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INHIBITORY EFFECT OF OAK LEAF TANNINS ON THE HYDROLYSIS OF PROTEINS BY TRYPSIN

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Abstract—Oak leaf tannin forms complexes with both casein and nettle leaf protein at pH 5·0 and the degree of complex formation depends on the ratio of protein to tannin concentrations and on the time of contact between the protein and tannin. When complexed with oak leaf tannin, casein is almost completely protected from hydrolysis by trypsin at pH 7·6. The pH of the mid-gut of larvae of the winter moth, *Operophtera brumata*, was found to be 9·2 and, at this pH, enzymic hydrolysis of complexes between casein and oak leaf tannins is increasingly inhibited as the proportion of tannin in the initial complex is raised, the inhibition being more marked with condensed than with hydrolysable oak leaf tannin. Reduction in casein digestibility may thus account for the inhibition by oak leaf tannins of larval growth of the winter moth, reared on artificial diets containing casein. When complexed with oak leaf tannins, nettle leaf protein is partially protected from hydrolysis by trypsin at pH 9·2. These observations may help to explain selection in some oak-feeding moth larvae for a high gut pH and for advance of the feeding period to avoid a high tannin content in the host-plant.

INTRODUCTION

LARVAE of the winter moth, *Operophtera brumata* (L.), reared on artificial diets containing casein as the chief protein source, grow more slowly and reach lower pupal weights when as little as 1·0 per cent tannin (from the leaves of the oak *Quercus robur* L.) is incorporated in their diet.[1] The level of tannins in *Q. robur* leaves increases from about 0·5 per cent dry wt. in April to about 5·0 per cent dry wt. in September. Inhibition of growth by tannins may thus be one factor causing selection for early larval feeding periods by the winter moth and perhaps some other species of Lepidoptera which feed on oak leaves.[2]

In view of the polyphenolic nature of tannins[3] and the acknowledged ability of many phenols to form complexes with proteins and to inhibit the action of enzymes,[4] it seemed that the most likely explanation for the inhibition of larval growth by oak leaf tannin might involve interference with the ability of the larvae to digest dietary protein. This paper describes experiments undertaken to determine whether oak leaf tannins have the ability to form complexes with casein and leaf protein and to what extent the protein in such complexes is susceptible to hydrolysis by trypsin. Where possible, the experimental materials and conditions were selected to simulate the known or probable biological parameters.

RESULTS AND DISCUSSION

Formation of Casein–tannin and Leaf Protein–tannin Complexes

It was found that the water-soluble fraction of oak leaf tannin has the ability to bind casein in the form of a complex, insoluble in water at pH 5·0. As expected, therefore, this

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[1] P. P. FEENY, *J. Insect Physiol.* **14**, 805 (1968).
[2] P. P. FEENY and H. BOSTOCK, *Phytochem.* **7**, 871 (1968).
[3] E. HASLAM, *Chemistry of Vegetable Tannins*, Academic Press, London and New York (1966).
[4] J. B. PRIDHAM (editor), *Enzyme Chemistry of Phenolic Compounds*, Macmillan, New York (1963).

tannin has protein-binding powers at the pH of macerated oak leaves. After 15 hr of contact between the casein and tannin, the weights of the complexes follow a linear relationship, indicating complete binding of all protein and all tannin, as far as the point corresponding to a protein–tannin ratio of 1:1 (Fig. 1). At higher relative initial tannin concentrations the curve drops below the theoretical line, indicating that not all the tannin had been complexed. Evidently all the casein was precipitated by half its weight of tannin but the resulting complex could also completely absorb a further equal amount of tannin within the same period. Weight of complex formed thus depends on the initial ratio of tannin to protein and also on the time of contact between casein and tannin, since for all three initial tannin concentrations the weight of complex was consistently lower after 2 hr than after 15 hr of contact (Fig. 1).

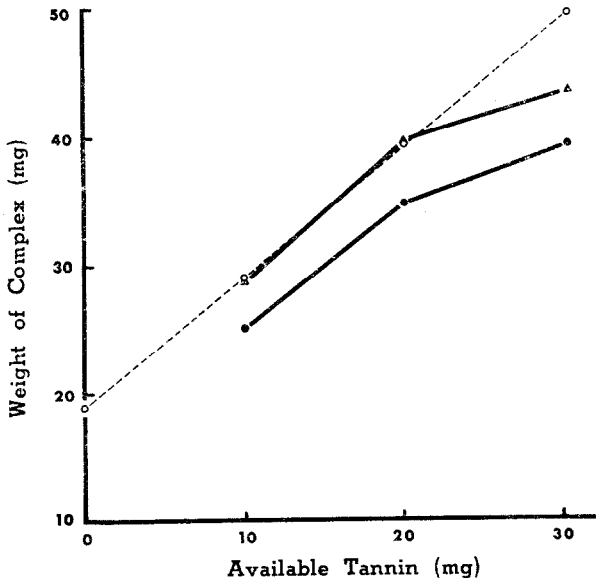

FIG. 1. WEIGHTS OF COMPLEXES FORMED BETWEEN 18·9 mg CASEIN AND INCREASING AMOUNTS OF OAK LEAF TANNIN AT pH 5·0 AFTER 2 hr AND AFTER 15 hr CONTACT AT 20°.

(Means of duplicate values.) ● = complex weights after 2 hr; ▲ = complex weights after 15 hr; ○ = theoretical points corresponding to complete precipitation of all casein and available tannin.

Of more interest from the ecological viewpoint, oak leaf tannin was shown to have the ability to form complexes with nettle leaf protein at pH 5·0. In spite of the short period allowed for tanning (10 min), to simulate mastication of leaf tissue by an insect larva, precipitation approached completion when the protein–tannin ratio reached 1:1 (Fig. 2). Below this equivalence point the curve drops sharply, suggesting that protein which had been tanned only partially is able to remain in aqueous solution. Above the equivalence point the weight of complex continues to increase with initial tannin concentration showing that, as with casein complexes, leaf protein–tannin complex is capable of absorbing further quantities of tannin.

It is likely that tanning for longer periods would result in complexes containing a relatively constant proportion of tannin. Such experiments were not considered relevant to the present biological situation, though the idea is supported by the constant composition of complexes

between tannin from oak leaf litter and nettle leaf protein after long periods of tanning (W. R. C. Handley, personal communication).

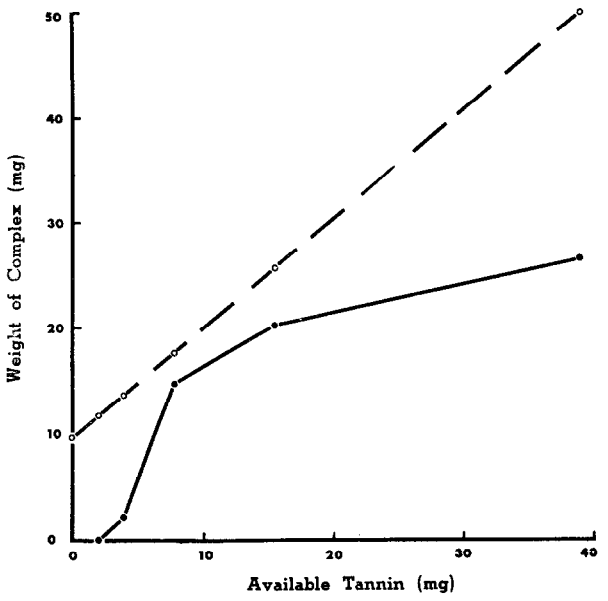

FIG. 2. WEIGHTS OF COMPLEXES FORMED BETWEEN 9·6 mg NETTLE LEAF PROTEIN AND INCREASING AMOUNTS OF OAK LEAF TANNIN AT pH 5·0 AFTER 10 min CONTACT AT 20°.

(Means of duplicate values.) ● = weights of complex found; ○ = theoretical points corresponding to complete precipitation of all protein and available tannin.

Hydrolysis of Casein–tannin and Leaf Protein–tannin Complexes by Trypsin

After incubation with mammalian trypsin for 3 hr at pH 7·6 and 26°, free casein was 82·8 per cent hydrolysed, whereas under identical conditions the same amount of casein in the form of a complex with oak leaf tannin was only 0·96 per cent hydrolysed (Table 1). Evidently the formation of a complex with excess oak leaf tannin at pH 5·0 has the effect of almost completely protecting casein from hydrolysis by trypsin at pH 7·6, within the enzyme's optimal pH range.

TABLE 1. PER CENT HYDROLYSIS OF FREE CASEIN AND OF CASEIN–TANNIN COMPLEX DURING DIGESTION WITH TRYPSIN FOR 3 hr AT pH 7·6 AND 26°

Substrate	TCA-soluble nitrogen (mg)*	Per cent hydrolysis
10 mg casein	1·196	82·8
10 mg casein, complexed with oak leaf tannin	0·014	0·96

* Unhydrolysed casein precipitated by 10% TCA and soluble nitrogen in supernatants determined by Kjeldahl analysis; means of triplicate values, after allowance for reagent controls containing inactivated trypsin (trypsin added after TCA).

It is known that tannin–protein complexes have a tendency to dissociate at high pH values.[5] Since such dissociation might be expected to reduce the inhibitory action of tannin on protein digestion, the previous experiment was repeated at pH 9·2, which was found to be the pH value prevailing in the mid-gut of the winter moth larva. The inhibitory effects of condensed and hydrolysable components were investigated separately. From a plot of per cent protein hydrolysed against initial tannin concentration (Fig. 3) it is apparent that the casein–tannin complexes became less readily hydrolysed as the amount of added tannin was increased. Inhibition for a given proportion of tannin was greatest at low tannin concentrations; as little as 10 per cent of condensed tannin caused a reduction of 20 per cent in hydrolysis. Casein complexed with its own weight of condensed tannin was almost 80 per cent less hydrolysed than the uncomplexed casein control.

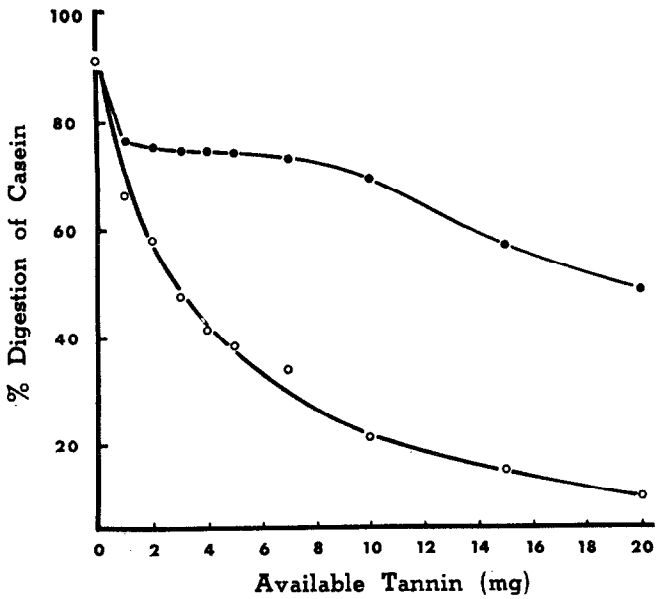

FIG. 3. PER CENT DIGESTION BY MAMMALIAN TRYPSIN AT pH 9·2 (26° FOR 3 hr) OF COMPLEXES FORMED AT pH 5·0 BETWEEN 10 mg CASEIN AND INCREASING AMOUNTS OF CONDENSED AND HYDROLYSABLE OAK LEAF TANNINS AFTER 30 min CONTACT AT 26°.

(Means of duplicate values.) ○ = condensed tannin; ● = hydrolysable tannin.

Hydrolysable tannin was clearly a much less effective inhibitor than was condensed tannin. Even in the presence of a 100% excess of hydrolysable tannin (20 mg per 10 mg casein), casein hydrolysis was still greater than half that of the uncomplexed control.

The final experiment revealed that the results obtained from a study of interactions between casein and oak leaf tannins are likely to be relevant to the ecological situation involving formation of complexes between the tannins and leaf proteins. Washed complexes formed at pH 5·0 between nettle leaf protein and oak leaf tannins were incubated with trypsin at pH 9·2 and the percentage hydrolysis calculated (Table 2). Whereas the uncomplexed leaf protein was 44·4 per cent hydrolysed under the experimental conditions, the same amount of protein was only 12·0 per cent hydrolysed in the presence of condensed tannin

[5] A. BOUDET and P. GADAL, *C. R. Acad. Sci. Paris* **260**, 4252 (1965).

and 20·8 per cent hydrolysed in the presence of hydrolysable tannin. Thus oak leaf tannins have the ability to reduce the digestibility of nettle leaf protein by mammalian trypsin at pH 9·2, though the inhibition is less marked than in the case of casein. As found with the casein complexes, hydrolysable tannin is a less effective inhibitor than is condensed tannin.

These results are in agreement with those of other authors. Loomis and Battaile[6] 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[7] found that inhibition of microbial hydrolysis of the proteins gelatin and gliadin by chestnut, *Castania sativa*, and wattle, *Acacia molissima*, bark tannins was more pronounced at pH 4·0 than at 7·0, and was also greater at a tannin–protein ratio of 4:1 than at a ratio of 1:4. Goldstein and Swain[8] 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

TABLE 2. PER CENT HYDROLYSIS OF NETTLE LEAF PROTEIN (3·4 mg) AND OF COMPLEXES BETWEEN NETTLE LEAF PROTEIN (3·4 mg) AND OAK LEAF TANNIN DURING DIGESTION WITH TRYPSIN AT pH 9·2 FOR 3 hr AT 26°

Substrate	TCA-soluble nitrogen (mg)*	Per cent hydrolysis
Nettle leaf protein	0·124	44·4
Nettle leaf protein, complexed with condensed oak leaf tannin	0·034	12·0
Nettle leaf protein, complexed with hydrolysable oak leaf tannin	0·058	20·8

* Unhydrolysed protein precipitated by 10% TCA and soluble nitrogen in supernatants determined by Kjeldahl analysis; means of duplicate values, after allowance for reagent controls containing inactivated trypsin (trypsin added after TCA).

restored more than 50 per cent of the enzyme activity. Goldstein and Swain[8] conclude that the condensed tannin–enzyme complexes may be held together by other, more stable, means in addition to hydrogen bonds.

Boudet and Gadal[5,9] have found inhibition of β-amylase by tannins from the leaves of the sessile oak, *Quercus petraea* Lieb. (=*sessilis* Ehrh.), which seem to be similar in chemical composition to the tannins from *Q. robur*.[2] They conclude that the inhibition is due to non-specific blocking of active sites by absorption of tannin onto the enzyme. The inhibition appeared to be irreversible, since on resuspending precipitated enzyme–tannin complex at pH 5·0, the enzyme regained only 5 per cent of its initial activity. However, on mixing enzyme and tannin solutions at higher pH values, Boudet and Gadal found that inhibition decreased to zero above pH 6·9, suggesting that stable amylase–tannin complexes are not formed under alkaline conditions. The present author has found that *Q. robur* leaf tannins almost completely inhibit the digestion of starch by α-amylase at pH 6·0.[10]

[6] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[7] J. BASARABA and R. L. STARKEY, *Soil Sci.* **101**, 17 (1966).
[8] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[9] A. BOUDET and P. GADAL, *C. R. Acad. Sci. Paris* **260**, 4057 (1965).
[10] P. P. FEENY, unpublished results.

It is not known to what extent the observed inhibition of casein and nettle protein hydrolysis may be due to inhibition of the trypsin by free tannin, released by partial dissociation of the protein–tannin complex, rather than to shielding of the protein substrate by combined tannin. Both inhibitory mechanisms may be operative at pH 7·6, though, in view of the observations of Boudet and Gadal (see above), it is likely that inhibition at pH 9·2 is due primarily to incomplete dissociation of (substrate) protein complexes, previously formed at a lower pH, rather than to (enzyme) protein complexes formed under alkaline conditions.

The unspecific nature of the interaction between tannins and proteins probably accounts for the widespread occurrence of such interactions in natural systems[4] and may render unlikely, in terms of natural selection, the development of specific metabolic resistant mechanisms. Whereas more or less irreversible complexes are likely to be formed between tannins and leaf proteins when mixed on the acid side of neutrality, one might expect selection to favour an alkaline gut pH in herbivorous insects to maximize the dissociation of such complexes and to minimize interaction between digestive enzymes and free tannin.

If digestibility of casein by the proteinases in the larval mid-gut is reduced by oak leaf tannins in a similar manner to digestibility by mammalian trypsin, it follows that the amount of nitrogen available for larval assimilation within a given time period is likely to be reduced by the presence of tannins in the diet. Such a reduction in the availability of nitrogen seems to be the most likely explanation for the slow growth rates and low pupal weights of winter moth larvae reared on artificial diets containing oak leaf tannins.[1]

Reduction by tannins of the digestibility of leaf proteins by trypsin suggests that the level of nitrogen available for insect growth on their natural diet of leaves may also be reduced in the presence of tannins. Evidence will be presented elsewhere to show that nitrogen is more likely to be a limiting factor for insect growth under natural conditions than is carbohydrate. It has been suggested[1] that the presence of increasing amounts of tannin in oak leaves as the season progresses may be one factor influencing selection for early larval feeding periods on oak leaves. 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 is possible that one of the functions of the high gut pH in the larvae of many phytophagous insects[11] is to increase the amount of nitrogen available from leaf protein–tannin complexes.

EXPERIMENTAL

Materials

A protein preparation from the nettle, *Urtica dioica* L. (kindly provided by Dr. W. R. C. Handley, Dept. of Forestry, Oxford University), was used for these experiments, owing to the considerable difficulty of extracting protein from oak leaves (these have a high content of interfering polyphenols). Handley extracted protein from nettles by homogenizing fresh leaves in distilled water, straining the homogenate through nylon cloth and centrifuging to remove cellular debris. Crude protein was precipitated by addition of citric acid solution at pH 4·5. Before use, the protein was washed by alternate solution and precipitation, brought about by adjustment of the pH, until the supernatant became colourless.

Tannins were extracted from September oak leaves (*Quercus robur*) by a modification of the salting-out procedure of Brown and Love.[12] The water-soluble and water-insoluble fractions of the crude tannin are referred to as fractions S and P, respectively. Fraction S was separated into its component condensed and hydrolysable tannins by chromatography on Sephadex columns and thick paper.[2]

Casein ("Light White Soluble") and mammalian trypsin were obtained from British Drug Houses (Poole, Dorset).

[11] V. B. WIGGLESWORTH, *The Principles of Insect Physiology*, 6th ed., Methuen, London (1965).
[12] B. R. BROWN and C. W. LOVE, Report on Forest Research, pp. 90–92, H.M.S.O., London (1961).

Formation of Complexes Between Proteins and Oak Leaf Tannin

Conditions for these two experiments were chosen to simulate approximately those at which protein–tannin interaction might be expected to occur under natural conditions when food is masticated by insect larvae. Several homogenates of May and July oak leaves in CO_2-free distilled water were found to have a pH of approximately 5·0.

Possible interaction between casein and oak leaf tannin was studied as follows. A solution of tannin of 6 mg/ml was made by stirring 0·36 g into 50 ml of distilled water. Since the tannin was not separated into S and P fractions (see above) 10 ml of acetone was added to complete solution. (This concentration of acetone was found not to precipitate the casein added later.) Sodium chloride (0·1 per cent), to facilitate complex formation, and L-ascorbic acid (0·1 per cent), to minimize oxidation of the tannin, were added. Solutions of 2 and 4 mg/ml were made by dilution of the original solution with distilled water.

Samples of 5 ml of the tannin solutions (containing 10, 20 or 30 mg) were brought to 20°. Casein in aqueous solution (18·9 mg in 4 ml) was added to each, followed by 3 ml of 0·1 M sodium citrate buffer of pH 5·0. After maintenance at 20° for 2 or 15 hr, the samples were centrifuged (3000 rev/min, 5 min). The sedimented complexes were washed with buffer, rinsed briefly with water to remove buffer, and dried at 85° for several days to constant weight. The weights of these complexes, corrected for controls in which tannin or protein was omitted, are plotted against the amount of added tannin in Fig. 1.

The experiment with nettle leaf protein followed a similar procedure, though natural conditions were simulated more closely by addition of the protein after the buffer and by reducing the tanning period to 10 min. (This prior addition of buffer probably results in reduced tanning, because some protein may precipitate before complete mixing with the tannin is achieved.) The weights of complexes derived from protein: tannin ratios from 5:1 to 1:4 are plotted against the amount of added tannin in Fig. 2.

Determination of the Mid-gut pH *of Winter Moth Larvae*

This was achieved by two methods. Firstly, fifteen larvae, reared to the 5th instar on oak and hawthorn (*Crataegus*) leaves, were dissected and the mid-guts immediately transferred to 0·5 ml CO_2-free distilled water. The suspended guts were thoroughly stirred and the suspension transferred rapidly to the micro-cell of a sensitive pH meter, which recorded a pH value of 9·2. After 10-fold dilution of the suspension with CO_2-free distilled water, the pH dropped only slightly, to 8·9, indicating that the original solution was well buffered and that the value of 9·2 was a reasonable estimate of the actual mid-gut pH of the larvae.

The second method followed closely that of Swingle.[13] Each of six 5th-instar larvae, reared on oak or hawthorn leaves, was carefully dissected on a microscope slide, revealing the intact gut. In each case, the mid-gut was pierced with a fine (0·3 mm bore) glass capillary tube (1 in. long) and the fluid gut contents allowed to rise into the tube by capillary action (without coming into contact with the atmosphere). When the fluid had risen halfway up the tube, the tube was quickly withdrawn and its lower end dipped into a drop of thymol blue indicator solution, some of which was 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 in which the gut fluid was replaced by standard sodium barbiturate/HCl buffer solutions (pH range 8·6–9·6). The pH of the guts of all six larvae was found to be 9·2–9·3, thus confirming the value found by the previous method. In the second procedure, unlike the first, there is no danger of the pH value being determined more by the tissue of the gut than by the contents of the lumen.

Enzymic Hydrolysis of Protein–Tannin Complexes

Three experiments were undertaken. The first was to determine the effect of tanning on the digestion of casein by trypsin when the enzyme operates at pH 7·6, within its optimum range for casein hydrolysis (pH 7·5–9·0).[14] Casein–tannin complexes were prepared as follows. Samples of tannin (fraction S only, 20 mg in 1 ml of distilled water) were brought to 26° after addition of 1 ml of 0·1 M sodium citrate/citric acid buffer of pH 5·0. Casein solution (10 mg in 1 ml of distilled water) was added and tanning allowed to proceed at 26° with occasional stirring, for 15 min. The complexes were then centrifuged and the supernatants found by Kjeldahl analysis[15] to contain a mean value of 0·029 mg of nitrogen, indicating that virtually all the casein had been incorporated in the complexes.

After dispersion of the complexes into 1 ml samples of distilled water, 0·1 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7·6, 2 ml) and trypsin (0·25 mg in 1 ml of 0·0025 N HCl) were added at 26° and hydrolysis allowed to proceed at this temperature. After 3 hr the reaction was stopped by addition of aqueous trichloroacetic acid solution (10% w/v) ("TCA"), the solutions were shaken periodically for 1 hr and then centrifuged. The

[13] H. S. SWINGLE, *Ann. Entomol. Soc. Am.* **21**, 469 (1928).

[14] J. H. NORTHROP, M. KUNITZ and R. M. HERRIOTT, *Crystalline Enzymes*, 2nd ed., Columbia University Press, New York (1948).

[15] R. B. BRADSTREET, *The Kjeldahl Method for Organic Nitrogen*, Academic Press, London and New York (1965).

supernatants were analysed by the Kjeldahl method[15] to determine the TCA-soluble nitrogen released by hydrolysis. An exactly similar procedure was followed simultaneously with samples of uncomplexed casein (10 mg in 1 ml distilled water) in place of casein–tannin complex (Table 1).

A second experiment was undertaken to determine separately the effects of hydrolysable and condensed tannins on digestion of casein by trypsin at pH 9·2, the mid-gut pH of winter moth larvae. To simulate more closely the natural situation, casein was tanned at pH 5·0 as described above, but the complexes were not sedimented from excess tannin prior to hydrolysis.

To samples of solutions of condensed tannin (from 0 to 20 mg in 3 ml distilled water) were added 0·01 M citrate buffer (pH 5·0, 1 ml) and, after equilibration at 26° for 30 min to permit tanning of the casein, the pH was brought to neutrality by addition of 0·1 M NaOH solution (1 ml). (The addition of NaOH was necessary to neutralize the citrate buffer and so prevent evolution of CO_2 on subsequent addition of the carbonate buffer.) 0·2 M carbonate buffer ($Na_2CO_3/NaHCO_3$, pH 9·2, 2 ml) was added immediately and the pH confirmed to be 9·2 (previous tests had shown that this reagent system gave a stable pH of 9·2, with good buffering capacity). Trypsin (0·5 mg in 1 ml 0·0025 N HCl) was added and hydrolysis allowed to proceed at 26° for 3 hr, after which the soluble nitrogen released by hydrolysis was estimated as before. An identical experiment was carried out simultaneously using hydrolysable instead of condensed tannin.

All Kjeldahl titres were corrected for controls (omitting either protein or tannin and with either active or inactivated trypsin) and converted to percentage of casein hydrolysed by trypsin (Fig. 3), using the value of 14·5 per cent for the nitrogen content of the casein (British Drug Houses specification).

In a third experiment, leaf protein complexes formed (*a*) with condensed tannin and (*b*) with hydrolysable tannin, and subsequently washed with distilled water to remove uncombined tannin, were separately exposed to the action of trypsin at pH 9·2. To samples of the tannin solutions (20 mg in 1 ml) were added 0·01 M citrate buffer (pH 5·0, 1 ml) and an aqueous solution of leaf protein (3·4 mg in 1 ml). After periodic shaking at 26° for 30 min, the solutions were centrifuged and the sedimented complexes washed with distilled water. The complexes were next dispersed into samples of 3 ml distilled water before addition of 0·2 M carbonate buffer ($Na_2CO_3/NaHCO_3$ at pH 9·2, 2 ml) and trypsin (0·5 mg in 1 ml 0·0025 N HCl). After incubation for 3 hr at 26°, hydrolysis was halted by addition of TCA (3 ml) and the supernatants decanted for Kjeldahl analysis. Samples of leaf protein solution, with distilled water as the only additive, were also analysed to determine the total nitrogen content of the leaf protein preparation (found to be 8·21 per cent).

After correction for reagent controls (omitting tannin or protein and with either active or inactivated enzyme), Kjeldahl titres were used to calculate the per cent hydrolysis of the leaf protein in each duplicate pair of samples (Table 2).

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