Polyphenol Interactions. Part 1. Introduction; Some Observations on the Reversible Complexation of Polyphenols with Proteins and Polysaccharides

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Early studies of the interactions between polyphenols and proteins are reviewed. The complexation of some simple phenols and a group of biosynthetically inter-related esters of gallic acid with bovine serum albumin (BSA) is examined by equilibrium dialysis and microcalorimetry. The phenomenon is pH dependent. The results indicate that molecular size and conformational flexibility of the polyphenol substrate lead to enhanced interactions with the protein. Preliminary studies with polysaccharides indicate that the binding here is pH independent. These studies suggest that whilst the binding of polyphenols to these macromolecules is influenced by similar structural features the ability of the polysaccharide to form structures which encapsulate the polyphenol is, in this instance, a further critical feature of the complexation.

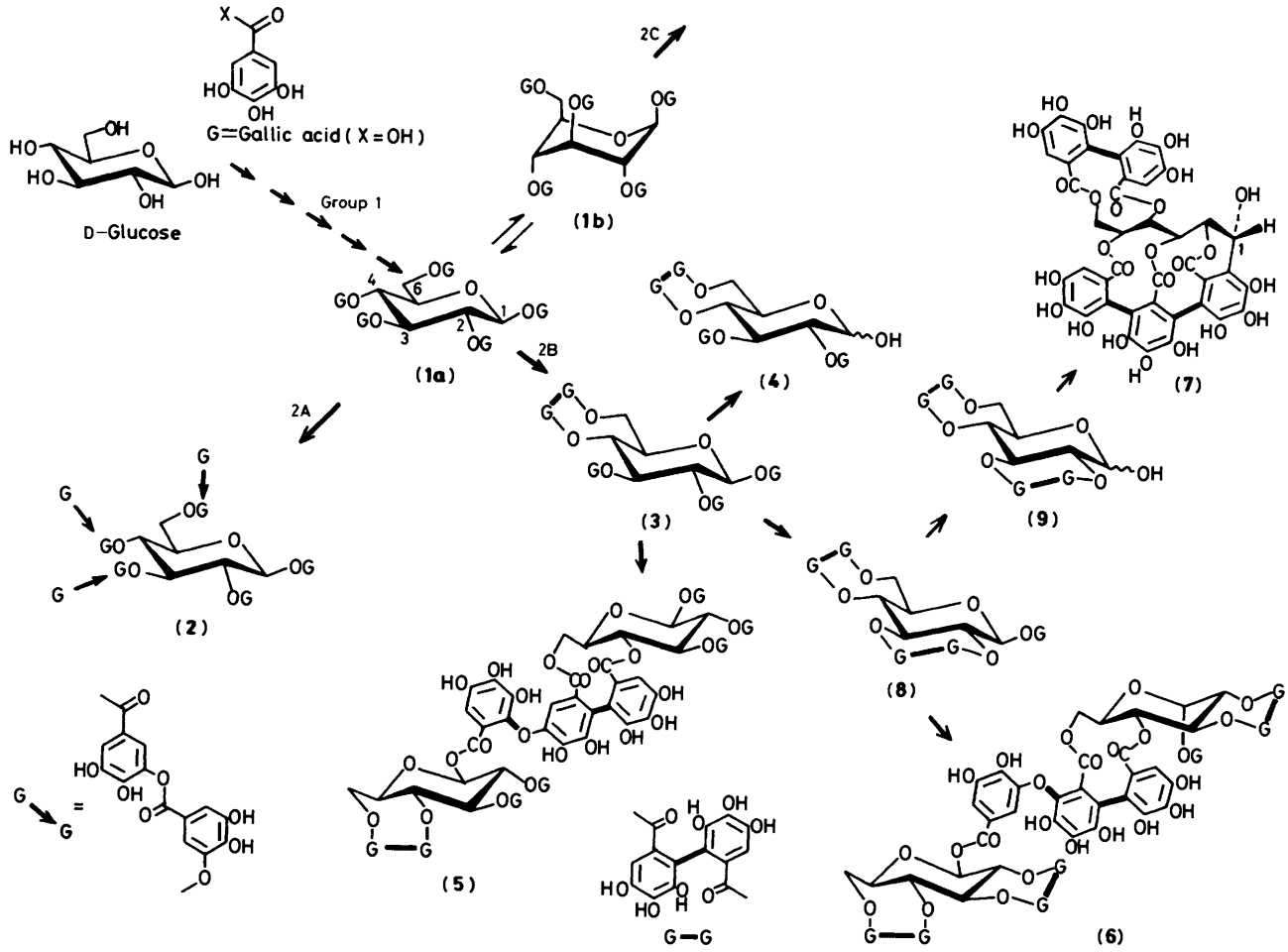
As organic chemistry has advanced it has maintained a consistent orientation towards the solution of important biological problems.[1] Paradoxically, one such problem which still remains unresolved is of particular interest to organic chemists. The roots of the subject lie firmly in the province of 'natural products,' alkaloids, terpenes, phenols, polyacetylenes, mycotoxins, pigments, *etc.*, and increasingly the question is asked: why do plants and micro-organisms synthesise these substances? Over the past 30 years this perplexing problem has stimulated conjecture and controversy; numerous quite speculative hypotheses have been advanced.[2–10] Intuitively perhaps, many biologists believe that products of metabolism are more likely, rather than less likely, to be usefully concerned with the life processes of an organism. However, the absence of factual evidence has generally inhibited approaches to this problem. Thus for instance information, *at the enzymic level*, which relates to the biosynthesis of many 'natural products' would permit an evaluation of the early, quite contrary, assertion that they are simply waste products of metabolism, vestiges of evolution, that may have no function whatsoever.

Polyphenols with molecular weights from 500 to 20,000 and beyond (vegetable tannins of the earlier chemical and botanical literature[11]) constitute a distinctive group of phytochemicals. They possess a great structural diversity, wide phylogenetic distribution and, according to Czapek's original definition,[12] are typical secondary plant products. The uniqueness of this group of secondary metabolites lies not only in their polyphenolic character and the substantial molecular weight range which they encompass but also in their ability to associate strongly with primary metabolites such as proteins and carbohydrates.[11] It was envisaged that a comprehensive study of these complexation phenomena might throw some light on possible roles of these compounds in higher plant metabolism and upon some of the hypotheses which have been advocated. Such studies are also of great practical utility since these interactions often substantially influence the properties of many plant products ranging from the taste, palatability,[13] and nutritional value of foodstuffs[14] to the microbial decomposition of vegetable matter in the formation of soils.[15,16] Yet again the peculiar geographical distribution of aesophogal cancer worldwide has been correlated[17] with the use of plant decoctions, as beverages and folk remedies, from plants with high concentrations of polyphenols. Particular attention in this respect has been give to 'teas' and their action on the mucous membrane of the mouth and alimentary tract. These and related observations point increasingly to the need for a much better fundamental understanding, at the molecular level, of the interaction between polyphenols and proteins, polysaccharides, and various nitrogenous metabolites.

The interaction between polyphenols and proteins may be either reversible or irreversible. Studies of the reversible association of polyphenols with proteins have a long history;[18] one of the first scientific papers on this subject is that of Sir Humphry Davy in 1803. Early work such as this demonstrated some of the macroscopic features of the complexation and enabled several empirical definitions of the term 'vegetable tannin' to be advanced.[18–20] Loomis[21] has succinctly summarised the main conclusions of this earlier work. The principal means whereby proteins and polyphenols are thought to reversibly complex with one another are *via* (i) hydrogen bonding,[22–23] (ii) hydrophobic interactions,[24] and (iii) ionic interactions. As, however, the chemical nature of plant polyphenols has been revealed it has become clear that ionic interactions play little, if any, part in the association. On the other hand, the relative significance of hydrogen bonding and hydrophobic interactions in the complexation is still uncertain. Whilst the tendency has been to emphasise the part played by intermolecular hydrogen bonding,[22,23] Hoff has drawn attention to the fact that hydrophobic effects may dominate the interaction.[24] However, until such time as structurally defined plant polyphenols became available, quantitative investigations were not feasible and as a physical phenomenon it still therefore remains poorly understood.

Results and Discussion

Recent studies[25,26] of gallic acid metabolism in higher plants has opened the way for a biosynthetically related study of the structure–activity relationships in the association of polyphenols with proteins, polysaccharides, and various alkaloids. The products of gallic acid metabolism permit a phylogenetic subdivision of plants (dicotyledons); they also provide a series of polyphenols of defined molecular shape and size. Some plants (Scheme, group 1) synthesise simple esters with D-glucose.[27] For others the elaboration of penta-*O*-galloyl-β-D-glucose (**1**) is, on the basis of circumstantial reasoning and preliminary biosynthetic experimentation, a metabolic watershed from which at least three broad distinctive pathways diverge,[28] (Scheme, groups 2A—C). The gallotannins [*e.g.* (**2**), group 2A] are formed by the esterification as *m*-depsides of additional

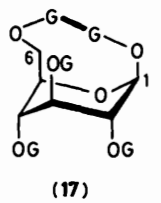


Scheme. Gallic acid metabolism in higher plants

gallic acid molecules to (**1**). These metabolites, like (**1**), adopt flexible disc-like conformations in which the galloyl ester groups are displayed on the periphery of the molecular surface. Two further pathways (Scheme, groups 2B and C) diverge to substances collectively designated as ellagitannins[28] by oxidative coupling of appropriately orientated galloyl ester groups in the intermediate (**1a**, **b**). Oxidative coupling to give esters of hexahydroxydiphenic acid may occur *via* (group 2B) the thermodynamically preferred $^4C_1$ conformation of penta-*O*-galloyl-β-D-glucose (**1a**) or less frequently *via* the higher energy $^1C_4$ conformation [(**1b**), group 2C]. In either case intramolecular covalent bonding of this nature produces, from (**1**), more compact and less conformationally mobile polyphenolic metabolites. Quantitative studies show that in leaf tissue the major biosynthetic thrust is invariably towards the synthesis of the condensed higher molecular weight polyphenols. It may therefore be assumed that the 'astringency' of such tissues is, in large part, attributable to the polyphenols indicated [Scheme, (**2**), (**5**)—(**7**).

Quantitative measurements of the binding of various phenols and polyphenols to the protein bovine serum albumin (BSA) were obtained by equilibrium dialysis.[29] Initially the interaction between BSA and four simple phenols, resorcinol, catechol, pyrogallol, and methyl gallate (**10**)—(**13**), was examined. For each system the number of moles of phenol bound per mole of protein (r) were obtained as a function of the unbound phenol concentration (m_f). The Scatchard analysis[30] was used to treat the data, plotting r/m_f *versus* r. The binding of resorcinol to BSA at pH 6.8 is much weaker than that for the other three phenols and over the range studied the data fit the one-site-binding model. Curvature of the remaining plots suggested that a two-site-binding model might be more appropriate. The analysis[31] gave values for the number of primary (n_1) and secondary (n_2) binding sites and the corresponding equilibrium constants K_1 and K_2. However, the intrinsic experimental problems, particularly adventitious phenol oxidation, encountered when operating at or near pH 7.0 finally led to the adoption of pH 2.2 for all subsequent measurements. Although the overall binding of phenols to protein at this pH is reduced the general trends are similar to those observed earlier[31] at pH 6.8. Experiments with the simple phenols (**10**)—(**13**) showed that the results were amenable to single-site-binding analysis.[30] Compared with the data obtained at pH 6.8 these data suggest that at more acid pH values hydrophobic interactions probably assume a greater significance.

The Scatchard analysis[30] assumes that the protein has a fixed number of independent binding sites each of which has the same propensity to bind ligands. The validity of this assumption when dealing with polyphenols is questionable and an alternative method of analysis was also employed. This leads to the evaluation of the free-energy of transfer of the protein from an aqueous solution to an aqueous solution containing ligand.[32] This quantity, $\Delta G^{\ominus,tr}$, is obtained from relationship (1). A discussion of the physical meaning of $\Delta G^{\ominus,tr}$ and its derivation is given in Appendices A and B. For a given phenol-



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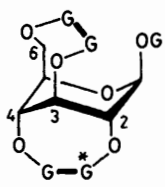
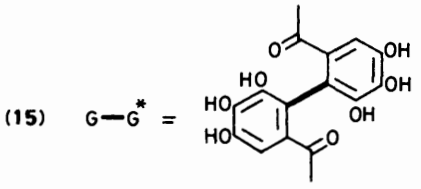
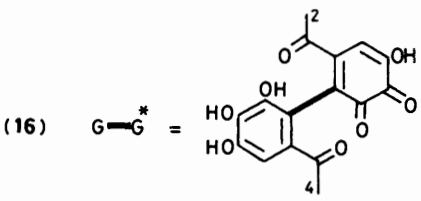
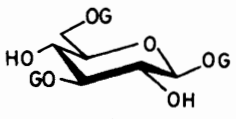




(15) G—G* =



(16) G—G* =



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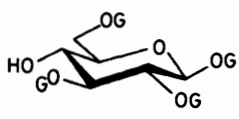

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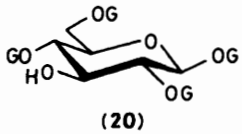

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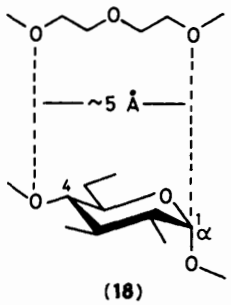

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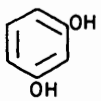

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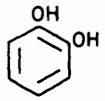

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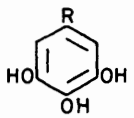

(12) R = H

(13) R = CO_2Me

$$\Delta G^{\ominus,tr} = -RT \int_0^{m_f} r \, d\ln m_f \qquad (1)$$

protein system, $\Delta G^{\ominus,tr}$ is obtained as a function of m_f by graphical integration. The free-energy of transfer thus gives a useful, direct, model-independent, and quantitative measure of the net interaction occurring between two associating species; the more negative the value the greater is the attraction between them. The dialysis experimental data for the simple phenols (**10**)—(**13**) treated in this way give the results shown in Table 1 and Figure 1. In general this procedure is preferred for the purposes of this work since it provides more consistent and reliable data.

Table 1. Association of phenols with bovine serum albumin (BSA): $\Delta G^{\ominus,tr}$ values; pH 2.2 and 6.5, 298 K, $-\ln m_f = 6.5$

Phenol	$-10^3 \Delta G^{\ominus,tr}$/kJ mol^{-1} pH 2.2	pH 6.0
1,2-Dihydroxybenzene (catechol)	3.21	15.63
1,3-Dihydroxybenzene (resorcinol)	3.15	10.88
1,2,3-Trihydroxybenzene (pyrogallol)	2.08	85.46

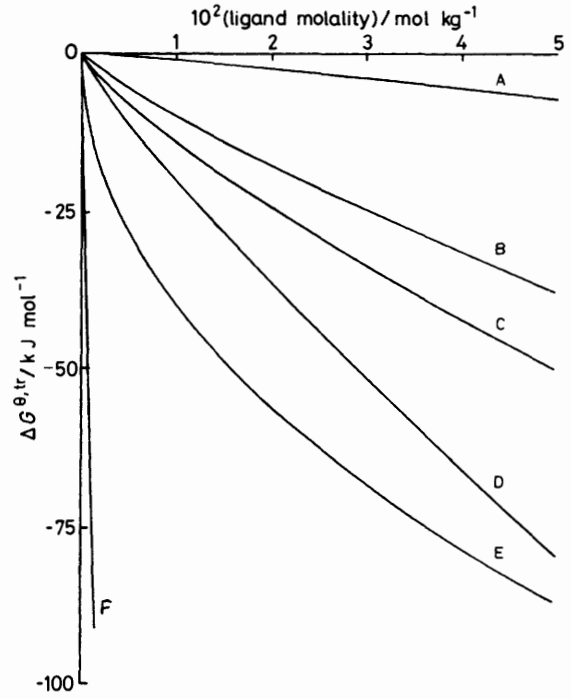

Figure 1. $\Delta G^{\ominus,tr}$ for simple phenol–BSA interactions: A (resorcinol, pH 6.5); B (pyrogallol, pH 2.2); C (resorcinol, pH 2.2); D (catechol, pH 2.2); E (catechol, pH 6.5); F (pyrogallol, pH 6.5)

Using this procedure the interactions of various polyphenolic metabolites (**1**)—(**9**) derived from gallic acid were examined. All the detailed measurements have been made with the protein bovine serum albumin (BSA). Although the work of Butler and Hagerman[33] has shown that both proline-rich and conformationally mobile proteins have high affinities for polyphenols and that on occasion such proteins may be preferentially precipitated in the presence of other proteins, there is no evidence, from a limited range of other experiments, to suggest that the *comparative* patterns of behaviour amongst the polyphenols examined is changed by variations of either pH or of the substrate protein. Data were analysed initially by the single-site-binding model enabling approximate values of n and K (the intrinsic association constant) to the obtained (Table 2). From the association constants K it is possible to discern a clear ranking order of affinities of the various polyphenols for the protein BSA. The molar free-energy of transfer changes, $\Delta G^{\ominus,tr}$, were also calculated for all nine systems over a range of free ligand concentrations and a diagrammatic comparison is shown in Figure 2. Molar enthalpies of transfer $\Delta H^{\ominus,tr}$ for four polyphenols were obtained from microcalorimetric measurements, and entropies of transfer ($\Delta S^{\ominus,tr}$) were determined using the $\Delta H^{\ominus,tr}$ and $\Delta G^{\ominus,tr}$ data. Plotting $\Delta H^{\ominus,tr}$ against $T\Delta S^{\ominus,tr}$ for these substrates gave a linear plot whose slope approximated

Table 2. Association of polyphenols with bovine serum albumin (BSA): Scatchard analysis and $\Delta G^{\circ,tr}$ values: pH 2.2, 298 K

Polyphenol	$10^3 K^a$/kg mol^{-1} [a]	$-\Delta G^{\circ,tr}$ [b]	$-\Delta H^{\circ,tr}$ [b]	$T\Delta S^{\circ,tr}$ [b]
(1)	25.8	26.9	246.3	219.4
(19)	6.77	9.1	146.7	137.6
(14)	0.40	0.9		
(3)	8.76	19.7		
(4)	0.43	2.8	18.2	15.5
(6) from *Rubus* sp.	7.77	11.3	163.3	152.0
(5) from *Filipendula ulmaria* and *Rosa* sp.	94.1	58.7		
(7)	0.87	1.0		
(2)	34.0	19.1		

[a] Determined by Scatchard analysis.[30,31] [b] Values of $\Delta G^{\circ,tr}$, $\Delta H^{\circ,tr}$, and $T\Delta S^{\circ,tr}$ all in kJ mol^{-1}: obtained at value of $-\ln m_f$ of 10.0 (m_f = 4.5 × 10^{-5} mol kg^{-1})

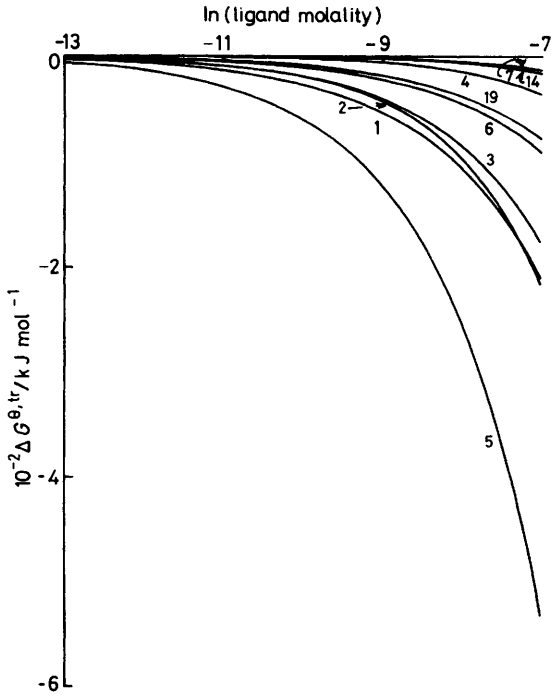

Figure 2. $\Delta G^{\circ,tr}$ for polyphenol–BSA interactions

to unity. This is indicative of enthalpy–entropy compensation[34] and accounts for the relatively small value of the free-energy changes, $\Delta G^{\circ,tr}$, which are observed (Table 2). This proportionality indicates that the trends in the magnitude of protein–polyphenol interactions as the structure of the polyphenol is varied may, with caution, be examined by reference to any one of these three thermodynamic functions.

Over the past 250 years there have been several attempts to define the chemical nature of a 'vegetable tannin.'[11,18] Of these that of Bate-Smith and Swain in 1962 has much to commend it.[19] These plant products were defined as 'water soluble phenolic compounds having molecular weights between 500 and 3 000 and, besides giving the usual phenolic reactions they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.' This empirical definition was broadly adopted from earlier ideas of the mechanism of vegetable tannage of hides and skins. Thus polyphenols which are effective in this respect must be able to associate with protein to form stable cross-linked structures but also of the appropriate molecular size to penetrate the inter-fibrillar regions of the collagen structure. The present results now permit some of the critical facets of polyphenol structure necessary for enhanced complexation with proteins in solution to be delineated for the first time; the analysis here is based on the comparison of $\Delta G^{\circ,tr}$ values.

Molecular size is important. Thus the efficacy of binding to BSA increases exponentially in the galloyl-D-glucose series with the addition of each galloyl ester group (tri ⟶ tetra ⟶ penta, Table 2.) It reaches a maximum in the flexible disc-like structure of penta-*O*-galloyl-β-D-glucose (**1**) and suggests that with molecules such as (**1**) there is a co-operativity in the binding of phenolic groups on the substrate to the protein surface. Addition of further depsidically linked galloyl ester groups to (**1**), as in the gallotannins [group 2A, (**2**)] although it increases the molecular weight of the polyphenol (940 ⟶ *ca.* 1 250, hepta–octagalloyl-D-glucose) does not lead to an increase in protein-binding capacity. Presumably in this instance the increase in molecular size and weight compared with (**1**) does not bring with it a concomitant increase in the number of groups on the surface of the molecule available to bond with the protein. These observations confirm earlier empirical studies[23] with β-glucosidase that in the simple galloyl-D-glucose series penta-*O*-galloyl-β-D-glucose (**1**) represents the optimum size and configuration for complexation with proteins. The importance of molecular size is, however, seen once again in the 'dimeric' species [(**5**), molecular weight 1 874] which, of the various metabolites of gallic acid encountered to date in higher plants, has proved to be the most efficient in its complexation with protein.

Equally significant as molecular size, however, is conformational flexibility and mobility in the polyphenolic substrate. This is demonstrated quite clearly amongst the galloyl ester metabolites of group 2B (Scheme) whose biosynthesis proceeds by intramolecular oxidative coupling of neighbouring (4,6 and 2,3) galloyl ester groups in the precursor (**1**). Thus as galloyl ester groups in (**1**) are constrained by oxidative coupling and the formation of intramolecular biphenyl linkages [*e.g.* (**3**), (**4**), (**6**), and (**7**)] then the reduced conformational flexibility of the polyphenolic substrate is reflected in a reduced capacity to bind to BSA (Table 2). The apotheosis of this effect is seen in the case of the unique open-chain D-glucose derivatives vescalagin (**7**) and its diastereoisomer (epimeric at C-1) castalagin, metabolites of *Quercus* sp.[28,35] These rigid propeller-shaped molecules are in a sense analogues of penta-*O*-galloyl-β-D-glucose (**1**) but on a molar basis they are bound some 30 times less effectively to BSA than (**1**). It is worthy of note that in the galloyl-D-glucose series, where comparisons are possible, the introduction of one biphenyl linkage into both penta- and tetra-*O*-galloyl-D-glucose reduces the molar free energy changes by *ca.* 7 kJ mol^{-1}. The effect is rather more dramatic in the larger polyphenol substrates. Thus, although there is a subtle difference in the nature

of the linkage between the two galloyl glucose units in (**5**) and (**6**), the major difference is the presence of two additional biphenyl linkages in the *Rubus* dimer (**6**). The free-energy changes for the two dimers differ by *ca.* 48 kJ mol^{-1}, *i.e.* 24 kJ mol^{-1} for each of the additional biphenyl linkages. In this context the comparatively lower astringency of the proanthocyanidins[20,36] may be explicable, in part, in terms of the conformational restraints imposed by restricted rotation about the repeating interflavan bonds.[37] Collectively these results fully complement those of Hagerman and Butler;[33] complementarity between the polydentate ligand (polyphenol) and receptor (protein) is maximised by conformational flexibility in both components. It is also of interest to note that these results lend no succour to one theory of the role of secondary metabolism much canvassed in recent years and based on the concept of plant–animal co-evolution.[5,6] This particular observation has been discussed elsewhere.[38]

There is conflicting evidence concerning the pH at which maximum interaction occurs between polyphenols and proteins.[39,40] Van Buren and Robinson[39] reported that for tannin acid (**2**)–gelatin complexes this occurs at or near the isoelectric point whilst other studies of the same system[40] showed an increase in the *number* of phenol molecules bound, as the pH falls from 4.8 to 3.5, but with a concomitant decrease in the strength of binding. The extent of interaction of (**2**) with BSA has been monitored by microcalorimetry over the pH range 2.4—6.8. Maximal interaction (as indicated by the enthalpy) occurred at *ca.* pH 4.0 where the N ⟶ F transition of BSA occurs.[41] This change corresponds to the separation of domains in the protein and hence presumably to an increase in the number of possible binding sites. A strong dependence on protein concentration for the binding of polyphenols has also been demonstrated in this study for both BSA and lysozyme. All the available evidence suggests that, in agreement with earlier observations,[11,20,39] polyphenols are multidentate ligands and at high protein concentrations can cross-link neighbouring protein molecules, rather than simply complexing with one, thus giving rise to a variable ligand–protein ratio in the precipitates.[31]

These observations derived from studies of the precipitation of BSA in the form of its complex with (**2**). The precipitation was dependent on pH (pH 4.0 maximum with BSA) and the stoicheiometry of the protein–polyphenol complex (determined by a turbidimetric titration procedure and by microanalytical measurements on the precipitates) showed a clear dependence on the initial protein concentration. At low protein concentrations (10^{-6} molal BSA) the stoicheiometry of the protein–polyphenol complex was *ca.* 1:12C but at higher protein concentrations (10^{-5} molal BSA) the composition was *ca.* 1:60. Precipitates left to stand in the presence of polyphenol solutions sequestered further polyphenol and this same propensity may also be inferred from the data of Van Buren and Robinson.[39] The precipitation of protein by polyphenols may be reversed by the addition of further protein solution and the complex, once formed, may also be dissociated by treatment with acetone without denaturation of the protein.[42]

The reversible association of polyphenols with proteins appears to be a nonspecific surface phenomenon in which complexation occurs *via* both hydrogen bonding of the polyphenol to the exterior ketoimide and polar groups on the protein and hydrophobic interactions. Present information does not permit an evaluation of the relative importance of these effects. When polyphenols precipitate proteins from solution two situations may be envisaged. At low protein concentrations the polyphenol associates at one or more sites on the protein surface to give a monolayer which is less hydrophilic than the protein itself [Figure 3(a)]. Aggregation and precipitation then ensue. Where the protein concentration is high the relatively

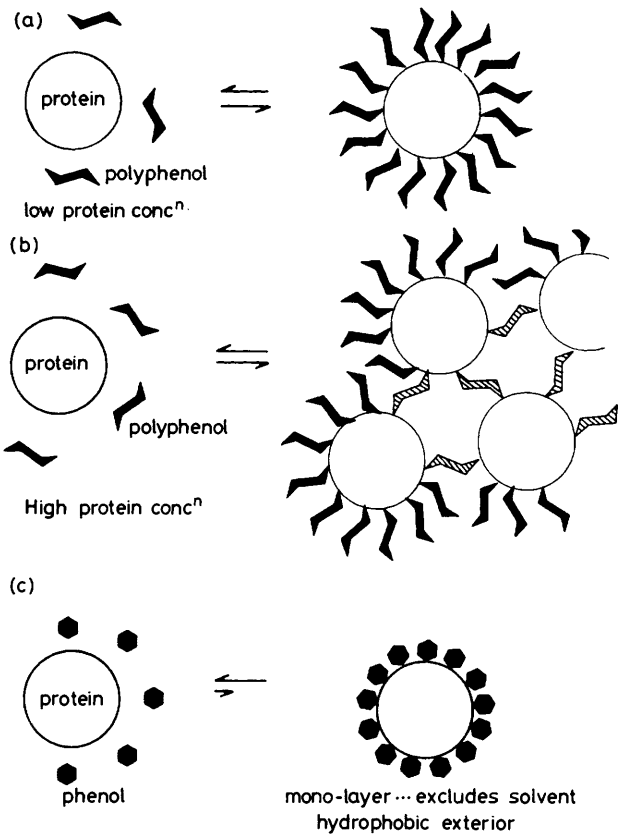

Figure 3. Protein–phenol, protein–polyphenol precipitation

hydrophobic surface layer is formed by complexation of the polyphenol onto the protein and by cross-linking of different protein molecules by the multidentate polyphenols [Figure 3(b)]. Precipitation then follows as above. This tendency to cross-link protein molecules at higher protein concentrations would explain the changing stoicheiometry of the aggregates. An interesting corollary of this hypothesis is that simple phenols such as pyrogallol and resorcinol should also be capable of precipitating proteins from solution if they can be maintained in solution at concentrations sufficient to push the equilibrium in favour of the protein–phenol complex and thus form a hydrophobic layer of simple phenol molecules on the protein surface [Figure 3(c)]. For many simple phenols the limit is provided by their solubility in water but it can be achieved with BSA (3×10^{-5} molal) and pyrogallol (1 molal) and resorcinol (2 molal).

Similar comparative studies of polyphenol–polysaccharide interactions have been hampered to some extent by the availability of water-soluble polysaccharides with clearly defined structure and molecular weight. Thus although some preliminary investigations have been completed in this area, the most satisfactory quantitative data, to the present, has been obtained using polysaccharides in the solid state [cellulose triacetate (in membrane form)] and the chromatographic adsorbents Sephadex G-25, G-50, and LH-20. Adsorption isotherms which relate to the binding of various polyphenols to cellulose acetate membranes (20—25 μm thickness, 6 000—8 000 and 12 000—14 000 molecular weight cut-off) have been obtained (Figure 4, Table 3) in which the relative slopes of the adsorption isotherms are correlated. These data show a remarkably similar pattern of binding to those presented earlier for the interaction of BSA with polyphenols (Figure 2, Table 2). The gradation in strength

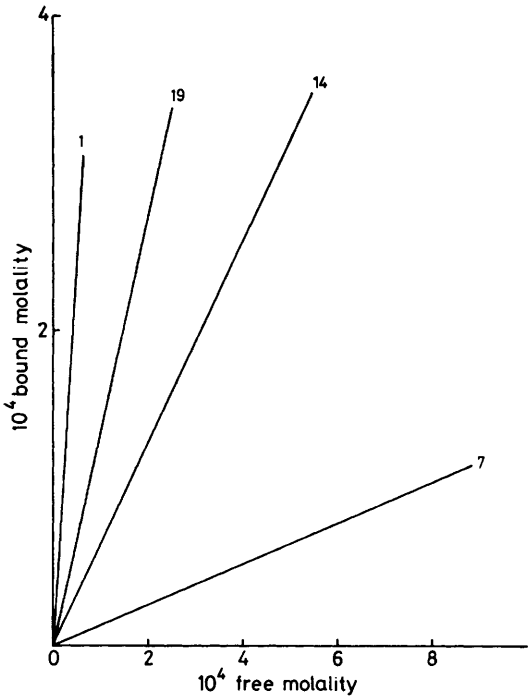

Figure 4. Binding of polyphenols to cellulose acetate–adsorption isotherms

of binding is strongly influenced by molecular size and conformational flexibility. The 'dimer' (**5**) is thus most strongly bound to cellulose triacetate and the highly condensed metabolites (**7**) (and its C-1 enantiomer) most weakly. However, the results strongly suggest that the formation of intramolecular hexahydroxydiphenoyl ester linkages (Scheme; group 2B), with the consequent freezing of elements of molecular mobility have, by comparison, a much more dramatic effect on complexation than was observed in solution with the protein BSA. Thus 1,3,6-trigalloyl-β-D-glucose (**14**) is, for example, more strongly bound to cellulose triacetate than either the 'dimer' (**6**) or vescalagin (**7**). Molecular flexibility is thus seen as a rather more critical characteristic in this series. Parenthetically it may be noted that astonishingly good resolutions of racemic mixtures may be achieved by chromatography on microcrystalline cellulose triacetate.[43] The lamellar arrangement of the polysaccharide in the manner of a crystal lattice acts as a two-dimensional molecular sieve making possible the inclusion of foreign molecules. Aromatic substrates are frequently well resolved by this procedure[43,44] and this indicates that hydrophobic interactions are probably of greater significance in some polyphenol–polysaccharide interactions.

Phenols and polyphenols may be readily separated by chromatography on the Sephadex cross-linked dextran gels and this has been the basis of the isolation of the majority of polyphenols used in this work.[26–28] The physical basis of these separations is not entirely clear but adsorption and partition chromatography probably play a more significant role than the customary molecular sieving.[45,46] Sephadex gel chromatography has been applied in this study and the rank order of binding for a range of phenols to Sephadex gels has been determined by using the apparent partition coefficient, K_{AV}, which is proportional to the more frequently employed distribution coefficient K_D.[47,48] Both Sephadex G-25 and G-50 were used with buffers of pH 2.22 (Cl^- containing 0.1M-NaCl) and acetate buffer pH 3.95. Change in pH does not markedly alter the rank order of binding of phenols although it appears that for each

Table 3. Adsorption isotherms: polyphenols and cellulose acetate. Slopes of plots of m_f (unbound polyphenol concentration) *versus* m_b (bound polyphenol concentration): m_b/m_f, 298 K

Polyphenol	Slope
(**5**)	15.5
(**1**)	5.2
(**2**)	2.7
(**19**)	1.4
(**3**)	1.2
(**14**)	0.65
(**4**)	0.24
(**7**)	0.13

Values obtained from equilibrium dialysis experiments using cellulose acetate membranes.

Table 4. Chromatography of polyphenols on 'carbohydrate' supports: (i) Sephadex G-25, pH 2.2 K_{AV} values, Cl^- buffer; (ii) Sephadex LH-20, MeOH–H_2O (19:1), K_{AV} values; (iii) cellulose–Whatman No. 2, R_F values, (a) 6% acetic acid, (b) butan-2-ol–acetic acid–water (14:1:5). All measurements at 298 K

			(iii)	
Polyphenol	(i)	(ii)	(a)	(b)
Phenol	1.1	1.13		
(−)-Epicatechin	1.83	1.96	0.37	0.50
Procyanidin B-3	2.36	3.04	0.43	0.34
(**14**)	4.43	2.49	0.12	0.44
(**4**)	8.74	3.60	0.48	0.40
(**3**)	53.9	6.2	0.35	0.37
(**20**)	16.25	6.17	0.11	0.45
(**19**)			0.09	0.45
(**16**)	5.89		0.17	0.21
(**15**)	6.18		0.41	0.25
(**7**)	2.5	2.28	0.53	0.02
(**17**)	7.54		0.15	0.21
(**8**)	7.57		0.29	0.26
2,6-Di-*O*-galloyl-1,5-anhydro-D-glucitol	4.43		0.26	0.55
2-*O*-Galloyl-6-*O*-digalloyl-1,5-anhydro-D-glucitol	5.83		0.13	0.59
Penta-*O*-galloyl-β-D-glucose (**1**)		8.56	0.08	0.50
(**6**)		24.8	0.29	0.02
(**5**)		56.7	0.30	0.14

substrate there is a slightly greater affinity for the gel at the higher pH. This is an interesting contrast with the corresponding behaviour with proteins and may be of great importance *in vivo*. These studies show (Table 4) that subtle changes in the polyphenol structure results in marked changes in affinity for the polysaccharide gel. Once again in the simple galloyl-D-glucose series molecular size and flexibility appear to be the two major criteria which most strongly influence the retention of polyphenols by the polysaccharide matrix. Thus the highly condensed rigid polyphenolic structures of vescalagin (**7**) (and its C-1 enantiomer castalagin) are bound weakly to the polysaccharide. It is also interesting to note that hexahydroxydiphenoyl-bridged polyphenols based on the $^4C_1$-D-glucopyranose conformation [*e.g.* (**3**) and (**8**)] are comparatively much more strongly bound to Sephadex than analogous phenolic metabolites based on the thermodynamically less favourable $^1C_4$ conformation [*e.g.* (**15**) and (**16**)] which have a much more compact molecular structure. Two further observations are also significant. The introduction of a second intramolecular hexahydroxydiphenoyl linkage into the $^1C_4$-D-glucopyranose metabolites produces a relatively small decrease

in the K_{AV} value [*cf.* (17) and (15)] whilst the corresponding change in more open $^4C_1$-D-glucopyranose derivatives [*cf.* (3) and (8)] produces a much more dramatic change in K_{AV}. Secondly, the small change in K_{AV} between geraniin (16) and its dihydro-derivative (15) suggests that in the $^1C_4$-D-glucopyranose metabolites the overall shape rather than the number of free phenolic groups is a much more important factor in determining the strength of binding to the polysaccharide.

As with the protein BSA, addition of galloyl groups in the simple galloyl-D-glucose series produces an exponential increase in binding to the polysaccharide (as measured by K_{AV}). Penta-*O*-galloyl-β-D-glucose (1) and the 'dimers' (5) and (6) were *not* eluted from Sephadex G-25 and G-50 with aqueous buffers under the conditions described. It is thus clear that molecular size here exerts a significant effect on the association with the polysaccharide gel. In the case of the 'dimer' (6) this factor over-rides any diminution in the strength of association which may result from restrictions on conformational mobility (*cf.* complexation with BSA, Table 2). In order to establish a relationship between these substrates and the simpler phenolics, experiments were conducted with the more hydrophilic adsorbent Sephadex LH-20 using 5% aqueous methanol as eluant. Determination of K_{AV} gave a relative order which showed a broad measure of correlation with the data obtained for G-25 and G-50 gels (Table 4). However, both 'dimers' are bound substantially more strongly to the polysaccharide than penta-*O*-galloyl-β-D-glucose (1) and this undoubtedly confirmed the view that in the binding of polyphenols to polysaccharide gels molecular size may well be a paramount factor.

Dextran is a polymeric glucan derived microbiologically by the action of *Leuconstoi mesenteroides* on sucrose and consists of α-D-(1 ⟶ 6)-linked units to an extent of 90—95%. Cross-linking by epichlorhydrin yields the gels such as Sephadex. The affinity of aromatic compounds for dextran-gels is well documented and the addition of hydroxy groups to the aromatic ring markedly increases that affinity—the 1,3 and 1,3,5 orientation of phenolic groups significantly produces the strongest association.[49] The origins of this affinity are uncertain. Several suggestions have been advanced:[50–52] interaction between the phenolic hydroxy and the ether groups of the cross-links, the phenyl group acting as electron donor to the hydroxy groups of Sephadex and inclusion within the pores of Sephadex. It is not possible, on present evidence, to attempt to fully rationalise the behaviour of natural polyphenols when chromatographed on Sephadex gels. If one assumes that all metabolites permeate the pores of the gel then the distinctive characteristics displayed by the higher molecular weight species [such as (5) and (6)] suggests that for these larger molecules interactions between the aryl ester groups of the solute and the interior of the pores of the gel may well be significant. The pore may for example provide an environment, akin to that of the cycloamyloses,[53] in which hydrophobic forces assume a much greater importance. In the context of the results, however, it is interesting to note the quite clear parallels and differences which are evident between the chromatographic characteristics of polyphenols on the various Sephadex gels and on cellulose (Table 4, R_F values in two solvent systems[26–28]). Chromatography on cellulose media is, it is presumed, essentially a surface phenomenon; that in 6% acetic acid is generally attributed to differential complexation to the fibre. In the simple D-glucopyranose galloyl ester series increase in the number of phenolic ester groups results in an enhanced affinity for the polysaccharide. Likewise the introduction of intramolecular hexahydroxydiphenoyl ester linkages into the $^4C_1$-D-glucopyranose form of the metabolites leads to a decreased binding to the cellulose. Both these observations are similar in type to those made with BSA and the Sephadex gels. However, significantly, the 'dimers' (5) and (6) have approximately the same

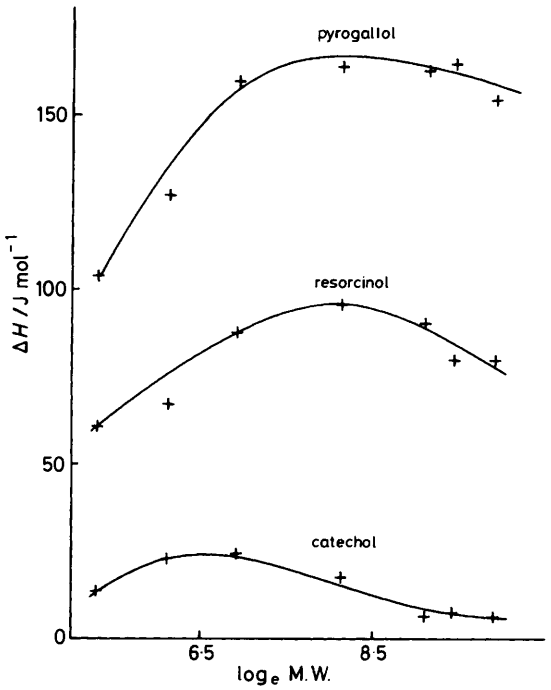

Figure 5. Heats of interaction of simple phenols and poly(ethylene oxide) (PEO) as a function of the molecular weight of the poly(ethylene oxide). The enthalpy change (ΔH) is per mol of PEO sub-units

affinity (as measured by R_F values) for cellulose as the monomers [(3) and (8), respectively] from which they are derived biosynthetically. The characteristics of polyphenols in organic media (BAW)[54] contrast sharply with those in 6% acetic acid although there are some similarities to those noted with the Sephadex gels, in particular the strong affinity of the 'dimers' (5) and (6) and highly condensed structures such as (7) and (15)—(17) for the cellulose support. The comparative patterns of chromatographic behaviour of polyphenols with cellulose and the dextran gels underlines the critical importance of the nature of the polysaccharide substrate. Where the association is primarily a surface effect then broad similarities are noted with the analogous complexation with proteins. Where the polysaccharide is cabable of forming inclusion complexes then significant departures from these patterns of behaviour may be expected.

These observations provide a background for studies of the complexation of polyphenols with polysaccharides in solution. Initial studies have been carried out with poly(ethylene oxide)s (PEO) and amyloses with varying molecular weights. Soluble PEOs have, from time to time, been used to bind and precipitate polyphenols from solution.[54] There is a marked structural specificity in the binding of simple phenols to PEO. Thus catechol (11) is bound much less strongly than resorcinol (10) or pyrogallol (12) which display a comparable affinity. For both pyrogallol and resorcinol the measured heats of interaction per ethylene glycol sub-unit ($-\Delta H_M$) show a rapid increase with PEO molecular weight until the PEO reaches a chain molecular weight of between 1×10^3 and 4×10^3. At this point the values of $-\Delta H_M$ reach a plateau (Figure 5). This has been attributed to a co-operative hydrogen-bonding interaction between the ether oxygen atoms of the PEO and the phenolic hydroxy groups of the pyrogallol or resorcinol substrate. A 7_2 helical structure has been established for some PEOs in the solid state[55] and it is tentatively proposed that the microcalorimetric data are consistent with a bridging of O(1) and O(3) in the incipient

helical form of the PEO by the 1,3-dihydroxyresorcinol (or pyrogallol) unit (**10**). This co-operatively both sets in train and stabilises the establishment of an ordered regular helical structure of the PEO in solution. The O(1) to O(3) separation in the PEO 7_2 helix maximises the hydrogen-bonding potential of the resorcinol (1,3) arrangement of phenolic groups. The O(1) to O(3) separation is *ca.* 5 Å and is similar to the glycosidic O(1) to O(4) distance in poly-(1-α-4-D-glucopyranose) polysaccharides such as amylose[56] (**18**).

Further studies are in progress to evaluate the nature of the binding of plant polyphenols to polysaccharides and to determine the extent of competition between polysaccharides and proteins for polyphenolic ligands.

Experimental

Polyphenolic Substrates.—Polyphenols were obtained by previously described methods: tannic acid [(**2**), Chinese gallotannin) from Chinese galls (*Rhus semidata*)[57] and penta-*O*-galloyl-β-D-glucose (**1**) by methanolysis of tannic acid;[57] 1,2,3,6-tetra-*O*-galloyl-β-D-glucose (**19**) by methanolysis of the phenolic extract from Turkish galls (*Quercus infectoria*)[58] and 1,2,3,6-tetra-*O*-galloyl-β-D-glucose from *Bergenia* sp;[28] 1,3,6-tri-*O*-galloyl-β-D-glucose (**14**) by partial hydrolysis of chebulinic acid;[58,59] the hexahydroxydiphenoyl esters (**3**,**4**) and (**5**) from meadowsweet (*Filipendula ulmaria*); the dimer (**6**) from *Rubus* sp. and vescalagin (**7**) and castalagin from oak galls (*Quercus robur*).[35,58]

Equilibrium Dialysis and Microcalorimetry.—A Dianorm equilibrium dialysis apparatus,[29] contained in an air thermostat at 25 °C, was used to determine the extent of binding of phenols to protein. The membranes used (Spectrapor; molecular weight cut-off 6 000—8 000) were preconditioned before use. The concentrations of phenols were determined spectrophotometrically.

The calorimetric investigations were performed using an LKB batch microcalorimeter operating at 25 °C. The amendments which have been made to this and the general operational procedures have been described elsewhere.[60] The experiments consisted of mixing known quantities of solutions containing phenol and protein and monitoring the enthalpy change. Corrections were made for the enthalpies of dilution of the phenols and protein.[61,62]

Chromatography.—Sephadex gel chromatography was performed using standard procedures[63] and elution volumes monitored using a Vivatron Universal photometer. Blue Dextran was used to obtain the void volume of the columns. The apparent partition coefficient (K_{AV}) was obtained from equation (2)[47] where V_e, V_0, and V_t are respectively the elution,

$$K_{AV} = (V_e - V_0)/(V_t - V_0) \qquad (2)$$

void, and total column volumes. It was experimentally confirmed that for the systems investigated, K_{AV} was independent of the column packing and dimensions.

Appendix A

The Free Energy of Binding of a Ligand to a Macromolecule and the Relationships between this and the Extent of Binding and the Free Energy of Transfer of the Macromolecule from Water to a Solution containing Ligand.—Suppose we consider a system consisting of 1 kg of solvent (which we shall consider to be water but might well also be a buffer solution) and a macromolecular species (P) at stoicheiometric molality m_P° and a ligand (L) at stoicheiometric molality m_L°. It is convenient to consider two situations, one which pertains to the real system and the many interactions occurring in this and the other where these interactions are 'switched off.' (This corresponds to what may be called the 'ideal' situation.) The difference between any property of the real and ideal situations can, in principle, report on the tendency of the ligand and macromolecule to interact, however we shall address ourselves here to changes in thermodynamic properties and in particular the Gibbs free-energy changes.

For the real system we assume that the molality of the macromolecule is sufficiently dilute so that macromolecule–macromolecule interactions are rare and consequently contribute a negligible amount to the system. In the real system, however, macromolecule–ligand interactions do occur and these interactions may be represented as a set of associative equilibria in which n is the maximum number of ligands which will bind to a macromolecule:

$$\begin{aligned} P + L &\rightleftharpoons PL \\ P + 2L &\rightleftharpoons PL_2 \\ &\vdots \quad\quad \vdots \\ P + iL &\rightleftharpoons PL_i \\ &\vdots \quad\quad \vdots \\ P + nL &\rightleftharpoons PL_n \end{aligned}$$

An equilibrium constant (K_i) can be written for each step [equation (A1) where m_{PL_i} is the molality of the PL_i complex

$$K_i = m_{PL_i}/m_P m_L^i \qquad (A1)$$

and m_P and m_L are the molalities of 'free' (*i.e.* uncomplexed) macromolecule and ligand, respectively]. The total free energy of the system (G^{real}) may formally be written as (A2) where

$$G^{real} = G_w^{real} + G_s^{real} \qquad (A2)$$

G_w^{real} is the free energy of 1 kg of solvent in the real system and G_s^{real} is the free-energy contribution from all of the solute species in the solution. The solvent contribution may be written as (A3)

$$G_w^{real} = G_w^\circ - RT\,m \qquad (A3)$$

with G_w° being the free energy of 1 kg of pure solvent and m being the osmolality, *i.e.* the total molality of all solute species. For the present systems as here equation (A4) applies. Consequently, the relationship (A5) holds. If we now turn to the contribution from the solute species we have (A6) where j

$$m = m_P + m_{PL} + m_{PL_2} + \cdots m_{PL_n} + m_L = m_P^\circ + m_L \qquad (A4)$$

$$G_w^{real} = G_w^\circ - RT\,(m_P^\circ + m_L) \qquad (A5)$$

$$G_s^{real} = \sum_j m_j \mu_j \qquad (A6)$$

represents any solute species and μ denotes the chemical potential. This gives equation (A7). Since for any species we have (A8) from the equilibrium condition, equation (A7) becomes (A9). The conservation equations (A10) and (A11) can then be substituted into equation (A9) to give (A12). This may be written as (A13) where $\mu_P^{\circ,w}$ and $\mu_L^{\circ,w}$ are the standard-

$$G_s^{real} = m_L\mu_L + m_P\mu_P^{real} + \sum_{i=1}^{i=n} m_{PL_i}\mu_{PL_i}^{real} \qquad (A7)$$

$$\mu_{PL_i}^{real} = \mu_P^{real} + i\mu_L^{real} \qquad (A8)$$

$$G_s^{real} = \mu_L^{real} m_L + \mu_L^{real} \sum_{i=1}^{i=n} i m_{PL_i} + \mu_P^{real} \sum_{i=0}^{i=n} m_{PL_i} \quad (A9)$$

$$m_P^\circ = m_P + m_{PL} + \cdots m_{PL_i} + \cdots m_{PL_n} = \sum_{i=0}^{i=n} m_{PL_i} \quad (A10)$$

$$m_L^\circ = m_L + m_{PL} + 2m_{PL_2} + \cdots i m_{PL_i} + \cdots n m_{PL_n} = m_L + \sum_{i=1}^{i=n} i m_{PL_i} \quad (A11)$$

$$G_s^{real} = m_P^\circ \mu_P^{real} + m_L^\circ \mu_L^{real} \quad (A12)$$

$$G_s^{real} = m_P^\circ (\mu_P^{\ominus,w} + RT \ln m_P) + m_L^\circ (\mu_L^{\ominus,w} + RT \ln m_L) \quad (A13)$$

state chemical potentials of the macromolecule and ligand in water. Consequently the free energy of the real system is obtained by addition of equations (A5) and (A13).

Turning now to the ideal system, the free ligand and free macromolecule molalities correspond to the stoicheiometric molalities and so by substitution of $m_L = m_L^\circ$, $m_P = m_P^\circ$ into equation (A14) we get (A15). The free-energy change arising

$$G^{real} = G_w^\ominus - RT(m_P^\circ + m_L) + m_P^\circ (\mu_P^{\ominus,w} + RT \ln m_P) + m_L^\circ (\mu_L^{\ominus,w} + RT \ln m_L) \quad (A14)$$

$$G^{ideal} = G_w^\ominus - RT(m_P^\circ + m_L^\circ) + m_P^\circ (\mu_P^{\ominus,w} + RT \ln m_P^\circ) + m_L^\circ (\mu_L^{\ominus,w} + RT \ln m_L^\circ) \quad (A15)$$

from interactions between solute species, *i.e.* from binding, is the difference between (A14) and (A15) [equation (A16) where m_L^b

$$\begin{aligned} \Delta G^{binding} &= G^{real} - G^{ideal} \\ &= RT[(m_L^\circ - m_L) + m_P^\circ \ln (m_P/m_P^\circ) + m_L^\circ \ln (m_L/m_L^\circ)] \\ &= RT[m_L^b + m_P^\circ \ln (m_P/m_P^\circ) + m_L^\circ \ln \{(m_L^\circ - m_L^b)/m_L^\circ\}] \end{aligned} \quad (A16)$$

is the molality of bound ligand].

If we now consider a solution where the total ligand concentration is much greater than the total protein concentration ($m_L^\circ \gg m_P^\circ$) and $m_L^\circ \gg m_L^b$. Under these circumstances the final term on the r.h.s. of equation (A16) becomes $-m_L^b$ and consequently equation (A16) reduces to (A18). If we now

$$m_L^b = \sum_{i=1}^{i=n} i m_{PL_i} = m_L^\circ - m_L \quad (A17)$$

$$\Delta G^{binding} = RT m_P^\circ \ln (m_P/m_P^\circ) \quad (A18)$$

substitute the equilibrium constant expressions [equation (A1)] into the conservation equation (A10) then we have (A19)

$$m_P^\circ = m_P \Sigma \quad (A19)$$

where Σ denotes the binding polynomial (A20). Consequently from equations (A18) and (A19) we get equation (A21) and the molar free energy change on binding is (A22).

The next objective is to relate the binding isotherm to the experimental quantities from equilibrium dialysis studies. In such studies the usual quantities monitored or obtainable are the free ligand molality and the total number of moles of ligand

$$\Sigma = 1 + \sum_{i=1}^{i=n} K_i m_L^i \quad (A20)$$

$$\Delta G^{binding} = -RT m_P^\circ \ln \Sigma \quad (A21)$$

$$\Delta G_m^{binding} = -RT \ln \Sigma \quad (A22)$$

bound per mole of macromolecule ($r = m_L^b/m_P^\circ$). We have from equation (A11) and the equilibrium constants, equation (A23) and differentiating equation (A20) gives (A24). Incorporation of equation (A24) into equation (A23) gives (A25).

$$m_L = m_L^\circ - m_P \sum_{i=1}^{i=n} i K_i m_L^i \quad (A23)$$

$$(\partial \Sigma/\partial m_L) = \sum_{i=1}^{i=n} i K_i m_L^{i-1}$$

or

$$(\partial \Sigma/\partial \ln m_L) = \sum_{i=1}^{i=n} i K_i m_L^i \quad (A24)$$

$$m_L = m_L^\circ - m_P (\partial \Sigma/\partial \ln m_L)$$

or

$$m_L^b = m_P (\partial \Sigma/\partial \ln m_L) \quad (A25)$$

Consequently, equation (A26) follows where equation (A19) has

$$\begin{aligned} &= m_L^b/m_P^\circ \\ &= [m_P (\partial \Sigma/\partial \ln m_L)]/[m_P \Sigma] \\ &= (\partial \ln \Sigma/\partial \ln m_L) \end{aligned} \quad (A26)$$

been used. Integration of equation (A26) and using this in equation (A22) gives (A27) and therefore the nett free-energy

$$\Delta G_m^{binding} = -RT \int_0^{m_L} r \, d \ln m_L \quad (A27)$$

change arising from macromolecule–ligand interactions can be obtained by appropriate integration of the experimentally determinable quantities.

Appendix B

The Free Energy of Transfer of Macromolecule.—The chemical potential of the macromolecule when present as in a dilute solution in water is given by equation (B1). The chemical

$$\mu_P^w = \mu_P^{\ominus,w} + RT \ln m_P^\circ \quad (B1)$$

potential of the macromolecule when it is dilute in a solution containing ligand may be written in three ways. First, we may express it with respect to the aqueous standard state [equation (B2) where all deviations from ideality of the macromolecule are

$$\mu_P^s = \mu_P^{\ominus,w} + RT \ln m_P^\circ + RT \ln \gamma_P^\circ \quad (B2)$$

expressed using an activity coefficient (γ_P°)]. Secondly, we have equation (B3), using equation (A13), and finally rather

$$\mu_P^s = \mu_P^{\ominus,w} + RT \ln m_P \quad (B3)$$

than use the aqueous standard state we may use, as a standard state for the macromolecule, the particular ligand solution considered and then equation (B4). We consider the 'thought'

$$\mu_P^s = \mu_P^{\ominus,s} + RT \ln m_P^\circ \quad (B4)$$

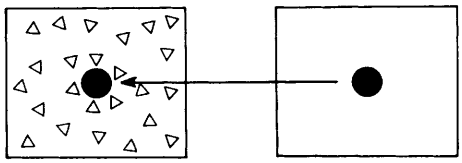

Figure 6. Free energy of transfer, $\Delta G^{\ominus,tr}$: the 'thought' experiment. The filled circle denotes macromolecule and the open triangles ligan molecules

experiment illustrated in Figure 6 in which one molecule of macromolecule, when present in a dilute solution in water, is transferred to a solution containing ligand. The free-energy change for this process may be termed the molecular free energy of transfer and the value for this when multiplied by Avogadro's constant is consequently the standard chemical potential of transfer (sometimes called the free energy of transfer) of the solute. So in terms of equations (B3) and (B4) we have (B5)

$$\begin{aligned}\Delta G^{\ominus,tr} &= \mu_P^{\ominus,s} - \mu_P^{\ominus,w} \\ &= RT \ln m_P/m_P^\circ \end{aligned} \quad \text{(B5)}$$

which, using the relationship given in equation (A19), becomes (B6). It is therefore apparent from this and equation (A22) that

$$\Delta G^{\ominus,tr} = -RT \ln \Sigma \quad \text{(B6)}$$

$\Delta G^{\ominus,tr}$ is identical to $\Delta G_m^{binding}$. It is worthwhile noting that other inter-relationships exist between the various quantities defined by equations and in particular expression (B7) links the

$$\gamma_P^\circ = 1/\Sigma \quad \text{(B7)}$$

formal activity coefficient of the macromolecule to the binding potential.

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