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PLANT POLYPHENOLS—SECONDARY METABOLISM AND CHEMICAL DEFENCE: SOME OBSERVATIONS

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Key Word Index—Polyphenols; secondary metabolism; chemical defence; structure–activity relationships; protein association; astringency.

Abstract—Secondary metabolism and theories of plant–animal co-evolution are briefly discussed. Current ideas concerning the role of polyphenols as mediators of a general form of chemical defence in plants are outlined. Studies are described of the association of a group of biosynthetically inter-related polyphenols based on gallic acid and D-glucose with the protein bovine serum albumin. The results are interpreted in terms of structure–activity relationships in protein–polyphenol complexation and are used to comment on the co-evolution theory and the position of plant polyphenols in higher plant secondary metabolism and defence.

INTRODUCTION

For almost 200 years studies of the chemistry of natural products have constituted a dominant theme of organic chemistry. With the flowering of biochemistry in the twentieth century there came the realization that for some natural products (e.g. L-amino acids, fatty acids, nucleotides) a distinctive role in the life of all organisms could be assigned. Natural products such as these occur in broadly similar patterns and the pathways by which they are synthesized are similar, if not identical, in most organisms. They are frequently referred to as primary or intermediary metabolites. In contrast, an infinitely greater body of natural products, principally of microbial and plant origin, such as alkaloids, terpenes, polyenes, polyacetylenes, phenols and mycotoxins, occur sporadically in nature; their presence indeed often constitutes something of a taxonomic speciality. Moreover, they appear to have no explicit function in the economy of the producing organism. For this reason they are often collectively designated as secondary metabolites [1].

Although this distinction between natural products is useful in a didactic way, the boundary between the two areas of metabolism is often imprecise and, as new discoveries are made, frequently transitory. Nonetheless the question of the function of secondary metabolism in plants and micro-organisms is one that has excited continued debate and speculation. Whilst several propositions centre on the suggestion that it is the processes of secondary metabolism and not, in the general case, the secondary metabolites themselves which are of importance to the organism [2] one theory which has gained increasing prominence is based on a theory of plant and animal co-evolution [3–6]. It focuses on the secondary metabolites and the premise that plants, as a response to an environmental and ecological challenge, have evolved the stratagem of a chemical armoury appropriate to the environmental pressures which they face. Two quotations [7, 8] from recent published work convey the general thrust of these ideas, which are based on the assumption that early in their evolution plants developed the characteristics which made them unpalatable: ". . . natural selection serves as a mechanism by which a population of herbivores may 'call forth *de novo*' the evolution of a biosynthetic pathway producing compounds toxic to the herbivore . . ." and ". . . much of the purpose of the synthesis of complex molecules of terpenoids, alkaloids, and phenolics lies in their use as defence agents in the plants fight for survival . . .".

In this context the purported role of polyphenols (syn. vegetable tannins) is widely quoted. The relevant physiological effect of polyphenols is considered to be that of astringency based on their ability to complex with proteinaceous materials [9]. This may render tissues unpalatable to a predator by precipitating salivary proteins or, by immobilizing enzymes, impede the invasion of the host tissues by microbial predators or parasites. Feeny [10] has speculated further on the function of polyphenols in relation to the suggested correlations between the concept of plant apparency and the nature of a plant's chemical defences. Based on findings with pedunculate oak (*Quercus robur*), he surmised that polyphenols (syn. vegetable tannins) are characteristic of the chemical defence of apparent plants. They act as quantitative, dosage-dependent barriers even to insects which normally feed on leaves containing them. The mode of action of polyphenols, he argued, arises from their general distastefulness to predators and from their ability to reduce the availability of food nitrogen by forming complexes with leaf protein. Feeny concluded that the adaptive emphasis in apparent plants, such as oak, is likely to favour retention of the most effective compounds for quantitative defence.

The undoubted attractiveness of these ideas has led to their wide acceptance but their effect has also been hypnotic. Thus despite the fairly well-defined roles proposed for polyphenols in plant defence (see above) much

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of the discussion concerning these substances has taken place in the virtual absence of any explicit physicochemical information relating to their ability to bind proteins—the fundamental property which underlies the proposals. Recent work concerning the pathways of metabolism of gallic acid in higher plants [11, 12] has opened the way for some facets of these hypotheses relating to polyphenols to be tested experimentally, *in vitro* if not *in vivo*. In particular, it permits a study of how the protein-complexing capacity of some natural polyphenols is related not only to their structure but also to their biosynthetic derivation and hence to the metabolic costs of their synthesis. Since, as Rhoades has commented [5], if these substances are the end-products of energy-demanding synthesis it is reasonable to assume that there has been positive selection for their production.

RESULTS AND DISCUSSION

A phylogenetic subdivision of plants (dicotyledons) is now possible on the basis of the products of gallic acid metabolism [11, 12]. In summary, some plants (Fig. 1, group 1) principally synthesize simple esters with D-glucose. For others, the formation of β-penta-O-galloyl-D-glucose (**1**) is, on the basis of circumstantial reasoning [13], a biosynthetic watershed and from this intermediate at least three broad distinctive pathways diverge (Fig. 1, groups 2A, 2B and 2C). One stream leads [13] to the gallotannins (e.g. **2**, 2A) by the acretion of further gallic acid molecules linked as depsides to **1**. Two further pathways (2B and 2C) diverge to ellagitannins [14, 15] by oxidative coupling of appropriately disposed galloyl ester groups in **1**. Oxidative coupling to give esters of hexahydroxydiphenic acid may occur (group 2B) via the thermodynamically preferred conformation of β-penta-O-galloyl-D-glucose (**1a**) or less commonly via the higher energy conformation (**1b**). Attention here is directed particularly towards those plants where the principal metabolites are derived from **1a**. One group of plants is able to bring about intramolecular oxidative coupling of the galloyl ester groups at positions 4 and 6 in **1a** to give the intermediate **3** from which the 'dimer' **5** is then derived. Other groups of plants are able to bridge both positions 4,6 and 2,3 oxidatively in **1a** to give the intermediate **8** from which the 'dimer' **6** is then derived by further intermolecular coupling. Plants of the genus *Quercus* alternatively metabolize from **8** unique open-chain glucose derivatives such as vescalagin (**7**) rather than form dimeric species characteristic of other plant groups. The leaf tissue of these various categories of plants has been examined in detail—all contain other low molecular-weight phenolic materials such as flavonoids—but quantitative studies at all stages of leaf growth show that the principal biosynthetic thrust is unchanging and is in-

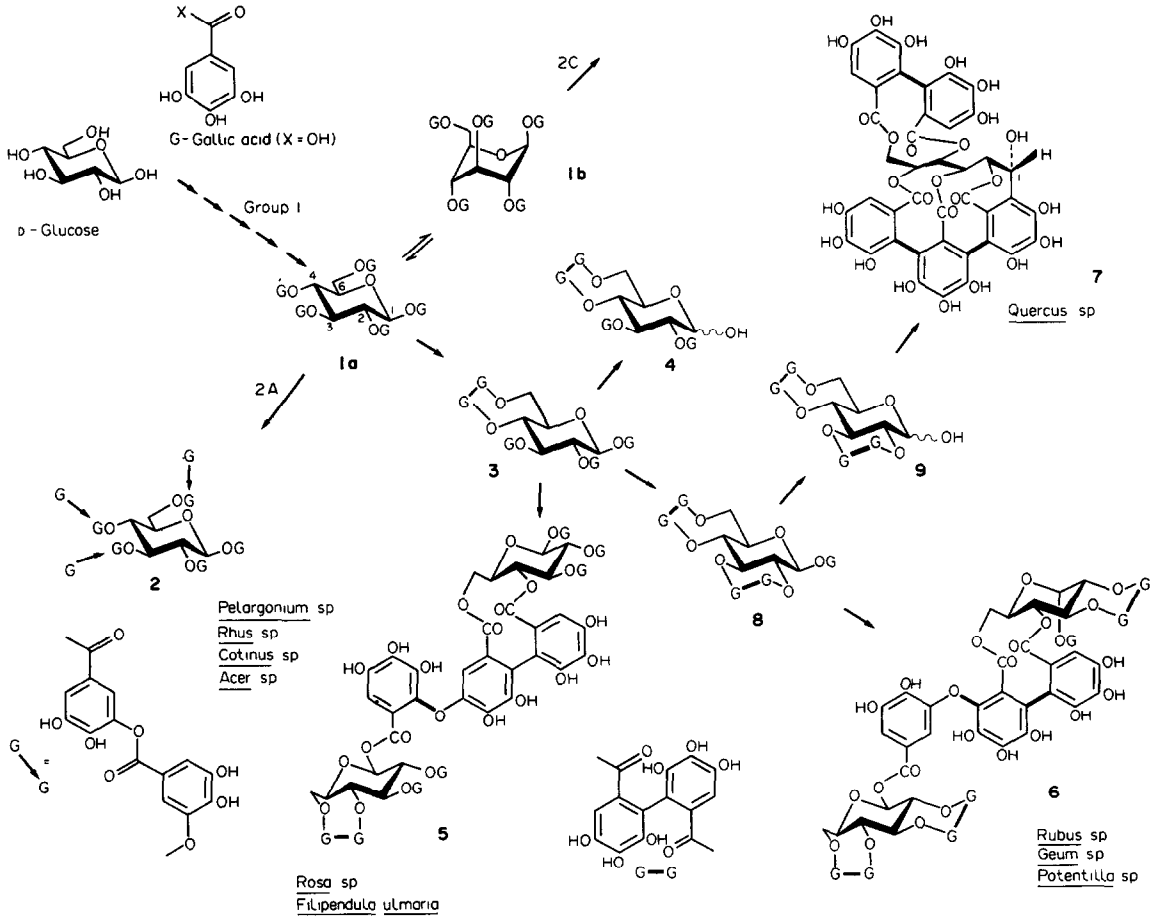

Fig 1. Gallic acid metabolism in higher plants.

variably towards the elaboration of the condensed and higher molecular-weight polyphenols—'end-products' of the biosynthetic pathways deriving from β-penta-O-galloyl-D-glucose (**1a**)—rather than the accumulation of intermediates such as **3**, **4**, **8** and **9**. Thus, for example, the following polyphenols overwhelmingly predominate in the leaf tissue of particular plants: **2**, MW ~ 1250, in *Rhus* sp., *Pelargonium* sp., *Cotinus* sp. and some *Acer* spp.; **5**, MW 1874, in *Rosa* sp. and *Filipendula ulmaria* (meadowsweet); **6**, MW 1870, in *Rubus* sp., *Potentilla* sp. and *Geum* sp.; **7** and its C-1 diastereoisomer, MW 934, in various *Quercus* sp. It must be assumed on this evidence that the astringency of these tissues is attributable, in very large part, to the individual polyphenols **2**, **5**, **6** and **7**. These molecules and their presumed biosynthetic precursor (**1**) have therefore been examined to determine the strength of their association with the protein bovine serum albumin (BSA) and to ascertain the validity of some of the ideas implicit in the theory of plant–herbivore co-evolution and the suggested role of polyphenols. For technical convenience, all measurements were carried out at pH 2.2. Although the overall binding of phenols to protein is weaker at this pH compared to physiological pH, the general trends are very similar. Experiments with simple phenols (with S. H. Gaffney) suggest that compared to higher pH values hydrophobic interactions probably assume a greater importance at this lower pH. Similarly, all measurements quoted have been made with the protein BSA. The work of Butler and Hagerman [16] has shown that both proline-rich and conformationally mobile proteins have high affinities for polyphenols and that such proteins may, on occasion, be preferentially precipitated in the presence of other proteins. However, there is no evidence from a range of experiments conducted with other proteins to indicate that the overall *relative patterns* of behaviour amongst the polyphenols **1**–**7** is changed in any way by variations of the pH or of the substrate protein. Quantitative experimental measurements of the binding of the various polyphenols to the protein BSA were obtained using equilibrium dialysis procedures (Dianorm apparatus, Teflon cells with cellulose acetate membranes 20–25 μm thickness, 6–8000 and 12–14 000 MW cut off [17]). For each system studied, the number of moles of polyphenol bound per mole of protein (r) was obtained as a function of the unbound polyphenol concentration (m_f). There are several methods of analysing the data obtained. The most common is that due to Scatchard [18] in which it is assumed that the protein has a fixed number (n) of independent binding sites, each of which has the same propensity to bind ligands. The data were treated in this manner and showed that, at least approximately, the number of sites was independent of the nature of the polyphenol ($n \approx 12$–14). However, in view of the fact that a presumed functional dependence of r and m_f is necessary for such analyses and since there is no evidence to presume that the independent site-binding model is entirely appropriate for the present systems, an alternative mode of analysis, which is model-independent, was also employed. This method leads to the evaluation of the free energy of transfer of the protein from an aqueous solution to an aqueous solution containing ligand ([19]; Lilley, T. H. and Tasker, I. R., unpublished observations) This quantity ($\Delta G^{\theta,tr}$) is obtained from the relationship:

$$\Delta G^{\theta,tr} = -RT\int_0^{m_f} r d\ln m_f$$

For a given polyphenol–protein system, $\Delta G^{\theta,tr}$ is obtained as a function of m_f by straightforward graphical integration. The free energy of transfer thus gives a useful, direct and quantitative measure of the nett interaction occurring between the two associating species—the more negative the value the greater is the attraction between them.

The results obtained by this procedure are shown in Fig. 2 and Table 1 shows values of the free energies of transfer for various polyphenols at three separate values of the free ligand concentration. The results show a clear ranking order, in terms of the ability of the polyphenols **1**–**7** to complex with the protein. The same general trends are evident in the analysis of the same data by the Scatchard procedure, although some minor differences are evident (Table 2). Micro-calorimetric studies of the complexation process were possible with the polyphenols shown in Table 2. These studies permitted the calculation of the molar enthalpy and molar entropy of transfer of the protein. Plots of $\Delta H^{\theta,tr}$ for each of the five polyphenols shown in Table 2 vs the calculated values of $T\Delta S^{\theta,tr}$ over a range of free ligand concentrations are linear with slopes approximating to 1. This is consistent with the comparatively small values of $\Delta G^{\theta,tr}$ observed and is indicative of enthalpy–entropy compensation in the process of ligand binding. It is an observation which substantiates the

Table 1. Free energies of transfer ($\Delta G^{\theta,tr}$, kJ/mol) of protein for integral values (8, 10, 12) of $-\ln m_f$ for polyphenolic ligands. Measurements at pH 2.2

	Polyphenol	Integral values $-\ln m_f$ 8	10	12
(i)	'Dimer' (**5**) (*ex Filipendula ulmaria*, *Rosa* sp.)	251.4	58.7	13.7
(ii)	β-Penta-*O*-galloyl-D-glucose (**1**)	106.2	26.9	6.8
(iii)	Tannic acid (**2**)	96.5	19.1	3.8
(iv)	β-1,2,3,-Tri-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**)	85.6	19 7	4.5
(v)	'Dimer (**6**) (*ex Rubus* sp.)	45.5	11.3	2.8
(vi)	β-1,2,3,6-Tetra-*O*-galloyl-D-glucose	38.4	9.1	2.2
(vii)	2,3-Di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (**4**)	15.4	2.8	0.5
(viii)	Vescalagin (**7**) (*ex Quercus* sp.)	5.5	1.0	0.18
(ix)	β-1,3,6-Tri-*O*-galloyl-D-glucose	6.3	0.92	0 13

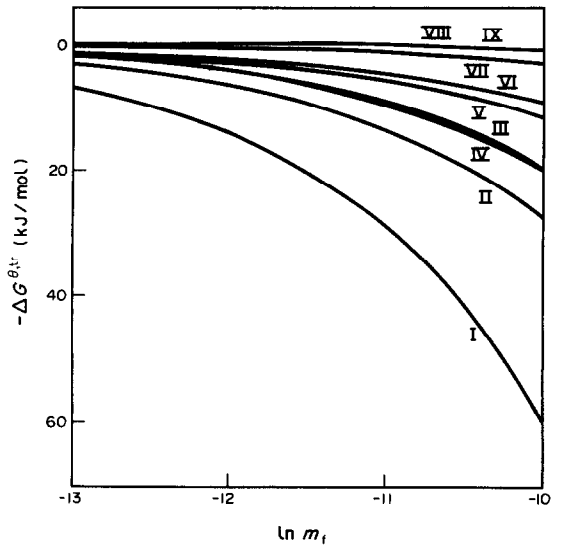

Fig. 2 Plots of free energies of transfer ($\Delta G^{\theta,tr}$, kJ/mol) of protein vs $-\ln m_f$ for various polyphenolic ligands. Notation as follows: (i) Dimer **5** (*ex Filipendula ulmaria, Rosa* sp.); (ii) β-penta-*O*-galloyl-D-glucose (**1**); (iii) tannic acid (**2**); (iv) β-1,2,3-tri-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**); (v) dimer **6** (*ex Rubus* sp.); (vi) β-1,2,3,6-tetra-*O*-galloyl-D-glucose; (vii) 2,3- di- *O*- galloyl -4,6(*S*) -hexahydroxydiphenoyl - D -glucose (**4**), (viii) vescalagin (**7**) (*ex Quercus* sp.); (ix) β-1,3,6-tri-*O*-galloyl-D-glucose.

earlier suggestion [20] that comparisons of protein–polyphenol interactions as between the same protein and different polyphenols may be made by a study of the variations in any one of these three thermodynamic functions.

The study of the interaction of proteins with polyphenols has a long history—one of the first scientific papers on this subject was that of Sir Humphry Davy in 1803 [21]. Early work such as this demonstrated some of the macroscopic features of the complexation and permitted several empirical definitions [22] of the term 'vegetable tannin' to be advanced. More recent studies [23–25] have delineated the principal means—hydrogen bonding, hydrophobic and ionic interactions—whereby polyphenols may associate with proteins. Analysis of the data presented here now enables some important 'structure–activity' correlations to be delineated. For the first time, some of the critical features of polyphenol structure essential for enhanced complexation with proteins are evident. Molecular size is important. Thus the 'dimer' **5**, MW 1874, is of the various metabolites of gallic acid encountered in higher plants, clearly the most efficient in its association with proteins. Similarly, the efficacy of binding to BSA increases incrementally in the galloyl-D-glucose series with the addition of each phenolic ester group (tri → tetra → penta). It reaches a maximum in the mobile flexible disc-like structure of β-penta-*O*-galloyl-D-glucose (**1**). However, addition of further depsidically linked galloyl ester groups to **1** as in the gallotannins (2A, **2**, syn. tannic acid), although it increases molecular size (MW 940 → ~ 1250, hepta-octagalloyl-D-glucose) does *not* lead to an increase in protein-binding capacity. Quite clearly the increase in molecular weight is not associated with a concomitant increase in the number of groups (most probably phenolic) on the periphery of the polyphenol molecule available to complex with the protein.

Just as significant as molecular size, however, is conformational mobility and flexibility in the polyphenol substrate. This is succinctly demonstrated by the various galloyl ester metabolites of group 2B (Fig. 1) whose biosynthesis proceeds by the progressive oxidative coupling of vicinal galloyl ester groups in the precursor (**1**). Thus as galloyl ester groups in **1** are constrained by the formation of intramolecular biphenyl linkages (e.g. **3**, **4**, **6** and **7**), then the reduced conformational flexibility is directly reflected in a reduced capacity to bind to BSA. The apotheosis of this effect is seen in the case of vescalagin (**7**) and its C-1 diastereoisomer castalagin, the principal polyphenols of oak leaves and galls. These unique propeller-shaped open-chain glucose derivatives are virtually inflexible. They are, in a sense, analogues of β-penta-*O*-galloyl-D-glucose (**1**) but on a molar basis they are bound substantially less effectively to BSA than **1**. In this context it is worth noting that the comparatively lower astringency, on a molar basis, of the proanthocyanidins compared to simple esters of glucose and gallic acid may well be explicable, in part, in terms of similar conformational restraints imposed by restricted rotation about the repeating inter-flavan bonds [26]. Collectively these results therefore fully complement those of Hagerman and Butler [16]; complementarity between the polydentate ligand (polyphenol) and receptor (protein) is very clearly maximized by conformational flexibility in both components.

The same patterns of results have also derived from two related investigations. Polyphenols act as inhibitors of many enzymes. A study of the effects of polyphenols **1**–**7** upon β-glucosidase activity (with I. Dring and K. Elmer) showed that the kinetics are complex but most satisfactorily analysed as mixed competitive (active-site binding)

Table 2. Molar enthalpy, free energy and entropy for the association of polyphenols with bovine serum albumin. Measurements at pH 2.2

Polyphenol	ΔH^θ (kJ/mol)	ΔG^θ (kJ/mol)	ΔS^θ JK/mol)
(i) β-Penta-*O*-galloyl-D-glucose (**1**)	−33.4	−25.2	−27.5
(ii) β-1,2,3-Trigalloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (**3**)	−67.6	−22.5	−151.3
(iii) β-1,2,3,6-Tetragalloyl-D-glucose	−65.6	−22.0	−146.3
(iv) 'Dimer' (**6**) (*ex Rubus* sp.)	−53.7	−22.2	−105.7
(v) 2,3-Di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-D-glucose (**4**)	−29.2	−15.0	−47.7

and non-competitive (binding at points remote from the active site) inhibition. However, the patterns of enzyme inhibition by polyphenols 1–7 are closely related to their capacity to bind to protein (see above). Polyphenols are also active against the schistosomiasis-transmitting snail *Biomphalaria glabrata*, intermediate host of *Schistosoma mansoni*. In a continuing study (with Professor K. Hostettman, University of Lausanne), various polyphenols (including 1–7) have been tested for their molluscicidal activity. Comparison of the threshold polyphenol concentration (ppm) to achieve LD_{100} (24 hr) shows a remarkably similar trend amongst polyphenols 1–7 to that noted (see above) for protein binding, such as to suggest that association with protein may be an important factor in the molluscicidal action of the polyphenols.

These results quite clearly add substantially to an understanding of the mechanism of association of polyphenols with proteins. They also now permit some comments to be made on the theory of plant–herbivore co-evolution.

The experimental data presented shows that there is a very wide variability in the protein-complexing capabilities of the polyphenolic biosynthetic 'end-products' (2–7). There is no consistent pattern nor is any correlation discernible between the metabolic cost to the plant of its synthesis of a particular polyphenol and the subsequent capacity of that polyphenol to bind to protein (astringency, etc.). Frequently, although not invariably, the ability of polyphenols **2**, **3**, **4**, **6**, **7** and **8** is diminished when compared to that of the central biosynthetic intermediate, β-penta-*O*-galloyl-D-glucose (**1**) from which they are derived. Particular note in this context may be taken of *Rubus* sp. and *Quercus* sp. where respectively **6** and **7** (and its C-1 diastereoisomer) are the principal polyphenols. In such cases the plant's subsequent oxidative chemical manipulation of the intermediate β-penta-*O*-galloyl-D-glucose (**1**) is quite clearly counterproductive *vis à vis* the synthesis of more astringent metabolites. If, as Rhoades has suggested [5], there has been positive selection for the production of such metabolites then this does not appear to be based solely on the capacity of these polyphenols to bind to protein.

Polyphenols constitute a group of natural products of very great structural diversity and wide phylogenetic distribution [22]. The evidence outlined suggests that although the retention of polyphenolic synthesis may confer an advantage, a secondary benefit, on the plant and may well be the basis of selective pressures, it does not appear to support the proposition that the purpose of polyphenol metabolism is to generate agents specifically for the plant's defence. In this context it is interesting to note that in a recent detailed comprehensive study of herbivory in a lowland tropical forest, Coley [27] observed that for mature leaves phenol measures are the least well correlated with herbivory and she suggested that the importance of phenolic content as a defence in earlier work may have been overemphasized. Whilst it may well be argued that polyphenol complexation with protein is not, as has been assumed, the critical property of polyphenols operative in plant defence, the ubiquity of polyphenols and the bewildering variety of their molecular forms may alternatively, and perhaps more reasonably, be viewed on the basis of present evidence, as Bu'Lock [2] has perceptively remarked in the analogous context of microbial secondary metabolism, "... as a result of there being very little selection pressure on their identity, i.e. the products bring no great advantage or disadvantage *per se*." Bu'Lock has speculated indeed that microbial secondary metabolism serves to maintain basic metabolism in circumstances not propitious (e.g. nutritional imbalances, etc.) for growth and replication. Whether the same is true in plants is not clear but the evidence presented above suggests that to simplify and reduce possible explanations of the origins and role of secondary metabolism to just one—based on the concept of co-evolution—is not at this stage justified.

EXPERIMENTAL

Polyphenols. Substrates were prepared by previously described procedures and usually involved final purification by chromatography on Sephadex LH-20. Tannic acid (**2**, Chinese gallotannin) was obtained from Chinese galls (*Rhus semialata*) [28]; β-penta-*O*-galloyl-D-glucose (**1**) was derived by methanolysis of tannic acid [28]; β-1,2,3,6-tetra-*O*-galloyl-D-glucose was obtained by methanolysis of the phenolic extract from Turkish galls (*Quercus infectoria*) (Haslam, E. H., unpublished results); β-1,3,6-tri-*O*-galloyl-D-glucose was isolated after hydrolysis of chebulinic acid [29]; β-1,2,3-tri-*O*-galloyl-4,6-[*S*]-hexahydroxydiphenoyl-D-glucose (**3**) and 2,3-bis-*O*-galloyl-4,6-[*S*]-hexahydroxydiphenoyl-D-glucose (**4**) were obtained from *Quercus infectoria* galls or meadowsweet (*Filipendula ulmaria*) [14, 15]; the dimer **5** was obtained from meadowsweet (Haslam, E. H., unpublished results); the dimer **6** was isolated from *Rubus* sp. (blackberry and raspberry) [14, 15], and vescalagin and castalagin (**7**) from oak galls (*Quercus robur*) ([30]; Haslam, E. H., unpublished results)

Methodology. (i) *Equilibrium dialysis.* The Dianorm equilibrium dialysis system was employed and consisted of 20 Teflon cells each with a half-cell volume of 5 ml [31]. Membranes were made from cellulose acetate Spectrapor tubing, 20–25 μm thickness, 6–8000 and 12–14 000 cut-off. Membranes were washed and equilibrated with the buffer soln (pH 2.20, Cl^- in conductance H_2O) before use. Cells were assembled by making a sandwich with two half-cells and a membrane and filled—one half with protein soln (3.5 ml), the other half with phenol soln (3 5 ml) using an Excalibur dispensing pipette. Control dialyses were carried out in which phenol was equilibrated with buffer to determine the adsorption of phenol by the membrane Cells were rotated in the carrier at 10 rpm, 298 K for 48 hr and the phenol content of each half-cell determined spectrophotometrically. The mathematical treatment and analysis of the data will be discussed elsewhere.

(ii) *Microcalorimetry.* Microcalorimetric experiments were carried out in buffer solns (pH 2 20, Cl^- in conductance H_2O) using an LKB 2107-010 Batch Microcalorimeter.

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REFERENCES

1. Bu'Lock, J. D. (1965) *The Biosynthesis of Natural Products*, p. 1. McGraw-Hill, London.
2. Bu'Lock, J. D. (1980) in *The Biosynthesis of Mycotoxins* (Steyn, P. S., ed.), pp. 1–16. Academic Press, London
3. Fraenkel, G. S. (1980) *Science* **129**, 1466.
4. Ehrlich, P. R. and Raven, P. H. (1965) *Evolution* **18**, 586.
5. Rhoades, D. F. (1979) in *Herbivores—Their Interactions with Secondary Plant Metabolites* (Rosenthal, G. A. and Janzen, D. H., eds.), pp. 3–54. Academic Press, London

6. Janzen, D H. (1979) in *Herbivores—Their Interactions with Secondary Metabolites* (Rosenthal, G. A. and Janzen, D. H., eds.), pp. 331–350. Academic Press, London.
7. Janzen, D. H. (1969) *Science* **164**, 415.
8. Harborne, J. B. (1977) *Introduction to Ecological Biochemistry*, p. 2. Academic Press, London.
9. Bate-Smith, E. C. (1973) *Phytochemistry* **12**, 907.
10. Feeny, P. P. (1976) *Rec. Adv. Phytochem.* **10**, 1.
11. Haslam, E. (1982) *Fortschr. Chem. Org. Naturstoffe* **41**, 1.
12. Haddock, E. A., Gupta, R. K., Al-Shafi, S. M. K., Layden, K., Magnolato, D. and Haslam, E. (1982) *Phytochemistry* **21**, 1049.
13 Haddock, E. A., Gupta, R K., Al-Shafi, S. M. K., Magnolato, D. and Haslam, E. (1982) *J. Chem. Soc. Perkin Trans.* 1, 2515.
14. Gupta, R. K., Al-Shafi, S. M. K., Layden, K. and Haslam, E. (1982) *J. Chem. Soc. Perkin Trans.* 1, 2525.
15. Haddock, E. A., Gupta, R. K. and Haslam, E. (1982) *J. Chem. Soc. Perkin Trans.* 1, 2535.
16. Hagerman, A. E. and Butler, L. G. (1981) *J Biol Chem.* **256**, 4494.
17. McManus, J. P. (1980) Ph.D. Thesis, University of Sheffield.
18. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660.
19. Schellman, J. A. (1975) *Biopolymers* **14**, 999.
20. McManus, J. P., Davis, K. G., Lilley, T. H. and Haslam, E. (1981) *J. Chem. Soc. Chem. Commun.* 309.
21. Davy Sir Humphry, (1803) *Philos. Trans.* in Nierenstein, M. (1932) *Incunnabula of Tannin Chemistry*, pp. 116–167. Edward Arnold, London.
22. Haslam, E. (1981) in *The Biochemistry of Plants* (Conn, E. E., ed.), Vol. 7, pp. 527–554. Academic Press, London.
23. van Buren, J. P. and Robinson, W. B. (1969) *J. Agric. Food Chem.* **17**, 772.
24. Loomis, W. D. (1974) *Methods Enzymol.* **31**, 528.
25. Oh, H. I., Hoff, J. E., Armstrong, G. S. and Haff, L. A. (1980) *J. Agric. Food Chem.* **28**, 394.
26. Haslam, E. (1977) *Phytochemistry* **16**, 1625.
27. Coley, D. (1983) *Ecol. Monogr.* **53**, 209.
28. Armitage, R., Bayliss, G. S., Gramshaw, J. G., Haslam, E., Haworth, R. D., Jones, K., Rogers, H. J. and Searle, T. (1961) *J. Chem. Soc.* 1842.
29. Schmidt, O. Th., Demmler, K., Bitterman, H. and Stephan, P. (1957) *Liebig's Annalen* **609**, 192.
30. Mayer, W., Bilzer, B. and Schauerte, K. (1971) *Liebig's Annalen* **754**, 149.
31. Weber, H. G., Schildknecht, H. and Kesselring, P. (1971) *Am. Lab.* **3**, 15.