TANNINS:

A BIOCHEMICAL RE-ANALYSIS

OF THEIR

IMPORTANCE AS ANTI-FEEDANTS

by

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ABSTRACT

Tannins have long been thought of as antifeedants owing to their presumed digestibility-reducing properties. In this thesis information, at the molecular level, is presented in a reassessment of this assumption and the apparency theory of plant chemical defence which is dependent upon it.

An introductory review provides chemical and opperational descriptions of tannins and a general outline of their ecological impact. Detailed attention is given to (i) tannin-protein complex formation and (ii), an assesment of *in vivo* evidence concerning the effects of tannins on herbivores. It is concluded that the evidence does not support the hypothesis that tannins uniformly reduce digestion, even though they do generally act as antifeedants.

A series of crude tannin-containing plant extracts were prepared and characterised by chemical analyses and by their ability to precipitate protein and inhibit pepsin/cellulase digestion of celulose. Results indicated between-tannin variation but not that the chemical properties of crude tannins might be used to predict their interaction with the other components of a herbivore's diet.

Experiments under conditions where soluble tannin-protein complexes formed and which modelled some digestive systems, showed that tannins could under varying circumstances, inhibit or promote the digestion of protein. Soluble

tannin-protein complexes were also formed in the presence of bile salts when they would otherwise have occured as precipitates. In these conditions clear relief from digestibility reduction was found. In the light of these results a new model describing the effects of tannins on digestion, consistent with results obtained *in vivo*, is proposed.

The influence of light intensity on tannin production was investigated in the field and their metabolic cost was examined using a radiochemical technique.

A concluding chapter draws together all the available information concerning the ecology and metabolism of tannins and proposes a unified hypothesis as to their production and distribution in plants.

Chapter One

Introduction

1.1 Tannins and their Interaction with Proteins

The term "tannin" came into use at the end of the eighteenth century to define those organic substances present in the water-soluble extracts of plants, which effect the transformation of raw animal hide into leather. The changes that occur during the tanning of hide result in the flexible properties of skin being preserved in leather which has considerable resistance to heat, water, abrasion and microbial decay. The mechanism of tannage is still imperfectly understood, but it is generally thought to occur through the bonding of tannins to otherwise bacterially degradable regions of collagen fibrils in hide (Gustavson, 1956; Haslam, 1966).

The resistance to putrefaction conferred on hide by tannins has long been recognised, for instance the grave-digger in Shakespeare's "Hamlet" observes that a particularly well preserved corpse is that of a tanner. It is the interaction of tannins with proteins and other biological macromolecules, particularly their interference with enzymic degradative processes, that provides the underlying focus for the work presented in this thesis.

1.1,1 An Operational Description and Definition of Tannins

Tannins may clearly be defined through their action in tannage, however this is not the only field of human endeavour in which they are of importance. They are responsible for haze formation in beer (Van Sumere et al., 1975), they influence the palatability of wines, ciders and various fruit and vegetable crops (Swain, 1962; Van Sumere et al., 1975; Lea, 1978) and they have been used as astringents in pharmaceutical preparations (Trease and Evans, 1978). In each instance tannins act through complex formation with protein e.g. those of the hops, mouth or skin. As in the case of beer haze, tannin-protein complexes tend to be insoluble and protein precipitation has been used to define and thus assay tannins (Horowitz, 1970).

Gustavson (1956) defined tannins as "that portion of the water-soluble vegetable materials which precipitates gelatin from solutions and which can be so completely removed from solution by hide powder that the residual solution will not precipitate gelatin from solution". The use of gelatin, a collagen derivative, and hide powder in the definition is important as the protein type influences the precipitation reaction between tannins and proteins and thus controls the quantification of tannins by precipitation assays. Gustavson (1956) also

recognised that agents which did lead to gelatin precipitation did not necessarily make good leather tanning materials.

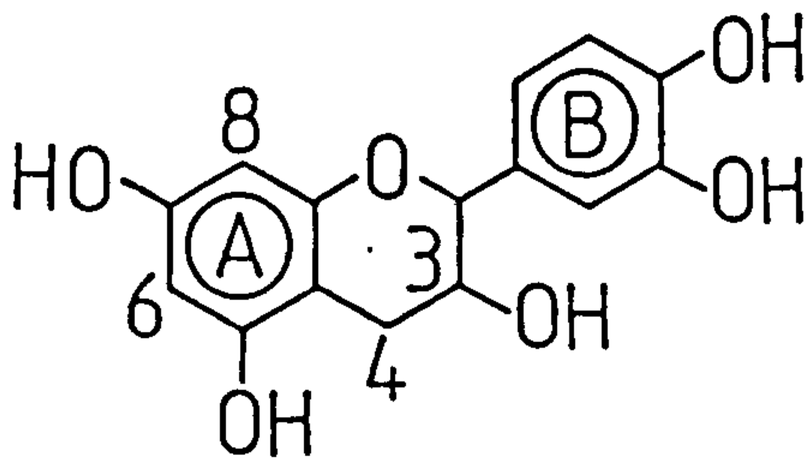
The definition of tannins in operational terms is thus not clear cut. Here they are considered to be "natural products able to cause the precipitation of proteins from aqueous solutions" and no reference to leather production is made. This ability to precipitate proteins has been termed astringency (Bate-Smith, 1973a), a word taken from the latin ad = to and stringere = to bind. In less technical contexts an astringent is a substance that stops bleeding and causes skin contraction, hence the medicinal use of tannins. Astringent substances are unpalatable to the human animal as they cause a puckering and drying sensation in the mouth; tannins may also be bitter in taste but this is considered a separate phenomenon (Bate-Smith, 1972; Lea and Arnold, 1978). This organoleptic astringency is another distinctive attribute of tannins (Joslyn and Goldstein, 1964), besides protein precipitation, and it has also occasionally been used as a measurement or diagnostic test (Ariga et al., 1981; Ariga and Asao, 1981).

1.1,2 The Chemical Nature of Tannins

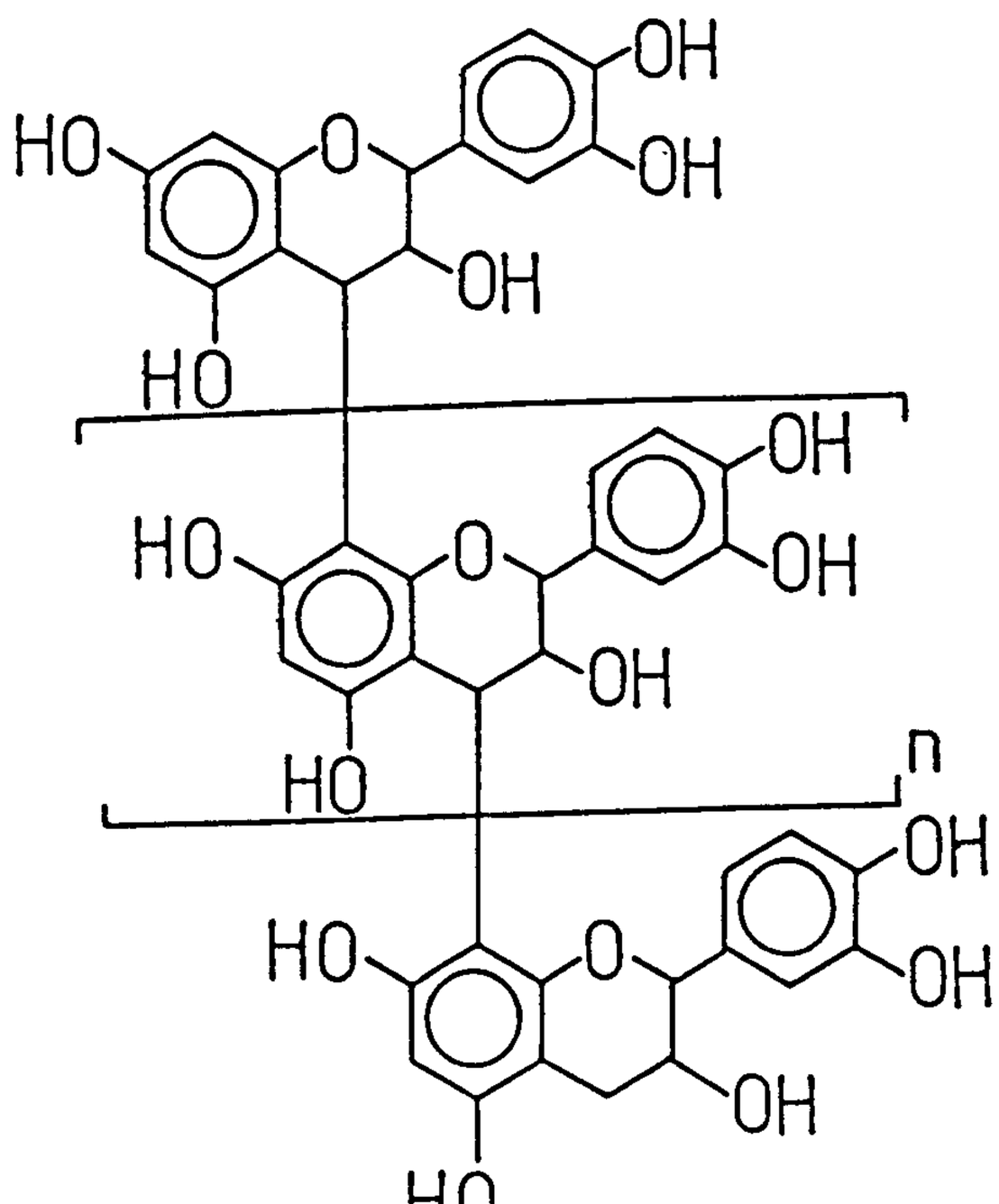
The tannins were first shown to be divisible into two groups of polyphenolic compounds by Freudenberg (Haslam,

1981). These groups are called the condensed tannins and the hydrolysable tannins, although both can undergo hydrolysis in aqueous media. They differ in their component subunits and the bonding between these. Condensed tannins are polymers derived from flavan-3-ols such as catechin (I) and or flavan-3,4-diols (Haslam, 1981). They may be typified by the structure (II). The important point to note is that the interflavan bonds are carbon-carbon bonds, C-4 to C-8 linkage is typical but C-4 to C-6 links (III) are also reported for the mollisacacidin (IV) derived tannins of Acacia mearnsii (Haslam, 1981). Given that the monomeric flavanol cannot act as a tannin then condensed tannins range in molecular weight from 560 upwards (two units).

In contrast hydrolysable tannins are typically derived from ellagic acid (V) and or gallic acid (VI) monomers or polymers which are esterified to a central glucose molecule, e.g. Tannic acid (VII). Hydrolysable tannins are thus a structurally variable group of substances which do not attain the high molecular weights of the longer condensed tannin polymers, remaining at about 10^3 in weight rather than exceeding 10^4 (Jones et al., 1976). This is noteworthy as molecular size may well be a factor influencing the relative abilities of tannins to precipitate proteins (Gustavson, 1956; Oh and Hoff, 1979).

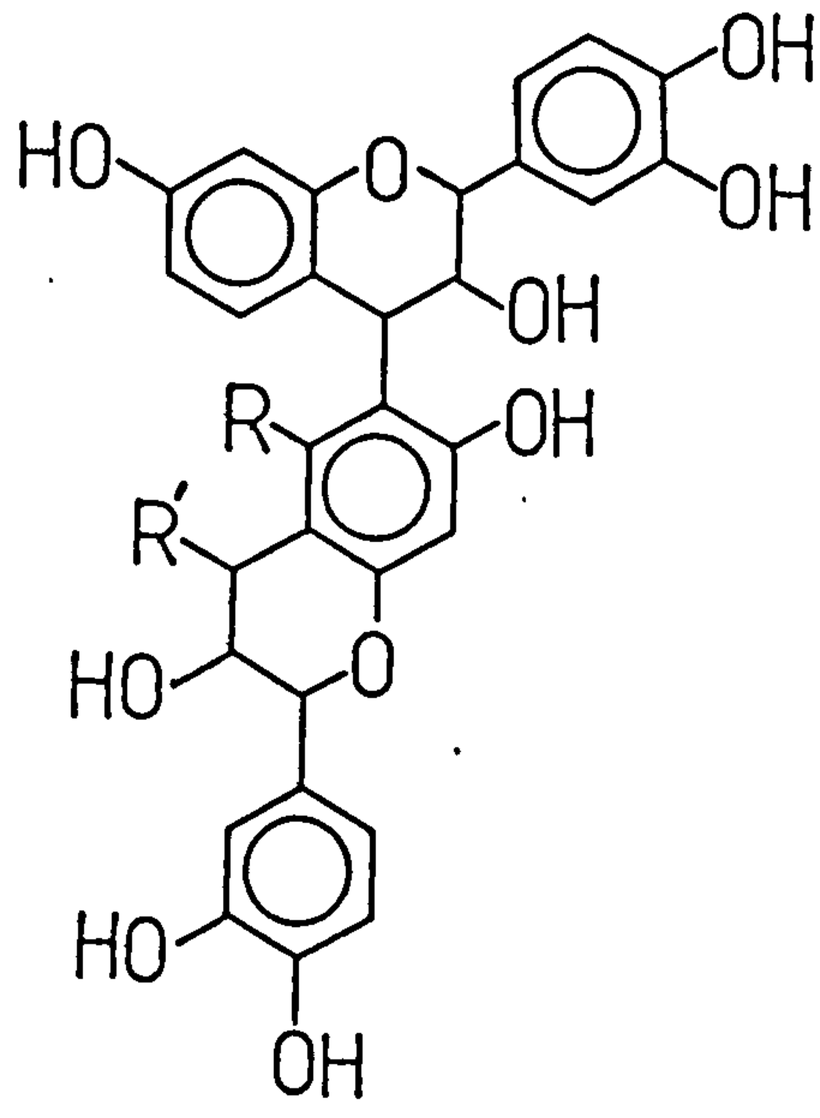


I Catechin, showing letters and numbers used to denote benzene rings and some carbon atoms.



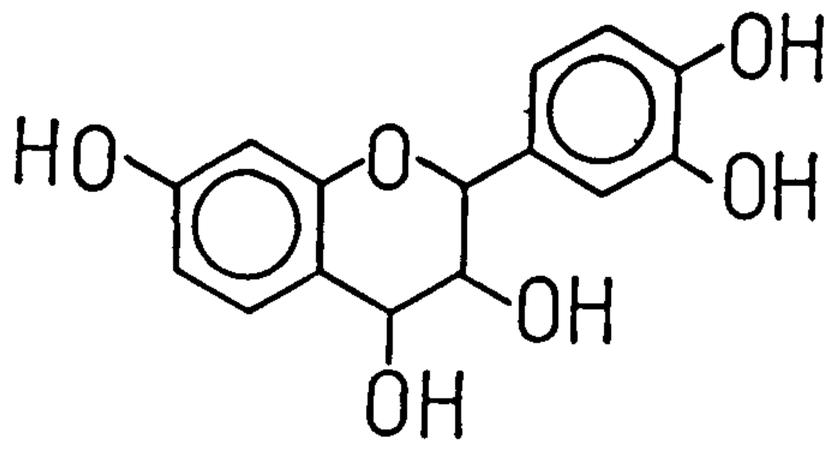
II A Condensed Tannin (procyanidin), n= 0,1,2,3...

structures I & II re-drawn from Haslam, \1981)



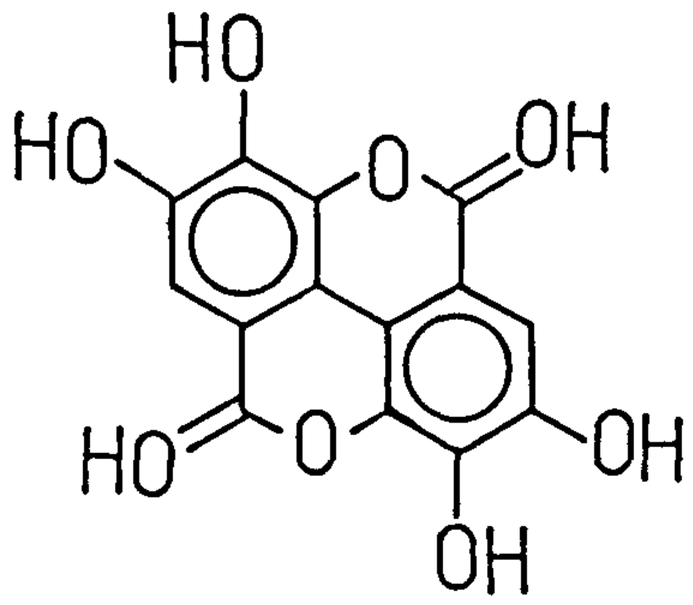
III A Condensed Tannin (from Acacia mearnsii)

R= OH and R'= H, or R= H and R'= OH.

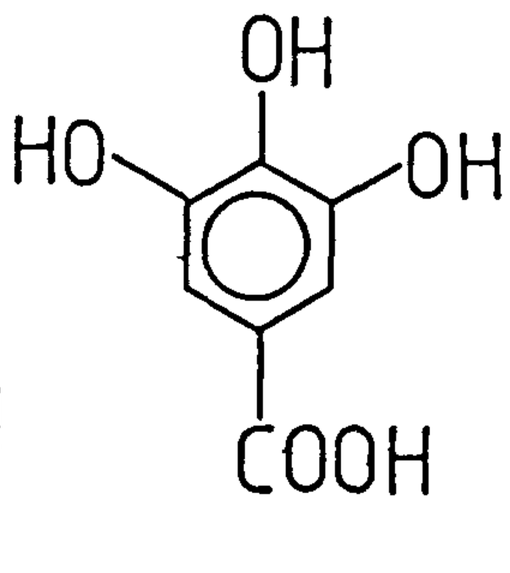


IV Mollisacacidin (precursor of III)

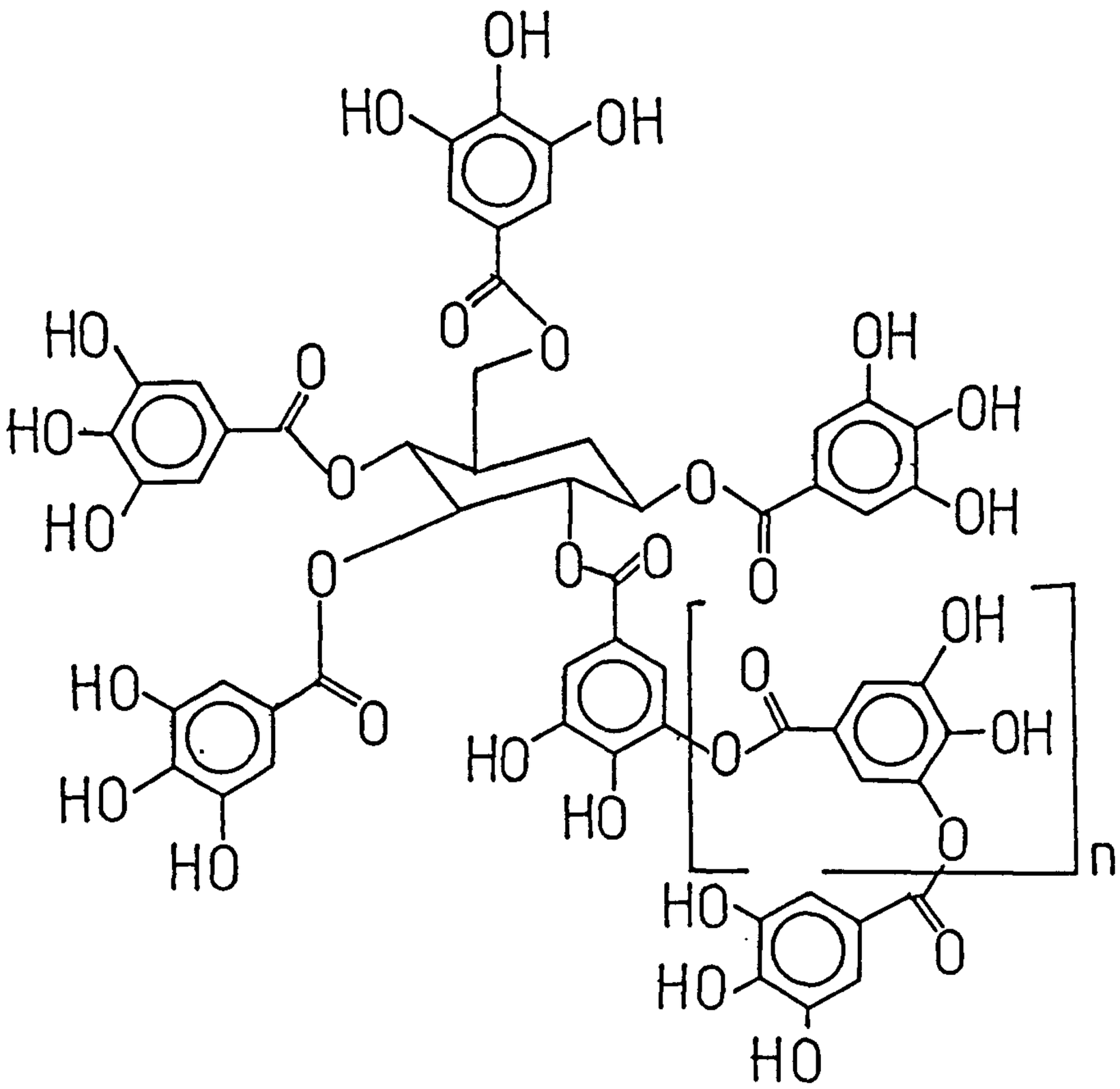
structures III & IV re-drawn from Haslam, (1981)



V Ellagic acid



VI Gallic acid

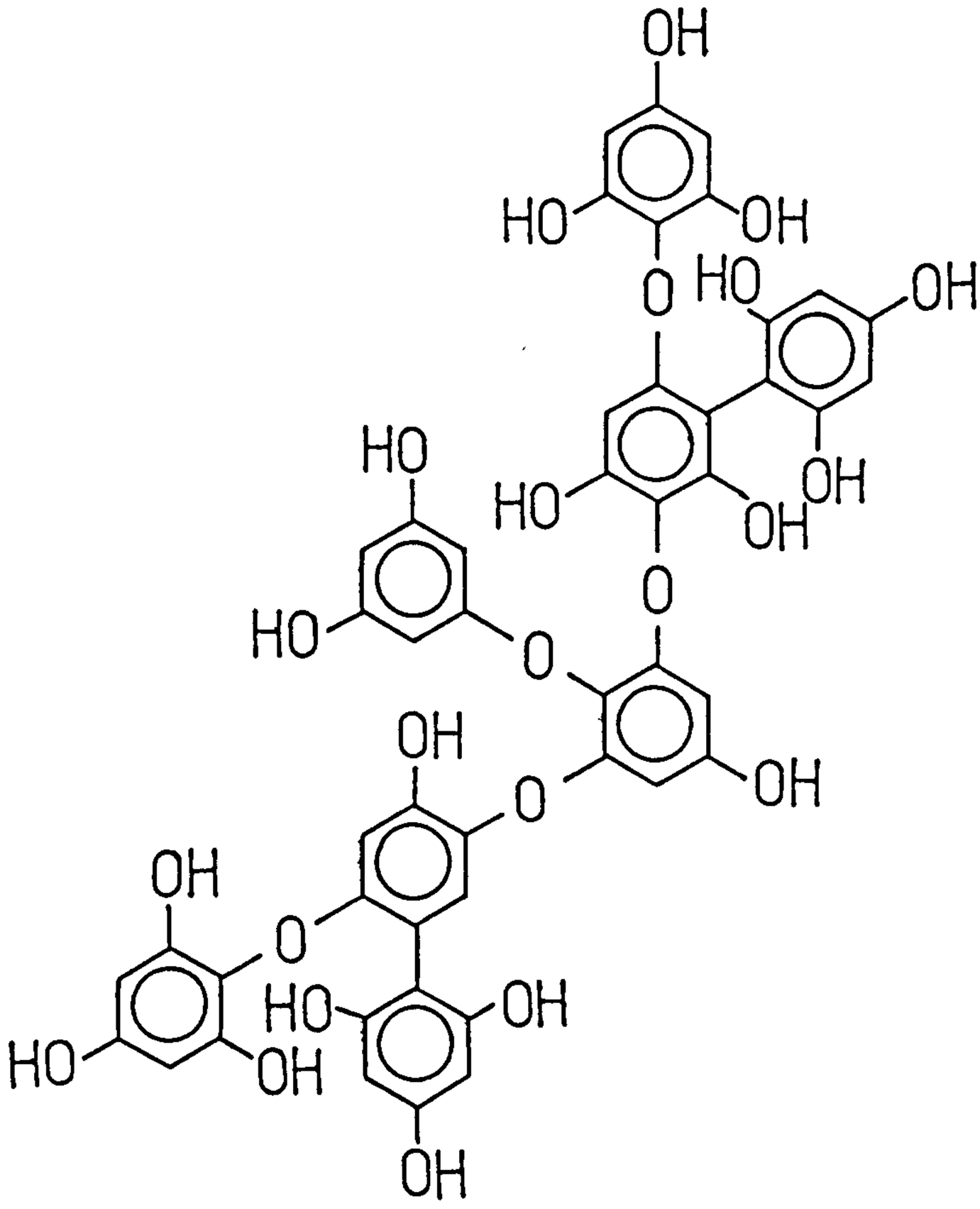


VII Tannic acid, n=0,1 or 2

structures V, VI & VII re-drawn from Haslam, (1981)

Whilst Freudenberg's classification remains in use, it does not encompass the whole range of functional tannins, nor is it exhaustive in describing tannins composed of the subunits listed above. For instance the galloyl or phenylpropanoid substituted condensed tannins (Nonaka et al., 1981, 1982; Takenaka et al. 1983) and non-glucose based hydrolysable tannins (Haslam, 1966) may indicate further types of operational tannins. Chemically quite unrelated tannins have been reported such as the phloroglucinol derived phlorotannins (VIII) of the Phaeophaceae (Ragan, 1981; Grosse-Damhus et al., 1983), which are the presumed cause of astringency in brown algae. The phenolic resins of Larrea tridentata (Rhoades, 1977a) have also been demonstrated to inhibit protease activity by complex formation with proteins.

Swain (1979a) has included all the above amongst a range of naturally occuring substances which he has classed as tannins. His criteria for defining tannins were threefold. Tannins are (i) of high molecular weight, typically 1000 to 3000, and (ii) they have sufficient phenolic hydroxyl groups to complex with proteins and other macromolecules containing -OH and $-NH_2$ groups, by forming hydrogen bonds at normal pH. These hydrogen bonds should (iii) be susceptible to auto or enzyme catalysed oxidation to form covalent linkages. On the basis of these criteria he recognised four sub-types



VIII A phlorotannin. Structure described by Grosse-Damhus et al. (1983). Some hydroxyls may be substituted by halogens in vivo, although smaller unsubstituted structures have been found (Ragan 1981).

of tannin: true tannins (condensed and hydrolysable tannins), prototannins (their precursors; e.g. catechin), β-tannins (low molecular weight substances which satisfy criteria ii and iii only) and oxytannins (oxidation products of small molecules which satisfy criteria i, ii and iii upon their formation). Swain (1979) carefully left protein precipitation out of his definition which usefully encompasses the Larrea resins of Rhoades (1977a) which have never been shown to precipitate proteins. Indeed these β-tannins are insoluble in water except in protein solutions into which they will partition out of ether (Rhoades, 1977a). Swain's definition is biologically useful for what it includes yet it is practically awkward in the three measurements required to define a substance as a tannin.

The present writer feels that it is more helpful from the practical point of view to define tannins (sensu strictu) as in section 1.1,1. Maintaining the historically important phenomenon of protein precipitation in the definition is also of great use in avoiding semantic confusion in more theoretical discussions of tannins and their effects. According the status of "pseudo-tannins" to other molecules which fall within Swain's definition, and which it may be biologically expedient to regard (sensu lato) as tannins is seen as the best way to approach these anomalies.

The problem of defining tannins is compounded by the general co-occurrence of several chemically distinct types of tannin in the same plant. The chemical complexity of commercial tanning materials has been demonstrated by White (White, 1957; Gustavson, 1956) who detected at least 27 phenolic spots on a bidirectional chromatograph of Mimosa tannin (Acacia mearnsii; wood, bark), a condensed tannin source, and 40 spots for Myrabolans (Terminalia chebula; fruit), a hydrolysable tannin source. Whilst the majority of the total phenolics present in these crude tannins may well be composed of related substances e.g. condensed tannins of different polymer lengths (Oh and Hoff, 1979), two problems arise when attempting to relate their chemical and protein precipitating properties. Firstly, proximate analyses for, say, condensed tannins, by anthocyanidin formation (Swain and Hillis, 1959; Gartlan et al. 1980), will not account for variations in astringency due to polymer length or interflavan bond type. This problem is soluble by isolating each structrally unique condensed tannin and studying it alone, which leads to the second problem, namely of potential synergistic interaction between the components of crude tannins. Low molecular weight components of tannins, perhaps not themselves able to precipitate proteins, may help solubilise large condensed tannins and or take part in bonding to protein

(Gustavson, 1956).

Where tannins in crude extracts from a large range of taxonomically unrelated plants are to be compared, exhaustive chemical analysis of each extract is impracticable. The development and use of a series of different proximate techniques may be the best way to relate the protein precipitating ability of these plant constituents to their chemistry. Further discussion of the chemical nature of tannins and the use of such means to characterise them is reserved for Chapter 2.

1.1,3 Tannin-Protein Complex Formation and Intermolecular Bonding

Tannins are operationally and strictly defined as being able to form precipitates with proteins in aqueous solutions, however, soluble complexes resulting from the interaction of tannins with proteins are also likely to be formed. Calderon et al. (1968) physically demonstrated that these were formed between gelatin and the hydrolysable tannin tannic acid by a light-scattering technique. Given the chemical nature and macromolecular size of proteins, and of many tannins, as well as the complexes formed between them, then their solutions will be colloidal systems where the components are dispersed in an aqueous medium. Processes leading to the formation of tannin-protein precipitates may then be separable as

nucleation and growth processes as generally described by Shaw (1970). Precipitation after nucleation is not the only alternative to continued dispersion; total solvent entrapment in the dispersed phase may occur by gel formation. Indeed, gel formation by the addition of tannin to viscous protein solutions has been observed by the writer. Viscosity, reactant concentrations, adsorption inhibitors, particle-particle aggregation and orientation effects are amongst factors controlling the growth of colloidal particles (Shaw, 1970). Not surprisingly these are also among the themes to be found in the literature on tannin-protein precipitation reactions (Van Buren and Robinson, 1969; Hagerman and Butler, 1978,1980a, 1980b; McManus et al., 1981).

In addition there are two other colloidal effects of importance here. Firstly the addition of a flocculant to a colloidal solution does not immediately cause precipitation, there is a "threshold effect", once sufficient binding has occured a floccus forms. Superoptimal flocculant concentrations can lead to the dissolution of the floccus (Shaw 1970). Both threshold precipitation effects (Schultz et al., 1981) and redissolution effects(Van Buren and Robinson, 1969) are found with tannin-protein systems. Secondly there is the idea of bridging. Large polyelectrolytes with adsorbable surface groups, e.g. hydroxyl or carboxyl, have been

found to make good flocculants (La Mer et al., 1956), this probably being due to their size and ability to form cross-links between molecules. McManus et al. (1981) considered that tannins acted by a bridging action, causing the aggregation of tannin-protein complexes, particularly at high protein concentrations as would be exemplified by gel formation. However, at lower protein concentrations the adsorption of tannins or other phenolics to the protein surface, making it more hydrophobic, was thought to be the more important process leading to precipitation. By this argument McManus et al. (1981) predicted and demonstrated the precipitation of proteins by the simple phenolics resorcinol and pyrogallol, which are not normally considered to be tannins.

The literature on tannin-protein interactions is almost entirely devoted to studies where the formation of a precipitate is the only phenomenon used to show an interaction. Factors demonstrated to influence precipitation may have acted at any stage in the colloidal process outlined above and do not necessarily relate to the interaction of single tannin and protein molecules. Additionally any haze or opaque suspension produced has generally been termed a precipitate whether it precipitated or not. Centrifuges have been used to achieve precipitation, an example of note being Martin

and Martin (1983) who used a centrifugal force of 19,000 g! The biological relevance of whether dense precipitates or hazy suspensions occur will be discussed in Chapter 4, here factors believed to influence tannin-protein bonding will be introduced with due regard to the foregoing comments.

Classically tannins are thought to bind to proteins by the formation of hydrogen bonds between their phenolic hydroxyl groups and the carbonyl oxygens and amido nitrogens of the protein's peptide bonds (Loumis and Battaile, 1966; Swain, 1979; Mc Manus et al., 1981). In a major review of the tannin-protein interaction Zucker (1983) pointed out that in alpha-helical protein structures the peptide bonds face inwards and are thus not clearly favorable sites for binding. Nevertheless Hagerman and Butler (1980a) showed that the hydrogen bond acceptor N-N-dimethyl formamide inhibited the precipitation reaction, as would be expected on a competitive basis. Such a hydrogen bonding dependent mechanism is also thought to account for the action of polyvinylpyrrolidone (PVP) in removing beer hazes (see Figure 1.1 and Anderson and Sowers, 1968).

Oh et al. (1980) showed that whilst nonionic and anionic detergents dissociated tannin-protein complexes, the cationic detergents, urea and guanidine hydrochloride were less active or ineffective in this regard. They

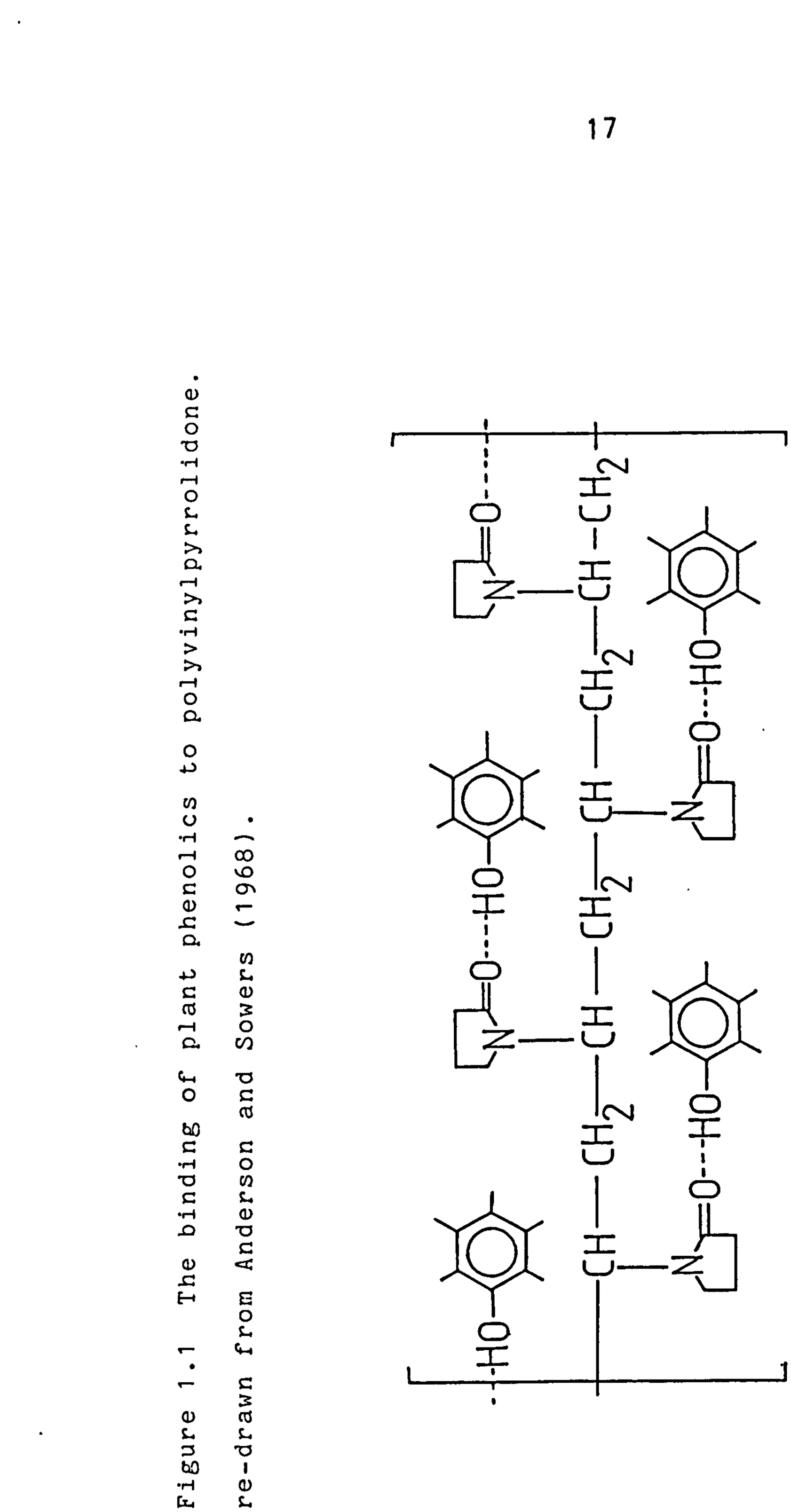


Figure 1.1 The binding of plant phenolics to polyvinylpyrrolidone re-drawn from Anderson and Sowers (1968).

found that the relative hydrophobicities of polyamino acid substrates positively influenced the ease with which they could be precipitated by tannins, as did increased ionic strength and reaction temperature. This evidence for hydrophobic bonding was further augmented by their demonstration of tannin adsorption by an uncharged polystyrene resin. Further evidence for hydrophobic bonding in tannin-protein complexes is given by Hagerman and Butler (1980a) where the non-polar solvent dioxane is shown to inhibit precipitation in contrast to methanol and ethanol which promoted precipitation.

Both hydrogen bonds and hydrophobic bonds are fully reversible and indeed so too are freshly formed tannin-protein precipitates (Hagerman and Butler, 1978). Mejbaum-Katzenellenbogen and Dobryszyka (1962) have even been able to regenerate immunochemically recognisable proteins from such complexes. This last point shows that the reversibility of tannin-protein interactions extends to regenerating apparently intact tertiary protein structures. Stronger bonding types have, however, been suggested such as salt (ionic) linkages and covalent bonds (Swain, 1965), and some examination for covalent links has been made. Covalent bonds between tannins and proteins are thought a likely conesequence following the oxidation of o-diphenols to quinones (Synge, 1975). Diphenoloxidases occur in many tannin rich plants and so

may readily effect this. Although quinones are important in leather tannage (Gustavson, 1956) there is little direct evidence for their occurrence in naturally formed tannin-protein complexes (Van Sumere et al., 1975). Zucker (1983) is also of this view, citing the ease with which tannins can be extracted from plants using aqueous acetone (Foo and Porter, 1980) as further evidence that tannins are not covalently bound to plant material after death. With the proviso that covalent links may assume progressively more importance with time and after complexation, bonds in tannin-protein complexes seem to be mostly hydrophobic and hydrogen bonds (Zucker, 1983). Thus it appears that Swain's (1979) definition of tannins needs revision to include hydrophobic bonding amongst the forces linking tannins to proteins.

The factors governing the stability of these tannin-protein links as outlined above may now be summarised as (i) tannin and protein concentrations, both relative and absolute, (ii) the ionic strength of their aqueous solutions and (iii) the presence of competitors for the formation of either major type of bond. Further effects such as the tannins' relative ability to cross-link complexes may additionally determine the occurence of precipitation or gel formation. The above are general considerations and Chapters 2 to 4 deal with the influences that individual tannins and proteins bring

to the system. At this point it is nevertheless convenient to introduce an additional factor, namely the effect of pH. At acid pH values and/or close to the isoelectric pH of a protein, precipitation is generally observable in a solution containing tannins and proteins. In an otherwise identical system it will generally not occur at more alkaline pH values (Loumis and Battaille, 1966; Hagerman and Butler, 1978). It is this observation that has played a major role in the ecological literature concerning tannin-protein complex formation.

1.1,4 The Binding of Tannins to Other Biological Macromolecules

Very little work has been carried out in this area although starch-tannin adsorption has been studied (Davis and Hoseney, 1979). Tannins also form complexes with cellulose, pectin, and other plant cell wall components as well as alkaloids (Swain, 1965). Zucker (1983) has noted the very different mobilities of condensed and hydrolysable tannins on paper chromatograms: he concluded that condensed tannins are inherently better able to bind to cellulose owing to their poor mobilities. In summary, tannins do seem able to at least bind to a range of other biochemicals besides proteins, although none of these have the same diversity of potential binding sites (Zucker, 1983), and our understanding of these processes

is extremely poor.

1.2 The Ecological Status of Tannins

Tannins are considered to fulfil the role of allelochemics (Whittaker and Feeny, 1971; Rhoades and Cates, 1976; Swain, 1979), that is to say chemicals that mediate ecological interactions between organisms of one species and another. As Whittaker and Feeny (1971) pointed out, the gross functioning of ecosystems may be described in terms of elemental nutrient cycles and fluxes of chemically available energy, but allelochemicals provide what they termed "color and design". The important processes in which tannins appear to play an allelochemical role are (i) the defence of living plants against herbivores and pathogens and (ii) the decay of dead plant material and the associated return of nutrients to living plants.

Non-allelochemical functions for tannins have also been proposed. McClure (1979) has reviewed evidence that tannins act at a physiological level as antagonists to giberellin production; however, from Section 1.1 it will be appreciated that tannins have the ability to antagonise many biochemical processes through their interaction with proteins. In agreement with McKey (1979) and Zucker (1983) it will be assumed here that tannins have no important *in vivo* role at the physiological level.

In considering the relation of plants to their

physical environment, operationally defined tannins such as the phenolic resins of Larrea tridentata may act as antidesiccants and ultraviolet screens (Rhoades, 1977b). By contrast, condensed tannins and hydrolysable tannins occur intracellularly and not on leaf surfaces and so would not be expected to act as antidesiccants. Furthermore, the UV spectra of condensed tannins show that they do not absorb at the required wavelengths and so would not screen underlying cytoplasm from its deleterious effects (Waterman et al., 1984).

Against this background only allelochemical roles for tannins are considered to be of importance in what follows. Whilst the major emphasis is put on their role in plant-herbivore interactions, a summary of their other effects is given to allow some comparisions of mechanisms to be made.

1.2,1 The General Allelochemical Effects of Tannins in the Environment

Polyphenolic compounds, including tannins, have wide ranging effects on ecosystems. They may, for instance, play a role in pedogenesis by the flocculation of clays or the cheluviation of ions especially iron (Bloomfield, 1957; Satchell, 1974). The directly antibiotic effect of tannins is strikingly illustrated by the blackwater river phenomenon (Janzen, 1974) where darkly coloured

polyphenol-rich river waters support a noticeably depauperate biota. The surrounding terrestrial vegetation is tannin-rich and produces a poorly decomposable litter, the phenolics of which are thought responsible for the podzolisation of the underlying soils as well as for seeping into and colouring the rivers.

Toxic effects of polyphenolics may account for the particularly small fish fauna of blackwater rivers (Swain, 1979), and their astringent properties may reduce the palatability of plant litter to cominuting detritivores (Lofty, 1974; Cameron and La Point, 1978). It is, however, the formation of insoluble complexes with proteins and plant cell wall polysacharides which has been viewed as their major means of exerting allelochemical effects.

Handley (1954) introduced the concept that resistant tannin-protein complexes, formed on plant cell death, mask cell walls from microbial attack and so inhibit the degredation of both cell walls and protein. He was able to physically demonstrate masking and histochemically demonstrate that tannin-rich litter followed a different mode of decomposition to tannin free litter, resulting in the production of mor and mull humus forms respectively. The observation of different humus forms in forest soils with the same litter input and turnover times (Tanner, 1981) clearly suggests the real ecological importance of

factors controlling the chemical mode rather than the rate of litter decomposition. Little work has been done on the fundamental processes whereby tannins may affect this. Handley (1961), did however, manage to demonstrate that nitrogen release to growing plants from plant-extract tanned protein was directly related to the ability of the plant to produce mull humus. Similar results for the release of mineralised nitrogen were also obtained by Fenton (1958). Handley (1961) made the point that lightly tanned protein was able to give a slower but more sustained release of nitrogen than untanned protein and so support better plant growth. This possibly beneficial action of tannins has more recently received support from Synge (1975) although the only independent line of biochemical evidence for the action of tannins in soils has come from Starkey and co-workers (Benoit and Starkey, 1968a, 1968b; Benoit et al., 1968; Lewis and Starkey, 1968). They examined the effects of tannin on a range of substrates (protein, chitin, starch and cell wall polysacharides) and their associated degradative enzymes. Tannins were, in all cases, found to reduce the rates of enzymic degradation of these substrates, thus further supporting the idea that tannins are generally able to inhibit biochemical reactions in the environment.

Tannins are also thought to leach out of leaf litter (Baldwin and Schultz, 1984) and, through an imprecisely

defined mechanism, inhibit the nitrification process in soils (Baldwin et al., 1983), particularly in climax communities (Rice, 1977). Whilst this effect may not be general (Lee et al., 1983) this and the recalcitrance of organic nitrogen to mineralisation may well control the growth and development of some plant communities (Jordan et al., 1979; Vitousek et al., 1982).

However, tannin-rich vegetation as exemplified by oak and beech forests does not become submerged in its own detritus i.e. leaf and woody litter, so clearly tannins and their complexes are biodegradable. Lewis and Starkey (1969) implicated the Deuteromycetes as the most likely decomposers of these complexes in the soil, although it remains the case that only one organism, Penicilium adametzi (Grant, 1976), has been unequivocally shown to utilise a condensed tannin as a carbon source with which to support growth. As Grant (1976) points out, the first enzyme in the relevant catabolic pathway for tannins is likely to have some unusual properties. Bocks et al. (1963 1964) had little success using fungi to break condensed tannins into smaller fragments for analysis although hydrolysable tannins were more prone to degradation. In the defence of living plants against pathogenic attack tannin-rich tissues should be relatively immune from successful attack, as tannins would be expected to inactivate most fungal enzymes and

viruses through complex formation (Swain, 1977; Konshi and Hotta, 1979). Levin (1971, 1976) has proposed that tannins may be of particular importance in the defence of woody tissues whilst Swain (1978) believes that tannins have been of major importance to the successful evolution of land plants through their allelochemical action on microbes. Recent evidence that shows tannins to be at least one important factor in resistance to fungi is provided by Donnelly (1983) who encountered a pronounced susceptibility to Rhizoctonia species in low tannin varieties of Lespedeza cuneata during plant breeding trials.

This anti-microbial action of tannins extends to some specialised plant-herbivore systems, as in the case of ruminant mammals which depend on microbial fermentation. Another particularly interesting case is afforded by attine ants which cultivate "fungal gardens" with leaf material, so that they can crop the fungus as food. Seaman (1984) has shown that not only do the ants avoid plant material likely to be rich in tannin, but that tannins act directly upon the fungus culture to reduce the yield of food to the ants.

1.2,2 The Effects of Tannins on Herbivores

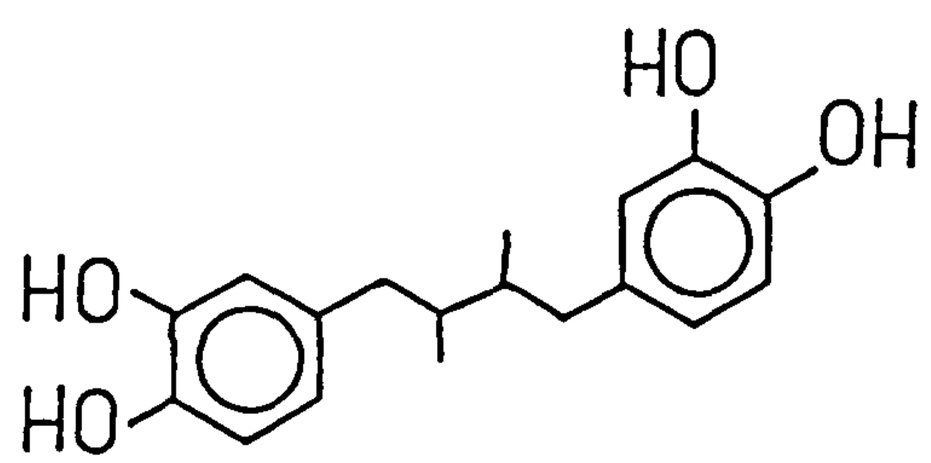
Tannins are thought to reduce the palatability of plant material to herbivores through astringency and to

reduce its digestibility through complexation reactions, primarily with plant proteins. Two studies have been particularly instrumental in establishing this view amongst ecologists.

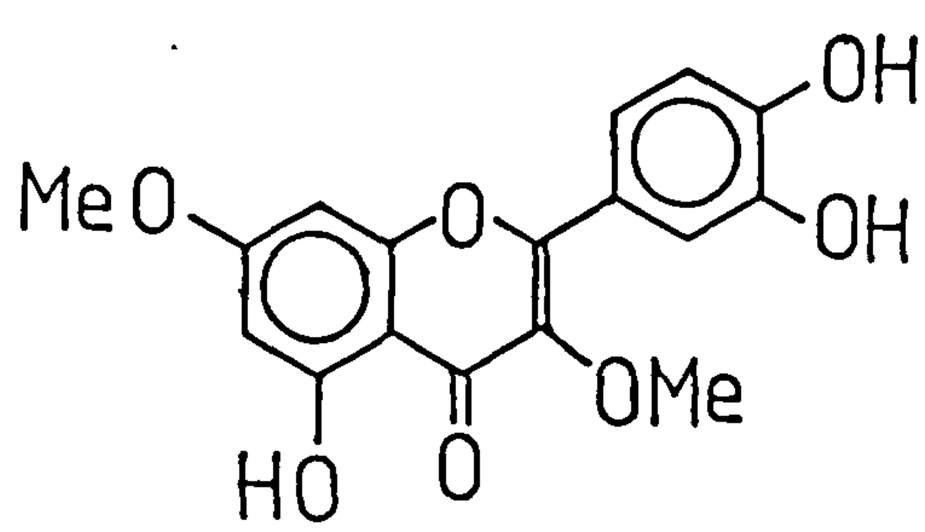
Feeny (1968, 1969, 1970; Feeny and Bostock, 1968) examined the effects of oak leaf tannins on the herbivorous lepidopterans which consume oak foliage, particularly the winter moth, Operophtera brumata. The oak is a climax dominant species for much of western Europe, whilst Feeny found the winter moth to be the dominant lepidopteran herbivore, in spring, on the plants he studied. In spring Feeny found that the foliage had its maximum level of nitrogen and minimum level of tannin on a dry weight basis, whilst as the leaves aged during the year levels of these constituents fell and rose respectively. Feeny argued that nitrogen was likely to be a limiting factor in the nutrition of insect herbivores and that the formation of indigestible complexes between tannins and proteins would reduce the nutritional quality of the foliage and so reduce the fitness of the herbivores consuming it. In spring foliage, tannins amounted to 0.66% dry wt., a level which rose to 5.50% by September, yet in studies of food selection only 1% was required to deter feeding by winter moth caterpillars consuming artificial substrates. Whilst this deterrent effect was probably due to adverse

palatbility, Feeny's main interest was to examine digestibility reduction. By an *in vitro* simulation of mastication and proteolysis he showed that the formation of insoluble tannin-protein complexes during mastication would probably reduce nitrogen nutrition *in vivo*. He then demonstrated that dietary tannin consumption by winter moth lavae reduced growth rates and pupal weights, both factors likely to reduce fecundity. Using ^{14}C labelled material Feeny showed that the tannins did not pass through the peritrophic membrane and so their action must be located within the gut. The conclusions drawn were that tannins were likely to act *in vivo* through forming indigestable tannin-protein complexes, presumably insoluble precipitates as seen *in vitro*, and do not act as toxins within the body *per se*.

Rhoades (1977a, 1977b; Rhoades and Cates, 1976) studied the antiherbivore chemistry of *Larrea tridentata*, a dominant of large areas of semi-arid vegetation in southern USA and Mexico. The leaves of this plant are covered in a phenolic resin, the major components of which include nordihydroxyguariaretic acid (IX) and flavonoids such as quercetin-3,7-dimethly ether (X). He found that this resinous coating tended to deter chewing insects from consuming foliage and that given a choice they would take the least resinous offered including that which had had the resin removed. A phenol oxidase system



IX Nordihydroxyguariaretic acid



X Quercetin-3,4-dimethyl ether

structures IX & X re-drawn from Rhoades, (1977a)

in the leaves was shown to enhance the action of the resin. Thus the plant contains β-tannins and oxytannins sensu Swain (1979). Like Feeny, in his study, Rhoades was able to demonstrate that the phenolic resin of L. tridentata formed a complex with proteins: the insoluble resin being found to partition out of diethyl ether into aqueous solutions of protein. Furthermore these complexes were poorly digestible by erepsin and proteases freshly prepared from the plant's natural herbivores. Phenoloxidases enhanced this digestibility reducing effect. Again like Feeny, Rhoades showed that during an in vivo study, the resin was responsible for reduced larval growth rates in the plant's natural herbivore Astroma quadrilobatus. Whilst still based on the assumption that nitrogen is the limiting factor in the herbivore's diet, this study supports the idea that tannins (sensu lato) may act primarily as digestibility reducing substances.

These studies of Feeny and Rhoades, as outlined above, depend on the assumption that nitrogen is a major limiting factor in the diet of the relevant herbivores, the limiting shortage of which is further exacerbated by tannins. Whilst Rhoades (1977a) was able to improve the palatability of Larrea by resin removal, Feeny (1970) could not so demonstrate that tannins actually served as the allelochemical agent in oak. Indeed, he considered

that increased leaf toughness was the chief proximal factor if not the ultimate cause of lowered herbivory in mature oak leaves (Feeny 1970).

1.2,3 Plants as Food for Animals

Considerable effort has been expended in assessing the utility of plant material as food for animals and in particular for insect herbivores, with particular attention being paid to the importance of nitrogen nutrition. The basic problem for insect herbivores is that leaves contain up to 90% water and only 1-3 % protein, indeed almost all plant tissues have markedly less protein than insect and mammalian tissues. Animal tissues are also relatively higher in gross energy content and phosphorus than plant tissues which lack or have insufficient levels of the particular requirements of animals, such as the amino acid methionine or the B vitamins. The net effect of this is that herbivores have to consume a disproportionately large amount of plant tissue per unit of animal tissue produced. The requirement for nitrogen is particularly clear when one considers that carbohydrates form the structural materials in plants whilst nitrogenous materials are involved in this role in animal tissues (Hodkinson and Hughes, 1982). Reviews by McNeil and Southwood (1978) and Mattson (1980) emphasise the fact that the levels of

nitrogen in plant tissues span the correct range of magnitude to control herbivore growth, in particular they cite evidence for positive associations between ecological conversion and digestion efficiencies with dietary nitrogen content. Given that tannins can prevent the efficient digestion of protein they would thus seem in a position to influence herbivore feeding success.

It should, however, be noted that plants are variable both within and between species as to their nitrogen content, as are different parts of the same plant, e.g. meristematic tissues versus mature foliage. Whilst low nitrogen nutrition can limit a plant's growth, low plant nitrogen contents which nevertheless support plant growth may in their own right act as antiherbivore defence mechanisms (Auerbach and Strong, 1981) in certain circumstances (Morran and Hamilton, 1980). Some plants may thus actually reduce the need for chemical defences in this way.

1.3 Tannins Set in Context with Other Antiherbivore Allelochemicals

Tannins are by no means the only allelochemicals involved in the defence of plants. Indeed they are but one of a wide range of plant secondary metabolites, i.e. substances not involved in primary and intermediate metabolism, which are known to confer protection upon plants against herbivores (Harborne, 1982). Table 1.1 provides a list of some of these other types of secondary metabolites together with their (potential/supposed) allelochemic activities. The list does not aim to be comprehensive, it does, however, aim to illustrate the chemical diversity of these substances and, most importantly, the range of physiological processes they may antagonise in animals. In contrast to almost all of the compounds listed in Table 1.1 the tannins would not be expected to penetrate into the body tissues because of their size and polar chemical nature. This observation led Feeny (1976) and Rhoades and Cates (1976) to propose two broadly similar hypotheses regarding the action and distribution of antiherbivore allelochemicals. They regarded tannins as having strictly digestibility reducing effects which were exerted within the digesta. By virtue of their restriction to the gut lumen tannins were not expected to be toxic to other physiological processes. The majority of other substances in Table 1.1

Table 1.1 Non-tannin potential antiherbivore allelochemicals*

Class of chemical	Examples	Activity
I Nitrogen containing		
Alkaloids	morphine	May have dramatic and deleterious effects on both
	strychnine	insect and mammalian physiology, generally through
	emetine	direct action upon nervous systems
Non-protein amino acids	pipecolic acid	May disrupt protein synthesis by their innapropriate
	canavine	binding to t-RNA and/or incorporation into proteins,
	mimosine	thus leading to generally inhibited metabolism.
Cyanogenic glycosides	limarin	Release cyanide gas as plant cells are disrupted by
	amygdalin	mastication, and so poison oxidative metabolism by
	lotaustralin	their action on cytochromes.
Amines	putrescine	Many smell repellant whilst some are halucinogenic,
	mescaline	phytophagy may then be reduced by either atribute.

Table 1.1 continued

Glucosinolates	sinegrin	Acrid and bitter tasting, some toxic.
Peptides and proteins	abrin, ricin viscotoxin	May be protoplasmic toxins, haemagglutinins or specific trypsin inhibitors.
Methylazomethanol glycosides	cycasin	powerful carcinogens, found in cycads.
II Non-nitrogenous		
Cardiac glycosides	ouabain oleandrin	Pharmacological activity on the heart, can also be emetic.
Triterpines	limonin	Bitter tasting substances.
Phytosterols	Estrone ecdysone	Hormone analogs which may disrupt animal reproductive and developmental physiology.
Saponins	yamogenin	Haemolyse blood cells, sometimes toxic in other ways

Table 1.1 continued

Diterpeniods	agathic acid	Widespread in resins and latex, some toxic.
	grayanotoxin-1	
Sesquiterpene	vermeerin	Can be toxic to mammals and insects by their action
lactones	arbusculin	on nervous tissue, contact dermititis also reported
Polyacetylenes	falcarinol	some toxic
Organic acids	oxalic acid	Both of these acids are toxic due to their ability
	monofluoroacetic	to inhibit the tricarboxylic acid cycle.
Flavonoids	rotenone	Insecticidal activity
Quinones	aloe-emodin	Cathartic action

* Compiled with reference to Harborne (1973, 1982); Bell (1978) and Burnett et al.

are thus toxic because they do pass into the body tissues and interrupt body functions. These toxins are best exemplified by cardiac glycosides or alkaloids. Such toxins generally occur at concentrations of less than 2% dry wt. in plant tissues whilst tannins have been known to reach levels of 60% dry wt. (Rhoades and Cates, 1976). In drawing these distinctions, antiherbivore allelochemicals could be divided into two groups: those which act at high concentration and in direct proportion to their concentration, and those whose action is at low concentration, not strongly concentration dependent, and due to specific biochemical qualities. These two groups have become known as the digestibility reducing substances and toxins of Rhoades and Cates (1976) respectivly, which correspond to the quantitative and qualitative defences of Feeny (1976).

Antiherbivore allelochemicals do not all fall into one or other of these two extreme categories. The trypsin inhibitors provide an example of a group of digestion inhibiting allelochemics which act at a low concentration similar to that of the catalyst they inhibit. This contrasts with the less specific protein complexing action of tannins which are generally presumed to act at higher concentrations, primarily on the enzyme's substrate. Whilst not digestibility reducers, resins and latexes which act to "gum up" insect mouth parts or legs

(Tingey and Gibson, 1978) probably act in a quantitative manner. So, too, may many toxins as it seems to be accepted that many animals can cope with a range of toxins provided they are encountered in small amounts (Feeny, 1976; Glander, 1983). Despite these difficulties, the division of chemical defences into essentially quantitative and qualitative types does seem to have been a helpful concept when attempting to formulate reasons for the different ecological strategies adopted by plants using different types of chemical defence.

1.3,1 The Apparency Theory of Plant Defence

In their studies of digestibility reducing substances both Rhoades (1977a) and Feeny (1970) used species which were dominant in their natural vegetation. Such vegetation dominants were considered to be "apparent" plants, that is to say "easily susceptible to discovery by whatever means their natural enemies might employ" (Feeny, 1976). Apparent plants are thus liable to be abundant, large, long-lived, evergreen, strongly scented, with conspicuous coloured flowers, etc. All these properties make them "predictable" in space and time in the sense of Rhoades and Cates (1976), who preferred not to include the host finding abilities of the herbivore in their definition. "Unapparent" plants tended to have

opposing characters and so to be "ephemeral" in space and time.

When they examined the evidence availble to them both, Feeny (1976) and Rhoades and Cates (1976) came to the conclusion that unapparent/ephmeral plants tended to contain chemically diverse qualitative/toxic defences in contrast to the quantitative/digestibility reducing defences of apparent/predictable plants. The application of apparency in time was used by both Feeny (1976) and Rhoades and Cates (1976) to account for the changes in the levels of chemical defence investment by oak and creosote bush leaves as they matured. The developmental stage exposed to herbivores for least time was seen to have the least defence investment in quantitative defences. Rhoades and Cates (1976) reinforced the point by citing evidence that as tannin levels increased in bracken as the fronds matured, there was a corresponding decrease in cyanogenisis, the toxic defence system which is most pronounced at the ephemeral young frond stage.

In explaining the divergent distribution of allelochemics between apparent and unapparent plants and plant parts both Feeny (1976) and Rhoades and Cates (1976) made assumptions concerning the relative metabolic costs of producing toxins and digestibility reducing substances. They both argued that chemical defence production should be costly in terms of plant resources

and that the rewards should be commensurate with the level of this defence investment. Both authors assumed that because of the large percentage dry weight investment in quantitative defences, this indicated a proportionately large metabolic cost. This assumption is clearly wrongly founded as one would not estimate the productivity of an ecosystem from its standing crop of biomass; similarly, deductions from quantities of metabolites ignore the influence of metabolic turnover and net carbon fluxes through the plants concerned. This point will be returned to in Chapter 7; here the development of the ideas of Feeny, Rhoades and Cates will be continued.

The assumed lower cost of toxins does fit well with the presumed escape in time of ephemeral plants from herbivores, i.e. they do not need to expend so much metabolic energy on chemical defence because of their life history patterns. The protein complexing substances developed as defences by apparent plants were seen by Feeny (1976) and Rhoades and Cates (1976) as the logical consequence of being unable to escape from herbivores and so having to have an effective defence against a wide range of predators. Tannins were thought impossible to circumvent completely over evolutionary time because of their general ability to disrupt the vital process of digestion. The non-specificity of their ability to bind

to protein was thought to be the very reason behind the lack of any evidence that tannins could be "detoxified". Toxins were seen as less effective because they could be subject to specific counter-adaptation. Many examples of this exist; some particularly well establised ones concern the Ragwort/Cinnabar Moth, Potato/Colorado Beetle, Cabbage/Cabbage White Butterfly and Legume Seed/Brucid Beetle interactions (Edwards and Wratten, 1980). In each of these plant-insect interactions the plant remains unpalatable to most herbivores yet in each case a particular insect species has evolved a detoxification mechanism for that particular plant's allelochemicals and thus become a sucessful herbivore on that plant. The allelochemicals concerned in the above examples are all different and the detoxification mechanisms are certainly not all common (Applebaum, 1964; Brattsten, 1979). All this variation represents a considerable genetic investment in plant-herbivore interactions by both the plants and animals over evolutionary time, a point made by Boxenbaum (1983) who noted that for mammals this is the very reason for the diverse range of xenobiotics that they are able to metabolise; i.e. the xenobiotic metabolism of animals is an inheritance resulting from considerable evolutionary adaptation to overcoming the potentially toxic effects of plant secondary products. Both Feeny (1976) and Rhoades

and Cates (1976) state their ideas explicitly as hypotheses deriving from and contributing to the theory of co-evolution (Ehrlich and Raven, 1964) between plants and insects. The genetic investment referred to above being the result of an evolutionary arms race involving plant allelochemicals and animal detoxification mechanisms. The most effective toxic type of defence is clearly one for which there is no detoxification mechanism, thus divergent and unusual toxins are liable to selection, whilst evolutionary convergence on digestibility reducers is expected on account of their infallibility. However, this latter strategy would be found economically viable only for those plants needing that level of defence investment. Put in these evolutionary terms, this is the explanation that Feeny (1976), Rhoades and Cates (1976) and Cates and Rhoades (1977) arrived at for what they concluded was the the likely distribution of plant chemical defences against herbivores.

The work of Feeny (1970, 1969, 1968, Feeny and Bostock, 1968) and that of Rhoades (1977a 1977b) together with the papers presenting the ideas outlined above (Feeny, 1976; Rhoades and Cates, 1976; Cates and Rhoades, 1977) are still very widely cited today. Rhoades (1979), using essentially the same evidence, stated that "tannins act by forming relatively indigestible complexes thereby

reducing the rate of assimilation of dietary nitrogen" and that "It is no accident that the most widespread form of defensive plant secondary metabolites, namely tannins and related phenolics, act mainly by inhibition of digestive processes, providing protection against all classes of enemies that acquire nutrients by the breakdown of plant tissues." These ideas have indeed been accorded theory status (e.g. by Rhoades, 1979; Bernays, 1981; Berenbaum, 1983; Coley, 1983; Zucker, 1983). A brief survey of their use in more general studies of both plant defence chemistry patterns and processes in community ecology will be given before a critical review of the evidence that tannins actually act as outlined above is presented in Section 1.4.

1.3,2 Apparency, Defence and Coevolution

Divorced from hypotheses about plant defence the concept that some plants are more apparent to herbivores than others has proved useful in studying the community ecology of phytophagous insects. Faunal species diversity on host plants tends to decline with declining apparency (trees > shrubs > perenials > weeds and annuals). Similarly, if the concept is extended to include successional status and taxonomic isolation as well as local abundance and seasonality, then again apparency has been useful in explaining differences in

community structures (Strong et al.,1984). It should be noted that species diversity is not a measure of herbivorous damage although the latter has been related to plant apparency in at least one instance (Abul-Fatih et al., 1979).

When hypotheses relating apparency to plant defences are introduced the concept does not seem to have been of such value. The palatability of early successional plants to generalist herbivores does seem to be greater in early versus late successional species (Cates and Orians, 1975; Reader and Southwood, 1981) but the data is very variable (Strong et al., 1984). Futuma (1976) has provided contradictory evidence to this data whilst Maiorana (1978) has argued that the concept of generalist herbivores is untenable. The lower levels of herbivore damage that might be expected to occur on ephemeral plants (that escape in time) have not been detected (Coley, 1980), and additionaly when Jung et al. (1979) examined the distribution of allelochemics in a stable community system they were unable to find the divergence in toxin type defences predicted.

The problems encountered with the theory may be attributable to its inception as an explanation for events resulting from coevolution in the strict sense of Ehrlich and Raven (1964). The general occurrence of such tightly coupled stepwise evolution has recently been

brought into doubt (Jermy, 1984; Strong et al., 1984), and it does seem that such closely linked interaction between plants and animals may well be the exception rather than the rule. A good case in point are the phytoectysteroids in ferns. Despite the obvious temptation to invoke a coevolutionary explanation for their presence, no allelochemical action has been seen when either adapted or non fern feeding insects were fed ferns, so just how and why did these plants evolve to contain phytoectysteroids? To maintain a coevolutionary explanation one has to resort to an invocation of the ghost of competition past (Connell, 1980) which is of course not verifiable.

Strong et al. (1984) conclude that the insect fauna which feeds on particular plants is likely to be a "pot pourri of the coevolved, preadapted and opportunistic in varied and unpredictable proportions". They believe that coevolution does not provide a general mechanism for the contemporary structure of phytophagous insect communities, but that it does in some individual cases. It seems reasonable to assume that coevolution may be a similarly weak general explanation for other phytophagous animal communities. Given the foregoing it would seem useful to examine the action of tannins on the widest range of herbivores possible so as to obtain general results for all taxonomic groups of herbivores. Feeny

(1970) and Rhoades (1977a) worked on only a few insect species and so counterbalancing evidence for mammalian herbivores is particularly needed. This will further the current process of re-evaluating the physiological effects of tannins in phytophagy and the ecological hypotheses dependent on them (Bernays, 1981; Martin and Martin, 1983).

1.4 Tannins as Deterrents to Herbivory: a Review of the Evidence

Since the early work of Feeny (1970) a considerable number of studies concerning the influence of tannins on the voluntary consumption and digestion of plant material have become available, although hitherto these have only been partially reviewed (Bernays, 1980, 1978; Kumar and Singh, 1984). The responses by animals to diets containing tannins range from a reduction in voluntary food intake leading eventually to starvation and death, through to increased food consumption over and above that found with a tannin free diet. This review attempts to be comprehensive and to this end some 74 studies of plant-herbivore systems have been examined. Brief summaries of these are given in Tables 1.3 and 1.4. which divide them into invertebrate and vertebrate studies respectively. No discussion of individual studies is given in the text which concentrates on examining the general trends that can be construed from this information.

1.4,1 Experimental Designs of Feeding Studies

The studies described in Tables 1.3 and 1.4 are concerned with 36 invertebrate and 38 vertebrate feeding interactions respectively. Many of these involve animals

fed artificial diets containing tannic acid or quebracho tannin, although tannins from some eleven plant families are represented in experimental diets and a still wider range in those studies reporting field observations. Tannins in the widest sense are included here (e.g. phlorotannins and protein-complexing phenolic resins), the presence of these atypical types being noted in the Tables and also in the text where conclusions depend critically on studies concerning them.

The information collated below falls into six categories. (i) Information concerning the individual experimental systems and their interpretation in ecological terms. Thereafter evidence for the following measurable effects of dietary tannin consumption has been sought, (ii) altered food consumption rates, (iii) altered growth rates, (iv) altered digestive efficiencies, (v) altered nitrogen nutrition, and finally (vi) metabolic or pathological effects.

No two groups of workers have used the same methodology in investigating the effects of dietary tannin consumption. Each study listed in the Tables concerns itself with a named species of herbivore (or group of herbivores) feeding upon a tannin containing diet. The observed relationship between tannin content and its presumed effects is based upon correlations. Some investigators have shown that the correlation is

strongest with tannin content versus other likely and measured variables (Table 1.3, Studies 23, 27, 30, 33; Table 1.4, Studies 30-34) yet by no means all investigations are so thorough.

Many of the diets are artificial and the tannin they contain is thought to be the only source of dietary allelochemicals. Except where stated these represent "no choice" feeding situations. In contrast whole plant diets will normally contain other allelochemicals as well as tannins (e.g. gossypol in cotton). Additionally, whole plant diets are less easily controlled for variation in other factors such as their fibre, nitrogen or water content. In these respects results from the consumption of whole plant material are less easily interpreted. The main advantage with whole plant studies is that they provide an ecologically realistic diet concerning which herbivore preference is usually known. Additionally many of the studies of animals consuming whole plants have been conducted in the field where the animals are feeding on the plant by choice, i.e. in contrast to most artificial diets. In Tables 1.3 and 1.4 such information as is known about the ecology of the situation is provided in the notes marked "A". Succeeding information in the tables provides data on the measurable effects of tannin consumption; these are discussed here in terms of their probable underlying

mechanisms.

The most frequent measurement or observation made is whether animals consume tannin-rich food in preference to tannin-poor (or tannin-free) food. In a "no choice" feeding situation this may be reflected in reduced food consumption when experimental animals are compared to control populations feeding upon a tannin-free diet. It should be noted that these two situations are not directly comparable as the "no choice" situation requires the animal to consume tannin to complete its other nutritional requirements, whereas the situation allowing the expression of a preference between food materials provides an escape from this. Tannins are also generally expected to reduce food consumption rate by virtue of the unpleasant (to humans) astringent sensation they produce in the mouth (Joslyn and Glick, 1964). It is widely assumed that for invertebrates, the activity of tannins on sensory receptors in the mouth parts will be similar in effect. Tannins released from plant tissue through masticaton are expected to complex with salivary mucoproteins as well as proteins of the mouth surface, and so to directly reduce the ease and comfort with which food is swallowed because of their effects upon buccal lubrication. Observations regarding palatability or consumption rate (CR) are recorded under notes "B" in the Tables. Definitions of nutritional indices used in the

Tables (e.g. CR) are given in the preceeding Table (Table 1.2).

Another frequent observation in tannin-related feeding experiments is that of animal growth rate (GR) in relation to tannin consumption; this was the measurement used by Feeny (1969). It is important to realise that animals may grow less well on a tannin-containing diet because they (i) eat less food, (ii) they digest the food they do eat less well or (iii) because they are being poisoned; indeed all three of these may apply. Observations relating to growth rate are recorded under notes "C" in the Tables. Whilst showing an important effect they yield no information on the possible mechanisms by which tannins may affect herbivores.

Particularly for work on insects, there is much useful information available on digestive efficiencies and this is also recorded under notes "C" in the Tables. Definitions for the terms "Approximate Digestibility" (AD), "Efficiency of Conversion for Digested food" (ECD) and their product "Efficiency of Conversion for Ingested food" (ECI) are provided in Table 1.2. Such data does indicate mechanisms whereby tannins may affect growth rates. Of major importance is the effect of tannins on AD as opposed to ECD. If AD is affected by tannin (e.g. reduced) then tannin is acting on the process of digestion as expected by Rhoades (1979); however, if ECD

is affected then the tannin is acting on the post digestive processes involved in the conversion of digested food into animal biomass. Whilst tannins might act in a quantitative manner on either component in ECI, there is no currently accepted mechanism to explain any effect on ECD, and such an effect would be at variance with the conclusions of Rhoades (1979). Nevertheless it is important to realise that the results for AD and ECD treat the gross features of digestion and with regard to AD, no distinction is made as to whether tannins influence the digestive enzymes or the substrates, and amongst substrates no differentiation is made between the digestion of protein and of other substances. It might thus be possible for AD not to be greatly affected whilst the specific digestion of protein was.

The possible influence of tannins in limiting nitrogen nutrition has been stressed in Section 1.2 from which it will be clear that the expectation is that for the protein component of ECI, it is AD and not ECD that should be affected. Information relating specifically to nitrogen nutrition is collated under notes "D" in the Tables. Measurments of excreted or faecal nitrogen (NEX) are frequently made. Two difficulties in interpretation arise, firstly the measurement of NEX for mammals is generally exclusive of the urinary component and secondly all measurements are inclusive of endogenous protein

losses. These endogenous losses may include enzymes, mucous or peritrophic membrane and tannins are likely to increase the loss of these thus making it impossible to measure undigested dietary protein as NEX. This proviso applies to all indices of nitrogen nutrition and needs to be circumvented by ^{15}N tracer experiments.

Finally there are several reports of metabolic or pathological effects consequent upon the consumption of tannins. These range in severity from unusual metabolites appearing in the urine of animals consuming tannins to records of outright death. Needless to say, reports of this nature are not expected for allelochemicals which are only supposed to inhibit digestion. For most of these effects the causal mechanisms point to the activity of tannins or their component phenolics from outside the gut lumen and within the animal's tissues. Information on these aspects of tannin consumption is presented under notes "E" in the Tables.

With the nature of the evidence to hand now established, the effects of tannins on invertebrate and vertebrate herbivores will be described separately, Therafter a concluding section will compare these and examine the differing effects dependent on the chemical nature of the tannins involved.

1.4,2 Tannins and Invertebrate Herbivores

The first two studies in Table 1.3 are concerned with marine invertebrates and indicate the probable antifeedant action of phlorotannins. The following discussion is, however, entirely devoted to studies of terrestrial invertebrate herbivores which, in all but three cases (studies 1-3), come from insect taxa. Of the nine orders of insect reported to contain phytophagous species (Strong et al., 1984) six are represented here (Orthoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera, Hymenoptera) for which there are six, two, three, two, eighteen and one reports respectively.

In the reports where tannins are consumed to some extent at least, increasingly high concentrations are reported to lead to decreasing rates of consumption in twenty studies (1-4, 6,7, 10-14, 16-18, 22-24, 26, 33, 36). Two of these reports indicate the presence of a threshold level of tannin below which there is no depression of CR (6, 18) and such effects cannot be ruled out by the information provided by the other studies, indeed these effects are probably typical and underline the need for field or whole plant diet studies before ecologically useful conclusions can be drawn. However, from these studies it seems that the evidence for the antifeedant effect of tannins is quite strong; only four reports have been unable to attribute any clear

Table 1.2. Definitions of terms indicated as abbreviations in Tables 1.3, 1.4 and the text.

I Nutritional indices

CR = Consumption Rate.

= Food ingested per unit time.

GR = Growth Rate.

= Biomass gained per unit time.

AD = Aproximate Digestibility.

= (Food ingested - faeces) / Food ingested.

ECD = Efficiency of Conversion of Digested Food.

= (Biomass gained) / (Food ingested - faeces).

ECI = Efficiency of Conversion of Injested Food.

= Biomass gained / Food ingested (= AD.ECD).

NEX = Nitrogen loss in faeces per unit time.

NUE = Nitrogen Utilisation Efficiency.

= Biomass gain (of N) / Nitrogen ingested.

II Phenolics.

TP = Total Phenolics. HT = Hydrolysable Tannin.

CT = Condensed Tannin. TA = Tannic Acid.

GA = Gallic Acid (a component molecule of TA).

Table 1.3, Studies of the Consumption of Tannins by Invertebrate Herbivores

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Key: headings indicate the herbivore(s) and source(s) of tannin in the diet (which is either artificial and contains a plant extract [E] or of whole plant material [P]). The findings of each study are then listed in categories A-E as described in the main text. Abbreviations used below are explained in Table 1.2

I Invertebrates, Marine Ecosystems

1 Marine Herbivores / Kelp (Alaria marginata; Phaeophaceae [P])

B: Most phenolics and least herbivory found in reproductive fronds. Phlorotannins can be expected to be amongst the phenolics on taxonomic grounds.

(Ragan, 1982; Steinberg, 1984)

2 Marine Epibionts / Sargassum natans (Phaeophacae [P])

B: Fouling of frond tips by various invertebrates was reduced by their high phlorotannin content. Positive tests for both protein precipitation and phenolics performed.

(Sieburth and Conover, 1965)

II Invertebrates (Arthropoda), Terrestrial Ecosystems

3 Red Spider Mite (Tetranychus pieroi; Arachnida) / Oil palm (Tenera sp. Palmae [P])

B: Herbivory shown to be inversely proportional to the level of condensed tannins present.

(Rajaratnam and Hock, 1975)

4 Insect Herbivores / Bracken (Pteridium aquilinum; Dentstaedtiaceae [P])

B: Tannins shown to protect mature fronds from herbivory. Although present their concentration is too low to protect immature fronds.

(Tempel, 1981).

5 Hylopedetes nigrithorax and Homeoastax dentata (Orthoptera) / Tropical forest ferns (13 species [P])

B: Palatability appeared to be chemically determined, yet was not significantly correlated with tannins, phenolics, fibre or nitrogen.

(Rowell et al., 1983)

6 Acrididae (Orthoptera), four species (Schistocerca gregaria, Zonocerus varigatus, Chortiocetes terminifera, Locusta migratoria) / quebracho tannin [E].

B: Only for over 10% tannin was CR reduced.

C: " " " " " was ECD reduced, AD unaffected.

E: no effects on mortality at less than 10% tannin. Tannins passed through the gut relatively unchanged although they exited in disproportionate association with the peritrophic membrane.

(Bernays et al., 1981).

7 Desert locust (Locusta migratoria; Orthoptera) / tannic acid [E]

A: Graminivorous, not ecologically used to high tannin levels.

B: CR depressed.

C: GR & ECD depressed, AD unaffected.

D: NUE unaffected.

E: Midgut lesions, peritrophic membrane penetrated by tannin. LD-50 at 1g/Kg. Faecal phenolics somewhat low indicating possible metabolism of TA.

(Bernays, 1978)

8 Schistocerca (3 species, Orthoptera) / tannic acid [E].

A: Polyphagous, naturally used to dietary tannin.

C: GR unaffected.

D: NUE unaffected.

E: Survival unaffected, peritrophic membrane not penetrated, TA accounted for in faeces although much as GA. (Bernays, 1978)

9 Anacridium melanorhodon (Orthoptera) / tannic acid [E].

A: Naturally feeds on woody plants including Acacia sp. which contain hydrolysable tannins.

B: Slight phagostimulation, increased CR.

C: increased AD, ECD & GR.

E: Mortality decreased.

These beneficial effects were not seen with quebracho tannin.

(Bernays et al., 1980; Bernays and Woodhead, 1982)

10 Orthoptera, three species (Cibolacris parviceps, Schistocerca americana, Astroma quadrilobatum) / Creosote Bush (Larrea tridentata; Zygophylleae [P]).

B: CR depressed. Phenolic resin present, also see section 1.3 for further details.

(Rhoades, 1977a, 1977b)

11 Green Peach Aphid (Mysus persicae; Hemiptera) / tannic acid [E]

B: Given the choice of food with and without TA, no feeding on TA was observed.

E: Given tannin-containing diets only 0.01M TA was lethal. An even lower lethal dose, 0.1mM, is recorded for another aphid, Schizaphis graminum.

(Schoonhoven and Dersen-Koppen, 1976; Bernays, 1981)

12 Tarnished Plant Bug (Lygus lineolaris; Hemiptera) / Cotton (Gossypium hirsutum; Malvaceae [E]).

A: Naturally feeds on cotton.

B: Cotton tannin found to inhibit feéding to a (ten times) greater extent than gossypol. Sinigrin was comparatively weak in deterrence.

(Hatfield et al., 1982)

13 Colorado Potato Beetle (Leptinotarsa decemlimeata; Coeleoptera) / tannic acid [E]

A: Fed potato leaves sprayed with tannin solution.

B: CR reduced to zero.

E: Starvation to death with 5mg TA / 100cm^2 leaf surface.

(Popsil, 1982)

14 Alfalfa Wevil (Hyspera postica; Coeleoptera) / tannic acid [E]

A: Fed TA sprayed alfalfa.

B: CR reduced.

E: Mortality increaced.

(Bennet, 1965)

15 Paropsis atomaria (Coeleoptera) / Eucalyptus, 13 species (Myrtaceae [P])

B: CR unaffected and high in response to low dietary nitrogen yet condensed tannins and resins present.

D: NUE unaffected and as high as for Pieris rapae feeding on cabbage.

E: Phenolic inputs accounted for in frass and thus not taken into the body.

(Fox and Macaulay, 1977)

16 Agromyzidae (3 species, Melanoagrmyza sojae, Ophiomyia centrosematis, O. phasioli; Diptera) / Soya Bean (Glycine max; Leguminosae [P])

B: Resistance between cultivars and wild varieties linked to the intra-stelear location of tannins. Hydrolysable tannins present.

(Chiang and Norris, 1983)

17 Apple Maggot (Rhagoletis pomonella; Diptera) / Crab Apple varieties (Malus sp.; Rosaceae [P])

B: Varietal resistance proportional to the phenolics present, presumed to be tannins on taxonomic grounds.

E: 1000 ppm TA prevented larval development and is the tentative explanation for the resistance.

(Pree, 1977; Lea et al., 1983)

18 Semiothisa colorata (Lepidoptera) / Creosote Bush (Larrea tridentata; Zygophyllaceae [P])

A: Monophagus on this host plant.

B: Phagostimulation at low resin levels, CR depressed at high levels.

NB Phenolic resin present.

(Rhoades, 1977a)

19 Egyptian Cotton Bollworm (Earias vittella; Lepidoptera) / Tannic acid [E]

C: GR depressed.

E: Mortality increased.

NB Hydrolysable tannins thought present in natural food i.e. cotton.

(Sharma and Agarwal, 1982)

20 Papilio polyxenes (Lepidoptera) / Liriodendron tulipifera (Magnoliaceae [E])

A: Umbellifer specialist, does not naturally consume L. tulipifera.

C: AD & GR unaffected.

D: NUE, NEX, NAR & NCR unaffected.

E: Mortality induced by tannins, rupture of gut walls and bacterial septicemia found in dying caterpillars.

(Berenbaum, 1983)

21 Papilio glaucus (Lepidoptera) / Liriodendron tulipifera (Magnoliaceae [E])

A: Generalist naturally eats L. tulipifera.

C: AD & GR unaffected.

D: NUE, NEX, NAR & NCR unaffected.

E: Mortality unaffected.

(Berenbaum, 1983)

22 Cabbage White Butterfly (Pieris brassicae; Lepidoptera) / quebracho tannin [E]

A: Non-adapted herbivore.

B: Deterrence to feeding on cabbage + quebracho at 0.1% (by fresh weight). The authors argued that CT alone could defend a plant such as bracken fromn this insect.

(Jones and Firn, 1979)

23 Egyptian Cotton Boll Worm (Earias vittella; Lepidoptera) / Cotton bolls (Gossypium hirsutum; Malvaceae [P])

A: Different genotypes of cotton were used which contained both tannin and gossypol in different concentrations, both relative and absolute.

B: For tannins CR decreased as tannin levels increased, the opposite trend was found for gossypol.

(Sharma and Agarwal, 1981)

24 Heliothis zea (Lepidoptera) / Cotton (Gossypium hirsutum; Malvaceae [E])

A: A cotton pest.

B: CR depressed.

C: GR depressed, AD & ECD unaffected.

E: Lowered sugar and protein in haemolymph.

N.B. CT demonstrated, as were similar correlations of the above factors with the astringency of the plant

(Klocke and Chan, 1982; Zummo et al., 1983)

25 Heliothis virescens (Lepidoptera) / Cotton (Gossypium hirsutum; Malvaceae [P])

A: Cotton pest.

C: Growth depression attributed to gossypol not CT. CT, gossypol and various flavonoid constituents considered.

(Hanny, 1980)

26 Orange Striped Oak Worm (Aniota senatoria; Lepidoptera) / Oak (Quercus species, several; Fagaceae [P])

A: Oak specialist, alkaline gut pH.

B: Slow growth on late season foliage attributed to reduced CR.

C: AD high and unaffected, ECD and ECI depressed. Notably high AD, ECD & ECI on Bur Oak which has the highest tannin.

D: NUE unaffected by tannin.

(Lawson et al., 1982)

27 Alsophila pometaria (Lepidoptera) / Oak (Quercus species [P])

A: Comparative study to the above, this species is also an oak specialist.

B: Higher CR than A. senatoria.

C: Higher GR than A. senatoria, due to CR only, not to AD or ECD.

D: CR independent of foliar N which thus controls growth, in contrast to A. senatoria whose GR is N independant owing to increased CR at low N. In summary, A. pometaria, which feeds on early succulent N rich foliage has a high CR plus low ECI strategy vs. A. senatoria (late season feeder) with low CR and high ECI.

N.B. tannin levels did not vary seasonally in the food, only N and water did.

(Lawson et al., 1984)

28 Checker Spot Butterfly (Euphydryas chalcedona; Lepidoptera) / Diplacus auranticus (Scrophulariaceae [P])

A: Seasonality of the interaction resembles that of the oak moth/oak in that early feeding on young leaves is important in the life history. Phenolic resin present.

C: GR, ECD & ECI depressed, AD unaffected.

D: NUE unaffected.

E: survivorship and laval size depressed.

(Lincoln et al., 1982; Williams et al., 1983).

29 Spodoptera littoralis (Lepidoptera) / British fern species [P]

A: Non-adapted insect herbivore used.

B: Tannins did not correlate well with food choice.

(Ottosson and Anderson, 1983)

30 Chain Dotted Measuring Worm (Lepidoptera) / Bog Ericad species (Kalmia polifolia, Ledum groenlanicum, Chamaedaphne calyculata; Ericaceae [P])

A: The plants are the major natural food sources.

B: Food selection between species dependent upon their pubescence and N content; all are rich in phenolic allelochemics.

(Reader, 1979)

31 Gypsy Moth (Lymastria dispar; Lepidoptera) / Grey Birch (Betula populifolia; Betulaceae) and Black Oak (Quercus velutina; Fagaceae [P])

A: A study examining the effects of defoliation induced defences on natural herbivores.

C: GR depressed.

E: Development time and survival declined.

(Wallner and Walton, 1979)

32 Winter Moth (Operophtera brumata; Lepidoptera) / oak tannin (Quercus petrea; Fagaceae [E]

A: Natural food source.

C: GR declined.

E: Mortality increased.

F: CT and some HT.

(Feeny, 1970; 1968; also see section 1,3)

33 Winter Moth (Operophtera brumata; Lepidoptera) / native British tree species [P]

A: Species used were naturally consumed as foods by the insect. Fibre, water content and total nitrogen were measured besides tannins.

B: CR depressed by tannins measured as enzyme inhibition. Other factors not significant.

C: GR depressed, same analysis as above. AD depressed by all four factors yet ECI not significantly affected by any variable.

E: Mortality not affected but birth rate the following year was decreased. This is probably the way low GR is translated into factors affecting population levels.

(Wint, 1983)

34 Euproctis chrysorrhoea and other phytophagous lepidopterans / tannic acid [E]

B: CR increased i.e. phagostimulation.

(Grevillius and Van Gornitz both cited in Bernays, 1981)

35 Hemileuca olivae (Lepidoptera) / Grass species, C3 vs C4, [P]

A: This herbivore naturally prefers feeding on C4 vs. C3 grasses

B: Relative palatabiliy difference due to low CT content of C4 grasses.

(Capinera et al., 1983)

36 Moth and Sawfly larvae (Lepidoptera and Hymenoptera) / Birch (Betula pubescens; Betulaceae [P])

C: GR typically depressed as measured by larval development times or lowered body weights. Nevertheless, some species were unaffected.

(Haukioja and Niemela, 1977, 1979)

antifeedant activity to tannins (5, 15, 29, 30). Of the latter two concern ferns and the others concern eucalypts and ericads and in each case interference by other allelochemics is not unlikely. Despite the evidence for threshold effects, "quantitative" feeding deterrence at higher tannin concentrations is not in dispute by the authors of the above studies.

The hallmark of allelochemical defences based upon tannins lies in their presumed complete dependability. Unlike toxic defences, phagostimulation in response to tannin containing diets should not occur. However, three reports of this do exist (9, 18, 34), that by Rhoades (1977a) does, however, indicate feeding deterrence at high resin concentrations. The remaining reports (9, 34) are both concerned with animals feeding on tannic acid-containing diets.

Despite these contradictory reports it does seem that tannins are typically able to deter or at least partially reduce herbivory by most invertebrate herbivores yet generalisations cannot be soundly made and individual plant/herbivore systems need individual examination. The effects of tannins on growth rate are not clear cut, eight studies (7, 19,24, 28, 31-33, 35) report depressed growth rate (GR) due to tannin consumption yet in three cases (8, 20, 21) no significant effects could be found. It must be pointed out that fewer reports on GR exist and

so any conclusions based on them are less well founded. What is remarkable is that in nine reports (6, 7, 9, 20, 21, 24, 26-27, 33) concerning AD, no significant reduction due to tannin consumption is evident in eight and where AD was reduced (33) no effect on ECI could be found. This is quite at variance with expectations based on Rhoades (1979); classical digestibility reduction is not supported by the available evidence. This point is reinforced by the four studies (6, 7, 26, 28) which also indicate lowered ECD in response to tannins. Thus, whilst the evidence is that tannins can reduce GR in some herbivores, this will be by reduced consumption (also see studies 24, 33) or post digestion effects revealed through ECD. In only one study (9) AD and ECD are both reported to rise but this is for the unusual case of an animal which also shows phagostimulation in response to tannic acid.

In section 1.4,1 the possibility was raised that the above evidence could not conclusively rule out a specific reduction in the digestion of protein. Nevertheless the six reports (8, 15, 20, 21, 26, 28) of unaffected nitrogen utilisation efficiency (NUE) are without contradiction and once more fail to support the mechanism of allelochemic action favoured by Rhoades (1979).

In seven studies (13, 14, 18, 20, 28, 31, 32) of animals consuming tannins and surviving on the diet, the

death rate was nevertheless increased in the populations of experimental animals although the reverse was found for animals exhibiting phagostimulation (9). Nil effect on mortality is reported in only two instances (21, 33). Given the preceding information that tannins do not seem to act as digestibility reducers in any strict sense, other possible causes of death have been noted. In two cases midgut lesions and subsequent infections have been noted (7, 20). Given this information, reports of the metabolism and uptake of tannic acid (9, 7) are not surprising although this is not always the case (8, 15). The one report (24) of altered haemolymph sugar and protein levels may be explained by the influence of gallic acid absorbed from tannic acid hydrolysis.

1.4,3 Tannins and Vertebrate Herbivores

Evidence derives from thirty-eight studies representing work on reptiles, birds and both marsupial and eutherian mammals. Most reports, however, concern birds (eight) and mammals (twenty-nine). Of the latter the following groups are represented: Phasocolarctidae, Lagomorpha, Rodentia, Artiodactyla and Primates. As with invertebrates the vast majoriy of reports in Table 1.4 (1, 2, 3, 6, 7, 8, 9, 11, 13, 14, 16, 22, 24, 25, 26, 27, 29, 31, 32, 34, 36, 38) indicate low palatability or reduced consumption for high tannin diets although the effect may be negligible or only a minor influence on food selection (see 20, 28, 30, 33, 37). There are no cases reported of mammals consuming lethal doses of tannin and at the opposite extreme there are no cases of phagostimulation in situations where an alternative tannin free diet is available. Nevertheless there are two reports of increased CR for no choice feeding on low levels of tannin (15, 17). Given that there are antinutritional effects resulting from tannin consumption, these reports of increased CR may be rationalised in terms of the animals making this good by the consumption of additional nutrients (again see 15,17).

With regard to growth rates there are no reports of enhanced positive growth rates for mammals consuming

dietary tannin. Of the nine reports (3, 5, 6, 7, 12, 16, 17, 18, 26) where GR has been measured four (3, 6, 7, 26) attribute a depressed GR to tannin consumption. The nutritional indices available for invertebrates are not generally available for vertebrates; however, there are two reports (17, 27) that indicate reduced AD or increased faecal calorific values. This has not been observed in all cases (28), a particularly interesting situation (26) being that of goats fed a starvation diet where the diet producing the greatest GR was that in which the animals consumed the most tannin. The conclusion of the authors (Provenza and Malechek, 1984) was that tannins did not affect digestion in a negative way, and that they normally acted upon CR. Given the available evidence tannins do seem to influence GR but no firm and general conclusions seem possible as to the mechanisms involved.

There is a useful amount of information relating faecal nitrogen to dietary tannin consumption. In all nine cases (3, 6, 10, 16, 17, 18, 21, 25, 27) faecal nitrogen increases. Where authors have expresed a view on the cause of this (16, 18, see also 21) they have indicated an increased loss of endogenous, not dietary, nitrogen. Thus there is no conclusive evidence that tannins act to inhibit protein digestion in vertebrates, indeed this does not seem particularly likely in view of

one report (23) suggesting that tannins may aid protein uptake in some circumstances. Only one report (17) of both faecal and urinary nitrogen measurement is available and this indicates compensation by lowering urinary loss to balance increased faecal loss

Measurements of LD-50 for tannic acid have required the use of stomach tubes to deliver lethal doses to rats and it seems inconceivable that any animal would naturally consume sufficient plant material to receive a toxic dose. However there is evidence (4, 6) that tannins affect avian egg hatching rates and chick mortality as well as producing skeletal deformities. Less drastic is the incidence of glandular atrophy and mucosal erosion in the gut or changed lipid levels in the liver (see 7,16). There are also several reports (7,15) of gluconuride or methyl gallic acid excretion associated with tannin consumption (see Section 1.4,4). In summary there is evidence of tannins producing deleterious effects on vertebrate herbivores, which, like those found with invertebrates, are not symptomatic of strict digestibility reducing agents.

Table 1.4, Studies of the Consumption of Tannins by Vertebrate Herbivores

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Key: headings indicate the herbivore(s) and source(s) of tannin in the diet (which is either artificial and contains a plant extract [E] or of whole plant material [P]). The findings of each study are then listed in categories A-E as described in the main text. Abbreviations used below are explained in Table 1.2

I Reptiles

1 Giant Tortoise (Testudo gigantea) / "Tortoise Turf" [P]

B: Rejection limits (<80% consumption) for tannin containing plants, 2.1mM and 0.4mM for CT and HT respectively.

(Swain, 1976)

II Birds

2 Quelea (Quelea quelea) and other birds / Sorghum (Sorghum bicolor; Graminae [P])

B: Crop damage i.e. grain consumption is inversely proportional to CT levels. Bird damage is greater than insect damage. The repellent action of Sorghum tannin to the quelea hes been demonstrated.

(McMillian et al., 1972; Bullard and Shumake, 1979).

3 Muscovy Ducks (Cairana moshata) / Beans (Vicia faba; Leguminosae [P])

B: CR depressed

C: GR "

D: NUE "

(Martin-Tanguy et al., 1977)

4 Egg laying hens / Beans (Vicia faba; Leguminosae [P])

E: Egg weight and hatching rate depressed.

(Martin-Tanguy et al., 1977).

5 Chickens / Carob pods (Ceratonia siliqua; Leguminosae [E])

C: GR depressed. CT and some HT present.

(Joslyn et al., 1968).

6 Chickens / Sorghum (Sorghum bicolor; Graminae [E])

B: CR depressed.

C: GR "

D: Reduced protein digestion.

E: Slightly elevates liver lipids and lipid metabolism. Some deleterious effects (not NUE) are methionine reversible. Growth deformities in the legs of some animals seen as well as reduced egg production in laying hens.

N.B. CT only.

(Potter and Fuller, 1968; Rostagno et al., 1973; Elkin et al., 1978a, 1978b; Herstad, 1979; Sell and Rogler, 1983, 1984).

7 Chickens / tannic acid [E]

B: GR depressed.

C: GR "

E: Slight increase in liver lipids; methionine and choline partially correct this, O-4 methyl GA found in urine.

(Potter and Fuller, 1968)

8 Ptarmigan species (Lagopus lagopus, L. mutus, L. lecurus) / Sub arctic browse [P]

B: Evidence for resin avoidance and that this relates to the protein complexing ability and digestibility ($p<0.001$) of the phenolic resin, which may thus act as a tannin. Resins also deter feeding by other sub-arctic browsers but the tannin-like action of the resins they consume is less well established.

(Bryant and Kuropat, 1980).

9 Canada Geese (Branta canadensis) / Coastal marsh vegetation [P]

B: CR depressed. Phenolics were found to be the major proximate cue rather than nutrient quality. Both quebracho tannin and TA gave similar results on captive animals.

(Buchsbaum et al., 1984).

III Mammals

10 Koala (Phasocolarctos cinereus; Phasocolarctidae) / Eucalyptus species (Myrtaceae [P])

A: Hindgut fermenter, only eats Eucalyptus sp.

D: High faecal nitrogen levels may be due to tannins.

(Hume, 1982).

11 Snowshoe Hare (Lepus americanus; Lagomorpha) / Sub arctic browse [P]

B: CR depressed in some circumstances, TP not well correlated to this but protein complexing phenolics are ($p<0.002$).

N.B. Resins, TP, and protein complexing phenolics separately measured.

(Sinclair and Smith, 1984).

12 Woodrat (Neotoma fuscipes; Rodentia) / Oak and other phenolic rich foliage [P]

A: Oak specialist, foliage contains 16% CT and 40% TP.

B: CR high despite tannin, indeed tannin-rich foliage preferred to other available plant material differently defended by other allelochemicals.

C: GR positive in contrast to the non specialist N. lepida which loses weight on an oak diet. Nevertheless AD low in contrast to tannin-free diets although processing rates fast.

D: High nitrogen retention on oak compared to N. lepida, nitrogen losses atributed to loss of endogenous protein not dietary protein.

(Astatt and Ingram, 1983).

13 Stephens Woodrat (Neotoma stephensi; Rodentia) / Juniper (Juniperus communis; Cupressaceae [P])

A: Specialist on juniper which is never less than 80% of the diet.

B: Selection for plant parts with low levels of allelochemical is made.

F: Both CT and terpenoids present.

(Vaughan, 1982)

14 Arctic microtine rodents (three species) / Labrador Tea extract (Ledum palustre; Ericaceae [E])

A: Component of naturally available vegetation.

B: CR reduced.

E: Reduced body fat, reduced litter size.

(Jung and Batzli, 1981)

15 Prarie Vole (Microtus ochrogaster; rodentia) / tannic acid [E]

B: CR increased for 6% TA over 3% TA where this can allow completion of dietary protein requirements.

E: Uronic acid excretion in response to TA yet high protein levels in the diet depress this, possibly because TA remains in the gut bound to protein.

(Lindroth and Batzli, 1983).

16 Laboratory rat / tannic acid [E]

B: CR depressed

C: GR "

D: NEX increased, attributed to endogenous protein loss, not dietary protein loss. Increased faecal levels of the mucoprotein component glucosamine reported.

E: Larger animals less likely to die, yet mortality rate did increase. LD-50 2.26g/kg. Fatty liver resulted from GA not TA per se which did not pass through the gut wall unhydrolysed. GA and O-4 methyl GA excreted in the urine. Methionine and choline did not alleviate the effects, blood composition unaltered. On a 3% diet intestinal mucus secretion rose yet this did not afford complete protection as glandular atrophy and mucosal erosion occurred.

(Joslyn and Glick, 1968; Glick and Joslyn, 1970a, 1970b; Singleton and Kratzer, 1973; Mitjavila et al., 1977)

17 Laboratory rat / tannic acid [E]

A: This study is separated from the above as it involved feeding chronically (27 weeks) very low levels (0.64 and 1.128 %) only.

B: CR slightly increased.

C: GR depressed, calorific values of faeces increased.

D: Faecal NEX increased, NUE depressed, but counterbalanced by decreased urinary nitrogen.

(Mitjavila et al., 1971).

18 Laboratory rat / Condensed tannin [E]

C: GR depressed, but not as much as for TA.

D: NEX increased, attributed to endogenous protein losses which may include additional bile salts.

(Glick and Joslyn, 1970b; Wursch, 1979).

19 Laboratory rat / Sericea tannin extract (Lespedesa cuneata; Leguminosae [E]

E: (i) 2% administered over extended periods gave no effect, TP not absorbed into the body.

(ii) Acute dose of 3g/kg body wt., by stomach tube delivery, killed rats within a week. Lumps of tannin-rich material found in stomach after death.

(Booth and Bell, 1968).

20 Buffalo (Syncerus caffer; Artiodactyla) / Savanah vegetation [P]

A: Tannins and oils in some grasses may reduce CR but only when the animal cannot switch to an alternative food source.

(Field, 1976)

21 Elk (Cervus elaphis) / Fireweed (Epilobium angustifolium; Onagraceae) and Maple (Acer saccharum; Aceraceae) [P]

A: results compared to a tannin-free hay plus alfalfa diet.

D: NEX increased. the relationships between faecal nitrogen (y) and dietary nitrogen (x) are y=0.77+0.486x with tannin and y=1.02+0.443x without nitrogen. For the 0 to 5% range of x employed in the experiments it is the constant factor which determines y not the term in x, which suggests non-dietary nitrogen loss is increased by tannins.

(Robins, 1983).

22 Sheep / Sericea (Lespedeza cuneata; Leguminosae [P])

A: The high-tannin variety had the most nitrogen and least fibre.

B: CR depressed by tannins irrespective of other factors.

(Wilkins et al., 1953).

23 Sheep / Lucerne extract [E].

D: Increased protein uptake attributed to dietary tannins in certain circumstances.

(Jones and Mangan, 1976).

24 Sheep (Orkney Isles breed) / Seaweeds [P]

A: The animals are adapted to this (staple) diet by virtue of their specialised rumen biota.

B: CR depressed by astringent seaweeds, the most astringent types (Ascophyllum nodosum, Fucus vesiculosus) not being consumed when others are available. Phlorotannins are the presumed cause of astringency.

(Paterson, 1984).

25 Goats (Artiodactyla) / Oak plus Alfalfa mixture. [P]

B: CR depressed

D: NEX increased

E: No toxic effects revealed through haematological tests.

(Nastis and Malechek, 1981).

26 Goats / Blackbush (Coleogyne sp.; Rosaceae [P])

A: Goats given either high or low tannin containing foliage. Foliar protein directly proportional to foliar tannin level in this instance.

B: CR depressed by high tannin levels.

C: Comparative increase in GR for animals on high tannin diets, i.e. tannins did not adversely affect the digestion and utilisation of nutrients in the high-tannin feeds.

(Provenza and Malechek, 1984)

27 Cattle / Sericea lespedeza (Lespedeza cuneata; Leguminosae [P])

A: Various high and low-tannin cultivars fed to cattle.

B: CR depressed by high CT.

C: AD " "

D: NEX increased.

N.B. Contains CT.

(Hawkins, 1955; Donnelly et al., 1971).

28 Cattle / tannic acid [E]

A: Results compared to those above for CT given at the same level.

B: CR unaffected.

C: AD unaffected.

(Hawkins, 1955).

29 Vervet Monkeys (Cercopithecus aethiops) / Acacia tortilis and A. xanthophloea (Leguminosae [P])

B: Tannins were consumed but nevertheless they were negative feeding cues.

E: Tannins may well have contributed to nutrient stress at a time when high mortality occurred. Tannins in faeces indicated their ability to pass through the gut unaltered. (Wrangham and Waterman, 1981).

30 Howler Monkey (Allouatta palida; primates) / Tropical rain forest [P]

B: Phenolics seem unimportant in food selection compared to protein and fibre.

(Milton, 1977).

31 Black and White Colobus Monkey (Colobus guereza) / Tropical rain forest [P]

B: Tannins consumed but there is definite food selection against tannin-rich foliage, some consumption may, however, be beneficial to avoid bloat.

(Oates et al., 1977)

32 Black Colobus Monkey (Colobus satanus) / Tropical rain forest [P]

B: Selects food low in tannins, but lignin and fibre are also major negative feeding cues.

(McKey et al., 1981).

33 Red Colobus Monkey (Colobus badius) / Tropical rain forest [P]

B: No clearly discernible role for tannin as a major determinant of food selection is evident.

(Choo, 1981).

34 South Indian Leaf Monkey (Presbytis johnii) / Tropical rain forest [P]

B: Consumes a diet low in fiber and tannin although neither is an absolute deterrent.

(Oates et al., 1980)

35 Chimpanzee (Pan troglodytes) / Fruits naturally consumed [P]

B: Suggestive evidence that condensed tannins may influence feeding on fruits.

(Wrangham and Waterman, 1983).

36 Yellow Baboon (Papio cynocephalus) / Acacia gums from A. tortilis and A. xanthophloea (Leguminosea [P]).

B: Gum with least tannin and most carbohydrate preferred.

(Hausfater and Bearse, 1976).

37 Western Gorilla (Gorilla g. gorilla) / Tropical lowland rain forest [P]

B: More CT eaten than the mountain gorilla yet it is not a major influence on food selection relative to lignin and fibre; tannins are, however, helpful in considering why particular plants are rejected.

(Calvert, 1985).

38 Mountain Gorilla (Gorilla g. berengei) / Tropical montane vegetation [P]

B: Astringent (tannin containing) plant parts tend not to be eaten.

(Bate-Smith, 1972a).

1.4,4 Vertebrate-Invertebrate and Tannin-Tannin Comparisons

Recent publications on tannins and their effects on herbivores have tended to consider effects on either vertebrate (Glander, 1982; Kumar and Singh, 1984) or invertebrate (Bernays, 1978,1980; Martin and Martin, 1984) herbivores, and draw slightly different conclusions. Put simply, these are that tannins do not act as effective digestibility reducers in insects, and that they have incompletely effective deterrent action against mammals.

Comparing the two previous sections reveals that the evidence for tannin-reduced CR is equally strong for both groups of herbivores and differences between these two groups seem to be very much a function of the kinds of experiments which have been performed with them. Particularly lacking (for ethical & practical reasons) are measures of ECI and its two component indices AD and ECD in vertebrate herbivores. Because of the gap this leaves in the available evidence it is not possible to conclude that vertebrates differ from invertebrates in the effects tannins produce on AD in contrast to ECD.

With respect to nitrogen nutrition, the question is comparatively more open for vertebrates, but nevertheless, given the available evidence for both groups of herbivores, it is more likely that

tannin-induced losses of endogenous protein are of greater importance than the reduced digestion of dietary protein. This coupled to the evidence for damage to the gut by tannins, their influence on mortality, and xenobiotic metabolism in both groups of herbivores, clearly suggests that tannins do not act predominantly upon digestibility.

There is good and repeated evidence that tannins do often reduce GR so it is not in doubt that they can exert an allelochemical action. Many early studies focused particularly on the negative effects of tannins on nitrogen nutrition in an attempt to explain this reduction in GR. Their promotion of endogenous protein loss coupled with their potential drain on methionine as a methylating agent for xenobiotic phenolics (Schline, 1978), does suggest that tannins may exacerbate conditions of limiting nitrogen nutrition. The mechanism whereby this may occur is not, however, likely to be by a reduction of protein digestibility. With regard to the process of digestion it is worth pointing out some beneficial effects of tannins. The explanation for the phagostimulation in A. melanorhodon induced by tannic acid (Bernays and Woodhead, 1982) was that the animal might possibly use the hydrolytically derived gallic acid in endogenous syntheses. This does not appear to be general among most insect species. For ruminants,

tannins have, however, been thought to be generally useful in preventing bloat (Reid, 1973) and perhaps also in protecting protein from digestion before its entry into the small intestine (Jones and Mangan, 1976). Further evaluation of the possible beneficial effects of tannins will be made in succeeding chapters.

The information in Tables 1.3 and 1.4 reveals differences between some of the important types of tannin. Tannic acid is unique for its presence in all reports of gut lesion and gastrointestinal damage (vertebrate or invertebrate). It is also the only tannin for which metabolism and excretion are reported. By contrast, condensed tannins are not thought to leave the gut lumen. Thus in contrast to tannic acid they would not be expected to drain one carbon metabolism and methionine resources. However the reports of Elkin (1978a, 1978b) and Ford (1977) of corrective methionine treatment for chicks fed a condensed tannin diet indicate that some condensed tannin enters the body (or that methionine reacts with tannin in the gut), so casting doubt on the received wisdom that the components of tannic acid are the only ones to enter the body. The conclusion can, however, be made that despite its hydrolysability, tannic acid does exert an allelochemical effect which is not abolished by hydrolysis.

Very little information exists on the phenolic protein

complexing resins except for that on Creosote Bush and some arctic plants. In contrast to condensed and hydrolysable tannins, these are typically ether soluble which may allow their entry into the body across cell membranes, so giving the potential for action outside the gut lumen. Reports for animals consuming seaweed reviewed above indicate that astringent protein precipitating agents (presumably phlorotannins, see Section 4.2) may be important in marine plant-herbivore interactions.

Despite chemical differences and the possible differences between tannins (sensu lato) in their effects on digestion, all share the common feature of astringency, and so almost certainly reduce plant palatability by this mechanism. Given the evidence that tannins seem to exert effects on GR principally by reducing CR, it is pertinent to ask whether their "bark is worse than their bite" The small but significant number of reports where tannins seem to be beneficial to animals consuming them and the paucity of results demonstrating GR reduction due to digestibility reduction or toxicity, make it all the more reasonable to pose this question. It remains to be proved that animals use astringency as a negative cue to avoid nutritionally dire consequences. On the evidence of feeding trials the effects of tannins would appear to be significant but not

acute, generally leading to a reduction of fitness as seen through a number of physiological effects which appear in animals feeding for prolonged periods on levels of tannin above average for their natural diet.

1.5 Formulation of Research Objectives

The material reviewed in the preceding section was entirely concerned with *in vivo* experimentation and observation. Nevertheless it has been results obtained *in vitro* that have been of fundamental importance in shaping our interpretation of these *in vivo* observations, as is clearly exemplified in the hypothesis of Feeny, Rhoades and Cates (see Section 1.2,2) where the *in vivo* effect of tannins was attributed to their interaction with protein *in vitro*. Tannin-protein complexes, soluble or insoluble, have never been demonstrated *in vivo* and so the validity of the *in vitro* models of digestion which have been used is an obvious target for investigation given the current mismatch between *in vitro* evidence for digestibility reduction by tannins and the observations *in vivo*. It is also clearly opportune to examine the activity of a range of tannins in the light of reported inter-tannin differences in herbivore or soil systems (see previous Sections).

The basic aim of the research presented here has been to attempt a re-assessment of the ecological status of tannins as the major form of quantitative plant defence chemical. Experiments have been conducted to ascertain whether inter-tannin differences can be seen in simple *in vitro* models of digestion, and related to their chemistry

as seen in Section 1.4,4. However the major part of the investigation has been to assess the potential effects of tannins in digestion through a more thorough and diverse examination of the variables affecting the interaction of tannins and proteins in digestive systems than has previously been attempted.

Two supporting studies were also undertaken. The first was an assessment of how factors in the physical environment affect the producton of tannins by plants, in contrast to production in response to biological factors such as herbivore pressure. The second was to investigate the cost of tannin production in terms of fixed carbon resources. Together with the work on digestion these studies combine to help assess the status of tannins as antiherbivore allelochemics in terms of their wider ecology briefly reviewed in Section 1.2.

Chapter Two

The Chemical Characterisation of a Series of Tannins

2.1 Introduction

This chapter concerns itself with the selection and development of techniques with which to measure some of the chemical characteristics of tannins, the aim being to relate these to the tannins' potential allelochemical behavior, as gauged from "in vitro model" experiments (see Chapters 4 & 5). In Section 1.1 the need to measure and quantify the phenolic nature of tannins, to assign them to particular chemical classes and to measure their molecular weights was illustrated. These represent progressively more difficult operations. The outcome of each measurement is critically dependent on the method by which the tannins are extracted from the plant material. The following describes the extraction and chemical description of a series of tannin-rich plant extracts, from a wide range of plant taxa. Only water soluble tannins, i.e. tannins defined in the strict sense, are considered in the following.

2.1,1 Extraction and the Choice of Extractant

There is no comparative survey of methods of tannin extraction available in the literature; most workers use their own methods. The abundance of experimental techniques has arisen as each typically aims to extract a sub set of the tannins and associated phenolics in the

material studied e.g. only hydrolysable tannins or only condensed tannins within a narrow molecular weight range. This study has aimed for 100% extraction of all water soluble tannins and to do this on a scale yielding up to 10 g of extract.

The most common extractants are water, methanol, acetone or mixtures of these. Cansfield et al. (1980) used water to extract tannins from field beans in a cold extraction (20 ^{o}C) lasting four hours, this being followed by a subsequent re-extraction and combination of extracts. Seed proteins were also extracted in this procedure, necessitating their removal by reverse osmosis (Cansfield et al. 1980). Their procedure illustrates the problems and hazards inherent in tannin extraction. Firstly extractions at low temperatures are used to avoid the possible alteration of tannins by polymerisation or other reactions at high temperatures (Foo and Porter, 1980). Lengthy extractions are used to improve yield, yet these still leave tannins open to oxidation and irreversible reaction with proteins. The separation of proteins and tannins from each other once they are in solution presents one problem but perhaps of prime concern is the possibility that the fraction of most ecological or nutritional interest, namely that which binds most strongly to proteins, never gets into solution owing to its consequent insolubility.

Water may be a good physiological extractant for ecological work but organic solvents generally seem to be thought more efficient. Methanol is particularly popular (Thompson et al. 1972; Hagerman and Butler, 1978; Matsuo and Itoo, 1981a), indeed extraction into methanol is essential for some chemical analyses (Burns, 1971). From personal observation, tannic acid (BDH) is entirely soluble in methanol but quebracho tannin (Harshaw Chemicals, Glasgow) is not and so the use of 100% methanol is automatically suspect. A 50% (aqueous) methanol extractant is used by Bate-Smith (1973b) and this does overcome the solubility problem with quebracho. Goldstein and Swain (1963) reported that compared to 100% methanol, 50% methanol is superior in removing high molecular weight condensed tannin polymers, both confirming this as a better extractant and illustrating the potential bias extraction procedures can introduce on polymer length analyses in particular.

Nonaka et al. (1981) used 100% acetone but achieved a high yield of high molecular weight tannins only after re-extraction in 100% methanol. Personal observation is that quebracho is even less soluble in 100% acetone than 100% methanol. However 70% (aqueous) acetone is often used (Strumeyer and Malin, 1975; Jones et al. 1976; Ariga et al. 1981) and Jones et al. (1976) have defended its use in competition with 50% methanol by reference to

Bate-Smith (1973b) who experienced difficulties in extracting certain types of condensed tannin that Jones et al. (1976) could extract with ease. Foo and Porter (1980) have also reported the superiority of 50-70% acetone noting its ability to disrupt tannin protein complexes in leather. This contrasts with aqueous methanol which is used in chromatography systems for tannins where such a high solubility is not displayed. My observation is that only a very small fraction of quebracho is left undissolved by 70% acetone at room temperature, none is left undissolved in 50-60% acetone in which tannic acid is also entirely soluble. Aqueous acetone has thus been adopted as the extractant in this study as it seems to offer the best means of aproaching the goal of total tannin extraction. It is of course the case that the initial tannin content of the plants cannot be known so neither can the true yield be obtained. However by initially extracting more phenolics than can be extracted with water, and so probably with saliva, then aqueous acetone solutions of these extracts should contain the allelochemically important components for studies of herbivory, even if the other polyphenolics remain insoluble. It is nevertheless true that the extractability of (not nature or quantity of) tannins may be an important variable (Asquith et al., 1983) in determining the resistance of a plant to a herbivore.

2.1,2 The Preparation of a Powdered Extract

Methods for the purification of tannins are even more diverse than those for their initial extraction. The needs of the present study were for a fast procedure which could be used with several litres of extract. This ruled out some attractive methods such as that using Sephedex (LH type) gel chromatography (Hagerman and Butler, 1978) where ascorbate could be used in the extractant as an antioxidant and then removed at the chromatography stage. Two options were considered as ways to purify the extracts, (i) specific removal of the tannins from other plant material, and (ii) non specific partitioning to at least partially remove some of the impurities. Matsuo and Itoo (1981a) used alkaloids as specific precipitants for tannins. However, 100% recoveries of tannic acid and quebracho could not even be approached using their method, a problem that the original authors had also encountered with some extracts (Matsuo and Itoo, 1981b). Polyvinylpyrrolidone was explored as an adsorbant for tannins but whilst able to do this, only detergents resolubilised the tannins succesfully and these then presented another separation problem.

Salting out techniques to remove solvents and other plant constituents have been used by Ariga et al. (1981) and Jones et al. (1976) but when tried, these led to

losses of phenolics. Finally it was decided to settle for the removal of pigments by partitioning the aqueous acetone extracts with diethyl ether, this having been found to be superior to petroleum ether. Partition was achieved by ensuring that the extract was 50-60% acetone. Some acetone is lost into the ether in this system so that the aqueous layer becomes more polar and some material precipitates out. Other material accumulates at the partition layer, much of which can be expected to be protein and this may explain the low amino nitrogen content of the extracts as this was discarded (see Section 4.2). After partition the aqueous layer was separated off and lyophylised to give a powdered extract. Practical details of the procedure employed are given in Appendix 1.1.

2.1,3 The Partition of Various Phenolics between 50% Acetone and Diethyl Ether

In order to confirm that the partition procedure adopted above would concentrate tannins in the aqueous phase, some simple partition experiments were carried out (methods in Appendix 1.2). Recoveries of 100% for tannic acid and quebracho were obtained in the water/acetone layer, in contrast to 55% and 65% for gallic acid and catechin. Ascorbate, a potentially interfering nonphenolic, was 100% recovered in the aqueous layer

whilst 97% of salicylic acid partitioned into the ether layer. Of the several other phenolics available for testing, hesperidin, a flavonoid glycoside, was insoluble in 50% acetone whilst coumaric, sinapic, ferulic and caffeic acids were only recovered to a maximum of 30% in the aqueous layer. These results illustrate that tannins are not lost at the partition stage but there will be a loss of some of the extract's "total phenolics".

2.1,4 The Tannins in the Survey.

The complete range of tannins used in the survey is listed in Table 2.1 which also gives details of their source, date of preparation and taxonomy. Including the four extracts not prepared at Strathclyde, the tabulation includes 17 extracts from a total of 14 species drawn from 12 families. Although not listed in Table 2.1, gallic acid and catechin have been included in the following analyses because of their role as component molecules in tannins. Each extract has been assigned a two-letter code (see Table 2.1) to identify it in subsequent figures and text.

Table 2.1 Tannins analysed

I Extracts prepared from fresh material at Strathclyde

Tannin source (1)	Code	Plant family (2)	Date and place of collection, notes
Aesculus hippocastanum	Ah	Hippocastanaceae	23/06/83 University grounds (Chesters)
Callistemon citrinus	Cc	Myrtaceae	07/02/83 Glasgow Botanic Gardens (GBG)
Diospyros ebenum	De	Ebenaceae	21/02/83 GBG
Loropetalum chinense	Lc	Hamamelidaceae	24/02/83 GBG
Myrtus obcordata	Mo	Myrtaceae	04/02/83 GBG
Pomaderris phylloides	Pp	Rhamnaceae	02/03/83 GBG
Pteridium aquilinum	B1	Dennstaedtiaceae	23/05/83 Queens view, near Drymen
Pteridium aquilinum	B2	Dennstaedtiaceae	13/06/83 Queens view, near Drymen
Pteridium aquilinum	B3	Dennstaedtiaceae	01/08/83 Queens view, near Drymen
Pteridium aquilinum	B4	Dennstaedtiaceae	26/09/83 Queens view, near Drymen
Quercus sp.	Qs	Fagaceae	04/10/83 Loch Lomond, shore near Balmah

Table 2.1 continued

Taxus bacata	Tb	Taxaceae	24/01/83	Kilbarchan
Vaccinium myrtilis	Vm	Ericaceae	15/06/83	Ben Each
II Tannins from comercial sources or recieved as gifts				
Tannic acid	TA	see note 3		BDH Ltd.
Quebracho tannin	Qb	see note 3		Harshaw Chemicals, Glasgow
Terminalia chebula	Tc	Combretaceae		Gift (4), Ground fruits used
Pinus radiata	Pr	Pinaceae		Gift (4), Extract from bark

Notes: (1) Native british species named according to Clapham *et al*. (1962), other species names are as given by the Glasgow Botanic Gardens. (2) Family names are as in Willis (1973). (3) TA is a relatively pure substance, not an extract whilst Qb is comercially prepared from *Schinopsis balansae* bark (Anacardiaceae). (4) Donated by D Marks, Pr originated from E. Haslam.

2.2 The Determination of Total Phenolic Content

The phenolic nature of tannins has already been noted as has the probable importance of phenolic hydroxyl groups in tannin-protein bonding. Haslam (1974) showed how increased protein precipitation resulted from increased galloyl substitution in hydrolysable tannins, the implication being that the increased complement of phenolics per tannin molecule increased its astringency. Haslam (1974) also found the astringency of a dimeric condensed tannin to be similar to that of digalloyl glucose whilst a trimeric condensed tannin has a higher astringency close to that of trigalloyl glucose. Despite the possible influence of bridging, and thus molecular size and weight, the total phenolic content of a tannin is a related and important variable as regards astringency.

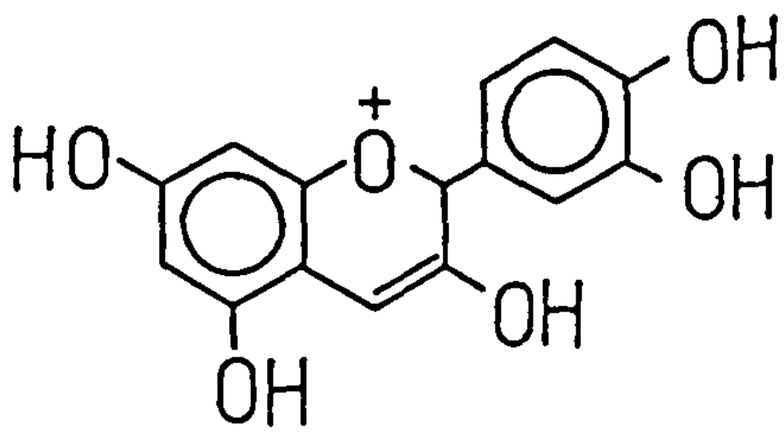
Molecular size and phenolics are related in hydrolysable tannins because an increase in phenolics is necessarily tied to an increase in galloyl or ellagyl groups. In condensed tannins the hydroxylation of the individual flavonoid units, particularly that of the "B" ring, is variable (but also compare structures II & III from Chapter 1) . Different B ring hydroxylation within structure II, gives the two important types of condensed tannins (i) the di-hydroxylated procyanidin tannins and (ii) the tri-hydroxylated prodelphinidin tannins. These

are named after the anthocyanidin pigments (XI & XII) generated upon their hydrolysis. Mixed types of condensed tannin also occur (Foo and Porter, 1980). Jones et al. (1976) have reported that astringency increases with the proportion of prodelphinidin in a tannin, these having more phenolic hydroxyls able to bind to proteins. This evidence, coupled with that of Haslam (1974) implying one protein binding site per condensed tannin monomer, supports the conclusion that the ortho oriented hydroxyls on the flavanoid B ring bind to protein, not the meta orientated ones of the A ring.

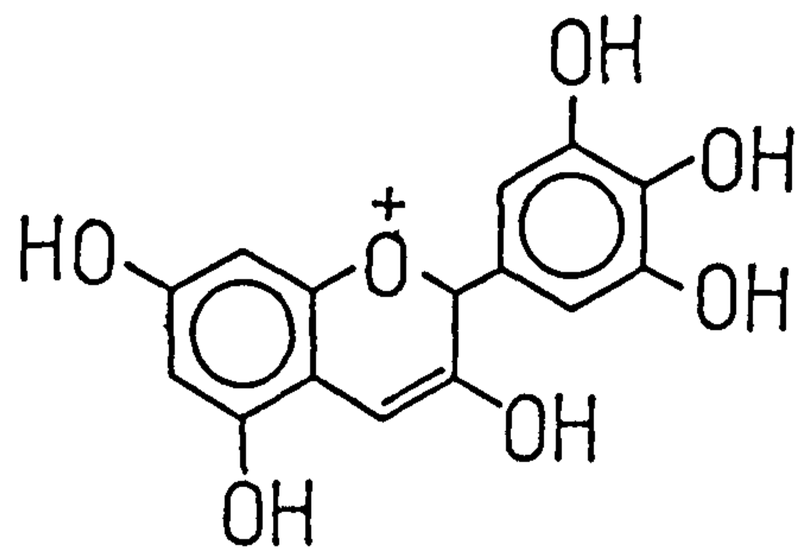
Given their clear influence on the ecologically important properties of tannins, a measurement of the "total phenolics" of each tannin-containing extract is a pre-requisite for their further study.

2.2,1 Selection and Description of Methods

Several techniques for the measurement of total phenolics have been published. These may be divided into those dependent on the oxidation of phenols in the analysis, such as the Folin-Denis, Folin-Ciocalteau, Prussian blue and Permanganate Index methods (Singleton and Rossi, 1965; Price and Butler, 1977; Ribereau-Gayon and Peynaud, 1958). Further groups of analyses are dependent on complexation reactions with iron (Maxson and Rooney, 1972; Hagerman and Butler, 1978) or on coupling



XI Cyanidin



XII Delphinidin

structures XI & XII re-drawn from Haslam, 1981

reactions with diazo compounds (Goldstein and Swain, 1963). The relative merits of most of these methods have been reviewed by Mbi (1979) and Choo (1981). Their work led to the adoption of the Folin-Denis method as the standard technique for total phenolics at Strathclyde. The procedure for this method as used in this study is that given by Choo (1981) and it is detailed in Appendix 1.3

In general the technique is able to detect all phenols, whether they occur as mono- or polyphenols, but it is susceptible to interference by nonphenolics such as ascorbate. The technique does also depend critically on two timed intervals between the addition of reagents, thus allowing room for added operator error. A practical advantage of the method when working on pigment containing extracts is that 1 ml samples of extract are added to 100 ml of reagents, the dilution removing the need for blank corrections. When analysing for total phenolics in pigment free extracts where a through put of over 100 samples per day was needed (see Chapter 3) the Folin-Denis procedure became unwieldly and a second technique was adopted, this being a slight modification of the method of Hagerman and Butler (1978) (see Appendix 1.4 for the experimental details).

In contrast to the Folin-Denis reaction where reduction of the reagents by the test substance produces

a colour, in this assay (Hagerman and Butler, 1978) the direct interaction of ferric ions with phenols produces a coloured complex. The production of coloured complexes between phenolics and iron chloride in solution is well known, and their differing colours may be used as a guide to the types of phenolics involved, typically these are green for condensed tannins and blue for hydrolysable tannins (Trease and Evans, 1978). Hagerman and Butler (1978) only worked on Sorghum tannins (procyanidin type) and noted the purple complex produced with ferric chloride in 5% triethanolamine. Although not previously tested on a range of tannin types, as tannic acid and quebracho both gave similar purple coloured complexes, as did all the other extracts listed in Table 2.1, the method has been adopted here. Its advantages are that by following the procedure detailed in Appendix 1.4 an instantaneous reaction producing a stable colour lasting several hours is achieved, without any timed or involved procedure. Moreover the standard curve is linear over a considerable absorbance range unlike that of the Folin-Denis reaction (see standard curves, Figs 2.1 and 2.2). The disadvantage of this technique is that it lacks sensitivity compared to the Folin-Denis procedure and can only be used on extracts after ether extraction of the pigments.

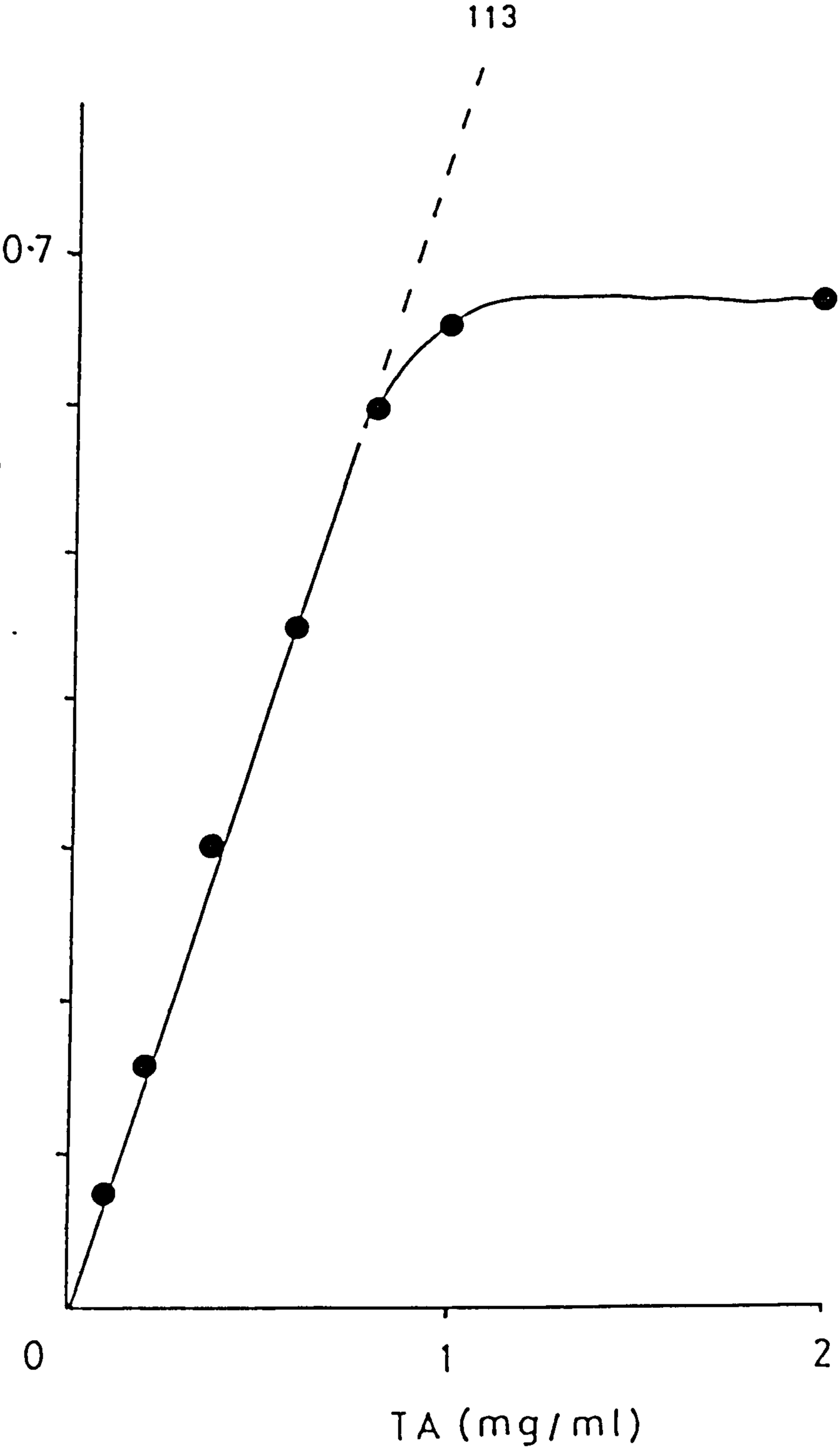


Figure 2.1 Standard curve for tannic acid measured by the Folin-Denis method for total phenolics. The curve illustrates the narrow range of absorbance (ABS) over which measurements must be made.

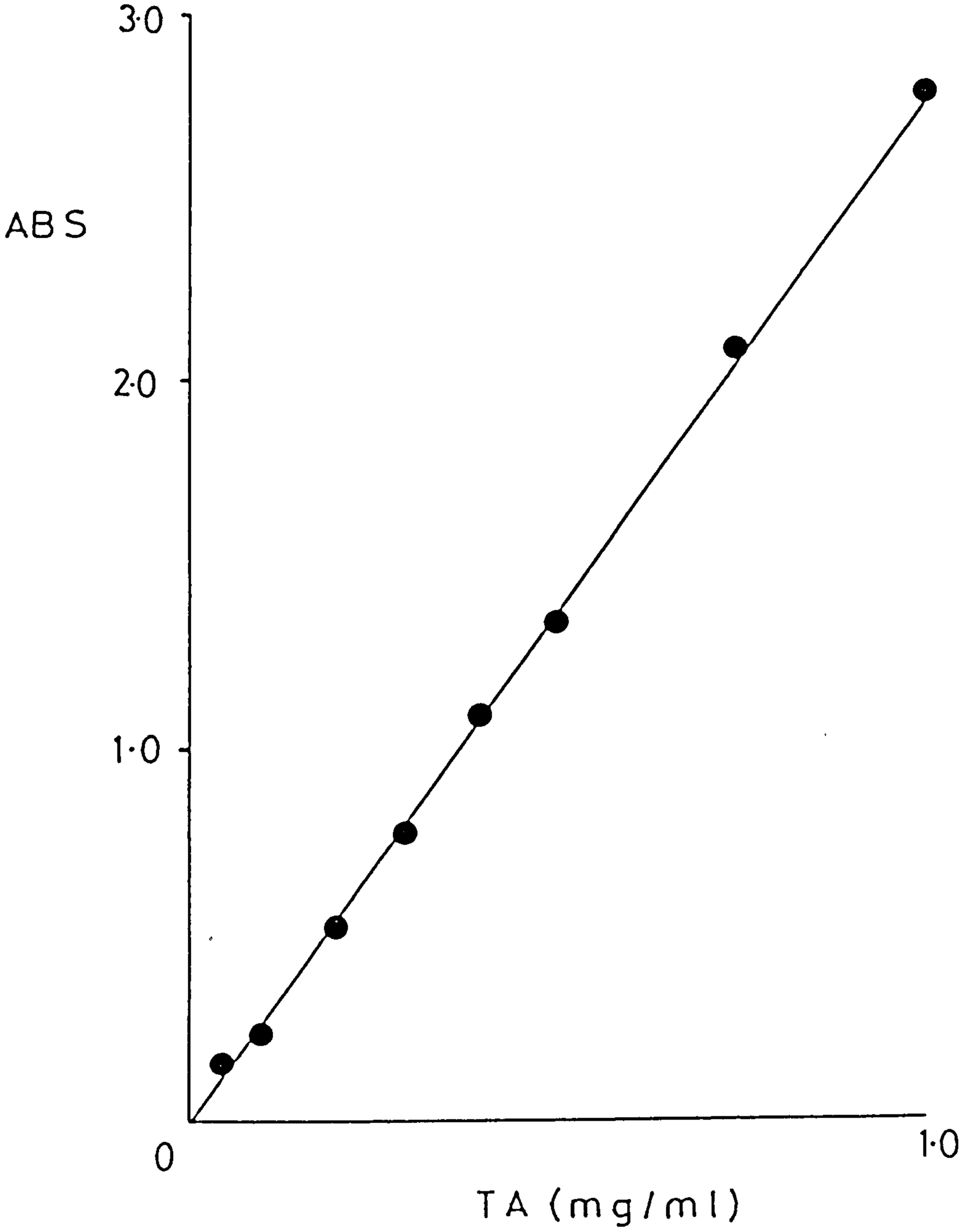


Figure 2.2 Standard curve for tannic acid measured by the Hagerman and Butler (1978) method for total phenolics. The curve illustrates the wide range of absorbance (ABS) over which measurements can be made.

2.2,2 Results for Total Phenolics

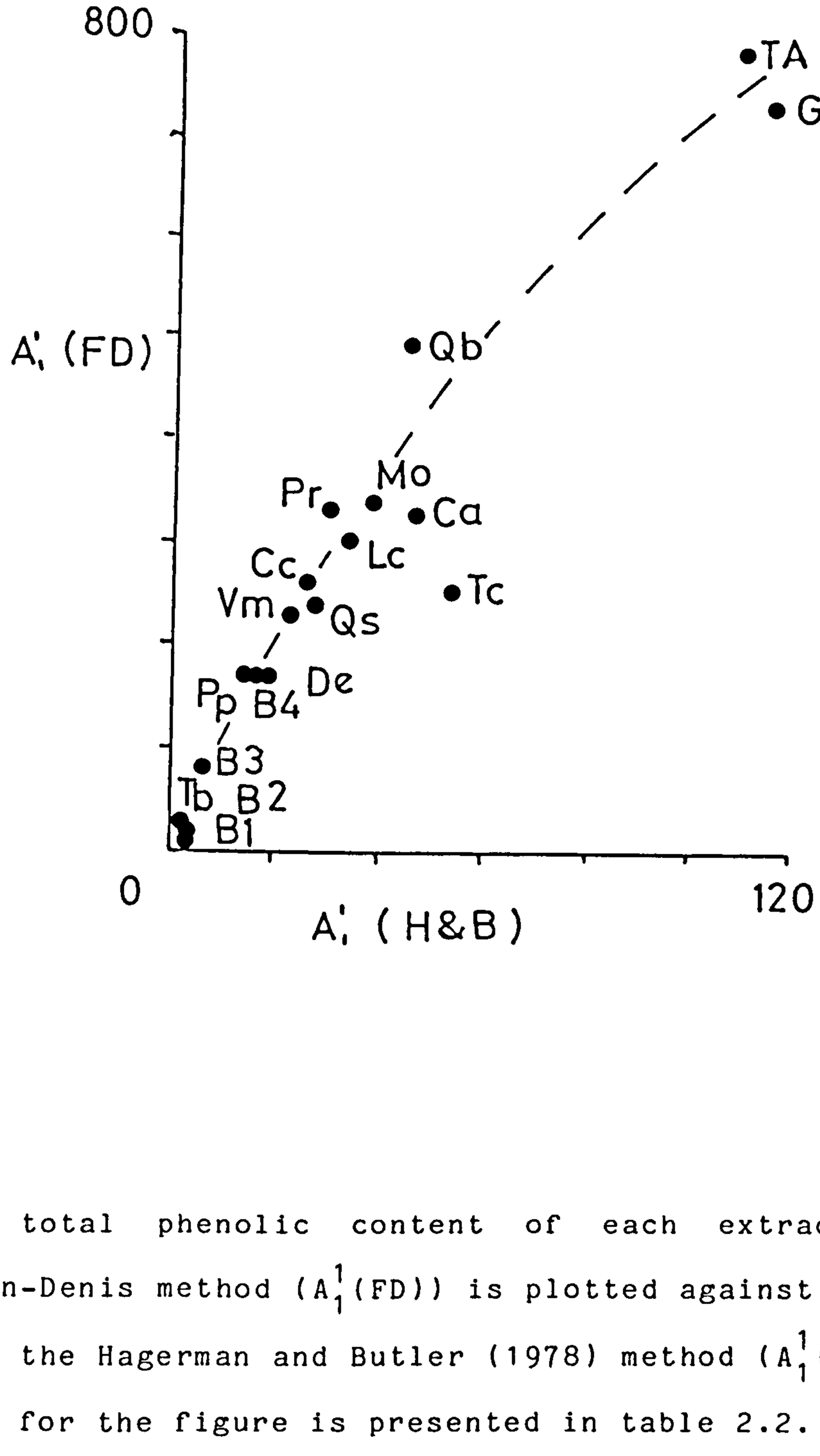
The absorbances (for 1% solutions through a 1cm path length, denoted A_1^1) for each extract listed in Table 2.1 together with data for gallic acid and catechin, are given for both assays in Table 2.2. Figure 2.3 shows this data and the correspondence between the two assays. This is not perfect, presumably owing to the different reaction processes leading to the production of the coloured complexes. Terminalia chebula (Tc) provides a notable outlier whilst the other points describe a curved distribution, indicating that the Hagerman and Butler (1978) assay progressively overestimates total phenolics (by Folin-Denis method) at higher absorbances. The other and important point illustrated by this Figure is the very large range in A_1^1 values and thus concentrations of phenolics, amongst the plant material extracted. Unfortunately some extracts were found to be so low in phenolics as to be near the limits of detection in the following assays, notably Ah, Tb, B1 & B2.

Table 2.2 Analyses (A_1^1 values) for Total Phenolics and Condensed Tannins*.

Tannin	Code	FD	H&B	CT	Vme
Aesculus hippocastanum	Ah	12	3.4	4.0	1.2
Calistemon citrinus	Cc	260	26	5.6	7.8
Diospyros ebenum	De	170	18	11	19
Loropetalum chinense	Lc	300	33	6.4	10
Myrtus obcordata	Mo	340	38	5.0	4.8
Pomaderris phylloides	Pp	170	14	68	29
Pteridium aquilinum	B1	17	3.0	0.3	0.0
Pteridium aquilinum	B2	17	2.4	0.3	1.2
Pteridium aquilinum	B3	79	5.8	7.0	4.4
Pteridium aquilinum	B4	170	15	36	17
Quercus sp.	Qs	240	27	23	4.8
Taxus bacata	Tb	26	2.0	8.4	6.6
Vaccinium myrtilis	Vm	230	22	32	11
Tannic Acid	TA	780	110	0.0	0.0
Quebracho Tannin	Qb	490	45	63	13
Terminalia chebula	Tc	250	54	0.0	0.0
Pinus radiata	Pr	330	30	91	32
Catechin	Ca	350	46	17	21
Gallic Acid	GA	750	115	0.0	0.0

*Notes: FD and H&B; Total Phenolics by Folin Denis and Hagerman and Butler methods respectively. CT and Vme; Condensed tannins by Proanthocyanidin and Vanillin (in methanol) methods respectively.

Figure 2.3 Comparison of methods for total phenolics



The total phenolic content of each extract by the Folin-Denis method (A_1^1(FD)) is plotted against the values from the Hagerman and Butler (1978) method (A_1^1(H&B)). The data for the figure is presented in table 2.2.

2.3 The Determination of Condensed Tannin Content

Drawing together the information presented in sections 1.1 and 2.2, condensed tannins have been shown to vary in their degree of polymerisation, the linkage between their monomeric constituents and the hydroxylation pattern of their constituent monomers. Following the measurement of total phenolics the next stage was to gauge the fraction of these present in condensed tannins.

2.3,1 Selection and Description of Methods

Two chemically specific methods are available for measuring condensed tannin content, these are the vanillin reaction (Burns, 1971) and the method dependent on anthocyanidin production (Swain and Hillis, 1959). Again, both Mbi (1979) and Choo (1981) have reviewed their relative merits. They adopted as their standard technique the "proanthocyanidin" method of Swain and Hillis (1959), modified for increased anthocyanidin yield as described by Horowitz (1970). This method depends upon the acid hydrolysis of condensed tannins in butanol to yield anthocyanidin pigments e.g. cyanidin or delphinidin, depending on the nature of the tannin. As the production of these pigments depends on the hydrolysis of the interflavan bonds, the reaction should not detect the non-tannin monomer catechin although it is

susceptible to interference by chlorogenic acid. Catechin does produce an intense yellow colour in the assay, presumably not an anthocyanin but nevertheless a reaction giving a non zero A_1^1 (see Table 2.2). Flavan-3,4-ol monomers (leucoanthocyanidins) may form anthocyanidins in the assay but their lability ensures their decay before absorbance is measured (Watterson and Butler, 1983). The greatest drawback of the method is that cyanidin and delphinidin have different absorbances in the assay, with that of delphinidin being significantly greater than that of cyanidin, leading to marked over-estimation of condensed tannins with a high prodelphinidin content.

Given this known problem with the assay, all the extracts were also assayed by the vanillin reaction. This second method is critically dependent on timing and reaction temperature (Dalby and Schuman, 1978) these being reasons for not using it for routine work. Its advantage is that vanillin reacts with the flavonoid A ring (at the C-6 position), forming a chromophore that is not influenced by B ring hydroxylation (i.e. procyanidin/prodelphinidin ratio). The specificity of the reaction conditions in the assay are for a reaction with flavonoids with free <u>meta</u> orientated A-ring OH groups, single C2-C3 bonding and with no carbonyl function at C-4 (Sarkar and Howarth, 1976). Whilst being

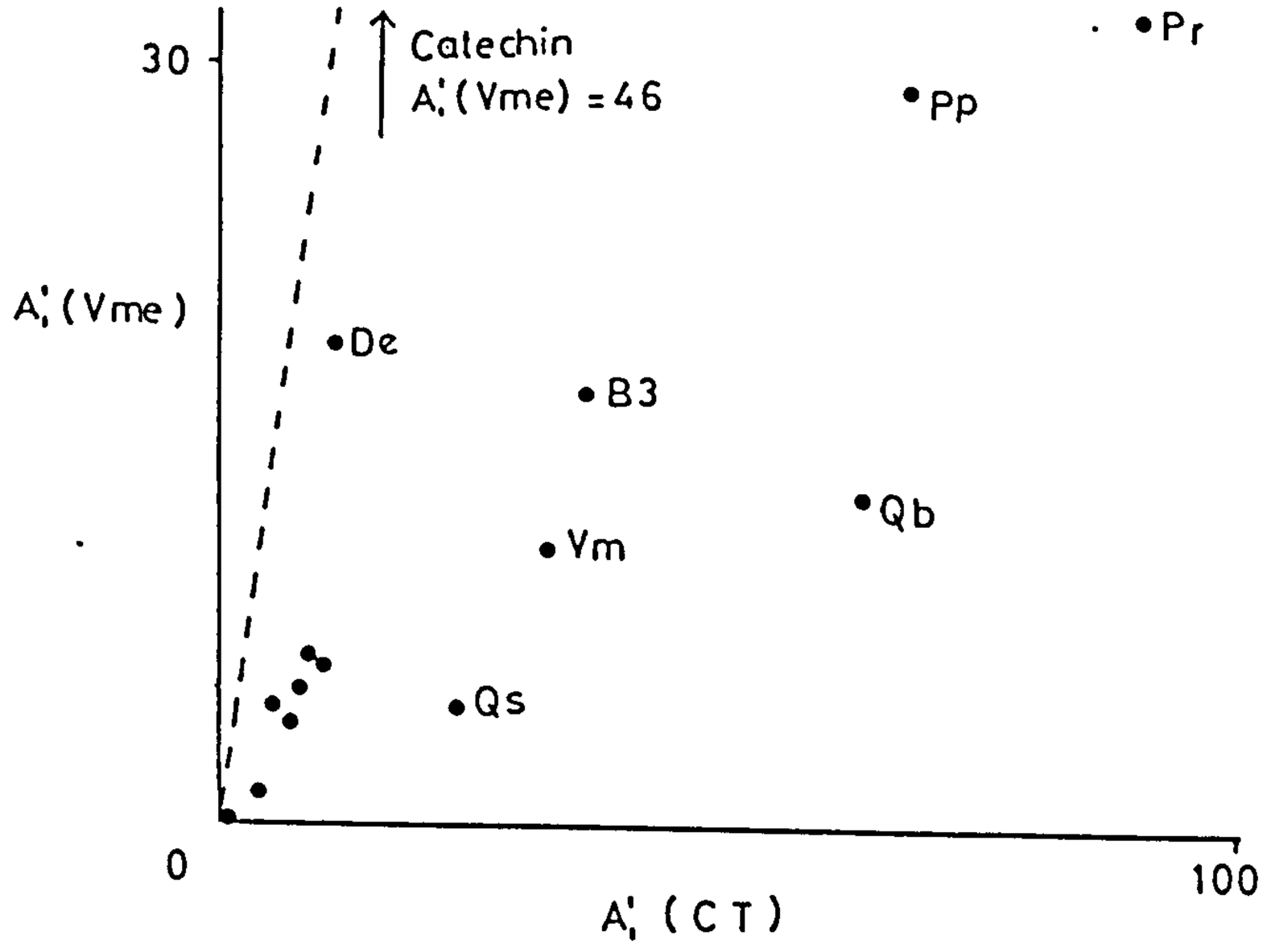
largely condensed tannin specific, the assay is susceptable to variation dependent on the type of interflavan bonding (e.g. C-6 to C-4 or C-4 to C-8, see Section 1.1,2). Like the proanthocyanidin method, the vanillin reaction is also susceptible to some chemical interference, in this case by dihydroxychalcones. Walton et al. (1983) have also found an interfering colour reaction in Sorghum which is due to the acid in the reagent, and therefore probably due to leucoanthocyanidins and the reaction will probably detect the phloroglucinol based phlorotannins too. Additionally the vanillin reaction has to be carried out in 100% methanol which is, as has been seen, a dubious solvent for condensed tannins.

The practical details for both assay methods are given in Appendices 1.5 and 1.6, the vanillin method used being that of Price et al. (1978).

2.3,2 Results for Condensed Tannins

The results of the analyses for each extract are given in Table 2.2. and as for total phenolics, the data for the two analyses used are plotted against each other, see Figure 2.4. Unlike total phenolics, there is evidence of a considerable divergence from any one to one relationship between these assays. The significance of this is discussed in the following section. The

Figure 2.4 Comparison of methods for condensed tannins



The condensed tannin content of each extract by the Vanillin method (A_1^1(Vme)) is plotted against the values from the proanthocyanidin method (A_1^1(CT)). The data for the figure is presented in Table 2.2. Points to the right of the hatched line, deviating from it by succesively larger Vme/CT values, are likely to have succesively longer polymer lengths. (explanation in text).

percentage of condensed tannin in the total phenolics is given in Table 2.3, with the calculations being set out in Table 2.4. The extract with the highest A_1^1 in the proanthocyanin assay and vanillin assay (Pr) has been used as the standard for both these means of quantifying the percentage of condensed tannin phenolics. This has led to "over 100%" values in the estimates %CT1 and %CT2 . This may be due to the variation introduced by differential reactions in the Folin-Denis assay and the influence of the relative procyanidin/prodelphinidin contents of the tannins. Despite this, inspection of the data indicates a positive association between these estimates. Values less than 100% indicate extracts where hydrolysable tannins might be sought. Particularly high values of %CT2 versus %CT1 could in part be due to high flavanol monomer concentrations in the extracts (see Ca, Tb, B2 and De).

Table 2.3 Analyses for Condensed Tannins: Polymer Lengths* and their Proportion (%) in the Total Phenolics*.

Tannin	Code	Vac	P1	P2	%CT1	%CT2
Aesculus hippocastanum	Ah	4.3	2.4	3.3	120	100
Calistemon citrinus	Cc	9.0	1.6	0.72	78	31
Diospyros ebenum	De	23	1.2	0.58	23	120
Loropetalum chinense	Lc	2.6	6.3	0.84	77	34
Myrtus obcordata	Mo	3.5	3.7	1.0	53	15
Pomaderris phylloides	Pp	20	8.7	2.3	150	180
Pteridium aquilinum	B1	0.54	1.4	300	6	0
Pteridium aquilinum	B2	2.3	0.33	0.25	6	72
Pteridium aquilinum	B3	4.6	3.9	1.7	32	55
Pteridium aquilinum	B4	12	7.7	2.1	77	100
Quercus sp.	Qs	9.6	6.2	4.8	35	20
Taxus bacata	Tb	3.9	5.5	1.3	120	260
Vaccinium myrtilis	Vm	25	3.3	2.9	50	49
Tannic Acid	TA				0	0
Quebracho tannin	Qb	20	8.1	4.8	47	27
Terminalia chebula	Tc				0	0
Pinus radiata	Pr	29	8.1	2.8	100	100
Catechin	Ca	234	0.19	0.81	17	62
Gallic Acid	GA				0	0

*Notes: Formulae for Polymer lengths (P1 and P2) and percentages of condensed tannins (%CT1 and %CT2) are given in Table 2.4. Vac is the A_1^1 value for the vanillin assay performed in glacial acetic acid.

Table 2.4 Formulae for calculating the polymer lengths of, and the percentage of condensed tannin in, the extracts

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I Polymer length by the method of Butler *et al*. (1982). For a sample of pure tannin, the length in catachin monomer units is given by:

Length= L = A_1^1(Vac) of catechin / A_1^1(Vac) of the tannin

As many of the present extracts are impure, a correction factor (CF) needs to be used to accuont for this. This is based on the assumption that the *Pinus radiata* tannin (Pr) is 100% pure condensed tannin.

CF = A_1^1(CT) of tannin / A_1^1(CT) of Pr

Polymer length (P1) is thus given by:

P1 = CF x L

II Polymer lengths estimated as the procyanidin/vanillin ratio (CT)/(Vme)

P2 = A_1^1(CT) of the tannin / A_1^1(Vme) of the tannin

Table 2.4 continued

III The percentages of condensed tannins in the extracts' total phenolics: these percentages have been estimated in two ways. In both cases total phenolics are measured by the Folin-Denis method and a Pinus radiata (Pr) standard is used as a reference point as an assumed 100% pure tannin. The first estimate (%CT1) is calculated as below using the proanthocyanidin method (CT) to measure tannin:

$$\%CT1 = \left[\frac{A_1^1(CT) \text{ of extract}}{A_1^1(FD) \text{ of extract}}\right] \times \left[\frac{A_1^1(FD) \text{ of Pr}}{A_1^1(CT) \text{ of Pr}}\right] \times 100\%$$

The second estimate (%CT2) is calculated by reference to the vanilin method for condensed tannins (Vme), and substituting A_1^1 values obtained by this tecnique, in the above equation, in place of those obtained by the proanthocyanidin method.

2.4 The Determination of Condensed Tannin Polymer Lengths

Condensed tannin polymers, irrespective of the interflavan bond type, are linear structures; branching is unreported. Their comparative variability in molecular weight and thus length should be reflected by variation in their abilities to cross link and precipitate proteins. Evidence that this is the case is almost entirely dependent upon indirect measurements of molecular size by gel permeation chromatography. Gustafson (1956) believed that tannins, on his definition, had weights between 300 and 3000. Quesnel (1968) examined cocoa tannins finding that 50% were dimeric (weight= 500) but that polymers of up to 5000 were present. Somers (1966, 1967) working on condensed tannins in wine, estimated the weight range at 2000-5000 but with some tannins exceeding this, and approaching weights of 50,000. At the other end of the scale Goldstein and Swain (1963) reaffirmed the lower limit of 500 for astringent tannins. This lower weight limit for astringency was also seen in the results of Weisen and Wetter (1978) although their tannins were probably of the hydrolysable type.

The range of molecular weights exhibited by condensed tannins is not actively disputed but it remains a point of contention as to whether condensed tannins at either

end of the reported weight range are astringent. Roux (1972) reported that dimeric condensed tannins were not astringent, whilst the results of Nonaka et al. (1981), who reported that they were, may be due to additional galloyl substitution in the tannins. As already noted, Haslam (1974) has reported astringency for procyanidin dimers, but this astringency is weak and on the present evidence condensed tannin dimers would seem to be right at the lower weight limit for astringency. At the other end of the scale the reduced solubility of the longer polymers (section 2.1) may reduce their ability to inhibit enzymes free in solution although this might enhance their protection of cell wall material from degradation. Oh and Hoff (1980) used affinity chromatography on immobilised serum albumin to fractionate grape tannins, and they demonstrated that astringency rose with molecular weight to a maximum at 2100 (approximately 8 monomer units) from a minimum of 900 (3-4 units). At higher molecular weights they found a decline in astringency. This finding of low astringency at high molecular weight had also been noted by Jones et al., (1976) working on legume tannins in the weight range 6,000-28,000.

2.4,1 Methods for Polymer Length Determination.

As has been pointed out, gel permeation chromatography

(GPC) has been the method of choice, initially using Sephadex G type gels (Somers, 1966) and more recently the hydrphobic LH gels (Jones et al. 1976; Hagerman and Butler, 1978; Cansfield et al., 1980; Ariga and Asao, 1981). These authors worked on a few extracts, extensively purified by involved procedures which do not lend themselves to the survey of a large range of tannins. Other similarly complex methods have used acetylated tannins for GPC on "Styragel" columns (Williams et al., 1983) and ^{13}C NMR spectroscopy of GPC eluted tannins, to calculate monomer to chain terminating unit ratios and procyanidin/prodelphinidin ratios (Czochanska et al., 1983). LH-20 gel chromatography in 50% acetone of the extracts listed in Table 2.1 was attempted but had to be abandoned as it proved impossible to completely elute samples within one bed volume of solvent, i.e. there was considerable evidence that phenolics were being adsorbed to the gel matrix.

A chemical method that would not be subject to interference by impurities in the extract was then sought. Lewak (1968) found that the ratio of the vanillin assay to that of the Folin-denis method (Vme/FD) gave a good correlation with the retention time of condensed tannins on LH-20 gel chromatograms. However, as in this study the extracts to be tested were not pure condensed tannins this was impracticable. Another assay

ratio, that between the proanthocyanidin and vanillin methods (CT/Vme) is also thought to relate to condensed tannin polymer length (Goldstein and Swain, 1963). The rationale for this is that both assays are oppositely influenced by the ratio of monomer to chain-terminating flavanoid units. Firstly the vanillin assay is reactive to monomers, whilst the proanthocyanidin assay should not be, as flavan-3-ols do not react to form anthocyanins in the assay. Secondly the longer the proanthocyanidin polymer is, the more anthocyanidin pigment that can be produced by hydrolysis, whilst the more steric hindrance there is to the vanillin reaction. Most authors assume that vanillin is equally able to interact at C-6 or C-8 which in reality means for a condensed tannin it is only able to react at the C6 position, the C-8 being blocked in polymer formation. Butler et al. (1982) found that A_1^1 values in the assay (Vme) of oligomers, exceeded that of catechin. Nevertheless consideration of Figure 2.4 shows that the condensed tannin-rich extracts deviate from the dotted line indicating the ratio of A_1^1 values expected for catechin containing extracts. In every case the deviation is toward increased CT/Vme as anticipated for polymerised flavan-3-ols.

Butler et al. (1982) examined the kinetics of the vanillin reaction in several solvents, and found that in glacial acetic acid under precisely defined conditions,

chromophore production was proportional to the number of polymer chain terminating units. This work was carried out using known pure compounds, but the use of the proanthocyanidin assay was suggested as an independent (of the vanillin reaction) means to gauge condensed tannin purity in impure samples. As both assays are relatively specific for condensed tannins, this was adopted as a second means to gauge condensed tannin polymer lengths, in addition to the vanillin/proanthocyanidin ratio method. The experimental details for the vanillin assay in glacial acetic acid (Vac) are given in Appendix 1.6

2.4,2 Results for Polymer Lengths

The results for the vanillin assay in glacial acetic acid are given in Table 2.3 together with the assay ratios P1 and P2 used to estimate polymer lengths. It should be noted that each estimate is of mean polymer length for the extract and cannot give any information on the distribution of lengths, as can be gained from the GPC methods. The calculations made for each estimate are set out in Table 2.4. Polymer length measure P1 indicates a range from near monomeric to octameric mean polymer lengths, putting the upper mean weight limit at about 2000, although of course there may well be longer polymers within the distribution. Inspection of the

Table shows a positive association with values for P2 but that this second measure is rather variable. The extreme value for B1 is probably due to inaccurate measurements at very low A_1^1 values. In spite of this, considering the more condensed tannin rich extracts (those marked out separately in Figure 2.4), these tend to have long (P1) polymer length estimates except for De which lies particularly close to the line of values expected for catechin.

2.5 The Determination of Hydrolysable Tannin Content

Measurements of hydrolysable tannin contents are notable by their absence in extensive surveys of plant foodstuffs (Gartlan et al., 1980; Oates et al., 1977; Coley, 1983). This might be seen as surprising given our present knowlege of hydrolysable tannin chemistry, particularly of their biogenesis, structure and interactions with proteins (Beart et al., 1985; Haslam, 1974). However given the belief that their hydrolysability may render them inactive in the gut, coupled with the lack of an effective assay or even diagnostic test for them, our relative ignorance about their ecological importance is understandable. Indeed virtually the only information on their action in vivo comes from laboratory studies using tannic acid in artificial diets fed to captive animals.

2.5,1 Existing Methods for the Assay of Hydrolysable Tannins.

Blazej and Suty (1977) have attempted to provide what amounts to a diagnostic test for hydrolysable tannins by direct UV spectroscopy. They claim to be able to detect hydrolysable tannins in the presence of condensed tannins, though not to quantify them accurately. This method was tried but found difficult to repeat, probably

because of other interfering plant phenolics in the extracts.

Bate-Smith (1972b) has used the reaction of hexahydroxydiphenic acid (HHDP) with nitrous acid, to form the basis of an assay; HHDP is the precursor of the dilactone ellagic acid (V) and is the form present in unhydrolysed ellagitannins (Haslam 1979). In the assay a transient blue colour is produced in anoxic experimental conditions, the maximum peak absorbance at 600 nm being recorded as it occurs. An attempt was made to follow this method with the extracts listed in Table 2.1. Only with Diospyros ebenum was there any reasonable and positive reaction. Whilst this extract may well be a source of ellagitannin, the strong reaction with quebracho and nil reaction with Terminalia chebula (an ellagitannin source; Haslam, 1966) led to the pursuit of the assay being abandoned. Indeed the only succesful use of this technique to date has been by Bate-Smith (1972c) and then only for material from within the genus Geranium.

By extending the work of Haslam (1965), Bate-Smith (1977) also devised a method for gallotannin estimation, using the reaction with potassium iodate in which a red colour is formed in aqueous acetone extracts. Again, this method was attempted with the extracts listed in Table 2.1. Colour reactions were detected, but not all

were unequivocably red. Thus interfering side reactions were present. Turbidity was also a problem, as reported by Bate-Smith (1977). The colours obtained are listed in Table 2.5, and the method used to obtain these results is described in Appendix 1.7. It is difficult to interpret these results with confidence, even as indications of hydrolysable tannin presence. It should also be noted that ellagitannins do react in the assay too, but more strongly than gallotannins (Bate-Smith, 1977).

Bate-Smith (1977) has used the technique to examine material from the genus Acer whilst Baldwin and Schultz (1983) have also used it to study changes in Populus x euroamericana tannins. The problems encountered here with both the above techniques are perhaps illustrative of the problems of using what are highly specific assays outwith the taxa they were developed upon.

2.5,2 A Novel Method for the Determination of Hydrolysable Tannins.

The difficulties encountered with the two published methods as outlined above led to the search for a single assay for both types of hydrolysable tannin which was sufficiently simple and general for surveys of a range of ecological material. It is well known that when they are added to ferric chloride solutions, condensed tannins form green coloured complexes in contrast to the blue

Table 2.5 Colour Reactions in Diagnostic Tests* for Hydrolysable Tannins.

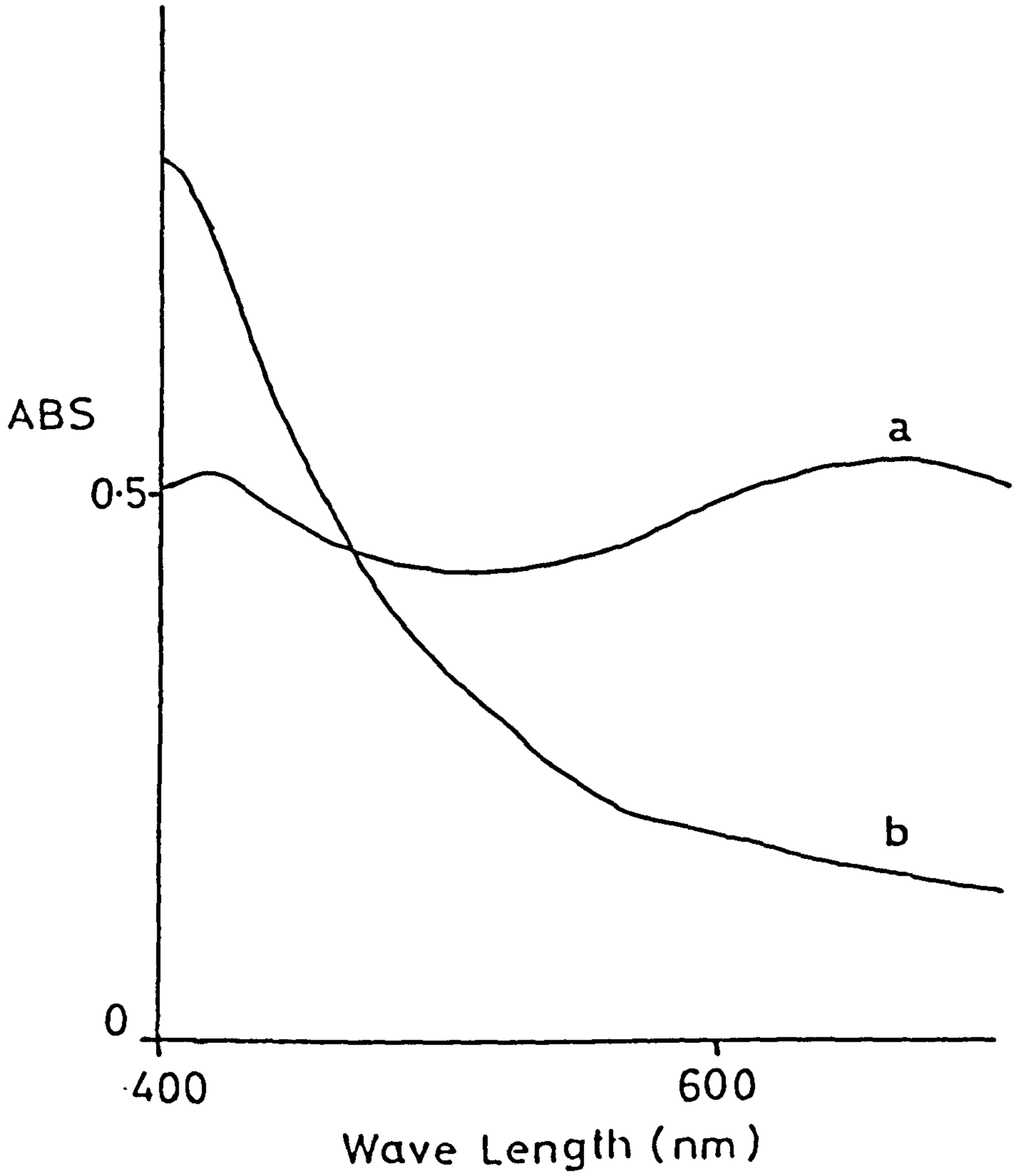
Tannin	Code	Iodate Reagent	$FeCl_3$ Reagent
Aesculus hippocastanum	Ah	red-brown	green
Calistemon citrinus	Cc	dark brown	blue-green
Diospyros ebenum	De	red (as for TA)	blue-black
Loropetalum chinense	Lc	deep red	blue-black
Myrtus obcordata	Mo	green-black	blue-black
Pomaderris phylloides	Pp	brown	green
Pteridium aquilinum	B1	brown	green
Pteridium aquilinum	B2	brown	green
Pteridium aquilinum	B3	brown	green
Pteridium aquilinum	B4	brown	green
Quercus sp.	Qs	dark brown	blue-green
Taxus bacata	Tb	red-brown	green
Vaccinium myrtilis	Vm	dark-brown	green
Tannic Acid	TA	red	blue-black
Quebracho tannin	Qb	dark brown	green-blue
Terminalia chebula	Tc	yellow-grey	green
Pinus radiata	Pr	dark brown	green
Catechin	Ca	deep orange	green
Gallic Acid	GA	yellow-orange	green

*Note: Methods as given in text, the initial colour (only) of the complexes formed with $FeCl_3$ is recorded

coloured complexes formed by hydrolysable tannins. This empirical observation seems to be without any detailed theoretial explanation (Trease and Evans, 1978) however the following simple method for hydrolysable tannins attempts to exploit this phenomenon, by employing suitable conditions for the formation of these differently coloured complexes, unlike Hagerman and Butler's (1978) method for phenolics.

Initial exploratory observations showed that when dilute tannin solutions were added to a ferric chloride solution (1.62 g $FeCl_3$ per litre of 0.001 M HCl), clear but coloured solutions could be produced (e.g. no problems with haze or precipitation). These were distinctly green or olive in colour for quebracho tannin, catachin and most importantly, gallic acid. Tannic acid gave a dark blue colour when mixed with ferric chloride although this faded somewhat with time. Ellagic acid is insoluble in water and so could not be tested. The colour reactions of the other extracts are listed in Table 2.5; all range between blue-black and green in colour. These observations suggested that hydrolysable tannins might be assayed in the presence of condensed tannins by measuring the loss in blue colour expected after the hydrolysis of the tannin to produce gallic acid or ellagic acid. Figure 2.5 shows that the visible spectrum of tannic acid does indeed exhibit a marked

Figure 2.5 Visible spectra for hydrolysed and unhydrolysed tannic acid in the presence of ferric chloride.



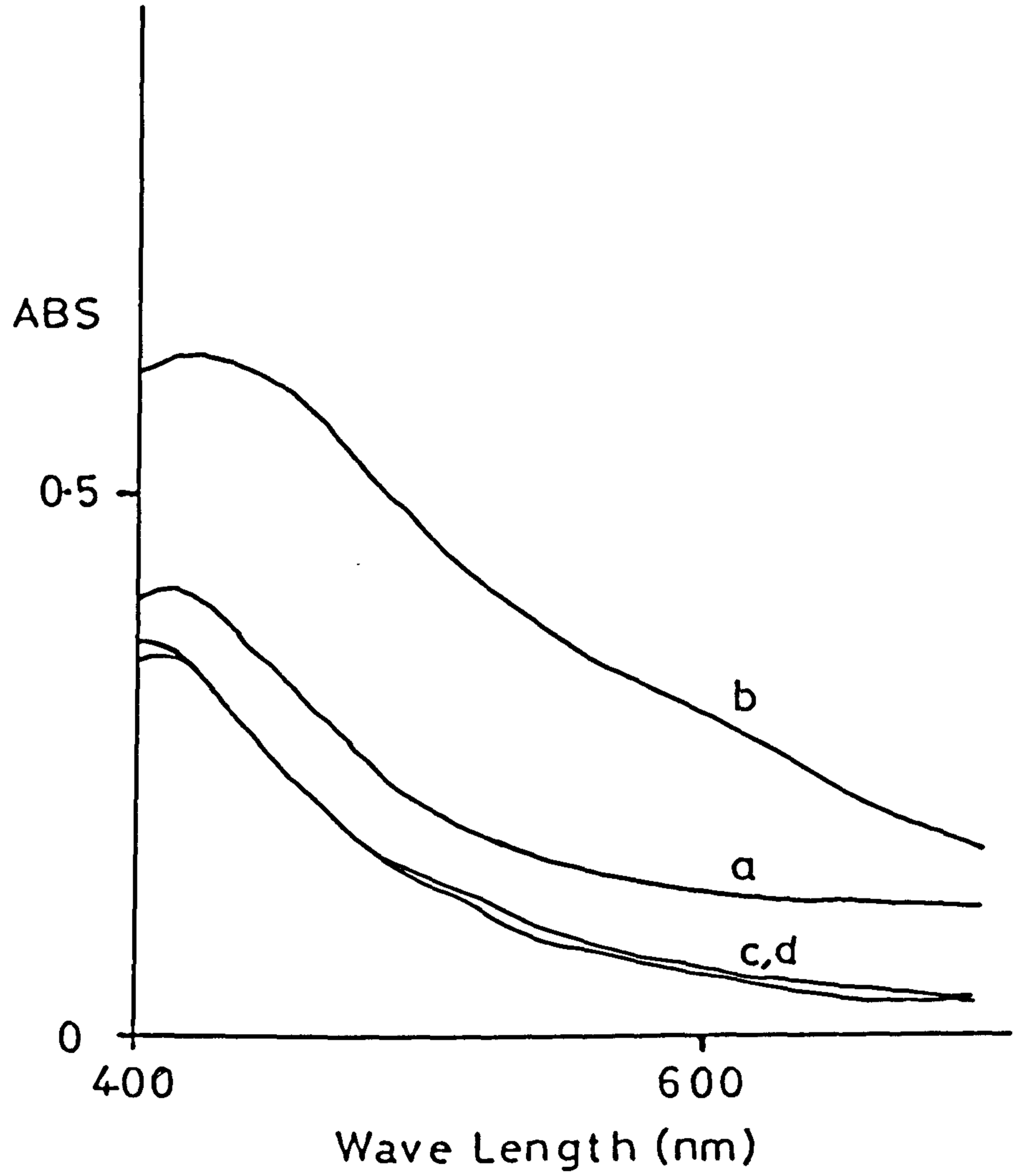
The curves show absorbance (ABS) as a function of wavelength for unhydrolysed (curve a) and hydrolysed (curve b) tannic acid, both present at the same concentration.

decrease in absorbance in the blue region (maximum change at 660 nm), consequent upon hydrolysis. In contrast, that for quebracho (Figure 2.6) shows an increase whilst gallic acid shows no change.

The blue complex with tannic acid was notably transient, decaying to a green hue over a few minutes as indicated by Figure 2.7. With a little practice, it was found to be entirely feasible to add a sample of tannin to the ferric chloride reagent in a cuvette, mix the contents and read the absorbance at 660nm, exactly fifteen seconds after adding the tannin sample. This was adopted as standard practice as the best means to maximise the absorbance readings. Measurements after hydrolysis were made using the same protocol although this was not strictly necessary as the absorbance of the green complex is relatively constant with regard to time.

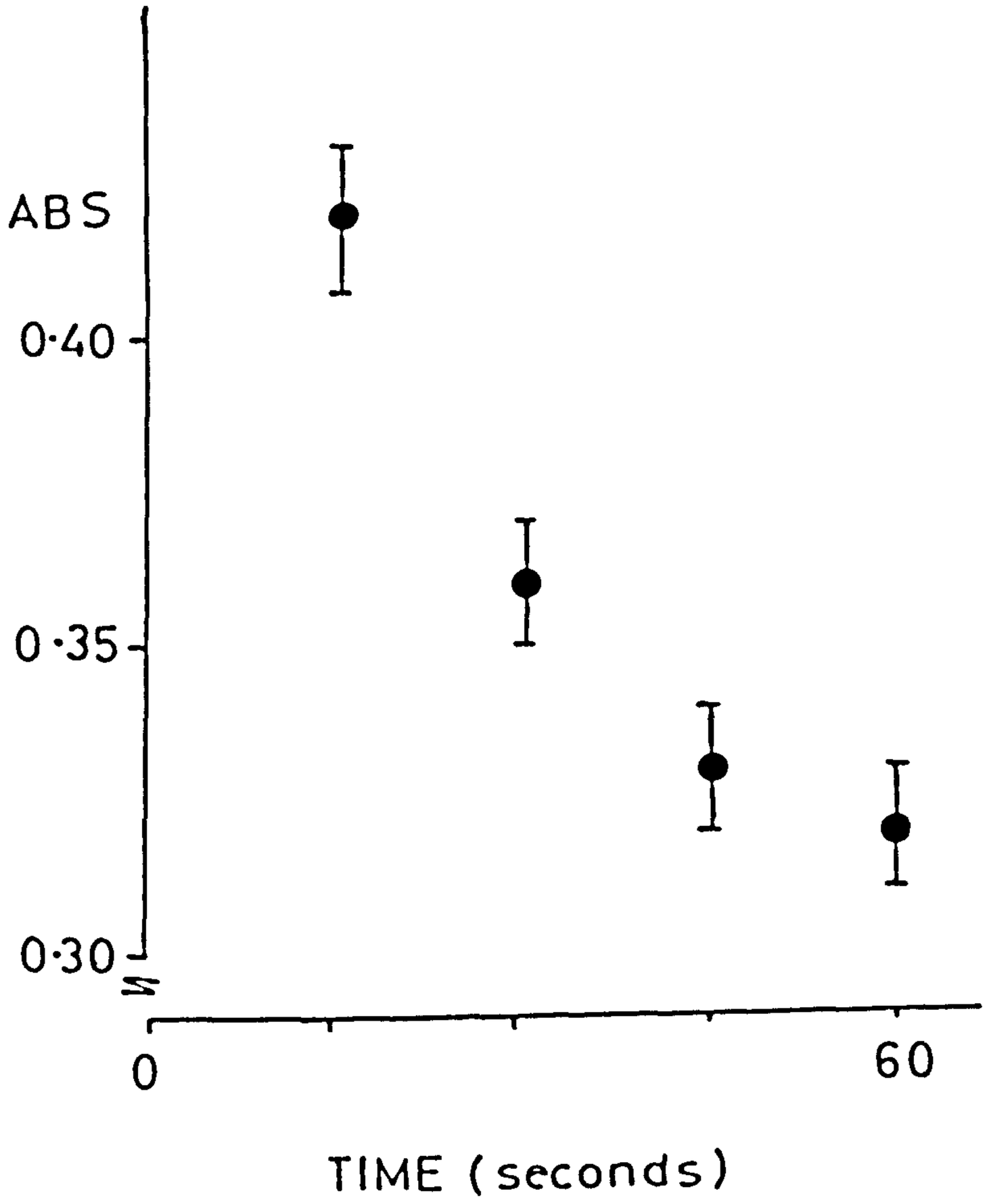
Temperature does affect the rate of decay of the blue complex, but not markedly over the room temperature range. At 5^{o}C complex formation is not instantaneous and takes longer to decay, whilst at 40^{o}C blue colour formation is instantaneous and its decay rate is also increased. Here the temperature factor has not been fully investigated as tannic acid absorbances during the experiments remained constant and thus acted as a control. Indeed whilst accuracy would be improved by developing the assay for use in a cold room (e.g. to

Figure 2.6 Visible spectra for "hydrolysed" and "unhydrolysed" quebracho tannin and gallic acid in the presence of ferric chloride.



The curves show absorbance (ABS) as a function of wavelength for unhydrolysed (curve a) and hydrolysed (curve b) quebracho, both present at the same concentration. Curves c and d are for gallic acid before and after hydrolysis.

Figure 2.7 Change in absorbance (ABS) at 660 nm with time, for tannic acid in the presence of ferric chloride.



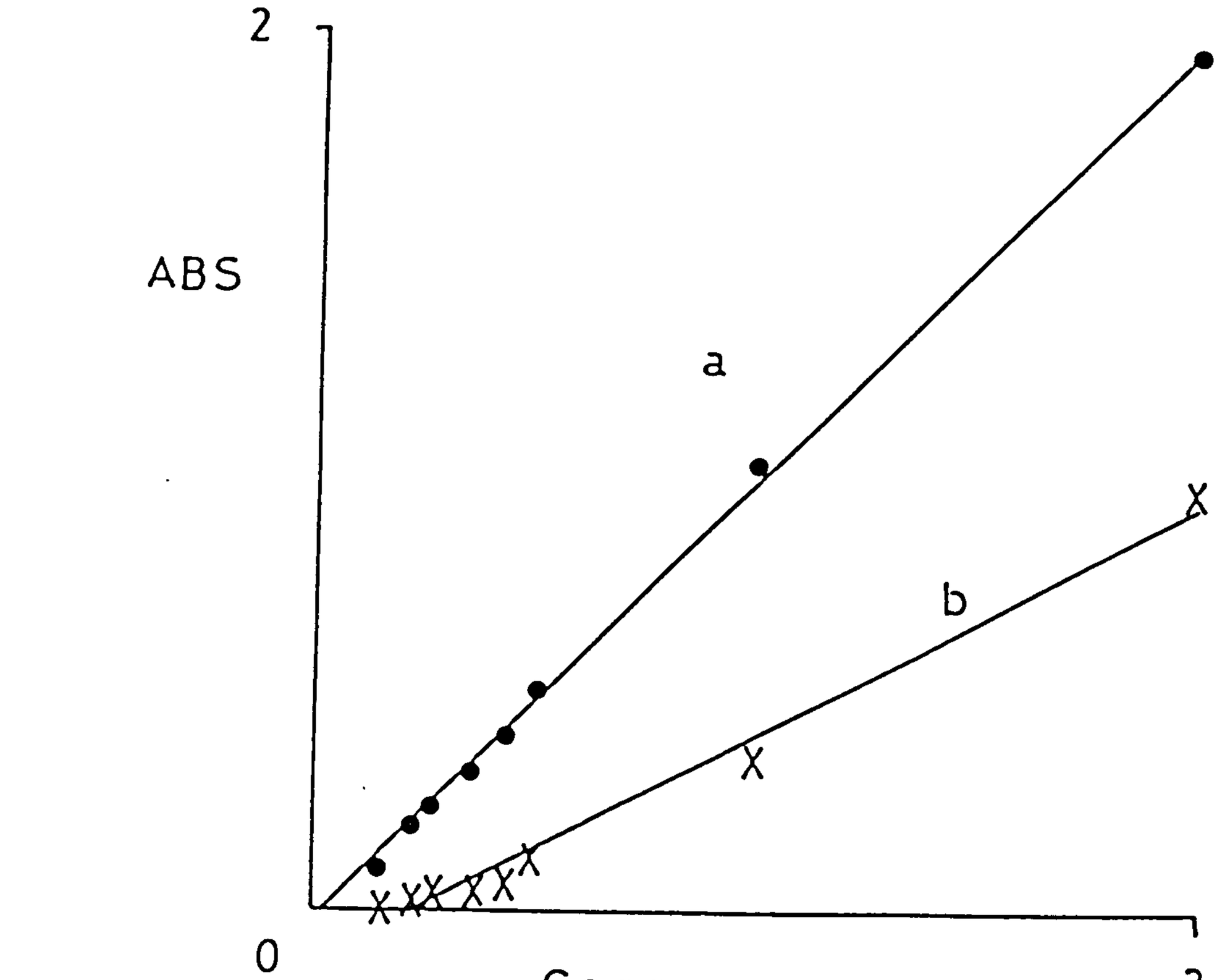
The absorbance change was recorded by the addition of tannic acid (200μl, 1mg/ml) to 3 ml of ferric chloride at time zero. Error bars indicate the range of values obtained in six replicate experiments.

catch the maximum absorbance change at 660nm), reference to a tannic acid standard is suggested as the best way to retain its simplicity.

The hydrolysis of tannic acid was attempted in both acid and alkaline conditions. The method adopted was to use an alkaline hydrolysis (see Appendix 1.8), which produced slightly more intense spots of gallic acid on a TLC of the hydrolysate than the reverse treatment employing the acid first. The hydrolysate was used directly in the re-assay with ferric chloride. No blue complex was seen in the post hydrolysis assays of tannic acid after this treatment.

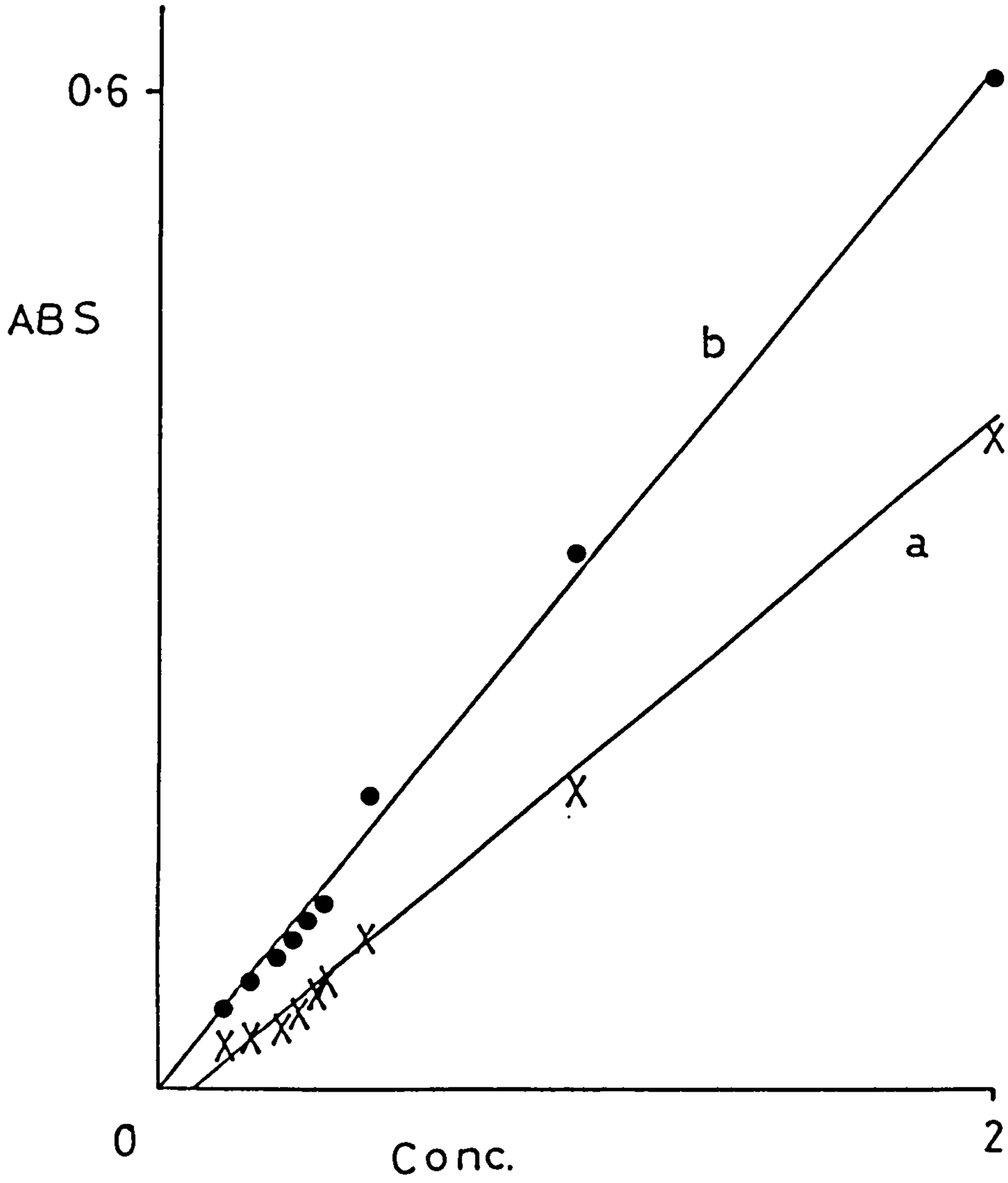
A full description of the method employed in the assay, which draws together the points made above, is given in Appendix 1.8, Figures 2.8 and 2.9 present standard curves for the absorbances produced for tannic acid and quebracho when assayed before and after hydrolysis. The important point is that they all represent linear functions (except at very low absorbances), thus the functions describing the pre-post hydrolysis absorbance for each tannin are also linear. The linear regression equations for the absorbance (y) produced for given concentrations (x) of tannin added in the assay are given below. The percentage R^2 is not a statistical test but does express the proportion of the total sum of squares in the regression ANOVA due to the

Figure 2.8 Standard curves for tannic acid in the assay for hydrolysable tannins.



Curve a is for unhydrolysed tannic acid, curve b for hydrolysed tannic acid. Concentration (Conc.) is measured in mg/ml of tannin in the 500μl sample assayed.

Figure 2.9 Standard curves for quebracho tannin in the assay for hydrolysable tannins.



Curve a is for unhydrolysed quebracho, curve b for hydrolysed quebracho. Note that the positions of curves a and b are reversed compared to Figure 2.8 and that all the absorbance values are lower. Concentration (Conc.) is measured in mg/ml of tannin in the 500μl sample assayed.

regression as opposed to the residual error. This result has been corrected for the appropriate number of degrees of freedom and the calculations follow Ryan et al. (1980)

Tannic acid, pre-hydrolysis

(1) $y = -0.213 + 1.00\ x \quad R^2 = 99.8\%$

Tannic acid post-hydrolysis

(2) $y = -0.116 + 1.51\ x \quad R^2 = 98.2\%$

Tannic acid, pre-post hydrolysis

(3) $y = 0.095 + 0.49\ x \quad R^2 = 96.4\%$

Quebracho, pre-hydrolysis

(4) $y = -0.0156 + 0.201\ x \quad R^2 = 99.7\%$

Quebracho, post-hydrolysis

(5) $y = 0.0028 + 0.308\ x \quad R^2 = 99.3\%$

Quebracho, pre-post hydrolysis

(6) $y = -0.0184 - 0.107\ x \quad R^2 = 91.3\%$

Whilst condensed tannins will interfere in the assay of hydrolysable tannins, on the above evidence, their contribution may be corrected for by the use of the proanthocyanidin assay results. The error in the equations 3 and 6 is higher than those of 1, 2, 4 and 5 (see R^2 values) butsimple arithmetic shows the equations that would be predicted by subtraction (e.g. eqns. 1-2

and eqns 4-5) are identical to those produced from the raw data.

2.5,3 Use of the Assay to Detect a Hydrolysable Tannin in the Presence of a Condensed Tannin.

The demonstrations of linear relationships between absorbance and tannin concentration in the assay, as outlined above, still leave the possibility that competitive or synergistic interactions between different tannins in complex formation may render the use of the method invalid. A series of measurements were made for tannin solutions ranging from 2mg/ml to 0.12 mg/ml in total tannin concentration. At each concentration absorbances in the assay were recorded for solutions containing 0% to 100% tannic acid, the balance to 100%, being made up by quebracho tannin. Regression equations derived from experimentally measured pre-post hydrolysis absorbance changes are given bellow. The variables x and y are as before, z is the percentage of tannic acid in the mixture of tannic acid and quebracho being tested.

x = 2mg/ml

(7) $y = -0.23 + 0.13\ z \quad R^2 = 98.9\%$

x = 1mg/ml

(8) $y = -0.17 + 0.0093\ z \quad R^2 = 99.6\%$

x = 0.5mg/ml

(9) $y = -0.084 + 0.0049\ z \quad R^2 = 98.9\%$

x = 0.44mg/ml

(10) $y = -0.091 + 0.0040\ z \quad R^2 = 98.2\%$

x = 0.33mg/ml

(11) $y = -0.072 + 0.0031\ z \quad R^2 = 97.8\%$

x = 0.23mg/ml

(12) $y = -0.0046 + 0.002\ z \quad R^2 = 97.6\%$

x = 0.12mg/ml

(13) $y = -0.017 + 0.0008\ z \quad R^2 = 89.2\%$

These results again showed linear behaviour, but the evidence of the R^2 statistic indicates that the error involved becomes increaseingly high at low tannin concentrations (x) and to keep the ANOVA residual error to less than 2.5%, systems with total concentrations of over 0.2mg/ml need to be used.

Linear behavior per se does not rule out deviation from results expected from the equations (3) and (6). The expected behaviour being the absorbance change in the assay given by the algebraic sum of the changes predicted from the individual functions for tannic acid and quebracho i.e. equations (3) and (6). Using these equations, where z = 100% and 0% respectively, equations in z for the constant terms were derived by substituting

values into a linear equation of the form y = a + bx,, at x = 1.0mg/ml. The resulting equations for the constants a and b were as follows:

(14) a = 0.018 + 0.0077 z

(15) b = -0.107 + 0.0060 z

By using these equations and the values of x and z used in the experiments generating equations (7) to (13), a set of "expected" equations was obtained.

From the sets of expected and experimental equations of the form y = c + d z, regression equations for c and d in x were calculated. These are given below.

Expected

(16) c = 0.0148 - 0.105 x

(17) d = 0.00076 + 0.0060 x

Experimental

(18) c = -0.02 - 0.11 x R2 = 92.7%

(19) d = 0.0010 + 0.0064 R2 = 94.3%

Considering the equation y = a + bx, when z = 0 this will be given by equations (16) and (18) which should be equal to equation (6). When z = 100% y should be given by the sums eqn.(16) + [100 * eqn.(17)] and eqn.(18) + [100*eqn.(19)]. These equations in x, which should equal

equation (3), are given below.

Experimental $y = 0.100 + 0.53\ x$

Expected $y = 0.091 + 0.49\ x$

The experimental match to the standard curve for quebracho is very close whilst the expected match to the tannic acid curve is very close. The converse comparisons are less good and the influence of quebracho on the assay of tannic acid cannot be ignored. Nevertheless as a semi-quantitative approach to detect and rank the contents of hydrolysable tannins in plant extracts, this seems to be a workable technique. The real test of its value in the ecological context will be if it can successfully predict high astringency in condensed tannin-deficient plant extracts.

In considering the possibility of chemical interference from other plant constituents, it is evident that these would be undetected only if they gave a blue complex in the assay that was labile to hydrolysis. The author is not aware of such a substance. However there are plant phenolics whose interference is likely but detectable. Salicylic acid does give a strong absorbance in the assay, but this is not labile to hydrolysis. Its glycoside, salicylin, is likely to produce interferance, but after hydrolysis, due to the release of salicylate. The major problem is likely to be phenolics which are not

detected as condensed tannins but which do interfere in the same way as condensed tannins, making the detection of small amounts of hydrolysable tannin particularly difficult.

2.5,4 Analysis of the Extracts for Hydrolysable Tannins

The values for the change in A_1^1 at 660 nm consequent upon the hydrolysis procedure are given in Table 2.6. Whilst insuficient sample was available for Pinus radiata tannin (Pr) to be used in place of quebracho in the experimentation reported above, this has been used as the standard upon which to estimate the bias due to condensed tannin in the assays (because it has the highest A_1^1 for condensed tannins; see Table 2.2). Using the standard curves presented in Figure 2.10. and the data for the proanthocyanidin assay (Table 2.2), estimates of the bias due to condensed tannin in the assay have been calculated as follows:

$$\text{bias} = \left[\frac{A_1^1 \text{ of sample (CT)}}{A_1^1 \text{ of Pr (CT)}}\right] * A_1^1 \text{ of Pr in HT assay)}$$

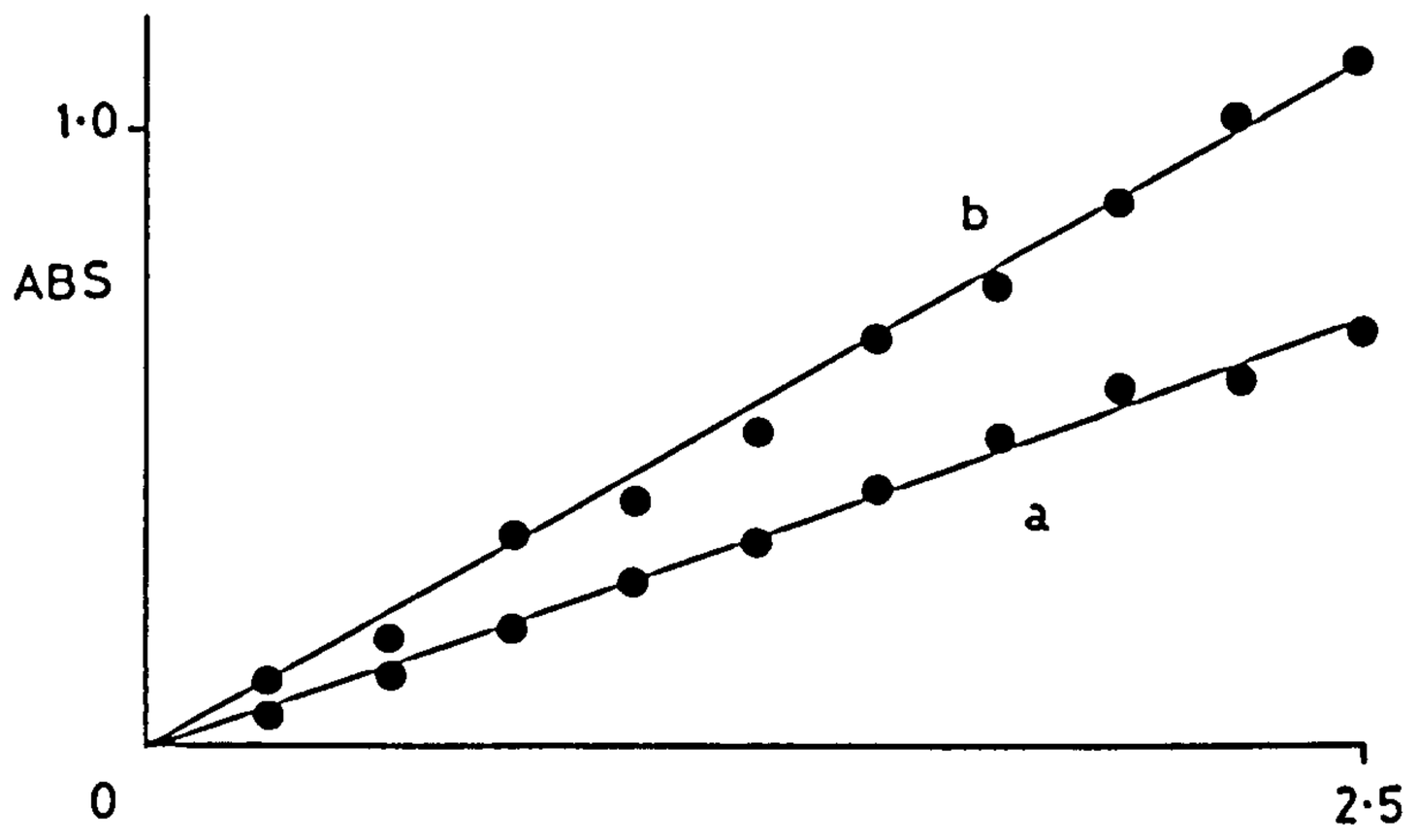
The subtraction of this source of bias still leaves negative values for most of the extracts, although De changes from negative to positive (Table 2.6) and thus becomes identified as a source of hydrolysable tannins. The gaps in Table 2.6 (for B1, B2, Tb) are for extracts

Table 2.6 Analysis for Hydrolysable Tannins*

Tannin	Code	A_1^1	HT
Aesculus hippocastanum	Ah		
Calistemon citrinus	Cc	-6.3	-5.9
Diospyros ebenum	De	-0.18	+0.55
Loropetalum chinense	Lc	-8.4	-8.0
Myrtus obcordata	Mo	-8.1	-7.7
Pomaderris phylloides	Pp	-16.1	-12.0
Pteridium aquilinum	B1		
Pteridium aquilinum	B2		
Pteridium aquilinum	B3	-1.3	-0.84
Pteridium aquilinum	B4	-2.9	-0.49
Quercus sp.	Qs	-6.6	-5.1
Taxus bacata	Tb		
Vaccinium myrtilis	Vm	-2.1	-0.017
Tannic Acid	TA	+37	+37
Quebracho tannin	Qb	-7.9	-3.7
Terminalia chebula	Tc	+2.8	+2.8
Pinus radiata	Pr	-6.0	0.0
Catechin	Ca	-0.98	0.15
Gallic Acid	GA	0.0	0.0

* Notes: A_1^1 is the difference in A_1^1 value between pre-hydrolysis and post-hydrolysis assays; HT, positive values indicate hydrolysable tannins, these being the A_1^1 values corrected for the influence of condensed tannins

Figure 2.10 Standard curves for Pinus radiata tannin (Pr) in the assay for hydrolysable tannins.



Curve a is for unhydrolysed Pr, curve b for hydrolysed Pr. Note that the positions of curves a and b are as expected from Figure 2.9. Concentration (Conc.) is measured in mg/ml of tannin in the 500μl sample assayed.

containing insuficient phenolics for absorption readings to be taken in the assay.

De was not the only extract expected to contain hydrolysable tannins on the basis of its colour reaction with ferric chloride, although it was the only extract to give the same colour reaction as tannic acid with iodate. The other extracts giving positive reactions with ferric chloride, presumably have insufficient hydrolysable tannin (if any) to overcome masking by other phenolics in either the iodate test or the new assay (NB the iodate reaction of Tc). Considering the ratio of total phenolics (FD) to the corrected A_1^1 in the hydrolysable tannin assay, the extract Tc appears to contain only 24% hydrolysable tannin in its total phenolics, relative to tannic acid (defined to contain 100%). De contains 7% using the above means of estimation. Considering its low polymer length estimate, it could be argued that the vanillin assay should be used to replace the proanthocyanidin assay in the above calculations. Accepting this raises the estimate to 43% hydrolysable tannins in the total phenolics of De. The use of either the vanillin assay or the proanthocyanidin assay in these estimates, erroneously shows catechin to contain some hydrolysable tannin.

2.6 Summary of the Extracts' Chemical Characteristics: Evidence from Paper Chromatography

On the basis of information from the chemical analyses, this concluding section groups the extracts according to their chemical characteristics. As both the components of condensed and hydrolysable tannins are detectable in chromatograms of their hydrolysis products (Choo 1981), chromatographic evidence is used here as a partial check on previous conclusions. With the exception that paper, rather than cellulose TLC plates, has been used as the stationary phase, the methods employed have followed Choo (1981). Results are given in Table 2.7, methods in appendix 1.9.

2.6,1 Extracts Containing Condensed Tannins Only.

The extracts falling into this category (Pr, Qb, Pp, B1, B2, B3, B4, Tb and Vm) do so because they give negative reactions in both the iodate and ferric chloride tests and show no trace of gallic or ellagic acid in chromatographic analyses. The one questionable reaction is the iodate test of Tb, but hydrolysable tannins would not be expected here on taxonomic grounds. All the above extracts do of course give positive assays for condensed tannins and give chromatograpic traces of at least one anthocyanidin. Indeed in each case cyanidin is

Table 2.7 Results of Chromatographic analyses‡.

Tannin	Code	Cy	Del	Fl	GA_h	GA_f
Aesculus hippocastanum	Ah	++		1	*	
Calistemon citrinus	Cc	++		1	*	
Diospyros ebenum	De	*	++	1	++	
Loropetalum chinense	Lc	++			++	
Myrtus obcordata	Mo	++	*	1	*	
Pomaderris phylloides	Pp	++	+	1		
Pteridium aquilinum	B1	++		1		
Pteridium aquilinum	B2	++		1		
Pteridium aquilinum	B3	++	*	1		
Pteridium aquilinum	B4	++	*	1		
Quercus sp.	Qs	++	+	2	*	
Taxus bacata	Tb	++	*	1		
Vaccinium myrtilis	Vm	++		1		
Tannic Acid	TA				++	++
Quebracho tannin	Qb	++		2		
Terminalia chebula	Tc				++	++
Pinus radiata	Pr	++	+			
Catechin	Ca					
Gallic Acid	GA				++	++

‡Notes: ++ Clear occurance; + Minor anthocyanin where both occur, * trace occurance, 1,2... number of flavanoids.

Cy, Cyanidin; Del, Delphinidin; Fl, other flavonoids

GA, Gallic Acid; subscripts: h, in hydrolysate; f, free.

essentially the only, or at least the major, (see Pp & Pr) pigment produced.

Whilst condensed tannins may be the only tannins present there is evidence for other phenolics. Although often used as a condensed tannin standard (Swain and Hillis, 1959; Gartlan et al., 1980) quebracho has a notably low proportion of condensed tannin in it's total phenolics (see %CT1 & %CT2, Table 2.3). Paper chromatography reveals two distinct and large "flavonoid" spots (Rf 0.71 & 0.52) on the criteria of Choo (1981), as well as much other material streaked between the origin and solvent front. The extracts Vm, B1, B2, B3 and B4 also have low values for %CT1 and %CT2. Chromatograms of these all show one "flavonoid". In contrast, extracts Pr, Pp and Tb should only contain condensed tannins in their phenolics. Nevertheless Tb (only) gives rise to a single flavonoid spot.

2.6,2 Extracts Likely to Contain Small Amounts of Hydrolysable Tannins.

These extracts (Ah, Cc, Lc, Mo and Qs) satisfy the positive criteria for containing condensed tannin used above; they do not, however, satisfy all the criteria used to exclude the possibility of their containing hydrolysable tannin. All give at least a partially blue colour with ferric chloride, except Ah which does,

however, give a red-brown iodate reaction. These extracts also all contain at least trace amounts of gallic acid in chromatograms of the hydrolysates whilst this was not present before hydrolysis. None give positive reactions in the hydrolysable tannin assay proposed above yet all but Ah can be expected to contain a large proportion of non-condensed tannin phenolics on the basis of the %CT data.

Considering all the available information, none of the data concerning Qs, Cc and Ah is strongly positive and thus the influence of hydrolysable tannins on the properties of these extracts, if present, should be negligible. Published information supports this as only trace amounts of hydrolysable tannins have been found in Ah (Bate-Smith 1977) and when present in Qs, these are minimal late in the year, when the sample used here was collected (see Table 2.1 and Feeny and Bostock, 1968).

The position is less certain for Mo and Lc. For Mo, the extracted total phenolics are low in condensed tannins and the ferric chloride reaction is clearly positive. However, the negative evidence of the iodate test and the hydrolysable tannin assay could indicate the presence of a non-tannin phenolic giving a blue ferric chloride reaction. For Lc, where a positive iodate reaction is present, and the extract is likely to contain hydrolysable tannins on taxonomic grounds, the novel

assay proposed is either insensitive or at fault.

2.6,3 Hydrolysable Tannin-Containing Extracts

Of the three remaining extracts (TA, Tc and De), TA is expected to be a pure hydrolysable tannin, although the detection of gallic acid in chromatograms of unhydrolysed TA suggests some impurity. Tc contains no condensed tannin, and does not give a positive iodate reaction, but does give a positive ferric chloride test and hydrolysable tannin assay. In the light of this and the expectation of hydrolysable tannins on taxonomic grounds, it is regarded as a hydrolysable tannin-containing extract here. In spite of this the assay (HT) indicates that tannins are a minor component of the phenolics of Tc. De gives positive reactions with all the tests used to determine hydrolysable tannin content, it also contains condensed tannins and the chromatographic evidence is that it is the only extract where prodelphinidins predominate in the condensed tannins.

2.7 Summary

It will be clear that the available techniques for the preparation and chemical characterisation of tannins all have drawbacks. Notably, the means to quantify one major type of tannin, hydrolysable tannin, cannot be used with confidence. In selecting plant taxa for this study, selection was exercised to maintain a high taxonomic diversity whilst largely avoiding hydrolysable tannins. The positive and negative evidence sumarised in Section 2.6 indicates that this has been largely achieved. Most of the extracts thus contain predominantly procyanidin-type tannins, and variation between extracts is largely due to polymer length differences and differences in the associated non-tannin phenolics.

Chapter Three

Tannin-Protein Complex Formation

3.1 Introduction to Factors Affecting Tannin-Protein Complex Formation

As a prelude to examining the interaction of proteins with the extracts described in Chapter 2, some exploratory investigations of tannin-protein reactions were undertaken, particularly those leading to precipitate formation. Whilst there is a substantial literature on this subject, some important results are unconfirmed or the subject of conflicting reports. In contrast to the discussion of section 1.1,3, the following is less concerned with a theortical appreciation of the nature of tannin-protein interactions, and more to do with the empirical description of the factors influencing them. This is necessary both to develop assays for tannins dependent upon their protein complexing properties and to assess the worth of experiments performed in vitro as predictors of the in vivo situation.

3.1,1 Ionic Strength and Buffer Salts.

The ionic strength of a solution is generaly denoted as μ and defined by the equation below where m = molality and z = valence.

$$\mu = 1/2 \sum mz^2$$

This factor is thought to influence tannin-protein precipitation reactions (Martin et al., 1983), however authors have almost without exception expressed their data in terms of the molarities of named salts, not explicitly in terms of ionic strengths. It is also the case that much less work has been carried out in this area of research compared to that into pH and reactant concentration.

Calderon et al. (1968) carried out tannin-protein reactions in unbuffered systems, to which the addition of 1.0 mM salt (NaCl) introduced observable effects. Whilst not greatly affecting the quantities of precipitate (of tannin and gelatin) produced by low levels of tannin, more tannic acid and less quebracho was precipitated in the presence of salt. Hagerman and Butler (1978) found that ionic strength had no effect on the ratio of precipitated tannin and protein ($R(_{p}$), defined in Table 3.2) where reactions were carried out in solutions ranging from 5M to 50mM NaCl in strength, a range extended down to 10mM for bovine serum albumin precipitation by Oh et al. (1980). Goldstein and Swain (1965) reported 10mM phosphate (i.e. higher μ) to be a threshold below which tannin induced enzyme inhibition was decreased. By contrast to BSA, Oh et al. (1980) found the turbidity of tannin-gelatin and tannin-polyproline systems to increase as ionic strengh

rose from 10mM to 1M. In summary, it does seem that at low ionic strengths this variable may be the controlling factor that determines the occurrence of precipitation. With dilute buffers the nature of the buffer salt and its concentration may then become critically important (Schultz et al., 1981).

The choice of buffer salts for experimenents with tannins presents problems beyond those indicated above as tannins may react directly with many routinely used buffers. For instance precipitates were seen to form between tannins and phthalate buffers. In the work presented here buffers have generally been made using inorganic salts at concentrations not greatly in excess of those required to ensure maximal precipitation reactions (see below).

There is a general lack of information about the in vivo concentrations of inorganic solutes in the digesta. However one exception to this is provided for insects by (Martin et al. 1985). Here it would seem that ionic strengths in vivo are above the thresholds so far identified as needed to ensure maximal precipitation.

3.1,2 pH and pI

The work of Feeny (see sect 1.2,2) relied on the study of Loomis and Battaile (1966) for its description of pH effects upon precipitation reactions. This study

concluded that hydrolysable and condensed tannins could be distinguished by the effect of pH upon their ability to precipitate protein. Condensed tannins precipitated protein to a constant degree at pHs less than 7, in contrast to hydrolysable tannins which had a maximum effect at pH 3 to 4 and no effect at pH> 5. However, Calderon et al. (1968) reported that protein precipitation was maximal at the isoelectric pH (pI) of gelatin in its reaction with both types of tannin. With the pI of gelatin at 4.8, in these experiments, the idea that protein precipitation would not occur at pH> 8 was not investigated and remained unchallenged for several years, during which time many of the basic hypotheses on the ecology of tannins were introduced (see Section 1.2, 1.3) This was unfortunate as the pI effect is of critical importance to the apparently adaptive role of high midgut pH in winter moth caterpillars (Feeny, 1970; Berenbaum, 1983). Hagerman and Butler (1978) reconfirmed the pI effect on tannin-protein precipitation for a range of five proteins including trypsin (pI = 10.1) and lysozyme (pI = 11.0). The present author has also observed the precipitation of both these enzymes at pH values between 8 and 10, i.e. in the range found in the midgut of several lepidopteran larvae (Berenbaum, 1980). Given that the trypsin-like proteases found in insects (House, 1974) have comparable isoelectric points to

mammalian trypsin, tannins could directly inhibit these digestive enzymes even if they could not indirectly inhibit them by reaction with their substrates, which might have more acid pI values and not bind tannins under these conditions.

Malamud and Drysdale (1978) have tabulated the isoelectric points of a wide range of proteins and Table 3.1 lists a number of these for some plant proteins and animal digestive enzymes. Almost all the plant proteins tabulated have acidic pI values, with ribulose bis phosphate carboxylase (RBPC), quantitatively the most important, having a pI of 4.9. The influence of pH on tannin induced precipitation of this particular protein was investigated by the preparation of a small amount of the protein by the method of Siegel and Lane (1975) and measuring the turbidity induced by adding tannic acid to it in solutions buffered to a range of pHs (recipies detailed in appendix 2.1). The results showed maximal turbidity at pH >3 and pH < 7.5, less at pH = 7.5 and none at pH > 7.5. These findings, for freshly prepared material, confirm those of Martin and Martin (1983). The conclusion that may be drawn from this evidence is that the typical gut pHs of mammals and many insect midguts (pH5 to pH7.5) would not be expected to prevent the formation of insoluble tannin-RBPC complexes. The importance of this result is that RBPC may account for up

Table 3.1 Isoelectric points of some selected proteins

I Digestive enzymes	pI
α-Amylase (human saliva)	6.2 - 6.5
α-Chymotrypsin (cow pancreas)	8.8
β-Galactosidase (pig intestine)	4.1
Lipase (pig)	4.0
Pepsin (pig)	2.2
II Other animal proteins	
Bovine serum albumin	4.7 - 4.9
III Plant proteins	
Ribulose bis phosphate carboxylase	4.9
Phosphoenolpyruvate carboxylase	4.9
Ribose phosphate isomerase	4.8
Carboxy peptidase C	4.5
Catalase	5.8
β-Galactosidases	8.4 - 6.1
Lipoxidase	5.7
Leghaemoglobin	5.1
Phosphodiesterase I	3.9

Note: These values have been extracted from the tabulation provided by Malamud and Drysdale (1978).

to 50% of a leaf's protein (Martin and Martin, 1984), and so this protein is thus particularly significant in the diets of folivores.

The arguments made for the midgut pH values of larval lepidopteran tree feeders (presumed particularly prone to tannin consumption) being adaptively high (Berenbaum, 1980) appear insupportable as their mean was 8.67 (range 7.6 to 9.5) versus 8.29 (range 7.0 to 9.6) in herb feeding phytophagous larval lepidopterans, a difference too small to have an impact on complex stability. Nevertheless the very high pHs reported (Berenbaum, 1980) could be adaptive and prevent in vivo protein precipitation by tannins. However, these high pHs require a special physiological mechanism to support them (Dow, 1984), thus there will also be an associated cost in terms of metabolic energy.

3.1,3 Reactant Concentration

This section is concerned with the effects of both absolute and relative tannin and protein concentrations on the amounts and stoichiometry of tannin-protein precipitation. The effects of concentration on precipitation thresholds and precipitate dissolution (section 1.1,3) are well established, yet the work of Van Buren and Robinson (1969) forms the only detailed information on systems where precipitates are continually

present. The following discussion concerns systems where tannins and proteins are present in a measured ratio (tannin/protein) designated R(t). Following their precipitation the ratios R(s) and R(p) identify the tannin to protein ratios in the supernatant and precipitate respectively. It is also be helpful to denote the ratio of supernatant/precipitate tannin as T. These abbreviations will be used frequently in the ensuing discussion and are set out again in Table 3.2 which is intended as a quick reference guide.

The initial work of Calderon et al. (1968) on pH and gelatine precipitation led to their modelling the reaction by the equation

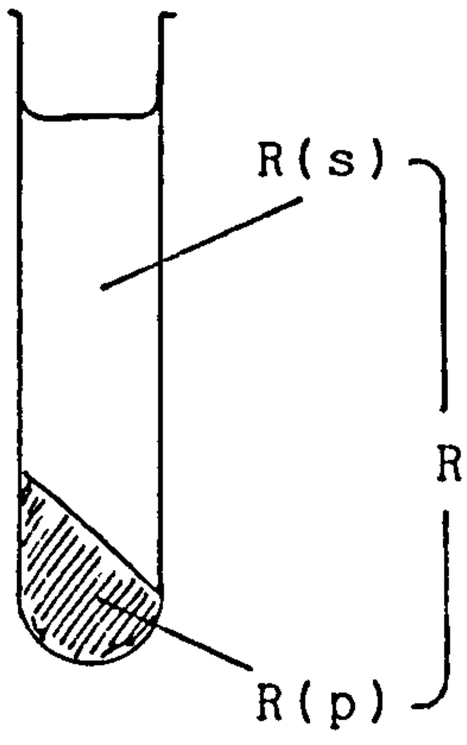
$$\text{Gelatin} + \text{tannin} \longleftrightarrow (\text{gelatin-[M.tannin]})n$$

They expected R(p) to be closely correlated to M, and also for M and n to be positively related to R(t) and each other, up to a point where excess tannin interfered with the crosslinking between protein molecules and initially formed soluble tannin-protein complexes. Van Buren and Robinson (1969) found that in a gelatin-tannic acid system where precipitation increased with R(t), at low absolute reactant concentrations, R(p) tended to exceed R(t). The opposite relationship between R(p) and R(t) occurred at high R(t) where total gelatin precipitation tended to occur thus giving high T values.

Table 3.2 Definitions and abreviations introduced in Chapter 3.

I Tannin-protein ratios

Consider a system containing tannin and protein such as that illustrated below.



Centrifuge tube containing tannin and protein in the form of a precipitate and supernatant solution.

R(t) Ratio of tannin/protein in the whole system (w/w).

T Ratio of tannin in the supernatant to that in the precipitate (w/w).

P Ratio of protein in the supernatant to that in the precipitate (w/w).

R(s) Ratio of tannin/protein in the supernatant only (w/w).

R(p) Ratio of tannin/protein in the precipitate only (w/w).

II Other abreviations

pI The isoelectric point of a protein (pH units).

M,n Numerical constants in equations (no units).

Whilst these results point to there being a limit to the range of R(p) values obtained in precipitating systems their work showed that precipitates did not have fixed stoichiometries. Further evidence for this was obtained by their confirmation of distinct precipitation maxima for tannic acid and gelatin in systems with constant concentrations of tannic acid but varied R(t), i.e. confirming the limit to the relation of R(t) and R(p) outlined above. However, their results for precipitation maxima were contradicted by Hagerman and Butler (1978) who reported a rise to a plateau as R(t) falls from initially high values in a system containing bovine serum albumin as the protein.

Van Buren and Robinson (1969) further examined the existence of soluble complex formation. They found 100% gelatin precipitation attainable, but could under no circumstances produce 100% tannin precipitation with gelatin, R(s) never approaching zero despite the use of a purified tannin. Indeed, for data from experiments at constant T, R(S) was found to be inversely related to gelatin concentration but directly proportional to the absolute concentration of the system, indicating that precipitation is closely related to soluble complex concentration.

The foregoing discussion of concentration effects has been made with the notable absence of any information

input from physiological or ecological studies. In marked contrast to pH, essentially nothing of direct use is known concerning the relative and absolute concentrations of tannins and proteins in the digesta of herbivores. Studies in the medical and veterinary literature do detail the composition and volumes of gastrointestinal secretions but constant recycling and reabsorption makes extrapolation to the digesta difficult. A more limited but achievable goal is to examine plant material and at least measure the relative amounts of tannin and protein entering the digesta. Such information is available for a wide range of species (see Section 5.2 and Coley, 1983; Gartlan et al. 1980) and it would seem that plants can contain more than sufficient tannin to precipitate their entire compliment of protein (Table 5.1) and others may have sufficient tannin to precipitate their protein several times over (Barry and Forss, 1983; Martin and Martin, 1983). The dilution of ingested plant material with saliva and other secretions may lead to the digesta becoming relatively fluid or remaining a semi solid paste. Experiments performed in test tubes run the risk of being over-dilute and uncontrolled for the effects of adsorbtion onto plant structural components present in the diet.

3.2 Salt Concentration Effects on Tannin-Protein Precipitation

Experiments were first undertaken to ascertain the existence of threshold precipitation effects and to establish the minimum salt concentrations required to ensure the precipitaton of proteins by tannins. This was to allow the elimination of salt concentration as a variable in subsequent experiments which deal with pH and concentration effects (Section 3.3). To this end tannin and protein concentrations in the experiments reported here were kept lower than those used subsequently.

3.2,1 Methods

Stock solutions of protein (5.0 mg/ml), either pepsin (Sigma) or α-amylase (Sigma) and of tannin, either tannic acid (2.5 mg/ml) or Quebracho (5.0 mg/ml) were made as unbuffered solutions in distilled water. Prior to the experiments two series of salt solutions (of either NaCl or $MgSO_4$) were prepared at concentrations of 0, 0.08, 0.16, 0.24, 0.32, 0.40, 0.80, 1.2, 1.6 and 2.0 molar of salt. Precipitates were then formed in centrifuge tubes containing 1ml of a salt solution, 1ml of protein solution and 1ml of tannin. Haze formation was seen in most tubes as tannin was added. The tubes were allowed

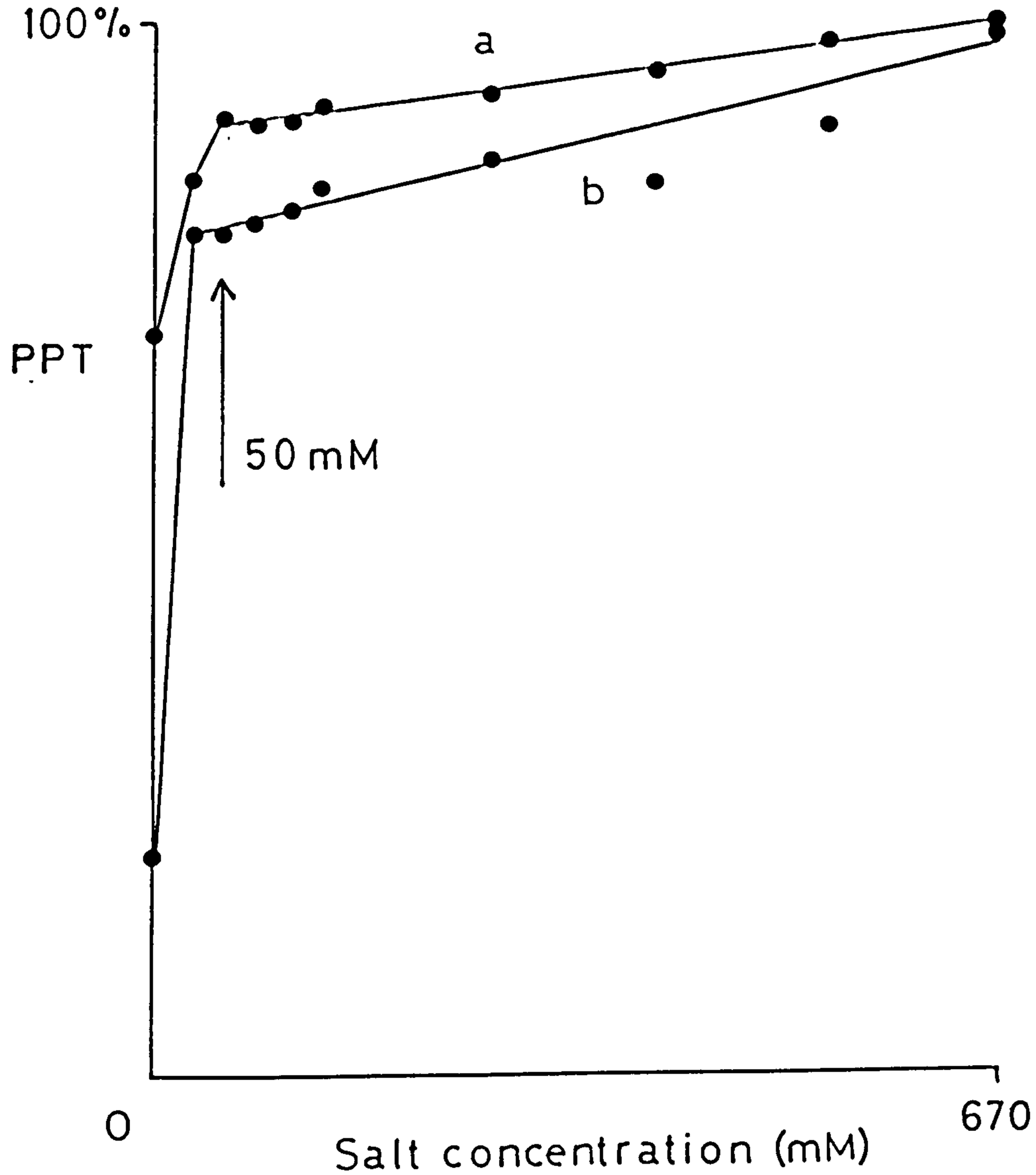
to stand 15 minutes before being centrifuged at the top speed of an MSE bench centrifuge for five minutes.

After centrifugation the supernatants were discarded and 4ml of SDS/triethanolamine reagent (see Appendix 1.4) were added to each tube. This procedure follows that of Hagerman and Butler (1978) for redissolving tannin-protein precipitates after their collection as a pellet by centrifugation. An aliquot of this solution was then assayed for phenolics by the procedure described in Appendix 1.4, with the modification that 1ml of sample (in SDS/triethanolamine reagent) was added to 1ml blank reagent and 1ml water to maintain the normal reagent concentrations used in the assay.

3.2,2 Results

No difficulties were found with redissolving tannin-protein precipitates by this method where the systems contained NaCl and either of the tannin and protein test materials. Expressing the total phenolics precipitated as a percentage of the maximum value (Figure 3.1), indicates a threshold concentration above which precipitation is nearly complete, as anticipated from the results discussed in Section 3.1,3. This threshold would appear to be about 50mM NaCl for the results presented for pepsin and this was also found to hold true in an identical experiment using α-amylase as the protein.

Figure 3.1 The influence of salt concentration on pepsin precipitation by tannins.



Precipitation of pepsin, expressed as a percentage of the maximum precipitated (PPT) is plotted against salt concentration for a system where the salt is NaCl. The upper curve (a) is for precipitation by tannic acid whilst the lower (b) is for precipitation by quebracho tannin.

The SDS/triethanolamine reagent did not redissolve those precipitates generated in systems where $MgSO_4$ was present. This suggests that this salt takes part in the tannin-protein precipitation reaction rendering it irreversible. This confirms the need for care in selecting buffer salts for work with tannins and proteins. The experiments were repeated to the point prior to centrifugation, but with the reaction mixtures being assembled in cuvettes, and a simple turbidimetric assay was made employing a spectrophotometer set at 900nm. This procedure was acceptable for tannic acid as precipitates did not settle during the measurements. No decrease in turbidity was detected at low salt concentrations although solutions were less cloudy in the absence of any salt. This seemingly higher threshold level is reasonable as the ionic strength of $MgSO_4$ is higher than NaCl owing to its divalent ions and also because it is apparently directly involved in the tannin-protein complex.

3.2,3 Conclusions

These simple experiments suggest two important conclusions. Firstly, that experiments on tannin-protein precipitation reactions need to be carried out in salt solutions which are of an ionic strength equivalent to at least 50 mM NaCl and secondly, that any salts

incorporated into the experiments (e.g. in buffers) must not interfere in tannin-protein interactions so as to make the results "salt specific". The only practical guard against this in terms of the work reported here was to screen each buffer used to ensure that tannins and proteins were soluble in it individually and that their precipitates were soluble in SDS/triethanolamine reagent. As a result of this inorganic buffers have been used throughout most of this work.

3.3 Tannin, Protein, pH and Concentration Effects.

These experiments were designed to investigate the effects of the factors listed above on simple tannin-protein precipitation experiments. As in Section 3.2 tannic acid, quebracho tannin, pepsin and α-amylase were used. Six pH conditions were employed at each of which, the attempt was made to produce and isolate tannin-protein precipitates. At each pH precipitation was examined at four protein concentrations, and at each concentration, over a ten point range of the tannin : protein ratio R(t). The total design thus comprised some 960 reactions.

3.3,1 Methods

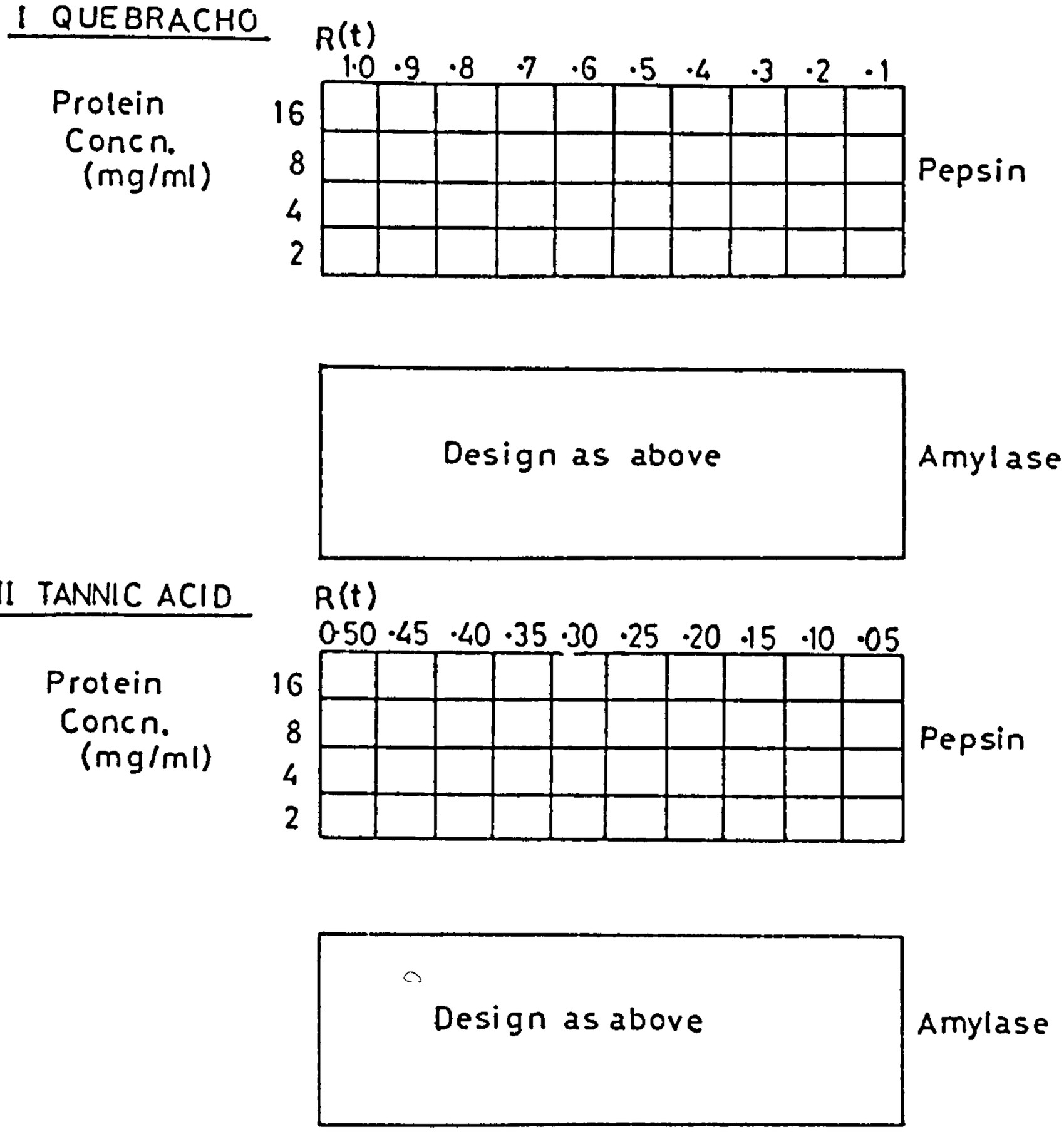
Each of the individual experiments in the design was carried out by the following standard method. In each batch of experiments a single protein, tannin and pH value were chosen. All solutions were then made in a buffer of the chosen pH, the six pH values used being 2.3, 3.4, 5.7, 7.6, 9.2, 10.8. Stock solutions of these buffers, made as described in Appendix 2.1, were used throughout the experiments. Whilst not of equal ionic strengths they were of strengths over the 50mM threshold identified in Section 3.2. and all satisfied the criteria (of Section 3.2) for not interfering in tannin-protein

complex formation.

At the start of the experiments a protein solution of 32mg/ml concentration was made. Tannin solutions were initially 32mg/ml for quebracho and 16mg/ml for tannic acid. 2ml aliquots of protein solution were then added to each of ten centrifuge tubes to which 2ml, 1.8ml, 1,6ml etc. to 0.2ml samples of one tannin solution were added in order. the volume in each tube then being made up to 4ml by the addition of additional "blank" buffer solution. The tannin and protein solutions were then diluted by half and the process was repeated to give, in total, four sets of ten reactions with R(t) ranging from 1.0 to 0.1 where quebracho was used or from 0.5 to 0.05 for reactions containing tannic acid and with the protein solution at 16mg/ml, 8mg/ml 4mg/ml and 2mg/ml in each set (after dilution with the tannin and additional buffer solution). This reaction scheme is presented diagramatically in Figure 3.2. Precipitation reactions appeared to be immediate. However, reactions were left for 15 minutes before the experiment was continued. At the end of this time the tubes were centrifuged for 5 minutes at the top speed of an MSE bench centrifuge.

After centrifugation the supernatant was decanted and total phenolics in the precipitate were measured as described previously in Section 3.2,1 and in Appendix 1.4, after first redissolving the pelleted precipitate

Figure 3.2 A diagramatic summary of precipitation experiments carried out at any one pH value. Each cell in the blocks of 40 represents one precipitation reaction.



with SDS/Triethanolamine reagent. No difficulties were encountered in this process or with any of the buffers employed. An aliquot of the supernatant was also assayed for total phenolics, after a suitable dilution, using the standard procedure described in Appendix 1.4.

It had been the intention to measure protein concentration in both the supernatant and precipitate fractions generated by the experiments and to this end the following methods were investigated. (i) The Biuret Method (Mokrasch et al. 1956): this requires the use of a strongly alkaline reagent which yields a dark brown/black colour with tannins which obscures the measurement of protein in their presence, and made the method of no use here. (ii) Lowry's Method (Lowry et al., 1951): this method is based on a redox reaction which is subject to interference by the oxidation of phenolics and was thus again of no use.

With these methods having proved patently useless for the needs of this study, the method of Bradford (1976) was adopted. This technique uses a dye binding reaction to detect proteins as a blue (dyed) complex. However, it is very sensitive to interference from SDS and could not be used on samples from redissolved precipitates. Bradford's method has been used by Martin and Martin (1983) to detect protein after its elution from a sephadex G-50 gel to which tannins remained bound. The

need for this separation phase quickly became apparent, as tannins are even more sensitively detected by Bradford's reagent than are proteins. Tannnins do, however, produce a markedly different shade of blue to that produced by proteins and so the interference is readily detectable. In this study tannins were removed from the samples of supernatant assayed by their adsorption onto polyvinylpyrrolidone (PVP), a procedure that proved satisfactory except in systems with very high T and a high total concentration of tannin. Full details of the methods used to measure supernatant protein concentration are given in Appendix 2.2

3.3,2 Results

Of the 960 individual (and unreplicated) observations made, not all produced precipitation reactions. Figure 3.3 summarises observations made for each of the two proteins. The occurrence of precipitation tannin at every R(t) value at a given pH and protein concentration was taken as the criterion for a positive entry in the two block diagrams presented.

Pepsin has a pI of 2.2 and α-amylase has a pI of 6.3, and as expected (Hagerman and Butler 1978) precipitation occurs at more alkaline pH for α-amylase. α-Amylase does not show any lack of precipitation on the acid side of its pI and in this respect the results of Loumis and

Figure 3.3 Presence/absence of precipitation for the two proteins in the presence of the two tannins at various pH values and concentrations of tannin and protein.

PEPSIN

Protein Concn. mg/ml	pH=2·3	3·4	5·7	7·6	9·3	10·8
16	Qb TA	Qb TA	Qb TA	Qb		
8	Qb TA	Qb TA	Qb TA	Qb		
4	Qb TA	Qb T	Qb TA			
2	Qb TA	Qb TA	Qb TA			

AMYLASE

Protein Concn. mg/ml	pH=2 3	3·4	5·7	7·6	9·3	10·8
16	Qb TA	Qb TA	Qb TA	Qb TA	Qb TA	Qb TA
8	Qb TA	Qb TA	Qb TA	Qb TA	Qb TA	Qb TA
4	Qb TA	Qb TA	Qb TA	Qb TA	Qb TA	Qb
2	Qb TA	Qb TA	Qb TA	Qb TA		

Qb and TA indicate the presence of precipitation for sysyems containing quebracho tannin and tannic acid respectively, see text for criteria used to asses this.

Bataille (1966) appear confirmed. However, they, unlike Hagerman and Butler (1978), would not have expected the existence of precipitates at pH > 7 and they would have expected some differentiation between the ranges of pH where the condensed and hydrolysable tannins produced precipitates. Interestingly, no clear diffferentiation between tannic acid and quebracho tannin can be seen here despite their very diferent chemical structures. Note, however, that on a weight for weight basis tannic acid is present at half the concentration of quebracho tannin. However, its higher total phenolic content (on a weight basis) and the less than 100% condensed tannin component in the phenolics of quebracho (see table 2.3) may approximately counterbalance this so it would be premature to assign greater activity to the tannic acid. This is an approximation that it is impossible to circumvent; any conclusions that might be drawn as to the differences between the protein precipitating capacities of tannins isolated as crude plant extracts will be subject to two separate sources of error introduced both by (i) the difficulties in their chemical characterisation, including those of separating tannins from non-tannin phenolics and (ii) by those due to the techniqes used to measure their protein precipitating capacity.

Consideration of the values of T averaged over the 40

values in each set of experiments with a given tannin and protein (see Figure 3.2) shows that these mean values are consistently lower for tannic acid-containing systems (see Table 3.3). On the basis of the preceedeing arguments, this could be due to non-tannin phenolics in quebracho being left in the supernatant whilst tannins were precipitated. Alternatively, the lower values of T for tannic acid could indicate that the precipitate in these systems is relatively unsaturated with tannin as less tannin has been added in the experiment. As previously noted, where chemically heterogeneous extracts are employed it will generally be impossible to distinguish between tannin and non-tannin phenolics in the supernatant.

A second feature seen in Table 3.3 is the tendency of T in quebracho-containing systems, to rise with pH. Indeed, in the absence of a precipitate, e.g. in strongly alkaline conditions, T is at infinity for both tannins. This trend to high T values in precipitating systems, and is especially clear when examining averaged data for groups of values differing in R(t), but with other factors constant. For α-amylase and quebracho, an ordered rise in T in response to both pH and decreased protein concentration is seen for the data plotted in Figure 3.4. Similar results are seen with pepsin and quebracho (Figure 3.5). The data for these figures and

Table 3.3 Mean values of T averaged for groups of observations with a single tannin, protein concentration and pH value.

I Pepsin containing systems

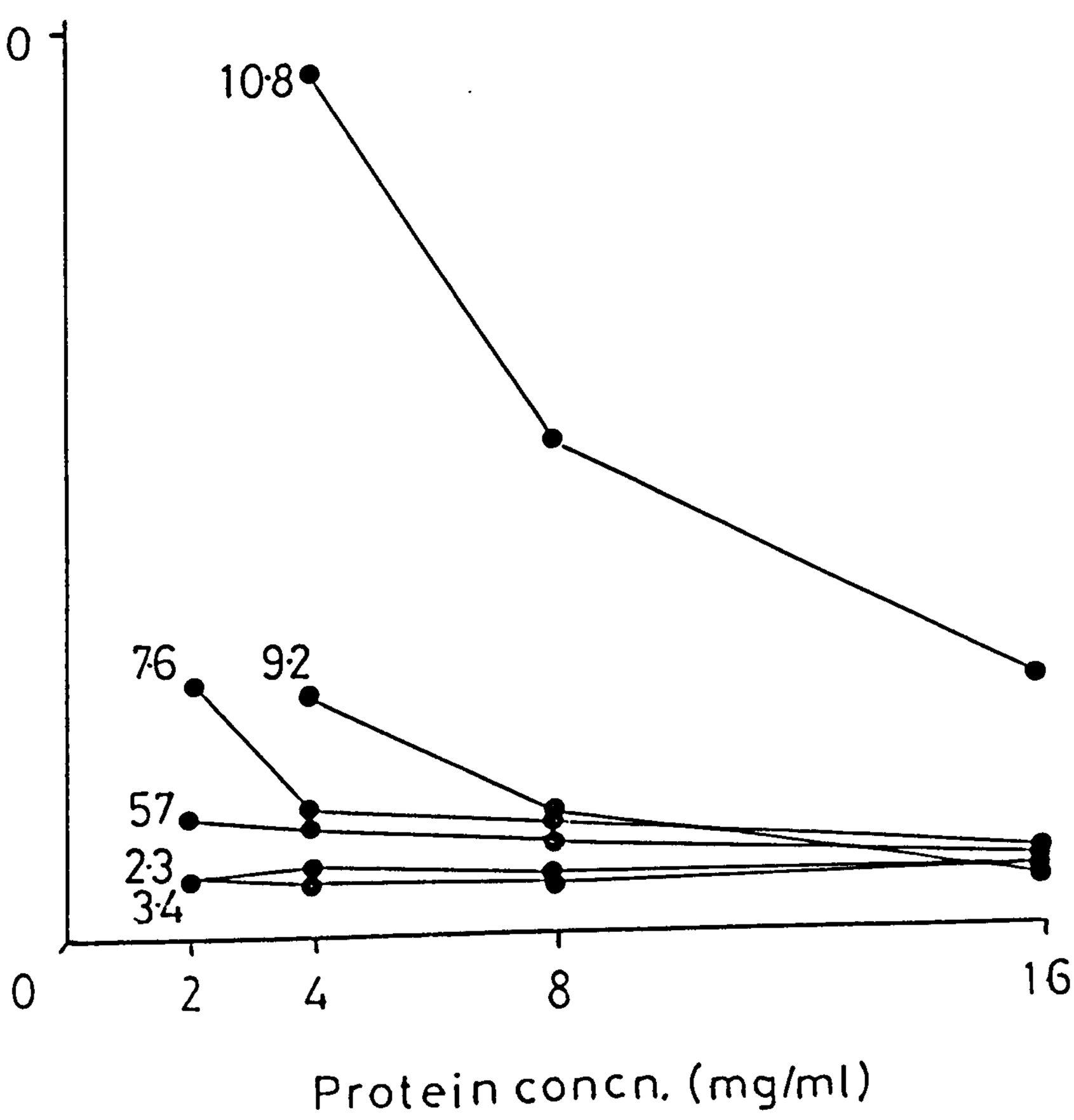
pH	Quebracho	Tannic acid
2.3	2.77 (0.12)	2.22 (0.17)
3.4	3.23 (0.30)	1.96 (0.28)
5.7	4.24 (0.48)	1.77 (0.26)

II Amylase containing systems

pH	Quebracho	Tannic acid
2.3	2.09 (0.25)	1.77 (0.14)
3.4	1.76 (0.09)	1.64 (0.27)
5.7	3.26 (0.35)	1.38 (0.12)
7.6	4.74 (0.85)	1.90 (0.15)

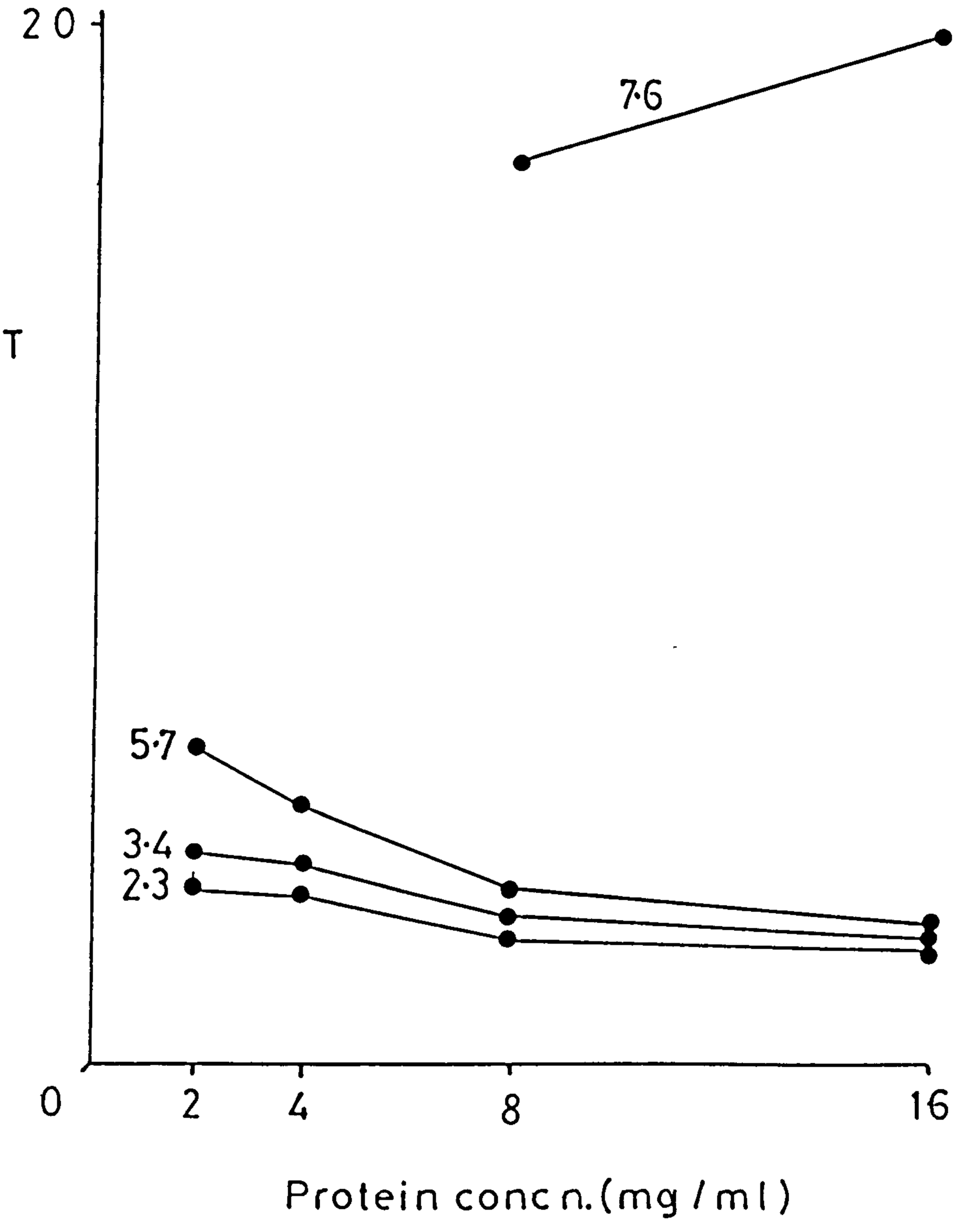
N.B. Figures in parentheses are standard errors.

Figure 3.4 T values plotted against protein concentration for the amylase-quebracho system.



Separate curves represent results from experiments at different pH values as indicated by the numerical values on the graph. Note that at zero protein concentration (nil precipitation) T becomes infinity at each pH value.

Figure 3.5 T values plotted against protein concentration for the pepsin-quebracho system.



Separate curves represent results from experiments at different pH values as indicated by the numerical values on the graph. Note that at zero protein concentration (nil precipitation) T becomes infinity at each pH value.

that for tannic acid is tabulated in Tables 3.4 to 3.6. Some further differentiation between tannic acid and quebracho can be seen in these data. The above trends for T are difficult to distinguish with tannic acid although there are some high values as conditions that do not favour precipitation are approached. This indicates that precipitation is much more of an all or nothing phenomenon with tannic acid, in contrast to quebracho where a wider range of conditions supports partial precipitation, as might be expected for a more heterogeneous material.

Re-computing the data in each block of 40 results provides average values of T at each R(t) and these are recorded in Tables 3.7 and 3.8. T is seen to fall with R(t) in all cases. The curves plotted (Figures 3.6 & 3.7) are for the data on quebracho-containing systems where curves for systems at different pH are separate as expected from the preceeding discussion. The fall in T with R(t) is expected on the assumption that R(p) can vary only within fixed limits. Where there is precipitation when R(t) is low, T must decline as a consequence of more tannin entering the precipitate.

The overriding combination of protein type and pH as the major controlling variables of precipitate formation have already been emphasised (see Figure 3.2). Of the two other variables R(t) and the total system

Table 3.4 Pepsin-containing reactions, Mean values of T at various protein concentrations and reaction pH values.

I Quebracho - Pepsin system

protein / pH= (mg/ml)	2.3	3.4	5.7	7.6
16	2.16 (0.18)	2.43 (0.22)	2.66 (0.22)	19.7 (1.08)
8	2.38 (0.17)	2.80 (0.24)	3.34 (0.24)	17.2 (0.73)
4	3.17 (0.17)	3.84 (0.24)	4.91 (0.23)	
2	3.36 (0.18)	3.86 (0.17)	6.04 (0.23)	

II Tannic acid - Pepsin system

protein / pH= (mg/ml)	2.3	3.4	5.7	7.6
16	1.29 (0.22)	1.34 (0.20)	1.19 (0.12)	
8	1.65 (0.16)	1.53 (0.18)	1.41 (0.18)	
4	2.66 (0.29)	2.41 (0.32)	1.88 (0.16)	
2	3.29 (0.21)	2.55 (0.19)	2.60 (0.27)	

N.B. Figures in parentheses are standard errors.

Table 3.5 Quebracho - Amylase containing reactions, Mean values of T at various protein concentrations and reaction pH values.

protein / pH (mg/ml)	2.3	3.4	5.7
16	2.01 (0.20)	1.78 (0.16)	2.27 (0.21)
8	1.95 (0.24)	1.67 (0.12)	3.10 (0.19)
4	2.31 (0.16)	1.81 (0.11)	3.62 (0.21)
2	2.07 (0.11)	1.78 (0.13)	4.05 (0.19)

protein / pH (mg/ml)	7.6	9.2	10.8
16	2.53 (0.16)	1.79 (0.16)	8.28 (0.20)
8	3.70 (0.26)	4.13 (0.22)	16.20 (0.10)
4	4.24 (0.18)	7.87 (0.61)	28.7 (3.07)
2	8.48 (0.52)		

N.B. Figures in parentheses are standard errors.

Table 3.6 Tannic acid - Amylase containing reactions, Mean values of T at various protein concentrations and reaction pH values.

protein / pH (mg/ml)	2.3	3.4	5.7
16	1.30 (0.15)	1.25 (0.12)	0.95 (0.13)
8	1.48 (0.16)	1.28 (0.11)	1.16 (0.13)
4	2.24 (0.26)	2.13 (0.26)	1.70 (0.19)
2	2.06 (0.14)	1.92 (0.16)	1.73 (0.18)

protein / pH (mg/ml)	7.6	9.2	10.8
16	1.58 (0.14)	0.99 (0.11)	5.89 (0.47)
8	1.48 (0.13)	2.44 (0.14)	7.63 (0.61)
4	1.53 (0.13)	7.98 (0.72)	
2	3.02 (0.20)		

N.B. Figures in parentheses are standard errors.

Table 3.7. Mean values of T in the amylase containing reactions, averaged for observations at each R(t) value, each pH and tannin.

I Quebracho containing reactions.

R(t)/pH=	2.3		3.4		5.7		7.6	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
1.0	2.89	0.331	2.58	0.17	4.45	0.301	5.41	1.31
	3 20	0.285	2.32	0.075	4.25	0.336	5.44	0.903
	2.73	0.110	2.22	0.104	4.01	0.376	4.98	0.994
	2.57	0.078	1.83	0.078	3.72	0.374	5.55	1.52
	2.19	0.238	1.92	0.047	3.36	0.500	5.11	1.51
	1.81	0.094	1.87	0.148	3.10	0.439	5.18	1.50
	1.55	0.105	1.59	0.096	2.85	0.905	4.25	1.55
	1.53	0.194	1.21	0.088	2.60	0.445	4.81	1.97
	1.37	0.275	1.03	0.062	2.50	0.371	4.03	1.80
0.1	1.04	0.222	1.06	0.260	1.76	0.339	2.26	0.195

II Tannic acid containing reactions.

R(t)/pH=	2.3		3.4		5.7		7.6	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
0.5	2.67	0.451	2.56	0.405	2.16	0.267	2.73	0.404
	2.55	0.381	2.42	0.419	2.17	0.430	2.51	0.501
	2.52	0.282	2.26	0.341	1.86	0.211	2.43	0.492
	2.38	0.179	2.10	0.272	1.99	0.163	2.25	0.483
	1.93	0.161	1.79	0.280	1.52	0.224	2.22	0.452
	1.69	0.363	1.55	0.191	1.31	0.241	1.90	0.249
	1.39	0.241	1.27	0.167	1.05	0.253	1.54	0.352
	1.15	0.242	1.24	0.300	0.795	0.123	1.50	0.407
	0.915	0.164	0.743	0.105	0.560	0.092	1.56	0.301
0.05	0.512	0.176	0.493	0.039	0.415	0.069	0.812	0.216

Note: S.E. = standard error for the mean ($\bar{x}$)

Table 3.8. Mean values of T in the pepsin containing reactions, averaged for observations at each R(t) value, each pH and tannin.

I Quebracho containing reactions.

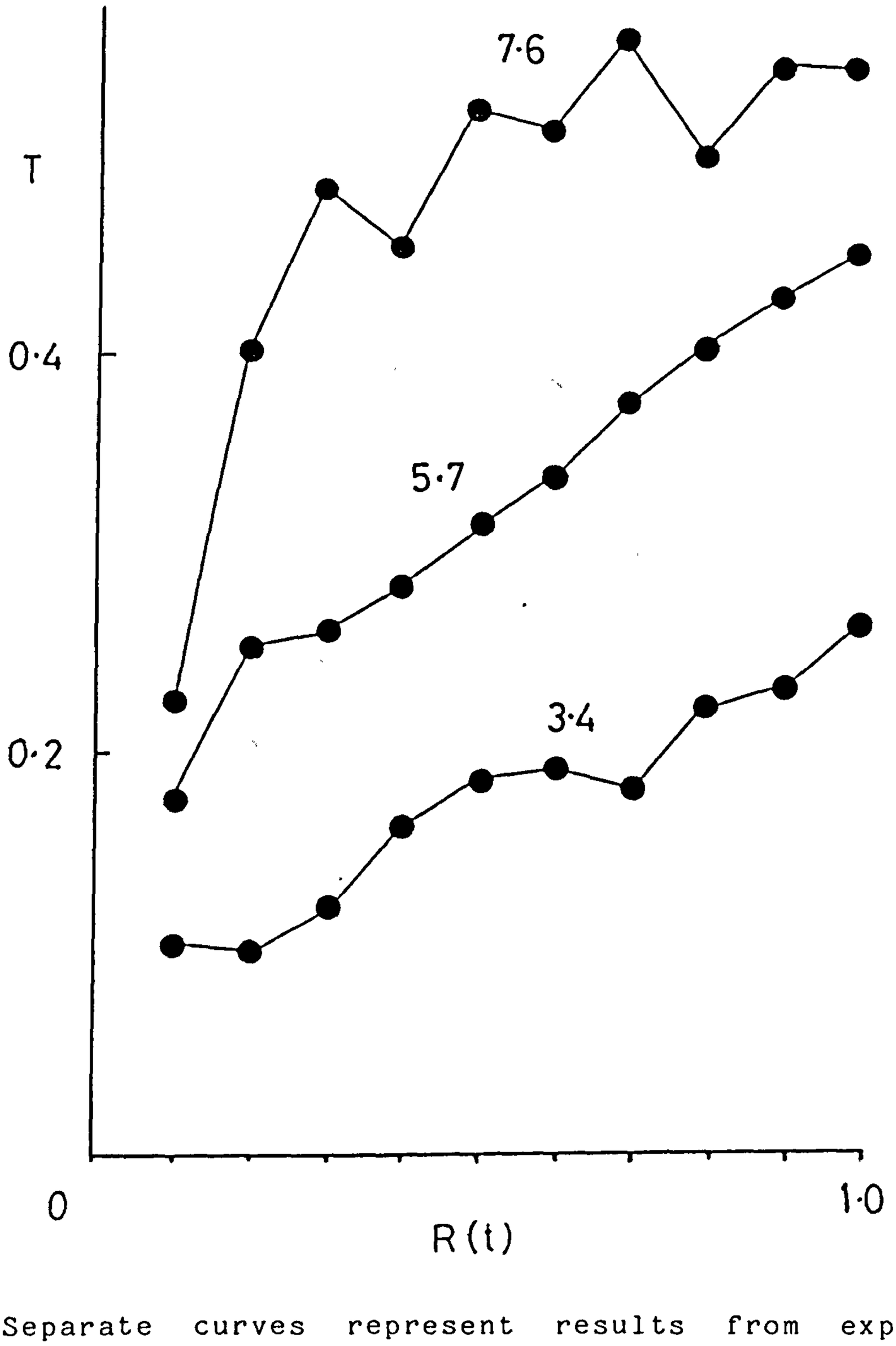
R(t)/pH=	2.3		3.4		5.7	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
1.0	3.34	0.042	3.97	0.034	5.07	0.735
	3.14	0.293	4.08	0.328	4.73	0.546
	3.36	0.290	3.81	0.309	4.61	0.740
	3.29	0.241	3.58	0.329	4.61	0.754
	3.01	0.339	3.30	0.465	4.40	0.754
	2.69	0.300	3.00	0.460	3.84	0.761
	2.45	0.350	2.85	0.423	3.89	0.874
	2.26	0.328	2.57	0.323	3.89	0.874
	2.17	0.337	2.45	0.374	3.79	1.11
0.1	1.97	0.250	2.69	0.074	3.63	1.05

II Tannic acid containing reactions.

R(t)/pH=	2.3		3.4		5.7	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
0.5	3.04	0.442	2.90	0.424	2.48	0.459
	2.99	0.447	2.74	0.304	2.57	0.467
	2.84	0.491	2.60	0.405	2.30	0.370
	2.68	0.494	2.29	0.365	2.16	0.338
	2.34	0.530	2.14	0.313	1.80	0.385
	2.20	0.625	1.83	0.324	1.63	0.256
	1.94	0.451	1.65	0.375	1.31	0.223
	1.46	0.517	1.37	0.268	1.22	0.208
	1.45	0.342	1.20	0.268	1.03	0.176
0.05	1.30	0.364	0.901	0.189	1.239	0.287

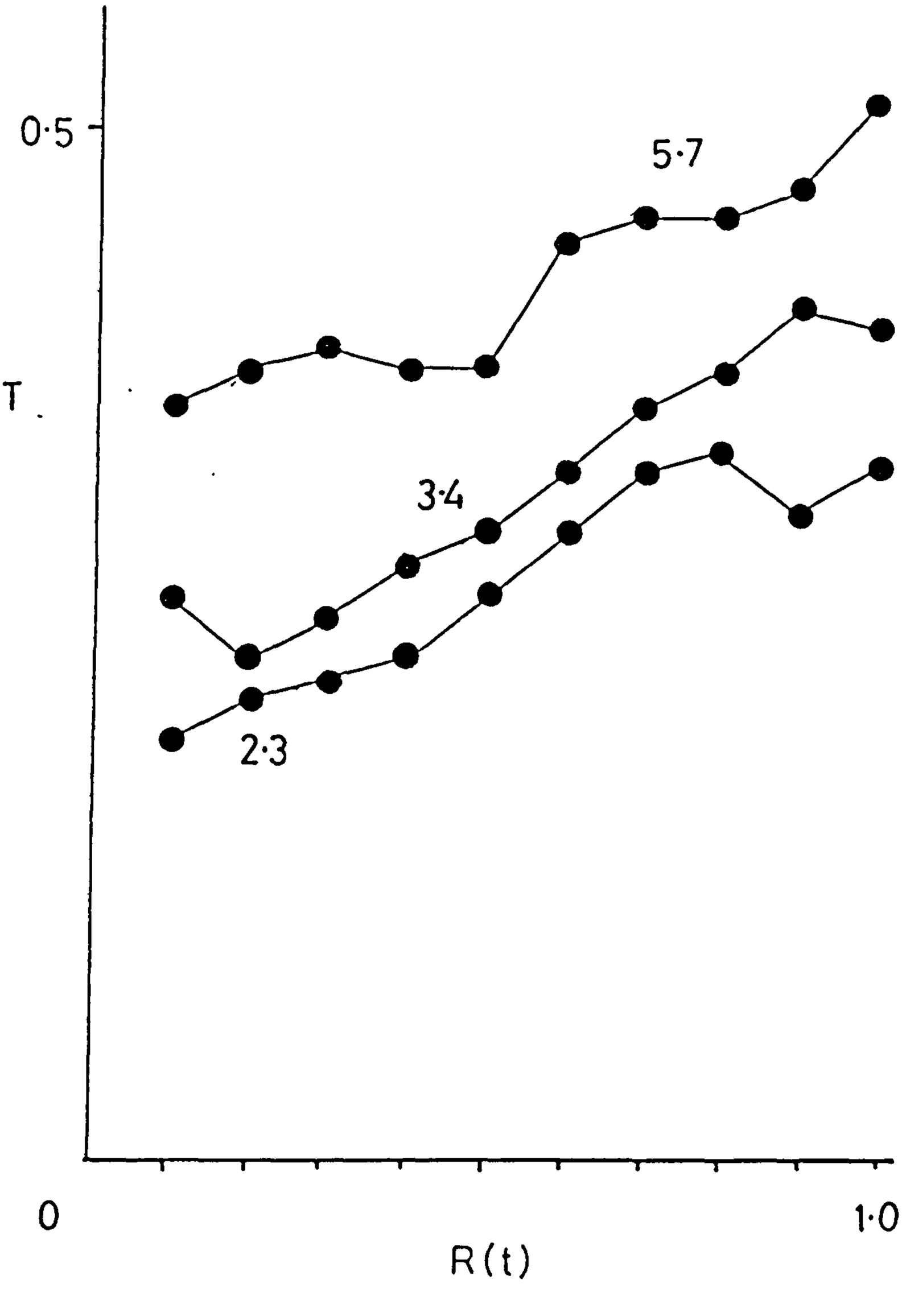
Note: S.E. = standard error for the mean (x).

Figure 3.6 T values plotted against R(t) for the amylase-quebracho system.



Separate curves represent results from experiments at different pH values as indicated by the numerical values on the graph.

Figure 3.7 T values plotted against R(t) for the pepsin-quebracho system.



Separate curves represent results from experiments at different pH values as indicated by the numerical values on the graph.

concentration expressed as protein concentration, some gauge of their relative importance in these experiments can be seen by an examination of the results of two way ANOVA analysis of each block of 40 results. These analyses are presented in Tables 3.9 & 3.10. Each shows these two factors to be generally highly significant sources of variation. For pepsin-quebracho systems it is notable that R(t) is consistently the least important source of variation (compare F values). This position also holds for tannic acid-pepsin systems. However, with α-amylase and tannic acid the position is reversed at low pH, yet where pH > pI, R(t) is again the lesser of two important sources of variation, thus paralleling results with pepsin where pH > pI in all experiments.

These results do show that the two tannins differ in their interactions with the two different proteins but that these differences are relatively subtle compared to the major variables determining whether precipitates form at all, namely pH and protein type (particularly protein pI). Within the limits imposed by these variables tannin type may modify the influence of R(t) and protein concentration on T.

The Bradford method used to detect protein in the supernatant fractions proved quite sensitive for α-amylase, although at high T and high pH interference by tannins was suspected and only data for R(p) in the pH

Table 3.9 The contribution of R(t) and protein concentration to variation in T for pepsin containing reactions at various pH values.

Values for the F statistic (and associated probabilities p) are given. These result from two way ANOVA computations for each set of 40 results with a given tannin and pH value. In the ANOVA degrees of freedom are allocated as folows: R(t), 9; protein concentration, 3; error, 27; giving a total of 39.

I Quebracho containing reactions

source/pH	2.3	3.4	5.7
R(t)	15.12 (.999)	6.92 (.999)	3.01 (.975)
Protein concn.	46.50 (.999)	32.77 (.999)	68.85 (.999)

II Tannic acid contaning reactions

R(t)	21.37 (.999)	24.97 (.999)	24.97 (.999)
Protein concn.	102.0 (.999)	46.86 (.999)	46.86 (.999)

Table 3.10 The contribution of R(t) and protein concentration to variation in T for amylase containing reactions at various pH values.

Values for the F statistic (and associated probabilities p) are given. These result from two way ANOVA computations for each set of 40 results with a given tannin and pH value. In the ANOVA degrees of freedom are allocated as folows: R(t), 9; protein concentration, 3; error, 27; giving a total of 39.

I Quebracho containing reactions

source/pH	2.3	3.4
R(t)	12.52 (.999)	16.52 (.999)
Protein concn.	1.50 (N.S.)	0.495 (N.S.)

source/pH	5.7	7.6
R(t)	55.83 (.999)	2.87 (.975)
Protein concn.	167.0 (.999)	47.77 (.999)

II Tannic acid contaning reactions

R(t)	17.80 (.999)	16.58 (.999)
Protein concn.	15.60 (.999)	16.56 (.999)

source/pH	5.7	7.6
R(t)	26.31 (.999)	21.00 (.999)
Protein concn.	23.08 (.999)	73.40 (.999)

range 2.3-7.6 is presented in table 3.11. For pepsin the method proved relatively insensitive and the following discussion is based entirely on results with α-amylase.

There is essentially complete protein precipitation at high R(t), with confident detections and measurements of protein only being made at low R(t) in most cases. Plots of the data thus tend to have a "reverse J" shape (Figure 3.8), and the large number of zero entries make the application of statistical tests generally impossible. Taken as a whole the data does follow the trend expected from the results for T, i.e. the opposite trend to T, with P rising as R(t) declines. With quebracho there is also some tendncy for P to rise with pH as would also be expected, in parallel to the change in T (see Table 3.12). Two sets of data were available where a two way ANOVA could be employed to examine the relative importance of the factors R(t) and protein concentration. The analysis showed that as with T, both are significant sources of variation although R(t) is the lesser of the source of variation. In conclusion, the results discussed above and the fact that almost all the protein is precipitated in most of the experiments, combine to indicate that the variable R(p) will be essentially dependent on variation in T, not T and P. R(s) is totally dependent on these other three variables and is not separately discussed.

Table 3.11. Mean values of P in the amylase containing reactions, averaged for observations at each R(t) value, each pH and tannin.

I Quebracho containing reactions.

R(t)/pH=	2.3	3.4	5.7	7.6
1.0	0	0	0.031	0.031
	0	0	0.042	0.056
	0.005	0	0.063	0.056
	0.005	0	0.063	0.066
	0.005	0	0.074	0.089
	0.008	0	0.087	0.102
	0.008	0	0.084	0.130
	0.074	0.021	0.099	0.205
	0.094	0.070	0.112	0.466
0.1	0.037	0.201	0.442	0.679

II Tannic acid containing reactions.

R(t)/pH=	2.3	3.4	5.7	7.6
0.5	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0.064	0	0	0
	0.092	0.047	0	0
	0.109	0.084	0	0
	0.213	0.124	0	0.297
	0.297	0.214	0.053	0.415
0.05	0.452	0.554	0.149	1.121

Note: Zero values indicate that no protein could be detected in the precipitates of all four reactions at each R(t) value. Standard errors are omitted due to the many zero values in the data set.

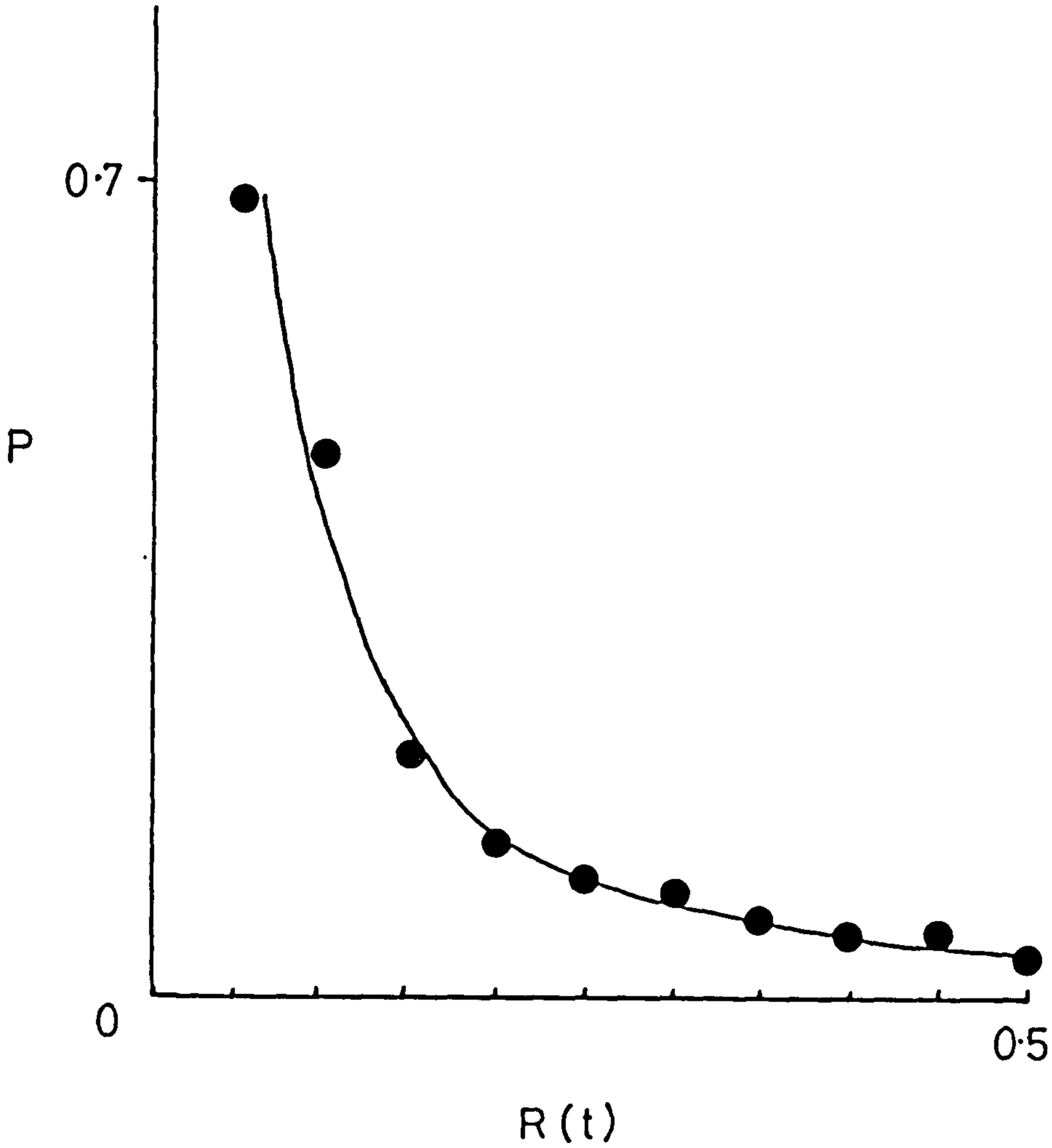


Figure 3.8 P values plotted against R(t) for the amylase-quebracho system at pH = 7.6.

Table 3.12 Mean values of P averaged within groups of observations made at different protein concentrations and pH values for amylase containing reactions.

I Quebracho containing reactions

protein concn./ pH=	2.3	3.4	5.7	7.6
16 mg/ml	0.0267 (0.0110)	0.0043 (0.0046)	0.0325 (0.0160)	0.0694 (0.0247)
8 mg/ml	0.0278 (0.0280)	0.0033 (0.0022)	0.0558 (0.0224)	0.0638 (0.0164)
4 mg/ml	0.0278 (0.0280)	0.0281 (0.0200)	0.109 (0.0356)	0.308 (0.116)
2 mg/ml	0.0976 (0.0544)	0.0812 (0.0554)	0.242 (0.0786)	0.406 (0.142)

II Tannic acid containing reactions

protein concn./ pH=	2.3	3.4	5.7	7.6
16 mg/ml	0.0300 (0.0193)	0.0216 (0.0160)	0.0043 (0.0037)	0.0606 (0.0420)
8 mg/ml	0.0840 (0.0460)	0.0657 (0.0446)	0.0149 (0.0145)	0.122 (0.0998)
4 mg/ml	0.0770 (0.0456)	0.106 (0.299)	0.0097 (0.0098)	0.136 (0.0875)
2 mg/ml	0.299 (0.0956)	0.227 (0.103)	0.0524 (0.0360)	0.450 (0.279)

Standard errors for the means are in parentheses.

The variable R(p) does show the variation expected above; the one way in which its behaviour is somewhat divergent from that predicted by T alone is in the decline in value with falling R(t). The rate of decline becomes somewhat more precipitous at low R(t) as rising values of P contribute significantly to its variation; this pattern can be seen in Table 3.13 and figure 3.9.

The discussion of results originating from these precipitation experiments has so far kept to the initial intention of describing factors determining the nature and occurrence of precipitates. However, the data can be used to obtain some insight into the nature of the reaction at the molecular level. Following the arguments of Van Buren and Robinson (1969), that in conditions where total protein precipitation has occurred, phenolics in the supernatant must represent unbound- tannin, the approach of Scatchard (Van Buren and Robinson, 1969) can be used to investigate the nature of the tannin-protein binding interaction. Where A represents the concentration of unbound ligand (i.e. tannin in the supernatant) and R (= R(p)) represents the ratio of bound ligand to protein, then for a situation where the protein has a number of identical independent binding sites for the ligand, the relationship given in the equation below holds and so plots of R/A versus R are linear.

Table 3.13. Mean values of R(p) in the amylase containing reactions, averaged for observations at each R(t) value, each pH and tannin.

I Quebracho containing reactions.

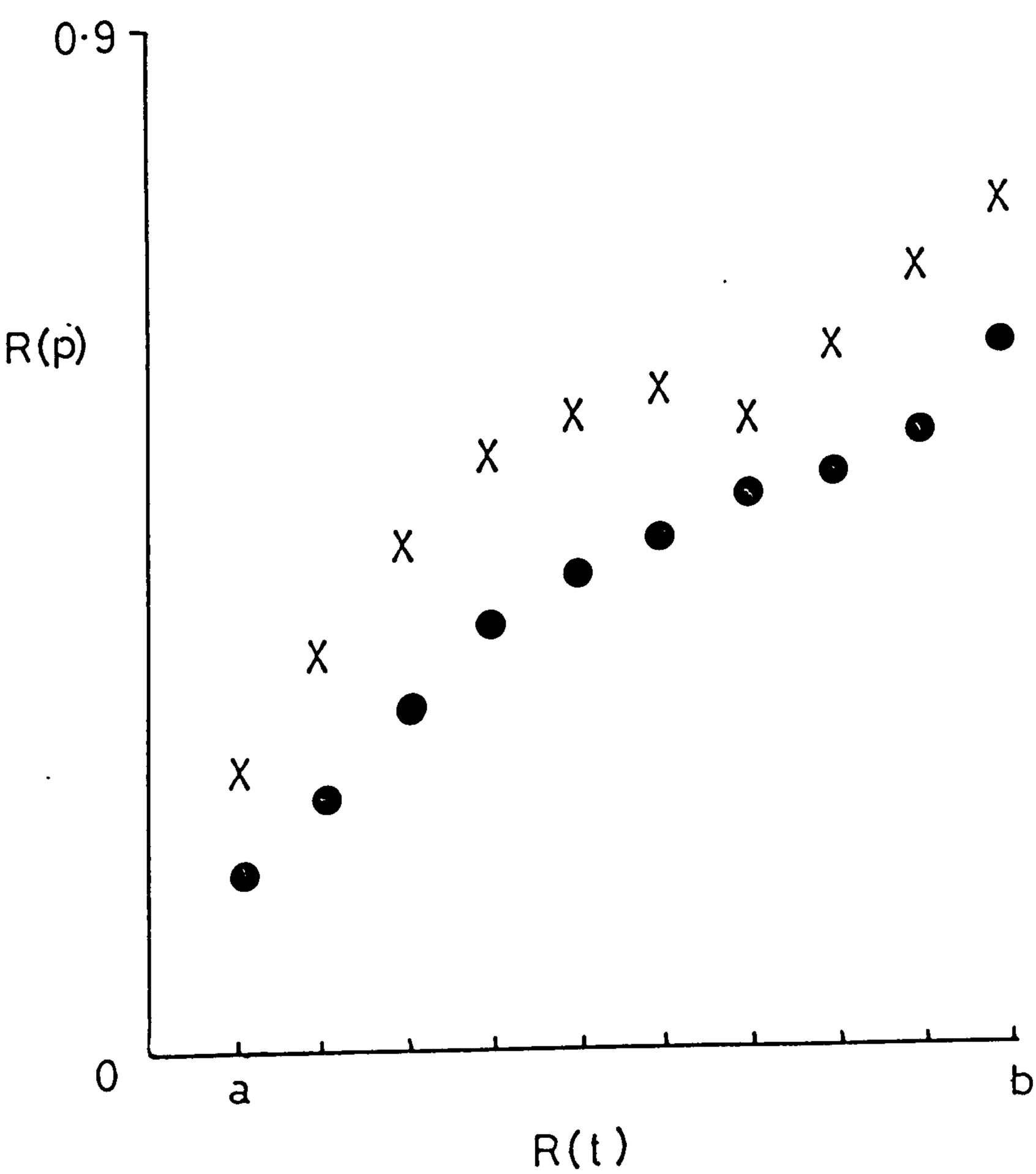
R(t)/pH=	2.3		3.4		5.7		7.6	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
1.0	0.608	0.051	0.651	0.034	0.441	0.014	0.305	0.090
	0 533	0.027	0.624	0.014	0.418	0.025	0.262	0.070
	0.501	0.017	0.587	0.015	0.401	0.025	0.258	0.074
	0.458	0.009	0.587	0.020	0.385	0.036	0.210	0.061
	0.445	0.031	0.455	0.008	0.353	0.033	0.193	0.058
	0.418	0.014	0.409	0.021	0.317	0.030	0.170	0.047
	0.369	0.015	0.359	0.014	0.270	0.026	0.157	0.047
	0.297	0.018	0.323	0.014	0.218	0.020	0.124	0.039
	0.219	0.039	0.186	0.049	0.150	0.014	0.114	0.038
0.1	0.156	0.013	0.142	0.014	0.122	0.009	0.106	0.063

II Tannic acid containing reactions.

R(t)/pH=	2.3		3.4		5.7		7.6	
	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.	$\bar{x}$	S.E.
0.5	0.754	0.091	0.738	0.089	0.840	0.075	0.715	0.065
	0 710	0.080	0.679	0.062	0.775	0.105	0.698	0.078
	0.653	0.062	0.604	0.047	0.716	0.065	0.633	0.070
	0.597	0.049	0.548	0.048	0.686	0.058	0.586	0.067
	0.571	0.055	0.570	0.038	0.631	0.057	0.505	0.058
	0.546	0.031	0.548	0.062	0.579	0.066	0.316	0.097
	0.497	0.026	0.522	0.030	0.530	0.067	0.427	0.046
	0.439	0.011	0.442	0.021	0.439	0.033	0.411	0.034
	0.364	0.012	0.349	0.021	0.351	0.014	0.338	0.008
0.05	0.264	0.026	0.246	0.025	0.209	0.112	0.300	0.072

Note: S.E. = standard error for the mean ($\bar{x}$).

Figure 3.9 R(p) values plotted as a function of R(t) for amylase containing reactions at pH = 2.3.



Points marked by dots are for quebracho containing reactions (x-axis, a = 0.1, b =1.0) whilst those marked X are for tannic acid containing reactions (x-axis, a = 0.05, b = 0.5).

$$R/A = kn - kR$$

k = equilibrium constant for the binding reaction

n = maximum amount of ligand bound per unit of protein.

As previously reported by Van Buren and Robinson (1969), the data for tannin-protein interactions does not fit such a simple theory. Figure 3.10 illustrates the clearest set of data provided by the present work, which like figure 8 of Van Buren and Robinson (1969) shows a concave curve. Whilst a convex curve could be interpreted as positive evidence for cooperative binding effects, a concave curve indicates either interference between binding sites or non-identical sites. These last two possibilities have also been indicated by other work dealing with the bonding of non-tannin phenolics to proteins (Morran and Walker, 1968; McManus et al., 1981).

3.3,3 Conclusions

The preceeding results presented in Section 3.3,2 deal with two proteins and one tannin not previously considered by Van Buren and Robinson (1969). The results obtained follow their findings for a tannic acid gelatin system without contradiction. This result is perhaps unremarkable in that protein precipitation is thought a general phenomenon exhibited by all proteins in the

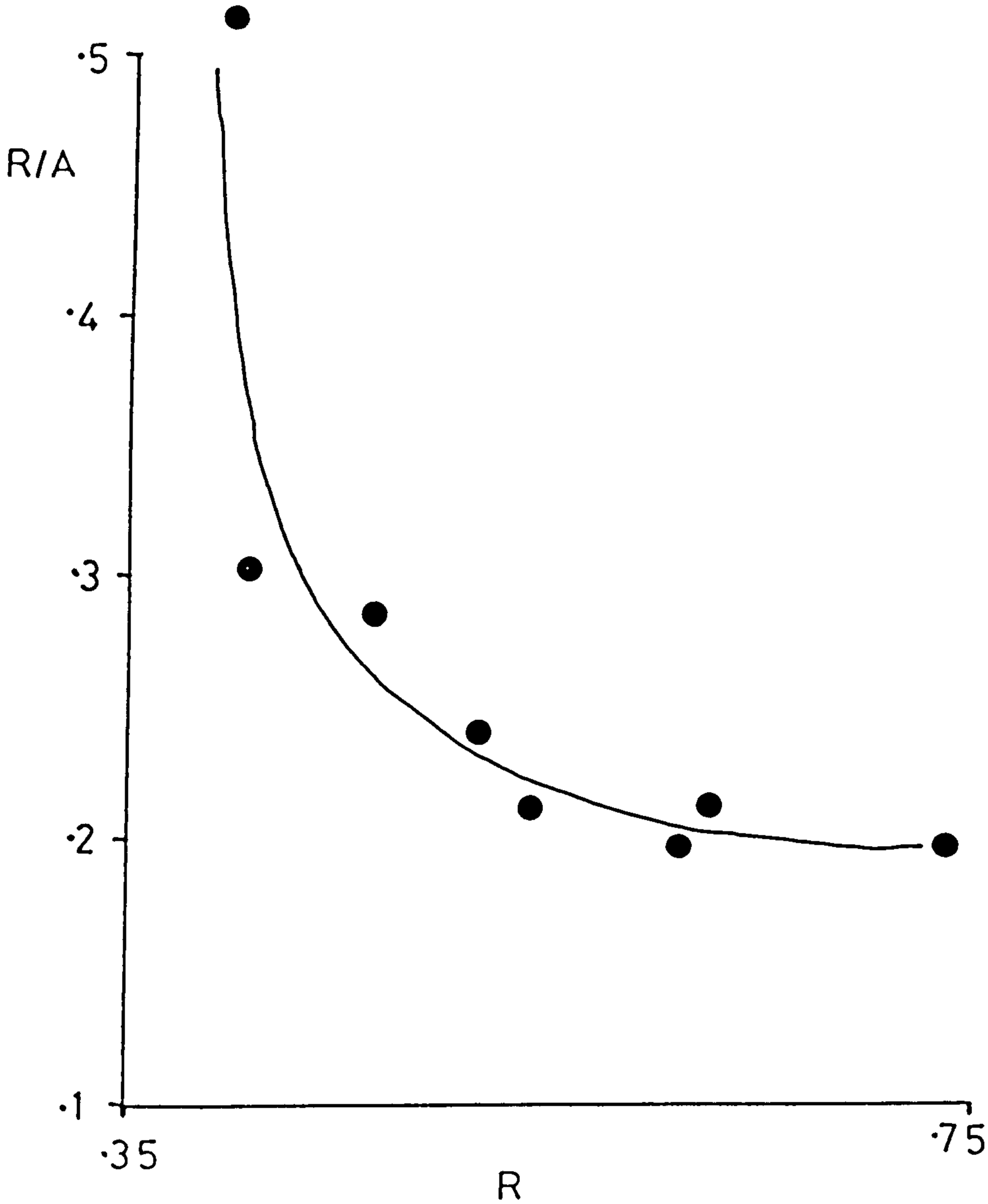


Figure 3.10 Scatchard plot of data for amylase and tannic acid at pH = 5.7 and with the protein concentration at 2mg/ml. Definitions of R and A given in the text.

presence of tannins. The variation that different tannins may bring to the system is the subject of the next chapter. Here it is notable that whilst individual proteins exert an influence on precipitation reactions through their pI, the pattern seen in the variables R(p) and T in response to R(t), protein concentration and pH is similar for pepsin, α-amylase and gelatin. Thus at this simple descripive level it seems reasonable to generalise about the effects tannins might have on one protein from information gained with another.

3.4 Implications for Measuring Tannins by Protein Precipitation.

The apparently general patterns displayed in tannin-protein precipitation reactions are particularly fortunate as practically no coherent body of information exists concerning the influence of protein structures on their precipitability by tannins. Collagen-like structures and other proline-rich proteins seem to have a high affinity for tannins (Hagerman and Butler, 1980b) whilst glycoproteins do not (Strumeyer and Malin, 1970). However, the removal of sugars from a glycoprotein has been seen to decrease its affinity for tannins even further (L. G. Butler, pers. comm.). In short, the absence of any detailed information about how phenols interact with protein structures at the molecular level precludes any real theoretical understanding of how tannins react with particular proteins. The probability that different proteins have similar patterns of reaction with tannins helps reduce one source of uncertainy in interpreting precipitation assays which, may then be more confidently used to show variations introduced by different types of tannin.

With regard to using protein precipitation as an assay technique for tannins, several conclusions can be drawn from the experimental work reported above. Firstly, precipitation reactions are likely to be most sensitive

to the presence of tannins when carried out at the pI of the protein and in solutions of an ionic strength in excess of 50 mM NaCl. In such a system precipitation will be controlled by R(t) and protein concentration. It has also been seen that total protein precipitation can occur but not total tannin precipitation, and so any phenols remaining in solution may not be chemically different to those in the precipitate (Van Buren and Robinson, 1969) and may not be said to be non tannin phenolics.

The ideal experiment to measure the tannin content of an aliquot of phenolics (e.g. of a plant extract) would seem to be to test constant samples of the phenols by their addition to increasingly concentrated solutions of protein. The expectation is that the amount of protein precipitated will rise to a maximum value indicative of the protein precipitating ability of the phenols. At this point the phenolics remaining in solution will comrise a minimum of unprecipitated tannins and non tannin phenolics. With due regard to the comments made above, the quantity of non-precipitating phenolics will give an indication of the abundance of tannins in the sample of phenolics. These two measures, but particularly that of precipitated protein, will then indicate the activity of the extract of phenolics as a tannin.

Chapter Four

Insoluble Tannin-Containing Complexes

4.1 Introduction

The biologically and ecologically important properties of tannins are thought to depend on their complexation with proteins and other components in animal and microbial diets (see Chapter 1). The work presented in this chapter has as its object the characterisation of the series of tannins described in Chapter 2 in terms of their complex-forming abilities. The reactions investigated here include precipitation with proteins and amino acids as well as the adsorption of tannins into solid cellulose matrices. The ultimate objective of this work is to assess variation in these "biochemical" characteristics of tannins as a function of their chemical variation as detected by the proximate chemical analyses used in ecological studies. This will help answer questions as to whether there is biochemical variation of potential ecological interest between compounds classed as tannins and whether simple chemical analyses can detect it.

Section 4.2 deals with measurements of protein precipitating ability. By definition, the precipitation of protein by tannins is the most dependable way of detecting them, however, as will be shown, quantifying tannins by this reaction has proved less than easy. Moreover, precipitate formation is not in itself a biologically or ecologically useful measurement, only the

effects of nullifing the function of proteins are of interest. Thus care must be taken in interpreting the effects of protein precipitation. For instance in digestive systems precipitation is normally assumed to lead to enzyme inhibition which is then taken to be a deleterious effect. The gastric enzyme, rennin, is well established as the agent responsible for insolubilising and slowing the digestion of milk proteins in young mammals (Orskov 1982), a presumably beneficial process for the animal! In short care has to be taken in interpreting all *in vitro* measures of the effects of tannins e.g. precipitation. This is because, as seen in the example with rennin, the presence of precipitation is not necessarily deleterious.

Section 4.3 provides an alternate assessment of tannin activity by examining their impact on an enzymic degradation of cellulose using a system (Jones and Hayward, 1975) which many investigators believe to be a useful one for modelling the *in vivo* process of digestion in the rumen. This together with data on the ability of some tannins to form precipitates with proteins and simple amino acids, will allow inter-tannin comparisons to be made for variation in their biochemical properties as well as a comparison of these with their chemical properties.

4.2 Quantification of Tannins by Protein Precipitation

A considerable effort has been made in recent years to quantify tannins by their interaction with proteins. Given that tannins have been defined in terms of precipitation behaviour, most workers have employed this phenomenon in designing techniques to measure tannins. Methods can be divided into those employing haemoglobin as the protein and those that use bovine serum album (BSA). The only recent variant on this theme is seen in the work of Hoff and Singleton (1977) who measured the adsorption of tannin to (BSA) immobilised on a solid support, and not free in solution.

The earliest attempt at the use of a protein precipitation technique for the routine measurement of tannins in samples collected for ecological or taxonomic purposes was made by Bate-Smith (1973b). He used freshly drawn human blood as the source of protein in his experiments. Haemoglobin from haemolysed blood cells was precipitated by tannin and the loss of haemoglobin was measured in a spectrophotometer at 578nm to gauge the "astringency" of the sample. Schultz _et al_. (1981) attempted to make the assay more convenient by using other sources of haemoglobin. However, they found the assay to be absolutely dependent on a fresh supply of blood. Additionally, the threshold level of tannin needed in the assay to initiate the precipitation of

haemoglobin was found to be undesirably high (Schultz et al., 1981). Asquith and Butler (1985) have also noted that many non-tannin plant constituents absorb at 578nm and so interfere with the assay.

Both Martin and Martin (1983) and Hagerman and Butler 1978,1980a) report that BSA is a suitable protein for this work but differ in their methods for measuring its reaction with tannins. The use of the method of Bradford (1976) by Martin and Martin (1983) was discussed in Chapter 3 and was found to be less than satisfactory in use. Hagerman and Butler (1978) initially measured the loss of tannin by precipitation from solution but whilst of potential use in indicating the amount of tannin present this measure alone does not quantify the ability of that tannin to precipitate protein. Hagerman and Butler (1980a) then developed a radiochemical method for detecting the precipitation of ^{14}C labelled BSA by tannins. By a combination of their two techniques Hagerman and Butler (1980a) were then able to measure the specific activity of a tannin in terms of protein precipitation on a "per total phenolics" basis. This was the first time such an analysis had been proposed. Unfortunately their radiochemical techniques require sophisticated instrumentation and have not been adopted in ecological surveys.

In the work described below the method of Hagerman and

Butler (1980a) is employed to the stage where a tannin-protein precipitate is generated for analysis. Thereafter the protein in the precipitate is analysed by an alkaline hydrolysis followed by a ninhydrin assay for amino acids in the hydrolysate. The use of an alkaline hydrolysis in 4M NaOH followed the recommended procedure for hydrolysing proteins (Mahler and Cordes, 1971). Alkali, not acid, was chosen for the hydrolysis because only alkali dissolved the tannin protein precipitate to give a clear solution at the start of the hydrolysis stage. It is of note that both Martin et al. (1985) and Butler (Asquith and Butler, 1985) have now changed their techniques for the measurement of protein in the presence of tannins to what they consider to be more satisfactory methods, although neither used a ninhydrin technique. However, Marks et al. (1985) have reported the suitability of the ninhydrin method for measuring proteins in the presence of tannins.

4.2,1 Methods

In following Hagerman and Butler (1980a), BSA (Sigma fraction V) was prepared as a 5mg/ml stock solution in a buffer of pH 4.9, which is the protein's isoelectric point. The buffer (170mM NaCl and 200mM acetic acid brought to pH 4.9 with NaOH) also contains more than sufficient salt to ensure that ionic strength does not

limit precipitation.

For each precipitation assay, two sets of eleven test tubes containing 0, 0.5, 1.0, 1.5, ... 5.0 ml of BSA stock solution were prepared, each tube being made up to 5.0ml by the addition of "blank" buffer solution. A sample of the tannin to be assayed was then dissolved in more buffer solution and any insoluble material was removed by decanting or centrifugation. 0.5 ml of the clear tannin solution was then added to each of the twenty-two tubes containing BSA/buffer solution. Fifteen minutes was then allowed for complete precipitate formation after which the tubes were centrifuged for 5 mins at the top speed of a bench centrifuge to pellet the precipitates. The supernatants were then assayed for phenolics as previously carried out in section 3.2, and thereafter they were discarded. The pelleted material was then re-dissolved in 4ml of 4M NaOH and heated for 4 hours in a boiling waterbath. At the end of this period the hydrolysate produced was neutralised with 4M HCl and assayed for amino nitrogen by the method of Moore and Stein (1954, 1968) as described in Appendix 2.3. It is of note that none of the extracts themselves (hydrolysed or unhydrolysed) gave detectable reactions for amino nitrogen when tested at the concentrations employed in the experiments.

Experiments were repeated with tannin solutions of an

appropriately altered concentration where previously either no precipitation occurred or where no levelling off in the amount of protein precipitated was seen as the higher BSA concentrations were reached. The 0-5mg/ml concentration range for BSA employed in the experinents was thus used as a "window" through which maximal levels of precipitation were found by adjusting the concentration of tannin added to the assays. This resulted in a considerable amount of trial and error experimentation, the reasons for which will be discussed in the following section.

4.2,2 Results

Of the eighteen extracts studied, the data for four are plotted in Figures 4.1 to 4.4. Each figure shows the data for the protein precipitated by the tannin as the protein concentration rises from 0.0 to 5mg/ml. Also shown is the concomitant change in the level of phenolics which remain in the supernatant solution. The expected outcome to the experiments is seen in the results obtained for quebracho tannin (Figure 4.1). Here there is a rise in the precipitation of protein in the experiment up to a plateau beyond which there is no more precipitation as the concentration of protein in the reaction increases, the excess remaining in solution. This is exactly the situation revealed by Hagerman and

Legend for Figures 4.1 to 4.4: Results for Specific activitiies

Key: The scales marked H&B and points marked as dots indicate the absorbance in the total phenolics assay of the supernatant fractions from the experiments. The scale marked NIN and points marked X indicate the absorbances of 100μl samples of the hydrolysed precipitate solutions in the ninhydrin reaction. The x axis indicates the volume of 5mg/ml BSA present in the assays. Horizontal bars indicate the data used for the calculations presented in table 4.1 & 4.2. See text for details of the assay and its interpretation.

Figure 4.1 Specific Activity Results for Quebracho tannin.

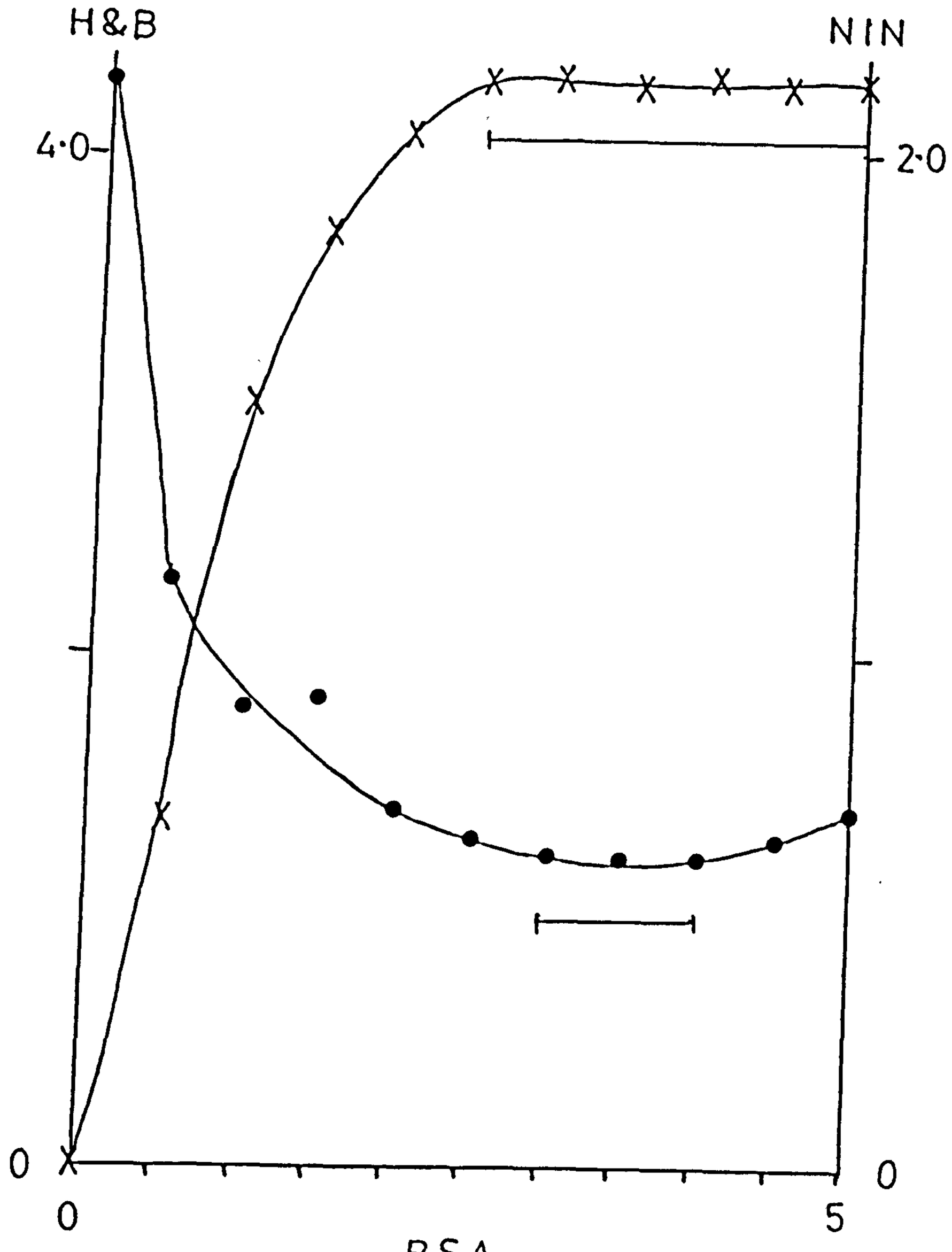


Figure 4.2 Specific Activity Results for Tannic acid.

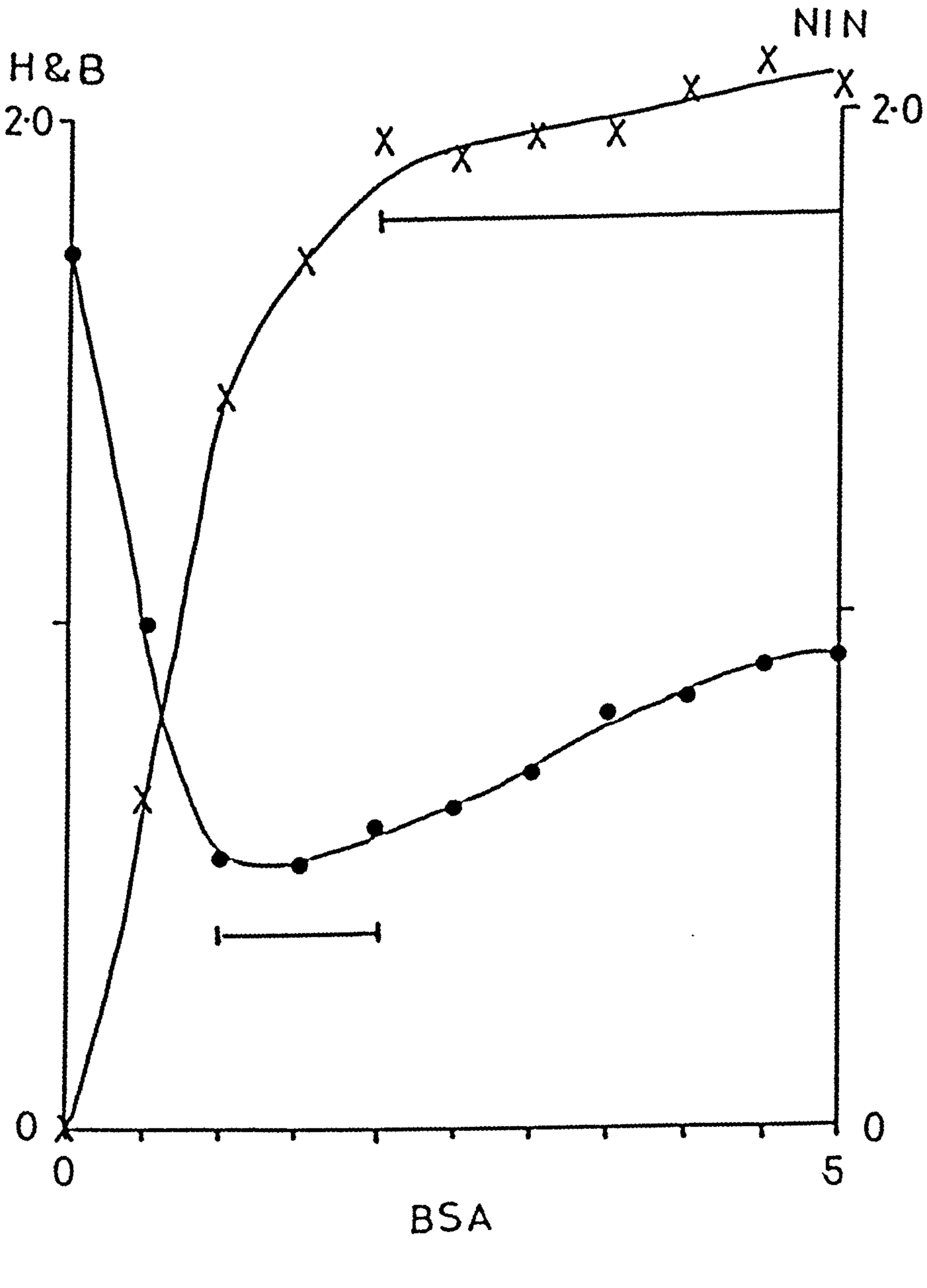


Figure 4.3 Specific Activity Results for

Pinus radiata tannin.

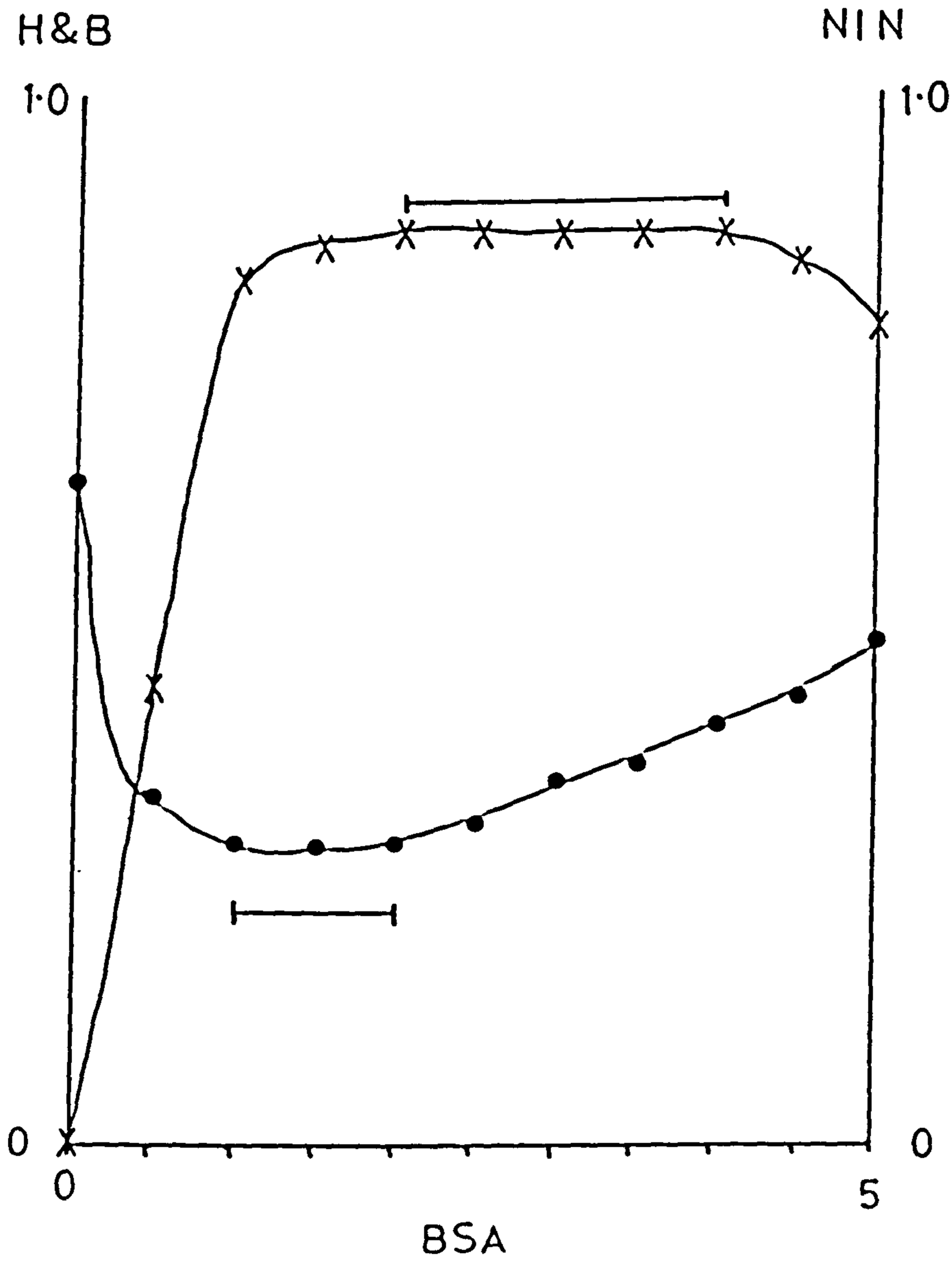
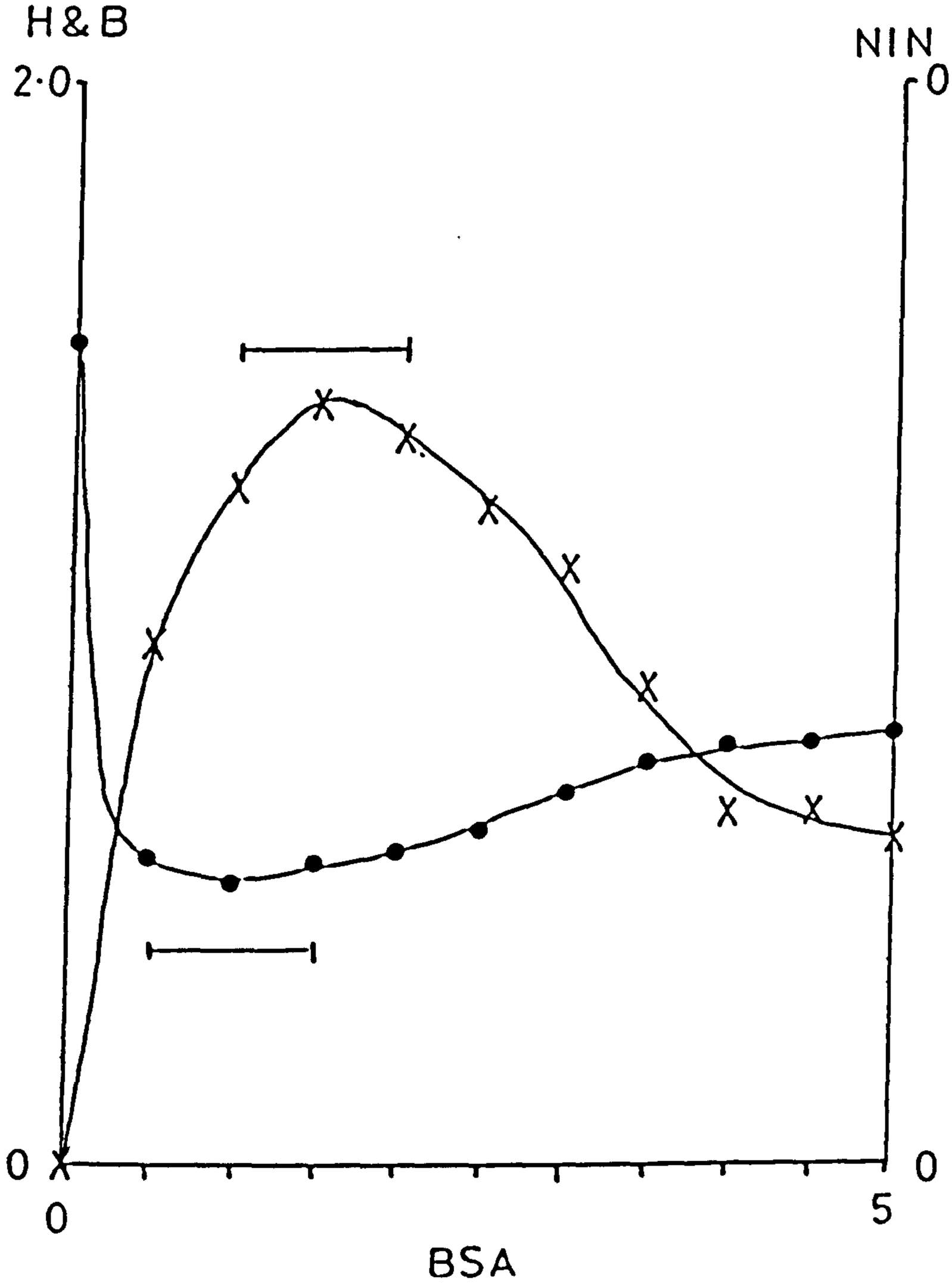


Figure 4.4 Specific Activity Results for

<u>Quercus</u> sp. tannin.



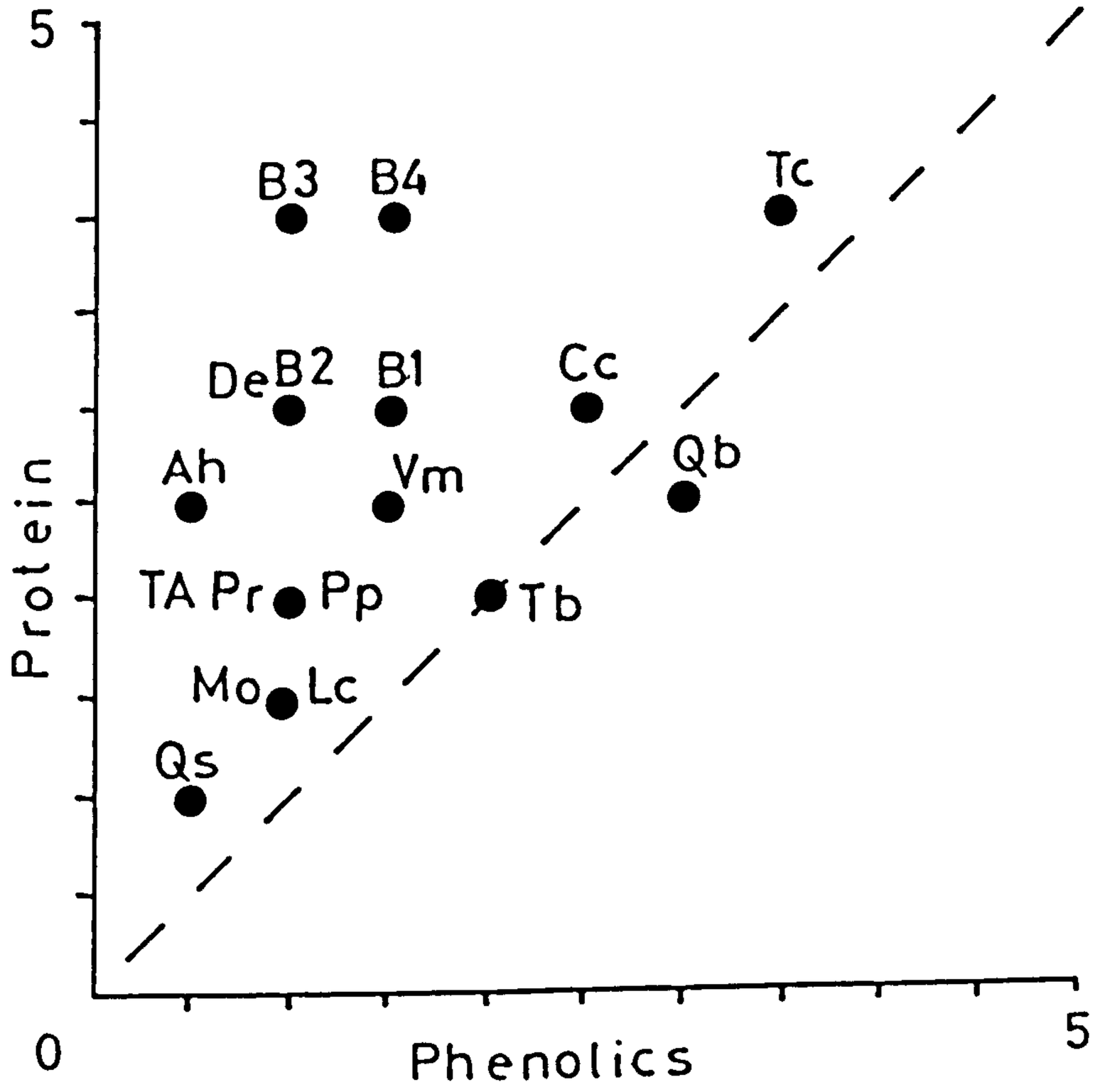
Butler (1980a). The parallel fall in soluble phenolics, which they did not record, does not exactly follow the pattern seen in the curve for protein. There is no flat minimum, but rather a curve which begins to rise at the higher levels of protein. This tendency indicates that at the higher protein concentrations, that protein which is in the precipitate is associated with less and less tannin whilst phenolics which were demonstrably tannins by virtue of their inclusion in a precipitate are tending to occur in solution with protein. Such a scenario is more in keeping with the results of Van Buren and Robinson (1969) in which they reported redissolution of tannin-protein complexes in the presence of an excess of either component. As a final observation, it should be noted that the level of maximum protein precipitation occurs at a lower concentration of protein than does maximum phenolic precipitation.

The results for tannic acid (Figure 4.2) are similar to the extent that there is a rapid rise in protein precipitation followed by a "levelling off", but not to a flat plateau. The curve for supernatant phenolics shows a clear minimum attained after an initial and steep fall. Thereafter there is a clear rise, again indicating that with rising protein concentration more protein is associated with less tannin in the precipitate. It proved impossible to manipulate experimental conditions

in order to obtain a flat plateau maximum for protein precipitation. Difficulty with this was also found with results for several other extracts and a possible cause is the entrapment of protein solution within the precipitates, some of which were quite bulky. Unlike quebracho, the minimum for supernatant phenolics occurs before the levelling off for protein precipitation.

Figures 4.3 and 4.4 show results for extracts Pr and Qs respectively and illustrate the range of results obtained in the survey. For Pr a plateau in protein precipitation is seen but there is a clear fall at high protein concentrations, presumably as the decline in the ratio R(p) (as deffined in Table 3.2) approaches a minimum where precipitates are unstable. Figure 4.4 shows an extreme case of this, where a clearly peaked response is seen in the curve for protein precipitation. This figure illustrates the same type of curve seen in Van Buren and Robinson's (1969) Figure 2. In both Figures 4.3 and 4.4 the minimum in supernatant phenolics again occurs before the maximum level in protein precipitation is reached. The decision as to where these maxima and minima occur is somewhat subjective; however, collecting the data for all the extracts is interesting in that there is a clear asymmetry to be seen in that Quebracho is the only extract where maximal protein precipitation is attained before maximum phenolic precipitation (see Figure 4.5 for

Figure 4.5 Plot of the lower limits to the ranges for precipitation maxima seen in specific activity experiments.



Vertical and horizontal axes are callibrated in ml of 5mg/ml BSA, i.e. identically to the x axes of figures 4.1 to 4.4. Lower limits to precipitation maxima for protein are plotted against those for phenolics.

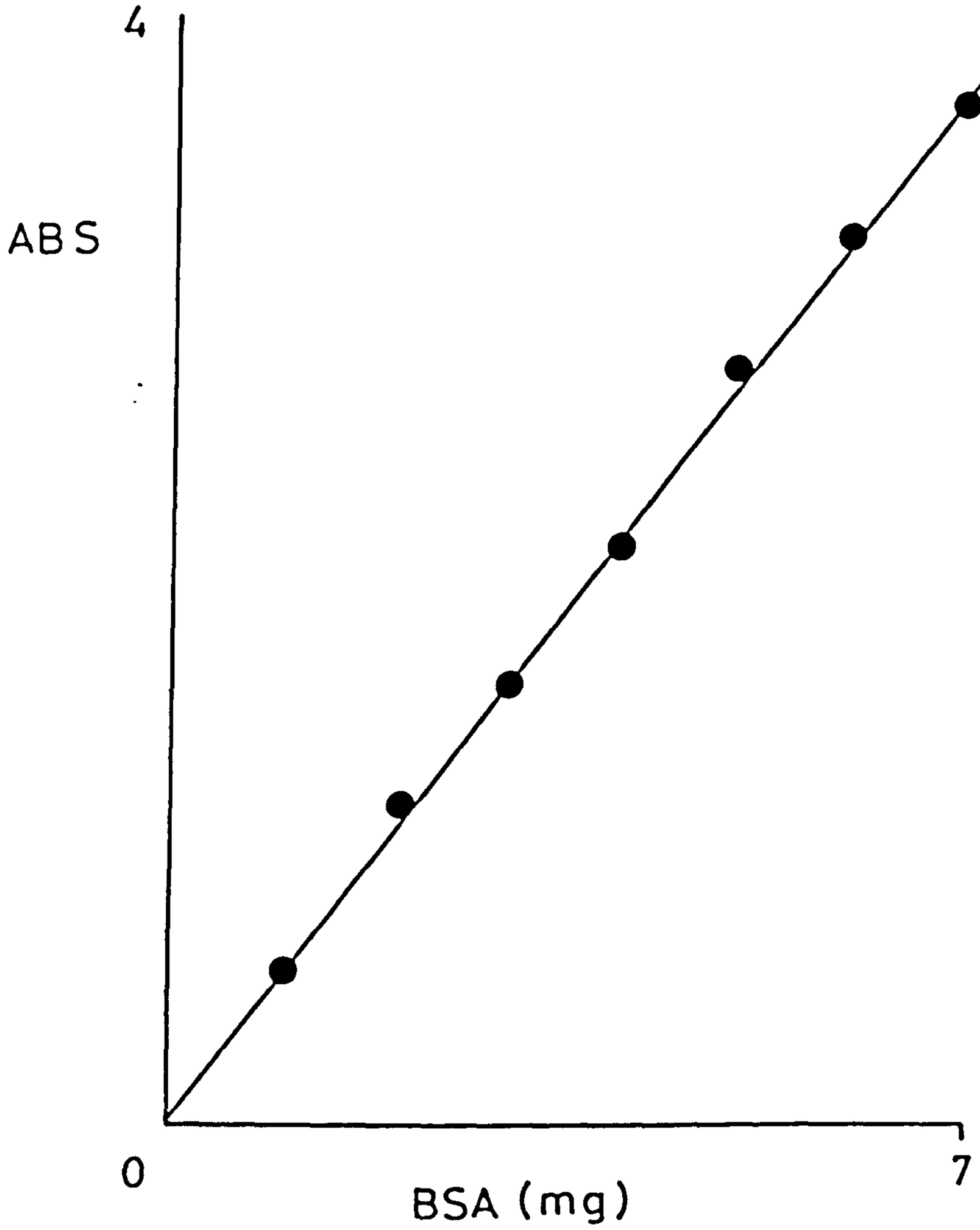
a scatter plot of the data). This reinforces the notion that declining R(p) occurs generally for all the tannins tested and that this will lead to the ultimate decline in precipitate formation as the excess of unprecipitated protein rises in the system. From a methodological point of view, the combination of threshold precipitation effects and precipitates redissolving in excess protein make this protein precipitation assay difficult when working with a range of tannins. For example, it proved quite impossible to conduct all the experiments using a concentration of tannin set at a given level of total phenolics and yet expect to obtain precipitation maxima within the range of protein concentrations used. The results of this can clearly be seen in Table 4.1 where the concentrations of phenolics employed with each extract tested are recorded.

The objective of this work was to estimate the specific activity of the extracts as tannins on a per total phenolics basis. To this end two measures for this have been calculated. The first follows Hagerman and Butler (1980a) and calculates specific activity (SA1) as the maximum amount of protein precipitated in the assay divided by the total phenolic content of the tannins present in the system (i. e. supernatant and precipitate), this last value (TPS) being obtained from their absorbance in the measurement (Hagerman and Butler,

1978) of phenolics. The value used for the protein precipitated is that of the ninhydrin assay for hydrolysed protein averaged over the values surrounding the highest level attained (as indicated in the figures) and converted into mg BSA by reference to a calibration curve (Figure 4.6). Table 4.1 provides the data used to calculate specific activities for all the extracts, as derived from plots of experimental data such as displayed in Figures 4.1 to 4.4.

Caution has already been expressed that the phenolics present in a precipitate with protein do not necessarily constitute all the phenolics capable of acting as tannins in the extract used to cause the protein to precipitate. Nevertheless, given that some non-tannin phenolics are likely to be present as impurities in the extracts, a second specific activity measure was calculated (SA2). SA2 uses values calculated for the minimum amount of soluble phenolics in the experiments (calculated at the precipitation maxima, see Figures and Table 4.1) these then being used to express the maximum proportion of the total quantity of phenolics present which enter into precipitates (%T). This achieves a best estimate of functional tannins. The estimate SA2 expresses the activity of the extract in terms of these phenolics alone and is calculated as (SA1 / %T) * 100%. The results for all these variables (SA1, SA2, %T, TPS) are presented

Figure 4.6 Calibration curve of ninhydrin reaction results as a function of hydrolysed bovine serum albumin (BSA).



The absorbance (ABS) recorded at 570 nm in the ninhydrin assay is plotted against the weight of hydrolysed BSA present in the solution sampled (e.g. 100μl samples of hydrolysed precipitate solutions in the specific activity assay).

Table 4.1 Specific activity assays, summary data. This is a tabulation of data derived from plots of experimental results as exemplified by figures 4.1 to 4.4

		Protein (NIN)					Phenolics (H&B)				
Tannin	Code	Range		$\bar{x}$	σ	n	Range		$\bar{x}$	σ	n
Aesculus hippocastanum	Ah	2.5	5+	0.17	0.000	5	0.5	2.5	0.17	0.008	5
Callistemon citrinus	Cc	3.0	5+	1.20	0.067	5	2.5	4.0	0.89	0.000	4
Diospyros ebenum	De	3.0	5+	1.13	0.019	5	1.0	2.5	0.23	0.028	4
Fucus vesiculosus (*)	Fv	1.5	5+	1.06	0.020	8	FD assay used, equiv. $\bar{x}$=0.012				
Loropetalum chinense	Lc	1.5	2.5	1.16	0.018	3	1.0	2.0	0.24	0.031	3
Myrtus obcordata	Mo	1.5	4.0	1.03	0.069	6	1.0	2.0	0.60	0.004	3
Pomaderris phylloides	Pp	2.0	5+	0.80	0.036	7	1.0	2.0	0.26	0.009	3
Pteridium aquilinum	B1	3.0	5+	1.43	0.040	5	1.5	5+	0.34	0.008	8
Pteridium aquilinum	B2	3.0	4.0	0.28	0.046	3	1.0	5+	0.77	0.018	9
Pteridium aquilinum	B3	4.0	5+	0.83	0.013	3	1.0	5+	0.25	0.016	9
Pteridium aquilinum	B4	4.0	5+	1.17	0.014	3	1.5	3.0	0.29	0.004	4

Table 4.1 continued

Quercus sp.	Qs	1.0	2.0	0.53	0.025	3	0.5	1.5	0.22	0.010	3
Taxus bacata	Tb	2.0	5+	0.80	0.045	7	2.0	5+	0.70	0.180	7
Vaccinium myrtilis	Vm	2.5	5+	1.13	0.015	6	1.5	5+	1.00	0.010	8
Tannic acid	TA	2.0	5+	2.00	0.060	7	1.0	2.0	0.56	0.030	3
Quebracho tannin	Qb	2.5	5+	2.15	0.010	6	3.0	4.0	1.22	0.010	3
Terminalia chebula	Tc	4.0	5+	1.98	0.060	3	3.0	5+	1.89	0.040	5
Pinus radiata	Pr	2.0	4.0	0.87	0.000	5	1.0	2.0	0.29	0.004	3

Notes: for both "protein" and "phenolics" the ranges of BSA concentrations in the initial reaction mixtures for which precipitation maxima/minima occur are given (identical to ranges indcated by horizontal bars on figures 4.1-4.4). The means ($\bar{x}$), standard deviations (σ) and number of data points used (n) are statistics calculated from the data within these ranges.

* The (equivalent) value for $\bar{x}$, calculated from a Folin-Denis assay, was used as the H&B method proved insufficiently sensitive to the very low phenolic content of the extract.

in table 4.2.

The first comment to be made is that the value of SA1 for tannic acid is very close to that reported by Hagerman and Butler (1980a), as seen when the present value is corrected by a dilution factor accounting for differing volumes of reagent used in the phenolics assay and thus becomes 2.78. Compared to the other extracts described in Chapter 2, the value of SA1 for TA is just over half the maximum value recorded, whilst the minimum value is less than 20% of the value for TA. There is thus a clear spread of values for the variable SA1 and inspection of Table 4.2 shows a similar range for SA2. Values of SA2 are all higher than those for SA1 as values of %T are all less than 100% (range: 22% to 72%). There is no correlation between SA1 and %T evident in the data, thus indicating that a tannin or extract may be active as a precipitant whilst not necessarily being subject to precipitation in proportion to this activity. Indeed, such a relationship would not have been expected. Comparing the data in Table 4.2 to that for chemical analyses tabulated in Chapter 2 shows that not a single chemical variable correlates well with the measures of specific activity. Of note is the absence of any relation with measures of polymer length or of the percentage of condensed tannin in the extracts. As it was concluded that procyanidin-rich condensed tannins

Table 4.2 Specific Activity Results*

Tannin	Code	TPS	%T	SA1	SA2
Aesculus hippocastanum	Ah	0.25	31	1.61	5.20
Calistemon citrinus	Cc	1.79	50	1.59	3.18
Diospyros ebenum	De	0.79	71	3.36	4.73
Fucus vesiculosus +	Fv	0.028	41	37.9	91.4
Loropetalum chinense	Lc	0.80	70	3.42	4.89
Myrtus obcordata	Mo	1.10	45	2.22	4.92
Pomaderris phylloides	Pp	0.54	52	3.49	6.72
Pteridium aquilinum	B1	0.64	47	5.27	11.2
Pteridium aquilinum	B2	1.30	40	0.51	1.27
Pteridium aquilinum	B3	0.37	32	5.30	16.6
Pteridium aquilinum	B4	0.70	59	3.94	6.68
Quercus sp.	Qs	1.51	63	0.83	1.32
Taxus bacata	Tb	0.96	27	1.96	7.25
Vaccinium myrtilis	Vm	1.29	22	2.66	12.1
Tannic Acid	TA	1.74	68	2.71	3.99
Quebracho tannin	Qb	4.30	72	1.18	1.64
Terminalia chebula	Tc	3.72	49	1.25	2.56
Pinus radiata	Pr	0.63	54	3.26	6.04

* Notes: TPS, %T, SA1 and SA2 are as defined in the text. catechin and gallic acid do not form precipitates.

were the predominant feature in the phenolic components of the extracts (Section 2.6) these results are disappointing.

It can be argued that despite the numerical variation in the values of SA1 and SA2 perhaps this is actually a narrow range in terms of the possible activities of tannins. This view is supported by results for an extract of the seaweed Fucus vesiculosus. Brown algae are known to contain phlorotannins (see Chapter 2) which are, from a chemical point of view, quite unlike hydrolysable and condensed tannins. Reports concerning these tannins (Ragan, 1981) have stressed their instability so a solution of phlorotannin was made fresh by homogenising some live seaweed in the pH 4.9 buffer used for the specific activity experiments and then proceeding to use this in the standard assay. The SA1 and SA2 values obtained were 37.9 and 91.4 respectively. These results demonstrate that the results previously obtained do not span the range detectable with the assay whilst also emphasising the similarity in specific activity of these other extracts, rather than their differences. However, the fact that fresh seaweed was used in these experiments may be causal as regards the SA1 and SA2 values.

Considering data for individual extracts does provide some helpful results. The Diospyros extract De was

suspected of containing hydrolysable tannin and so the low %CT1 value (=23%) should not have indicated low astringency and indeed both SA1 (=3.36) and %T (=71) indicate a substantial amount of tannin present in the extract. In contrast, extract Tc, with a (suspected) low concentration of hydrolysable tannin and no condensed tannin, has lower specific activities than TA and De. The data is also clearly indicative of the presence of other precipitable phenolics in some extracts though these do not necessarily add to specific activities. For example, consider the data for B1 (%CT1=6%, %T=47%, SA1=5.27) and B2 (%CT1=6%, %T=40%, SA1=0.51). Conversely, extracts with %CT1 and %CT2 values of around 100% (Ah, Pp, Tb, Pr) have comparitively low proportions of precipitable phenolics (%T values).

4.2,3 Discussion

The results outlined above confound the belief that satisfactory interspecific comparisons of plant tannins can be made by the use of chemical analysis if the ultimate objective is to make inferences concerning tannin-protein interactions. This follows because of the range in specific activities found for extracts of equal phenolic concentrations, and owing to the non-correlation between these activities and other chemical variables.

These results which make use of a comparatively

diverse range of chemical techniques, reinforce points previously made by Martin and Martin (1983) in justfying the use of protein precipitation assays to measure tannins. This is not to say that intraspecific measurements cannot be based on chemical measurement techniques, but here errors will obtain if the components making up the plant phenolics change betwen samples.

It could be argued that if chemically pure and known sources of tannin had been used in this work, then some correspondence between (say) condensed tannin polymer length and specifc activity might have been obtained. This does indeed seem probable (see Section 1.1) however, this is to miss the point that herbivores do not consume pure chemicals but crude extracts. The importance of this work has been to find out if these differences can be distinguished against the background of the other phenolics present in the plant. Having failed to do this, there is no justification from an ecological point of view in examining the specific activites of the component phenolics of an extract individually unless the commitment is made to do this exhaustively for the whole extract and to consider synergism betwen components.

On the positive side the results indicate a successful method for measuring the activities of tannin-containing extracts and several important sources of tannin-tannin variation have come to light. While the difference in

activity between the algal and tracheophyte tannins is of considerable interest, there are also differences within the group of tracheophyte tannins needing further consideration. The existence of peaked responses seen in the curves for protein precipitation indicates that for some tannins the conditions where precipitates form are quite narrow. Given that the assay conditions are designed to be very favourable to precipitate formation then perhaps values for SA1 and SA2 are not the only biologically important variables, and parameters such as %T and threshold precipitation levels (very approximately indicated by TPS) should be included when assesing the potential effect of a tannin in a biological environment. Such an investigation would not only need eventual in vivo testing, but also its utility is crucially dependent on the notion that precipitation phenomena are central to investigating the role of tannins in the environment, an assumption brought into question in Chapter 5.

4.3 Tannins in Cellulose/Cellulase Systems

In contrast to the protein precipitation techniques which make for the most accurate detection and determination of tannins in terms of their operational definition, this section is concerned to measure the impact of tannins on digestive systems. Whilst the notion that tannin-protein precipitates are indigestible is long standing (Feeny, 1969; Rhoades, 1977a) it is curious that measurements of tannins in proteolytic or other digestive systems have been entirely abandoned in favour of protein precipitation techniques. By returning to a digestive system, the divide between the *in vitro* and *in vivo* situations is narrowed and inferences about the latter from the former may be more reasonably made.

The choice of a cellulolytic system is not immediately obvious especially as a range of other enzyme inhibition methods has been tried and tested. An early method was that of Goldstein and Swain (1965) who studied the inhibition of β-glucosidase and its hydrolysis of aesculin. Although not a reaction of such nutritional importance as trypsin proteolysis, this study was important as it indicated that by forming a precipitate with the enzyme, tannin inhibited its activity, but a point overlooked by most later workers was that when resuspended (but not dissolved) the precipitated

tannin-protein complex retained 60% of its activity (Goldstein and Swain, 1965). Indeed, this phenomenon has been exloited industrially in the use of tannins bound to solid media to adsorb and immobilise enzymes for large scale biomimetic syntheses (Watanabe et al., 1979). The clear message here is that the immoblisation of an enzyme will not necessarily inhibit its activity if it can still interact with its substrate. As protein-protease systems appear different from the above systems in that both components of the reaction may be immobilised by the tannin, they are not directly invstigated here. Nevertheless, the work of Hagerman and Butler (1981) indicating the clear specificity exhibited by tannins in binding to proteins, shows that even in systems containing two or more proteins only one may be involved in tannin binding. Such two protein systems are the subject of investigation in the next chapter, here the object has been to concentrate on single protein systems of nutritional importance.

One such system considered was the starch-amylase reaction. However, this has two major drawbacks as a technique. Firstly, tannins adsorb on to starch and inhibit the reaction in this way (Davis and Hoseney, 1979). Secondly, methods devised to examine the influence of tannins on this reaction (Wint, 1983; Cooke et al., 1985) suffer from the interference of tannins in

the measurement of starch by the iodine indicator, making the course of the reaction difficult to follow.

Cellulolysis is of major importance not just for soil microbial degradation but also for its role in ruminant and ruminant-like digestive systems. Indeed, such digestive systems are reported in a diverse range of mammalian herbivores (Moir, 1968). Not only is the reaction nutritionally important but it has practical advantages for study. These include the use of the insoluble nature of the cellulose substrate to compare the inhibitory effects of tannins adsorbed to it separately from those free in solution.

A convenient method for measuring the saccharification of cellulose by cellulases is by substrate weight loss, and indeed this is the method used in studies measuring forage digestibility in cellulase solutions (Jones and Hayward, 1975). A well established procedure involves a pepsin digest as a pretreatment prior to cellulase digestion. This method (Jones and Hayward, 1975) has been applied to assessing the digestibility of a wide range of herbivore forrage and browse and has been generally reported as a good method for indicating ruminal digestability in both agricultural and natural systems (Choo, 1981; Faithful, 1985).

4.3,1 The Pepsin/Celulase Digestibiliity Assay

The assay described below follows the procedures employed by Choo (1981) who in turn adopted the method of Jones and Hayward (1975). The basic procedures are detailed in Appendix 2.4. Where this work differs from that previously reported is in artificial rather than natural substrates being subject to digestion.

Several sources of cellulose were tried as substrates in the assay, these being various papers and cellulose chromatography media. Whatman 3M chromatography paper was found to lose an easily measurable weight (see below) and yet provide a residue of solid which could conveniently be collected by Buchner filtration onto preweighed filter papers. Also as the substrate was present as one piece of paper, not ground material, enzyme solutions could be changed by carefully decanting off one solution and replacing it with another.

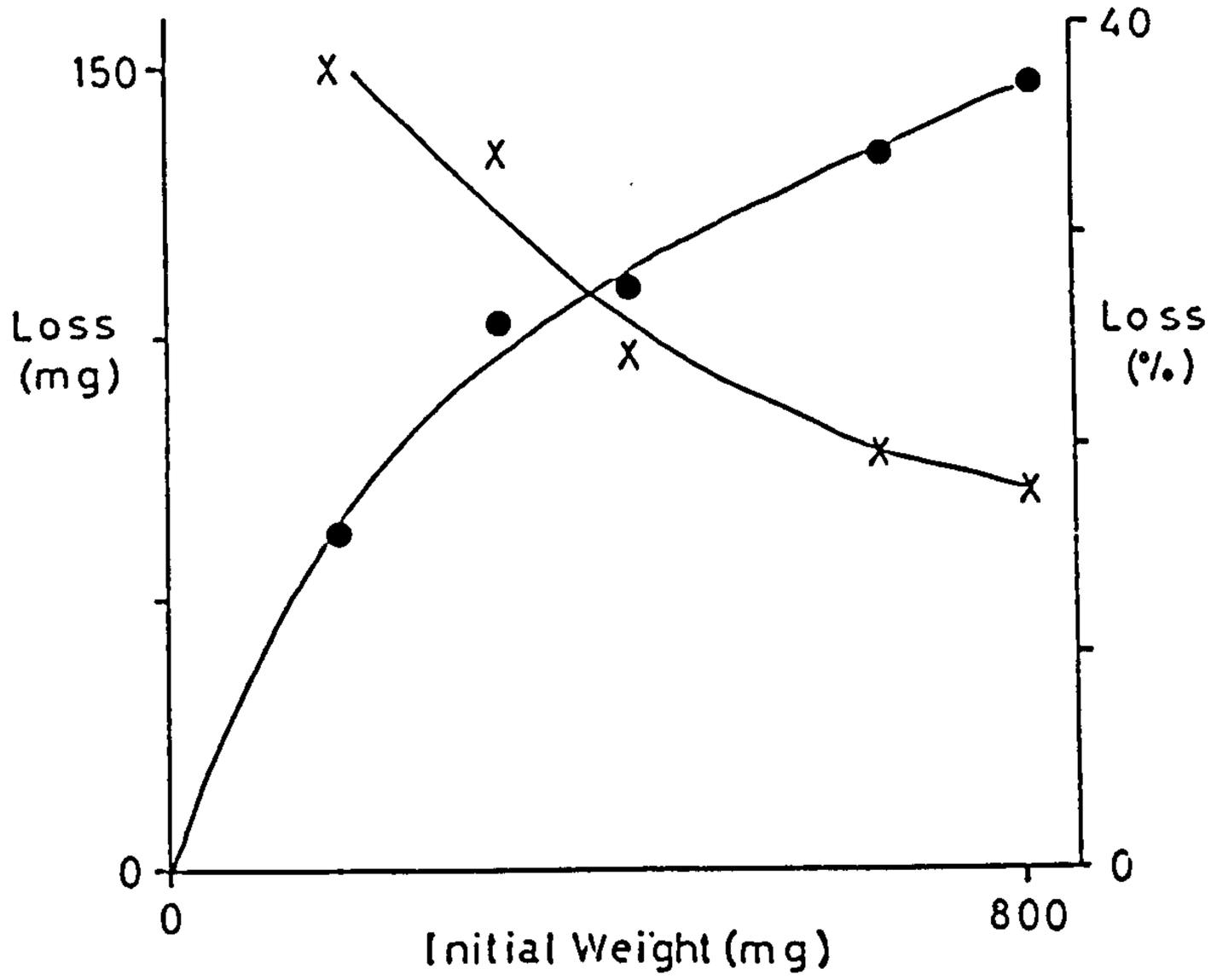
The first set of experiments carried out were digests of untreated chromatography paper (hereafter referred to as "paper"). The object was to find out if the 200mg samples of material employed by Choo (1981) saturated the 20ml of cellulase enzyme used in the assay. To this end strips of pre-weighed paper of different weights were digested in the standard cellulase stage of the assay and their percentage weight loss was recorded as a function of their initial weight. The results of this experiment

are diplayed in Figure 4.7. While increasing net weights of paper were digested as sample sizes increased, the proportion of paper digested decreased. Ideally the experiment should have been extended to a level where no increased weight loss was seen as sample size was increased. This limit was approached in the experiments but larger samples of paper were difficult to put into the sample tubes and cover with enzyme solution.

As noted by Choo (1981) the fungal cellulase preparation used here is a crude product made from Trichoderma viride, and a source of various hydrolytic cellulases, exo- and endo- β-gluconases and some hemicelulases. Clearly the cellulase solution could not entirely degrade the paper but increasing the paper sample size above 200mg did increase the net weight loss and so a 200mg sample did not saturate the enzyme with degradable material. As enzyme inhibition is most effectively measured when the enzyme is substrate saturated and as larger weight changes are more easily measured, substrate sizes used in the experiments were set at between 0.8g and 1g, with data for percentage weight loss being used in the ensuing computations. Digests of untreated paper lost 18-20% in the assay.

After making this adjustment to aid sensitivity a second set of preliminary experiments were made to investigate the role of the pepsin pretreatment. It

Figure 4.7 Weight loss of cellulose in cellulase digestion as a function of initial sample weight

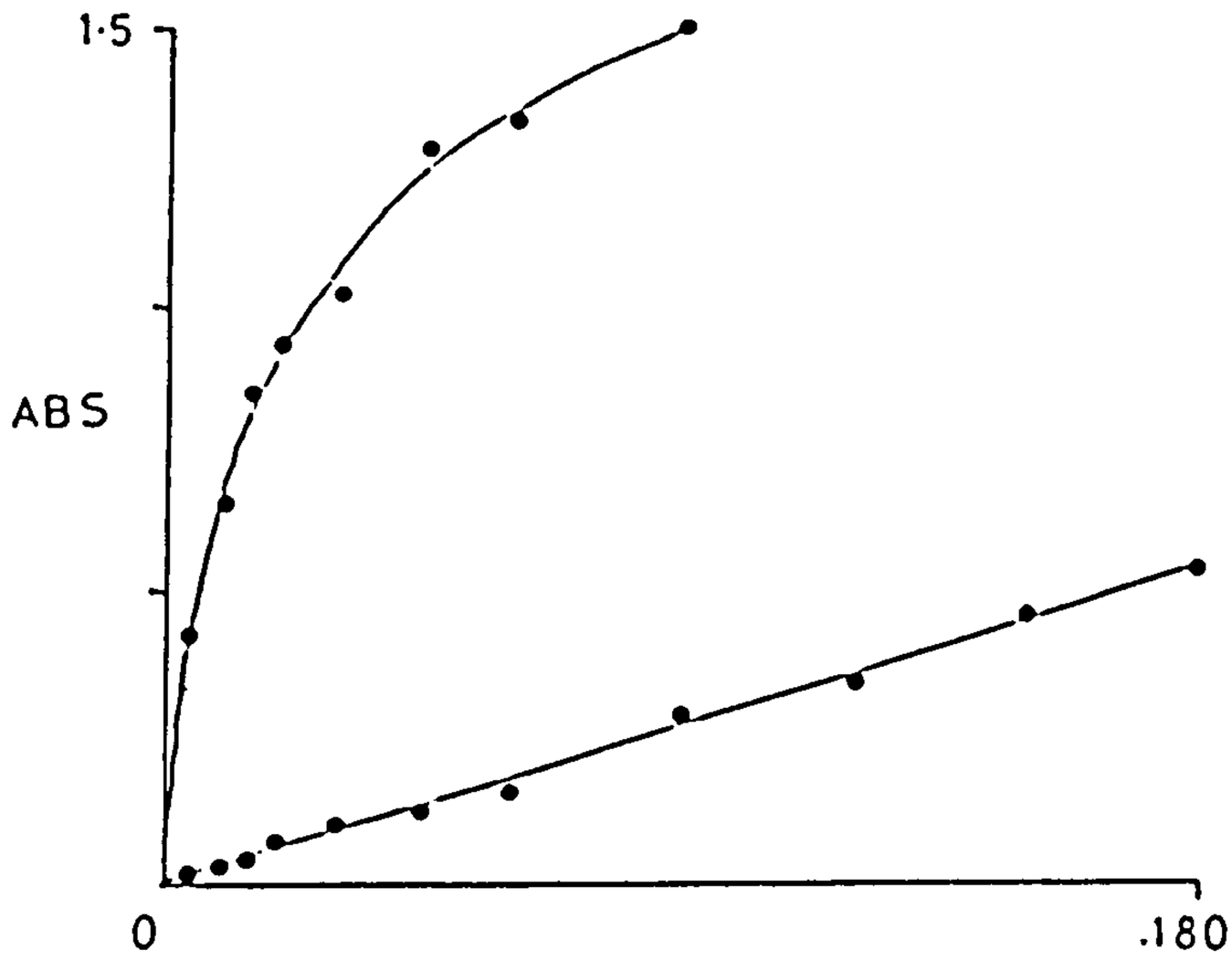


Weight loss expressed in mg and also as a percentage of the initial weight of the sample.

seemed curious that pepsin pretreatment could be of use for two reasons: firstly, because plant protein is likely to be revealed to proteases after, not before, cell wall degradation and, secondly, because residual pepsin trapped in the samples shculd hinder the action of the cellulase. Jones and Hayward (1975) provided two explanations as to why pepsin was of use. They argued that pepsin did remove protein and reveal cellulose to the cellulase and, further, that pepsin in some other way altered the surface nature of the cell so as to aid its subsequent degradation. The latter argument seemed particularly implausible both because of the function of pepsin as a protease and because they could provide no evidence for it. The former argument does, however, seem to warrant attention. Indeed, Jones and Bailey (1972) had previously examined this when comparing freeze dried versus oven dried herbage samples in cellulase-only digestion. However,even though oven drying did denature and insolubilise leaf protein no differences in cellulase digestibility were found, the comparability being attributed to protease activity found in the cellulase used.

The present experiments used the same supply of enzyme (BDH) and its protease activity was compared to that of the pepsin used in the pretreatment. Figure 4.8 shows the results of a digestion of BSA by the pepsin and

Figure 4.8 The digestion of bovine serum albumin (BSA) by the enzymes used in the pepsin/cellulase digestibility assay.



The absorbance (ABS) of trichloroacatic acid soluble products from proteolysis assayed using the ninhydrin method (as in Section 5.2) is plotted against time for a reaction incubated at 30°C. The upper curve is for a solution containing 20 mg/ml BSA and 2mg/ml pepsin at pH=2. The lower is for a solution containing 20 mg/ml BSA and 5 mg/ml T. viride "cellulase" at pH=4.8.

"cellulase" enzymes. Whilst the pepsin is by far the stronger source of protease activity, it was calculated that after a 48 hour digest over 150 mg of BSA would be degraded, far more protein than was likely to be found in a 200mg sample of leaf material. In the light of these results it seemed quite unreasonable to regard pepsin pretreatment as necessary to remove interfering protein from the leaf.

Recalling comments made in Section 1.2, concerning the occurrence of cellulose masking by tannin-protein complexes which render plant cell walls resistant to decay, this phenomenon seemed a suitable reason for pepsin pretreatment. Although not previously acknowleged to have such a role (Faithful, 1985), in such circumstances additional protease treatment might aid the necessary removal of the mask. Given that the pepsin stage is carried out in a buffer of pH 1.35 where tannins will precipitate pepsin (pI=2), then its protease activity will almost certainly be inhibited to some extent. However, given the high specificity between tannin and pepsin there is a real possibility that the pepsin solution aids the dissolution of tannin from the sample. This tannin is then removed with the pepsin solution before incubation with the cellulase, and so removed as a cause of variation in the cellulolytic weight loss.

After making the above analysis, experiments were designed to (i) investigate how tannins might inhibit cellulase digestion and then to (ii) compare the ability of the tannins characterised in section 4.2 to inhibit cellulase.

4.3,2 Cellulase Inhibition and Cellulose Masking

The first set of experiments introduced tannins into the assay using paper impregnated with either tannic acid or quebracho tannin. As a variation a replicate set of substrates was made with paper first impregnated with a protein solution (casein). The procedure used to prepare the substrates was as follows. Pieces of paper were cut into 15 x 15 cm squares and paper used for untreated "control" experiments were soaked in a tray containing 150ml of distilled water which covered the base of the tray in a shallow layer. The tray was kept agitated by being placed on an orbital mixer and allowed to soak for 10 minutes, at the end of which time the paper was removed, drained of excess fluid and then placed flat in an oven to dry at 80^{o}C. For those papers to be impregnated with tannin the water was replaced by a solution of tannin. Papers receiving a pre-treatment with protein were first soaked in a casein solution for 10 minutes, drained and then transferred to the tannin solution without drying. "Protein only" treated papers

were dried immediately after the soak in casein solution. The rationale behind this process was to mimic the production of dried leaves, the normal material used in the pepsin/cellulase digestibility assay. Making a dry material also gave the advantage of being able to store the substrate and use the same preparation in several experiments. Strips of the papers of about 2.5cm wide were weighed and then roled into cylinders and placed into specimen bottles to which the enzyme solutions were added in the experiments. Adding the enzyme let the paper uncoil and no problems were seen with uneven digestion.

In the following experiments the casein solution used was 1% in concentration and both tannins were used at the following solution concentrations 2.0%, 1.5%, 1.0%, 0.5% 0.25%. Each substrate prepared was tested in three treatments: (i) the standard assay with a pepsin pretreatment before the cellulase, (ii) with a buffer only pretreatment at pH 1.35, (iii) without any pretreatment, i.e. cellulase only. The substrates prepared were (i) either with or without protein, (ii) either with tannic acid or with quebracho and (iii) prepared at one of the five tannin concentrations. This then provided for a set of 3 (treatments) x 2 (tannin) x 2 (protein) x 5 (concentration) = 60 assays, the results of which were analysed by ANOVA techniques.

Dividing the 60 assays according to the tannin they contained, the mean percentage weight loss for quebracho impregnated papers was 12.9% (σ=2.4%) versus 4.6% (σ=2.4%) for those containing tannic acid, this difference being highly significant ($p>.999$). This source of variation accounted for 76% of the total in the ANOVA making further comparisons within the entire data set difficult.

Considering the blocks of 30 observations on each tannin separately, and considering weight loss as a function of tannin concentration, weight loss decreases as more tannin is present in the bathing solution in which the paper was prepared (see Table 4.3 for summary data). Even where quebracho is at double the concentration of tannic acid and therefore at a similar level of total phenolics, quebracho-containing substrates are more digestible. This difference is the reverse of that expected by Zucker (1983) who believed that condensed tannins were likely to be better at binding to cellulose and therefore potentially more inhibitory to its degradation. When the influence of the various treatments and the presence or absence of protein is examined (see Table 4.3) further sources of variation are uncovered. In the experiments carried out with quebracho, those substrates containing casein showed the higher weight losses. The cause of this could have been

Table 4.3 Effects of tannin concentration, protein and experimental treatment on cellulose digestion.

I Mean weight losses (%) for substrates grouped by the tannin and then by (i) tannin concentration and (ii) the experimental treatment given.

(i) Tannin concentration*	Quebracho	Tannic acid
0.25	14.0 (0.87)	7.7 (0.23)
0.50	14.3 (0.20)	4.4 (0.69)
1.00	13.1 (0.61)	3.8 (0.46)
1.50	12.0 (0.81)	4.7 (0.61)
2.00	11.1 (0.89)	2.4 (0.46)
(ii) Experimental Treatment		
pepsin + casein	13.9 (0.26)	5.0 (0.60)
buffer only + casein	15.2 (0.25)	4.5 (0.70)
cellulase only + casein	12.7 (0.70)	3.2 (0.79)
pepsin + no protein	12.1 (0.82)	6.3 (0.66)
buffer only + no protein	11.9 (1.04)	5.3 (0.53)
cellulase only + no protein	11.4 (0.66)	3.4 (0.66)

ln1

N.B. Figures in parentheses are standard errors.

* Concentrations (% w/v) reffer to the solutions used to preparae the substrates.

II ANOVA treatment of the data

source	df	SS	MS	F	p
TA v.s. Qb	1	0.102525	0.102525	179.4	0.9999
Error	58	0.033147	0.000572		
Total	59	0.133147			

Note: ANOVA calculations follow Campbell (1974).

the simple elution of protein from the paper, but interestingly, the reverse is seen with tannic acid i.e. notwithstanding any elution loss, the presence of protein promotes tannic acid in its inhibition of cellulose digestion. A more efficient use of the data can be made when a two way ANOVA is employed. The result of such an analysis is presented for each tannin in Table 4.4. Considering the presence/absence of protein and the treatments as one source of variation and tannin concentration as the other, both sources are seen to contribute significantly to the variation in the data as detected in the ANOVA.

With the apparent detection of cellulose masking seen in the tannic acid plus casein impregnated paper, a re-examination of the whole data set (60 values) was made in a two way ANOVA to examine the contribution of tannin and protein as sources of variation. The results of this analysis are presented in Table 4.4 and as expected tannin type is a highly significant contributor to the variation in the results. Presence or absence of protein as anticipated by Jones and Bailey (1972), is not a significant contributor but the interaction term between the two sources of variation is a significant source of variation. In summary, this experiment provides an indication that cellulose masking can occur and enhance the inhibitory effect of tannins on cellulase activity.

Table 4.4 Factors influencing pepsin/cellulase digestion. ANOVA of data from experiment on substrates impregnated with quebracho and tannic acid.

I Quebracho containing substrates

source	df	SS	MS	F	p
Tannin concn.	4	0.004466	0.001117	3.364	0.95
Treatment	5	0.005128	0.001026	3.090	0.95
Error	20	0.006637	0.000332		
Total	29	0.016231			

II Tannic acid containing substrates

source	df	SS	MS	F	p
Tannin concn.	4	0.008937	0.002234	9.841	0.999
Treatment	5	0.003415	0.000683	3.009	0.95
Error	20	0.004545	0.000227		
Total	29	0.016896			

III All data combined

source	df	SS	MS	F	p
TA or Qb	1	0.102525	0.102525	195.6	0.999
+ or - protein	1	0.000765	0.000765	1.459	N.S.
Interaction	1	0.003028	0.003028	5.779	0.95
Error	56	0.029355	0.000524		
Total	59	0.135672			

Note: ANOVA calculations follow Campbell (1974).

However, tannins may differ both as to their ability to inhibit cellulase activity and more subtly in the way their interaction with proteins modifies this action.

Following these initial results a further investigation of the interaction of tannins and proteins in determining cellulolytic activity was made. Two separate sets of experiments were performed, one with the tannin concentration used to soak the paper set at 1.0% and the other with the concentration set at 0.1%. Where papers were pretreated with a protein solution the same concentration of 1.0% was used in both experiments, the design of each set of experiments being identical to that reported above. Papers were either soaked in tannic acid (T), quebracho (Q) or neither tannin (-). The protein pretreatment was in casein (C), BSA (B) or not given (-). This provided for nine types of substrate, and using the above codes, these were as below.

--	T-	Q-
-C	TC	QC
-B	TB	QB

Digests on each substrate were run in duplicate, and the same set of three experimental treatments was employed as before (i.e. plus pepsin, plus buffer, no pre-treatment) thus giving 9 x 2 x 3 = 54 digests at each tannin concentration.

The results of the experiments carried out at the higher level of tannin showed that virtually all the variation in the assay results could be ascribed to the presence or absence of tannin. Making the most efficient use of all the data in a three way ANOVA showed only one highly significant source of variation, namely tannins (see Table 4.5) The only other significant factor contributing to the results was the interaction between treatments and tannins.

Eliminating the effect of tannin and considering the data for the sets of 18 results for each tannin treatment (-,T,Q) in a two way ANOVA for protein and treatment effects traced the influence of treatments seen previously to the tannin-free controls, not the tannin-containing systems (see Table 4,6). In short, cellulose masking could not be demonstrated at this high tannin concentration using this experimental design. Differences in the results due to pretreatments were seen in the absence of tannin and these were not dependent on the presence of protein, thus confirming earlier results (Jones and Hayward, 1975; Jones and Bailey, 1972). Nevertheless these effects were very minor compared to those of the tannins as seen in the three way ANOVA presented earlier. In conclusion, the concentrations of tannin employed here were probably at such an inhibitory level that masking by tannin-protein complexes did not

Table 4.5 Factors influencing pepsin/cellulase digestion

Exhaustive three way ANOVA for all factors.

source	df	SS	MS	F	p
Tannin (-,T,Q)	2	0.06352	0.031760	143	0.999
Protein (-,C,B)	2	0.00110	0.000558	2.48	N.S.
Tannin x Protein	4	0.00110	0.000275	1.238	N.S.
Treatment	2	0.00061	0.000305	1.374	N.S.
Treatment x Tannin	4	0.00310	0.000775	3.491	0.95
Treatment x Protein	4	0.00240	0.000600	2.702	N.S.
Treatment x Tannin x Protein.	8	0.00210	0.000263	1.185	N.S.
Error	27	0.00599	0.000222		
Total	53	0.07992			

Note: ANOVA calculations follow Campbell (1974).

Table 4.6 Factors influencing pepsin/cellulase digestion

Separate analyses for substrates by tannin content.

I Containing tannic acid.

source	df	SS	MS	F	p
Protein (-,C,B)	2	0.001295	0.000648	1.766	N.S.
Treatment	2	0.000464	0.000232	0.632	N.S.
Interaction	4	0.002792	0.000688	1.874	N.S.
Error	9	0.003302	0.000367		
Total	17	0.007814			

II Tannin free control.

source	df	SS	MS	F	p
Protein (-,C,B)	2	0.000695	0.000347	2.409	N.S.
Treatment	2	0.002067	0.001034	7.180	0.99
Interaction	4	0.000849	0.000121	1.472	N.S.
Error	9	0.001292	0.000144		
Total	17	0.004903			

III Containing quebracho tannin.

source	df	SS	MS	F	p
Protein (-,C,B)	2	0.000210	0.000105	0.695	N.S.
Treatment	2	0.001179	0.000590	3.907	N.S.
Interaction	4	0.000897	0.000224	1.483	N.S.
Error	9	0.001359	0.000151		
Total	17	0.003645			

Note: ANOVA calculations follow Campbell (1974).

appreciably add to the inhibition of cellulase.

In the experiments performed at the lower tannin concentration the results did indicate more subtle interactions between variables, including the masking effect, and so the data analysis has been made somewhat differently to expose this. Within each digest pre-treatment group (see Table 4.7) significant differences ($p>.99$) were once again seen to be dependent on the presence or type of tannin. In each case the tannic acid-containing substrate had the lowest digestibility ($p>.95$) and the result for quebracho was intemediate between tannic acid and the tannin-free controls. In no case was there any significant contribution to the variation in the results by the presence/type of protein used. Proteins only assumed significance when considered together with tannins.

Considering the whole data set (54 values) no interactions in two way ANOVA's were found between treatments and tannins or treatments and proteins, but such an analysis of the data did reveal a significant interaction between tannin and protein which was much more important than that due to proteins <u>per se</u> (see Table 4.7).

Returning to the ANOVA's for each treatment alone (see Table 4.8) several interesting trends are present. If the total variation in each is examined, as indicated by

Table 4.7 Effects of tannin and protein on cellulose digestion.

I Mean weight losses (%) for substrates grouped according to tannin content and experimental treatment.

Tannin/Treatment	Cellulase only	Buffer and Cellulase	Pepsin and Cellulase
No tannin	18.1 (0.75)	18.4 (0.40)	17.5 (0.61)
Tannic acid	8.5 (2.55)	11.0 (1.51)	12.1 (1.63)
Quebracho	16.3 (0.47)	15.3 (1.02)	13.9 (0.45)

N.B. Figures in parentheses are standard errors.

II ANOVA treatment of the data

source	df	SS	MS	F	p
Tannin	2	0.050976	0.025488	45.11	0.999
protein	2	0.003971	0.001985	3.513	0.95
Interaction	4	0.018524	0.004631	8.196	0.99
Error	45	0.025445	0.000565		
Total	53	0.098916			

Note: ANOVA calculations follow Campbell (1974).

Table 4.8 Factors influencing pepsin/cellulase digestion

Separate analyses for differently digested substrates.

I Cellulase only treatment.

source	df	SS	MS	F	p
Tannin (-,T,Q)	2	0.030773	0.015386	29.36	0.999
Treatment	2	0.005297	0.002649	5.055	0.95
Interaction	4	0.011899	0.002975	5.677	0.95
Error	9	0.004716	0.000524		
Total	17	0.052868			

II Buffer pre-treatment.

source	df	SS	MS	F	p
Tannin (-,T,Q)	2	0.016589	0.008294	21.37	0.999
Treatment	2	0.000212	0.000106	0.27	N.S.
Interaction	4	0.006869	0.001717	4.427	0.95
Error	9	0.003490	0.000338		
Total	17	0.027160			

III Pepsin pre-treatment.

source	df	SS	MS	F	p
Tannin (-,T,Q)	2	0.009158	0.004579	6.274	0.95
Treatment	2	0.000542	0.000271	0.397	N.S.
Interaction	4	0.0 2945	0.000736	1.081	N.S.
Error	9	0.006130	0.000681		
Total	17	0.018775			

Note: ANOVA calculations follow Campbell (1974).

the total sums of squares (TSS), then this declines in the order cellulase > buffer only > pepsin. This appears to reaffirm the results of Jones and Hayward (1975) that a pepsin pre-treatment reduces the variability of the assay and so improves the correlation with other variables. The way in which the TSS divides between tannins, proteins and their interaction also changes with treatment. Where there is no pre-treatment and the masking effect is liable to be strongest, all three sources of variation are significant. Given a buffer-only treatment which will help alleviate some of the effect, protein loses significance. With pepsin neither protein nor its interaction with tannin provide for significant sources of variation.

To summarise so far, the interaction of protein with tannin could play a role in ruminant digestion through the masking effect, however, this is only likely to be a significant component of cellulase inhibition when tannin concentration is low and the direct effects of tannin on the enzyme reaction are lessened.

The final set of experiments reported here investigates the ability of the extracts characterised in section 4.2 to inhibit the cellulase reaction in substrates prepared with and without a BSA pre-treatment. The tannin solutions used were all made up to contain a total phenolic concentration equivalent to that of 0.1%

tannic acid.

First a set of BSA-containing substrates were given a buffer only pre-treatment with 19ml of the cellulase buffer. At the end of the pretreatment time the buffer was not discarded but added to a fresh samples of untreated paper in a replicate set of vials. To these 1ml of 20 fold concentrated cellulase solution was added to constitute a standard cellulase digestion system for the paper, but one containing the fraction of "free" tannin leached out of the substrate. The set of leached substrates were also given a normal cellulase digest which would be inhibited by the tannin which remained "bound" to the solid substrate. This system thus allowed for a distinction to be made between what may be characterised as "direct" versus "substrate level" inhibition of cellulase.

In a second set of experiments cellulase only digests of substrates prepared with and without BSA were performed to see if cellulose masking could be seen with the range of tannin types. All the digests made on the series of tannins were performed in duplicate and additionally a set of tannin free controls were made for comparison. All the digests were performed simultaneously with the same batch of cellulase and are therefore directly comparable.

The results from the first experiments on the full

range of extracts (see Table 4.9) showed that most substrates were degraded at a rate close to that of the control (20%). The weight loss with extract Ah is anomalously high from the leached substrate, and almost certainly due to the elution of non tannin material incorporated into the substrate from this (very phenol-deficient) extract. Weight losses clearly less than control are exhibited by three extracts (B4, De and TA) for systems where cellulase is inhibited in solution and by cellulose-bound material. Indeed the precipitation of some cellulase could be seen as the enzyme was added to the buffer containing "free" tannin in solution. It is notable that these three extracts do not have unusually high specific activities and that the cellulase inhibition by extracts that do (e.g. Fv) is not distinguishable from the average. Inspection of the specific activity data thus reveals that protein precipitating ability is, on this evidence, not a good predictor of an extract's ability to inhibit cellulase.

Comparing the effects of the presence/absence of protein in this cellulase inhibition system indicates that the presence of protein does increase the inhibitory effects of tannin (see Table 4.10). Otherwise the results are similar to the previous set with De and B4 still the most inhibitory extracts.

Considering all four sets of data shows,

Table 4.9 Effects of extracts on cellulose digestion I

Data shows means and standard errors for weight loss %

Tannin	Code	"Bound tannin"		"Free tannin"	
		x	S.E.	x	S.E.
Aesculus hippocastanum	Ah	28.6	0.23	16.2	0.82
Calistemon citrinus	Cc	21.1	0.11	20.5	0.23
Diospyros ebenum	De	6.7	1.20	12.2	0.81
Fucus vesiculosus	Fv	20.5	0.22	22.2	1.86
Loropetalum chinense	Lc	15.9	0.98	16.6	1.95
Myrtus obcordata	Mo	14.2	1.78	22.9	0.81
Pomaderris phylloides	Pp	15.5	0.35	20.2	0.81
Pteridium aquilinum	B1	20.5	4.30	23.6	0.97
Pteridium aquilinum	B2	23.0	0.17	18.9	2.12
Pteridium aquilinum	B3	21.9	1.14	18.0	0.98
Pteridium aquilinum	B4	1.7	0.57	15.4	1.39
Quercus sp.	Qs	23.1	0.11	18.6	0.97
Taxus bacata	Tb	20.1	1.20	22.1	0.74
Vaccinium myrtilis	Vm	22.4	0.96	16.6	0.20
Tannic Acid	TA	11.9	0.11	15.2	3.38
Quebracho tannin	Qb	19.5	0.74	17.8	0.84
Terminalia chebula	Tc	19.3	2.35	18.9	0.40
Pinus radiata	Pr	18.1	0.62	20.1	0.22
Catechin	Ca	20.1	2.23	21.5	0.56
Gallic Acid	GA	17.3	2.23	19.6	0.46
Mean Value	-	18.0		18.1	

"bound" and "free" tannins are as described in the text.

Table 4.10 Effects of extracts on cellulose digestion II

Data shows means and standard errors for weight loss %

Tannin	Code	"Masked"		"Unmasked"	
		x	S.E.	x	S.E.
Aesculus hippocastanum	Ah	27.5	0.62	23.5	3.25
Calistemon citrinus	Cc	19.9	4.32	18.3	3.72
Diospyros ebenum	De	0.9	0.0	14.3	0.49
Fucus vesiculosus	Fv	13.7	1.69	18.3	0.35
Loropetalum chinense	Lc	13.7	5.01	18.0	0.50
Myrtus obcordata	Mo	14.6	1.69	17.5	0.14
Pomaderris phylloides	Pp	21.1	2.76	17.6	1.41
Pteridium aquilinum	B1	19.2	3.51	18.9	3.53
Pteridium aquilinum	B2	19.9	0.56	23.4	1.37
Pteridium aquilinum	B3	1.1	0.25	18.4	0.96
Pteridium aquilinum	B4	13.5	0.05	15.3	2.79
Quercus sp.	Qs	13.8	2.13	15.9	3.15
Taxus bacata	Tb	17.1	0.43	19.4	5.22
Vaccinium myrtilis	Vm	13.4	0.0	19.7	1.41
Tannic Acid	TA	20.3	0.94	21.7	0.79
Quebracho tannin	Qb	11.5	1.57	21.6	2.87
Terminalia chebula	Tc	20.3	1.88	19.9	0.13
Pinus radiata	Pr	14.1	2.19	20.9	0.44
Catechin	Ca	15.4	1.94	19.5	2.04
Gallic Acid	GA	18.2	0.44	20.9	0.13
Mean Value	-	15.5		19.1	

"Masked" and "unmasked" substrates described in the text.

not surprisingly, that the cellulase-only digest of BSA-masked material produced the lowest weight losses. Also the correlation betwen these results and those for the digest inhibited by cellulose-bound tannin is very high (+.92, $p<.001$). All four sets of results indicate that tannin type contributes significantly to the variation in the results yet there is clearly additional variability introduced by the way tannin is combined in the cellulose substrate. None of the results correlate well with their chemical characteristics as described in Chapter 2.

4.3,3 Discussion

Once again the idea that results from a simple system such as a cellulase inhibition assay might correlate well with the chemical nature of the tannins present as inhibitors is confounded. The two most inhibitory extracts span the range for angiosperm tannins i.e. B4 contains procyanidin tannin whilst De contains hydrolysable tannin and prodelphinidin tannin. The positive gain from this work has been to demonstrate cellulose masking sensu Handley (1954) and to do so with a range of tannins. The implications of these results are more fully discussed in Section 4.5.

4.4 The Amino Acid Precipitating Properties of the Extracts.

It is generally thought that tannins produce precipitation reactions with proteins, and not with their constituent free amino acids. Handley (1954) did, however, show that certain amino acids precipitated some tannins from solution. Generally these reactions involved amino acids containing non-alpha amino groups, and tannins from species whose litter tended to form mor humus. Handley's observations were based upon the behaviour of freshly extracted unbuffered plant extracts to which amino acid solutions were added dropwise. Here the attempt has been made to confirm these findings and also to examine the influence of pH on the reaction. The methods used have been set out in Appendix 2.5 and the results obtained are presented in Figure 4.9.

Handley's observations are confirmed by these results in that the amino acids which do give precipitates are of the expected type (i.e. basic). No conclusion can be drawn as to the type of tannin most likely to produce precipitation, whilst the absence of precipitation cannot be taken as evidence that no interaction between the tannin and amino acid has taken place (see Chapter 1). The absence of precipitation at high pH indicates that ionisation of the non-alpha amino group may be important to the precipitation reaction. All precipitates formed

Figure 4.9 Formation of tannin-amino acid precipitates over a range of pH values

Extract	pH 2	3	4	5	6	7	8	9	10
TA	A	A	A	A	A	A			
Qb etc.*									
Qs	A	A	A	A					
De	A	A	A	A	A	A			
Pp	A	A	A						
B4	A H	A H	A H	A H	A H	A H			
Mo	A	A	A	A	A	A			
Lc	A	A	A	A	A	A	A		

A = precipitation with arginine; H = precipitation with histidine.

* All other extracts, like Qb, did not form precipitates at any pH with any amino acid tested.

were fully pH reversible.

The conclusion has to be drawn that there is no simple relationship between the chemical structure of tannins and their interaction with single amino acids. Tannin-protein interactions can thus be expected to prove at least as complex in terms of tannin and protein structure, although proteins with a high content of basic amino acids may interact particularly strongly with tannins, especially those giving positive reactions above. Ecologically the interaction of single amino acids with tannins may act to inhibit the amino acid nutrition of herbivores by imparing specific amino acid absorption in the gut, thus extending the allelochemical action of tannins beyond that envisaged by Feeny (1969).

4.5 Insoluble Tannin-Containing Complexes in Digestion

The purpose of this Section is to draw together the information presented in this and the preceeding Chapters, which indicates the occurrence and probable impact of insoluble tannin-protein complexes in animal digestive processes. Despite the crude nature of the extracts characterised in Chapter 2 all those which could be said to contain condensed or hydrolysable tannins on chemical grounds did cause protein precipitation. This was the case without exception, and although it proved impossible to relate the variation in these chemical characteristics to their protein precipitating ability, nevertheless all these materials are envisaged as likely to precipiitate protein in the diet.

For some of the materials the conditions in which precipitates occur maximally are somewhat narrowly defined and may not obtain in vivo. For instance the buffer used in the specific activity experiments is unpleasantly salty in taste. During mastication ionic strength may well be rather lower than that used in these experiments and perhaps other conditions will not be optimal for precipitate formation. Nevertheless these factors are more likely to reduce rather than abolish precipitate formation (e.g. see Figure 3.1). The pH used in the specific activity and the cellulase inhibtion

experiments is somewhat more acid than the values reported for insect midguts (see Section 3.1) but certainly relates well to rumen conditions (Moir, 1968). As precipitation also tends to occur in conditions more acid than protein pI then the results here may also hold for the mammalian monogastric stomach. Thus it may be reasonable to consider that tannin-protein precipitates occur in vivo at pH<6 but at alkaline pH values this is much less likely. Chapter 5 considers insect and mammalian intestinal digestion where pH is typically alkaline.

Given this rather restricted set of digestive processes to consider, can tannins exert an effect on animal nutrition? Given evidence that (human) nitrogen balance can be maintained after gastrectomy (Freman and Kim, 1978) then even if tannins were to exert a digestibility-reducing effect in this portion of the digestive tract then in whole animal terms it may not be of great importance at this stage of digestion. The importance of the stomach may lie in its role as a sterilising pretreatment stage. As tannin-protein complexes may redissolve on entry to the duodenum, then protein from both the stomach and rumen may be satisfactorilly digested here, and indeed protection of proteins (by tannins) from prior digestion may be of benefit (Jones and Mangan, 1976).

Another point raised during earlier discussion of Goldstein and Swain's work (Section 4.3) was the enzyme activity present in suspensions of tannin-protein precipitates. In test-tubes precipitates range in structure from densely solid coagula to finely suspended hazes. Gastric motility could act to keep all insoluble tannin-protein complexes finely divided and therefore available as substrates for digestion. The lack of any hard evidence for digestibility reduction by tannins becomes more understandable in these terms.

However, for an adult ruminant maintaining nitrogen balance by urea recycling, then the supply of its gross energy requirement by cellulolysis assumes particular importance, as this is not a reaction found in the lower and more alkaline portions of the digestive tract. Considerable attention has been paid to ruminant nutrition and the cellulase digestibility technique has been widely used in these studies. In is therefore worth considering the results obtained in section 4.3 in the light of other work. With regard to nitrogen assimilation from forages by rumen microbes, proteolysis is thought to be the rate-limiting factor (Nugent and Mangan, 1978). Trypsin inhibition has been ascribed to tannins in forages (Tamir and Alumot, 1969; Milic et al., 1972) yet this may not have much relevance to (trypsin free) fermentative digestion. However, depressed protein

digestion has been attributed to tannins in one instance (Ramachandra, 1977). More importantly generally depressed dry matter loss (Tripathi, 1978; Palo et al., 1985; Griffiths and Jones 1977) has also been atributed to tannins, although there may be a threshold effect (Sandarandan and Arora, 1979). These digestibility reducing effects have been found with condensed and hydrolysable tannins, and the positive correlation between brown algal astringency and reduced in vitro rumen fluid digestibility (Greenfield et al., 1983a,b) suggests that phlorotannins may also reduce ruminant digestion. As Griffiths and Jones (1977) pointed out there is as yet no evidence as to the mechanism for this effect, which could be by toxicity to rumen microbes, direct enzyme inhibition or by binding to enzyme substrates. The present work demonstrates the potential for both of the molecular level effects and also indicates how there may be an interaction between proteolysis and cellulolysis in dry matter digestibility. Such a link between tannin-protein complex digestibility and the supply of gross energy requirements may be a important factor both ecologically and agriculturally (Barry and Manley, 1985), where the correct utilisation of tannin-containing forages is of economic importance.

Chapter Five

Soluble Tannin-Protein Systems

5.1 Introduction

The work reported in Chapters 3 and 4 has made reference to, but not explored, the importance of soluble tannin-protein complexes. These form the focal point of this chapter, which examines their impact on protein-protease systems. The stability of solutions containing tannin and protein may depend critically on their pH and the specificity of the tannin-protein interaction (Hagerman and Butler, 1978, 1981), and these features are given due consideration in the following. An additional factor is also introduced, namely that of surfactants. Martin and Martin (1984) showed that substances with a detergent nature present in the insect gut may solubilise tannin-protein interactions. Here the possible role of such substances in extending the range of conditions in which soluble tannin-protein complexes may occur in mammalian systems is explored.

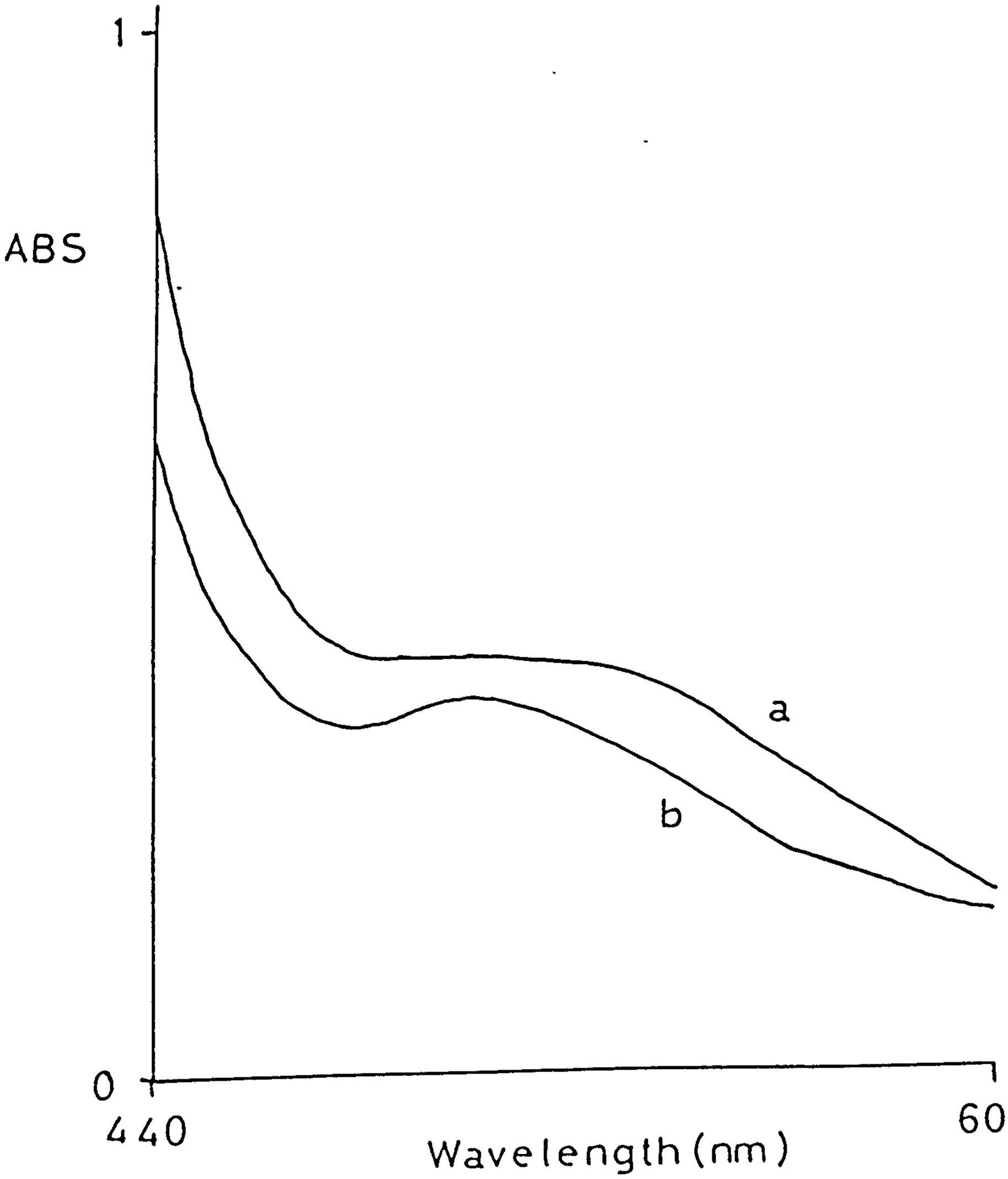
As a prerequisite for this work it was felt necessary to examine the evidence that tannin-protein complexes actually occur in soluble forms. The evidence of Van Buren and Robinson (1969) has already been introduced (Chapter 1), and two other independent lines of evidence also exist. Fishman and Neucere (1980) have demonstrated such complexes by gel filtration whilst Zanobini _et al_. (1967) used shifts in the visible spectrum of haemoglobin to detect tannin-haemoglobin binding in entirely soluble

systems, an approach also taken by Woof and Pierce (1968) who used differential UV spectroscopy in non-coloured systems.

Zanobini et al. (1967) further demonstrated that the tannin-protein bonding was reversible by the addition of the surfactant "Tween 80", the criterion for reversibility being the recovery of the original ("haemoglobin only") spectrum on the addition of the surfactant to the tannin-haemoglobin solution. The experimental system used by Zanobini et al. was technically simple and the attempt was made to repeat this work. The method was to record the spectrum of a haemoglobin solution in distilled water, from 440nm to 600nm. Re-recordings of the spectrum were then made on the addition of tannic acid and then of Tween 80. Whilst it proved possible to repeat the spectral shift obtained by the addition of tannic acid (see Figure 5.1), no reversal of this shift was obtained with Tween 80. Indeed the addition of 50μl of Tween 80 to a cuvette containing 4ml of a solution 1mg/ml tannic acid and 2mg/ml haemoglobin (in distilled water), caused the immediate precipitation of the contents from what had previously been a clear non-turbid tannin/protein solution. Needless to say this totally contradicts the earlier work of Zanobini et al. (1967).

As other investigators have reported, results with

Figure 5.1 Visible spectrum of haemoglobin with and without the presence of tannic acid.



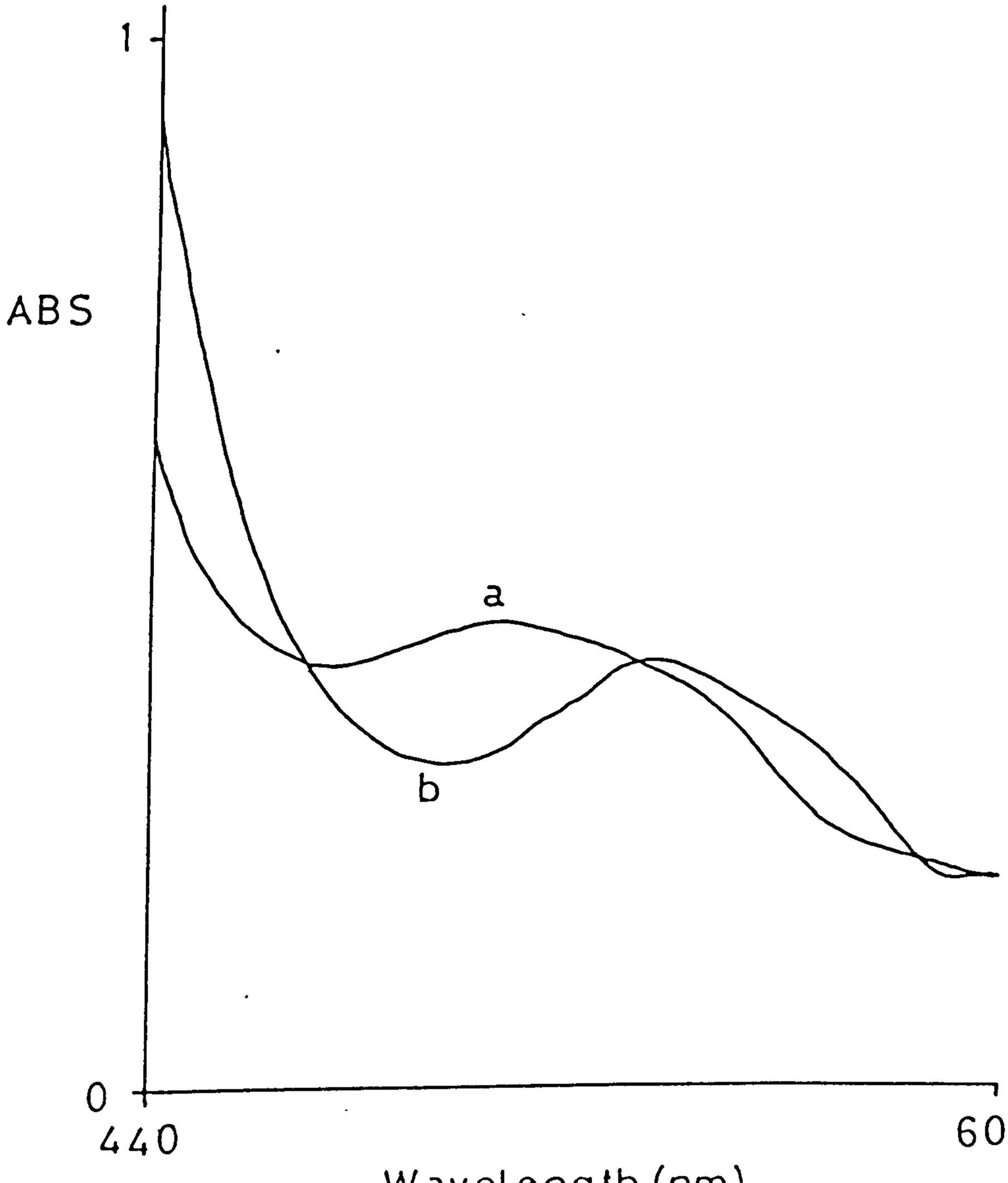
ABS= absorbance; curve a, with tannic acid.

curve b, without tannic acid.

haemoglobin depend critically on the protein preparation used (Bate-Smith, 1973a; Schultz et al., 1981). The failure here to repeat the results with Tween 80 may only indicate that the results of Zanobini et al (1967) are not general in that differences in the nature of a single protein (let alone differences between proteins) can influence results. It is suggested that the cause of the precipitation reaction with Tween 80 was that the protein's structure was altered by the surfactant, decreasing its solubility when in association with tannin. This follows because haemoglobin in the absence of tannic acid did remain in solution in the presence of Tween 80.

Sodium Dodecyl Sulphate (SDS) was tried as a second surfactant. This gave an alteration of the spectrum of haemeoglobin when present in solution with the protein alone. Adding SDS to the tannic acid-haemoglobin system used above did not produce precipitation but a spectral shift, leaving a spectrum that was neither that of pure haemoglobin nor that of haemoglobin in association with SDS (see Figure 5.2). This is again evidence that surfactants may not dissociate tannins from proteins and also indicates the possibility that Martin and Martin (1984) may have been wrong in suggesting that the action of surfactants in dissolving tannin-protein precipitates necessarily removed the interaction. Indeed, surfactants

Figure 5.2 Visible spectrum of haemoglobin in the presence of sodium dodecyl sulphate (SDS), with and without the presence of tannic acid.



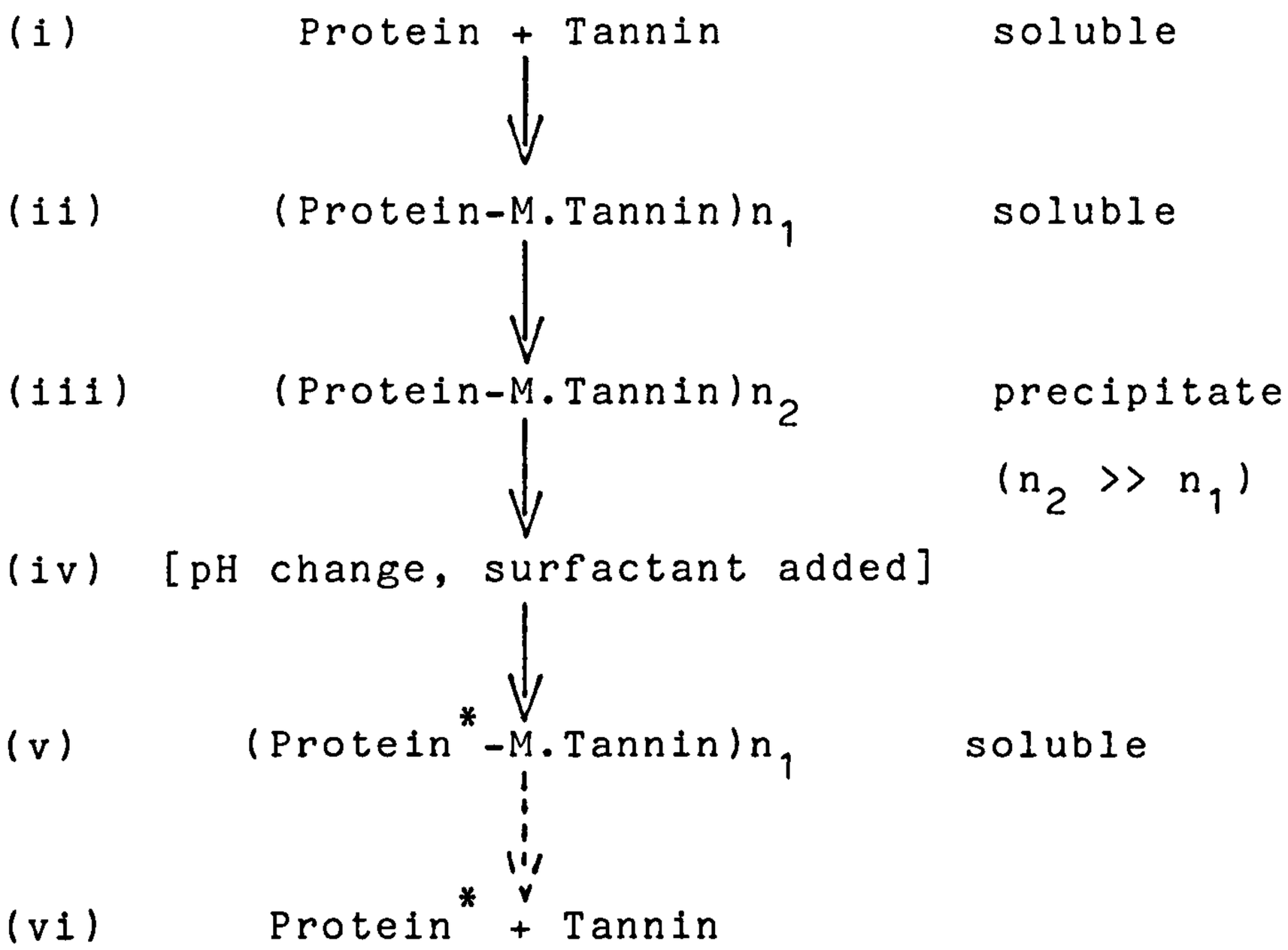
ABS= absorbance; curve a, with tannic acid.

curve b, without tannic acid.

may cause the formation of soluble tannin-protein-surfactant complexes and not produce free tannin and protein in solution.

Whilst not wishing to dismiss the idea that gastrointestinal surfactants may be important in tannin-protein interactions, a working model is proposed as illustrated in Figure 5.3, which incorporates the possibilities explored above. This conceptual framework expands the simpler model of Van Buren and Robinson (see Section 3.1) and its utillity is tested in the interpretation of the results presented in the next two sections.

Figure 5.3 A model of tannin-protein interactions.



The terminology here is similar to that used by Van Burren and Robinson (1969) except that the model is generalised to include any protein and any tannin (i). As previously, M is the ratio in which tannin and protein combine, combination initially being in the form of soluble complexes (ii). These may be a transitory stage which, given suitable conditions, may be followed by the aggregation of complexes to form precipitates (iii). Thereafter, if conditions change (iv), precipitates may redisolve into soluble complexes (v). At some stage in the preceeding processes the protein involved is likely to have become denatured (Protein*). There is no available evidence to suggest that tannins and proteins can be completely separated after complexes have formed (vi).

5.2 The Influence of Tannins on Protease Activity in Soluble Systems

The object of the work reported in this section was to find conditions in which tannins, proteases and substrate proteins could coexist in solution, without precipitate formation, and then to examine the impact of tannins on protease activity. This has been done using trypsin as the protease with a view to producing results interpretable in terms of both mammalian and insect digestion.

The importance of trypsin as a protease lies in its action as both a major mammalian digestive enzyme and in the occurrence of trypsin-like proteases in insects (Yang and Davies, 1971). Some caution must be excercised in the extrapolation of results with trypsin to insects as non-serine proteases may be the major enzymes in the guts of some insects. However, the importance of trypsin in intestinal proteolysis is further supported by evidence that it is the intestinal and not the gastric stage of protein digestion that is important in maintaining mammalian (human) nitrogen balance (see Section 4.5).

No published information could be found on the actual levels of trypsin present in the digesta of any herbivore and as the series of experiments was first intended to mimic digestion in insects, trypsin was employed at a

level within the range reported for "tryptic activity" in simuliids (Yang and Davies, 1968). Better information is available concerning the relative (but not absolute) amounts of tannin and protein in herbivore diets. A table of such ratios (R(t) as defined in Table 3.1) has been computed from the work of Coley (1983) and are given in Table 5.1. The range of R(t) used in the experiments falls in this range.

5.2,1 Materials and Methods

The tryptic digestion of Bovine Serum Albumin (BSA, Sigma fraction V; Trypsin, Sigma type II) was studied in the presence of various concentrations of tannin, at 37^{o}C. Two tannins were used in separate sets of experiments: tannic acid and quebracho tannin, the influence of both tannins being studied in each proteolytic system described below.

Proteolytic digestion of the BSA or BSA-tannin complex was carried out in a total volume of 25ml containing 50mM carbonate-bicarbonate buffer (see Appendix 2.1) adjusted to pH 10, and trypsin and BSA in quantities detailed below. The object of working at this pH was to mimic the digestion at the extreme alkaline pH of conditions reported for insect digestive tracts (Berenbaum, 1983; Dow, 1984). Thereafter more moderate pH conditions were employed to examine digestion at pHs more typical for

Table 5.1 Tannin-protein ratios (R(t)) for some tree leaves

Plant taxa Family	Mature Leaves			Young leaves		
	a	b	c	a	b	c
Anacardiaceae	0.23			0.50		
Annonaceae	0.33	1.31	6.45	0.08	1.16	9.12
Apocynaceae	0.08			0.09		
Bignoniaceae	0.12	0.89		0.15	0.14	
Bombacaceae	8.60	0.88		7.69	1.44	
Burseraceae	4.39	5.22	0.10	7.33	1.98	0.18
Caesalpinaceae	5.63	0.02	7.20	1.63	nill	9.12
Chrysobalanaceae	6.10			13.6		
Euphorbiaceae	0.83	4.99	0.13	nill	14.8	nill
Flacourtiaceae	0.24	1.08		0.06	0.06	
Guttiferae	11.3			12.7		
Melostomaceae	0.58			1.42		
Meliaceae	1.75	4.46	7.26	5.2	5.7	11.4

Notes: The above data has been calculated from Coley (1983) who provides a tabulation of condensed tannin and protein contents for leaves classed as either young or old, from tropical rainforest vegetation. Each figure tabulated above is for a single species (distinguished as a, b or c). Paired R(t) values for both young and mature leaves are given.

those reported in insects and mammals. This series of experiments at less alkaline pH values began by examining tryptic proteolysis in a system run in 25ml of 50mM citrate-phosphate buffer (Na_2HPO_4 adjusted to pH 7.5 with citric acid).

In all experiments proteolysis was performed with BSA at 4mg/ml, and trypsin at 200 units per ml in the 25ml buffer present. Control reactions (tannin-free) and reactions with R(t) set betwen 0.05 to 2.0 were used. Solutions of tannin and protein were made separately and mixed 15 mins. before the start of the experiments to ensure time for full complexation. At zero time trypsin (in 5ml of 0.001 M HCl) was added to the tannin-protein mixture making it up to volume. 1ml portions were removed after 1 minute, and at timed intervals for the following 2 hours, and added to 2ml 5% trichloracetic acid (TCA) which precipitated the undigested protein. 250 μl portions of the supernatant TCA solution were then assayed for amino nitrogen by the method of Moore and Stein (see Appendix 2.3). Proteolysis experiments were performed in triplicate with at least one control per batch. At the end of each reaction the remaining solution was checked with a pH meter to ensure that there had been no pH change

The procedure used in the basic proteolysis experiments described above was modified in subsequent

proteolysis experiments as follows. In experiments to examine the influence of heat denaturation on tannin free proteolysis, the BSA solution used in the experiment was heated to 90°C for 1 min. and then allowed to cool to room temperature before use.

Two proteins other than BSA were used in parallel lines of experiments, these being γ-globulins (Sigma, Bovine, Cohn fraction II) and haemoglobin (Sigma, Human). For these experiments the buffer pH was changed to 8.5 (50mM boric acid adjusted to pH with sodium hydroxide).

In experiments on all three substrate proteins a procedural modification was used to mimic events in mastication. In these experiments doubly concentrated unbuffered tannin and protein solutions were mixed to form precipitates which were then redissolved by the addition of an equal volume of doubly concentrated buffer to give the standard initial system. The final modification was to repeat experiments on BSA in pH 6.2 buffer (50mM Na_2HPO_4 adjusted to pH 6.2 with citric acid) where precipitation occurs in buffered conditions. Precipitates were then redissolved by the addition of cholate (cholic acid solution, Sigma) to a final concentration of 8.0 mM in the standard system.

5.2,2 Results and Discussion

The initial experiments at pH 10 indicated that there

was some interference by tannins in proteolysis at this high pH. In this system there was no turbidity or precipitation, and remarkably, at R(t) = 1.0 tannic acid actually aided the proteolytic reaction. Figure 5.4 shows a set of results for these experimental conditions, in which some enhancement of proteolysis is seen at R(t) = 1.0, although no effect due to tannic acid at R(t) = 0.1 was detected nor was any effect at all seen with quebracho tannin. These results support Feeny's suggstion that high gut pH could be adaptive in the avoidence of the effects of tannin. The evidence for the possible presence of positively beneficial effects was, however, wholly unexpected.

The first series of experiments investigating proteolysis at pH values more typical of those found in insect and mammalian herbivores was conducted at pH 7.5 where, once again, tannin and BSA do not form precipitates (Hagerman and Butler, 1978). A typical set of results using quebracho tannin is shown in Figure 5.5. For R(t) = 0.1 a clearly enhanced rate of proteolysis was seen at digestion times of less than one hour and this declined towards the reaction rate of the control at longer times. At R(t) = 0.1 this pattern was also observed for tannic acid. By contrast at R(t) = 2.0, quebracho tannin showed a marked inhibition of proteolysis (Figure 5.5) and this was also observed at

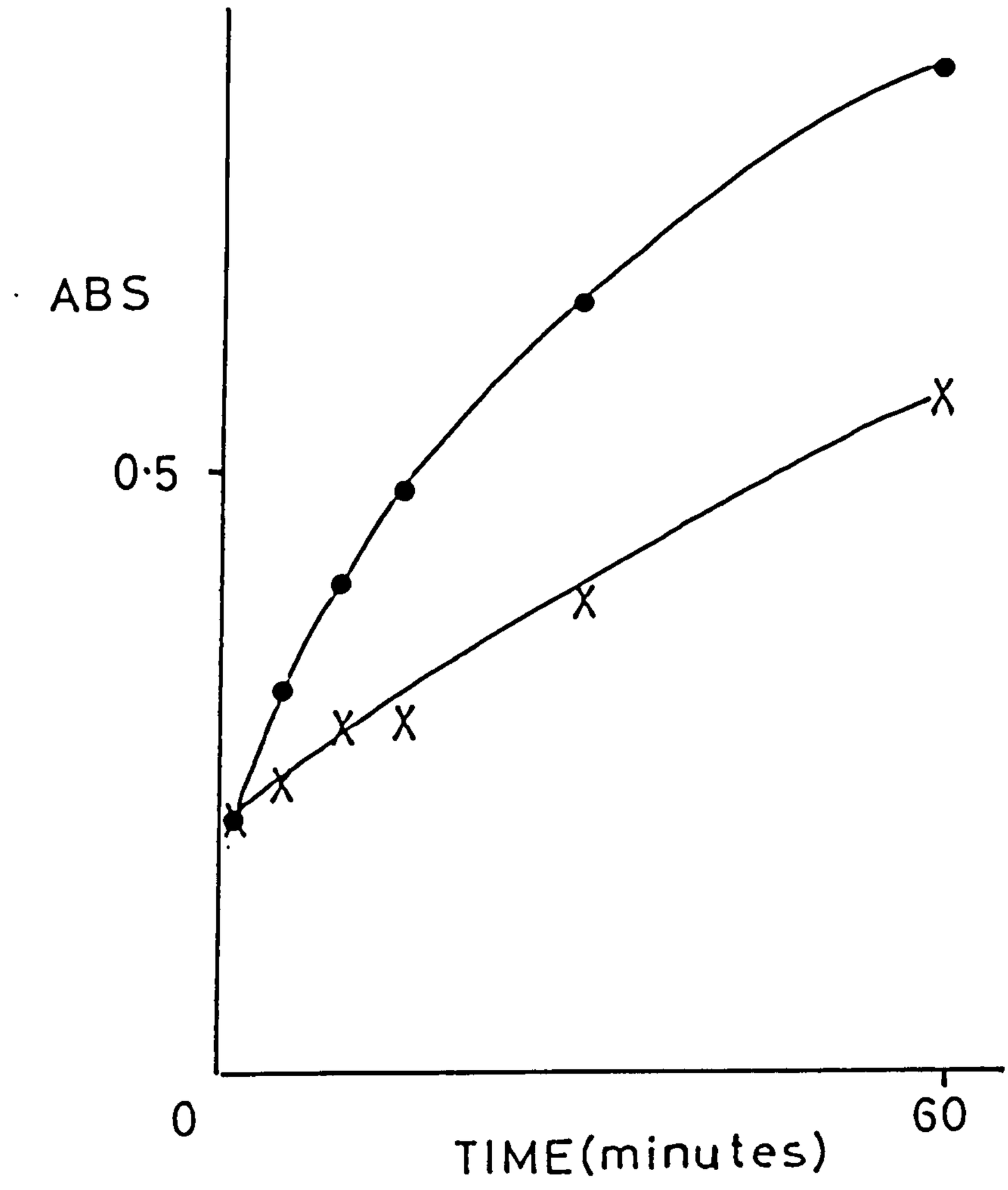


Figure 5.4 Tryptic proteolysis of BSA in the presece of tannic acid at pH= 10.0 The upper curve (points as dots) is for a reaction containing equal ammounts of tannin and BSA (R(t)= 1.0. Points marked X are for a tannin-free control.

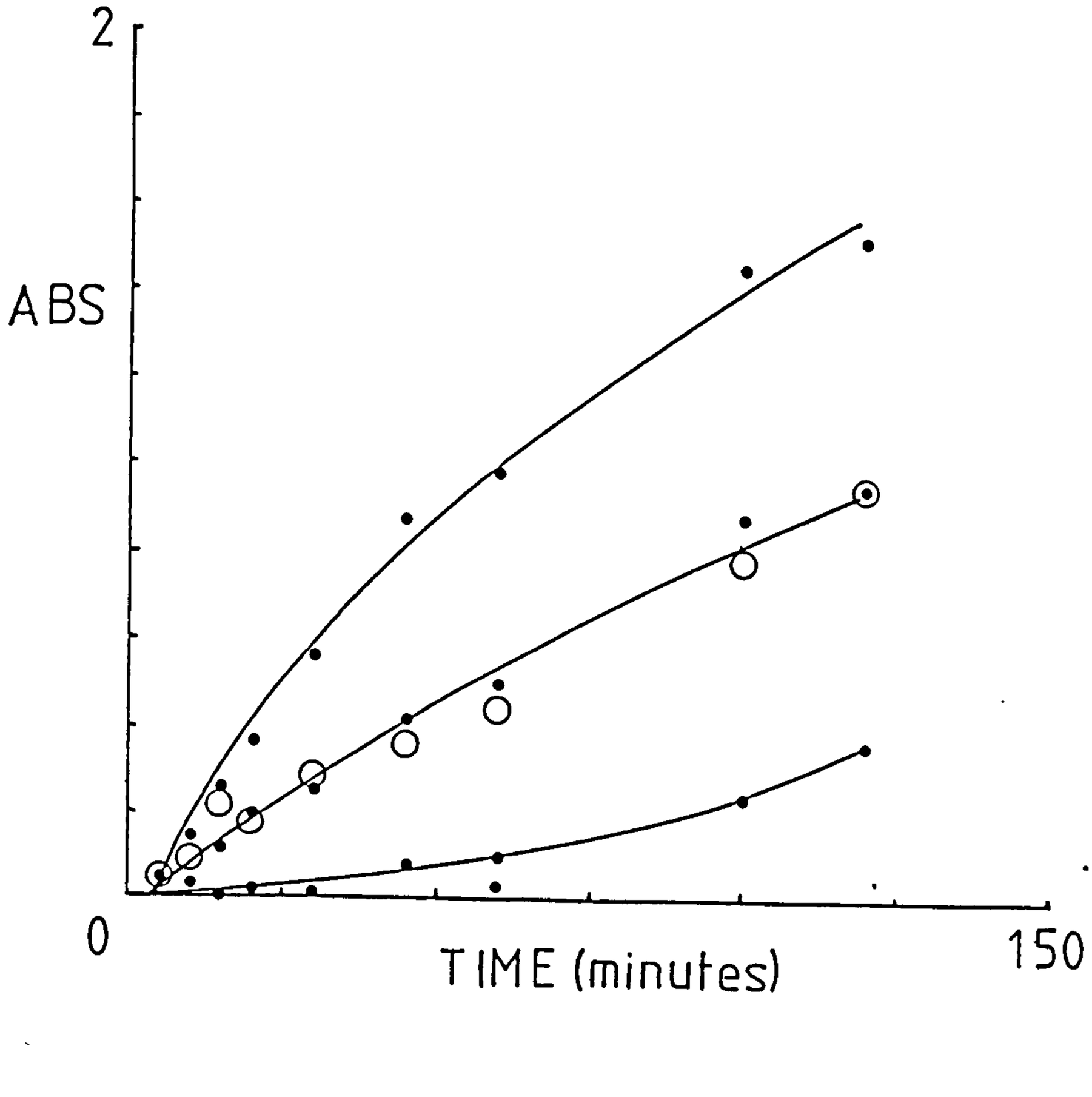


Figure 5.5 Tryptic proteolysis of BSA in the presece of Quebracho tannin at pH= 7.5. The upper, middle and lower curves (points as dots) have tannin-protein ratios (R(t)) of 0.1, 1.0 and 2.0 respectively. Points marked by open circles are for a tannin-free control.

R(t) = 1.0 for tannic acid. At R(t) = 1.0 quebracho tannin had no effect on proteolysis; this null point was close to R(t) = 0.5 for tannic acid.

It has long been known that both low molecular weight phenolic compounds (Green and Neurath, 1954) and high molecular weight polyelectrolytes (Dellert and Stahman, 1955; Morawetz and Sage, 1955) can enhance the rate of tryptic proteolysis. Spensley and Rogers (1954) proposed that polyanions of high molecular weight with a filamentous structure would be the most effective enhancing agents, e.g. polyacrylic and polyglutamic acids. At high pH, with phenolic groups ionised, the similarity between condensed tannins and these structures is striking (Haslam, 1981).

In the case of the polyelectrolytes the mechanism of stimulation of proteolysis is thought not to involve trypsin directly; but conformational changes in the structure of the substrate protein are believed to render the latter more readily available to the protease (Green and Neurath, 1954; Dellert and Stahman, 1955; Spensley and Rogers, 1954). This idea is supported by observations showing that tryptic autolysis is enhanced when this protein interacts with negatively charged surfaces (Johnson and Whateley, 1972). It is proposed that the mechanism of enhancement of BSA proteolysis observed here could also be caused by conformational

changes in the structure of the protein, brought about by its interaction with tannin. At higher R(t) the inhibition of proteolysis is assumed to be due to polyphenolic compounds covering the protein surface (McManus et al., 1981) leading to interference with the interaction of enzyme and substrate.

Heat-denatured BSA also showed increased rates of proteolysis compared with those of undenatured controls, as expected from earlier work (Linderstrom-Lang, 1938). However, addition of tannin to this system (R(t) = 0.1) gave no further stimulation of proteolysis at R(t) = 0.1 (see Figure 5.6) and hence results support the hypothesis that tannins induce conformational changes promoting tryptic attack. This is because if tannins aided proteolysis in other ways , independent of the native state of the protein, then heat denaturation of BSA should not abolish the observed effect.

With γ-globulin and haemoglobin, both of which have more alkaline isoelectric points than BSA, completely soluble tannin-protein systems (i.e. no precipitation or turbidity) were produced only at higher pH values,>8.5 (Hagerman and Butler, 1978). Proteolysis experiments repeated at this pH with these proteins gave quite different results to the first series with BSA. From R(t) = 0.1 to R(t) = 2.0 there was no observable stimulation or inhibition, and it concluded that there is

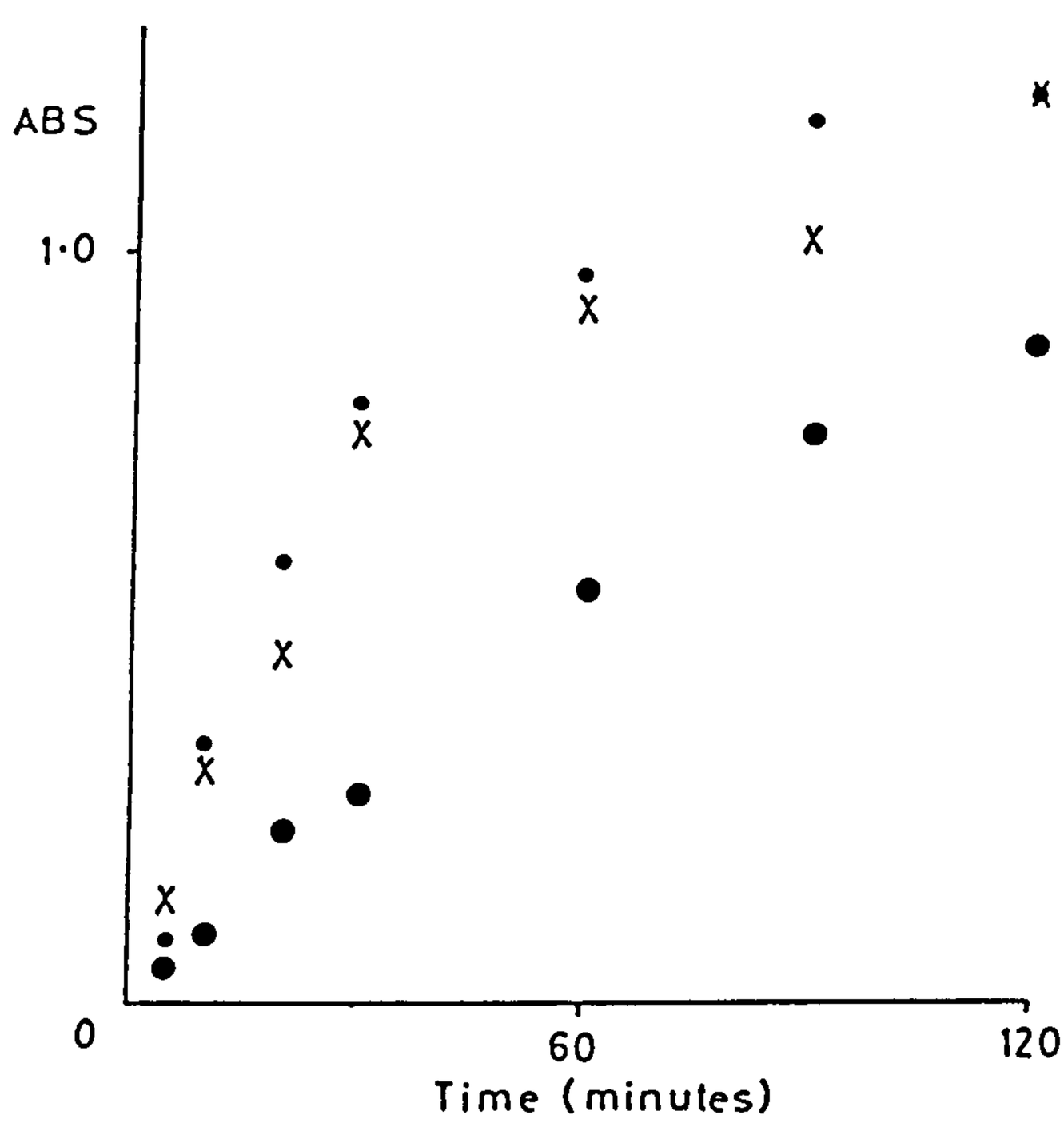


Figure 5.6 Tryptic proteolysis of BSA in the presence of tannic acid at pH= 7.5 The upper curve (points as dots) is for a reaction with R(t)= 0.1. Points marked X are for an otherwise identical reaction where the BSA has been heat denatured prior to the experiment. The lower curve (large dots) is for the normal control reaction which is tannin free and which used BSA without any denaturing pretreatment.

no complex formation between tannin and substrate or enzyme at this pH using these conditions.

The binding of tannins to substrate protein appeared to cause the observed changes in proteolysis, although the possibility that "third party" intermediates such as buffer cations took part in this effect was not ruled out experimentally. The initial procedure was then changed to that where precipitates were formed in unbuffered aqueous solutions, as would be expected to occur during mastication, and these precpitates were then redissolved. This procedure gave increased rates of digestion of both γ-globulin and haemoglobin (Table 5.2). Under these conditions stimulation of proteolysis was found at higher values of R(t) than in the original experiments with BSA and, moreover, this was greater than at low values of R(t) where there was either less stimulation or no observable effect.

When this more realistic procedure was applied to BSA at pH 7.5 and 8.5 inhibition of proteolysis was found at all values of R(t) from 0.1 to 2.0. Thus, as with the other two proteins, there appears to be a difference in the tannin-protein interactions in the soluble and precipitated complex. In the formation of insoluble tannin-protein complexes there may well be stronger interaction between tannin and protein than in cases where no precipitation occurs. BSA is reported (Hagerman

Table 5.2 Tryptic hydrolysis of various substrate proteins: effects of tannin and cholate

Substrate protein	pH	Experimental treatments[a]	Tannin present[b]	Tannin/protein ratio (T/P)	Ninhydrin assay absorbance ratio[c] at 30 mins	at 60 mins
BSA	7.5	NP; -C	TA	0.1	2.69	2.28
				1.0	0.12	0.19
			Q	0.1	2.53	2.29
				2.0	0.46	0.38
γ-globulin	8.5	RD; -C	TA	0.1	1.38	1.35
				1.0	1.75	1.62
			Q	0.2	1.40	1.33
				2.0	1.81	1.56
Haemoglobin	8.5	RD; -C	TA	0.1	1.40	1.31
				1.0	1.60	1.32
			Q	0.2	1.00	1.00
				2.0	1.46	1.09
BSA	8.5	RD; -C	TA	0.1	0.72	0.80
				1.0	0.56	0.62
			Q	0.2	1.00	1.00
				2.0	0.54	0.78

Table 5.2 continued

BSA	7.5	RD;	-C	TA	0.1	0.56	0.69
					1.0	0.60	0.58
				Q	0.2	0.82	0.91
					2.0	0.50	0.53
BSA	6.2	RD;	+C[d]	TA	0.1	0.93	0.93
					1.0	0.47	0.54
				Q	0.1	0.93	0.91
					1.0	0.57	0.62
BSA	6.2	NP;	-C[d]	-	-	0.31	0.35
BSA	6.2	P;	-C[d]	TA	0.1	0.64	0.76
					1.0	0.14	0.09
				Q	0.1	0.66	0.74
					1.0	0.20	0.16

[a] NP = not precipitated; RD = precipitated and redissolved; P = precipitated; +C = contains 8.0 mmolar cholate; -C = cholate absent.

[b] TA = tannic acid; Q = quebracho tannin.

[c] The ratio of the absorbances produced by the proteolysis products of experimental versus control treatments when samples at the times indicated.

[d] The control reaction contained 8.0 mmolar cholate.

and Butler, 1981) to have a relatively high affinity for tannins, certainly much greater than that of either γ-globulins or haemoglobin. If, because of this stronger interaction, some of the BSA becomes so tightly bound that it is unavailable to trypsin after dissolution this could have the effect of masking the enhanced proteolysis occurring in that portion of the BSA that remains available. This finding illustrates the complexity of any consideration of tannin-protein interactions in protein nutrition; clearly different results can be obtained by using the same substrates and different complexation conditions. The difference between BSA and the other two proteins is probably be due to their different affinities for the tannin and the experimental conditions selected. Furthermore, the method by which the protein has been prepared is also likely to be important and ideally should reflect the process of mastication.

It is suggested that enhanced and unaffected rates of tryptic digestion in the presence of tannins are dependent on the nature and native state of the protein substrate and the precise conditions under which it interacts with tannin. Recently Martin and Martin (1984) have pointed out that surfactants contained in insect digestive fluids, and their ability to hinder protein precipitation, must be taken into consideration in

studies of tannin-protein interactions under physiological conditions. They have further illustrated this point by demonstrating the unfavourability of insect gut conditions for tannin-protein precipitation using a good *in vitro* simulation for a composite lepidopteran gut fluid based on results for four species (Martin *et al.*, 1984).

The observations here suggest that Martin and Martin and others (Feeny, 1976; Rhoades and Cates, 1976; Hagerman and Butler, 1978) may be mistaken in correlating the absence of protein precipitation with non-interference of tannins with proteolysis. In this context it would appear that surfactants do have an important role in protein digestion in the presence of tannins, and this led to the investigation of the modifying effects of the mammalian bile acid, cholic acid, on the systems already studied. It was found that a precipitate of BSA and tannic acid at pH 6.2 was redissolved by 8 mM cholate, a concentration which corresponds to that found for cholic acid and derivatives in the human small intestine (Sjoval, 1959). Once a precipitate with R(t) = 1.0 is solubilised by this concentration of cholate, digestion proceeds at a much faster rate than in the absence of cholate although there is still a marked inhibition in comparison with a control reaction (Table 5.2). At R(t) = 0.1 tannic acid formed a

precipitate with BSA which subsequently redissolved (van Buren and Robinson, 1969), but in this instance cholate did not greatly influence the rate of proteolysis. It is important to note that in the latter case proteolysis with and without cholate proceeded almost as fast as the control containing cholic acid i.e. at an increased rate compared with the control without cholate. Whilst seemingly unrecorded in the medical literature (Hofmann, 1968; Passmore and Robson, 1976), bile surfactants may have a role in denaturing proteins in normal mammalian digestion, and this could explain why no additional tannin-induced digestive enhancement is seen when cholate is used in situations where tannins and proteins would otherwise complex, leading *per se* to the equivalent denaturing of the protein.

The relative effects of surfactants from mammals and insects on proteolytic systems remains to be investigated. Mammalian gut surfactants are synthesised outside the gut whilst those of insects appear to be generated in the lumen of the gut (Turunen and Kastari, 1979), potentially in the presence of tannins.

It is clearly of crucial importance to use a protein in its native undenatured state as the substrate in experiments of this type. Casein, used by Feeny (1969) in his original investigations of tannin-protein interactions, has a notably loose and randomly coiled

tertiary structure (White et al., 1978) so that the absence of any observation of enhanced tryptic digestion by Feeny is not surprising.

Ideally, studies in vitro attempting to investigate the role of tannins as feeding deterrents, should use plant proteins, particularly ribulose bis phosphate carboxylase isolated as fraction-1 leaf protein. Feeny (1969) did attempt experiments with an acetone powder prepared from leaves but the preparation of this material may well have caused the protein to become denatured as would the heat treatment method used in bulk preparation of leaf protein (Jones and Mangan, 1976). The most suitable commercial product (Sigma) has a carboxylase activity (0.01-0.03 units mg^{-1}) much less than that retained (1.48 units mg^{-1}) by freshly salted out material (Siegel and Lane, 1975). Thus, at present it does not appear that an acceptable preparation of fraction-1 leaf protein exists. Salting out would appear to be the method of choice for obtaining leaf protein for studies of this type but in the course of this investigation it has been possible to isolate only small quantities and to confirm, by turbidity measurements, the solubility of products of the tannin-protein interaction.

The implications of these results are that ecologically relevant conditions could occur under which an herbivore can experience increased tryptic digestion

from a diet containing a mixture of tannins and proteins when compared with a tannin-free diet with the same protein content. For example, half of the species reported by Coley (1983; see Table 5.1) have foliar R(t) ratios of less than 1.0 and could be considered as candidates for causing enhanced tryptic digestion in the gut of an insect herbivore! For some insects, reports attributing a phagostimulatory role to tannins and evidence of an ability of these animals to deal with and even thrive on diets rich in tannins are found in the literature (Bernays, 1981; Bernays and Woodhead, 1982). Although mammalian digestion differs importantly in that the high gut pH's of some insects do not occur, it is of interest that Jones and Mangan (1977) have attempted to explain observations of increased nitrogen retention from tannin-containing diets in sheep on the basis of dissolution of tannin-protein complexes making protein available for tryptic digestion.

5.3 Tannins and Tryptic Proteolysis: Enzyme Inhibition or Substrate Deprivation?

The preceeding experiments, in common with all those previously performed by other workers, investigate the effect of tannins on protease activity by measuring the rate at which proteolysis products are produced. Such an approach prevents any information being gained as to whether the tannin combines directly with the enzyme, as may be the case with pepsin, or whether the tannin affects proteolysis more by binding to the substrate protein.

Here the direct effects of a tannin upon the activity of trypsin in the presence of a protein substrate are reported for the first time. These effects have been studied by observing the rate of autolytic (i.e. self) degradation of trypsin (at pH 7.5) in the presence of a substrate protein (Bovine Serum Albumin; BSA), a tannin (tannic acid; TA), a bile salt (Sodium Glycocholate; GCo) and combinations of these. Trypsin activity in the various systems was measured by following its esterolytic reaction with an artificial substrate (α-N-Benzoyl-L-Arginine Ethyl Ester; BAEE) which is not precipitated by tannins in the conditions employed in the assay. This system thus allows for the measurement of (i) the loss of trypsin activity upon the addition of

tannin to the system and (ii) the influence of BSA, TA & GCo upon autolysis of trypsin, itself a proteolytic reaction.

5.3,1 Materials and Methods

The method employed for performing the basic autolysis reaction follows that of Whateley (1973), whilst the assay of trypsin esterase activity follows Schwert and Takenaka (1955).

A trypsin stock solution (20 mg/ml, Sigma type III) was made up in 0.001M hydrochloric acid to prevent autolysis. At time zero 1 ml of this solution was added to 10 ml of a calcium free buffer (0.05M NaCl, 0.01M Tris, 0.002M EDTA, pH 7.5) in order to initiate autolysis. Control reactions contained no other additions. The experimental variations made consisted of including the following substances in the buffer as follows: BSA, (Sigma, fraction V) to a concentration of 10 mg/ml; TA, (BDH Ltd) to 1 mg/ml and GCo, (Sigma, grade I) to 8mM. All glassware coming into contact with trypsin was silanised using "Repelcote" (Hopkin & Williams Ltd) to prevent adsorption catalysed autolysis. The reactions were contained in test tubes placed in a waterbath set at 25 oC.

In order to assay the tryptic activity of the reactions, 50μl samples were taken at timed intervals and

added to 3 ml of BAEE reagent (0.333 mM α-N-Benzoyl-L-Arginine Ethyl Ester.HCl, Sigma; 0.1 M tris; 5.0 mM CaCl ; pH 8.0) contained in a cuvette. The rate of esterolysis was measured immediately by recording the rate of change in the absorbance at 254 nm using a spectrophotometer coupled to a chart-recorder. All experimentally treated systems were run concurrently with a control reaction for comparison. Within the experiments each assay of tryptic activity was made in duplicate and each set of experiments was replicated and so the points plotted in the figures are derived from the mean values of these observations. The error bars surrounding the points indicate 95% confidence limits.

5.3,2 Results

The change in tryptic activity measured during the course of autolysis is expressed in terms of the activity of the control reaction at time zero (A_o) divided by the activity (A_x) for a particular time and system. For the experimental conditions employed, this method of data analysis gives linear functions for the control reaction and other reactions with comparable kinetics (Johnson and Whateley, 1972). As defined above, the control reaction has a constant A_o/A_x of 1.0 at time zero. The vertical shift upwards (A_o/A_x at time zero is significantly greater than control $p < 0.001$) for the reaction

containing TA only (Figure 5.7) can be explained by an immediate loss of some trypsin by precipitation consequent upon its addition at time zero. This vertical shift is eliminated by the inclusion of BSA in the system, an effect explicable in terms of competition in which BSA exhibits a much greater specificity for tannic acid than does trypsin.

The autolytic rate of trypsin self degradation in the TA (only) containing system is markedly faster than that in the other systems, despite the concentration of the enzyme in solution being lower. This apparent faster autolytic rate could be artifactual owing to progressive enzyme inhibition by binding with TA. However, most authors regard tannin-protein binding as being a rapid process occurring over 10-15 minutes (Goldstein and Swain 1965; Van Buren and Robinson 1969; Hagerman and Butler 1978; Martin and Martin 1983), which implies that loss of activity due to tannin binding should have taken place before the second observation at 40 minutes. An alternative explanation for the increased autolysis in the presence of TA is that conformational changes have been induced by the tannin binding to trypsin and that this directly promotes autolysis, as occurs when trypsin is adsorbed on (negatively charged) silica surfaces (Whateley, 1973).

The effect of adding GCo and then trypsin to TA

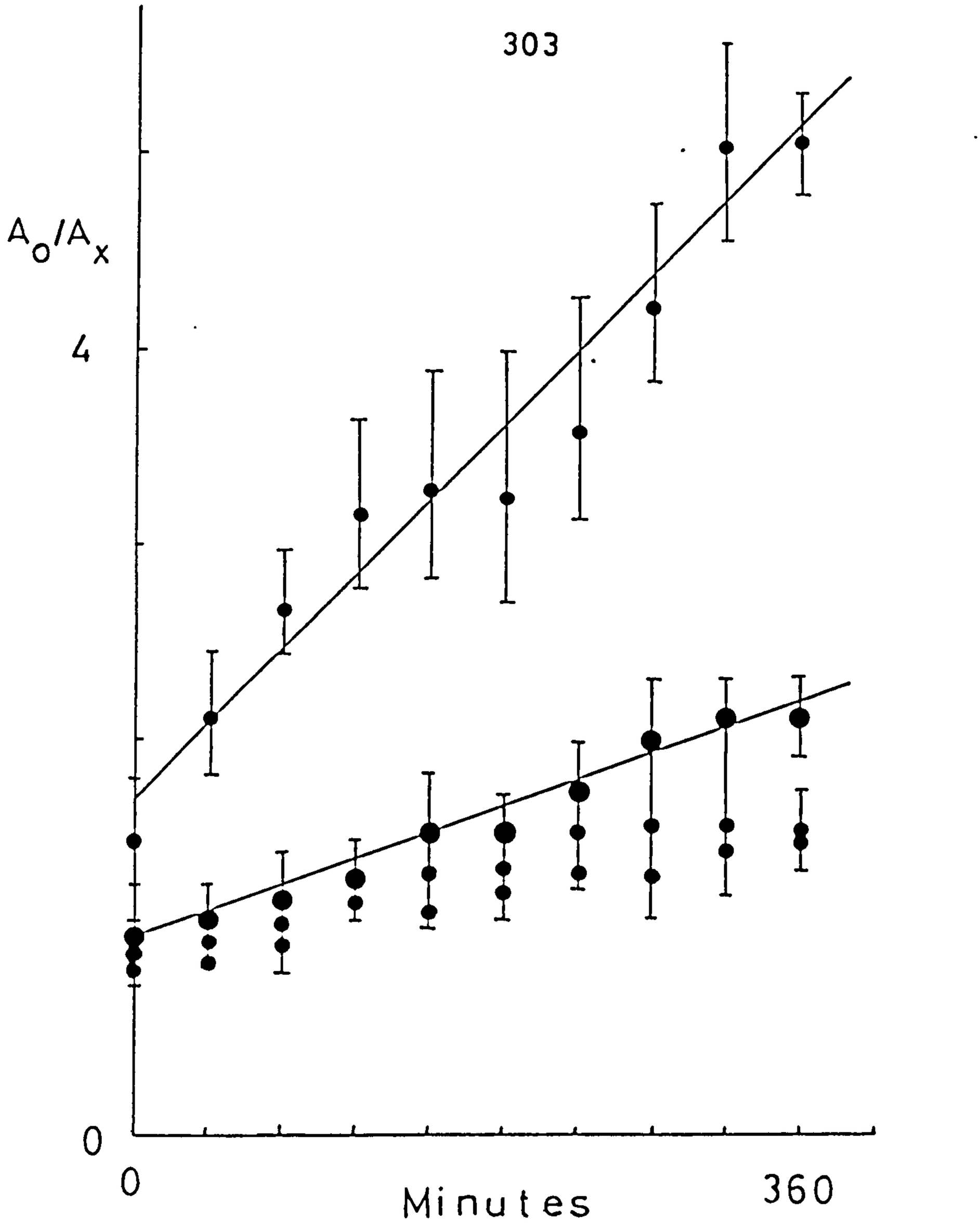


Figure 5.7 The ratio (A_o/A_x) of the time zero trypsin esterase activity for the control reaction (A_o), to the trypsin esterase activity for particular times and systems (A_x), is plotted against time in minutes. The uppermost curve is for a tannic acid containing reaction whilst the (lower) curve drawn through the enlarged dots is for the control reaction. Below the control curve points for reactions containing BSA and BSA plus TA are plotted. Whilst confidence limits for these and those of the control reaction overlap, the lowest point plotted is in each case that for the BSA (only) containing reaction.

(Figure 5.8) is to totally prevent trypsin-TA precipitate formation, leaving an optically clear solution. However the failure of this surfactant to eliminate the initial inhibition of trypsin by TA (A_o/A_x at time zero is significantly greater than control $p<0.001$) or to reduce the TA-induced increase in autolytic rate clearly indicates that the surfactant effect of GCo is limited to preventing tannin-protein complexes aggregating into a flocculum, and does not prevent tannin-protein complexation and consequent protein denaturation per se. Reactions containing GCo and trypsin only were found to be indistinguishable from those without, as were reactions containing all three additives together (i.e. TA, BSA & GCo).

5.3,3 Discussion

These results confirm the work of Hagerman and Butler (1981) by demonstrating again the specificity of tannin-protein interactions. Here, in the presence of competition from BSA, trypsin is seen to be an ineffective competitor for complex formation with a hydrolysable tannin. In terms of modelling digestion the significance of this observation is to confirm that the effect of TA is to deprive trypsin of substrate rather than to act directly upon it, so distinguishing TA from specific trypsin inhibitors (Ryan, 1979). Interestingly,

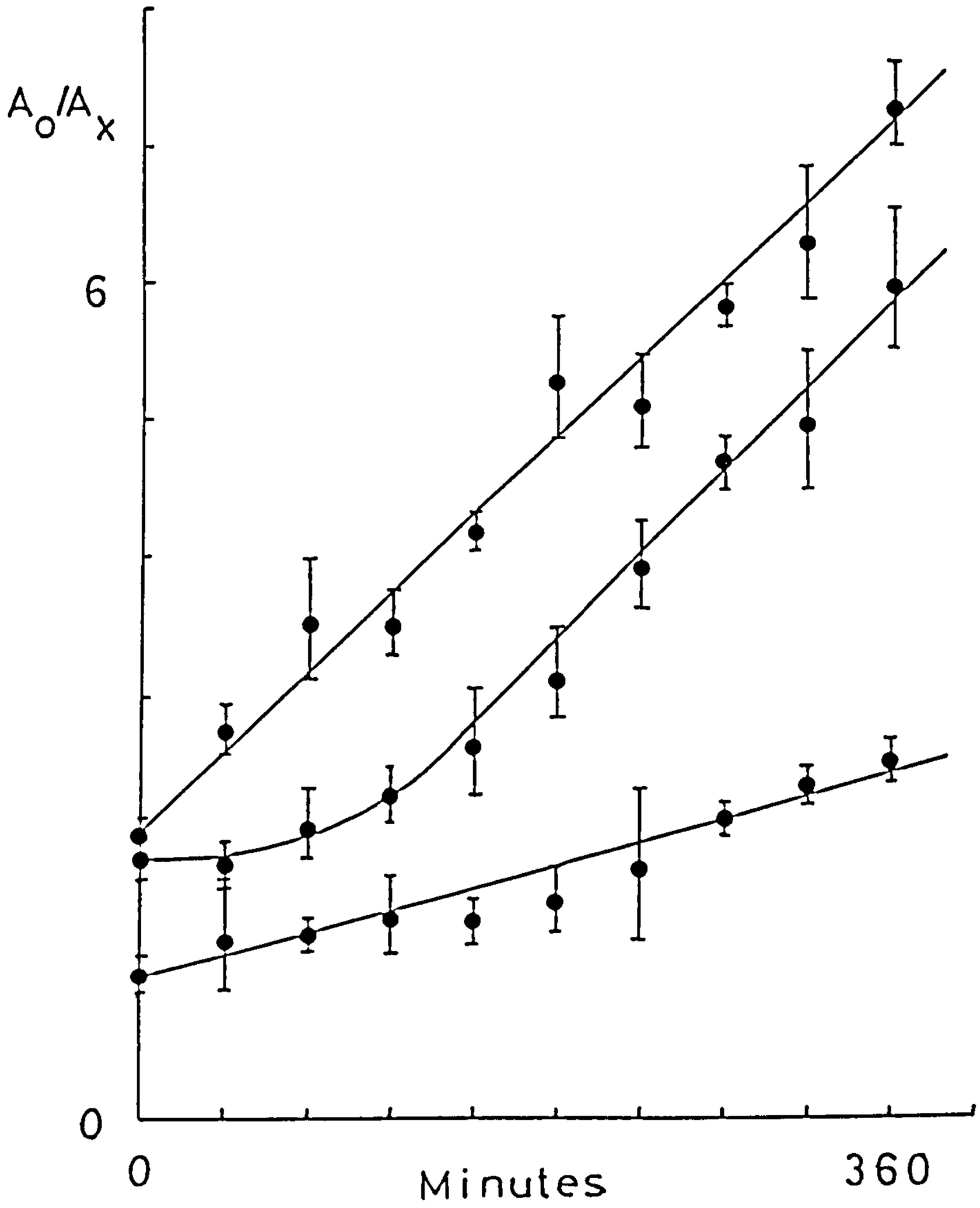


Figure 5.8 The ratio (A_o/A_x) of the time zero trypsin esterase activity for the control reaction (A_o), to the trypsin esterase activity for particular times and systems (A_x), is plotted against time in minutes. The uppermost curve is for a tannic acid containing reaction whilst the lowest curve is for the control reaction. Between these lies the curve for a reaction containing both tannic acid and glycocholate.

the importance of the competitive effect between trypsin and BSA is also found to extend to bile salt solubilised systems in which, despite the lack of a precipitate, there is evidence for a BSA-tannin interaction leading to the relief of trypsin from inhibition. This provides complementary evidence to the earlier report concerning the tryptic digestion of soluble BSA-tannin complexes (Section 5.2).

The general ecological significance of these observations is to re-emphasise the need to study proteolysis in those digestive systems where gut surfactants may be preventing the insolubilisation of proteins by tannins. With the probable exception of the rumen, the effects of bile salts in mammals and gut surfactants in insects (Martin and Martin, 1984) need to be accounted for in the prediction of ecological effects arising from dietary tannin consumption.

5.4 Soluble Tannin-Protein Complexes in Digestion

The results presented in this chapter do not claim to be based upon a perfect *in vitro* model of digestion and as the error involved in translating results obtained *in vitro* to the *in vivo* situation was the impetus for this work the attempt will be made to avoid repetition of such errors here.

The central points made by the results reported here are that if we wish to understand the influence of tannins upon digestion we must (i) consider the effect of tannins on enzyme-substrate systems (i.e not settle for measures of precipitation) and (ii) examine the system at a realistic pH and ionic strength and at the correct relative and absolute concentrations of tannin, enzyme and substrate. These principals are not very new although they have hitherto rarely been observed in practice.

The results of the present chapter considerably extend the fine detail involved in fulfilling these conditions where proteolytic systems are concerned. The enzyme substrate should be in the same state as that found in the gut, as the history of its extraction and treatment affect the results (N.B. the effect of precipitate redissolution seen in Section 5.2). Ideally protein in its native state needs to be the starting material for

the experiment and this should then be taken through procedures mimicking mastication and gastric pre-treatment. The role of the relative specificities of different proteins for binding tannins was revealed in section 5.3 and so models of digestion need to control for the effects of gut wall proteins in interfering with the proteolysis of dietary protein, otherwise even if the correct protease and substrate protein are used results may be misleading. Finally, if all these factors are accounted for misleading results may still be obtained if intestinal surfactants are omitted from the equation.

In short, the accomplishment of a model that might be used to study the effects of tannins in protein digestion with confidence is a considerable technical problem, particularly where leaf proteins such as ribulose bis phosphate carboxylase are to be used as the substrate. Indeed, the cost and effort involved may not be worthwhile when the in vivo evidence from feeding trials is examined. This is because of the clear lack of evidence to substantiate claims that tannins act as digestibility reducers. What the results of this and the preceeding chapter indicate are reasons why these effects may not be seen and why animals may be able to exploit tannin rich foods (e.g. Table 1.3 studies 9, 18, 23, 33, Table 1.4 studies 10, 11, 12). Except for ruminant systems, where they are probably absent, surfactants may

be a major factor in alleviating the effects of tannins. The only supporting evidence available so far from whole animal studies is that of Wursch (1979) who found increases bile salt losses for rats on high tannin diets and that of Freedland et al. (1985) who found that saponins removed the effects of tannins in the diet.

The other probable major factor to be considered is the specificity with which tannins bind to proteins. Work by Butler and co-workers (Hagerman and Butler, 1980b; Mehansho et al. 1983) indicates that tannins show a very strong preference for binding to proline-rich proteins and that human and rat saliva contains such proteins. In rats their production can be enhanced by high tannin diets or by the β-agonist isoproterenol. As yet unpublished work (Butler pers comm.) indicates that these proteins can substantially reduce the effect of tannins in the diet of a rat but if a β-antagonist is administered, (e.g. propanalol) weight and high faecal nitrogen losses occur in the animal. The occurrence of such proteins in the saliva is a mechanism whereby both trypsin and dietary protein may experience at least partial relief from the effect of tannin in the diet as envisaged in Section 5.3. This is particularly important as trypsin, together with enterokinase, is vital to the generation of intestinal endopeptidases, a process that occurs in the presence of tannin, and which is the key to

the "cascade" type of mechanism whereby these proteases are generated.

Chapter Six

Factors Affecting the Production of Tannins

6.1 Why Look at Tannin Production?

The introduction to this thesis laid considerable stress upon the supposed role of tannins in plant defence against herbivores, and indeed this is the best researched aspect of their ecological impact. It was nevertheless concluded that the evidence from *in vivo* studies supporting a clear digestibility-reducing or toxic effect for tannins is less than convincing. The experimental work on digestibility-reducing aspects of tannins, which was concluded in Chapter 5, supports the view that tannins can act as digestibility-reducers but may not work effectively as digestibility reducing allelochemicals under many "natural" situations. The *a priori* assumption is that plants or plant parts with the capacity to produce tannins and which are more exposed to herbivory, will be those likely to produce most tannins. This production of tannins is a response at the level of plant metabolism, and even given the assumption that tannins have no metabolic or physiological role (Section 1.2), it is in terms of the constraints upon the flux of fixed carbon through metabolism that explanations for tannin production will have to be sought.

It is thus worth considering the constraints upon tannin production in plants whilst trying to evaluate their function. This follows because a plant product has an associated cost, in terms of fixed carbon resources,

to the plant that produces it. Whilst the production of tannins might be at some constant constitutive level in each plant, there is evidence that the levels produced are variable and under control (Schultz, 1983; Baldwin and Schultz, 1983). On the assumption that this variaton is of adaptive value in terms of plant fitness, an understanding of the conditions which control the production of tannins may help us understand their role.

6.1,1 Tannin Production in Response to Herbivory.

There is now evidence from several and varied sources that herbivory can effect plant fitness and the dynamic properties of vegetation (Miles, 1979; Whittaker, 1982; Piggot, 1983). Given that the level of herbivory is important to a plant's performance, then any change in a plant's suceptibility to herbivory will be reflected in its vigour and abundance. Given that tannins are defence compounds, then from the plant's point of view tannin production should cost less than the damage that would occur in the absence of its production. An inducible element in tannin production in response to herbivory is from this view point a reasonable response to herbivory, in that resources are not squandered in the absence of herbivores.

Baldwin and Schultz (1983) have provided evidence that trees may be able to respond to herbivore damage in

neighbouring trees by a sensitivity to a gaseous chemical messenger (so far undefined), and so increase levels of allelochemicals in advance of any increased herbivore attack that they themselves may suffer. Evidence for a similar response in willow is provided by Rhoades (1983). Other and better substantiated evidence shows that plants can increase their production of tannins (or at least phenolics) once attacked. This has been independently demonstrated for birch by Finnish workers (Haukioja and Niemela, 1976, 1977, 1979; Haukioja, 1980) and by a separate group working in Britain (Edwards and Wratten 1982). The effect has also been seen in oak (Schultz and Baldwin, 1982) and so may turn out to be a general effect. Damage seen to induce tannin production has included both that by insect herbivores and that produced artificially by mechanical means.

Taken at face value this can be interpreted as strong evidence that induced tannin production is important in an antiherbivore defence role. However, after more prolonged and critical studies the Finnish group of workers have begun to reinterpret this effect as being at least in part a more general response to environmental stress. They have shown that soil conditions and plant nutrient stress were also factors affecting the quantity of tannins (by implication, see Haukioja and Niemela, 1976) produced and the duration of the response (Haukioja

and Niemla, 1976, Tuomi et al., 1984). In their most recent publication (Haukioja and Neuvonen, 1985) they have thus been unable to discount the hypothesis of nutrient stress as a factor in the response of trees to defoliation.

Studies describing the influence of soil nutrient availability on tannin production now form quite a large body of information which shows that the production of phenolics, and tannins in particular, is a general response by plants to mineral nutrient deficiencies. This will be dicussed in Section 6.3. The experimental work presented in this chapter is aimed at further investigating the notion that tannins are produced in response to factors other than herbivory. Here evidence is presented that tannin production is considerably influenced by the ambient light intensity in a plant's environment.

6.2 Light Intensity and Tannin Production I

This Section and the following (6.3) describe a field study which investigates the effect of ambient light intensity on the production of tannins and associated phenolics by the leaves of four West African tree species. The field work was conducted jointly by J. A. M. Ross and myself, with the help of four undergraduate field assistants. Decisions as to the species to study and material to sample were made with Ross, and all the numerical data introduced in Section 6.2 is from field measuremants or laboratory analyses carried out by her. Section 6.2 describes the work necessary as a background to my own studies which are reported in Section 6.3.

6.2,1 Introduction

The starting point for this work was an earlier report (Waterman et al., 1984) which showed that individuals of the tree species Barteria fistulosa (Passifloraceae) growing in forest clearings in the Douala-Edea Forest Reserve, Cameroun, respond to the higher light intensity of that environment by the increased production of phenolics in their foliage. Specifically, the condensed tannin component of foliar total phenolics was shown to exhibit this response to high light intensity. Barteria

fistulosa receives considerable protection against herbivory through an obligate mutualistic association with pseudomyrmicine ants (Janzen, 1972) so in this instance the production of condensed tannins seems to be attuned to the physical (i.e. light) environment rather than any need for a chemical defence (Waterman et al., 1984). Woodhead (1981) has shown that the total phenolic content of the condensed tannin containing species Sorghum bicolor (Graminae) likewise increases with high light intensity.

If it were to be shown that these are not isolated cases and that a plant's physical environment exerts a major influence on the production of these presumed chemical defences, then the impact of herbivores in determining the level of investment in plant secondary metabolism may not be as generally great as has been thought (Fraenkel, 1959; Janzen, 1979; McKey, 1979), at least for these phenolic compounds. In addition to examining the hypothesis that light intensity can influence the quantity and qualitative composition of phenolic allelochemicals in leaves, the analysis was extended to cover responses to light within individual plants. The reason for the latter lies in the many reports indicating the adaptive within-plant distribution of chemical defences (McKey, 1979; Choo, 1981). It is thus important to demonstrate a response to the physical

environment on a similarly small scale.

The species chosen for the study came from both closed and open canopy vegetation types, within which relativly shaded and unshaded plants were found. They were as follows. (i) Acacia pennata (L.) Willd. (Mimosaceae), a spiny climber found growing in light gaps in recently regenerated secondary forest. (ii) Cynometra leonensis Hutch. & Dalz. (Caesalpinaceae), a large forest tree which produces abundant epicormic shoots and thus provides easily accessible foliage. (iii) Diospyros thomasii Hutch. & Dalz. (Ebenaceae), a small understorey tree growing to between 5 and 10 metres high. (iv) Trema guineensis (Schum. & Thonn.) Ficalho (Ulmaceae), a small tree found in open secondary regrowth over disused farm land.

6.2,2 Materials and Methods

The study site was located in Sierra Leone, on Tiwai Island (12000 ha), which is located (7^{o} 33'N 11^{o} 21'W) in the Moa River, some 60 km inland from the Atlantic Ocean. The Island carries no permanent settlement. About half it's area supports 40-60 year old secondary high forest (Cole, 1968), in which leguminous species predominate (e.g. Cynometra leonensis, Piptadeniastrum africanum, Pentaclethra macrophylla). The remaining vegetation is a mosaic of swamp, riverine and young secondary forest

types, together with some recently farmed land. Mean annual rainfall in the area is 3300mm. Our species identifications were made with reference to identified specimens growing on site and to Hutchinson and Dalziel (1958) and Savil and Fox (1966).

For the measurements of light intensity individual plants were selected for their possession of branches in relatively sunny and/or shaded positions, which could easily be reached from a 6m ladder. Once located suitable groups of leaves were tagged for reference. Whenever possible, shaded and unshaded leaves were chosen from the same plant, but this was not always feasible and single sets of leaves were taken from individual plants some of the time. These sets of leaves were made the object of light intensity measurements for a sample of five days. The methods used for this are described in Appendix 2.6 and they yield an approximate measure of light intensity on a continuous but not necessarily linear scale.

On the completion of light intensity measurements leaves were collected and sun dried, the drying process being initiated within one hour of collection. For T. guineensis, which has simple alternate leaves borne on long straight branches with the youngest leaves at the single apex, leaves were separated into the following age classes: "Young", the six leaves over 2cm long closest to

the apex; "Mature", the next six older leaves and "Old", the remaining older leaves if present. Dried leaves were sealed in air-tight bags and returned to the laboratory for chemical analysis. In the laboratory samples were ground to give a maximum particle size of 1mm before chemical analysis.

Samples were assayed for their content (% dry wt.) of total phenolics (TP), condensed tannins (CT) and nitrogen (N). Total phenolics were estimated using the Folin-Denis method on extracts prepared in 80% aqueous methanol under reflux and are expressed in terms of tannic acid. Condensed tannins were estimated using a sample of the same extract and the proanthocyanidin method, with results expressed in terms of quebracho tannin. The details of the assay procedures have been described in Chapter 2 and by Gartlan et al. (1980) as have their applicability to dried material (Waterman et al. 1984). The nitrogen content of ground samples was measured by the Kjeldahl method with crude protein (PROT) being estimated as N% X 6.25.

6.2,3 Results

The four species studied were sampled from a range of light environments varying from open areas to closed forest. On average D. thomasii received the least percentage of available light (approximately 7%), with C.

leonensis, the other closed forest tree species , receiving slightly more (approximately 15%). A. pennata, which was sampled from light gaps in the forest received slightly more available light than the preceeding two species (approximately 28%), whilst T. guineensis received by far the most with shade leaves averaging 81% of the available light and the sun leaves receiving by definition 100% (see Apendix 2.6). The data for the shade leaves of T. guinensis (sampled exclusively from the basal foliage of the trees) is particularly approximate as inclement weather prevented a full five day sampling program. However, on the available data a statistical analysis (Mann-Witney U test) revealed that the median values of available light (LIGHT) were significantly different for every possible inter-species comparison ($p < 0.05$).

The following analysis is a species by species description of the relationships found between light intensity and the chemical measurements made on the samples of whole leaf material.

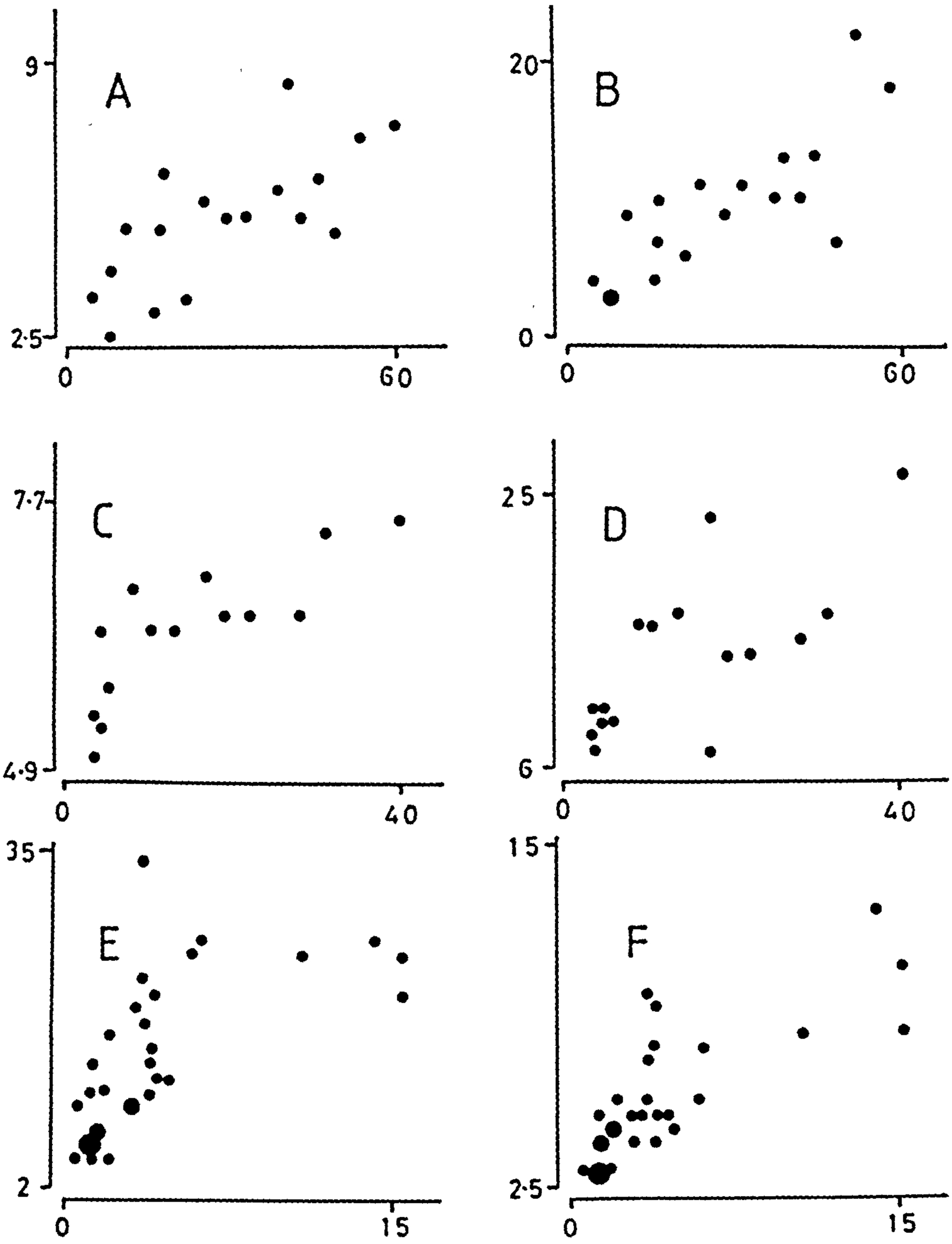
Acacia pennata. Samples were taken from a total of ten individual plants which provided eight paired samples for within plant comparisons of the effect of light intensity on leaf chemical composition. Scatter graph plots of the data for light intensity (LIGHT) versus total phenolics (TP) and condensed tannins (CT) are

presented in Figure 6.1 a&b. Positive correlations are clearly evident i.e. phenolic production increases as light intensity increases. The correlation coefficients between these three variables are (i) LIGHT versus TP, r = 0.73; (ii) LIGHT versus CT, r = 0.81 and (iii) TP versus CT, r = 0.86. All these correlations are associated with a high degree of statistical confidence ($p < 0.001$). The above analysis could simply reflect gross inter-plant differences. However, paired samples taken from the same plant (paired sample t-test, Campbell, 1974) show that there are significantly more total phenolics and condensed tannins in the leaves receiving the higher light intensity ($p < 0.01$) within any given individual. No correlations were found between any of the variables and foliar protein content (PROT), no value of the correlation coefficient, r, exceeded an absolute value of 0.2.

Cynometra leonensis. A total of eleven individual trees, which again provided eight paired samples for within plant comparisons, were studied. Scatter graph plots for the whole data set are given in Figure 6.1 c&d, where positive correlations are again evident between the three variables LIGHT, TP and CT (see Table 6.1) and the analysis of the paired data also showed the same within plant variations as before ($p<0.01$). However, for this species, significant negative correlations (in the whole

Figure 6.1 Scattergraphs of Foliar Condensed Tannin (CT) and Total Phenolic (TP) Contents (% dry weight) against Light Intensity (LIGHT) for Acacia pennata (plots A & B), Cynometra leonensis (plots C & D) and Diospyros thomasii (plots E & F).

Key: In each of the six plots LIGHT (%) is measured allong the x axis. For plots A, C and E the y axes indicate total foliar phenolic (TP) contents whilst in plots B, D and F the y axes indicate condensed tannin contents (CT).


A
9
2·5
0
60
B
20
0
0
60
C
7·7
4·9
0
40
D
25
6
0
40
E
35
2
0
15
F
15
2·5
0
15

data set) were found between protein and total phenolics (r = -0.65, p < 0.05) and between protein and condensed tannins (r = -0.62, p < 0.05) but not between available light and protein (r = -0.30).

Diospyros thomasii. Samples were taken from twenty-five trees with twelve paired samples. The scatter graphs and correlation coefficients for the data set are presented (Figure 6.1 e&f and Table 6.1), and the same pattern seen for A. pennata and C. leonensis is repeated. The analysis of paired samples again confirmed the results seen previously ($p<0.01$). No significant relationships were found between protein and any other variable.

Trema guineensis. In the absence of light measurements on a continuous scale, paired samples of leaves were taken from "sun" and "shade" positions on each of eight trees. Table 6.2 gives summary statistics for leaves in each light environment and for each of the age classes previously defined. Irrespective of leaf age class, "sun leaves" have greater quantities of total phenolics and condensed tannins than "shade leaves". With the single exception of condensed tannins in "old leaves", all these differences are statistically significant ($p < 0.05$). The correlation coefficient between total phenolics and condensed tannins was again high ($r = 0.73$ $p < 0.001$), for the whole data set.

Table 6.1 Correlation Coefficients Between Light Intensity (LIGHT) and Condensed Tannin Content (CT) and Total Phenolic Content (TP) for Foliage Samples of Three Species.

Species	LIGHT v.s TP	LIGHT v.s CT	CT v.s TP
Acacia pennata	0.73	0.81	0.86
Cynometra leonensis	0.69	0.72	0.84
Diospyros thomasii	0.65	0.79	0.74

Statistical Confidence (Campbell, 1974) for r=0, $p > 0.001$ for Acacia pennata, $p > 0.05$ for other species.

Table 6.2. Means and 95% Confidence limits (in parentheses) for the Total Phenolic (TP), Condensed Tannin (CT) and Protein (PROT)[1] contents[2] of foliage samples[3] taken from Trema guineensis, together with the correlation coefficients (r) between the two measures of Phenolcs

		"Sun Leaves"	"Shade Leaves"
"Young Leaves"	TP	2.72 (0.39)	1.42 (0.46)
	CT	14.11 (2.73)	5.96 (1.72)
	r	0.51	0.72
	PROT	18.59 (2.21)	18.03 (1.47)
"Mature Leaves"	TP	2.32 (0.22)	1.75 (0.57)
	CT	9.56 (1.30)	5.54 (2.04)
	r	-0.25	0.33
	PROT	14.46 (1.23)	15.30 (2.54)
"Old Leaves"	TP	2.03 (0.43)	1.30 (0.40)
	CT	7.99 (2.01)	6.19 (2.05)
	r	0.90	0.29
	PROT	11.10 (1.53)	12.55 (1.84)
All Ages Combined	TP	2.36 (0.20)	1.49 (0.20)
	CT	10.55 (1.46)	5.90 (2.51)
	r	0.73	0.35
	PROT	14.72 (1.36)	15.50 (1.55)

(1) PROT= N x 6.25. (2) % dry weights. (3) Eight paired "sun" and "Shade" Samples (from each age group) taken from eight separate plants were used in this analysis.

The correlation coefficients for the age/light classes show considerable variation (Table 6.2).

With T. guineensis not only does the light environment affect the quantity of foliar polyphenolics within age classes but it also appears to influence differences between age classes. For "sun leaves", "young leaves" have more condensed tannins than either of the other two age classes ($p < 0.01$ in both cases), and more total phenolics, significantly so in the case of "old leaves" ($p < 0.03$). In marked contrast there is no consistent variation in the "shade leaves". In the analysis of foliar protein content no statistically significant differences between "sun" and "shade" leaves were found for any age class. Irrespective of the light environment "young leaves" contained higher levels of protein.

6.2,4 Discussion

The results show that for all four species studied there is, without exception, an increase in the level of total phenolics and condensed tannins with increased light intensity. This occurs both within and between individual plants. These differences are found at near maximal, and relatively low, ambient light intensities, and thus the relationship would seem to hold for both extremes of the light environment. This was also found to hold amongst individual trees which would all be

classed as shaded by the subjective standards of the B. fistulosa study (Waterman et al., 1984).

Waterman et al., (1984) assumed that the response to the light environment in B. fistulosa was not due to genetic differences between individual plants. Clearly, for the four species studied here this assumption is correct because the response has been traced to the within plant level. It would thus seem that the phenomenon observed in this study, and probably those of Waterman et al. (1984) and Woodhead (1981), is a physiological response by individual leaves or branches to their microclimate.

The results for T. guineensis show that the response occurs for leaves of all ages and that, on a more subtle level, the light environment appears to affect the difference in chemical composition between leaves of different ages. Unfortunately, T. guineensis was the only species whose leaves could be assigned to age classes with confidence. It would be interesting to know if this response to light holds true for the other species.

6.3 Light Intensity and Tannin Production II

The study of Waterman *et al*. (1984) equated the increase in phenolics found in more highly insolated leaves with an increase in condensed tannins. In Chapter 2 it was explained that owing to the chemical variability of condensed tannins it is not possible to make a reliable estimate of the proportion of condensed tannins in an extract's total phenolics using the results of the proanthocyanidin and Folin Denis assay results alone. Nevertheless the ratios of the CT and TP values can give some indication of this proportion, particularly for intraspecific analysis of data where the hazards in this kind of analysis are likely to be much reduced. An analysis of such data, gained from the work presented in Section 6.2, is given below so as to introduce the rationale behind the subsequent more detailed analysis of the phenolic material contained in the trees studied.

6.3,1 Introduction

The significant correlations between LIGHT and CT, and TP and CT, reported above, are strongly suggestive that the increase in total phenolics is one of condensed tannins. However, a first indication that this does not satisfactorily explain the whole variation, is seen in the wide variability in the correlation coefficients

between CT and TP for the different light/age classes of T. guineensis (Table 6.2). This clearly suggests changes in other phenolics may well be involved for this species at least. Given that condensed tannins are not the only phenolic components of plant material, then if only condensed tannins increased with light, the ratio of the two variables (CT/TP) should also increase, as condensed tannins become a greater proportion of the total phenolics. For the three species where such an analysis is possible, the correlations of LIGHT versus (CT/TP) are as follows: A. pennata $r = 0.77$, $p < 0.001$; C. leonensis $r = 0.66$, $p < 0.01$; D. thomasii $r = 0.12$, NS. In the first two instances it would indeed seem to be the case that the proportion of condensed tannins increases in higher light intensity, whilst for D. thomasii no such correlation occurs. In the latter case it may be that there is a more general and coordinated increase in the production of phenolics, such as might include hydrolysable tannins (thought to occur in D. ebenum, Section 2.6). The conlusion that the increase in other phenolics is a coordinated one follows because a disproportionate increase in other phenolics would have led to a negative value of r.

Table 6.3 presents mean values for the CT/TP ratios for all four species, including the different age/light classes of T. guineensis. By inspection it can be seen

Table 6.3 The ratios between the Condensed Tannin (CT) and Total Phenolic (TP) contents (CT/TP) of the leaves sampled from the four species studied, including values for each light/age class of Trema guineensis sampled.

Species	CT/TP Ratio Mean, 95% Confidence Limit	
Acacia pennata	2.44	0.77
Cynometra leonensis	2.63	0.68
Diospyros thomasii	0.99	0.26
Trema guineensis	4.35	1.46 (whole data set combined)
" "	5.23	0.99 (young sun leaves)
" "	4.18	0.83 (mature " ")
" "	3.89	0.53 (old " ")
" "	4.31	0.98 (young shade ")
" "	3.42	1.39 (mature " ")
" "	5.06	1.82 (old " ")

that for T. guineensis the values for "sun leaves" are not consistently higher than those of "shade leaves". So, applying the arguments used above once again, other phenolics may be involved in the response to light in this species as well as in D. thomasii. Of the four species, T. guineensis has the greatest proportion of condensed tannins amongst its phenolics, with the two leguminous species being intermediate and D. thomasii having by far the least. On this basis, if other phenolics are involved with T. guineensis then light dependent changes in other phenolics cannot be ruled out for any of the other three species, not just for D. thomasii. To summarise, for all four species, condensed tannins may not be the only component of the total phenolics which increase in the light.

In order to make a more detailed examination of the changes in phenolics involved, it was felt necessary to apply the methods of Chapter 2 and work on pigment-free extracts of material. Such extracts were prepared from the dried leaf material remaining from the analysis presented in Section 6.2. In the case of D. thomasii additional material collected from plants more widely dispersed over Tiwai island was also used to increase the information on plant to plant variation. This material was collected on sites adjacent to the transect lines of a soil survey of the island. Given that soil conditions

showed relatively little variation, then little variation in plant phenolics is likely to be attributable to this factor which is henceforth discounted. Collections of material for other species were made from within sites sufficiently small for variability in soil type not to be a likely cause of variation in plant phenolics.

6.3,2 Materials and Methods

Extracts were prepared by a variant of the method reported in Section 2.1. 10-20 ml of the milled dry leaf material used in the above analyses was added to a 100ml conical flask and briefly shaken with 25ml of 70% aqueous acetone to wet the material. Then 25ml of diethyl ether was added and the flask was stoppered and set to shake on an orbital mixer for 24 hours. At the end of this time the mixture was filtered free of solid material and then separated with the lower aqueous layer being returned to the flask and freeze dried whilst the upper ether layer was discarded. The resulting powdered extract was then analysed by the same methods as used in Chapter 2. A major problem encountered in the analysis was that for A. pennata and T. guineensis in particular foliage samples did not always provide sufficient material and so as extracts were consumed results for some analyses are incomplete.

6.3,3 Results

The first step in the analysis of the extracts was to to examine their acid hydrolysates by paper chromatography (methods as for Section 2.6), and a species by species analysis of the results follows.

Acacia pennata. As only 19 extracts were available for analysis each was analysed. In every case distinctive traces for cyanidin and delphinidin were evident and typically of equal colour intensity (relative to each other). This means that for the whole species sample the CT/TP ratio over-estimates the quantity of condensed tannin present, but that for plant to plant comparisons this measure is not greatly biased by the prodelphinidin/procyanidin ratio (Pdel/Pcy) of the tannins present. Four extracts yielded an overwhelming predominance of delphinidin. Two of these samples were from highly insolated leaves and two from relatively shaded leaves, these representing collections from four individuals in which the paired collection did not exhibit a correspondingly high Pdel/Pcy ratio. This ratio thus constituted a real but unassignable cause of variation. All the extracts also gave a faint spot which was tentatively identified as ellagic acid (see Appendix 1.9 for criteria) and thus indicated the presence of a trace amount of ellagitannin in the extract.

Cynometra leonensis. A random sample of 15 extracts

was selected for chromatography and in each case a distinct trace for cyanidin was observed together with a very faint trace for delphinidin. This suggests that prodelphinidins do not influence the chromatographic analysis of these tannins to any great extent.

Diospyros thomasii. Again a subset of extracts was analysed and all gave the same pattern. Unlike D. ebenum (Section 2.6) no trace of delphinidin was seen and only a clear spot for cyanidin was produced, so once again prodelphinidins will not influence the analysis of tannins. The presence of ellagic acid was seen and additionaly the chromatogram gave an intense purple colour under UV light below Rf = 0.6. This area also turned brown after exposure to ammonia for several minutes. This indicates the presence of gallic acid which in turn is indicative of hydrolysable tannins in the extract

Trema guineensis. Extracts from this species gave the same patern for anthocyanins as C. leonensis. The spot indicativie of ellagic acid was also present in all the extracts but paper chromatographs of unhydrolysed T. guineensis extracts also showed this and clearly so. T. guineensis may thus contain free ellagic acid, not esterified to sugar as a tannin. Chromatography of unhydrolysed extracts for A. pennata and D. thomasii failed to show this material, and so the predictions made

concerning ellegitannins in these extracts remain valid.

Condensed Tannin Polymer Lengths

To complete the analysis of condensed tannins in the extracts, their polymer lengths were estimated by the methods developed in Section 2.4, so as to produce the measures P1 and P2 used previously. The gaps in the data for this are due only to deficiencies in the quantities of material available for analysis, these gaps are reflected in the sample sizes (n) given for each statistic reported in the text and tables.

Data for mean polymer lengths of the condensed tannins grouped according to species, irrespective of other variables (e.g. light or leaf age) are given in Table 6.4. The first point to notice is the considerable variability of the data as seen from the standard deviations. This applies particularly to D. thomasii tannins. Whereas the single extracts of each species studied in Chapter 2 were investigated simply for their chemical properties, here an indication of the within species variability of these properties is the goal of the investigation. The data shows that owing to the inherent variability in condensed tannin polymer lengths, interspecific variation is not generally significant, the only significant difference being between A. pennata and C. leonensis for P1.

Table 6.4 Condensed Tannin Polymer Lengths (P1 and P2*)

		P1	P2
Acacia pennata	$\bar{x}$	0.77	12.3
	σ	0.09	2.99
	n	18	18
Cynometra leonensis	$\bar{x}$	14.8	16.5
	σ	2.54	5.76
	n	23	25
Diospyros thomasii	$\bar{x}$	8.8	6.88
	σ	1.22	9.65
	n	51	52
Trema guineensis	$\bar{x}$	8.57	29.9
	σ	2.12	10.9
	n	32	29

*P1 and P2 are as Defined in Section 2.4.

On inspection of the data no clear trend in polymer lengths could be seen to depend on plant light environment for any of the species. However, with T. guineensis leaf age had been recorded as well as light intensity and when the interaction between these two factors was taken into account a more subtle pattern in polymer length variation was uncovered. Given that the data set was incomplete an ANOVA analysis was not possible and so differences in polymer length (ΔP1 or ΔP2) were calculated between leaf samples of the various age and light classes taken from individual trees. Table 6.5 gives the mean difference in polymer length (ΔP1 or ΔP2) within each sun/shade class for leaf age (i.e. young-mature and mature-old). Also given are the means for sun-shade differences within the three leaf classes. From this data it can be seen that for both measures of polymer length, there is a more marked difference in polymer length according to leaf age in sun leaves, with the youngest leaves having the longer polymers. The cause of this can be traced to the sun/shade comparisons within each age class. Young leaves tend to have the longer polymers in the sun yet older leaves tend to heve longer polymers in the shade.

The current hypothesis (Haslam, 1977) that the polymerisation of these tannins occurs as an unregulated non-enzymic reaction may be due for review as Butler

Table 6.5 Condensed Tannin Polymer Lengths: Further Analysis of Data for Trema guineensis.

Mean Differences in Polymer Length (P1 and P2) between Younger and Older Age Classes of Leaves within groups of "Sun" and "Shade" leaves.

		mean	std. deviation	replicates
P1	"Sun Leaves"	8.10	2.63	15
	"Shade Leaves"	4.55	2.75	11
P2	"Sun Leaves"	11.4	4.98	13
	"Shade Leaves"	-7.23	7.99	10

Mann-Witney U tests (Campbell, 1974) for differences between the means for "sun leaves" and "shade leaves" indicate significance (p> 95%) for both sets (P1 and P2) of data.

Mean Differences in Polymer Length (P1 and P2) between "Sun" and "Shade" leaves within age groups.

		mean	std. deviation	replicates
P1	"Young Leaves"	21.8	3.45	7
	"Mature Leaves"	1.37	0.35	5
	"Old Leaves"	-5.04	1.57	5
P2	"Young Leaves"	5.49	1.59	5
	"Mature Leaves"	3.80	7.25	7
	"Old Leaves"	-6.04	5.69	7

(pers comm.) has found (*in vitro*) "procyanidin polymerase" activity in *Sorghum* grain. It is thus untimely to attempt a mechanistic interpretation of these results in biochemical terms. However, in the present context, their importance lies in that they provide the first indication that a plant's light environment affects more than simply the quantity of condensed tannin produced.

Hydrolysable tannins

Chromatographic analysis of the extracts led to the expectation that *D. thomasii* tannins comprised both condensed and hydrolysable tannins. Indeed, this was the only species whose extracts gave positive results with the iodate and ferric chloride tests for hydrolysable tannins. In addition to the collection of material for the sun/shade study a considerable amount of other *D. thomasii* foliage had been collected and where appropriate this has been included in the following analysis which in total studied 61 collections. Of their extracts, 59 gave decreases in absorbance at 660nm after the hydrolysis stage in the assay for hydrolysable tannins (Section 2.5), this being before applying a correction for condensed tannins which typically increased the A_1^1 by further a 10%, to give a species mean of 4.9 (s=1.8, n=61). This suggests that *D. thomasii* is a rather rich

source of hydrolysable tannins compared to D. ebenum (see Chapter 2). Relatively few of the extracts analysed (27) were from leaves sampled in known light environments but these did provide a sufficient sample for an analysis to take account of this variable.

In order to estimate the quantities of hydrolysable tannin in the leaves by analysing their extracts, some compensation had to be made for variation in the quantities of material extracted from the leaves (i.e. variation in the quantity of soluble components versus insoluble, e.g. cell wall, in each leaf). The solution to this problem was to use the condensed tannin assay results which were available for both leaf and extract material alike, to provide a means of reference. Condensed tannins were known to increase as a function of the light intensity incident upon the leaves and if hydrolysable tannins were to increase in step with this, then the CT/HT ratio should be a constant, independent of LIGHT. This ratio between the condensed and hydrolysable tannin assays (CT/HT) was calculated for the extracts and its dependence on LIGHT investigated

The mean CT/HT ratio for the 13 extracts exposed to the lowest light intensisy was 4.34 (s=1.49, n=13) and for the leaves exposed to the highest light intensity the mean CT/HT was 3.20 (s=1.58, n=14). So the indication is that in the sun the quantity of condensed

tannins actually becomes smaller in proportion to hydrolysable tannins and an even greater rise in hydrolysable tannins (versus condensed tannins) occurs in the leaves! However, the difference between the means for CT/HT just fails significance at 5%, and so it can only be concluded that net hydrolysable tannin production increases approximately in proportion to condensed tannin production as ambient leaf light intensity increases. This is the first study to report a relation between light intensity, or indeed any other environmental factor, and hydrolysable tannin production.

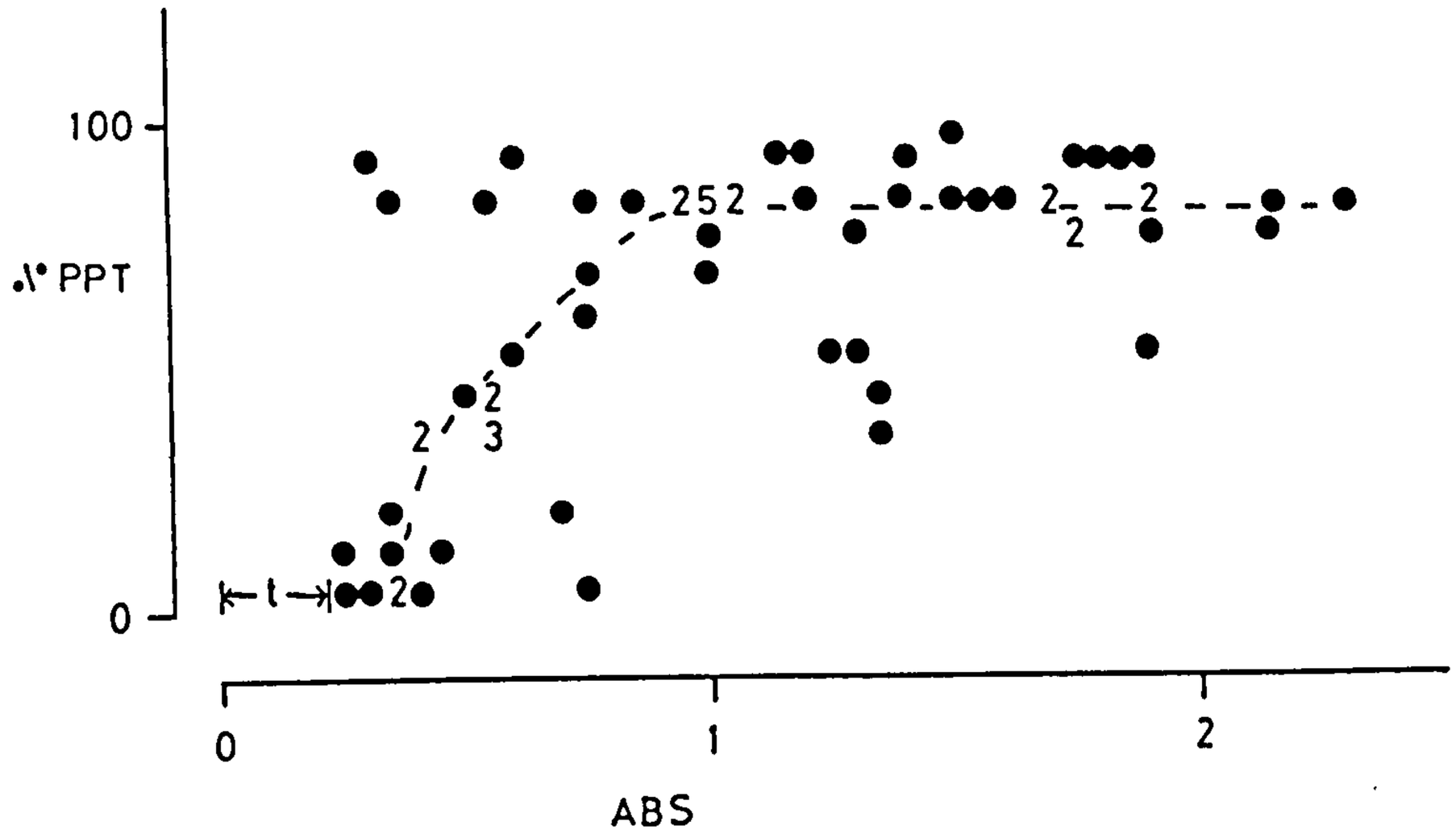
Protein Precipitating Activity

In Chapter 4 it was seen that chemical measures of tannins did not relate well to their ability to precipitate proteins and so an analysis of the astringency of the extracts was made. It was clear from the outset that it was impractical to perform a specific activity assay on each extract, both for reasons of time and because of the small quantities of material available, so the following compromise was adopted. A sample of each extract was made up as an aqueous solution in 5ml of water (concentrations in mg/ml were uncontrolled and results are expressed in terms of concentration of total phenolics) and 0.5ml aliquots of this were added to each of two centrifuge tubes, one

containing 2ml of "blank" pH 4.9 buffer (see Section 4.2) and the other containing 2ml of the BSA solution (pH 4.9) as used for the specific activity assays (see Section 4.2). Tubes containing the BSA were subsequently centrifuged and the results of total phenolic assays (Section 4.2) of the supernatant solutions were compared with total phenolic assays of the alternate solutions of the extracts added to "blank" buffer so as to calculate the proportion of phenolics present which were not precipitated by BSA. Assays for the proportion of BSA precipitated (%PPT) were also made by the Moore and Stein method (see Appendix 2.3). After being used for this (and the hydrolysable tannin assay), the aqueous solutions of the extracts whch remained unused were bulked for each species and freeze dried again to allow for their use in a standard specific activity assay, where the need to vary the phenolic concentration at will necessitated the use of a powdered extract as starting material.

Figure 6.2 shows the data on the percentage of protein precipitated by solutions of D. thomasii extracts of various total phenolic concentrations (each point is for a different extract). This species was chosen for illustration because the extract solutions showed a sufficient range in concentration of phenolics to give protein precipitations from the maximum to near a zero

Figure 6.2 Precipitation of Bovine Serum Albumin by Diospyros thomasii extracts.



Key: The percentage of the maximum recorded amount of protein precipitated (%PPT) is plotted against the absorbance (ABS) of the phenolics present as measured by the Hagerman and Butler assay for total phenolics. Dots represent single results, numbers indicate numbers of coincident points. The letter t indicates a possible threshold level of phenolics below which there is no precipitation.

value. The variability of astringency as a function of a single chemical measure is again seen here. In this case it is presumably intraspecific variation in the qualitative make up of plant phenolics which obscures any consistent relation between the two variables. The logic of this proposition is evident from considering the comparitively low variability of results for the specific activity assay employing the same phenolics (bulked mixtures) and the same protein (see Figure 6.3).

Not all the extract solutions showed the same range of phenolic concentrations when made up and Table 6.6 gives the results (percentages) for protein precipitation (%PPT) and non-precipitating phenolics (NPP), obtained with the extract solutions of each species which fell within two ranges of total phenolic concentration. As can be seen none of the C. leonensis extracts gave phenol-rich solutions and the reverse was true for the A. pennata extracts. Nevertheless, using the Table (6.6) to compare all four species with each other, there are no consistent and significant differences between the species to be seen.

Table 6.7 gives the data obtained in the specific activity assays of the pooled extracts. The reults for SA1 and SA2 are all rather high compared with the data in Chapter 4 (Table 4.2) and this may be due to the use of dried plant material in this work. The error, if it is

Figure 6.3 Specific Activity Results for

Diospyros thomasii.

Key: The scale marked H&B and points marked as dots indicate the absorbance of the total phenolics in the supernatant fractions of the assay. The scale marked NIN and points marked X indicate the absorbances of 100µl samples of the hydrolysed precipitate solutions in the ninhydrin reaction. The x axis indicates the volume of 5mg/ml BSA present in the assays. Horizontal bars indicate the data used for the calculations presented in table 6.7. See Section 4.2 for detatils of the assay and its interpretation.

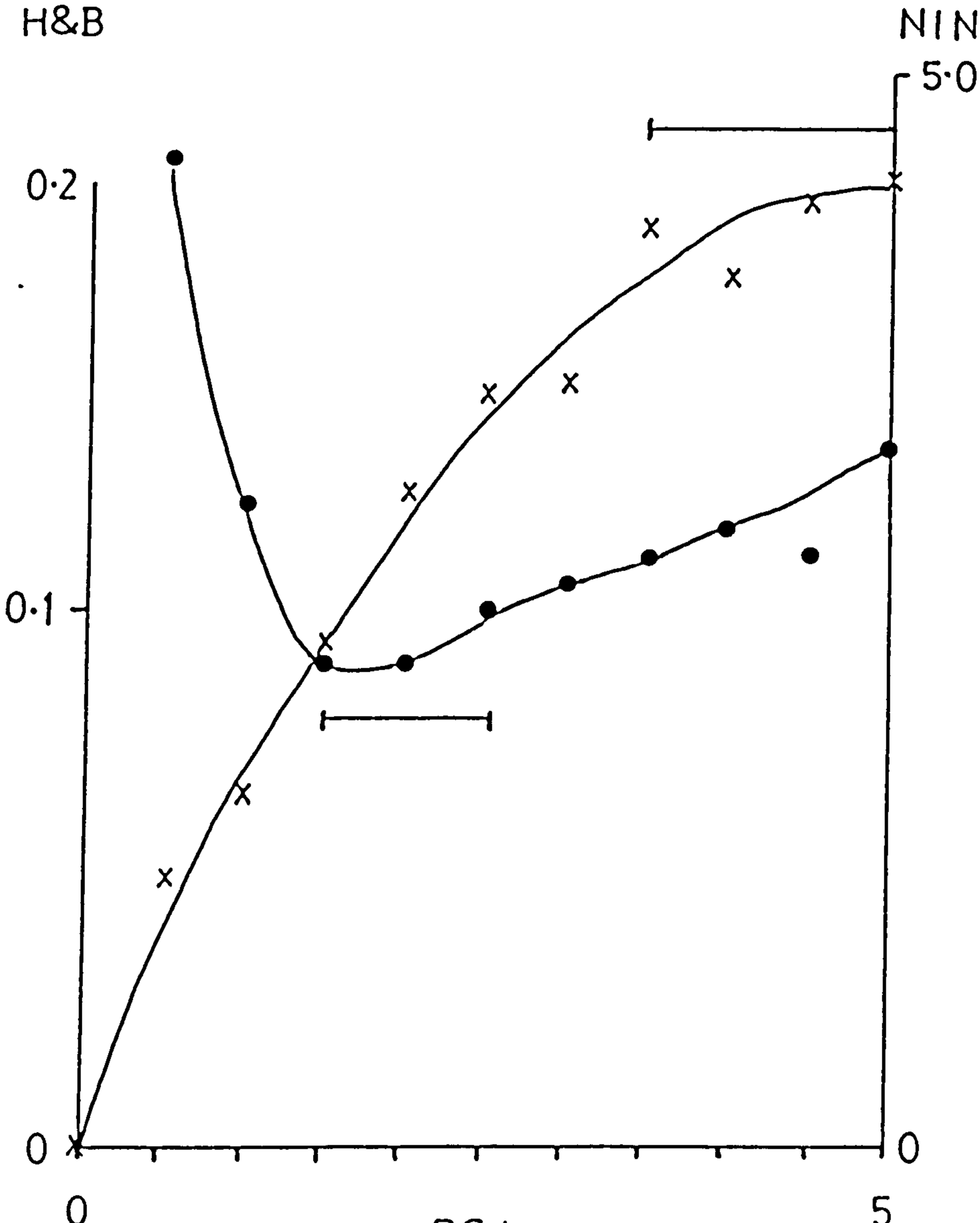

H&B
NIN
5·0
0·2
0·1
0
0
BSA
5

Table 6.6. Means and 95% confidence limits (in parentheses) for the phenolic contents (H&B) of extract solutions, the quantities of BSA they precipitate (PPT) and the proportion of non precipitating phenolics (NPP) in the extracts.*

I Results for phenolics present in concentrations producing absorbances in the range 0.75 to 1.25 in the H&B assay.

species	H&B	PPT(%)	NPP(%)
<u>Acacia pennata</u>	1.00 (0.32)	71 (26)	41 (8)
<u>Diospyros thomasii</u>	1.00 (0.10)	87 (5)	19 (12)
<u>Trema guinieensis</u>	0.87 (0.10)	66 (23)	40 (16)

II Results for phenolics present in concentrations producing absorbances in the range 0.75 to 0.25 in the H&B assay.

species	H&B	PPT(%)	NPP(%)
<u>Cynometra leonensis</u>	0.36 (0.09)	11 (18)	57 (53)
<u>Diospyros thomasii</u>	0.34 (0.05)	30 (34)	78 (24)
<u>Trema guinieensis</u>	0.38 (0.08)	19 (14)	79 (18)

* Note See text for definitions of the above quantities (H&B, PPT, NPP).

Table 6.7 Specific activities

Species	Code	TPS	%T	SA1	SA2
Acacia pennata	Ap	0.32	55	8.50	15.5
Cynometra leonensis	Cl	0.45	13	4.79	36.9
Diospyros thomasii	Dt	0.36	74	14.42	19.9
Trema guineensis	Tg	0.24	32	10.67	33.3

Basic data

	Protein (NIN)				Phenolics (H&B)			
Code	range	x		n	range	x		n
Ap	3.0-5+	2.27	0.109	5	1.5-2.5	0.14	0.003	3
Cl	2.5-4.5	1.83	0.119	5	2.0-3.0	0.39	0.010	3
Dt	3.5-5+	4.31	0.195	4	1.5-2.5	0.093	0.0058	3
Tg	3.5-5+	2.71	0.06	4	1.0-2.0	0.163	0.0029	3

Notes The quantities tabulated above are give in the same format as tables 4.1 and 4.2. and these are defined in section 4.2,2.

such, appears to be a factor of two and is taken into account in what follows.

It is clear from the preceeding account that interspecific comparisons of astringency which ignore plant-plant variation are meaningless in ecological terms. However, at an approximate level the results for Specific Activity (SA1) coupled with the results for foliar protein analyses introduced in Section 6.2 (PROT) can be used to estimate the amount of tannin required to precipitate the entire complement of foliar protein, (expressed as a condensed tannin-protein ratio). The data and calculations for this are set out in Table 6.8. The salient point revealed in the Table is that in each species the quantity of condensed tannin present is well in excess of that required to precipitate all the protein in the leaf, even in D. thomasii which also contains hydrolysable tannins too.

Despite the possible errors and the biological variation involved it seems reasonable to expect that there is at least sufficient tannin in each plant (independent of its light environment) to precipitate all its foliar protein. This is not to deny that there is a positive relationship between the condensed tannin/protein ratio (CT/PROT) and LIGHT as might be expected from the results of Section 6.2. However, if the actual range in CT/PROT is examined (see Table 6.8)

Table 6.8 Calculation of the R(t) Value at which Foliar Protein is Totally Precipitated by Foliar Tannin.

Principle: the calculation of the R(t) value (for a given species) assumes that foliar protein is equivalent to the bovine serum albumin used in the specific activity assay and that foliar tannins have the same A_1^1 in the assay of phenolics as quebracho tannin. By use of the A_1^1 values for quebracho tannin in the following expression, together with the specific activity (SA1) value for the species concerned, R(t) is given as follows.

$$R(t) = (SA1 \times [A_1^1/10])^{-1}$$

By dimensional analysis, this equation can be seen togive R(t) in terms of mg tannin/mg BSA.

Calculated data for the minimum value of R(t) at which foliar protein is completely precipitated by foliar tannins.

Acacia pennata,	0.026
Cynometra leonensis,	0.046
Diospyros thomasii,	0.016
Trema guineensis,	0.028

Table 6.8 Continued

Actual values of R(t) calculated from foliar condensed tannin (CT) and protein (PROT) contents. (R(t)=CT/PROT)

Species	mean	range
Acacia pennata,	0.70	1.57 to 0.16
Cynometra leonensis,	0.74	1.47 t0 0.29
Diospyros thomasii,	0.44	1.25 to 0.20
Trema guineensis ("sun, young")	0.76	1.06 to 0.36
" " (" mature)	0.67	0.82 to 0.30
" " (" old)	0.71	1.31 to 0.34
" " ("shade, young ")	0.33	0.50 to 0.15
" " (" mature)	0.36	0.62 to 0.09
" " (" old)	0.49	0.70 to 0.19

then in the terminology of Chapter 3 the variation in R(t) is small and always in excess of that previously used to give precipitation (e. g. precipitates were formed with quebracho at R(t)=0.1; Section 3.2). This evidence therfore supports the conclusion drawn from SA1 data that light induced variation in tannin production does not alter the plant's capability to precipitate its protein content in full. Even if the plants were subjected to predation by folivores which were able to solubilise tannin protein complexes, R(t) appears to be insufficiently variable between light environments to greatly affect digestibility as envisaged by the mechanims discussed in Chapter 5. In short, if tannin-protein complexes are soluble <u>in vivo</u>, there is little evidence for much increased plant protection by tannin production in the sun and if protein precipitation is the important phenomenon in plant defence by tannins then there is no evidence that the increased production of tannins in the sun aids defence through increasing the plant's ability to insolubilise foliar protein.

6.3,4 Discussion

The final results given above, which concern the ability of the extracts to precipitate protein, cast doubt about the adaptive significance of any increased production of tannins in the sun. Caution must be

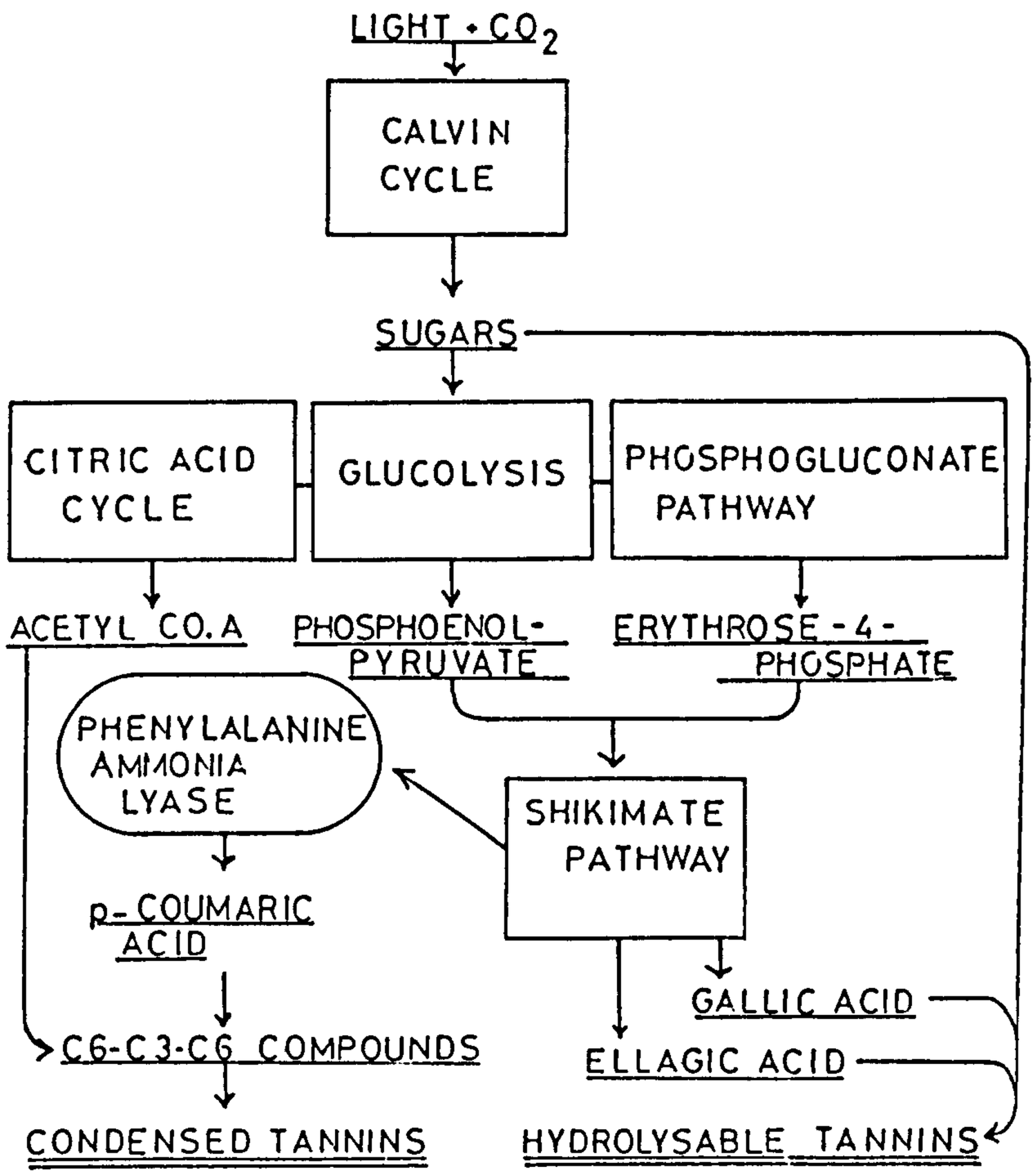
exercised in interpreting these results in terms of the *in vivo* or field situation but even if taken as only "corrobrative evidence" they add weight to the main argument presented in this Chapter, namely that the response to light intensity indicates that production of tannins is not strictly linked to their allelochemical function.

To tie the response to LIGHT into being an adaptive response in terms of plant defence, it would have to be argued that there is more herbivory in the sun than in the shade and that by using light intensity as a cue, an adaptive within plant distribution of tannins is achieved. The literature on the relative impact of herbivores in the sun and the shade is not great and there is no quantitative evidence on this subject for the present study. However, there was no gross difference between the level of herbivory in the sun and shade leaves evident during sampling, and what literature evidence there is does not support the notion that damage is disproportionately greater in the sun than in the shade. Indeed, the harsh microclimate on strongly insolated leaves (e.g. heat and water stress) has been cited as a reason against this (Edwards and Wratten, 1980) and in one study, Maiorana (1981) reported that shaded leaves were comparatively unpalatable compared to those in the sun. The observations of Fennah (1955) may

be of more general value. He found that on shaded cocoa trees, thrip infestation was densest on the upper and outer branches but on plants exposed to full sunlight, he found the reverse pattern of infestation. Here the response to LIGHT, as reported in Section 6.2, was found for four tree species growing in environments ranging from open to densely shaded forest sites. The increse in tannin production with light was independent of this variety in the plants' light environment, so even given insect behaviour such as that shown by thrips, the distribution of tannins in these plants does not seem uniformly adaptive. Furthermore, the microclimatic gradients imposed by sunshine, which might control the distribution of herbivores during the day, will not apply to nocturnal herbivores!

At this point it becomes reasonable to consider other reasons why plants might generally increase their production of phenolics in the sun, particularly as the effect is now established for species in five different plant families (Ebenaceae, Graminae (Woodhead, 1981), [Caesalpinaceae and Mimosaceae grouped as legumes], Passifloraceae (Waterman et al. 1984), and Ulmaceae). Figure 6.4 shows how the pathways involved in the synthesis of phenolics are related to the three major routes of intermediate metabolism (i.e. the glucolytic and phosphogluconate pathways and the citric acid cycle).

Figure 6.4 Sumary chart for the metabolic pathways involved in the photosynthetic prodution of phenolics, and tannins in particular.



The relationships between these processes and the two major types of tannin are slightly different. Hydrolysable tannins contain glucose and so may draw directly on the products of primary metabolism (i.e. the Calvin cycle) whilst the phenolic constituents of these tannins are derived from an early side branch of the shikimate pathway. In contrast, condensed tannins are produced by the further metabolism of the C6-C3 product of the shikimate pathway and this consumes metabolites from the citric acid cycle. Of additional note is the fact that most of the carbon in condensed tannin must pass through the reacton catalised by the enzyme phenylalanine ammonium lyase (PAL). This reaction is in metabolic terms irreversible and the enzyme is thought to be a point at which metabolic control is exerted (Camm and Towers, 1973).

Hanson and Havir (1981) note that up to 20% of fixed carbon may be subject to metabolism by PAL and that the enzyme is subject to coarse control (i.e. at the level of its synthesis). Some of the factors thought to influence the level of PAL activity are fungal infection, wounding, sugar levels and light quantity and quality (i.e. both intensity and wave length). The above factors are all positively correlated with PAL activity but only the response consequent upon wounding would be expected from results concerning induced defence reactions (see Section

6.1 and Urtani, 1971). The other responses show the link to light intensity to be either a direct one or an indirect one due to high levels of sugar accumulating in the leaves. With respect to the latter point, Margna (1977) provides evidence to suggest that PAL is under substrate level control, which, if it were the case, would explain an increased level of tannin production in high light intensity when there is abundant fixed carbon available. The available evidence is thus favourable to the notion that phenolics, including both types of tannin, could build up in highly insolated plants or plant parts for purely "economic" reasons.

The possible mechanism involved in the response to light, indicated above, corresponds to the notion of Haslam (1985) that secondary metabolites may be the result of an "overflow metabolism" into which excess metabolites may be shunted in times of stress. Haslam (1985) cites evidence that metabolic disorders such as those caused by metal-ion imbalances can result in the accumulation of metabolites, particularly organic acids. An early example of this was provided by Chesters and Robinson (1951), who regarded the build up of citrate in a zinc deficient fungal culture as evidence of the organism's inability to completely oxidise carbon to CO_2; this being taken to be a symptom of metabolic malfunction. Specifically with regard to tannins, boron

deficiency can result in their accumulation (Rajaratnam and Hock, 1975) possibly because this element is involved in the regulation of 6-phosphogluconate dehydrogenase and thus in the regulation of carbon flux into the phosphogluconate, and subsequently, into the shikimate pathways (Rajaratnam and Hock, 1975). The similarity of this situation to that reported by Chesters and Robinson (1951) is striking from the metabolic point of view. Further direct evidence that plant nutrient status is important in regulating tannin production is provided by Wilson (1955) and Barry and Forss (1983) who studied tannin production as a function of fertiliser applications to forage legumes. More circumstantially the evidence that vegetation on poor soils is likely to be rich in phenolics (Janzen, 1974; Waterman, 1983) also supports this case.

Considering the effects of both light and mineral nutrition on phenol production, a scenario can be envisaged where a plant subject to an external stress which results in imbalances in carbohydrate metabolism might then accumulate an overflow metabolite (sensu Haslam 1985). As such, the accumulation of tannin would fit the need for an oxidised product (e.g. compared to lipid production) and, unlike most simple organic acids, tannins may have beneficial effects as allelochemicals. In engineering terminology tannins are thus viewed as the

strain evident as a result of some external stress. Such a view of plant responses to the environment has been proposed by Ayres (1984) and it is one that seems to provide an attractive framework for considering tannins.

The evidence presented here is not intended to suggest that all secondary products are overflow metabolites produced in time of stress or that once produced that phenolics represent a waste of fixed carbon resources to the plant. Both the possible allelochemical function and possible re-entry into metabolism, via turnover, make such assumptions about secondary products unwarranted. Also the production of metabolites as allelochemicals in circumstances of low metabolic stress is not inconsistent with their also being accumulated as overflow metabolites during stress. In short, the present exercise has shown that both hydrolysable and condensed tannins may be produced for reasons independent of their allelochemical function.

Chapter Seven

The Metabolic Cost of Tannin Production

7.1 Introduction

In the hypotheses proposed by Feeny and by Rhoades and Cates (Section 1.3), the assumption was made that tannins are a relatively costly form of plant defence. It was noted in Section 1. that their reasoning for this was invalid because they ignored metabolic turnover when comparing the investment of fixed carbon in different types of allelochemicals, i.e. they made their estimate of production costs from the "standing crop" of biomass invested in tannins.

Whilst the metabolic turnover of some qualitative defences has been studied, notably for alkaloids (Seigler, 1977; Seigler and Price, 1976), it appears that no attempt has been made to provide comparative data for tannins or indeed any other quantitative defence. The work presented in this Chapter attempts to shed some light on the question of whether tannins are actively metabolised in plant cells once they are synthesised and whether their maintenance is a continual drain on fixed carbon and energy, or whether they are, as is often supposed, metabolically inert once produced and therefore much less costly to maintain.

7.1,1 Current Evidence Concerning the Cost of Tannin Production.

The claim that no work has been performed on the turnover of quantitative defences does need some substantiation as ^{14}C tracer techniques have been used in attempts to investigate carbon allocation to resins and tannins. Of such experiments, those which are numerically the most important are the type initiated by Mooney and co-workers and used subsequently by others (Mooney, 1972; Mooney and Chew, 1974; Bryant et al., 1983; Prudholm et al., 1983). This approach has involved labeling plants with $^{14}CO_2$ in the field with the cropping of material at various times after the label application. By analysing the quantity of label that remains in various fractions of the tissues after an extensive period of time, that is, when a near constant level of label is being maintained in each fraction, a measure of what is termed the "carbon use priority" for that fraction is made. This indicates the proportion of carbon fixed at the time of labelling which is ultimately and permenently allocated to each fraction (e.g. tannins, resins, cell walls, sugars). This does, however, only indicate allocation to pools of those products which are not actively metabolised, and so this only applies to the carbon allocation to part of the plant's biomass. This part of the plant's biomass may well account for the majority of the standing crop, since active

metabolic pools may well be separate and small by comparison (MacLennan et al., 1963). Nevertheless, by ignoring the loss of carbon through respiration and turnover nothing is revealed about the production costs of each fraction as a function of total carbon fixed and the associated energy investment involved.

Prior to the work reported above, Feeny had conducted what were, in effect, similar experiments, in which he enclosed a 360g branch of an oak tree in a bag containing 130 l of air (0.02% CO_2) labeled with 1.0 mCi of ^{14}C. He detected measurable levels of activity in tannins one week after the 12 hour photoperiod used to ensure fixation of the label. His work clearly showed that there was an active production of tannins in oak. It also suggested that, once formed, at least some tannin entered a pool which was not subject to rapid turnover and degradation, i.e. one which was possibly a metabolic sink. An explanation for this could be that tannin metabolism was active during labeling (due to stress and elevated CO_2 levels) but thereafter the metabolism of tannins was reduced when the stress and elevated CO_2 level were removed.

An interesting line of support for this is given by Baldwin and Schultz (1983) who show uptake and subsequent loss of ^{14}C in the total phenolics of damaged poplar seedlings whilst a group of undamaged controls show no

uptake of label. Whilst they showed that the plants concerned contained both condensed and hydrolysable tannins they did not trace the change in labelling specifically to either type of tannin. This evidence is, however, consistent with (i) metabolic turnover in tannins and (ii) stress induced elevation of tannin metabolism, including turnover.

The only other work of importance in the present context is that of Holzel and Stranch (1977) and Eastmond and Gardner (1974) who demonstrated the incorporation of ^{14}C into tannins from an acetate label infiltrated into the plant tissues. Like Feeny (1970) and Mooney and Chew (1974) these studies used labelling periods lasting from 12 hours to one week. The previous use of long labelling pericds and labels of 1 mCi and over in activity suggests that tannins may actually be hard to label (i.e. because of slow synthesis) whilst the proven utility of two different ^{14}C sources allows for useful variations in the experimental treatment of plant material to be made during labelling. These points are taken into account in the following section, which describes the design of experiments to investigate whether tannins are subject to metabolic turnover <u>in vivo</u>.

7.2 The Metabolism of Condensed Tannins in *Hymenaea coubaril*

The experimental material chosen for this study was the Costa Rican tree species *Hymenaea coubaril* (Leguminosae) used in the form of two-month-old seedlings. The experiments performed were of the "pulse-chase" type where plant tissues were fed a ^{14}C labeled substrate for a known time and then allowed to continue metabolism on a chemically identical but unlabelled substrate. Measurements of the label incorporated were then made on plant tissues sampled immediately after the pulse and chase periods. This then allows an estimation of how much carbon enters various fractions, e.g. tannin, and how much is lost from them by turnover during the chase period.

Although simple in concept, such experiments are subject to three major problems, all of which are particularly difficult to overcome when the study concerns tannins. Firstly, any experimental manipulation of tissues necessary to incorporate label may alter their metabolism; clearly any stress on the tissues during labelling is particularly likely to promote the incorporation of label into phenolics, as seen in Chapter 6. Secondly, the success of such experiments depends critically on the demonstration that label has actually

entered the compound of interest; with tannins their co-extraction with protein, starch and some amino acids is likely. Thirdly, separation and fractionation of material must be clean and without loss so as to give 100% accounting for label distribution; the propensity of tannins to precipitate out of solution or polymerise into insoluble forms tends to frustrate this goal in the analysis of labelled material.

Given the possibility that tannins may be formed slowly and also non-enzymically (Haslam, 1977) and are perhaps not subject to metabolism once formed, a substantial level of labelling was desirable to be sure to detect any changes that might result from the chase treatment. The experiments were thus designed to use high specific activity labels in conformity with previous workers (Section 7.1) so as to maximise the chance of achieving this. Both CO_2 and acetate labels were employed to ensure that results were independent of the particular treatment of plant tissues needed to incorporate ^{14}C. This does not mean that the factor of stress is eliminated in the experiments, as the use of cut leaves was necessary in the acetate feeding experiment and some heat and temperature stress was inevitable in the CO_2 feeding experiments. However, the potential for aberrant results is reduced in this way. Other precautions and problems are described below.

7.2,1 Materials and Methods

The seedlings of H. coubaril were grown at the Botanic Gardens in Glasgow, in humid glasshouses maintained at 70°C. After about two months, in which they attained a height of about 40cm, rapid growth appeared to cease, and plants were observed to produce new leaves at infrequent intervals only. The plants used in the experiments were at this stage of growth and were transferred to the laboratory 24 hours before the experiments, during which time they were kept warm and constantly illuminated under bench lamps. This was found to raise the dark respiration rate of the plants (during preliminary experiments) and was done to help ensure that the tissues used were in a state of active metabolism. Procedures for the application of the label for acetate and CO_2 are described below. Thereafter the methods for killing and extracton of labelled material are described; these were the same for material labelled in either way.

Acetate Labelling

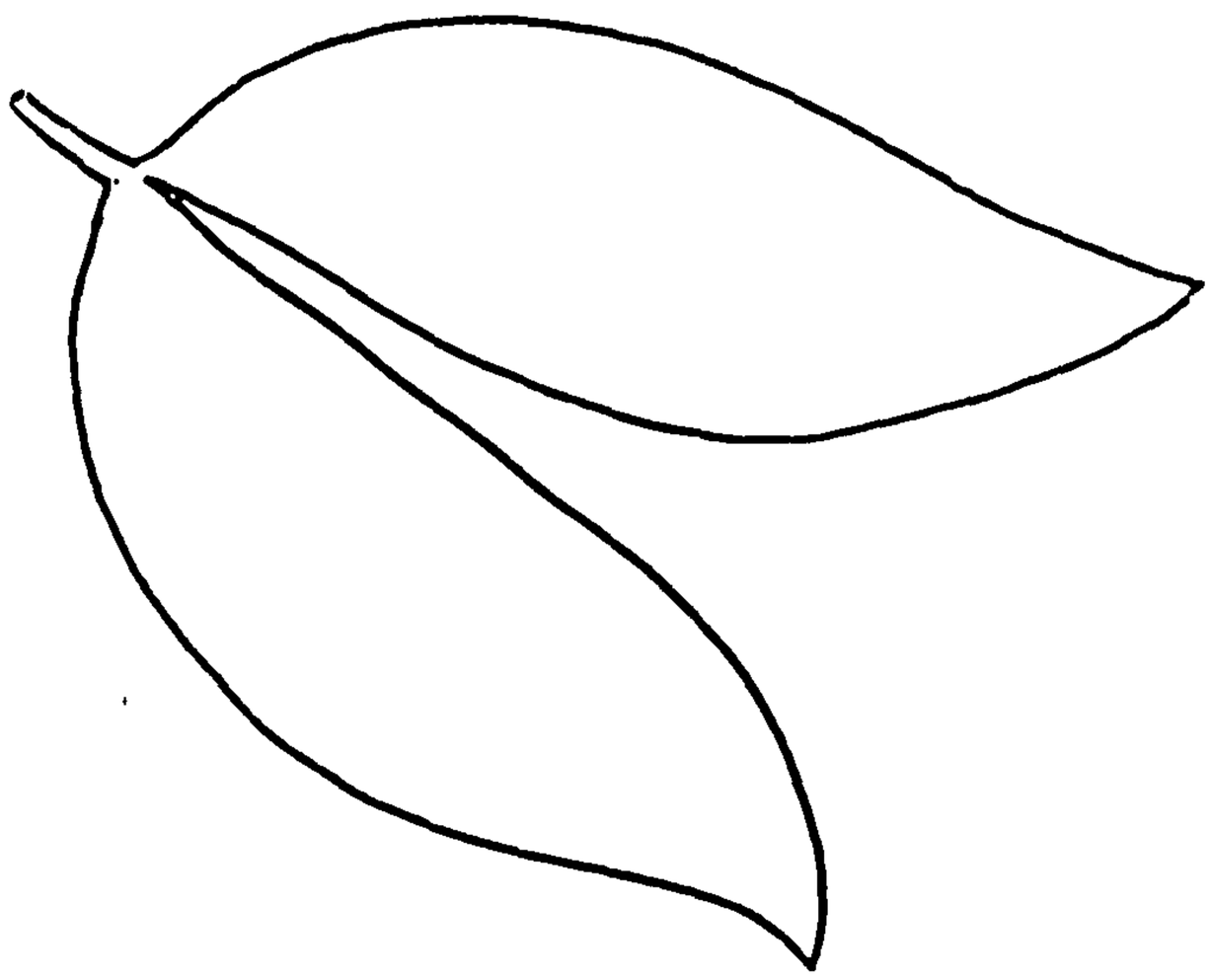
The label for the acetate feeding experiments was 250 μCi [2-^{14}C] sodium acetate (Amersham International) which was dissolved in 300ml of potassium phosphate buffer (50mM KH_2PO_4 taken to pH 5.2 by the addition of HCl). This buffer was chosen as it has previously been used with success in acetate labelling experiments (Grant and

ap Rees, 1981).

For label application the solution made up as above was divided into two 150ml portions and each was poured into shallow 20x20 cm^2 trays. These were placed on an orbital mixer platform set at a low speed to keep their contents gently agitated. The leaves of two plants were then cut at the base of the midrib and each leaf was then divided into its two component leaflets (see Figure 7.1). Each leaflet was then cut into strips of tissue (across the midrib) which were about 1 to 0.5 cm wide. Material from one half of each leaf was then floated (adaxial surface uppermost) on the surface of the liquid in one tray and that from the other half on the liquid in the other tray. As the leaves were symmetrical this procedure meant that the plant tissue in one tray was in all practical respects identical to that in the other. The two trays containing the leaf material were then illuminated by bench lamps for a total of 60 minutes. At the end of this time the label solution was strained off from the leaf strips, frozen (to prevent microbial contamination) and retained for future re-use, which was possible as only a small fraction of the ^{14}C was taken up by the plant tissue. Both halves of the labelled leaf material were rinsed to remove any remaining label, using three rinses with unlabelled buffer.

One half of the leaf material was then killed

Figure 7.1 Leaf of Hymenaea coubaril.



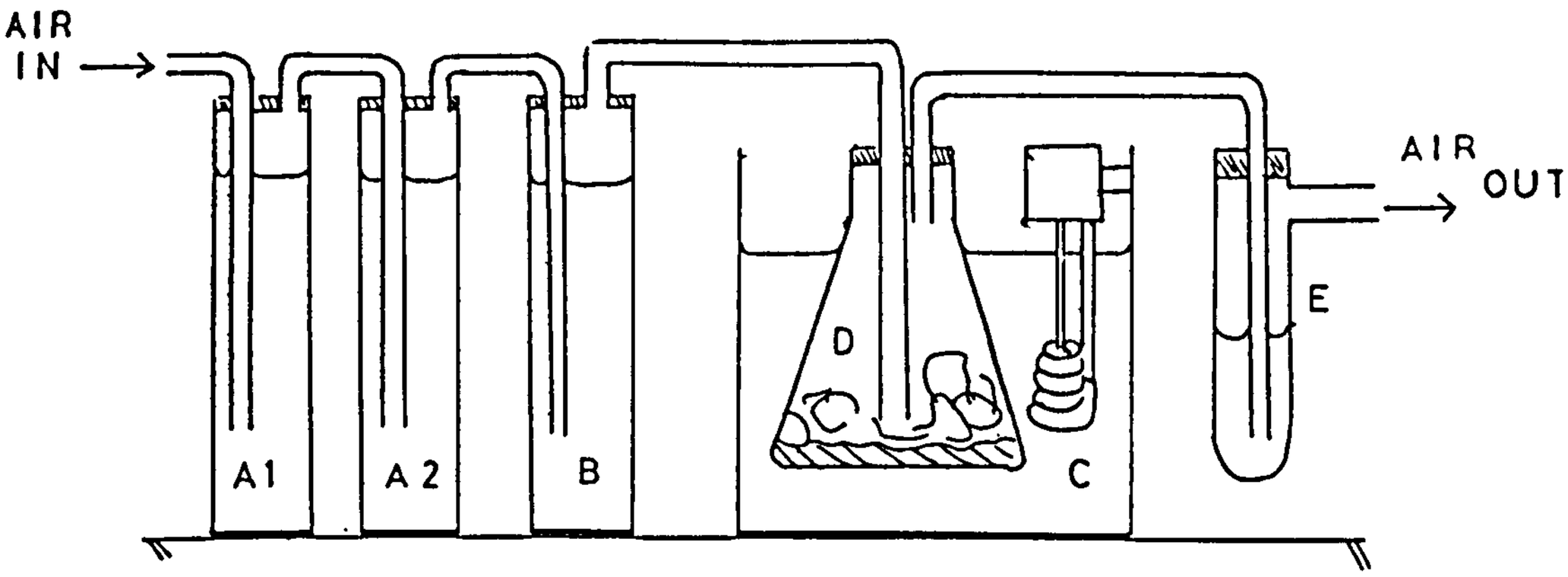
1.0 to 0.5 times life size.

immediately (see below) whilst the other was given a 180 minute "cold chase" in the apparatus illustrated in Figure 7.2. Here the leaf strips were placed on a bed of tissue paper soaked in "cold" buffer (50mM KH_2PO_4 taken to pH 5.2 with acetic acid) and allowed to metabolise the acetate in the dark. CO_2 free air was passed over the plant tissue during this time so as to remove respired CO_2 which was then trapped by precipitation as $BaCO_3$. The weight of this $BaCO_3$ was subsequently obtained as a measure of the extent of dark respiration and its radioactivity was also determined (see below) to gauge the contribution of label to respired CO_2. After the 180 minute chase this material was also killed to stop any further chemical reactions. These experiments thus yielded two samples of killed material for analysis (i) that given a pulse only treatent (designated P/-) and (ii) that given a pulse followed by a chase (designated P/C).

CO_2 Labelling

For the experiments where $^{14}CO_2$ was the label, whole plants were sealed into the apparatus depicted in Figure 7.3. At the beginning of the pulse stage in the experiments, the inverted glass trough covering the plants was partially evacuated by opening tap T3, turning on the vacuum pump, and allowing the mercury to rise 7cm

Figure 7.2 Apparatus for Cold Chase.



A1 and A2 100 ml measuring cylinders containing 10% KOH to absorb CO_2 from the air.

B Measuring cylinder full of $Ba(OH)_2$ as an indicator that air entering flask D is free of CO_2

C Thermostatically controlled waterbath maintained at $30^{o}C$

D Flask containing leaf material on a bed of filter paper soaked buffer solution.

E Tube containing $Ba(OH)_2$ solution to trap respired CO_2 as $BaCO_3$. The air outlet was attatched to a suction pump to draw air through the system.

Figure 7.3 Apparatus for CO_2 Labelling Experiment.

A Inverted 28l glass trough able to contain up to three plants in their pots.

B Mercury barometer used to check the pressure inside the apparatus.

G Grease seal arround join between trough and plastic base plate.

K 2l of KOH to trap CO_2 drawn out of A.

L Lump of solid $Ba^{14}CO_3$

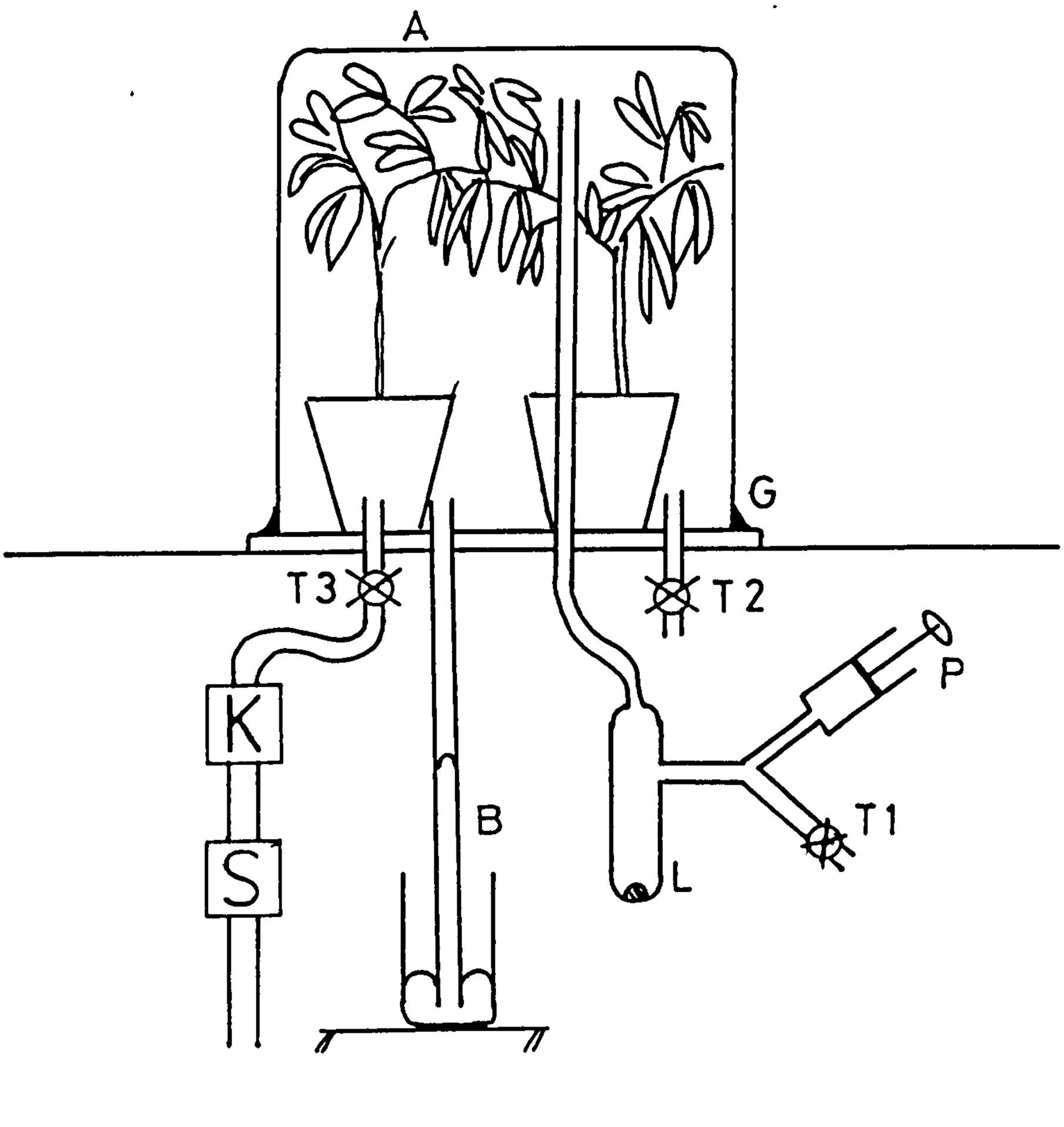
P Syringe containing conc HCl, used to generate $^{14}CO_2$ from L at time zero.

S Suction pump used to partially evacuate/ draw air through the system.

T1 Tap opened briefly to draw air and $^{14}CO_2$ into the partially evacuated apparatus at the begining of the experments (closed therafter) .

T2 Tap opened at the end of the experiments to help flush $^{14}CO_2$ out of the system.

T3 Tap used to seal off KOH and vacuum pump from the system during the labelling process.


A
G
T3
T2
P
K
S
B
T1
L

in the barometer. Tap T3 was then closed, concentrated hydrochloric acid was added to the barium [^{14}C] carbonate label (2.5 mCi; Amersham) and the CO_2 produced, which was generated instantly, was swept into the apparatus by opening tap T1. This was closed when atmospheric pressure was restored inside the apparatus, thus sealing in the label for a pulse time of two hours. During this time the plants were kept illuminated by bench lamps and the temperature clearly rose inside the apparatus. This was indicated by condensation which appeared on the inside of the trough and signs of heat damage seen on a few leaves at the end of the pulse (such leaves were not sampled for extraction). After the pulse time had elapsed unlabelled air was flushed through the apparatus to remove the $^{14}CO_2$ and the plants were then removed from the apparatus. The leaves of one plant were then removed and each was divided into two wherupon one set of leaflets was killed immediately. The other set was given a cold chase in the dark for two hours in the same apparatus as used previously (Figure 7.2), after which this set was also killed. The other plant or plants that had been labled at the same time were kept alive for one week in the lab before they were killed and extracted.

These experiments thus provided P/- and P/C material analogous to the acetate label experiments; they also provided material exposed to a week-long chase

(designated CH in what follows).

Killing and Extraction

Leaf material was killed by being cut into thin strips, if this had not been already carried out (i.e. as in the acetate label experiments), and then by being immersed in boiling 70% aqueous acetone for a period of 10 minutes. In the CO_2 label experiments the shoot and root material was also killed after washing the soil from the roots and cutting the (leafless) plants into 1 cm lengths, by adding this material to boiling acetone. For these experiments separate samples of leafy and woody tissues were prepared (i.e. fractions P/-(L), P/-(W), CH(L) and CH(W)) and so the fate of label translocated out of the leaves could be accounted for.

Extraction began after the above samples of dead tissue had been allowed to cool. Firstly an (Ultra-Turax) homogeniser was used to turn the tissue into a slurry of finely divided fibrous material whilst still in the 70% acetone solution. This was then boiled for a further 20 minutes before the liquid was decanted off and filtered. 100% acetone was then added to the bulk of the solid material for a further 20 minutes' extraction under reflux after which this supernatant was also decanted off, filtered and bulked with the initial acetone extract. The remaining solid material was given

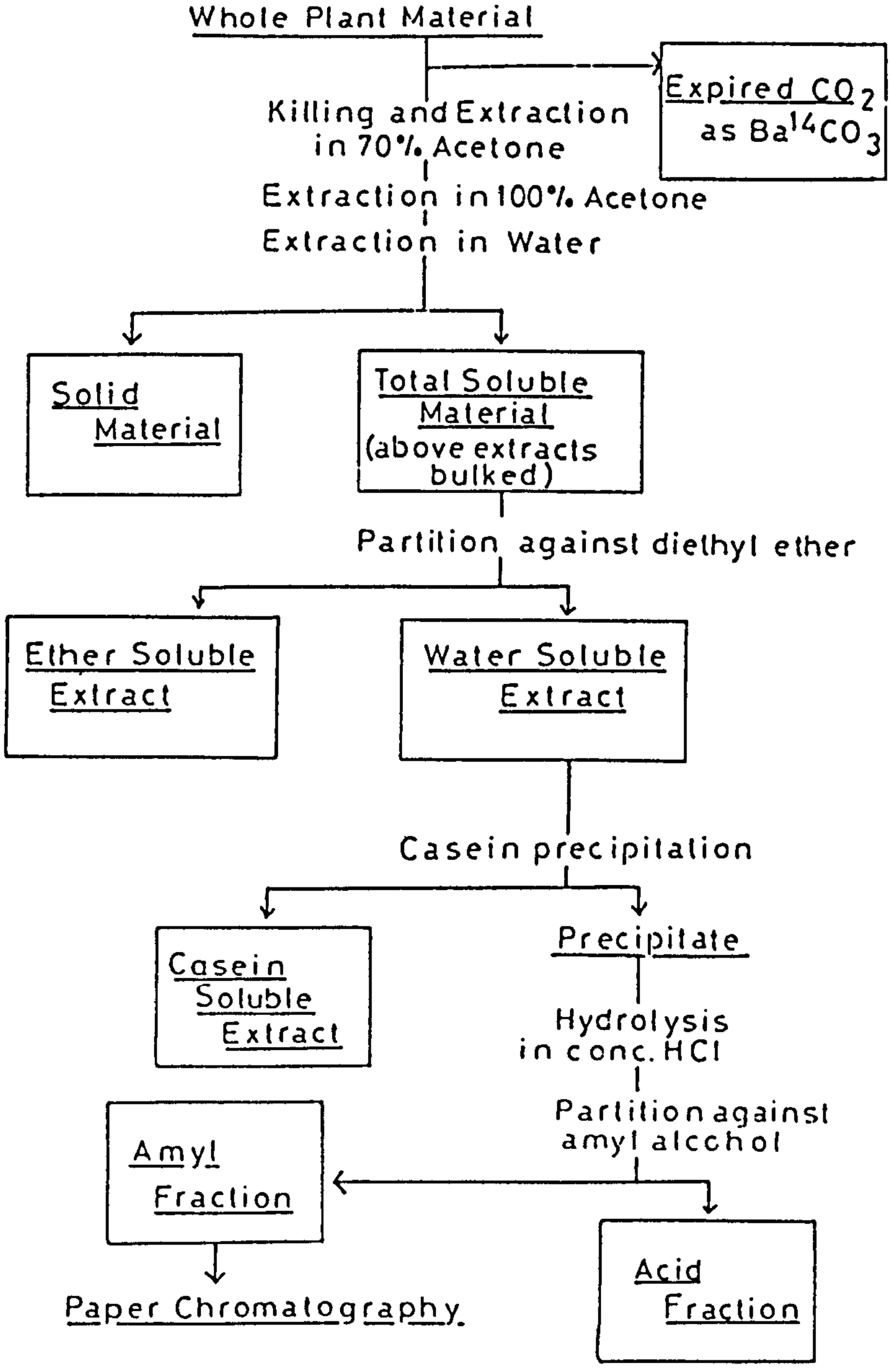
a final 20 minute extraction in boiling water after which it was filtered. In this way the plant material was divided into an "insoluble fraction" and a "soluble fraction". The insoluble material was then oven dried and retained for weighing and radiochemical analysis.

The soluble fraction was sampled for radiochemical activity, as were all subsequent fractions (see below), then this material was partitioned against diethyl ether in a separating funnel so as to divide it into "ether soluble" and "water soluble" fractions. Both these were concentrated by rotary evaporation at temperatures below 30^{o}C, a procedure in which acetone is mostly removed. Samples of the water soluble fraction were then added to 5% casein solutions (1:1 volume:volume), which with every sample of water solubles produced turbidity or precipitation. To aid precipitation 1M $CaCl_2$ solution was added (12.5% v/v). In the absence of tannin this turned clear casein solutions into milky white suspensions which did not precipitate, but in the presence of tannins dense coagula were produced, so enhancing the recovery of protein binding phenolics. These precipitates were collected by centrifugation. The supernatant solutions were collected and termed the casein soluble fractions. The casein/tannin pelleted material was found to be soluble in concentrated hydrochloric acid (but not HCl diluted with water). This

concentrated acid solution was then boiled for 30 minutes to hydrolyse the casein and the tannin. After that time the solution was diluted with an equal volume of water, little precipitation occurring, indicating the probable lack of any remaining tannin-protein complexes. What precipitation there was indicated the probable presence of phlobaphenes (Swain, 1959). The hydrolysis solution was then partitioned against amyl alcohol which gave a deep red coloured "amyl fraction" and a less coloured "acid fraction".

The rationale behind this procedure may be summed up as follows, (i) efficient extractants (for tannin in particular) are used. (ii) Condensed tannins are isolated in terms of their functional properties (i.e. precipitation by protein in water) and furthermore by their chemical properties (anthocyanins partition into amyl alcohol from acid solution; Harborne 1972). This means that radioactivity in the amyl fraction should be a good indicator of that in tannins, be they defined operationally or chemically. Further proof of the location of radioactivity in anthocyanins was also provided by paper chromatography of the amyl fraction in Forestal (see Appendix 1.9 for methods). A scheme for the whole procedure is set out in Figure 7.4 and this provides a reference as to how each fraction was generated.

Figure 7.4 Summary Scheme for the Extraction Procedure.



Radiochemical and Chemical Analysis

Liqiud scintillation counting (LSC) was used to measure the radioactivity present in each of the liquid fractions. This was achieved by using 100μl samples in 10 ml of scintillant (ES 299; Packard) counted in a (Tricarb 460CD; Packard) counter. The counter had been previously adjusted to correct for chemical quenching and by the use of internal standards it was found that no measurable colour quenching occurred with any of the coloured fractions (i.e. ether and amyl fractions).

For solid samples attempts were made to dissolve the insoluble fraction of cell wall material in NCS tissue solubiliser but this failed and so LSC could not be used. Instead small amounts (5-10mg) of this finely divided material were mounted on planchettes for counting with a GM tube (PANAX). This procedure was also used for the samples of respired CO_2 fixed as $BaCO_3$ in the dark chase given to the P/C material. The obvious drawback to this technique is internal absorption within the sample. The significance of this point is noted in the discussion of the results.

7.2,2 Results

Three replicate experiments using the acetate label were carried out and these are referred to as experiments A1, A2 and A3. The mean respiratory activity of the three sets of tissues, as measured by the weight of CO_2 respired at the P/C stage, was found to be 193 nmoles per minute per gram fresh weight of tissue. This statistic indicates a good level of respiration but hides the variation seen in the individual experiments (see Table 7.1), where experiment A3 shows a notably high value.

The label fixed in the insolubles at the P/C stage does, however, show an increase over that originally fixed at the P/- stage, for all the experiments (A1, A2, A3). The proportion of label in the solubles declines during the chase in each case but this is not just due to its entry into insoluble material, which does nevertheless appear to function as a metabolic sink. ^{14}C is also lost as CO_2 and the design of the experiment provided two ways to measure this loss. The direct method was to count the activity off the filter paper holding the $BaCO_3$ originating as respired CO_2 trapped in $Ba(OH)_2$. The indirect method was to calculate the net loss in activity, in both soluble and insoluble fractions, between the P/- and P/C stage and ascribe this to loss as CO_2. This is valid as the P/- and P/C fractions derive from the alternate halves of each leaf

Table 7.1. Acetate Label Experiment, Results I

Respiratory Rates for P/C Tissues. (n moles of CO_2 per minute per gram fresh weight of tissue)

A1, 123 A2, 69 A3, 386 mean, 193

Distribution (%) of ^{14}C Amongst the Major Fractions.

		A1	A2	A3	mean
P/-	Soluble Material	80	80	59	73
	Insoluble Material	20	20	41	27
P/C	Soluble Material	50	58	42	50
	Insoluble Material	35	22	35	32
	Respired CO_2	15	20	18	18

Distribution (%) of ^{14}C Between the Water and Ether Soluble Fractions.

		A1	A2	A3	mean
P/-	Water Soluble	79	92	92	88
	Ether Soluble	21	8	8	12
	Recovery*	97	104	91	
P/C	Water Soluble	93	89	93	91
	Ether Soluble	7	11	7	9
	Recovery*	78	84	85	

* Expressed as Percentages of the Soluble Material.

and so they should each have identical metabolism (on a fresh weight basis). This second method gave numerically larger results and so was used in place of the direct method which seemed to give an under recovery. The results for $^{14}CO_2$ loss are given in Table 7.1 and these show the quantitative importance of this other route by which ^{14}C is lost from soluble metabolites during the chase.

The next stage in the analysis was to consider whether all the fractions of soluble material, including these thought to contain tannins, were subject to this turnover. Table 7.1 shows the distribution of label between the ether and water soluble fractions. Clearly in both P/- and P/C material there is a similar division of label with most occurring in water soluble substances. The values for the recovery of label in these fractionations show one of the major drawbacks of this work, the recoveries are variable and systematically low for the P/C. The problem here appeared to be in the formation of a coagulum of material at the ether/water interface during partition. This solid layer enters neither fraction and sticks to the wall of the separating funnel as fluid is withdrawn. It seems likely that a heavily labelled fraction has been lost from the P/C material in this way.

Further analysis of the water soluble fraction showed

that it did contain tannins but that most of the label was not precipitated by casein and that this proportion did not change during the chase. Nevertheless the fraction that did precipitate was a significant one (see Table 7.2). Of that fraction at least half tended to enter the amyl fraction after hydrolysis, strongly indicating that this was label in condensed tannin. Considering the evidence, there is little indication that label in the amyl fraction rises in the chase (as a proportion of the total label in the water solubles). Multiplying out the mean proportions of label, to trace this back to the proportion in the whole leaf, the proportion of label in the amyl fraction falls from 7.7% (P/-) to 6.8 (P/C) during the chase. It would be wrong to place any emphasis on this owing to the poor recoveries mentioned, nevertheless compared to the clear rise of label in insoluble material and CO_2, tannins do not appear to be metabolic sinks for ^{14}C relative to these, from which activity does not seem to be lost during the chase. Further evidence, however, based on experiments with better recoveries is needed to substantiate this.

Three replicate experiments with the CO_2 labelling technique were planned, two were accomplished in full (C1, C2) but a lack of plant material meant that CH stage material only was produced for the third (C3).

Table 7.2. Acetate Label Experiment, Results II

Distribution (%) of ^{14}C Amongst the Water Soluble Fractions.

		A1	A2	A3	mean
P/-	Casein Soluble	80	80	71	77
	Acid Soluble	13	9	12	11
	Amyl Alcohol Soluble	7	11	17	12
	Recovery*	101	99	98	
P/C	Casein Soluble	81	72	75	76
	Acic Soluble	7	12	9	9
	Amyl Alcohol Soluble	12	16	16	15
	Recovery*	100	94	102	

* Expressed as Percentages of the Soluble Material.

Respiration in C1 and C2 P/C material was again active (see table 7.3) if different in the two plants used, and this may well explain the difference in the proportion of $^{14}CO_2$ respired by the two sets of tissue. Nevertheless the pattern of change in label distribution seen during the chase in experiments A1-A3 repeats itself in C1 and C2. That is to say, label incorporated into soluble material during the pulse (P/-) is lost to CO_2 and insoluble material during the chase, as seen in Table 7.3.

A major reason for using whole plant material in these experiments was being able to keep it alive for an extended chase period, even if this did not allow P/- and CH tissues to be as evenly matched as was possible when using leaf halves (i.e. P/- and P/C). Table 7.4 gives the label distributions in both fractions (L and W) of this material for comparison with those at the P/- stage. Despite the lack of paired material, it is clear that even more label from the soluble material was lost during the chase, particularly from the leaf tissues. The label distribution between the water and ether soluble fractions is close to that previously seen in experiments A1-A3 as is the distribution of label within the water soluble fraction (see Tables 7.4 and 7.5). However, one exception to this was for CH(L) material where there was a relatively high proportion of label in the amyl

Table 7.3. CO_2 Label Experiment, Results I

Respiratory Rates for P/C Tissues. (n moles of CO_2 per minute per gram fresh weight of tissue)

		C1, 291	C2, 66		
Distribution (%) of ^{14}C Amongst the Major Fractions.					
		C1	C2	C3	mean
P/-	Soluble Material	82	81		81
(L)	Insoluble Material	18	19		19
P/-	Soluble Material	77	44		60
(W)	Insoluble Material	23	56		40
P/C	Soluble Material	64	58		61
(L)	Insoluble Material	18	34		26
	Respired CO_2	18	8		13
CH	Soluble Material	45	5	20	23
(L)	Insoluble Material	55	95	80	77
CH	Soluble Material	43	40	26	36
(W)	Insoluble Material	57	60	74	64

Table 7.4. CO_2 Label Experiment, Results II

Distribution (%) of ^{14}C Between the Water and Ether Soluble Fractions.

		C1	C2	C3	mean
P/-	Water Soluble	98	98		98
(L)	Ether Soluble	2	2		2
	Recovery*	(+)	100		
P/-	Water Soluble	99	99		99
(W)	Ether Soluble	1	1		1
	Recovery*	99	94		
P/C	Water Soluble	98	99		98
(L)	Ether Soluble	2	1		2
	Recovery*	95	100		
CH	Water Soluble	90	98	95	95
(L)	Ether Soluble	10	2	5	5
	Recovery*	93	102	87	
CH	Water Soluble	82	96	99	89
(W)	Ether Soluble	18	4	1	11
	Recovery*	100	72	97	

* Expressed as Percentages of the Soluble Material.

\+ Spillage of Soluble Material, no Recovery Available.

Table 7.5. CO_2 Label Experiment, Results III

Distribution (%) of ^{14}C Amongst the Water Soluble Fractions

		C1	C2	C3	mean
P/-	Casein Soluble	90	90		98
(L)	Acid Soluble	5	6		5
	Amyl Alcohol Soluble	5	4		5
	Recovery*	96	99		
P/-	Casein Soluble	89	89		89
(W)	Acid Soluble	6	9		7
	Amyl Alcohol Soluble	5	2		4
	Recovery*	103	104		
P/C	Casein Soluble	89	91		90
(L)	Acid Soluble	5	6		5
	Amyl Alcohol Soluble	6	3		5
	Recovery*	68	98		
CH	Casein Soluble	84	82	81	82
(L)	Acid Soluble	7	6	6	6
	Amyl Alcohol Soluble	9	12	13	12
	Recovery*	75	97	106	
CH	Casein Soluble	82	87	85	85
(W)	Acid Soluble	8	8	8	8
	Amyl Alcohol Soluble	10	5	7	7
	Recovery*	100	72	97	

* Expressed as Percentages of the Soluble Material.

fraction, compared to P/-(L). No such rise is seen between P/- and P/C fractions. Recoveries of label during separation stages were again variable. Nevertheless the exercise of tracing the fraction of label in the amyl alcohol soluble material back to the whole plant context was repeated. The clear fall in label in soluble materials (nearly all water soluble) between the P/- and P/C stages and the nil change in label distribution seen between the acid and amyl fractions means that a net loss of activity in condensed tannins is again implicated.

The final point which remains is to calculate whether the rise in the amyl labelling of CH(L) water solubles (not seen in CH(W)) was more than counter balanced by the fall in total solubles. Considering the whole plants, in order to avoid errors due to the translocation of label (see Tabel 7.6), in P/- material there was 3.88 (C1) and 1.24 (C2) times as much label in soluble material versus insoluble material. In the CH material this position reverses and the excess of label in insolubles versus solubles is 1.16 (C1) and 10.12 (C2). As there will also be losses of label from soluble material to CO_2 during the chase too, then even if amyl fraction label doubled in both woody and leafy tissues, these results support the hypothesis of turnover in condensed tannins noted previously.

Table 7.6. CO_2 Label Experiment, Results IV

Radioactivity (dpm) Incorporated in the Plants (per gram fresh weight).

		C1	C2	C3
Soluble Material				
	P/-	4.06×10^{-7}	9.06×10^{-6}	
	CH	1.31×10^{-7}	6.86×10^{-6}	7.70×10^{-6}
Insoluble Material				
	P/-	1.05×10^{-7}	7.26×10^{-6}	
	CH	1.53×10^{-7}	6.94×10^{-7}	3.07×10^{-7}
Soluble/Insoluble Ratio				
	P/-	3.88	1.24	
	CH	0.856	0.099	0.251

In the description of experimental methods it was indicated that paper chromatography of the extracts in Forestal solvent was used to finally demonstrate the presence of label in condensed tannins. Table 7.7 gives the distribution of label on such chromatograms. Much more label remains at the origin for the material from experiments A1-A3 than with C1-C3. This was due to excessive heating of A1-A3 extracts producing what were presumed to be phlobaphenes. It was reduced by heating the C1-C3 amyl fractions less strongly. The recovery of label in the Rf range associated with cyanidin may seem disappointingly low but in fact this is good as even in optimal conditions the acid hydrolysis of condensed tannins is not expected to give more than a 10% yield (Swain, 1959). Owing to the variability added at the hydrolysis stage Table 7.7 is only presented as further evidence that label has entered tannins, and calculations as to the proportion of the activity in the amyl fraction derived from tannins are not made.

7.2,3 Discussion

The results from all the experiments, independent of the labelling technique, suggested that tannins were subject to further movement or metabolism followng their synthesis in H. coubaril. This was true for the lablled material in amyl alcohol extracts and also for all the

Table 7.7. Amyl Alcohol Soluble Fractions: Distribution (%) of Radioactivity on Paper Chromatograms Run with Forestal Solvent.

Fraction/Rf		0 to 0.1	0.1 to 0.5	0.5 to 0.75	0.75 to 1.0
ln1					
A1	P/-	59	5	6	40
	P/C	73	4	5	18
A2	P/-	80	4	3	13
	P/C	84	4	3	9
A3	P/-	80	6	3	11
	P/C	73	7	5	15
C1	P/- (L)	45	12	11	32
	P/- (W)	31	13	31	25
	P/C	63	6	9	22
	CH (L)	30	12	20	38
	CH (W)	33	13	18	36
C2	P/- (L)	32	9	15	44
	P/- (W)	14	14	11	41
	P/C	21	7	8	64
	CH (L)	30	5	3	62
	CH (W)	29	12	8	51
C3	CH (L)	50	14	3	29
	CH (W)	26	18	15	41

protein precipitated material. This follows as there were no disproportionate increases in chase labelling of this material compared to the amyl fractions alone. The results agree with those of Baldwin and Schultz (1983) but where their work is limited by their failure to trace label to tannins, as defined chemically, here the poor recoveries of label during the fractionation procedures limit the reliability of the conclusions drawn above. Additionally there is no conclusive proof that all labelled tannins were extracted from insoluble material, although this is not thought to be a source of error. To summarise, the available evidence suggests the possibility of catabolic pathways for tannins in plants, although rapid catabolism is certainly not indicated. These pathways may not be expressed in all plants and other explanations have been advanced to account for declines in extractable levels of condensed tannins in plant tissues in the past. These include polymerisation to insoluble nonastringent forms (Hillis and Swain, 1959; Goldstein and Swain, 1963) and deastringency by reaction with aldehydes as seen in maturing fruit (Matsuo and Itoo, 1982).

Notwithstanding these explanations it now seems worth while to consider the possible costs to plants which such turnover of tannins might represent. To be costly in terms of fixed carbon, fast turnover in undamaged tissue

would have to be demonstrated. However, Baldwin and Schultz (1983) could not find evidence for this and there are no undamaged controls in the present experiments. So it remains possible that turnover, if a general phenomenon, may be a damage induced/enhanced phenomenon.

Plants appear to waste considerable quantities of photosynthate in other ways too, for instance marine algae may lose 30%-40% of their primary production by the leaching-loss of phenolics or photorespired glycollate into the sea (Sieburth, 1983). With respect to land plants, both photorespiration and cyanide resistant respiration are well established phenomena, but it is still a controversial issue as to whether these processes represent a waste. Lambers (1982, 1985) has suggested that cyanide resistant respiration may be adaptive to deal with situations of energy overflow. If he is correct, then the flow of carbon through pathways synthesising allelochemicals may be similarly adaptive at the level of plant intermediate metabolism, whilst the size of stored pools of these metabolites may be regulated as an adaptaton to ecological constraints i.e. it is these pools which are the allelochemicals measured in ecological work.

Chapter Eight

A Consideration of Factors Influencing Allelochemic Distribution in Plants

8.1 Introduction

After the proposal by Fraenkel (1959) that plant secondary products were primarily produced for defence, considerable research and even more speculation has been directed towards lending support to this hypothesis. In the case of tannins, it is now clear that they do have at least some anti-feedant properties toward many herbivores (see Section 1.4). However, their status as quantitative defences (sensu Feeny, 1976), which ought not to be subject to evolutionary counteradaptation, is now seriously compromised by the evidence reported and reviewed in Chapters 1-5. The hypothesis that tannins are primarily produced in response to herbivore pressure as an adaptive counter measure is also open to question, as is the view that they are relatively costly to produce in terms of the plant's energy ecconomy (see Chapters 6 and 7). In this final Chapter the work presented previously is drawn together in a reassessment of the ecological status of tannins and other allelochemicals.

8.2 The Evolutionary History of Tannins

Tannins do not leave a fossil record, nevertheless much can be gained by making the assumption that the present day secondary chemistry of plants reflects that of their antecedents. All the major modern plant groups (angiosperms, gymnosperms and pteridophytes) include representatives which produce condensed tannins. Using such chemotaxonomic information as is available from the extant flora together with that which can be gleaned from the fossil record, Swain (1978) attempted to achieve an evolutionary chronology which included the emergence times of the major tannin-containing taxa and the major groups of herbivores. His scheme indicated the Carboniferous as the period when tannin-containing plants first arose (condensed tannins only). At this time, plant biomass production patently outstripped herbivory and decomposition processes as the Carboniferous period is noted for the production of coal and oil deposits. Tannins may well have been important in plant resistence to decay at that time and so may have contributed to the laying down of these deposits. Of more importance is the indication that all the major orders of modern insect and mammalian herbivores arose during or after the carboniferous period, in an environment in which plants containing condensed tannins were present.

Within the modern angiosperms the production of

tannins is considered to be a primitive trait and this holds when the notion of primitiveness is considered objectively in terms of an advancement index (Sporne, 1954). On this basis, Bate-Smith (1962) demonstrated that condensed tannins were present in the most primitive dicotyledonous taxa, whilst hydrolysable tannins (only found in dicotyledonous taxa) were typical of more advanced taxa. By contrast the most advanced taxa tended not to have tannins of either type. This suggests that the lack of tannins in advanced dicots is a secondarily evolved feature, whilst the absence of tannins in algae and bryophytes is primative; the occurrence of algal phlorotannins may be considered in terms of parallel evolution.

Some of the earliest land plants may have produced lignin but are not thought to have produced tannin and there are many modern taxa capable of this. However, within the dicotyledons there is a very clear association between woodiness and the presence of tannins in plant tissues so that the more advanced taxa tend to be herbs in habit (Bate-Smith, 1962; Gardner, 1977). The distribution of tannins anticipated as a consequence of apparency is thus that seen through the chemotaxonomy of these plants, a distribution that may be explained without reference to any plant-herbivore interaction. It is thus worth enquiring as to the closenss with which

tannin production and woodiness are tied to each other, in order to see if this presents a better explanation of the distribution of tannins than does plant apparency. It can be argued that tannins are not so likely to occur in herbaceous plants, as their metabolism is much less geared to phenol production in general because they lack the requirement for massive lignin synthesis. This in turn reasonably leads to the expectation that plants in predominantly herbaceous taxa will contain a higher proportion of non-phenolic toxins (Swain, 1978).

Such arguments based on chemotaxonomy do not, however, provide a satisfactory explanation of plant antiherbivore chemistry, any more than do those based on apparency theory. Problems arise because tannins are not always produced constituatively; polymorphisms exist (Ma and Bliss, 1978; Ross and Jones, 1983), even if they are infrequently reported. Where tannins are constitutively produced, they are also cleary subject to gentically controlled variation (Butler, 1982). These factors indicate that tannin production can be regulated independently of other metabolic processes in a plant: if tannin production were an obligate overflow from other metabolic processes then such regulation, particularly +/- tannin polymorphism, would not be expected.

In summary, tannins may be primitive from an evolutionary standpoint and associated with the woody

habit, yet they do not appear to be accumulated in an uncontrolled or constitutive fashion by all plants which are either woody or of primitive taxonomy.

8.3 Bracken: A Case History

If the production of tannins is at least to some extent controlled by plants, then it is of interest to consider the ecology of a tannin-containing plant for which there is considerable information available, so as to make the attempt at understanding the role of tannins in the total ecology of that species. Bracken (Pteridium aquilinum) is such a plant, and it is of interest as one of a group of ferns which appear to have evolved concurrently with the angiosperms (Page, 1976) and so to share much of their history as regards predation from herbivores.

Bracken contains a wide range of potential allelochemical constituents including sesquiterpene lactones, phytosterols, thiaminase inhibitors, cyanogenic glycosides and tannins (Evans 1976; Cooper-Driver 1976). Not all of these are proven to be effective (e.g. phytsterols) and not all are always present (polymorphism for cyanogenesis; Cooper-Driver et al., 1977). Nevertheless the near nil acceptability of bracken as a forage makes it an economically important pest in sheep farming areas (Rymer, 1976).

Historically bracken was a shade-tolerant woodland species, but with forest clearance it expanded its range into open areas and seems to have been able to utilise the additional light available to boost primary

productivity. The basis for this assumption lies in the way bracken clones in open areas add considerably to the litter layer of the soil so that its depth builds up to the extent that the bracken rhizomes lose contact with the mineral soil and the plant's vigour declines. Thus the centres of bracken clones tend to die out and the plants become "a victim of their own success" (Watt 1976).

Given the problems of litter accumulation, the presence of tannins may actually be a hindrance in some circumstances if the ideas of Handley (see Section 1.2) are accepted. A comparison of tannin levels in fronds from sun and shade environments might be very revealing if it showed that light was a major cause of tannin accumulation in plants growing in open areas. Herbivores do not seem, on this basis, to be the limiting factor to bracken growth, and the array of secondary metabolites present suggests that tannins are not the sole cause of the relative escape of bracken from predation.

In highly insolated plants, tannin accumulation in cyanogenic individuals might also be positively detrimental to defence as they might inhibit the enzymic reaction involved in cyanide production (Goldstein and Spencer, 1985). In short, when the whole ecology of a plant is considered it is not necessarily a simple matter to equate the presence of tannins with the notion that

they are of adaptive significance, even if this does seem an attractive hypothesis at first sight.

8.4 The Present-Day Ecology of Tannins

From a consideration of the evolutionary history of tannins (Section 8.2) and their role within a particular species (e.g. bracken), it appears that generalisations about their current ecological role(s) may be difficult to make. The hypothesis of Feeny, and Rhoades and Cates (see Chapter 1) did attempt to construct a general framework within which to view the distribution of plant antiherbivore allelochemicals. Tannins were envisaged as playing a fundamental role as the archetypal form of defence investment that was to be expected for apparent plants. Perhaps because apparency is immeasurable, particularly as defined in terms of herbivore perception, the general framework of plant herbivore defence chemistry proposed by Rhoades and Cates (1976) has been much discused but not generally accepted. Here a fresh look at the factors likely to determine plant defence strategies is made with a view to re-examining the question of whether there is any particular kind of strategy which would be expected to include a role for tannins.

8.4.1 Strategies for Plants and their Defence

Feeny (1976) explicitly recognised that unapparent and apparent plants were r and K selected respectively (sensu Pianka, 1970). However, a more recent analysis of the vegetative and reproductive strategies employed by plants (Grime 1979) proposes three, not two, fundamental responses by plants to the environment. The characteristic life histories which comprise these strategies are described as "ruderal" (R), "stress tolerant" (S) and "competitive" (C). These are defined with respect to their characteristic environments in Table (8.2), following Grime (1979). Some characteristic features of plants adopting these strategies are given in Table 8.3, again following Grime (1979).

An examination of these clearly shows that R-strategists possess the features associated with unapparency/ephemerality, and indeed these were equated with r-selection by Grime (1979). Examination of the environmental (Table 8.1) and anatomical (Table 8.2) features associated with C and S plants indicates that both could be described as including apparent features, yet only S plants were equated with K selection by Grime (1979). C-strategists were considered to exhibit intermediate reproductive characteristics between those of r and K-selection.

The work of Grime (1979) was based on an analysis of plant responses to climate, weather, soil mineral

Table 8.1 Morphogeninic responses to desiccation, shading and mineral nutrient stresses of competitive, stress-tolerant and ruderal plants and their ecological consequences in three types of habitat* (taken from Grime 1977).

Strategy	Response to stress	Consequences
Competetive	Large and rapid changes in root:shoot ratio, leaf area, and root surface area.	H1: Tendency to sustain high rates of uptake of water and mineral nutrients to maitain dry matter production under stress and to succeed in competition. H2: Tendency to exhaust reserves of water and or minerals both in the rhizosphere or the plant; etiolation in response to shade increases susceptibility to fungal attack. H3: Failure to rapidly produce seeds reduces chance of rehabilitation after disturbance.

Stress-tolerant	Changes in morphology often slow and of small magnitude.	H1: Overgrown by competitors. H2: Conservative utilisation of resources allows survival over long periods in which little dry matter production is possible. H3: Failure to rapidly produce seeds reduces chance of rehabilitation after disturbance.
Ruderal	Rapid curtailment of vegetative growth and diversification of resources into seed production.	H1: Over grown by competitors. H2: Chronically low seed production fails to compensate for high mortality rate. H3: Rapid production of seeds ensures rehabilitation after disturbance.

* Habitats. H1: The early successional stages of productive undisturbed habitats. H2: Late succession in productive habitats or chronically stressed environments (e.g. harsh climate or poor soil). H3: Severely disturbed (e.g. by periods of extreme weather) but potentially productive habitats.

Table 8.2 Some morphological characteristics of competitive, stress-tolerant and ruderal plants (taken from Grime, 1977).

		Competitive	Stress tollerent	Ruderal
1	Life forms	Herbs, shrubs and trees.	Lichens, herbs, shrubs and trees.	Herbs
2	Morphology of shoot.	High dense canopy of leaves, extensive latteral spread.	Extremely wide range of growth forms.	Small stature, limited lateral spread.
3	Leaf form	Long or relatively short.	Often small, leathery or needle like.	Various, often mesomorphic.
4	Longevity of established phase.	Long or relatively short.	Long to very long.	Very short.
5	Longevity of leaves and roots.	Relatively short	Long	Short

Table 8.2 continued

6	Leaf phenology.	Clear peaks of production, occur in periods of potential maximum growth.	Evergreens with various patterns of leaf production.	Short phase of leaf production in period of high potential growth
7	Flowering	Regular and yearly	Irregular and intermittant	Very frequent, early in life history.
8	Regeneration	Vegetative, seasonal in gaps, numerous wind borne seeds, and by seed bank,	Vegetative, and by a persistent seedling bank.	Seasonal regeneration in gaps, by seed bank and by numerous wind borne seeds/spores.

Note: this is a shortened version of Table 6 of Grime (1977) which lists additional features ascribed to these plants.

nutrients and competition from other plants. Herbivore pressure was of minor consideration. In short his work collated information from the study of classical plant ecology and his results, though subjective, may reasonably be taken as unbiased by theories of antiherbivore defence chemistry.

An important first step in producing a coherent hypothesis is to consider whether herbivores also exhibit strategies in their life history patterns. Rhoades (1985) provides evidence that they do, and he identifies two strategies termed "stealthy" and "opportunistic". An insect species that exhibits stealth shows an even and low level use of food resources so as not to trigger the production of enhanced levels of plant defences. K-selection is thus characteristic of these species. "Opportunism" is a strategy whereby plants are heavily exploited at times when they are unable to actively defend themselves (e.g. following some temporary environmental stress). Such insects show r selection. The evidence Rhoades provides for this is based largely on entomological observations, and independent of information from plant population dynamics. This state of affairs arises because the integrated study of plant and herbivore dynamics, examining reciprocal interactions between plants and herbivores, is an area of research where only limited information is yet available (Crawley,

1983).

By examining the interface between the studies of Grime (1979) and Rhoades (1985) some likely patterns in plant defences become evident.

R-Strategists

These plants are seen as having an exclusively herbaceous habit. It is also particularly likely that they will be subjected to opportunistic herbivory as (i) their ephemerality makes them an unlikely resource for long term stealthy exploitation and (ii) their vegetative stage is geared to fast growth and reproductive success not stress resistence, so they may be prone to weakening by factors such as weather or competition from other plants. Within the dicotyledons the evidence is that herbaceous plants do tend to have toxic allelochemical defences, and without considering either apparency or production costs, this may make sense on two counts. Firstly, where effective, toxins may promote complete avoidance or, alternatively, the minimum of consumption by herbivores unadapted to the large scale detoxification of toxins. Secondly, small amounts of toxins may be physiologically easy to store in fast growing cells, whereas tannins may be too bulky, and are also likely to be recalcitrant to degradation and translocation. Indeed all quantitative defences either chemical (e.g. tannin

or resin) or physical (spines, leaf toughness) would seem to constitute a difficult system for a rapidly growing plant to maintain. Ruderal (r-selected) plants are thus still expected to possess predominantly toxic/qualitative defences, but for reasons other than or additional to those to do with their apparency.

There is little evidence currently available to test these ideas comprehensively, even with respect to tannins. Most herbaceous species do not contain tannins or even belong to families where the formation of tannins is likely on taxonomic grounds (see Section 8.2). Nevertheless there are tannin-containing herbs and their ecology needs to be examined to see if they confound the ideas expressed above. Some headway can be made by considering the life histories of herbaceous species in plant families known to include tannin-containing species. For example, Urtica dioica (Urticaceae), Portulaca oleracea (Portulacaceae), and Euphorbia lathyrus (Euphorbiaceae) are all (at least partially) ruderal plants in families in which tannin containing species occurr but they do not contain tannins themselves (Bate-Smith, 1962).

The following, (all thought to contain hydrolysable tannins; Bate-Smith, 1962) are examples of species which superficially appear to be comparrable but which actually exhibit some stress tollerant features. Geum urbanum

(Rosaceae) is shade tolerant whilst Fillipendula ulmaria, Geranium sylvaticum (Geraniaceae) and Lythrum salicaria (Lythraceae) all tend to grow in wet areas. Other species may use storage organs to maintain their presence at a site (Circea lutetia; Onagraceae) or compete for space by vegetative spread (Fragaria moshata; Rosaceae) or by growing in tall dense stands (Chamaenerion angustifolium; Onagraceae). Considering species that contain condensed tannins, a similar analysis can be made. Two good examples of stress tolerant plants being Drosera rotundifolia (Droseraceae) and Sarracenia purpurea (Sarracaeniacae). These are herbs characteristic of bog vegetation.

Despite the incomplete evidence, it seems from what is avalible that many tannin-containing herbs will fit into the scheme without there being an intolerable abundance of exceptions to the rule i.e. tannin-rich extreme R-strategists.

S-Strategists

Unlike ruderal plants, these are found in persistant environments, in which soil or climatic factors create continually harsh conditions compared to those in which C strategists will predominate. The key factor here would appear to be stress, and, more precisely, long-term

chronic stress.

It is suggsted that the response of both plants and animals to these conditions will, in part, shape the plants' defence strategies. S-strategists appear likely to be predominantly exposed to predation by stealthy herbivores as they represent food resources that, compared to ruderal plants, will be continually available. Given the typically long vegetative stage in their life cycles, necessitated, at least in part, by the likelihood that particularly extreme environmental conditions may weaken them and delay reproduction, S-strategists will need a constitutive and high level of defence against both stealthy and occasional opportunistic attack. In short, a kind of "fortress" defence such as might be thought to be provided by quantitative defences is called for.

Furthermore, if S-plants continually experience some form of physiological stress, due to their physical environment, it is very likely that their defence chemicals will be primarily derived from phenolics as increased phenol metabolism may well be generally symptomatic of stress (see Chapter 6). In the specific case of mineral shortages, carbon- oxygen- and hydrogen-containing, but nitrogen- and sulphur-free, allelochemics (see Table 1.1) are to be expected in any case, (whether they are phenolic or not). A further

reason why quantitative defences, chemical or physical, should prove suitable systems for stress tolerant plants is that tissue growth and defence production in S-stratagists should proceed in step without the former process having priority as in a ruderal species. Support for these ideas can be seen in the general occurrence of xeromorphic features in stressed vegetation, even in rain forests (Buckley, et al. 1980) and in the acceptance of leaf toughness as an important part of plant defence even in phenolic-rich species (Lowman and Box, 1983; Rausher, 1981).

Here the conclusion reached is that these plants should have similar defences to those proposed by Feeny for apparent (i.e. for K-selected) plants, namely high concentrations of constituative, dose-dependent defences such as tannins, and also sclerophylous leaves, spines etc. The cost of producing these substances has not been a factor in this argument and whilst their apparency may play a part in the defensive strategy of these plants, it is the constraints on defence production imposed by constant physiological stress which are here considered critical.

The defence chemicals of many S-strategy plants may be costly to produce but this is not a necessary feature of the argument presented here. In this hypothesis tannins are still thought to act in high concentrations compared

to toxins, and in a dose-dependent way (see Section 1.4). It is on these two counts only that tannins and similar defences may still be regarded as quantitative in effect. This position holds true whether they act by influencing N-retention, reducing food consumption or poisoning the animal through their hydrolysis products, (i.e. the quantitative effect is not dependent on the mechanism of action.)

If stress is an important factor then these predictions regarding defence strategy ought to hold regardless of plant growth form and in this context some consideration of herbaceous species is of interest. Grime (1979) indicated that ruderal plants would be herbaceous, but that herbs were not exclusively ruderal. The question thus arises as to whether herbaceous plants in families that are considered to be tannin-free, can exhibit a stress tollerant strategy and if so, do they produce the anticipated forms of defence even if they are non-phenollic by nature?

In the British flora, salt marsh plants of the Chenopodiaceae provide some examples of this (Bate-Smith, 1962). These plants have no tannins or highly fibrous tissues, yet Salsola kali has developed spines and Atripex hastata may well be unpalatable through being salt encrusted. In other floras spines have been developed by stress tolerant members of other tannin-free

families (Compositae, Cactaceae).

C-Strategists

The above analysis closely follows Feeny, Rhoades and Cates in equating toxic defences with extreme r (i.e. R) selection and quantitative defences (i.e. bulky and dose dependent) with extreme K (i.e. S) selection, but for diferent reasons. To make the present analysis more productive, a separate C-strategy for defence remains to be identified. Considering stress, C environments are supposed to be continually productive and so there should be no mineral (i.e. nitrogen or sulphur) or other stress limitation present on the types of allelochemics synthesised. As regards growth form and rate, there are no constraints due to these factors that indicate the production of particular allelochemics either. The likely distinguishing features of C-strategists become clearer when the dynamic properties of plant responses to herbivore attack are brought into the picture.

Schultz (1983) believes that inter and intra plant variability in plant defence, notably that induced in response to herbivore attack, is the key to stability in plant-herbivore systems. He does not believe that the induction of additional allelochemical production <u>per se</u>, as a response to herbivory, is the factor that directly controls herbivore populations as this could be counter

adapted just as static levels of constitutive defences may be subject to counteradaptation. Schultz sees the effect of induced enhancment in the levels of allelochemical defence as one that may weaken herbivore resistance to predators or disease, particularly if the foraging costs rise in a variable environment where suitable food becomes rarer. In short a cascade effect is set in train by induced changes in plant defence which themselves may not have a large effect on herbivore fitness but which are reflected in significant effects that do regulate herbivore populations.

Returning to R, C and S-strategies, Edwards and Wratten (1985) have argued that C plants are more liable to have inducible defences than S-plants on account of S-plants having a supposedly more constant need for defence. Here it is argued that variability in a herbivores environment, notably that in food resources, is an important population stabilising factor whether the plant consumed exhibits an R, C or S-strategy. In contrast to Edwards and Wratten (1985) the proposal made here is that for C-strategists, this variability will be predominantly herbivore-induced whilst other sources of variaton may contribute to changes in R and S-plants.

With R-plants, their short life span, fast generation time and large reproductive output may allow for both considerable genotypic variation in defences and for year

to year herbivore selected defence variations. Coupled with a potentially lower level of "stealthy" predation these factors should reduce (although not eliminate) the importance of inducible defences as a stabilising contol of herbivore pressure. For S-plants, which should be more particularly liable to long term stealthy predation, high levels of constitutive defences may maintain plant resistence in periods of extreme stress (i.e. extreme weather as opposed to climate). This may well make plants harder to exploit if herbivores are also influenced by environmental conditions. Environmental fluctuations complemented by some induced resistence may thus provide stabilising variation in stress tolerant plant communities. However, it must be pointed out that to appreciably increase the level of defence in a plant which already has a high constitutive level of a quantitative defence, will require the induced production of a considerable amount of allelochemic. Unless an S-plant's induced defence was of a qualitatively different type (e.g. an alkaloid), and there is no evidence for this type of defence switch ever happening, then the occurrence of significant induced defence production in these plants is unlikely from a physiological standpoint.

With C-plants in persistent populations subject to "stealthy" and "opportunistic" predation, induced

variation may assume more importance than in communities of predominantly R-selected plants. Slower generation times will rule out response to herbivore pressure through genotypic variation; if the vegetation is less subject to extremes in the physical environment then, as with all the other factors listed above, if plant variability is the key to stability in plant-herbivore systems then for C-plants this variation will have to be predominantly induced within single generations of plants.

It is of course clear that these ideas depend on the notion that chemical changes in plants are inducible by herbivores and that herbivores are affected by them. Fowler and Lawton (1985) have taken a devil's advocate position on this and after critically reviewing the considerable literature in which such effects are reported, they find only two studies that satisfy their conditions for proof (Karban and Carey, 1984; Raup and Denno, 1984). Much of the remaining work has, in their opinion, been subject to poor statistical analysis and though they do not challenge the results as such, they do challenge the level of confidence that can be placed upon them. Taking the available literature as a whole, this study expects that induced changes in plant chemistry that affect animal performance will eventually be demonstrated with statistical confidence (at least in

laboratory conditions). The more important and also generally unproven hypothesis is that these changes are in themselves, or in concert with factors such as predation or disease, able to regulate herbivore populations.

8.4,2 Summary

By considering the dynamic properties of plant and herbivore populations separately from each other and in relation to their physical environment, a reassesment of plant defence strategies has been proposed which provides a context in which a plant's defence chemistry can be related to its life history pattern in general. The information given in Table 8.3 does not rely on apparency as a factor but will generally be consistent with observations based on plant apparency. The other major departure from the work of Feeny, and Rhoades and Cates, is the removal of production cost as a factor in the equation determining which allelochemical a plant produces. Even if R, C and S-strategies like apparency/ephemerality cannot be measured on a numerical scale, they are at least defined in terms of a number of measurable factors outwith the plant-herbivore interaction (e.g. seed procuction and longevity. See Table 8.2).

With respect to tannins the ideas presented above

Table 8.3 Sumary of defensive strategies hypothesised for R,C and S strategists

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I Ruderal

i Disproportionately attacked by opportunistic herbivores compared to C & S-strategists

ii Tendency to accumulate small amounts of toxin type allelochemics.

iii Inducible defences may be present but not a major contribution to inter-plant variation

iv Very low contribution by physical defences.

v High genotypic variability in defences coupled with fast generation time provides a response mechanism to herbivore pressure

vi Typically unconstrained by mineral resources as regards which allelochemic are produced

II Competitive

i Disproportionately attacked by stealthy herbivores compared to R-strategists

ii May adopt either quantitative or toxic defence chemicals or both.

iii Highly inducible response to herbivory.

iv May or may not possess physical defences.

Table 8.3 continued

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v Low genotypic contribution to varyability in defence owing to slow generation time.

vi Typically unconstrained by mineral resources as regards which allelochemic are produced

III Stress-tolerent

i Disproportionately attacked by stealthy herbivores compared to R-strategists

ii Quantitative allelochemicals stored.

111 Inducible defences may be present but most defences are constituitive.

iv High dependence on physical defence.

v Environmental factors (allong with induced changes), add variation to plant defences investment.

vi Stress metabolites feature as allelochemicals.

provide a framework in which to explain why "unapparent" plants such as herbs growing on a forest floor may have tannins and why not all "apparent" plants (e.g. C-strategists) produce tannins or other quantitative defences. However a necessary feature of the above system is that phenol and perhaps tannin production should be induced to a significant degree in plants exhibiting a competitive strategy. This point needs investigation, but the fact that much of the evidence for induced defences derives from studies of trees is encouraging, given that forests are generally present in continuously productive environments and as trees can be seen as exhibiting a competitive strategy in such vegetation.

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Appendices

Appendix One: Materials and Methods for the Preparation and Chemical Characterisation of Tannins.

A 1.1 Method Used in Preparing Tannin-rich Plant Extracts.

METHOD: Fresh plant material was collected and freed from other contaminating plants and soil. Leaf or frond tissue only, was used in the following stages.

The material was cut or broken into short sections and homogenised in an "ATOMIX" blendor (MSE) using the minimum volume of water. This produced a coarsely broken mass of fibres and leaf fragments.

An "Ultra-Turax" (Janke & Kunkel KG) homogeniser was then used to turn the above into a fine slurry. Microscopic examination showed that this did not rupture every cell, however the level of homogenistion was considerable and seemed largely independent of plant structure.

After homogenisation acetone was added to the extract to double its volume (i.e. to make it 50% v/v acetone). The extract was then stirred with a magnetic stirrer for one hour and at the end of this time solid material was removed using a Buchner filter funnel. Re-extraction of the solid material in 70% acetone released considerable amounts of plant pigments and gave a comparativly colourless solid residue. Whilst not contributing

significantly to the yield of phenolics this was nevertheless routinely performed.

Both extracts were combined and kept cool in a refrigerator whilst aliquots were purified in a continuous liquid/liquid partition apparatus, by partition against diethyl ether.

When all of an extract had been so treated it was placed under vacuum from a bench suction pump, in order to boil off as much solvent as possible and the remainder was then lyophylised to a powder in a freeze dryer (Edwards High Vaccume Ltd, model 30P.2/764).

A 1.2 Methods used for the Partition of Phenolics between 50% Acetone and Diethyl Ether

REAGENTS: 50% v/v aqueous acetone; Diethyl ether; Tannic, Ascorbic and salicylic acids (all from BDH); Hesperidin, catechin, sinapic, coumaric, ferulic and caffeic acids (all from Koch-Light Ltd); Quebracho tannin (Harshaw Chemicals); Gallic acid (R. N. Emmanuel Ltd).

METHOD: In each partition experiment a known amount of a phenolic compound was weighed out into a tared 100ml conical flask which was then filled with 40ml of 50% acetone followed by 40ml of diethyl ether. The stoppered flasks were then placed on an orbital shaker (set at a slow speed) and shaken overnight. The following day the

ether layer was removed by means of a separating funnel and the aqueous phase was returned to the flask and lyophylised. The resulting powder was then reweighed to calculate the recovery in the aqueous layer.

A 1.3 The Folin-Denis Method for Total Phenolics

REAGENTS: The Folin-Denis reagent (Choo, 1981) was prepared by the addition of 100g of sodium tungstate (BDH) and 20g of phosphomolybdic acid (BDH) to a 2l flask to which was then added 50ml of orthophosphoric acid (BDH) and 750ml of distilled water. The mixture was then set to reflux for 2hr, allowed to cool, made up to 1l by adding water and then stored in a dark cupboard before use. The second reagent used was saturated sodium carbonate (BDH) solution.

METHOD: A solution of lyophylised plant extract of known concentration was made using distilled water, any insoluble material being allowed to settle. The clear solution (1 ml) was added to a 100ml volumetric flask containing approximately 60 ml of water. At time zero 5ml of Folin-Denis reagent was added, followed by 10ml of saturated sodium carbonate solution after exactly three minutes. The contents of the flask were made up to 100ml with distilled water and mixed thoroughly by shaking. After exactly 20 minutes the absorbance (A) was measured

at 760nm. The spectrophotometer was zeroed on a reagents only blank reaction prior to the measurement. Measurements outside the range $0.10 < A < 0.50$ were repeated, and either more extract or a diluted extract was used to obtain results within this range (of linearity). All measurements on each extract were replicated until three values within a range of 0.01 absorbance units relative to each other were obtained, these being used to calculate A_1^1 values. Figure 2.1 shows a standard curve for this method using tannic acid.

A 1.4 The Hagerman and Butler Method for Total Phenolics

REAGENTS: The SDS/triethanolamine reagent was an aqueous solution containing 1% w/v of sodium dodecyl sulphate (BDH) and 5% v/v triethanolamine (BDH) in distilled water. The ferric chloride solution contained 1.62g $FeCl_3$ (anhydrous, BDH) per litre of 0.001M HCl.

METHOD: The test solution used was prepared as for the Folin-Denis method. 1ml of this solution or 1ml of a dilution in water was added to a vial containing 2ml of the SDS/triethanolamine reagent and 1ml of the ferric chloride solution. The contents of the vial were mixed, poured into a cuvette and the absorbance was read at 510nm within 1hr. It should be noted that colour formation is immediate and maximal using this method

(N.B. the addition of reagents in the above order is essential). Measurements were made in the range 0.1 < A < 2.0 and replication was as above. Reagent only blanks were used to zero the spectrophotometer. Figure 2.2 shows a standard curve for this method using tannic acid.

A 1.5 The Proanthocyanidin Method for Condensed Tannins

REAGENT: The butanol-HCl reagent was prepared by dissolving 0.700g ferrous sulphate heptahydrate (BDH) in 25ml conc. HCl and a little n-butanol (BDH), the resulting solution being made up to 1l with more n-butanol.

METHOD: The solution of extract to be tested was made up in 80% methanol. The methanol concentration does affect the assay and 80% was chosen to follow the standard procedure used in the Strathclyde laboratory. 1ml of the extract solution was added to 10ml of the butanol-HCl reagent in a test tube, the reaction mixture then being heated for 2hr in a boiling waterbath. After being allowed to cool the absorbance of the solution was measured at 550nm against a reagent only blank. For A > 0.75 a dilution of the reaction mixture was made with n-butanol. Replications were made as above.

A 1.6 The Vanillin Methods for Condensed Tannin Content and Polymer Length

REAGENTS: The vanillin reagents were prepared immediately before use. Both contained 4% HCl and 0.5% vanillin (BDH), 100% methanol being the solvent used in the simple analysis of condensed tannins (section 2.3) and glacial acetic acid being used in the polymer length assay (section 2.4)

METHOD: Solutions of the extract to be tested were made up in 100% methanol for the assay of condensed tannin content (carried out in methanol solution). For the polymer length assay (in glacial acetic acid) the test extract was first dissolved in a minimum amount of methanol (following Butler et al., 1982), glacial acetic acid was then added to dilute this. Care was taken to ensure that methanol concentration was kept to less than 6% v/v in the test solution.

The assay procedure for both vanillin reactions consisted of adding 1ml of test solution to 5ml of the reagent (made up in the appropriate solvent), which had already equilibrated to 30^{o}C in a water bath. The reaction was maintained at this temperature for 20 minutes and 5 minutes for the reactions in methanol and in glacial acetic acid, respectively. At the end of these incubation periods the absorbances were read at

500nm and 510nm, respectively. The normal number of replicates were made, reagent blanks were used to zero ths spectrophotometer, and repeat measurements on diluted extract solutions were made for A > 2.0.

A 1.7 The Iodate Reaction for Gallotannins.

REAGENT: Saturated aqueous potassium iodate solution

METHOD: Solutions of extracts were made up in 70% acetone and 1.5ml of KIO_3 solution was added to 3.5 ml of extract solution. All solutions were kept at approximately $15^{o}C$ in a large water bath for one hour during which time observations were made on the colours produced by the solutions.

A 1.8 Method for Hydrolysable Tannins

REAGENTS: Ferric chloride solution; 1.62g ferric chloride (anhydrous, BDH) disolved in 0.001M HCl to give a total volume of 1l. 4M sodium hydroxide (NaOH), 4 M hydrochloric acid (HCl).

METHOD: Extract solutions were made up in water and divided into two portions. Two drops of 4M NaOH were added to one portion of approximtely 5ml contained in a test tube. This was then placed in a boiling water bath for 2hr before the extract was cooled and neutralised by

the addition of two drops of 4M HCl. Samples of both the hydrolysed and unhydrolysed extracts were then assayed as follows. At time zero 500 μl of the extract was added to 3.5 ml of the ferric chloride solution in a cuvette, the contents of which were then rapidly mixed and the absorbance was then read at 600nm, 15 seconds after the addition of the extract solution. Reagent only blanks were used to zero the spectrophotometer. Replication was as normal, with A being read in the range 0.05 - 1.0.

A 1.9 Method for Paper Chromatography of the Extracts.

REAGENTS: Amyl alcohol (BDH), 2M HCl, Forestal chromatography solvent system (glacial acetic acid, water, conc. HCl in the ratio 30:10:3), Whatman No.3 paper.

METHOD: Approximately 100mg of lyophylised extract was dissolved in approximately 4ml of 2M HCl, and then heated to 100oC for 30 minutes and subsequently cooled. Amyl alcohol (1 ml) was then added to each extract solution, which was then shaken. After partition, a drawn out cappilary tube was used to spot samples of the upper amyl layer onto a paper chromatogram which was then developed in Forestal solution. Red spots were marked and recorded as anthocyanins if they turned blue in ammonia fumes; cyanidin (Rf 0.55 to 0.45) or delphinidin (Rf 0.37 to

O.30). Delphinidin spots were also recognisably more purple in hue than those of cyanidin. Further unidentified fllavonoids were identified as spots (below gallic acid) which flouresced yellow under UV light folllowing Choo (1981).

Ellagic acid could be detected as a spot intermediate in Rf between cyanidin and delphinidin, which appeared blue under UV-light and yellow under UV light after brief exposure to ammonia. Gallic acid was run as an authentic marker on the chromatograms. It was detected as a brown spot which developed after prolonged exposure to ammonia (30 minutes). For extracts where gallic acid was detected in the hydrolysate, a repeat chromatogram was run of an unhydrolysed sample of extract to check for the presence of free gallic acid in the extract.

Appendix Two: Materials and Methods for Experiments Investigating Tannin-protein Interactions and for Fieldwork

A 2.1 Buffer Solutions

The following lists the recipies used to prepare buffer solutions employed in the experimental work presented in Chapter 3 and following chapters (except where these have been given in the text). The buffer systems used are all taken from Gomori (1955).

REAGENTS: All the salts listed in the recipies were technical grade and supplied by BDH.

METHODS: For the buffers listed below, a solution of a given pH was made by the addition of one stock solution to another using a glass electrode pH meter to monitor the process until the correct pH was attained.

pH=2.0 and pH=2.2. 50 ml of 0.2 M KCl taken to the correct pH by the addition of 0.2 M HCl; the solution was then diluted to a final volume of 200 ml.

pH=2.5. 50 ml of 0.1M citric acid taken to pH by the addition of 0.2 M Na_2HPO_4; the solution was then diluted to a final volume of 100ml.

pH=3.0. As above but starting with 40 ml citric acid.

pH=3.4 and pH=3.5. As above but starting with 35 ml citric acid.

pH=4.0 and pH=4.5. As above but starting with 30 ml citric acid.

pH=4.6. 11 of (0.1M) citric acid taken to pH with 0.4 M Na_2HPO_4 before dilution to 21 final volume.

pH=5.0. As above but starting with 25 ml citric acid.

pH=5.7 and pH=6.0. As above but starting with 20 ml citric acid.

pH=6.5. As above but starting with 15 ml citric acid.

pH=7.0. As above but starting with 7 ml citric acid.

pH=7.5, =7.6, =8.0, =8.5, and pH=9.0. 50 ml of 0.2 M boric acid taken to pH with 0.05 M borax; the solution was then diluted to a final volume of 200ml.

pH=9.2. 50 ml of 0.2 M $NaHCO_3$ taken to pH with 0.2 M Na_2CO_3; the solution was then diluted to a final volume of 200 ml.

pH=9.5. As above but starting with 40 ml of $NaHCO_3$.

pH=10.0. As above but starting with 23 ml of $NaHCO_3$.

pH=10.8. As above but starting with 5 ml of $NaHCO_3$.

A 2.2 Bradfords Method for Proteins

REAGENT: 100 mg of Coomassie Brilliant Blue G-250 (BDH) was dissolved in 50 ml of 95% (v/v) aqueous ethanol. To this solution 100 ml of 85% (v/v) aqueous orthophosphoric acid (BDH) was added followed by dilution with distilled water to a final volume of 1l.

METHOD: The test (protein) solution (100 µl was added to 5 ml of the reagent in a test tube, the contents of which were then mixed by inversion. Absorbance was measured at 595nm after two minutes and before one hour, in a 3 ml cuvette, against a reagent blank prepared from 5 ml reagent and 100 µl of the (buffer) solution in which the protein was disolved. Absorbance measurements were made in triplicate and averaged. Protein contents were obtained from absorbance readings by reference to standard curves made using test solutions containing betwen 10 and 100 µg protein. Separate curves were prepared for each protein used, and all absorbance masurements were made within the range determined by the standard curve for the protein under test.

A 2.3 Method for the Ninhydrin Assay for Amino Nitrogen

REAGENT: This was bought preprepared from Sigma, made up in DMSO according to the Moore and Stein (1968) formula.

METHOD: Between 50 and 250 µl samples of hydrolysed protein solution were added to 500 µl aliquots of the reagent at time zero. The reaction mixture, contained in glass specimen bottles, was then heated in a boiling waterbath for 15 minutes. At the end of this time the reaction was stopped and the reaction mixture was diluted by the addition of 5.0 ml of cold 50% aqueous ethanol.

Absorbance of the resulting solution was measured at 570 nm. A standard curve was prepared to convert these values to weights of unhydrolysed protein (see Figure 4.6). 50% ethanol was used to further dilute the reactions where necessary before absorbance measurements were made.

A 2.4 Method for the Pepsin/Cellulase Digestibility Assay

REAGENTS: Pepsin Solution was prepared by dissolving 600 mg of pepsin (Sigma) in 300 ml of 0.1 M HCl (N.B. the acid solution alone was used in the "buffer only" treatments refered to in Chapter 4). A cellulase solution was prepared by dissolving 1.875 g Trichoderma viride cellulase (BDH) in 600 ml of a citrate/phosphate buffer (pH=4.6; see Appendix 2.1).

METHODS: Preweighed strips of substrate (paper) were placed in glass specimen bottles. For the pepsin pretreatment 20 ml of pepsin solution was added to the bottle which was then stoppered and place in a waterbath, set at 37^{o}C, for 24 hours. At the end of this time the pepsin solution was poured away and replaced with 20 ml of cellulase solution after which the bottle was returned to the water bath for 48 hours. At the end of this incubation with cellulase the contents of the bottle were poured over a disc of (weighed) filterpaper on a buchner

filter funnel. The cellulase solution was then filtered off and the residue was oven dried, allowed to re-equilibrate to ambient conditions for 24 hours on a laboratory bench, and then finally weighed. The variations to this technique used in Chapter 4 were to (i) omit the pepsin from the pretreatment solution and (ii) to omit the pretreatment altogether by beginning the reaction with the addition of cellulase.

A 2.5 Methods for Amino-acid Precipitation Reactions

REAGENTS: Amino-acid solutions were made up in water. Following Handley (1954), these were of 20mg/ml or of saturated solutions if the amino acid was less soluble than this. The amino-acids tested were lysine, arginine, histidine, leucine, isoleucine, valine, glycine, proline, and alanine. Buffers were those of pH=2.0, 2.5, 3.0, (0.5 unit intervals) to pH=10.0, as described in Appendix 2.1. All amino acids and salts were from BDH.

METHOD: Approximately 200mg of extract was disolved in 20ml water, 2ml of this solution was then combined with 2ml of each buffer. Amino acid solutions were then added dropwise to these solutions and the occurence of precipitation or cloudiness was noted.

A 2.6 Method for the Measurement of Ambient Light Intensity for Foliage.

METHOD: Individual plants were selected for their possession of branches in relatively sunny and/or shaded positions within the forest and which could easily be reached from a 6m ladder. Once located suitable groups of leaves were tagged for reference. Wherever possible shaded and unshaded leaves were chosen from the same plant, but this was not always feasible and single sets of leaves were taken from individual plants some of the time. These sets of leaves were made the object of light intensity measurements for a sample of five days. On each day measurements of the light incident upon each set of leaves studied were made by placing a piece of white card over them and recording the f-stop and shutter speed required to correctly expose 50 ASA film in a Minolta X300 camera fitted with a 50mm lens and aimed at the card. These measurements were timed at each set of leaves, and taken at intervals of about two hours throughout the daylight period. They were used for comparison with recordings made at 10 minute intervals during the day using an identical camera aimed at an identical piece of card placed in a large open clearing (recently disused farmland). This second set of readings was defined to be a measure of total light incident upon the vegetation. By first converting camera settings to

foot-candles, using a Weston light metre dial, this data allowed an estimation of the percentage of available light (LIGHT) which reached the leaves under study. This percentage of available light was not constant throughout the day for a given sample of leaves, as it was dependant upon solar movements in relation to openings in the surrounding canopy. However by averaging all readings taken during each day, for all five days sampling, an estimate of the percentage of available light reaching each group of leaves was calculated. This is not intended as an absolute measure, or even one linearly related to solar irradiance, but one that should allow variation in light intensity to be assessed on a continuous scale.