Polyphenol Interactions. Part 4.[1] Model Studies with Caffeine and Cyclodextrins

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Studies of the reversible complexation of a range of phenols and natural polyphenols in aqueous media with caffeine and related heterocycles, and with α- and β-cyclodextrin, using a range of physical methods (^1H and ^{13}C NMR spectroscopy, microcalorimetry, and X-ray crystallographic analysis) are reported. Values of the association constants (K) for the formation of 1:1 complexes between caffeine and a range of natural polyphenols have been determined (K 15–138 $dm^3 mol^{-1}$). Amongst galloyl esters there is a dependence for strong binding on molecular size, conformational flexibility, and the 'free' galloyl ester group content of the polyphenol. With phenolic flavan-3-ols, association is enhanced by galloylation at C-3. The extent of precipitation of polyphenols by caffeine is related to the association constants (K), the molecular size of the polyphenol, and the initial concentration of both substrates. Polysaccharide cavity sequestration of polyphenols has been studied by means of the model substrates α- and β-cyclodextrin. Compared with that of the natural galloyl esters (K 76–340 $dm^3 mol^{-1}$) the binding of phenolic flavan-3-ols to β-cyclodextrin is strong (K 210–6232 $dm^3 mol^{-1}$). Models are proposed for encapsulation within the cyclodextrin cavity. The results of these model studies are discussed in terms of the relative significance of hydrophobic effects and hydrogen bonding in polyphenol complexation. They provide a basis for the interpretation of the behaviour of polyphenols in their association with proteins, polysaccharides and other macromolecules.

Polyphenols are a unique group of water-soluble plant secondary metabolites notable for their molecular weight range (500–5/6000 daltons), the abundance of phenolic groups within their structure (12–15 per 1000 relative molecular mass), and the variations in shape and conformational flexibility which they display[2]—characteristics which endow polyphenols with the ability to complex strongly with a wide range of other metabolites. One of the first recorded uses by man of plants rich in polyphenols (*syn.* vegetable tannins) was in the conversion of raw animal hides and skins into durable, non-putrescible leather.[3] Polyphenols are similarly responsible for the taste and astringency of many beverages and for the physiological and pharmacological effects which underlie the efficacy of many herbal medicines.[4] The broad consensus of opinion is that all these properties devolve to a greater or lesser extent on the complexation of polyphenols with proteinaceous or carbohydrate materials, whether they be in the animal skin (*e.g.*, collagen[3]) or in the human body.[4] One view is that all these association phenomena are best described[4,5] by the 'hand-in-glove' metaphor, *i.e.*, there is not a static matching of donor and acceptor groups, but the complexation is time dependent and dynamic, although the required segmental mobilities of host and guest are probably small (a few ångstrøms) and not energetically prohibitive.

The binding energy, in this model, derives from the summation of a relatively large number of contacts developed in a time-dependent manner. The 'hydrophobic effect' is generally believed to be the major driving force for macromolecular association[6,7] of this type, with direct hydrogen bonding contributing little binding energy but enhancing specificity. An additional and intriguing problem, particularly in so far as the unresolved question of the metabolic role of polyphenols is concerned,[8] is whether the complexations with proteins display specificity or selectivity, (*i.e.*, the protein contains discrete binding sites in accessible locations) or are random processes (in which the protein surface provides a continuum of overlapping epitopes). Hagerman and Butler[9] were the first to demonstrate that proline-rich proteins (PRPs) and polymers have an unusually high affinity for natural polyphenols, lending support to the first of these propositions. In order to probe these questions, and in particular to consider the relative significance of hydrophobic effects and hydrogen bonding in polyphenol complexation, model studies with caffeine and cyclodextrins have been conducted. Preliminary discussions of some aspects of this work have been presented.[4,5,8,10,11]

Caffeine.—In aq. media polyphenols readily associate with several alkaloids (*e.g.*, brucine, cinchonine, cinchonidine, quinine, strychnine, and caffeine), leading to precipitation.[12,13] The complexes formed in tea between caffeine and polyphenols are thought to reduce or eliminate many of the known undesirable physiological actions of caffeine.[14] When precipitated, these particular complexes constitute the 'tea cream' which gives a measure of certain desirable attributes (strength and briskness) of the beverage.[15] Structurally, caffeine **1** has features which are reminiscent of peptides, particularly those associated with imino acids such as proline [*viz.*, the two –CO–N(Me)– groups]. The researches of Mejbaum-Katzenellenbogen[16,17] have shown that caffeine competes effectively with proteins for polyphenolic substrates and that it is possible to regenerate proteins, in a biologically active state, from insoluble protein–polyphenol complexes by treatment with caffeine.

Caffeine forms complexes of variable stoicheiometry with polyphenols. These may precipitate from solution and the precipitates are readily dissociated by the addition of detergents and organic solvents (MeOH, EtOH, Me_2CO, *etc.*). Under standardised conditions the extent of precipitation of different polyphenols by caffeine is dependent on molecular size (Table

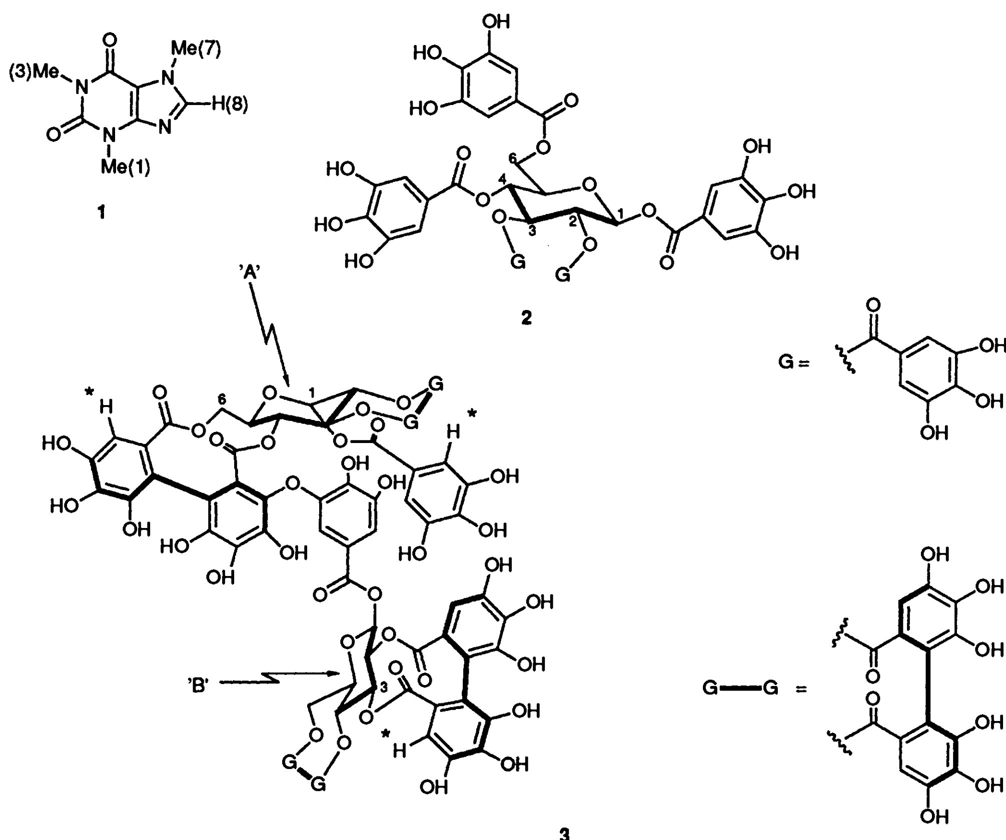


Table 1. Stoicheiometry of polyphenol–caffeine 1 in precipitated complexes: comparative extent of polyphenol precipitation.

Polyphenol	Polyphenol–caffeine quotient	% Polyphenol precipitated
(−)-Epigallocatechin gallate (32)	3.3	15
(+)-Catechin gallate (33)	2.2	41
1,3,6-Tri-*O*-galloyl-β-D-glucopyranose	3.0	70
1,2,6-Tri-*O*-galloyl-β-D-glucopyranose	2.5	65
1,2,4,6-Tetra-*O*-galloyl-β-D-glucopyranose (10)	2.6	79
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranose (2)	2.7	100
Davidiin (6)	2.1	66
Sanguin H-6 (3)	7.0	34

Initial concentrations—polyphenol (1.5×10^{-2} mol dm^{-3}) caffeine (3×10^{-3} mol dm^{-3}).

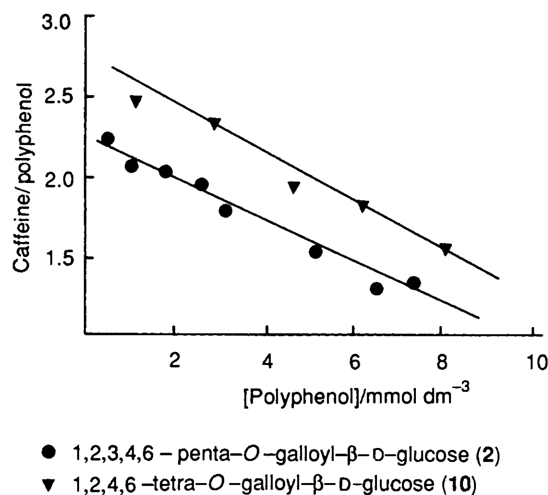


Fig. 1. Caffeine–polyphenol precipitation. Variation of caffeine content with initial polyphenol concentration (caffeine concentration 5×10^{-2} mol dm^{-3}).

1). The ratio of caffeine to polyphenol in the precipitates is variable (Table 1) and is related to the initial concentrations of both caffeine and polyphenol (Fig. 1). In competitive experiments the extent of precipitation (complexation followed by aggregation) is broadly, but not directly, related to the strengths of the initial association between caffeine and the particular polyphenols (Table 2).

Association constants for the formation of 1:1 complexes between caffeine and a range of phenols and natural polyphenols in aq. solution have been determined by microcalorimetry (Table 3) and by ^{1}H NMR spectroscopy (Tables 4 and 5). Batch microcalorimetry provides a convenient method for the measurement of the heat change consequent upon caffeine–polyphenol association. The method allows both the equilibrium constant and the enthalpy of interaction to be calculated simultaneously. The entropy of interaction may be calculated using the appropriate thermodynamic relationship. The data were analysed by a non-linear least-squares fitting procedure (see the Experimental section).

The complexation of caffeine with simple phenols has been studied in solution by using various spectroscopic techniques

(IR, UV—R. Martin, Ph.D. Thesis, University of Sheffield, 1986). X-Ray crystallographic analysis of the complexes of caffeine with methyl gallate (Figs. 2 and 3),[10] 3-nitrobenzoic acid,[10] 5-chlorosalicylic acid,[18] pyrogallol,[19] and potassium chlorogenate[20] show that these crystalline, intermolecular complexes are characterised by a layer-lattice structure in which the caffeine and the aromatic substrate are stacked in alternating layers, approximately parallel, with an interplanar separation of 3.3–3.4 Å (Fig. 2). For methyl gallate this stacking struture is complemented by an extensive in-plane system of hydrogen bonding between the three phenolic hydroxy groups (donors) and the two keto amide groups and the basic N-9 of caffeine (acceptors), Fig. 3. This propensity of caffeine and phenolic substrates to form 'stacking' structures[21] was exploited in the use of ^{1}H NMR spectroscopy to investigate complexation in aq. media. In all the cases examined the four singlet signals of caffeine exhibited significant, and frequently substantial, upfield chemical-shift changes ($\Delta\delta$) upon complexation with polyphenols, consistent with this model. Invariably, the 8-H of caffeine showed the largest chemical-shift change whilst the protons of the N-1 methyl group usually displayed the smallest displacements. The values of the association constants (K) for the formation of 1:1 complexes between caffeine and various polyphenols have been calculated (Tables 4 and 5) using the chemical-shift changes observed for 8-H of caffeine, by employment of a standard non-linear least-squares curve-fitting program and by taking note of the self-association of both polyphenols[1] and caffeine[22] (see the Experimental section). The constants calculated on the basis of the chemical-shift changes for each of the three *N*-methyl groups of caffeine are, however, generally in good agreement with these values (Tables 4 and 5); *e.g.*, 1,3,6-tri-*O*-galloyl-β-D-glucopyranose, K 37.1, 36.5, 35.9 and 36.1 dm^3 mol^{-1} based on the chemical-shift changes of 8-H, NMe(7), NMe(3) and NMe(1), respectively. Significantly these results show exactly the same trends as those which were observed earlier in studies of polyphenol–protein complexations.[23] There is thus a very strong dependence on 'free' galloyl ester group content and molecular size of the polyphenol, *cf.* 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose **2**, sanguin H-6 **3**,[1] and rugosin D,[24] and on its conformational flexibility, *cf.* compound **2**, tellimagrandin-2 **4**,[24,25] casuarictin **5**,[26] and davidiin **6**.[27] These results thus strongly reinforce the view that polyphenol–caffeine association not only mirrors in many of its facets polyphenol–protein complexation but also provides a good model system in which to examine some of the general mechanisms which are operative in complexation. Particularly striking therefore are the depressive effects on association which result from the restriction of conformational mobility by the biosynthetic formation of the hexahydroxydiphenoyl ester group from two galloyl ester groups (Fig. 4), the strong temperature dependence of association (indicative of significant entropy changes on complexation), and the overall weaker affinity which typical flavan-3-ols and procyanidins[2] display for caffeine (and proteins) as compared with galloyl ester derivatives.

In related studies the association of 1,3,6-tri-*O*-galloyl-β-D-glucopyranose, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose **2**, and (−)-epigallocatechin 3-*O*-gallate **32** with other purine and pyrimidine bases (adenine, adenosine, disodium adenosine monophosphate, guanosine, cytidine, and thymidine) were investigated. Although the pattern of results was similar, none of these substrates showed a comparable affinity for polyphenols as that displayed by caffeine **1**. The observed capacity to bind to polyphenolic substrates was, however, generally greater for the purine as compared with the pyrimidine derivatives. Similar studies with various simple peptides, including the octapeptide angiotensin and the linear nonapeptide hormone bradykinin (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg), showed little evidence for significant association, although for penicillin-G **7** and the synthetic peptide sweetener aspartame **8** complexation occurred selectively but weakly in the region of the phenyl rings of both substrates.

Employing the 'reverse' experimental technique (*i.e.*, maintaining a fixed concentration of the polyphenol, increasing that of the caffeine and measuring the chemical-shift changes,

Table 2. Selective precipitation of polyphenols by caffeine.

(i) 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucopyranose **2**, 1,3,6,-tri-*O*-galloyl-β-D-glucopyranose (TGG), davidiin **6**; association constants with caffeine in water at 60 °C: 82, 37 and 24 dm^3 mol^{-1}, respectively. Initial polyphenol concentration 1.5 × 10^{-3} mol dm^{-3}.

Caffeine concentration (mmol dm^{-3})	Polyphenol precipitated (%)		
	2	TGG	**6**
4.5	83	45	23
9.0	94	62	47
13.5	100	79	56
18.0	100	87	63

(ii) 1,2,3,4,6-Penta-*O*-β-D-glucopyranose (**2**), tellimagrandin-2 **4**, and 1,3,6-tri-*O*-galloyl-β-D-glucopyranose (TGG); association constants with caffeine in water at 60 °C: 82, 53 and 37 dm^3 mol^{-1}, respectively. Initial polyphenol concentration 1.5 × 10^{-3} mol dm^{-3}.

Caffeine concentration (mmol dm^{-3})	Polyphenol precipitated (%)		
	2	**4**	TGG
4.5	82	74	28
9.0	91	88	45

(iii) 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose **2**, (+)-catechin gallate **33**; and (−)-epigallocatechin gallate **32**; association constant of compound **2** with caffeine at 60 °C: 82 dm^3 mol^{-1}; for compounds **33** and **32** at 45 °C: 38 and 53 dm^3 mol^{-1} polyphenol concentration 1.5 × 10^{-3} mol dm^{-3}.

Caffeine concentration (mmol dm^{-3})	Polyphenol precipitated (%)		
	2	**33**	**32**
4.5	83	30	17
9.0	92	46	31
14.0^a	78	30	20

^{a} Concentration of compound **32** in this experiment 7.5 × 10^{-3} mol dm^{-3}.

Table 3. The association of polyphenols with caffeine in water at 25 °C, determined by batch microcalorimetry.^{a}

Polyphenol	K/dm^3 mol^{-1}	$\Delta H^\ominus$/kJ mol^{-1}	$\Delta S^\ominus$/J K^{-1} mol^{-1}
Methyl gallate (**24**)	39	−44	−118
1,3,6-Tri-*O*-galloyl-β-D-glucose	100	−48	−124
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**2**)	331	−44	−101

^{a} LKB Batch Microcalorimeter 2107-010.

Table 4. Phenol–caffeine association constants determined by ^{1}H NMR spectroscopy (250 MHz) at 22° C.

Substrate	K ($dm^3\ mol^{-1}$)
4-Nitrophenol[a]	22.6 (±0.7)
3-Nitrophenol[a]	7.9 (±0.5)
3,5-Dinitrophenol[a]	25.8 (±1.2)
4-Nitrophenol[b]	20.5 (±0.85)
3-Nitrophenol[b]	25.1 (±0.60)
2-Nitrophenol[b]	35.7 (±3.20)
4-Methoxyphenol[b]	14.2 (±0.4)
4-Chlorophenol[b]	4.0 (±0.6)
Phloroglucinol[b]	18.9 (±1.1)
Resorcinol[b]	15.6 (±1.3)
Pyrogallol[b]	38.1 (±1.5)

Solvents: [a] $CDCl_3$, [b] D_2O.

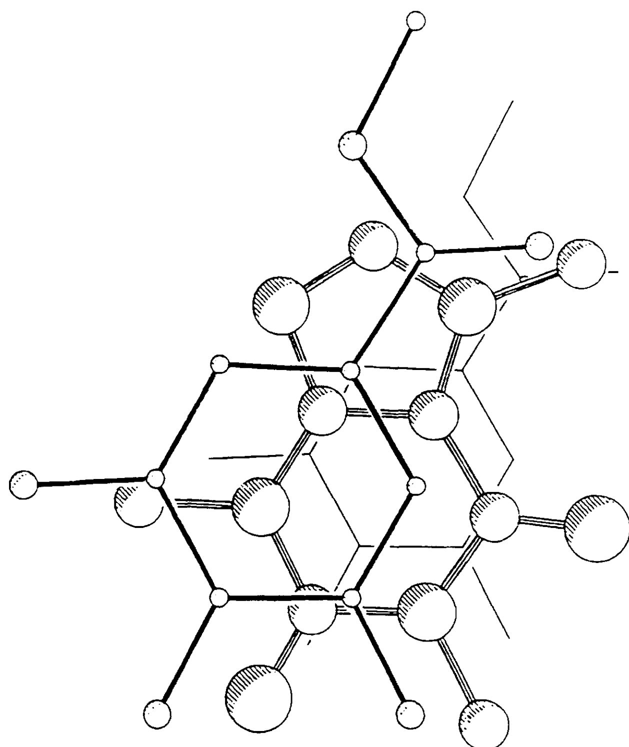


Fig. 2. Caffeine–methyl gallate complex: layer-lattice structure.

Table 5. Polyphenol–caffeine association constants determined by ^{1}H NMR spectroscopy (250 and 400 MHz) in deuterium oxide.[a]

Polyphenol	K/$dm^3\ mol^{-1}$ 45 °C	60 °C
(+)-Catechin (**30**)	26.1 (±0.8)	
(+)-Gallocatechin (**31**)	30.9 (±0.9)	
(+)-Catechin gallate (**33**)	38.2 (±1.2)	
(−)-Epicatechin (**28**)	34.5 (±1.0)	
(−)-Epigallocatechin (**29**)	35.6 (±1.1)	
(−)-Epigallocatechin gallate (**32**)	52.8 (±1.6)	
Procyanidin B-2 (**34**)	26.2 (±0.8)	
Procyanidin B-3 (**35**)	22.4 (±0.7)	
Procyanidin B-4	22.4 (±0.7)	
Methyl gallate		11.3 (±6.3)
1,3,6-Tri-*O*-galloyl-β-D-glucose		37.1 (±1.7)
1,2,6-Tri-*O*-galloyl-β-D-glucose		42.6 (±1.3)
1,2,4,6-Tetra-*O*-galloyl-β-D-glucose (**10**)		53.4 (±1.6)
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**2**)		81.6 (±2.5)
Corilagin		16.8 (±0.5)
Davidiin (**6**)		24.7 (±0.8)
Tellimagrandin-2 (**4**)		61.9 (±1.9)
Tellimagrandin-1		22.6 (±0.7)
Casuarictin (**5**)		19.9 (±0.6)
Rugosin A (**9**)		42.0 (±1.5)
Sanguin H-6 (**3**)		63.0 (±1.9)
Rugosin D		138.0 (±4.1)
1-*O*-galloyl-2,4:3,6-bishexahydroxydiphenoyl-β-D-glucose		15.0 (±0.5)

[a] Determined using chemical-shift changes for 8-H of caffeine (**1**).

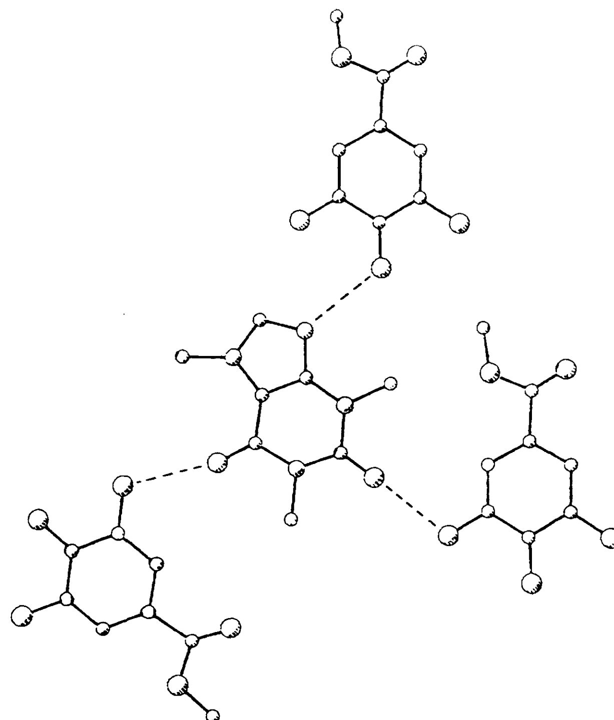
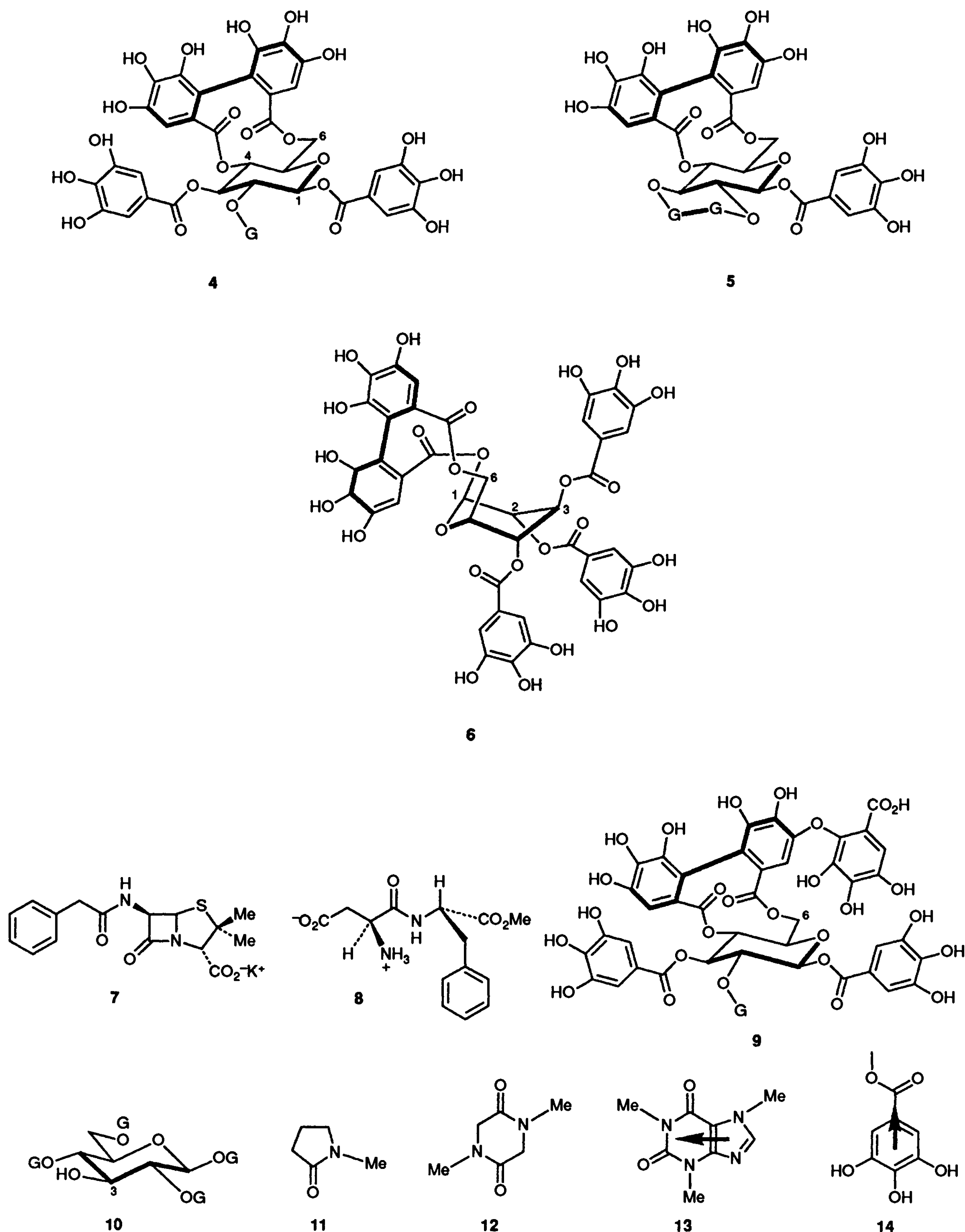


Fig. 3. Caffeine–methyl gallate complex: in-plane hydrogen bonding.

Δδ, of the protons of the polyphenol) permitted assignments to be made of the preferred sites of complexation with caffeine. These assignments were made on the basis of the earlier studies [1] using 2-dimensional $^{1}H-^{13}C$ correlation methods to identify particular phenolic ester groups in a natural polyphenol and the determination of partial association constants for these sites. In the galloyl D-glucose series each galloyl ester group in 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (**10**) binds caffeine independently and to broadly similar extents. The galloyl ester groups at positions 1 and 6 in 1,2,6-tri-*O*-galloyl-β-D-glucose, 1,2,4,6- and β-1,2,3,6-tetra-*O*-galloyl-β-D-glucose and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose **2** are, however, in these substrates, the overwhelmingly preferred sites for caffeine complexation. Confirming earlier observations the hexahydroxydiphenoyl ester group (Fig. 4) has a substantially reduced affinity for caffeine and in polyphenolic substrates containing this group caffeine generally binds selectively at positions bearing a galloyl ester group. However, for the 'dimeric' species sanguin H-6 (**3**) caffeine binds highly selectively in the region of the galloyl ester group * at C-1 (ring A) and the two polyphenolic nuclei * at C-6 (ring A) and C-3 (ring B). Such an observation is readily explicable if the 'dimeric' polyphenol naturally adopts a 'sandwich type' conformation.[1] Molecular models indicate that, in such a conformation, the three phenolic rings delineated form a pocket into which the caffeine may fit. In phenolic flavan-3-ols **27**–**31**, rings A and C provide the general site for caffeine association, but in the flavan-3-ol gallates **32** and **33** the galloyl ester becomes the preferred site for complexation.



Clearly, caffeine **1** possesses a number of features which optimise its effectiveness as a small molecule for complexation with polyphenolic substrates. The phenolic group is a good proton donor but poor acceptor in hydrogen-bonding systems.[28] The phenolic groups of galloyl esters and their derivatives would be predicted to possess enhanced proton-donor capabilities due to the influence of the ester carbonyl group. Various lines of evidence, albeit indirect, with substrates such as **11** and **12**, support the contention that the tertiary amide carbonyl [-CO(NMe)-] groups of caffeine are likewise good proton acceptors.[28–31] It thus seems probable that, in the complexation reactions of caffeine with polyphenols, hydrogen bonding between the polyphenol (proton donor) and the caffeine (proton acceptor) may ultimately make specific contributions to the stability of the complex (as in Fig. 3), presumably through the entropy gain as bound water molecules of solvation of both substrates are released.

The hydrophobic contribution is nevertheless probably the most important single factor influencing caffeine (and protein)–polyphenol interactions in aq. media. A distinctive feature of all

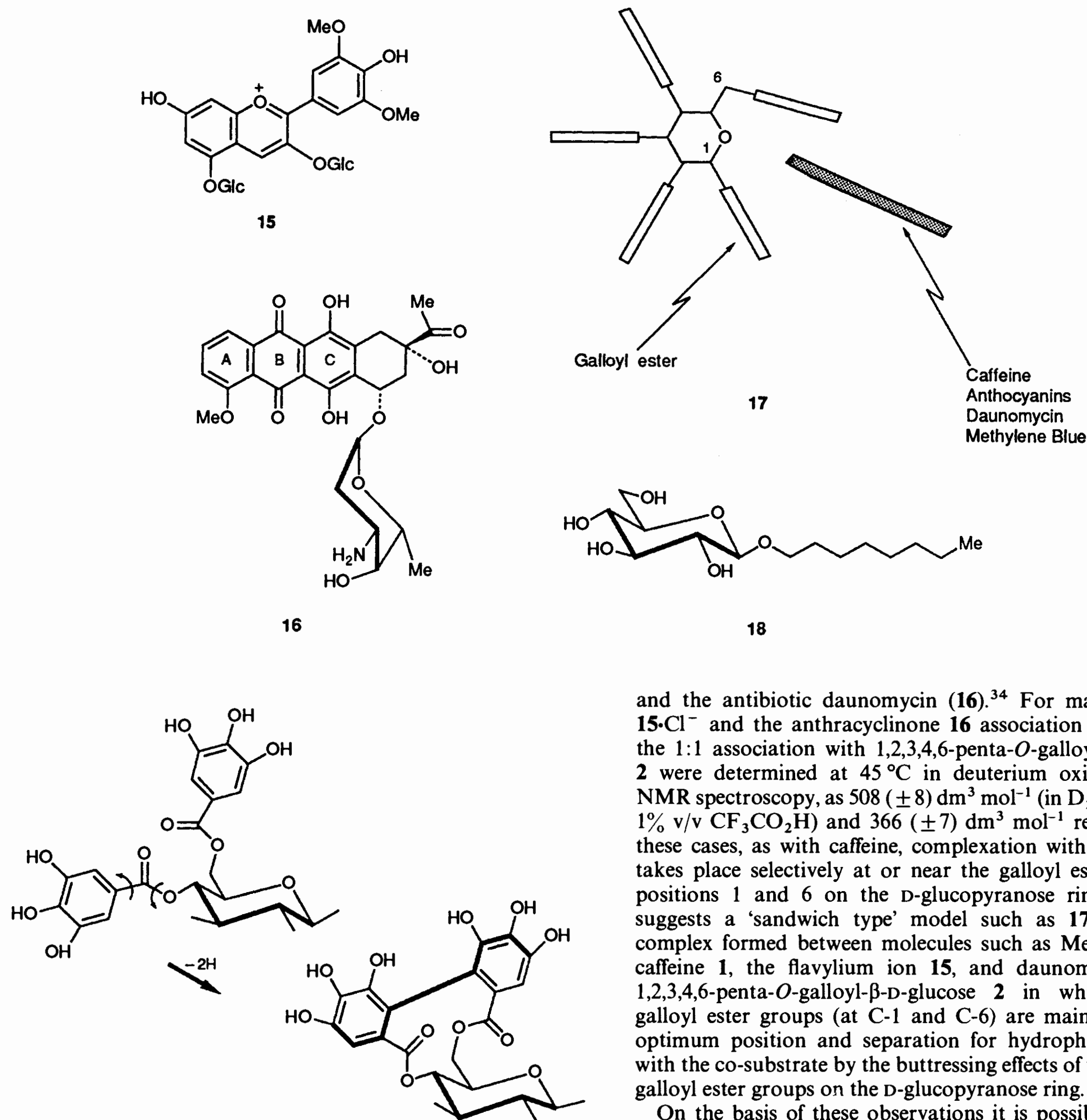


Fig. 4. Biosynthesis of the hexahydroxydiphenoyl ester group—loss of conformational mobility.

the crystalline phenol–caffeine complexes examined (X-ray) is the relative orientation of the planar caffeine and the phenolic partner in the layer lattice. The term 'polarisation bonding' has been coined [32] to describe the weak interactions between polar groups of one component and a polarisable region in the second in the formation of intermolecular complexes. Its principal feature is the juxtaposition of the polarising groups of one molecule and the polarisable region of the second. It is therefore of some significance to note that in the various phenol–caffeine complexes the phenolic groups and the associated aromatic nuclei are generally stacked above the 6-membered ring of caffeine (Fig. 2). This feature suggests that in this form of association the two molecules develop complementary interacting dipoles (*e.g.*, **13** and **14**). It should also be noted that polyphenols complex effectively with other species which can develop similar complementary dipolar characteristics, *e.g.* Methylene Blue,[33] the anthocyanin flavylium ion [*e.g.*, **15**],[34] and the antibiotic daunomycin (**16**).[34] For malvin chloride **15**$\cdot$Cl$^-$ and the anthracyclinone **16** association constants for the 1:1 association with 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose **2** were determined at 45 °C in deuterium oxide, using ^{1}H NMR spectroscopy, as 508 (±8) dm^3 mol^{-1} (in D_2O containing 1% v/v CF_3CO_2H) and 366 (±7) dm^3 mol^{-1} respectively. In these cases, as with caffeine, complexation with compound **2** takes place selectively at or near the galloyl ester groups at positions 1 and 6 on the D-glucopyranose ring of **2**. This suggests a 'sandwich type' model such as **17** for the 1:1 complex formed between molecules such as Methylene Blue, caffeine **1**, the flavylium ion **15**, and daunomycin **16** and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose **2** in which the two galloyl ester groups (at C-1 and C-6) are maintained in the optimum position and separation for hydrophobic stacking with the co-substrate by the buttressing effects of the remaining galloyl ester groups on the D-glucopyranose ring.

On the basis of these observations it is possible to suggest that the polyphenol–caffeine aggregates which form in solution, sufficient to induce precipitation (*vide supra*), take the form shown (Fig. 5). In this model, caffeine molecules cross-link (by hydrophobic stacking and/or hydrogen bonding) separate polyphenol molecules, rather as the latter cross-link proteins in the analogous protein–polyphenol precipitation processes.[35]

Cyclodextrins.—In an early study of the association of simple phenols with proteins Hansch *et al.*[36] observed that the association of the phenols very closely paralleled the transfer of the phenols from the water phase to octan-1-ol. Although there are similar parallels for polyphenols, the overall correlation between the affinity for proteins and the distribution between octan-1-ol and water was not consistent.[1] However, in studies of the association of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose **2** and (−)-epigallocatechin 3-*O*-gallate **32**, 0.003–0.021 mol dm^{-3}, with octyl β-D-glucoside [**18**; above (1.6%), and below (0.4%), the critical micelle concentration] in deuterium oxide monitored by ^{1}H NMR spectroscopy the protons of the D-glucopyranose ring of compound **18**, excepting the anomeric proton 1-H, were little affected whilst all those of the alkyl chain and 1-H experienced major upfield chemical-shift

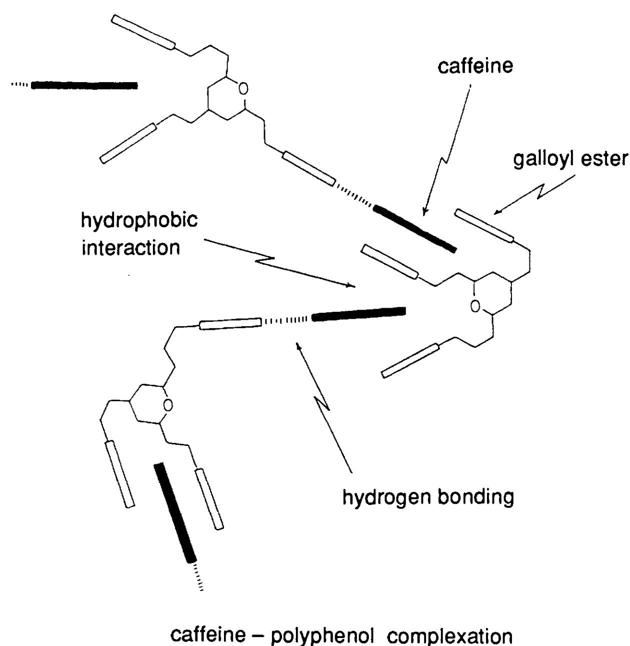


Fig. 5. Polyphenol–caffeine precipitation model.

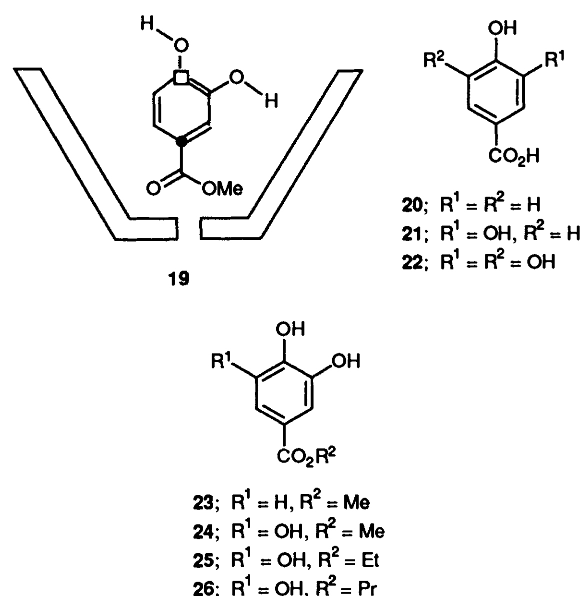
Table 6. Phenol–α-cyclodextrin association constants determined by ^{1}H NMR spectroscopy (250 and 400 MHz) in deuterium oxide.[a]

Phenol	Association constant K dm^3 mol^{-1}	
	20 °C	45 °
p-Nitrophenol	160 (±4.8)	
p-Hydroxybenzoic acid (**20**)	1361 (±40)	473
3,4-Dihydroxybenzoic acid (**21**)	702 (±21)	
2,4-Dihydroxybenzoic acid	28.5 (±0.9)	
3,4,5-Trihydroxybenzoic acid (**22**)	215 (±6.5)	
Methyl 3,4-dihydroxybenzoate (**23**)	448 (±13)	138 (±4)
Methyl 2,3,4-trihydroxybenzoate		10.0 (±0.3)
Methyl 3,4,5-trihydroxybenzoate (**24**)	15.2 (±0.5)	

[a] Determined using the chemical-shift changes for 3-H of the α-cyclodextrin.

changes. This suggests that in aq. media the polyphenols associate preferentially with compound **18** in the region of the hydrocarbon chain.

It has been known for some time that polysaccharides can adopt a variety of ordered shapes in the condensed phase and in several instances such conformations persist in solution.[37] Polyphenols bind strongly to some polysaccharides of this type (*e.g.*, starch) and studies of the behaviour of polyphenols with Sephadex gels point[23,38] to encapsulation within the pores of the gel as a factor which strongly influences chromatographic behaviour. The general significance of this form of cavity sequestration has been further investigated in model studies with α- and β-cyclodextrin. One of the most distinctive properties of the cyclodextrins is their ability to include substrates in the cavity formed by the six (α) or seven (β) 1-α-4 linked D-glucopyranose residues.[39,40] X-Ray crystallographic analyses of many aromatic–cyclodextrin complexes have been performed;[41–45] in the majority of these the phenyl ring penetrates into the cyclodextrin cavity. With the *para*-substituted phenols, the phenolic group is usually excluded from the cavity but the orientation is reversed in permethylated α-cyclodextrins.[46] In solution, complexation with aromatic



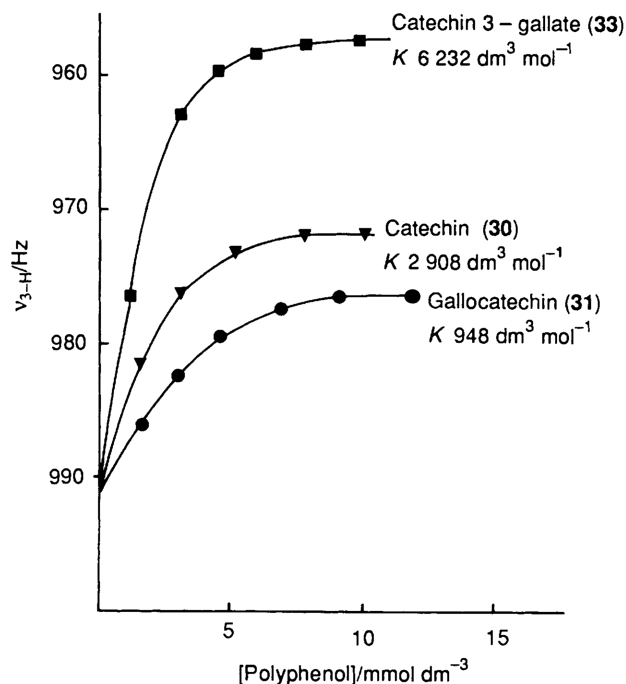


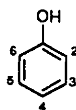
Fig. 6. Polyphenols–β-cyclodextrin complexation–binding curves. Plots of chemical shift (ν) *versus* polyphenol concentration, (β-cyclodextrin concentration 3 × 10^{-3} mol dm^{-3}, measurements at 250 MHz).

substates has been probed by following the ^{1}H and ^{13}C NMR chemical-shift changes, induced by the magnetic anisotropy of the phenyl ring as it penetrates the cavity, on the two concentric rings of C–H groups (at C-3 and C-5) in the cavity;[47–51] less frequently by following changes in the chemical shifts of proton and carbon nuclei in the substrate.[50,52]

The interactions of a range of simple phenols with α-cyclodextrin in deuterium oxide have been measured, and association constants determined (Table 6) using both ^{1}H and ^{13}C NMR spectroscopy. Invariably the protons 3-H of the α-cyclodextrin displayed a strong upfield shift and those at 5-H showed a downfield shift upon inclusion of the aromatic guest. The ^{1}H chemical-shift changes experienced by the guest phenols (Table 7) are also consistent with earlier measurements,

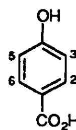
Table 7. Proton chemical-shift displacements (250 MHz; $\Delta\delta$ in Hz), for phenolic substrates, induced by α-cyclodextrin at 20 °C. Phenolic substrates 6×10^{-3} mol dm^{-3}; α-cyclodextrin 1.2×10^{-2} mol dm^{-3}.

Phenols



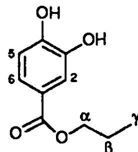
Phenol	2-H	3-H	4-H	5-H	6-H
Catechol		−0.6	−1.9	−1.9	−0.6
Resorcinol	4.7		−0.2	1.2	−0.2
Quinol (hydroquinone)	−2.5	−2.5		−2.5	−2.5
Phloroglucinol	1.2		1.2		1.2
Quinol β-D-glucoside	−4.0	−1.1		−1.1	−4.0
p-Nitrophenol	−23.2	−40.9		−40.9	−23.2
p-Nitrophenyl β-D-galactoside	−29.9	−40.8		−40.8	−29.9

Phenolic Acids



Phenolic acid	2-H	3-H	5-H	6-H
p-Hydroxybenzoic acid (**20**)	−63.2	−16.2	−16.2	−63.2
3,4-Dihydroxybenzoic acid (**21**)	−80.8		−11.0	−69.5
2,4-Dihydroxybenzoic acid		−14.2	−9.5	−18.0
3,4,5-Trihydroxybenzoic acid (**33**)	−56.3			−56.3

Phenolic esters



	2-H	5-H	6-H	α-H	β-H	γ-H
Methyl protocatechuate (**23**)	−69.9	11.6	−59.8	−0.8		
Methyl 2,3,4-trihydroxybenzoate		−3.0	−4.1	2.8		
Methyl gallate (**24**)	−35.3		−35.3	−3.4		
Ethyl gallate (**25**)	15.6		15.6	−6.9	−7.7	
Propyl gallate (**26**)	7.6		7.6	−6.2	−19.9	−12.5

indicating that, wherever possible, the phenolic groups are excluded from the cyclodextrin cavity **19**. For methyl protocatechuate this orientation **19** was confirmed by ^{13}C NMR measurements which showed a shielding of the *ipso* C atom **19**; ●, $\Delta\delta_1$ 0.876 ppm, compared with the deshielding of the corresponding *para* C atom **19**; □, $\Delta\delta_4$ 0.91 ppm.[49] A noticeable feature of these results is the decrease in association constant (Table 6) in passing from *p*-hydroxybenzoic acid **20**, through protocatechuic acid **21** to gallic acid **22** and similarly with the corresponding methyl esters. It is presumed that this pattern reflects the increasing size of the solvation shell associated with the phenolic groups **20**–**22** which increasingly inhibits cavity penetration.

Similar trends and observations followed studies with simple

Table 8. Phenol–β-cyclodextrin association constants determined by ^1H NMR spectroscopy (250 and 400 MHz) in deuterium oxide.[a]

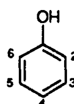
	K dm^3 mol^{-1}	
Phenol	20 °C	45 °C
Catechol	109 (±3)	
Resorcinol	117 (±4)	
Quinol (hydroquinone)	100 (±3)	
Phloroglucinol	110 (±3)	
p-Nitrophenol	407 (±12)	
p-Hydroxybenzoic acid (**20**)	1616 (±48)	
3,4-Dihydroxybenzoic acid (**21**)	459 (±14)	
2,4-Dihydroxybenzoic acid	1330 (±40)	
3,4,5-Trihydroxybenzoic acid (**22**)	114 (±3)	
Methyl protocatechuate (**23**)		280 (±8)
Methyl 2,3,4-trihydroxybenzoate		212 (±6)
Methyl gallate (**24**)		120 (±4)

[a] Determined using the 5-H chemical-shift changes in β-cyclodextrin.

phenolic substrates and β-cyclodextrin (Table 8). This cyclodextrin possesses a larger cavity,[39] which it is believed permits deeper penetration of the aromatic guest into the cavity. Consistent with this view are the greater proton chemical-shift changes now observed for 5-H as compared with 3-H of the cyclodextrin and the reversal of sign of the proton chemical-shift changes of the guest (Table 9) as compared with those observed with α-cyclodextrin. A number of features command immediate attention in the studies of polyphenol–β-cyclodextrin complexation. Compared with that of galloyl esters the binding of flavan-3-ol substrates **27**–**33** is quite strong (Table 10, Fig. 6). It is seemingly dependent on the stereochemistry of the hydroxy group at C-3 in the flavan and is diminished by successive hydroxy-group substitution in the B-ring (cf. **27**–**29**]. Complexation is enhanced by galloylation at the 3-hydroxy group; modestly (~2×) in the case of (+)-catechin **30** *vs.* **33**, but substantially (~9×) with (−)-epigallocatechin **29** *vs.* **32**. Significantly, substitution at C-4 by additional flavan-3-ol molecules to give the typical proanthocyanidin[2] structures **34** and **35** virtually suppresses completely the ability to complex with the cyclodextrins. For the flavan-3-ol phenolic substrates **27**–**33**, the expectation is that several modes of association are probably in operation involving the insertion of both rings A and B into the cyclodextrin cavity (Fig. 7a, b and c). That association occurs in part by method (c)—Fig. 7—is indicated by comparison of the association constants for (−)-epiafzelechin **27**, (−)-epicatechin **28** and (−)-epigallocatechin **29**. Increasing hydroxylation in ring B (increasing size, and increasing size of the solvation shell) steadily reduces the affinity of the phenolic flavan-3-ol substrate for β-cyclodextrin. Molecular models point to possible explanations for the influence of the stereochemistry of hydroxy-group substitution at C-3 upon the complexation and its enhancement by gallate ester formation at C-3. This latter effect is envisaged, at least in part, as being due to the pendant 3-galloyl ester group acting as an anchor (by hydrogen-bond formation) to the peripheral hydroxy groups at C-2 and C-3 on the top rim of the cyclodextrin cavity. Likewise the disposition of the hydroxy group at C-3 in the (+)-catechin series (3*S*)-**30** is such as to make hydrogen bonding possible (in a similar fashion) to the cyclodextrin, whilst that in the (−)-epicatechin (3*R*)-**27** series is not. Support for this general picture was also derived from intermolecular NOE experiments[53] (Table 11). However, a distinctive feature of the ^1H NMR studies conducted with all the flavan-3-ol substrates **27**–**33** is the large chemical-shift changes experienced by 6-H_2 of the D-glucosyl residues of the

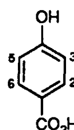
Table 9. Proton chemical-shift displacements (250 MHz; Δδ in Hz), for phenolic substrates, induced by β-cyclodextrin. Phenolic substrate 6×10^{-3} mol dm^{-3}; β-cyclodextrin 1.2×10^{-2} mol dm^{-3}.

Phenols[a]



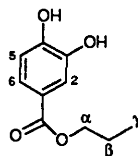
Phenol	2-H	3-H	4-H	5-H	6-H
Catechol		5.5	5.9	5.9	5.5
Resorcinol	13.1		17.1	18.9	17.1
Quinol (hydroquinone)	10.8	10.8		10.8	10.8
Phloroglucinol	26.7		26.7		26.7
Quinol β-D-glucoside	13.8	−0.7		−0.7	13.8
p-Nitrophenol	9.7	−3.8		−3.8	9.7
p-Nitrophenyl β-D-galactoside	8.4	−15.6		−15.6	8.4

Phenol carboxylic acids[a]



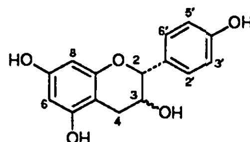
Phenolic acid	2-H	3-H	5-H	6-H
p-Hydroxybenzoic acid (**20**)	8.3	0.9	0.9	8.3
3,4-Dihydroxybenzoic acid (**21**)	1.4		13.8	15.2
2,4-Dihydroxybenzoic acid		−8.0	2.1	11.0
3,4,5-Trihydroxybenzoic acid (**22**)	3.0			3.0

Phenolic esters[a]



Phenolic ester	2-H	5-H	6-H	α-H	β-H	γ-H
Methyl protocatechuate (**23**)	3.1	23.4	13.3	−10.3		
Methyl 2,3,4-trihydroxybenzoate		8.0	8.4	2.8		
Methyl gallate (**24**)	7.8		7.8	−4.5		
Ethyl gallate (**25**)	10.0		10.0	−6.6	−7.9	
Propyl gallate (**26**)	12.8		12.8	−15.2	−10.2	−8.9

Phenolic flavan-3-ols[b]



Flavan-3-ol	2-H	3-H	4-H[α]	4-H[β]	6-H	8-H	2′-H	3′-H	5′-H	6′-H
(+)-Catechin (**30**)	−13.0	16.0	50.6	−27.7	20.1	3.3	43.7		41.0	33.2
(+)-Gallocatechin (**31**)	6.2	20.3	13.7	−24.1	28.6	4.5	32.3			32.3
(+)-Catechin 3-gallate (**33**)	7.8	33.1	−50.8	1.9	89.8	2.6	−24.9		−24.9	−4.3
(−)-Epiafzelechin (**27**)	16.8	15.0	18.4	−6.8	31.8	13.8	13.1	0	0	13.1
(−)-Epicatechin (**28**)	25.5	13.1	5.4	−8.7	30.5	14.8	13.2		6.2	0.5
(−)-Epigallocatechin (**29**)	24.2	9.3	−10.6	−13.0	20.6	12.9	1.6			1.6
(−)-Epigallocatechin 3-gallate (**32**)	52.9	46.7	−4.7	−59.2	25.8	36.5	−48.8			−48.8

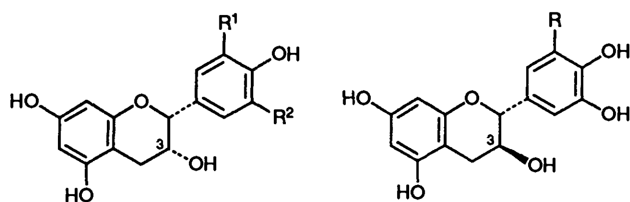
[a] Measured at 20 °C. [b] Measured at 45 °C.

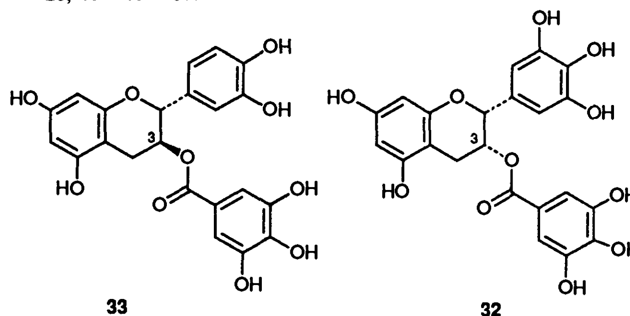
cyclodextrin. In all the cases investigated these were comparable to those shown for 5-H. Additionally the ratio of Δδ(1-H):Δδ(4-H) lay in the region (4–20:1). This suggests the possibility that the cyclodextrin cavity may also (in part) undergo penetration from the primary hydroxy group face, leading to a change in the preferred orientation of the $-CH_2OH$

Table 10. Polyphenol-β-cyclodextrin association constants determined by ^1H NMR spectroscopy (250 and 400 MHz) in deuterium oxide.[a]

Polyphenol	$K/dm^3\ mol^{-1}$	
	45 °C	60 °C
(+)-Catechin (**30**)	2908 (±87)	
(+)-Gallocatechin (**31**)	948 (±28)	
(+)-Catechin gallate (**33**)	6232 (±187)	
(−)-Epiafzelechin (**27**)	793 (±24)	
(−)-Epicatechin (**28**)	464 (±14)	
(−)-Epigallocatechin (**29**)	208 (±6)	
(−)-Epigallocatechin gallate (**32**)	1889 (±55)	
Procyanidin B-2 (**34**)	63 (±2)	
Procyanidin B-3 (**35**)	101 (±3)	
1,3-6-Tri-*O*-galloyl-β-D-glucose		340 (±10)
1,2,4,6-Tetra-*O*-galloyl-β-D-glucose (**10**)		316 (±10)
1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (**2**)		339 (±10)
Davidiin (**6**)		76 (±2)

[a] Determined using chemical-shift changes for 3-H and/or 5-H in β-cyclodextrin.





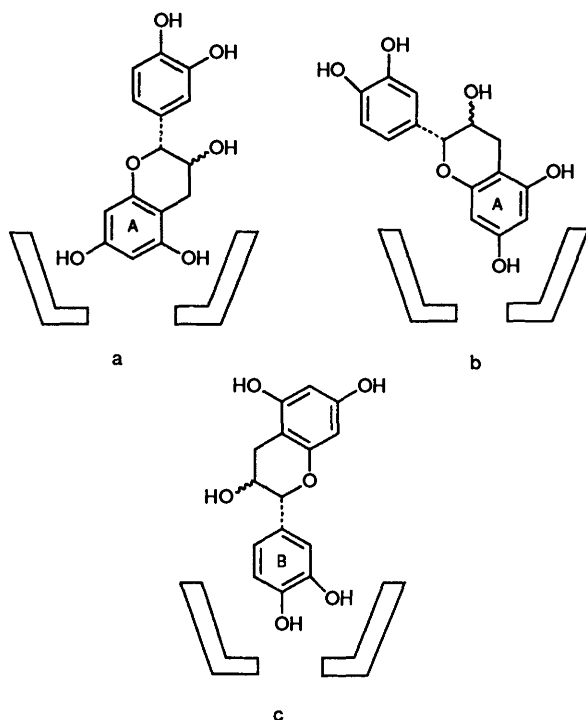


Fig. 7. Polyphenol–β-cyclodextrin complexation model.

group around the C(5)–C(6) bond,[41,48] and to a relative change in the environment of the protons 1-H and 4-H.

Loss of astringency is one of the major changes which takes place during the ripening of fruit.[54] One explanation which has been advanced is that other macromolecular species (*e.g.*, pectins) become available (soluble) during the ripening process and that these subsequently modify and disrupt the ability of polyphenols to bind to glycoproteins in the mouth when the fruit is tasted. Support for this theory is the observation that the cyclodextrins relieve the induced polyphenol inhibition of the enzyme β-glucosidase.[55] Further support for this mechanism has now been derived in the model ternary system polyphenol–caffeine–β-cyclodextrin in which the caffeine acts as a peptide model and the cyclodextrin as a polysaccharide model. In the presence of the β-cyclodextrin the complexation of typical flavan-3-ols with caffeine **1** is substantially reduced (Table 12 *cf.* K_a and K), indicating that complexation of the phenolic substrate with caffeine is inhibited by its partial inclusion within the cyclodextrin cavity as shown in structure **36**.

These results form the basis for the interpretation of the wide ranging behaviour of polyphenols in their complexation with natural macromolecules (*vide supra*) and these studies will be reported in future work.

Experimental

General Methods.—Polyphenols were isolated and purified as previously described.[1,56–63] NMR experiments were carried out using a Bruker WH-400 spectrometer equipped with an Aspect 2000 computer, or a Bruker AM-250 spectrometer. Temperatures were accurately measured using a Comard series 5000 electronic thermometer attached to a copper–constantan thermocouple in an NMR tube containing the chosen solvent.

Caffeine–Methyl Gallate Complex (1:1).—A mixture of methyl gallate (184 mg, 1 mmol) and caffeine (194 mg, 1 mmol)

Table 11. Intermolecular NOEs for polyphenol–β-cyclodextrin complexes. Measured at 45 °C in deuterium oxide at 400 MHz. Polyphenol, β-cyclodextrin; 2×10^{-2} mol dm^{-3}.

β-Cyclodextrin irradiation

Polyphenol	Proton(s) irradiated	Observed NOE (polyphenol)
Methyl gallate (**24**)	3-H	2-H, 6-H (2.4%)
(+)-Catechin (**30**)	3-H	2′, 5′-, 6′-H (2.4%)
(+)-Catechin gallate (**33**)	3-H	2′-, 5′-, 6′-H (0.2%)
(−)-Epigallocatechin gallate (**32**)	3-H	2′-, 6′-H (2.9%); 6-, 8-H (1.1%)
	5-H	6-, 8-H (3.6%)

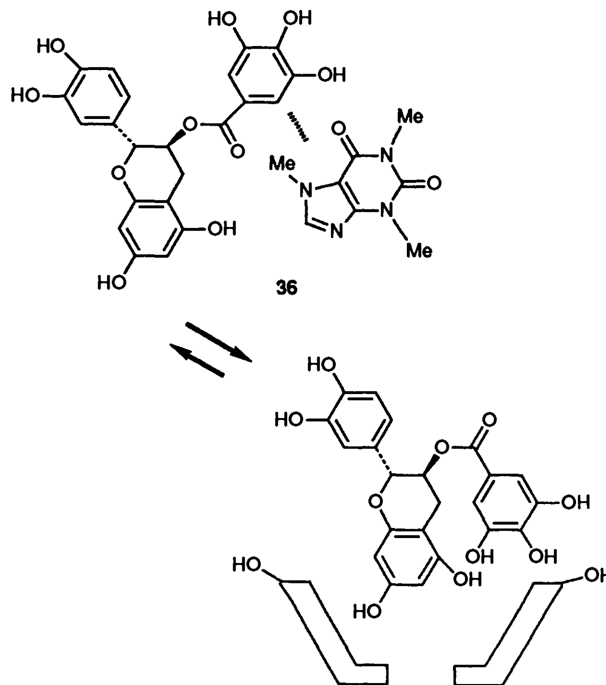
Polyphenol irradiation

Polyphenol	Proton(s) irradiation	Observed NOE (cyclodextrin)
Methyl gallate (**24**)	2-, 6-H	3-H (1.3%)
(+)-Catechin (**30**)	2′-, 6′-H	3-H (1.7%); 5-H (1.5%)
	5′-H	3-H (1.1%); 5-H (0.6%)
(+)-Catechin gallate (**33**)	2′-, 5′-, 6′-H	3-H (1.1%); 5-H (0.2%)
(−)-Epigallocatechin gallate (**32**)	2′-, 6′-H	3-H (0.8%); 5-H (0.6%)
	6-, 8-H	5-H (0.9%)

Table 12. Polyphenols–caffeine. Apparent association constants K_a measured in the presence of β-cyclodextrin[a] in deuterium oxide at 45 °C by ^{1}H NMR spectroscopy (400 MHz).

Polyphenol	K/dm^3 mol^{-1} [b]	K_a/dm^3 mol^{-1}
(+)-Catechin gallate (**33**)	38.2	4.4
(−)-Epigallocatechin gallate (**32**)	52.8	12.6

[a] Concentration of β-cyclodextrin 3.0×10^{-3} other conditions as specified in Table 5. [b] K values from Table 5.



was dissolved in the minimum of hot (70 °C), glass-distilled water. The solution was allowed to cool to room temperature and the precipitated complex was collected. The complex was recrystallised from glass-distilled water. The solution was allowed to cool to room temperature over a period of 30 h by immersion in water (60 °C) contained in a thermos flask. The *caffeine–methyl gallate* complex formed long needles (250 mg), m.p. 201–202.5 °C (Found: C, 50.7; H, 4.8; N, 15.0. $C_{16}H_{18}N_4O_7$ requires C, 50.8; H, 4.8; N, 14.8%).

Potassium Chlorogenate-Caffeine Complex.—The crude complex was isolated from green coffee beans by the procedure of Gorter.[63] The complex was recrystallised five times from ethanol–water (4:1, v/v), using decolourising charcoal at each stage. The complex was obtained as pale yellow rhombs (Found: C, 46.6; H, 5.0; N, 9.0. $C_{16}H_{17}KO_9{\cdot}C_8H_{10}N_4O_2{\cdot}2H_2O$ requires C, 46.9; H, 4.7; N, 9.0%). X-Ray crystallographic analyses of these two complexes have been reported[10,20] and atomic co-ordinates are available on request from the Director of the Cambridge Crystallographic Data Centre.*

Caffeine–Polyphenol Precipitation.—A polyphenol (5–15 mg) was weighed directly into a centrifuge tube (10 cm^3) and a fixed volume (5 cm^3) of caffeine (15×10^{-3} mol dm^{-3}) in glass-distilled water was added. The solution was warmed to 60 °C and then allowed to cool to 20 °C during 24 h. The solution was centrifuged and the supernatant was removed by decantation. The precipitate was washed with cold water (2 cm^3; 10 °C). The supernatant and aq. washings were combined and evaporated to dryness under reduced pressure. The supernatant residues and precipitate were dissolved separately in acetone and evaporated to dryness at 60 °C. The final complex (precipitate) and supernatant were analysed by ^{1}H NMR spectroscopy after dissolution in [$^2H_6$]acetone; galloyl esters were estimated by integration of the anomeric proton signal,[1] and flavan-3-ol derivatives by integration of the 2-H signals; caffeine was estimated using integration of the 8-H signal (Tables 1 and 2).

* See footnote 5.6.3 of Instructions for Authors, January issue.

Caffeine–Polyphenol Association Constants.—(a) *Caffeine constant.* Samples were prepared in deuterium oxide. For each polyphenol system a caffeine solution was prepared (3×10^{-3} mol dm^{-3}) and an aliquot (1 cm^3) was added to a precisely weighed sample of the polyphenol to give final concentrations of the polyphenol in the range 0–12 $\times 10^{-3}$ mol dm^{-3}. Samples were allowed to spin in the NMR spectrometer probe (set at 45 or 60 °C) for 20 min to reach thermal equilibrium before spectra were recorded. Measurements of chemical shift (8-H, caffeine) were made with respect to an external standard (sodium trimethsilylpropionate, TSP) contained in a reference capillary. Operational errors were estimated as ± 0.37 Hz.

Association constants for the formation of a 1:1 complex were determined using equation (1), incorporated into a standard non-linear least-squares curve-fitting program, where

$$K = \Delta\delta/[\Delta\delta^{\circ} - \Delta\delta)(P_0 - C_0 \cdot \Delta\varepsilon_0)] \quad (1)$$

(i) $\Delta\delta = \delta - \delta^{\circ}c$ and $\Delta\delta^{\circ} = \delta^{\circ}cp - \delta^{\circ}c$; (ii) δ is the experimentally measured chemical shift of the caffeine signal (8-H); $\delta^{\circ}C$ and $\delta^{\circ}cp$ are the chemical shifts of the proton in caffeine and the complex, respectively, and (iii) C_0 and P_0 are the initial concentrations of the caffeine and polyphenol, respectively. Results are shown in Tables 4, 5 and 12 for β-cyclodextrin.

(b) *Polyphenol constant.* In the reverse type of experiment the polyphenol solution concentration was set at (3.0×10^{-3} mol dm^{-3}) and the concentration of caffeine was varied over the range (0–16 $\times 10^{-3}$ mol dm^{-3}). Measurements of the chemical-shift change of particular galloyl ester protons in the polyphenol substrate[1] were monitored to give partial association constants for particular sites in the polyphenol.

Cyclodextrin–Polyphenol Association Constants.—A solution of the cyclodextrin (α or β) in deuterium oxide was prepared (3×10^{-3} mol dm^{-3}). An aliquot of this solution (1 cm^3) was added to a precisely weighed amount of the polyphenol to give final concentrations of the polyphenol in the range 0–15 $\times 10^{-3}$ mol dm^{-3}). Measurements of chemical shift (3-H for α-cyclodextrin, 5-H for β-cyclodextrin) and data analysis were carried out as discussed earlier for caffeine. Results are shown in Tables 6, 8 and 10.

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