6*	10.6*
(E 20)	28.6	57.7	66.9	49.5	36.2*	31.9*	31.5*	31.4*	31.1*	30.7*

* Pupa ( ) Larvae excluded from calculation of Means (see text).

219(e).

Appendix 20 (continued)

1) The variance of the two samples were first compared by an F-test (Bailey, 1959), as already described in Appendix 7 (p.170).

For the A series (oak leaves),

$\bar{x}$ = 30.8 n = 15 s^2 = 20.49

For the B series (basal artificial diet),

$\bar{x}$ = 30.3 n = 7 s^2 = 31.09

Therefore F = 1.52. The value of F, corresponding to 1% significance and 6 and 14 degrees of freedom, was found from standard tables to be 5.26 (Pearson and Hartley, 1962). No significant difference can therefore be detected between the sample variances.

2) The difference between the pupal weight means on the two diets was now tested for significance, using the t-test for small samples of equal variance (Bailey, loc. cit.), already described in Appendix 7 (p.170).

Pooled variance = 23.672

Therefore, t = 0.224

The value of t corresponding to 10% significance and 20 degrees of freedom was found from standard tables to be 1.725 (Bailey, loc. cit.). No significant difference can therefore be detected between the pupal weights attained by larvae fed on fresh oak leaves and those attained by larvae fed on the basal plain artificial diet.

4. Comparison of pupal weights attained by winter moth larvae fed on tannin diets and those attained on the basal artificial diet.

The mean pupal weights attained by larvae fed on the three

Appendix 20 (continued)

tannin diets and by those fed on the basal artificial diet (Table 10, p.866)) were compared as follows by analysis of variance (Bailey, loc. cit.) to determine whether any of the four means was significantly different from any of the other three. The data for June 5th were again used. The relevant data for the analysis are given in Table 33 (p.2216).

G = Total sum of all observations = 212.1 + 148.7 + 269.1 + 370.6

= 1,000.5

N = Total number of observations = 40

Therefore, $\frac{G^2}{N} = 25,025.006$

The total sum of squares about the general mean

$= (25.9^2 + 32.8^2 + \ldots.) + (28.8^2 + \ldots) + (22.9^2 + \ldots) + (14.4^2 + \ldots)$

$- 25,025.006$

$= 1,490.60$

The sum of squares between varieties

$= \frac{(212.1)^2}{7} + \frac{(370.6)^2}{14} + \frac{(269.1)^2}{12} + \frac{(148.7)^2}{7} - 25,025.006$

$= 405.31$

TABLE 43 Data for analysis of variance of pupal weight means (1964 experiment)

Diet :	B	C	D	E
Observed	25.9	28.8	22.9	14.4
pupal	32.8	21.4	29.1	20.9
weights	27.2	24.5	20.3	39.2
(mg.)	30.9	17.1	17.9	29.6
June 5th.	23.2	14.1	22.5	27.5
	31.9	21.4	15.4	30.7
	40.2	21.4	20.6	22.6
			22.5	30.2
			20.8	28.1
			26.3	24.4
			24.3	27.9
			26.5	21.4
				36.1
				17.6
Sample size :	7	7	12	14
Sum Σx	212.1	148.7	269.1	370.6
Mean $\bar{x}$	30.3	21.3	22.4	26.5

B = Basal artificial diet (control).
C = Diet containing 10% oak leaf tannin.
D = Diet containing 1.0% oak leaf tannin.
E = Diet containing 0.1% oak leaf tannin.

Appendix 20 (continued)

The analysis of variance table can therefore be set out as follows:

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Varieties	405.31	3	135.10	4.48
Residual (by difference)	1,085.29	36	30.15	-
Total	1,490.60	39	-	-

The values of F obtained from standard tables for 3 and 40 degrees of freedom were found to be 2.84 for 5% significance and 4.31 for 1% significance. It can therefore be concluded that there exists a real difference between at least two of the four sets of pupal weights.

Differences between each of the mean pupal weights attained on the tannin diets were now compared with the mean pupal weight reached on the basal plain diet by using the Student's t-test (see Appendix 7 for method) and making use of the pooled variance (30.15), calculated above from all the available data.

a) Comparison of B series (plain diet) and E series (0.1% tannin diet)

$$t = \frac{30.3 - 26.5}{5.49\sqrt{\frac{1}{7} + \frac{1}{14}}} = 1.495, \text{ with 36 degrees of freedom.}$$

Therefore the difference between the pupal weights attained on the plain and 0.1% tannin diets is not significant.

Appendix 20 (continued)

b) Comparison of B series (plain diet) and D series (1.0% tannin diet)

$$t = \frac{30.3 - 22.4}{5.49\sqrt{\frac{1}{7} + \frac{1}{12}}} = 3.029, \text{ with 36 degrees of freedom.}$$

Therefore the difference between the pupal weights attained on the plain and 1.0% tannin diets is statistically significant ($P < 0.01$)

c) Comparison of B series (plain diet) and C series (10% tannin diet)

$$t = \frac{30.3 - 21.3}{5.49\sqrt{\frac{1}{7} + \frac{1}{7}}} = 3.064, \text{ with 36 degrees of freedom}$$

Therefore the difference between the pupal weights attained on the plain and 10.0% tannin diets is statistically significant ($P < 0.01$)

5. Regression analysis

Using the data from June 5th (Table 10, p.86(b)), the regression of $\log_{10}$ pupal weight on $\log_{10}$ tannin concentration in the diet was analysed as follows (Bailey, loc. cit.)

The tannin concentrations (0.1, 1.0 and 10%) are in the ratio 1 : 2 : 3 when expressed as logarithms and, for convenience, these were taken as the values for x. The data for the analysis, derived from the pupal weights for June 5th, given in Tables 30, 31 and 32, are as follows:

Appendix 20 (continued)

Diet :	Series E (0.1% tannin)	Series D (1.0% tannin)	Series E (10% tannin)
x :	1	2	3
y (log. pupal weights) :	1.1584	1.3598	1.4594
	1.3201	1.4639	1.3304
	1.5933	1.3075	1.3892
	1.4713	1.2529	1.2330
	1.4393	1.3522	1.1492
	1.4871	1.3181	1.3304
	1.3541	1.4200	1.3304
	1.4800	1.3856	
	1.4487	1.4232	
	1.3874	1.3522	
	1.4456	1.1875	
	1.3304	1.3139	
	1.5575		
	1.2455		
$\sum y$:	19.7187	16.1368	9.2220

From this data, using the method described by Bailey (loc. cit.), the calculated regression line was found to be:

$$y = 1.452 - 0.0479x$$

The calculated regression line, together with the original data, is shown in Figure 28 (p.88(a)).

A Student's t-test (Bailey, 1959) was used to determine whether or not the calculated gradient of the regression line ($- 0.0479 \pm 0.0226$) was significantly different from zero (i.e. horizontal). The value of t was found to be -2.117. Therefore the difference between the regression gradient and the horizontal is significant at the 5% level,

Appendix 21: Comparison of fresh weight increase of winter moth larvae fed on different diets - experimental procedure and results, 1965 season

1. Experimental procedure

Tannin was extracted from May and July oak leaves (stored in the deep-freeze since the previous year) by the method of Brown and Love (1961). About 2 gm. of tannin was obtained from each batch of leaves, after dialysis and freeze-drying. Fractions (P) and (S) were not separated.

(+)-catechin was obtained from Koch Light Ltd., and gallic acid from British Drug Houses, Ltd.

500 gm. of artificial diet were prepared by the method described in Appendix 19, including the modifications mentioned in Chapter 5 (p. 89). 100 gm. of the diet was allowed to set at once. The remainder was used to prepare diets containing 1% May tannin, 1% July tannin, 1% (+)-catechin and 1% gallic acid, following a similar procedure to that used in 1964 (Appendix 20). These diets were then allowed to set in separate autoclaved containers.

125 of the standard plastic pots, previously disinfected with 80% ethanol, were labelled as follows: Series M (May tannin diet), Series J (July tannin diet), Series C (catechin diet) and Series P (plain basal diet). 25 pots were assigned to each series and 0.5 gm. of each diet placed in all the pots of the appropriate series.

From 150 winter moth larvae, reared from eggs in the laboratory on young oak leaves, 125 were removed on May 11th, 1965, weighed and

Appendix 21 (continued)

placed individually at random in the 125 pots, so that, in all, 25 larvae were placed on each of the five diets. The pots were intermingled and placed in the constant humidity chamber (p. 92), at room temperature, out of direct sunlight.

At intervals of a few days, the larvae were re-weighed and replaced on their diets. In spite of precautions, fungus growth developed on the diets and was not eliminated by replacement of the affected diets. A complete new series of containers and diets were therefore prepared and these, after disinfection by ultraviolet light, were used to replace the original set of pots on May 28th.

2. Results

The weights of the larvae from each series and on each weighing date are shown in Tables 34 to 38 (pages 226a to 226e). Mortality was such that weighing was discontinued after June 4th. The mean weights on the five diets (up to June 1st) are shown in Table 11 (p. 93(a)). Only those larvae which survived until at least May 24th were included in the means and in the subsequent analysis.

3. Comparison of fresh weight increase by winter moth larvae fed on the five diets

The mean 'growth increments' of the larvae on each of the five diets were compared by analysis of variance (Bailey, 1959), following the method described in Appendix 20. The growth increments were calculated from the data in Tables 34 to 38 as the difference between the peak fresh weight, reached by each larva, and its fresh weight at the

TABLE 34 Fresh weight change (mg.) of winter moth larvae fed on diet containing 1% (+)-catechin (C Series; 1965)

Date:	11/5	18/5	21/5	24/5	28/5	1/6	4/6
C1	8.8	22.4	24.9	26.0	27.2	27.6	18.7
C2	6.0	17.6	19.6	19.5	14.2	d	
(C3)	4.3	10.7	10.7	d			
C4	6.6	20.0	19.2	21.8	12.6	d	
(C5)	7.6	18.5	d				
(C6)	4.7	d					
C7	10.8	33.0	34.9	30.3	15.9	15.4	d
C8	10.4	36.0	34.3	32.1	28.0	d	
C9	6.2	14.0	17.7	18.9	16.3	d	
(C10)	5.0	d					
C11	7.5	21.4	26.2	29.6	13.5	d	
(C12)	13.8	10.3	d				
(C13)	9.2	d					
C14	6.8	15.0	10.3	18.0	d		
C15	7.9	23.3	27.7	24.9	11.3	10.2	d
C16	8.1	21.5	24.8	24.8	12.7	d	
C17	7.2	16.0	24.2	24.7	25.2	21.8	18.9
C18	5.2	15.6	17.5	18.3	17.8	12.9	d
C19	8.4	20.0	26.2	23.4	23.9	19.8	d
C20	7.7	22.8	27.8	27.1	16.4	12.7	d
C21	9.8	28.9	34.0	34.3	33.7	24.7	d
C22	5.9	16.8	18.4	16.4	14.2	d	
(C23)	4.7	d					
C24	6.3	18.0	21.4	17.7	11.4	d	
C25	5.2	14.8	18.7	16.0	9.6	d	

d = dead; ( ) = Larva excluded from analysis

TABLE 35 Fresh weight change (mg.) of winter moth larvae fed on diet containing 1% Gallic acid (G Series; 1965)

Date:	11/5	18/5	21/5	24/5	28/5	1/6	4/6
(G1)	5.1	10.6	d				
G2	10.0	27.8	31.1	34.3	19.5	14.6	12.7
(G3)	6.2	16.2	16.7	d			
G4	6.8	15.0	15.8	18.3	16.3	14.7	10.8
G5	7.6	17.2	23.3	21.0	15.0	d	
G6	7.9	20.3	23.4	23.8	22.2	11.3	d
G7	10.5	32.0	35.0	37.2	37.8	32.3	19.6
(G8)	5.0	14.1	d				
G9	8.3	19.7	25.9	22.8	18.7	d	
G10	7.4	18.0	22.0	21.4	21.9	d	
G11	8.6	24.6	30.3	31.1	d		
G12	6.8	16.4	22.5	25.5	16.9	11.9	d
(G13)	7.4	12.0	17.3	d			
G14	8.7	30.8	32.3	30.5	20.8	17.4	d
G15	5.6	10.4	10.0	10.3	12.2	10.5	8.2
G16	8.7	18.0	25.9	23.8	23.6	18.3	d
(G17)	6.4	20.0	d				
G18	5.7	15.3	18.5	21.3	22.4	18.0	13.8
(G19)	4.6	d					
G20	4.9	14.2	16.0	17.9	19.1	16.2	d
G21	4.6	10.7	12.2	13.4	13.4	d	
(G22)	5.7	10.7	12.0	d			
(G23)	4.5	7.9	d				
G24	10.8	32.4	39.5	33.2	d		
G25	9.0	20.0	27.3	34.5	26.6	18.4	d

d = dead; ( ) = Larva excluded from analysis

TABLE 36 Fresh weight change (mg.) of winter moth larvae fed on diet containing 1% May oak leaf tannin (M Series; 1965)

Date:	11/5	18/5	21/5	24/5	28/5	1/6	4/6
M1	10.6	30.0	27.9	24.9	21.9	19.7	d
M2	7.7	21.4	18.2	14.1	d		
M3	6.9	14.7	15.2	14.7	11.3	d	
M4	10.6	33.6	29.9	25.8	24.3	14.3	d
M5	9.5	36.8	41.9	34.9	24.8	20.7	18.6
(M6)	6.5	18.6	d				
M7	7.3	17.8	18.2	16.0	14.2	14.2	12.2
M8	11.9	51.3	45.3	44.0	26.9	23.4	d
M9	8.7	21.8	24.6	26.9	19.4	14.9	d
(M10)	6.4	d					
M11	11.7	39.4	34.2	36.8	d		
M12	7.6	32.3	26.0	23.2	12.4	d	
M13	5.2	14.3	15.1	16.3	14.5	10.8	d
M14	4.3	10.0	12.7	13.9	12.2	9.9	d
M15	5.9	17.0	17.2	13.1	d		
M16	6.3	12.8	15.1	11.9	7.5	d	
(M17)	4.6	10.9	10.9	d			
M18	5.2	17.9	15.7	15.2	13.7	d	
M19	5.1	16.2	20.3	20.0	13.3	d	
M20	6.3	17.4	20.2	18.2	13.6	d	
M21	6.1	15.2	20.1	16.7	21.8	19.4	13.7
M22	7.6	20.0	23.8	22.4	20.4	d	
M23	5.7	20.5	22.6	18.6	18.9	15.8	d
M24	4.2	13.0	16.0	16.2	17.1	12.1	9.5
M25	3.6	13.1	14.9	17.6	13.7	10.6	8.9

d = dead; ( ) = Larva excluded from analysis

TABLE 37 Fresh weight change (mg.) of winter moth larvae fed on diet containing 1% July oak leaf tannin (J Series; 1965)

Date:	11/5	18/5	21/5	24/5	28/5	1/6	4/6
J1	6.7	15.9	18.6	15.8	8.3	d	
J2	10.0	30.0	29.8	23.7	15.0	14.1p	12.6p
J3	6.8	19.6	18.0	14.9	d		
J4	4.7	13.0	15.2	15.8	12.4	10.4	d
J5	5.7	13.2	13.9	16.7	12.0	d	
J6	6.0	15.8	18.3	20.2	20.0	16.8	d
(J7)	6.1	d					
J8	9.0	22.0	19.5	17.3	12.3	d	
J9	6.0	17.3	17.0	15.5	11.5	d	
J10	8.0	25.3	25.4	24.9	23.4	14.3	d
J11	7.0	14.4	10.8	17.3	16.7	12.4	d
J12	9.8	37.8	40.9	42.1	29.6	28.8	d
J13	6.0	16.0	21.8	25.2	22.7	d	
J14	6.7	22.0	21.1	21.2	16.2	d	
J15	6.3	18.3	21.5	21.4	15.0	d	
J16	5.8	14.8	21.2	18.6	22.6	17.9	d
J17	7.4	21.7	24.1	24.3	20.5	13.9	d
(J18)	4.5	d					
J19	6.9	15.2	21.8	18.4	15.5	d	
J20	4.0	14.6	17.9	15.7	d		
J21	6.5	14.1	15.8	18.2	18.8	16.4	d
J22	7.1	16.4	21.4	22.0	20.6	16.8	d
J23	4.6	13.7	20.7	23.9	19.4	d	
J24	9.6	31.4	28.1	27.1	22.2	d	
J25	4.7	10.5	12.6	14.8	10.3	d	

p = pupa; d = dead; ( ) = Larva excluded from analysis

TABLE 38 Fresh weight change (mg.) of winter moth larvae fed on plain basal artificial diet (P Series; 1965)

Date:	11/5	18/5	21/5	24/5	28/5	1/6	4/6
P 1	5.8	15.8	19.3	21.8	22.5	14.9	14.0
(P 2)	5.4	d					
P 3	11.2	35.6	35.6	25.1	18.7	d	
P 4	8.4	12.9	17.2	23.2	21.8	14.3	d
P 5	9.8	36.2	42.0	35.9	26.4	24.2	d
P 6	11.9	38.6	41.0	31.4	29.0	20.0p	19.7p
P 7	5.2	15.6	20.3	24.2	21.9	21.4	16.2
P 8	6.9	14.0	22.1	24.9	29.3	25.9	22.4
P 9	8.5	21.5	24.7	19.1	19.1	15.6	d
P 10	9.8	33.5	36.2	33.1	27.5	d	
P 11	10.9	35.3	50.2	50.3	33.7	32.8	d
P 12	8.0	25.5	30.7	36.0	23.0	16.7	15.4
P 13	5.6	11.0	14.8	17.9	17.7	12.2	d
P 14	8.3	23.2	32.0	31.7	d		
P 15	5.4	14.3	17.2	23.2	20.8	18.8	15.0
P 16	5.2	14.5	16.8	15.3	d		
P 17	4.8	11.5	11.0	12.4	9.9	d	
P 18	7.6	25.1	26.2	29.8	22.8	13.4	d
P 19	9.6	32.2	40.7	44.7	27.0	22.0	d
P 20	4.8	13.4	13.6	17.2	18.7	12.3	d
P 21	7.3	22.0	31.9	31.9	22.2	17.0	d
(P 22)	5.6	d					
P 23	6.1	12.9	16.7	23.1	26.3	24.4	13.4
P 24	5.7	13.6	15.2	17.0	22.1	d	
P 25	5.8	16.0	22.8	28.3	25.4	18.2	15.9

p = pupa d = dead ( ) Larva excluded from analysis.

Appendix 21 (continued)

start of the experiment. As with the calculation of the weight means, unhealthy larvae, which did not survive until May 24th, were eliminated from the analysis.

The growth increments, calculated for all the larvae eligible for analysis, are shown in Table 12 (p. 95(a)), together with their means. Using these data, the analysis of variance table was drawn up as follows, using the same procedure as that adopted the previous year (Appendix 20):

Source of Variation	Sum of squares	Degrees of Freedom	Mean Square	Variance Ratio
Varieties	464.51	4	116.13	2.729
Residual	4,170.22	98	42.55	
Total	4,634.73	102		

The value of the variance ratio corresponding to 5% significance and 4 and 60 degrees of freedom was found from the standard table to be 2.53 (Bailey, loc. cit.). It was concluded, therefore, that there exists a real difference between at least two of the five mean growth increments.

The differences between the mean growth increments achieved on each of the four diets containing additivies and the value attained on the basal artificial diet (control) were now examined by the Student's t-test, using the pooled variance (42.55), calculated above from all the available data.

a) Difference between increments on July tannin diet and control

$$t = \frac{21.6 - 15.5}{6.523\sqrt{\frac{1}{23} + \frac{1}{23}}} = 3.171, \text{ with 98 degrees of freedom}$$

Appendix 21 (continued)

The difference between the fresh weight increase of winter moth larvae fed on the 1% July tannin diet and the fresh weight increase attained on the basal control diet is therefore statistically significant ($P < 0.005$).

b) Difference between increments on May tannin diet and control

$$t = \frac{21.6 - 17.1}{6.523\sqrt{\frac{1}{23} + \frac{1}{22}}} = 2.314 \text{ with } 98 \text{ degrees of freedom}$$

The difference between the fresh weight increase of winter moth larvae fed on the May tannin diet and the fresh weight increase achieved on the control diet is therefore statistically significant ($P < 0.05$; 2-tailed test).

c) Difference between increments on catechin diet and control

$$t = \frac{21.6 - 17.6}{6.523\sqrt{\frac{1}{23} + \frac{1}{18}}} = 1.949 \text{ with } 98 \text{ degrees of freedom}$$

The difference between the fresh weight increase of the larvae fed on the (+)-catechin diet and the increase achieved on the control diet is therefore statistically significant ($P < 0.05$; 1-tailed test). (The use of a one-tailed test was considered to be valid, since a decrease, rather than an increase, in fresh weight increments on the catechin diet was expected.)

Appendix 21 (continued)

d) Difference between increments on gallic acid diet and control

$$t = \frac{21.6 - 18.2}{6.523\sqrt{\frac{1}{23} + \frac{1}{17}}} = 1.630 \text{ with 98 degrees of freedom}$$

No significant difference can therefore be detected between the fresh weight increments on the gallic acid and control diets.

Appendix 22: Effect on the fresh weight increase of winter moth larvae of tanning the dietary protein - experimental procedure and results, 1966 season

1. Experimental procedure

a) Preparation of casein/tannin complex.

7.0 gm. of casein ('Light White Soluble', British Drug Houses, Ltd.) were dissolved in 50 ml. of distilled water and mixed with a solution of 3.5 gm. oak leaf tannin (Fraction (S) only, extracted from July oak leaves by the method of Brown and Love, 1961), in 20 ml. of distilled water. The resulting solution was brought to pH 5.0 by adding, dropwise, a saturated aqueous solution of citric acid, while monitoring the acidity with a pH meter. The resulting suspension of brown flocculent complex was stirred for 5 minutes and then sedimented in a centrifuge (4,000 r.p.m. for 5 minutes). The supernatant was found to give no further precipitate with citric acid, even at pH 3.5, showing that all the casein had been incorporated in the sedimented complex. Furthermore, the supernatant was pale brown in colour, indicating the presence of excess tannin. The precipitate of casein/tannin complex was washed with citric acid solution at pH 5.0, before incorporation in the experimental diet.

b) Preparation of diets

100 gm. of experimental and control diets were prepared, using the methods already described in previous appendices. The new modified diet, derived from the Cambridge and Canadian formulae, was used.(Chapter 5 and

Appendix 22 (continued)

Appendix 19). The only differences between the control and experimental diets were as follows:

	Control Diet	Experimental diet
Casein	7.0 gm.	7.0 gm.[1]
Oak leaf tannin	-	3.5 gm.[1]
Cellulose powder	5.8 gm.	2.3 gm.

([1] In the form of casein-tannin complex)

It can be seen that the content of cellulose powder in the control diet was increased by 3.5 gm. to compensate for the tannin included in the experimental diet. The tannin content in the experimental diet was probably slightly less than 3.5 gm., since a little tannin remained unprecipitated during the preparation of the complex. An extra 4 ml. of distilled water was added to the control diet, to compensate for the presence of approximately this amount of water in the sedimented casein-tannin complex.

The diets differed otherwise from the new modified formula only in that potassium hydroxide and formaldehyde were omitted. The diets were prepared by the new blending technique, though the casein and casein-tannin complexes were both included in group (a) as usual (p. 98). The usual precautions were taken against infection by micro-organisms. After pouring into autoclaved glass jars, both diets set to comparatively rigid gels.

Two series of 78 plastic pots, as used for previous experiments, were thoroughly washed and disinfected by immersion for several hours

Appendix 22 (continued)

in 80% ethanol. Small slabs of approximately 0.5 gm. of diet were then placed in each container, making two sets of 78 complete rearing pots, one set containing control diet and the other set the experimental diet. The pots were labelled T 1 to 78 (experimental diet) and C 1 to 78 (control diet). The diets were finally disinfected once more by exposure to ultraviolet light for one hour in a sterilised cabinet, to reduce any surface contamination.

The stock of winter moth eggs reserved the previous winter for this experiment was unfortunately not available, owing to circumstances beyond the author's control, so that it was necessary to collect larvae from the field. 200 3rd and 4th instar winter moth caterpillars were collected from oak and hazel trees in Wytham between May 8th and 15th, 1966. On May 15th, 156 of these larvae were individually weighed, placed in separate containers and arranged in a series of increasing weight. Beginning with those of lowest weight, successive pairs of larvae were removed from this series and assigned by the throw of a dice to the two diets. In this way, the experimental and control series could be made as comparable as possible, while including a randomised assignment of larvae.

All the larvae were surface-disinfected with a brush, dipped in 80% ethanol, and then transferred to their containers. A disc of Whatman No. 1 filter paper was then placed over each pot and the lids snapped into position. The pots were intermingled and placed on trays on a laboratory bench, away from direct sunlight.

Appendix 22 (continued)

The larvae were re-weighed at intervals of a few days and all diets and pots were renewed on June 1st. It was found that the diets showed very little tendency to dry out and faecal pellets did not tend to adhere to the surface of the diets. The experiment was discontinued on June 6th, since mortality had risen to such a level that no further useful information could be gained.

2. Results

The weights of the larvae on each of the six weighing dates are shown in Tables 39 and 40 (pages 233c and 233d). The mean weights on each date, with other statistical data, are given in Table 13 (p.1046). In calculating the means and in the subsequent analysis, only those larvae surviving until May 27th (i.e. for at least 12 days) were considered.

3. Comparison of fresh weight increments on control and experimental diets

Fresh weight increments were calculated for each larva as the difference between the maximum recorded weight attained up to and including May 27th and the fresh weight at the start of the experiment. They are listed in the final columns of Tables 39 and 40 for all the larvae eligible for inclusion in the analysis. The mean values of each of the two series of fresh weight increments were calculated and compared by the standard method based on the normal distribution for large samples (Bailey, 1959). The relevant data were as follows:

TABLE 39 Fresh weights (mg.) of winter moth larvae fed on basal artificial diet (control) in 1966, together with larval fresh weight increments (initial to peak weight).

Date:	15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increments
1	1.0	2.2	3.4	7.6	8.6	d	6.6
2	1.2	2.3	3.6	9.3	10.1	d	8.1
3	1.2	3.3	3.3	6.7	7.8	d	5.5
4	1.6	3.0	5.7	9.7	11.4	d	8.1
5	1.7	2.9	d.0				-
(6)	1.7	2.9	d				-
(7)	1.9	d					-
8	2.3	3.3	4.6	7.5	8.8	9.0	5.2
9	2.4	8.0	11.3	15.8	17.6	d	13.4
10	2.7	5.6	11.7	20.4	24.6	17.1[p]	17.7
11	2.7	4.7	7.9	11.6	10.8	d	8.9
(12)	2.7	d					-
13	2.8	4.9	9.3	17.0	16.8	d	14.2
14	3.0	5.1	9.4	16.3	16.6	d	13.3
15	3.1	4.9	10.6	16.7	19.3	14.5	13.6
16	3.2	4.6	7.3	11.4	19.2	14.7	8.2
17	3.2	7.0	10.8	9.0	12.2	12.5	7.6
18¾	3.3	8.3	13.1	27.3	25.8	d	24.0
19	3.7	9.5	15.6	36.8	38.7	22.3[p]	33.1
20	3.7	6.4	14.5	29.5	21.1	d	25.8
21	3.8	7.7	10.2	18.6	20.3	14.5	14.8
22	4.1	7.1	14.6	26.0	29.7	33.9	21.9
23	4.4	6.5	13.5	26.6	28.9	19.4	22.2
24	4.5	12.4	21.3	45.9	38.9	23.5[p]	41.4
25	4.5	11.8	20.8	35.1	27.9	d	30.6
26	4.5	12.6	20.7	32.2	26.1	16.9[p]	27.7
27	4.6	8.4	16.4	43.2	44.2	22.2	38.6
28	4.7	12.8	21.3	43.5	43.4	d	38.8

contd.

TABLE 39 (contd.)

	Date: 15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increment
29	4.8	13.9	22.6	34.5	21.2	d	29.7
30	5.0	10.9	20.6	33.2	34.6	23.2	28.2
(31)	5.1	15.1	6.7	d			-
32	5.1	15.0	28.1	54.5	36.2	d	49.4
(33)	5.2	d					-
(34)	5.3	12.6	16.8	d			-
35	5.4	5.6	8.2	10.7	13.9	d	5.3
36	5.4	7.5	10.0	13.6	17.6	19.6	8.2
37	5.8	12.0	22.5	35.2	29.3	17.4[p]	29.4
38	5.9	17.5	28.1	43.4	34.0	26.1[p]	37.5
39	5.9	10.6	19.0	33.5	27.1	d	27.6
40	6.1	11.9	18.9	27.2	16.5	d	21.1
41	6.3	13.3	18.3	18.8	d		12.5
42	6.6	13.8	25.8	33.6	26.7	16.2[p]	27.0
43	6.8	12.5	16.0	32.4	30.9	d	25.6
44	7.1	8.8	6.8	9.5	5.9	d	2.4
45	7.5	13.8	23.8	33.4	30.7	27.5	25.9
46	7.7	17.4	27.8	49.6	39.6	25.8[p]	41.9
47	7.7	16.0	24.9	35.3	25.2	d	27.6
48	7.7	14.4	23.9	27.6	17.3	d	19.9
(49)	7.8	15.1	d				-
50	7.8	14.3	24.4	38.7	31.3	22.6[p]	30.9
51	7.8	20.7	50.5	58.9	34.7	d	51.1
52	8.0	18.3	22.7	40.2	37.6	21.2[p]	32.2
53	8.1	14.8	30.6	35.2	23.7	d	27.1
54	8.1	17.5	45.2	50.1	24.0	d	42.0
55	8.2	16.7	22.7	26.8	23.5	d	18.6
(56)	8.3	17.7	16.5	par.			-
(57)	8.9	7.9	5.1	d			-

contd.

TABLE 39 (contd).

Date:	15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increment
(58)	8.9	18.0	d				-
59	9.0	15.3	37.6	24.5	19.8	d	28.6
60	9.0	16.4	30.4	37.2	21.2	d	28.2
61	9.3	14.8	23.0	29.2	25.4	d	19.9
62	9.4	15.8	33.0	46.2	31.4	25.6^{p}	36.8
63	9.4	18.8	17.0	34.4	21.3	d	25.0
64	9.6	17.4	41.7	47.3	27.8	d	37.7
65	9.9	19.5	46.2	45.8	29.3	23.2	36.3
66	9.9	17.0	34.8	40.4	27.3	d	30.5
67	10.1	16.9	36.4	31.7	22.9	d	26.3
68	10.5	21.1	41.2	59.1	44.1	d	48.5
69	11.2	20.0	45.1	50.5	31.4	d	39.3
70	11.4	18.9	39.4	48.3	26.6	d	36.9
(71)	11.7	par.					-
72	12.6	30.9	40.0	28.4	25.3	d	27.4
73	12.7	21.8	45.6	38.7	24.8	d	32.9
74	13.0	25.7	45.7	35.1	24.1	d	32.7
75	13.2	18.7	42.6	52.3	29.8	d	39.1
76	14.4	26.3	54.2	43.3	28.9	26.3^{p}	39.8
77	15.6	22.3	41.6	28.6	21.9	d	26.0
78	18.2	30.4	45.0	33.3	29.7	d	26.8

d = dead; ^{p} = pupa; () = larva excluded from analysis;
par. = parasite pupa (Angita exareolata).

TABLE 40 Fresh weights (mg.) of winter moth larvae fed on diet containing casein-tannin complex in 1966, together with larval fresh weight increments (initial to peak weights)

Date:	15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increment
(1)	0.8	1.1	d				-
2	1.2	2.1	2.9	3.8	3.6	5.3	2.6
3	1.4	3.4	4.5	4.0	d		3.1
41	1.6	2.3	2.4	3.3	4.1	3.4	1.7
5	1.7	2.8	2.0	2.0	d		1.1
(6)	1.8	2.3	d				-
7	2.3	4.3	4.7	5.3	3.4	d	3.0
8	2.3	4.3	4.7	5.3	3.4	d	3.0
9	2.5	3.6	5.5	5.8	4.7	d	3.3
(10)	2.6	3.6	d				-
11	2.7	2.7	4.5	6.2	d		3.5
(12)	2.7	3.0	d				-
13	3.0	3.5	4.9	3.0	d		1.9
14	3.1	4.9	7.6	7.9	7.3	d	4.8
(15)	3.2	2.7	d				-
16	3.2	4.3	5.4	5.0	d		2.2
17	3.2	5.5	6.8	8.3	5.6	d	5.1
18	3.2	7.1	11.3	10.8	9.3	d	8.1
19	3.7	5.0	3.7	3.0	d		1.3
20	3.8	6.8	10.2	8.1	d		6.4
21	4.0	9.8	15.2	12.2	9.0	d	11.2
(22)	4.0	d					-
23	4.1	15.8	13.2	8.8	d		11.7
24	4.4	6.7	10.8	10.3	7.1	d	6.4
25	4.5	11.8	10.4	14.6	13.3	d	10.1
26	4.6	11.5	13.8	14.7	d		10.2
(27)	4.6	9.4	11.3	d			-
28	4.7	12.7	16.3	21.4	19.9	d	16.7

contd.

TABLE 40 (contd.)

Date:	15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increment
29	5.0	10.1	11.3	7.4	d		6.3
30	5.0	10.3	16.8	10.4	d		11.8
(31)	5.1	12.2	12.4	d			-
32	5.2	10.3	15.5	15.3	10.9	d	10.3
33	5.3	13.6	17.6	17.4	12.8	d	12.3
34	5.4	9.1	13.5	9.9	d		8.1
35	5.4	12.5	14.7	12.7	d		9.3
36	5.5	9.4	11.4	9.7	d		5.9
(37)	5.7	4.8	d				-
38	5.8	12.7	13.2	11.3	d		7.4
39	6.0	8.1	10.4	11.1	12.4	d	5.1
40	6.3	12.7	11.4	12.9	9.4	d	6.6
(41)	6.6	12.0	par.				-
42	6.7	11.3	13.9	23.5	20.3	d	16.8
43	6.8	14.0	13.5	14.3	9.6	d	7.5
44	7.3	14.7	18.4	19.4	15.4	d	12.1
45	7.4	11.4	15.3	16.3	12.1	d	8.9
46	7.5	13.8	14.4	17.0	15.1	d	9.5
47	7.7	14.3	17.1	16.8	d		9.4
48	7.7	15.3	23.9	28.8	23.6	d	21.1
(49)	7.8	17.9	16.7	par.			-
50	7.8	14.0	17.9	16.7	17.6	d	10.1
51	8.0	14.5	21.6	27.2	24.5	d	19.2
52	8.0	16.7	23.2	25.4	16.4	10.9p	17.4
53	8.1	16.1	21.2	26.5	24.0	12.4	18.4
54	8.2	17.8	12.9	23.0	24.9	20.5	14.8
55	8.6	9.8	par.	24.5	25.4	d	16.3
(56)	8.6	9.8	par.				-
57	8.9	15.9	26.1	32.5	21.8	14.9	23.6

contd.

TABLE 40 (contd.)

Date:	15/5	19/5	23/5	27/5	31/5	6/6	Fresh weight increment
58	8.9	14.8	31.2	28.9	25.0	d	22.3
59	8.9	19.1	19.1	18.9	15.8	d	10.2
60	9.0	14.3	18.9	23.3	20.8	d	14.3
61	9.19	15.6	20.0	22.9	20.6	d	13.8
62	9.3	14.9	32.9	21.9	21.8	d	14.5
63	9.4	14.4	10.0	14.4	14.7	15.3	5.0
64	9.6	13.9	26.2	36.6	20.4	d	27.0
(65)	9.8	17.7	d				-
(66)	9.9	15.9	d				-
67	10.5	19.5	40.4	39.5	36.1	15.6P	29.9
68	11.2	15.9	21.8	20.5	16.2	d	10.6
69	11.3	20.8	32.3	19.7	13.1	d	21.0
70	11.5	18.2	24.3	21.4	9.8	d	12.7
71	11.8	20.7	29.5	30.7	20.1	d	18.9
72	12.1	12.5	21.3	31.8	23.7	14.8	19.7
73	12.7	17.3	31.4	42.9	22.3	16.6	30.2
74	13.1	27.3	39.7	30.3	21.7	19.5P	26.6
75	14.0	24.3	40.3	28.3	18.6	d	26.3
76	15.1	16.8	33.6	42.6	26.6	20.4	27.6
77	15.7	17.3	17.3	14.0	d		1.6
78	15.7	25.7	29.5	22.3	d		13.8

d = dead; P = pupa; ( ) = larva excluded from analysis;
par. = parasite pupa (Angitia exareolata)

Appendix 22 (continued)

	Control Diet	Experimental Diet
$\sum x$	1,690.4	751.4
n	67	64
$\bar{x}$	25.23	11.74
$\sum x^2$	52,721.72	12,682.64
s^2	152.62	61.28

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} = \frac{13.49}{\sqrt{\frac{152.62}{67} + \frac{61.28}{64}}} = 7.499$$

The value of d corresponding to 0.1% significance was found from the standard table to be 3.291 (Bailey, loc. cit.). It was therefore concluded that the difference in fresh weight increment, achieved by winter moth larvae fed on the diet containing tannin-casein complex, and the increment achieved on the control diet was statistically significant ($P < 0.001$).

Appendix 23: Quantitative investigation of oak leaf tannin-casein complexes.

1. Materials

A solution of casein ('Light White Soluble') was made up in distilled water, at a concentration of 5 gm./litre (i.e. 20 mg./4 ml.).

0.1 Molar sodium citrate/citric acid buffer at pH 5.0 was prepared.

0.375 gm. September oak leaf tannin (extracted by the method of Brown and Love, 1961) was stirred into 50 ml. distilled water. Since both (P) and (S) fractions were present, the tannin was incompletely dissolved, so that 10 ml. acetone had to be added to obtain complete solution. Such an addition of acetone had previously been employed in experiments of this kind by Handley (personal communication), and a control solution of acetone in the same concentration of distilled water was found not to precipitate casein from solution. The tannin solution as prepared therefore contained 31.25 mg./5 ml. 0.1% sodium chloride, to facilitate complex formation, and 0.1% L-Ascorbic acid, to minimise any oxidation, were added to the tannin solution. Aliquots of this solution were diluted with distilled water to obtain tannin solutions equivalent to 2/3rds and 1/3 of the original concentration (i.e. 20.8 mg. and 10.4 mg./5 ml., respectively).

2. Procedure

A series of glass centrifuge tubes was thoroughly cleaned in a chromic acid bath, rinsed in distilled water and dried at 85°C for

Appendix 23 (continued)

several days to constant weight. 5 ml. of each tannin solution were then pipetted into three tubes each, making nine tubes in all. To all these tubes were then added 4 ml. of the casein solution, equivalent to protein-tannin ratios of approximately 1 : 0.5, 1 : 1 and 1 : 1.5. 3 ml. of buffer (pH 5.0) were then added to each tube and the tubes placed, after shaking, in a water bath at 20°C, to allow tanning to proceed. Two sets of controls were treated simultaneously. The first contained casein and buffer, but tannin solution was replaced by acetone water (10 : 50, as used for tannin solution). The second contained 5 ml. of the most concentrated tannin solution, together with buffer, but the casein was replaced by distilled water (Table 41, p.236(a)).

Two tubes from each triplicate set (one in the case of the duplicate tannin control), were allowed to stand in the water bath for 15 hours, but the remaining tube in each set was removed after 2 hours. In both cases, the tubes were centrifuged (3,000 r.p.m., 5 minutes) immediately after removal from the bath. The sedimented complexes were washed with buffer, re-centrifuged and briefly rinsed with distilled water to remove buffer. They were then dried in an oven at 85°C for several days to constant weight. The weights of the residues were then calculated by difference, using the dry weights of the clean tubes, previously recorded.

3. Results

The weights of the residues found in each tube are recorded in Table 41. It is evident that the casein control yielded an average

TABLE 41 Weight of complexes formed by casein with different initial quantities of oak leaf tannin.

Tube no.	Casein (soln.[1] ml.)	Tannin soln. (mg./5ml.)	Water (ml.)	Citrate buffer[2]	Duration of tanning (hrs.)	Weight of residue (mg.)	Corrected complex wt.[3] (mg.)	Maximum complex wt.[4] (mg.)
1	4	-	5[5]	3	15	1.0		
2	4	-	5[5]	3	15	1.2		
3	4	-	5[5]	3	2	1.1		
4	-	31.3	4	3	15	0.8		
5	-	31.3	4	3	2	0.7		
6	4	10.4	-	3	15	30.4	29.0	29.0
7	4	10.4	-	3	15	29.7	28.3	29.0
8	4	10.4	-	3	2	26.3	25.0	29.0
9	4	20.8	-	3	15	39.8	38.1	39.1
10	4	20.8	-	3	15	42.6	40.9	39.1
11	4	20.8	-	3	2	36.3	34.7	39.1
12	4	31.3	-	3	15	44.8	42.9	49.4
13	4	31.3	-	3	15	46.3	44.4	49.4
14	4	31.3	-	3	2	41.0	39.2	49.4

[1] = 20 mg./4 ml.; [2] = 0.1 molar citrate buffer (pH 5.0); [3] = corrected for controls; [4] = theoretical value, calculated for complete incorporation of both tannin and casein in complex; [5] = acetone/water; 10/30.

Appendix 23 (continued)

residue of 1.1 mg. The amount of truly soluble casein in each tube was therefore reckoned as 18.9 mg., rather than 20 mg. It was also found that the tannin control yielded a residue of 0.8 mg. from the original 31.3 aliquot, so that the amount of truly soluble tannin was reckoned as 30.5 mg./5 ml. in the concentrated solution and 10.1 mg. and 20.3 mg. in the remaining two tannin solutions.

The relevant casein and tannin control weights were subtracted from the residue weights, to give the corrected weights of the tannin-protein complexes (Table 41). Also shown in the table are the theoretical maximum complex weights, calculated for complete precipitation of both casein and tannin.

Appendix 24: Preparation of leaf protein

1. Protein from oak leaves

a) On May 4th, 1966, 850 gm. of young oak leaves were collected from a tree in Wytham Wood and transferred in the polystyrene cold box to the laboratory cold room (4°C). 100 gm. of the leaves were homogenised in 200 ml. 0.1 Molar phosphate buffer (pH 7.5) in the cold room, using an 'Atomix' blender. The homogenate was squeezed through muslin cloth and returned to the blender for similar homogenisation of three further 100 gm. leaf samples. The process was repeated for the remaining 450 gm. of oak leaves, using a fresh sample of cold phosphate buffer. The resulting 400 ml. of crude green homogenate, which tended to turn brown, even at 0°C, were then centrifuged at 30,000 r.p.m. for one hour in a M.S.E. 'Superspeed' machine, maintained at 0°C. The resulting clear brown supernatant was heated rapidly to 95°C for 30 minutes to inactivate enzymes and then re-cooled to 0°C. Citric acid was then added to pH 4.0, using a pH meter, when crude impure protein was precipitated. This was centrifuged down and the clear deep brown supernatant decanted. The crude protein was again taken up in phosphate buffer at pH 7.5, giving a brown solution. Further precipitation with citric acid failed to remove the brown material, which was precipitated along with the protein, suggesting complex formation between protein and either degraded pigment material or tannin. The yield of crude protein was only 3 gm. This figure was much lower than expected from 850 gm. of leaves and suggests that much of the leaf protein was not extracted, possibly owing to complex formation.

Appendix 24 (continued)

An attempt to purify the leaf proteinwas made, using a Sephadex column. Grade G-50 (fine) Sephadex was selected, since this has a molecular exclusion limit of 10,000, and would be expected to separate leaf protein from any pigment or polyphenolic molecules likely to be present. 5 ml. of oak protein solution, in buffer at pH 7.5, were applied to the head of a 15 by 1 cm. column, made up in the same phosphate buffer, which was also used as eluent. Brown material was held at the top of the column, while a barely visible pale yellow band moved rapidly downwards withthe eluent front. After some minutes, the flow through the column ceased. It was suspected that this might be due to a reduction in pH at the head of the column, resulting from accumulation of the brown material and possibly causing complex formation. A few drops of sodium hydroxide were therefore added to the top of the column, whereupon flow resumed and the dark brown band started to move rapidly downwards.

The pale yellow band emerged from the column with the eluent front, and was collected. On acidification to pH 4.0 with citric acid, a cream-coloured precipitate appeared, which could be re-dissolved on increasing the pH. It was considered that this material might represent leaf protein. After a further column volume of eluent, the dark brown band emerged from the column and was also brought to pH 4.0 with citric acid. However, no precipitate was formed, indicating that no protein was present and confirming that this material was involved in complex formation with the leaf protein during the initial stages of extraction.

After three days delay, a further sample of the crude leaf protein (1 gm.), which had been stored at 4°C, was applied to the top of a new

and larger Sephadex column under the same conditions as used in the preliminary experiment. On this occasion, however, the protein band was markedly brown instead of pale yellow, indicating that the protein had become inextricably complexed with the brown material. The sample was therefore abandoned.

It was clear from this initial extraction that some oak leaf protein could be obtained in a reasonably pure condition, if extraction was carried out speedily and immediately followed by Sephadex separation. Evidently complexes between the protein and the brown material can be separated on Sephadex, provided that complex formation has not proceeded too far. It is also clear that storage at 0°C does not prevent continued formation of complexes between protein and the brown material.

b) The yield of protein was so small and the date so far advanced that it was considered impracticable to extract sufficient quantities of oak protein for use in place of casein in the 1966 insect rearing experiment. However, a second attempt to prepare oak leaf protein was undertaken to provide sufficient material for inclusion in enzyme digestion experiments.

500 gm. of oak leaves were collected from Wytham on May 17th, 1966, and homogenised in 0.1 Molar phosphate buffer (pH 7.5) in the cold room, immediately on return to the laboratory. 0.2 Molar sodium ascorbate was added to the buffer before use, to reduce oxidation of materials in the leaves. This seemed to be successful, since there was no tendency for the green leaf extracts to turn brown, as was found in the previous extraction. The homogenate was squeezed through muslin as before, giving 300 ml. of crude extract. This was centrifuged at

Appendix 24 (continued)

30,000 r.p.m. for one hour at 0°C in a M.S.E. 'Superspeed' machine to sediment cell debris, mitochondria, chloroplasts, etc. (Bonner, 1950). The pale yellow supernatant was stored for one day in the deep-freeze (- 20°C) in a sealed polythene bottle.

When the protein preparation was removed from the deep-freeze, it was noticed that the upper 1 cm. of the solid had turned pale brown, showing that changes continue to occur in the extract material in contact with air even in the deep-freeze. After removal of the brown surface material, the container was inverted over a conical flask, immersed in a boiling water bath, so that each drop of solution was transferred rapidly from 0°C to 100°C as it melted. After heating for 30 minutes, the solution was cooled and freeze-dried, to give 16 gm. of a yellow hygroscopic semi-crystalline solid, consisting of crude leaf material and sodium chloride.

Sephadex separation was not considered necessary, since the brown material, encountered during the last extraction, did not seem to be present. 2 gm. of the yellow material were shaken with phosphate buffer at pH 7.6, but only a fraction dissolved. Addition of sodium hydroxide, in which leaf proteins are reported to be more easily soluble than in phosphate buffer (Handley, W.R.C., personal communication) did not appear to increase solubility appreciably, though the suspension turned bright yellow above pH 9.0 - a colour change which was found to be reversible on reducing the pH.

A further 8 gm. of the pale yellow freeze-dried material were

Appendix 24 (continued)

suspended in phosphate buffer at pH 7.6 and then centrifuged (4,000 r.p.m., 10 minutes). The supernatant was treated with citric acid, bringing the pH to 4.0, when a white precipitate was deposited. This was recentrifuged and taken up in fresh buffer three times, to remove soluble material, and finally stored under citric acid solution at pH 4.0 in a refrigerator, in the presence of a little chloroform.

The yield of this white material, possibly pure oak leaf protein, was too small (less than 1 gm.) to be of use in experiments during 1966, though it seems that leaf protein can be prepared from young oak leaves, if suitable precautions are taken. The insolubility of much of the protein in this extraction was probably due to denaturation during the heating. It would, therefore, be desirable to use a different method for inactivating enzymes (such as the proteinases) in the protein extracts, or to curtail the heating.

By the end of May, the leaf protein level of oak has dropped considerably (Chapter 3) and the tannin content has begun to rise. It was therefore considered unlikely that further proteincould be extracted from oak leaves during 1966 and it was reluctantly decided to substitute nettle leaf protein, kindly made available by Dr. Handley, for the projected enzyme experiments.

2. Protein from nettle leaves

Handley has successfully used a protein extract from nettle leaves, in connection with experiments on the digestibilityof leaf-protein - tannin complexes to soil animals. Although not entirely pure, the

Appendix 24 (continued)

protein/retains at least some of its ability to combine with tannins and is digestible by trypsin. Handley selected the stinging nettle Urtica dioica L. as a source of leaf protein since the leaves are apparently lacking in polyphenols.

The method of extraction used by Handley (personal communication) was as follows: Fresh succulent nettle leaves from non-flowering shoots growing under conditions of plentiful light were collected and macerated in water in a blender. The dark brown homogenate was strained through nylon cloth and subsequently centrifuged to remove cell debris. Citric acid was then added to bring the pH to 4.5 in order to precipitate the protein and so prevent the activity of any inherent leaf proteinases. A few drops of chloroform were added to inhibit microbial decomposition and the extract was then stored in the refrigerator at 4°C in glass-stoppered bottles.

3. Purification of the nettle leaf protein preparation

Handley had previously suspected that the brown material associated with the nettle protein preparation was non-phenolic and probably due to degradation of plant pigments, and, in particular, chlorophyll. Attempts were therefore made to gain further information about the nature of this material and, if possible, to separate it from the leaf protein.

a) Sephadex separation

In view of the partial success obtained in preparing pure oak leaf protein by means of Sephadex chromatography, it was decided to discover whether the technique could be successfully applied to the

Appendix 24 (continued)

nettle extract. A sample of the extract (10 ml., containing approximately 3 mg. of dissolved material) was applied to the top of a 30 x 2 cm. column of Sephadex G-50 (fine), made up in phosphate buffer (Na_2HPO_4/NaH_2PO_4, 0.1 Molar, pH 7.6). The protein in the extract had previously been sedimented in a centrifuge and washed several times until the supernatants became colourless. The column was eluted with the same buffer at pH 7.6. A dark brown band immediately began to move down the column with the solvent front, followed by a paler brown band, much wider and incompletely separated from the first band. After 1 column volume, the first fraction was eluted (37 ml.). The second fraction, containing the pale brown band, was then collected and had been completely eluted after three column volumes. A further experiment, using a sample of only 0.5 ml., also resulted in lack of separation of the two bands, suggesting that the pale brown material was continually being released from the material in the first, dark brown, band. However, repetition of the Sephadex chromatography in NaOH/Glycine buffer at pH 9.2, when it was hoped that dissociation would be more complete, produced identical results, giving rise to the same fractions 1 and 2, unseparated.

b) Investigation of fractions 1 and 2

Fraction 1 was found to give a precipitate with citric acid at pH 4.0, in the same way as the original extract, showing that protein was present in this fraction, though evidently complexed to the brown material.

Fraction 2 gave no precipitate with citric acid, revealing the

Appendix 24 (continued)

absence of protein. It also failed to produce a precipitate with gelatin solution (1% gelatin, 10% sodium chloride) and did not give a dark blue colour with potassium ferricyanide/ferric chloride solution, showing that the brown material was not polyphenolic. Samples of both fractions were run on two-way paper chromatograms in 2% Acetic acid and BAW. Spraying with the ferricyanide reagent gave negative results, confirming the absence of phenols, but fraction 1 produced a spot in the chlorophyll position (R_f 1.0 in BAW; 0 in acetic acid), which fluoresced red in ultraviolet light, confirming the presence of chlorophyll and/or degradation products complexed to the protein in the extract. The fluorescence was weaker in fraction 2, but sufficient to show that some of the pigment material is removed by Sephadex.

c) Electrophoresis

Several attempts were made to separate the brown material, considered to contain pigment degradation products, from the nettle leaf protein by electrophoresis, using a variety of buffers at different pH values. Although the leaf protein was mobile, it was found to be impossible to separate it from the contaminating material.

Since all attempts to purify the nettle protein were unsuccessful, it was eventually decided to proceed with the planned experiments involving protein-tannin complexes by making use of the crude protein extract. It must be borne in mind, however, that the presence of adsorbed pigment material is likely to influence the ability of the protein to form complexes with tannins.

Appendix 25: Quantitative investigation of tannin-nettle protein complexes

1. Materials

Leaf Protein: Aliquots of the nettle leaf protein extract (Appendix 24) were evaporated to dryness to estimate the approximate concentration of protein material. The extract was then diluted with distilled water to a concentration corresponding to approximately 10 mg./3 ml. 3 ml. aliquots in triplicate were then dried at 85°C to constant weight in tubes which had previously been weighed after prolonged drying at this temperature. It was found that 3 ml. of the extract solution contained 9.6 mg. of leaf protein material.

Tannin: 400 mg. of oak leaf tannin, extracted from September leaves by the method of Brown and Love (1961), were stirred into 32 ml. of distilled water. It was necessary to add a little acetone to effect complete solution, since the tannin had not been separated into fractions (P) and (S). The concentration of this tannin solution was therefore approximately 50 mg./4 ml. Aliquots were then diluted to provide tannin solutions equivalent to approximately 2.5, 5, 10 and 20 mg./4 ml. 4 ml. aliquots of the tannin solution equivalent to 20 mg./4 ml. were then dried to constant weight at 85°C in tubes of known clean weight, when it was found that the actual weight of tannin present was 16.1 mg./4 ml. The true concentrations of the tannin solutions were therefore 2.0, 4.0, 8.1, 16.1 and 40.3 mg./4 ml.

Buffer: 0.1 Molar sodium citrate/citric acid buffer was prepared to yield a pH of 5.0.

Appendix 25 (continued)

2. Procedure

4 ml. of each of the five tannin solutions were pipetted into separate pairs of glass centrifuge tubes, which had been previously dried at 85°C to constant weight, after thorough cleaning in a hot chromic acid bath (tubes 5 to 14, Table 42, p.246(a)). In addition, 4 ml. of the most concentrated tannin solution (40.3 mg./4 ml.) were pipetted into a further pair of tubes (15 and 16). The tubes were placed in a water bath at 20°C. After about 30 minutes equilibration, 4 ml. of buffer solution were added to each tube, immediately followed by 3 ml. of protein solution, both protein and buffer having been previously brought to 20°C. The protein solution contained 9.6 mg. in all cases except tubes 15 and 16, in which the concentration was halved to 4.8 mg./3 ml., to achieve a tannin-protein ratio of approximately 80 : 1. After shaking to achieve complete mixing, the tubes were left to stand in the water bath for 10 minutes, after which they were centrifuged (3,000 r.p.m. for 5 minutes) and the precipitates washed, as in the previous experiment, before being dried to constant weight at 85°C for several days. The weights of the residues were then calculated by difference, using the known weights of the clean tubes.

Two pairs of control tubes were treated in the same way as the experimental tubes. The first pair (tubes 1 and 2) contained protein, but water in place of tannin. The second pair (tubes 3 and 4) contained tannin solution (16.1 mg.), but water in place of protein (see Table 42).

TABLE 42 Weights of complexes formed by nettle leaf protein with different initial quantities of oak leaf tannin.

Tube No.	Leaf protein (mg./3 ml.)	Tannin (mg./4 ml.)	Water (ml.)	Buffer (pH5.0)	Residue (mg.)	Corrected wt. of complex	Maximum complex weight[1]
1	9.6	-	4	4	0.1		
2	9.6	-	4	4	0		
3	-	16.1	3	4	0.7		
4	-	16.1	3	4	0.6		
5	9.6	2.0	-	4	0.2	0	11.6
6	9.6	2.0	-	4	0	0	11.6
7	9.6	4.0	-	4	2.2	2.0	13.6
8	9.6	4.0	-	4	2.4	2.2	13.6
9	9.6	8.1	-	4	15.0	14.6	17.7
10	9.6	8.1	-	4	15.5	15.1	17.7
11	9.6	16.1	-	4	20.9	20.2	25.7
12	9.6	16.1	-	4	20.8	20.1	25.7
13	9.6	40.3	-	4	28.6	26.8	49.9
14	9.6	40.3	-	4	28.2	26.4	49.9
15	4.8	40.3	-	4	20.9	19.1	45.1
16	4.8	40.3	-	4	21.1	19.3	45.1

[1] Theoretical value, calculated for complete incorporation of both tannin and casein in complex.

Appendix 25 (continued)

3. Results

The residue weights from all the tubes are shown in Table 42 (p.246a). The protein control tubes were found to yield a negligible residue, but the tannin controls yielded residues of 0.6 and 0.7 mg. The experimental residue weights were therefore reduced proportionately to allow for tannin precipitated other than by complex formation, giving the corrected weights of the complexes. Also shown in Table 42 are the theoretical maximum complex weights, equivalent to complete precipitation of both protein and tannin. It was unfortunately not possible to determine from this experiment the proportion of the sedimented tannin in the control which would have been involved in complex formation. However, the correction is small in proportion to the complex weights, and unlikely to affect any conclusions from the results. In calculating the complex weights, therefore, it was assumed that none of the sedimented tannin, estimated in the controls, was involved in complex formation, even though at least a proportion of it probably was.

Appendix 26: Determination of the pH of the mid-gut of winter moth larvae

The pH of the mid-gut of winter moth larvae was determined by two different methods.

1. Determination by pH meter

15 winter moth larvae, reared to the 5th instar, on oak and hawthorn leaves, were dissected in the Biochemistry Department of the Radcliffe Informary, Oxford, on May 8th, 1965. The mid-guts were immediately transferred to 0.5 ml. of CO_2-free distilled water and thoroughly stirred, to allow the gut contents to escape into solution. The mid-gut suspension was then transferred rapidly to the micro-cell of a sensitive pH meter, kindly made available by Dr. Esnoff, and the pH value determined. The value recorded was 9.2. After ten-fold dilution of the suspension with CO_2-free distilled water, the pH value dropped only slightly, to 8.9, indicating that the initial value of 9.2 was probably a reasonable estimate of the actual mid-gut pH of the larvae.

2. Determination by indicator solution

The pH of the mid-gut of winter moth larvae was also determined by the method of Swingle (1928). Six 5th instar larvae, reared on hawthorn or oak leaves, were examined. Each larva was carefully dissected on a microscope slide, revealing the intact gut. The mid-gut was then pierced with a fine (0.3 mm. bore) glass capillary tube (1 inch long), when the fluid gut contents were immediately drawn into the tube

Appendix 26 (continued)

(without coming into contact with the atmosphere) by capillary action. When the level of fluid had risen about half-way up the capillary tube, the tube was quickly withdrawn and its lower end dipped into a drop of thymol blue indicator solution, some of which was then drawn into the tube by capillary action. The indicator colour at the junction of the gut fluid and indicator solution was then compared under a binocular microscope with the colours produced by repeating the procedure with further glass tubes, replacing the gut fluid by standard sodium barbiturate/hydrochloric acid buffer solutions, which covered the pH range from 8.6 to 9.6. The pH of the mid-guts of all six larvae was found to be 9.2-9.3, thus confirming the value found by the earlier method.

Appendix 27: Inhibition of trypsin by oak leaf tannin at pH 7.6

1. Materials

Casein: 1 gm. casein ('Light White Soluble') was dissolved in 100 ml. of distilled water, giving a concentration of 10 mg./ml.

Tannin: 140 mg. of tannin (fraction (S) only), extracted from July oak leaves by the method of Brown and Love (1961), were dissolved in distilled water, to give a concentration of 20 mg./ml.

Buffers: 0.1 Molar phosphate buffer (Na_2HPO_4/NaH_2PO_4) was made up at a pH of 7.6. 0.1 Molar sodium citrate/citric acid buffer (pH 5.0) was also prepared.

Enzyme: Mammalian trypsin (British Drug Houses, Ltd.) was dissolved in 0.0025 Normal hydrochloric acid, to give a concentration of 0.25 mg./ml.

TCA: A 10% (w/v) solution of trichloracetic acid in water was prepared.

2. Procedure

a) Preparation of casein-tannin complex.

1 ml. of the tannin solution was pipetted into each of five glass centrifuge tubes, which had been cleaned previously with chromic acid. After equilibration of the tubes in a water bath at 26°C, 1 ml. of the citrate buffer (pH 5.0) was added to all five tubes. 1 ml. of the casein solution was then added to three of the tubes and 1 ml. of distilled water to the remaining two (control) tubes. After shaking, followed by 15 minutes in the water bath to allow tanning of

Appendix 27 (continued)

the protein to proceed, the tubes were centrifuged (3,000 r.p.m. for 5 minutes) and the five supernatants carefully decanted, with distilled water washings, into Kjeldahl flasks. The precipitated casein-tannin complexes were used for the subsequent digestion experiment. Meanwhile, Kjeldahl analyses were carried out on the five supernatants, together with water blanks, to confirm that all the casein (10 mg.) had been precipitated in the complexes. The results are shown in the following table:

Casein solution	Tannin solution	Water	Buffer (pH 5.0)	Kjeldahl Titre[2]	Average mg. Nitrogen[1]
-	-	2 ml.	1 ml.	0.40	(reagent blank)
-	-	"	"	0.38	"
1 ml.	1 ml.	-	"	0.81	0.029
"	"	-	"	0.81	
"	"	-	"	Lost	
-	"	1 ml.	"	0.88	0.032
-	"	"	"	0.84	

[1] After allowance for blank readings.

[2] ml. 0.005 N sulphuric acid.

It can be seen that the amount of nitrogen inthe supernatants from the casein-tannin complexes was slightly lower than that in the control tubes, containing tannin, showing that the casein had been completely precipitated during preparation of the complexes. The titres obtained from the tannin control tubes were used to calculate

Appendix 27 (continued)

the percentage of nitrogen in the tannin, found to be 0.16%.

b) Hydrolysis of casein-tannin complex by trypsin

The remainder of the tannin solution was used to prepare two further casein-tannin complexes, following exactly the procedure described above. All five complexes, each containing 10 mg. of casein, were dispersed into 1 ml. aliquots of distilled water in the original centrifuge tubes, now labelled 6-10 (see Table 15, p.122(a)). Into 5 further tubes, labelled 1-5, were pipetted 1 ml. aliquots of the uncomplexed casein solution (also containing 10 mg.). 2 ml. of the phosphate buffer (pH 7.6) were then added to each of the ten tubes, which were left to equilibrate in a water bath at 26°C. 1 ml. of the trypsin solution was then added to tubes 3, 4, 5, 8, 9, and 10 and hydrolysis allowed to proceed for three hours at 26°C. The reaction was then stopped by addition of 3 ml. of TCA solution to all 10 tubes, after which 1 ml. trypsin was added to the reagent control tubes, 1, 2, 6 and 7. The tubes were shaken and allowed to stand for one hour before being centrifuged (3,000 r.p.m., for 5 minutes). The supernatants were carefully decanted, together with distilled water washings, into Kjeldahl flasks for nitrogen analysis.

c) Results

The Kjeldahl titres for the nine supernatants (one of the 10 was accidentally lost during Kjeldahl distillation) are shown in Table 15 (p.122a). After the titres had been corrected for the relevant reagent controls, the mean values were used to calculate the amount of nitrogen released from 10 mg. casein in the two cases.

Appendix 28: Inhibition of trypsin by oak leaf tannins at pH 9.2

1. Materials

Casein: 1 gm. casein ('Light White Soluble') was dissolved in 100 ml. distilled water to give a concentration of 10 mg./ml.

Tannins: Condensed and hydrolysable tannins from August and September oak leaves were separated from the crude oak tannin using Sephadex column chromatography, as described in Chapter 4 (p.63). The condensed tannin corresponded to the streak 'C' and the hydrolysable tannin to streaks 'D' and 'H' (Figure 17, p. 506). About 300 mg. of each chromatographically pure tannin sample was available. Solutions of these tannins were made up in distilled water at a concentration of 20 mg./ml. Aliquots from these solutions were diluted with water to give a range of tannin solutions containing from 1 to 15 mg./ml.

Trypsin: 500 mg. of trypsin (Mammalian, British Drug Houses, Ltd.) were dissolved in 1 litre of 0.0025 Normal hydrochloric acid.

Leaf Protein: Nettle leaf protein preparation, as described in Appendix 24 (p.238), was kindly supplied by Dr. Handley. The protein was precipitated by citric acid, centrifuged and washed with distilled water, before being redissolved by addition of a few drops of sodium hydroxide solution. The precipitation and washing was repeated several times until the supernatant became colourless and free of the brown pigment material (Appendix 24). 1 ml. aliquots of the final leaf protein solution were dried to constant weight at 85°C in 3 previously dried and weighed glass centrifuge tubes. The weights of protein

Appendix 28 (continued)

material were found to be 3.2, 3.6 and 3.3 mg.; the concentration of the leaf protein solution was therefore taken to be 3.4 mg./ml.

Buffers: 1 litre of citric acid/sodium citrate buffer (0.01M) was made up at pH 5.0. 1 litre of sodium carbonate/sodium bicarbonate buffer (0.2M) was made up to give pH 9.2 at 26°C. This was found to be the only convenient nitrogen-free buffer available for this pH region.

Finally, solutions of sodium hydroxide (0.1 Molar) and trichloracetic acid (10% aqueous) were prepared.

2. Method

Two 1 ml. aliquots of each tannin solution were pipetted into pairs of glass centrifuge tubes, previously cleaned thoroughly in a hot chromic acid bath. The concentrations of the tannins in the tubes were 1, 2, 3, 4, 5, 7, 10, 15 and 20 mg./ml. in tubes 3 to 20 (condensed tannin) and 25 to 42 (hydrolysable tannin, see Table 43, p.254a). 2 ml. of distilled water, 1 ml. of citrate buffer (pH 5.0) and 1 ml. of the casein solution (10 mg.) were then added to each tube, after equilibration at 26°C in a water bath. The tubes were shaken and allowed to stand for 30 minutes, after which the pH was brought to neutrality by addition of 1 ml. 0.1 Molar sodium hydroxide solution. (The addition of sodium hydroxide was necessary in order to neutralise the citrate buffer and prevent evolution of carbon dioxide on addition of the carbonate buffer). 2 ml. of the carbonate buffer were then added without delay, and the pH confirmed to be 9.2 with indicator

TABLE 43 Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Casein (10mg/ml)	Water (ml.)	Tannin (1 ml.)	Citrate Buffer (ml.)	NaOH (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre 2	mg. of N. 2	% Hydrolysis.
1	1 ml.	3	-	1	1	2	1 ml.	3	20.2	1.34	92.6
2	1 ml.	3	-	1	1	2	1 ml.	3	19.8	1.31	90.4
3	1 ml.	2	1 mg.[5]	1	1	2	1 ml.	3	Lost	-	-
4	1 ml.	2	1 mg.[5]	1	1	2	1 ml.	3	14.84	0.96	66.2
5	1 ml.	2	2 mg.[5]	1	1	2	1 ml.	3	13.24	0.85	58.7
6	1 ml.	2	2 mg.[5]	1	1	2	1 ml.	3	12.92	0.83	57.2
7	1 ml.	2	3 mg.[5]	1	1	2	1 ml.	3	10.56	0.66	45.6
8	1 ml.	2	3 mg.[5]	1	1	2	1 ml.	3	11.23	0.71	49.0
9	1 ml.	2	4 mg.[5]	1	1	2	1 ml.	3	10.00	0.62	42.8
10	1 ml.	2	4 mg.[5]	1	1	2	1 ml.	3	9.46	0.58	40.0
11	1 ml.	2	5 mg.	1	1	2	1 ml.	3	9.18	0.56	38.6
12	1 ml.	2	5 mg.	1	1	2	1 ml.	3	9.18	0.56	38.6

TABLE 43 (Cont.) Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Casein (10mg/ml)	Water (ml.)	Tannin (1 ml.)	Citrate Buffer (ml.)	NaOH (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
13	1 ml.	2	7 mg.[5]	1	1	2	1 ml.	3	8.23	0.50	34.5
14	1 ml.	2	7 mg.[5]	1	1	2	1 ml.	3	8.20	0.50	34.5
15	1 ml.	2	10 mg.[5]	1	1	2	1 ml.	3	5.32	0.29	20.0
16	1 ml.	2	10 mg.[5]	1	1	2	1 ml.	3	5.80	0.33	22.8
17	1 ml.	2	15 mg.[5]	1	1	2	1 ml.	3	4.48	0.24	16.6
18	1 ml.	2	15 mg.[5]	1	1	2	1 ml.	3	4.00	0.20	13.8
19	1 ml.	2	20 mg.[5]	1	1	2	1 ml.	3	3.14	0.14	9.7
20	1 ml.	2	20 mg.[5]	1	1	2	1 ml.	3	3.49	0.17	11.7
21	1 ml.	3	-	1	1	2	1 ml.[1]	3	1.12		
22	1 ml.	3	-	1	1	2	1 ml.[1]	3	1.09		
23	-	3	20 mg.[5]	-	-	-	-	-	1.02	0.044[4]	
24	-	3	20 mg.[5]	-	-	-	-	-	1.10		

254(6).

TABLE 43 (Cont.) Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Casein (10mg/ml)	Water (ml.)	Tannin (1 ml.)	Citrate Buffer (ml.)	NaOH (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
25	1 ml.	2	1 mg.^{6}	1	1	2	1 ml.	3	15.42	1.00	69.0
26	1 ml.	2	1 mg.^{6}	1	1	2	1 ml.	3	17.01	1.11	76.6
27	1 ml.	2	2 mg.^{6}	1	1	2	1 ml.	3	16.72	1.09	75.3
28	1 ml.	2	2 mg.^{6}	1	1	2	1 ml.	3	15.74	1.02	70.3
29	1 ml.	2	3 mg.^{6}	1	1	2	1 ml.	3	16.72	1.09	75.2
30	1 ml.	2	3 mg.^{6}	1	1	2	1 ml.	3	16.68	1.09	75.2
31	1 ml.	2	4 mg.^{6}	1	1	2	1 ml.	3	16.74	1.09	75.2
32	1 ml.	2	4 mg.^{6}	1	1	2	1 ml.	3	16.79	1.10	75.8
33	1 ml.	2	5 mg.^{6}	1	1	2	1 ml.	3	16.43	1.07	73.8
34	1 ml.	2	5 mg.^{6}	1	1	2	1 ml.	3	16.50	1.08	74.5
35	1 ml.	2	7 mg.^{6}	1	1	2	1 ml.	3	16.34	1.07	73.8
36	1 ml.	2	7 mg.^{6}	1	1	2	1 ml.	3	16.18	1.05	72.5

TABLE 43 (Cont.) Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Casein (10mg/ml)	Water (ml.)	Tannin (1 ml.)	Citrate Buffer (ml.)	NaOH (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
37	1 ml.	2	10 mg.[6]	1	1	2	1 ml.	3	15.62	1.02	70.3
38	1 ml.	2	10 mg.[6]	1	1	2	1 ml.	3	15.28	0.99	68.3
39	1 ml.	2	15 mg.[6]	1	1	2	1 ml.	3	13.04	0.84	57.9
40	1 ml.	2	15 mg.[6]	1	1	2	1 ml.	3	12.64	0.81	55.9
41	1 ml.	2	20 mg.[6]	1	1	2	1 ml.	3	11.21	0.71	49.0
42	1 ml.	2	20 mg.[6]	1	1	2	1 ml.	3	11.00	0.69	47.7
43	-	3	20 mg.[6]	-	-	-	-	-	1.00	0.040	
W	-	4	-	-.	-	-	-	-	0.40		
W	-	4	-	-	-	-	-	-	0.46		
W	-	4	-	-	-	-	-	-	0.42		
W	-	4	-	-	-	-	-	-	0.44		
B	-	5	-	1	1	2	-	3	0.47		

TABLE 43 (Cont.) Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Casein (10mg/ml)	Water (ml.)	Tannin (1 ml.)	Citrate Buffer (ml.)	NaOH (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
B	-	5	-	1	1	2	-	3	0.45		
B	-	5	-	1	1	2	-	3	0.49		
B	-	5	-	1	1	2	-	3	0.44		

[4]

Tube No.	Leaf Protein (1 ml.)	Tannin (1ml)	Water (ml.)	Citrate Buffer (ml.)	Water (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
44	3.4 mg	20mg.	1	-	4	-	-	-	4.38		
45	3.4mg	-	1	-	-	-	-	-	4.46	0.279[4]	
46	3.4mg	-	1	1	3	2	1 ml.	3	2.58		
47	3.4mg	-	1	1	3	2	1 ml.	3	2.34	0.124[4]	44.4[4]

TABLE 43 (Cont.) Effect of oak leaf tannins on protein hydrolysis by trypsin at pH 9.2

Tube No.	Leaf Protein (1 ml.)	Tannin (1ml)	Water (ml.)	Citrate Buffer (ml.)	Water (ml.)	Carbonate Buffer (ml.)	Trypsin (½mg/ml)	TCA (ml.)	Kjeldahl titre (2)	mg. of N. (3)	%Hydro-lysis.
48	3.4 mg	20mg.[5]	-	1	3	2	1 ml.	3	1.17	0.034	12.0
49	3.4 mg	20mg.[5]	-	1	3	2	1 ml.	3	Lost		
50	3.4 mg	20mg.[6]	-	1	3	2	1 ml.	3	1.53	0.058[4]	20.8[4]
51	3.4 mg	20mg.[6]	-	1	3	2	1 ml.	3	1.50		
52	3.4 mg	-	1	1	3	2	1 ml.[1]	3	0.70		
53	3.4 mg	-	1	1	3	2	1 ml.[1]	3	0.68		

1 = Trypsin added after TCA. 2 = ml. 0.01N H_2SO_4.

3 = After allowance for relevant controls (see text). 4 = Average of the two values.

5 = Condensed Tannin. 6 = Hydrolysable Tannin.

Appendix 28 (continued)

papers (previous tests with larger quantities of solutions, using a pH meter, had shown that the reagent system used gave a stable pH at 9.2, with good buffering capacity). 1 ml. of the trypsin solution (0.5 mg.) was then added to each tube. After shaking, the tubes were covered with 'Parafilm' and allowed to stand in the water bath at 26°C for three hours. Hydrolysis was then stopped by adding 3 ml. of the TCA solution to all tubes.

In addition to the main sets of experimental tubes, several other samples were similarly treated. Duplicate samples of the casein solution (Tubes 1 and 2) were treated identically with the exception that an extra 1 ml. of water was added, in place of tannin solution. Two further samples of casein solution (tubes 21 and 22) were similarly treated with distilled water in place of tannin, but trypsin was not added until after the TCA, so providing a reagent blank. To check the nitrogen content of the tannin samples, 1 ml. (20 mg.) aliquots of condensed tannin (tubes 23 and 24) and hydrolysable tannin (tube 43) were also included in the experiment, withwater as the only added material. Eight control tubes were also included; four of these (labelled W in Table 43) merely contained 3 ml. distilled water; the other four (labelled B) were treated with all reagents except casein, tannin and trypsin, which were replaced by an additional 3 ml. of water.

All tubes were centrifuged one hour after addition of TCA (not added to the tubes specified above) and the supernatants carefully decanted into Kjeldahl flasks, together with distilled water washings, for nitrogen analysis in the usual way.

Appendix 28 (continued)

In addition to the samples so far mentioned, 10 tubes were treated simultaneously to determine the effect of tannins on digestion of leaf protein (Table 43). 1 ml. aliquots of both tannin solutions (20 mg.) were pipetted into pairs of duplicate tubes (condensed tannin in tubes 48 and 49; hydrolysable tannin in tubes 50 and 51). To each of these four tubes, 1 ml. of the leaf protein solution (3.4 mg.) was then added, after 1 ml. of citrate buffer at pH 5.0. The tubes were allowed to stand at 26°C in the water bath for 30 minutes, after which they were centrifuged and the precipitated complexes washed with distilled water to remove surplus tannin. The precipitates were then dispersed in 3 ml. of water, before addition of the carbonate buffer and trypsin, at the same time as these solutions were added to the tubes containing casein. Of the 6 remaining leaf protein tubes, two (44 and 45) received addition of water only and were not treated with TCA, so as to estimate the total nitrogen content of the leaf protein. Two further tubes (46 and 47) were digested in a similar way to the tannin complexes, but contained no tannin. Finally, two reagent blanks were run (tubes 52 and 53) in which the trypsin was added after the TCA. Details of the additions to each tube are given in Table 43 (p.254a). Digestion was carried out at the same time as the casein experiment and after the three hours at 26°C, TCA was added to all the tubes except 44 and 45 and Kjeldahl analysis was carried out on the supernatants along with those from the casein experiment.

Appendix 28 (continued)

3. Results

The Kjeldahl titres for each sample are shown in Table 43 (p.254a). From the titres obtained from tubes 23, 24 and 43, using the water blanks 'W', the nitrogen content of the condensed tannin was found to be 0.22% and that of the hydrolysable tannin 0.20%. These figures are too small to affect the experimental results significantly, especially since virtually all the tannin in the experimental tubes was bound to the protein precipitated by TCA and would therefore not have contributed to the nitrogen content of the supernatants. The Kjeldahl titres were therefore not reduced by the titre expected from the tannin content, but only by the average titres of the reagent controls (tubes 21, 22, 52 and 53). These corrected titres were then used to calculate the amount of nitrogen present in the supernatants, and these values are also shown in the Table.

The casein used in this experiment contains 14.5% nitrogen (B.D.H. specification) so that complete digestion of 10 mg. would yield 1.45 mg. of soluble nitrogen. Using this figure, therefore, the percentage of the maximum digestion was calculated for each of the experimental tubes and these percentages are shown in Table 43. They are also plotted graphically against tannin concentration in Figure 35 (p.125a).

After subtraction of the average reagent control titre, obtained from tubes 52 and 53, the titres from digested uncomplexed leaf protein (tubes 46 and 47) were used to calculate the amount of nitrogen released

Appendix 28 (continued)

by digestion of leaf protein under the conditions of the experiment.

The total amount of nitrogen in the leaf protein was calculated, from the titres in tubes 44 and 45, after subtracting the mean water control 'W', to be 0.279 mg. This is equivalent to a nitrogen content of 8.21%, which is somewhat higher than that normally given for plant protein (6.25%), suggesting that the brown pigment material, associated with the protein, contributes disproportionately to its nitrogen content.

Using the figure obtained for total nitrogen, the percentage digestion of the leaf protein was then calculated to be 44.4%. This is about half the value found for casein, as expected from previous information (Handley, personal communication). Using the reagent control, the titres from the tannin-complex samples (tubes 48 to 51) were also converted to soluble nitrogen equivalent, and hence to percentage digestion of the leaf protein. The results are shown in Table and are also indicated on Figure 35 (p.125a) for comparison with the results from the casein digestion.

Appendix 29: Determination of activity of ^{14}Carbon-labelled tannin samples

The beta activity of the tannin samples, obtained from oak leaves which had been enclosed in an atmosphere containing $^{14}CO_2$, was estimated by dissolving weighed samples of each tannin fraction in acetone/water (50 : 50) and counting automatically the number of disintegrations from three 0.01 ml. aliquots of each solution, during a 10 minute period, in a Series 724 Nuclear Chicago liquid scintillation system. The aliquots were transferred to small discs of filter paper, which were thoroughly dried before being placed in small pots containing the scintillation fluid, prior to counting. Background activity was determined by counting the disintegrations from 0.01 ml. aliquots of pure acetone/water solvent, treated in the same way as the tannin samples.

The average number of disintegrations found for each weighed sample was used to calculate the total activity of each tannin fraction in millimicrocuries (1 curie is defined as a disintegration rate of 3.7×10^{10} disintegrations/second). The calculated values for the activity of the tannin fractions are given in Chapter 7 (p. 139).

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