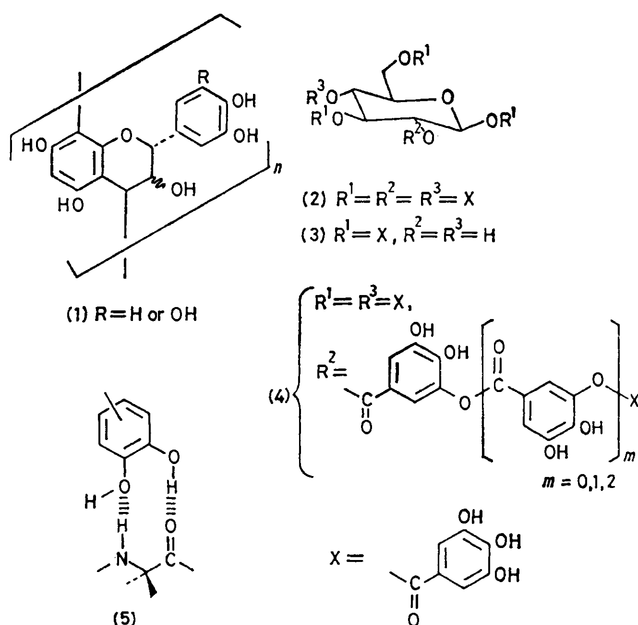
The Association of Proteins with Polyphenols

By JOHN P. MCMANUS, KENNETH G. DAVIS, TERENCE H. LILLEY,* and EDWIN HASLAM*
(*Department of Chemistry, University of Sheffield, Sheffield* S3 7HF)

Summary The complexation of phenols with proteins has been quantitatively examined using a range of physical methods; a theory is put forward for the precipitation of proteins from solution by complex polyphenols and by simple phenols.

Polyphenols are a distinctive group of higher plant secondary metabolites.[1] Two important classes of polyphenols {the proanthocyanidins[2] (**1**) and the esters of gallic acid[3] [*e.g.* (**2**), (**3**), and (**4**)]} possess the unique property of precipitating natural macromolecules (particularly proteins) from solution.[4] This phenomenon has long been recognised as being responsible for the astringency of unripe fruit and various beverages,[5] the impaired nutritional quality of some cereals and herbiage crops,[6] and the inactivation of enzymes and viruses.[7] The property has likewise been widely applied (*e.g.* in medicine, in the conversion of hide into leather,[8] and in the purification of enzymes[9]). However, quantitative investigations of the property have, until recently, not been possible and, in consequence, as a physical process it remains poorly understood.



The association of a range of simple phenols (resorcinol, catechol, pyrogallol, and methyl gallate) with bovine serum albumin (BSA, R.M.M. 69,000) has been studied using equilibrium dialysis[10] and microcalorimetry[11] in order to determine the influence of hydroxy-group configuration on the complexation. The data obtained with BSA using equilibrium dialysis gave Scatchard plots whose analysis was amenable to a multiple site binding treatment,[12] (Table).

Table. Binding parameters for the interaction of simple phenols with BSA. The parameters n_1, K_1 and n_2, K_2 were obtained from Scatchard plots assuming two-site binding.

	n_1	K_1 (kg mol^{-1})	n_2	K_2 (kg mol^{-1})
Resorcinol	10	9·2	—	—
Catechol	1	$3{\cdot}5 \times 10^4$	8	$4{\cdot}6 \times 10^2$
Pyrogallol	6·6	$1{\cdot}0 \times 10^4$	58·0	$4{\cdot}3 \times 10^2$
Methyl gallate	4·3	$1{\cdot}5 \times 10^4$	6·3	$4{\cdot}8 \times 10^2$

The derived values for the number of secondary sites (n_2) on the protein molecule accessible to complexation with the phenol is obtained by extrapolation and in all cases it probably represents the minimum value. These data are, in consequence, best viewed in a comparative rather than an absolute sense. Thus the affinity of resorcinol (1,3-dihydroxybenzene) for BSA is clearly weak but with catechol and pyrogallol, which have respectively two and three *ortho* disposed phenolic groups, the binding to protein is considerably enhanced both in its strength (K_1 and K_2) and in the number of primary (n_1) and secondary (n_2) sites on the protein molecule which are available for complexation. These observations give quantitative support to the earlier suggestion[13] that the *ortho*-dihydroxyphenolic groups in natural polyphenols are the primary points for the association with protein and that isolated phenolic groups do not participate to any significant extent. The complexation may occur primarily *via* a bidentate hydrogen bond formation with the keto-imide groups on the protein (**5**).

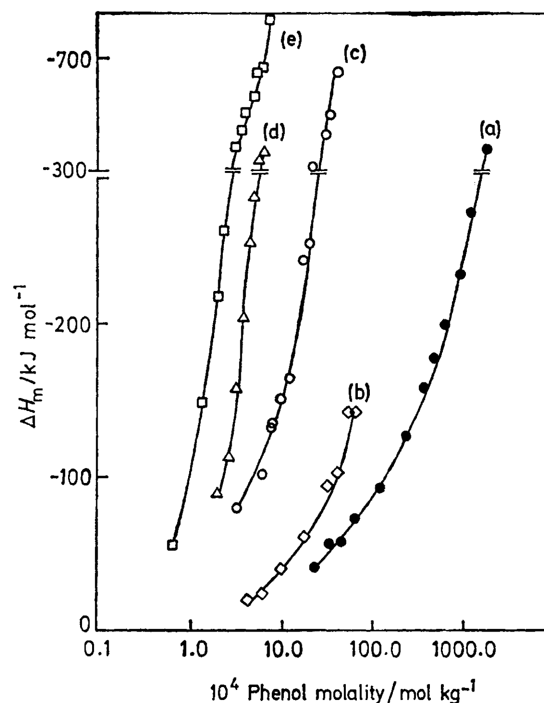


Figure 1. Calorimetric isotherm; the enthalpy change per mole of BSA plotted against phenol molality. (a) Catechol, (b) procyanidin B-3 (**1**; $n = 2$), (c) β-1,3,6-tri-*O*-galloyl-D-glucose (**3**), (d) tannic acid (**4**), and (e) β-penta-*O*-galloyl-D-glucose (**2**). Measurements at pH 6·8 and 298·15 K. Protein concentration in systems (a) and (b) was *ca.* 10^{-4} molal and in systems (c), (d), and (e) was *ca.* 3×10^{-5} molal.

Similar data were obtained, at fixed protein concentrations, for a range of natural polyphenols [*e.g.* (**1**; $n = 2$) (**2**), (**3**), and (**4**)] of increasing molecular size and complexity and Figure 1 shows the isotherms obtained for the enthalpy of association of these phenols with BSA. It is significant to note that the optimum level of association in the galloylglucose series occurs with β-pentagalloyl-D-glucose (**2**) and not the higher molecular weight ($\simeq$ heptagalloyl) tannic acid (**4**). The greater degree of binding to protein displayed by these polyphenols was not amenable to a Scatchard analysis and their associative behaviour displayed further distinctive features. Thus *lowering* the protein concentration (10^{-5} to 10^{-6} molal BSA) leads to an *increased* molar enthalpy of interaction with tannic acid (generally some 4 to

10 times greater) at a fixed tannic acid concentration. In addition, at concentrations where the polyphenol-protein complex is precipitated little or no heat change[14] due to the precipitation process was observed and this favours the proposition that the structure of the protein-polyphenol complex in the solid state closely resembles that in solution.

The precipitation was dependent on pH (pH 4·0 maximum with BSA), and the stoicheiometry of the protein-polyphenol complex required for precipitation (determined by a turbidimetric titration procedure and by micro-analytical data on the precipitates) also showed a similar dependence on the initial protein concentration. At low protein concentrations (10^{-6} molal BSA) the stoicheiometry of the protein-polyphenol complex was *ca.* 1 : 120 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 proclivity may be inferred from the data of van Buren and Robinson.[15] 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.[9]

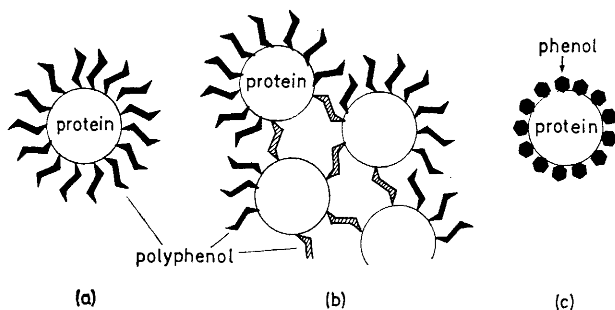


Figure 2. Protein precipitation by phenols. (a) Polyphenols, low protein concentrations. (b) Polyphenols, high protein concentrations. (c) Simple phenols.

These observations now permit a hypothesis to be advanced to explain the propensity of some polyphenols such as tannic acid (**4**) and the higher oligomeric proanthocyanidins (**1**; $n > 2$) to precipitate proteins from aqueous 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 mono-layer which is less hydrophilic than the protein itself [Figure 2(a)]. Aggregation and precipitation then ensue. Where the protein concentration is high the relatively hydrophobic surface layer is formed by complexation of the polyphenol onto the protein and by cross-linking of different protein molecules by the multi-dentate polyphenols [Figure 2(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 2(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).

The mode of formation and properties of the protein-phenol complexes also suggests a putative role for some phenols, and in particular the higher molecular weight polyphenols, in plant metabolism. The ability of these polyphenols reversibly to coat the surface of proteins may be a means at the plant's disposal to modify and control the activity of particular proteins in the plant cell at certain points in its development.

The authors thank the A.R.C. for financial support.

(*Received, 9th January* 1981; *Com.* 024.)

[1] J. B. Harbone, 'Secondary Plant Products,' vol. 8, 'Encyclopaedia of Plant Physiology,' eds. E. A. Bell and B. V. Charlwood, Springer-Verlag, Berlin-Heidelberg-New York, 1980, p. 239.
[2] E. Haslam, *Phytochemistry*, 1977, **16**, 1625.
[3] R. D. Haworth and E. Haslam, *Prog. Org. Chem.*, 1964, **6**, 1.
[4] T. White, *J. Sci. Food Agric.*, 1957, **8**, 378.
[5] E. C. Bate-Smith, *Food*, 1954, **23**, 124.
[6] L. G. Butler and M. L. Price, 'Polyphenols in Cereals and Legumes,' ed. J. H. Hulse, International Development Research Centre, Ottawa, 1980, p. 39.
[7] D. E. Hathway and J. W. T. Seakins, *Biochem. J.*, 1958, **70**, 158.
[8] F. N. Howes, 'Vegetable Tanning Materials,' Butterworth, London, 1953.
[9] S. Hestrin, D. S. Feingold, and M. Schramm, *Methods Enzymol.*, 1955, **1**, 234.
[10] H. G. Weder, J. Schildknecht, and P. Kesselring, *Am. Lab.*, 1971, **3**, 15.
[11] I. Wadsö, *Acta Chem. Scand.*, 1968, **22**, 927.
[12] G. Scatchard, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 660; F. Karush, *J. Am. Chem. Soc.*, 1950, **72**, 2705.
[13] E. Haslam, *Biochem. J.*, 1974, **139**, 285.
[14] M. N. Jones and P. Manley, *J. Chem. Soc., Faraday Trans.* 1, 1980, **76**, 654.
[15] J. P. van Buren and W. B. Robinson, *J. Agric. Food Chem.*, 1969, **17**, 772.