Polyphenol Interactions. Part 2.[1] Covalent Binding of Procyanidins to Proteins during Acid-catalysed Decomposition; Observations on Some Polymeric Proanthocyanidins

Jill E. Beart, Terence H. Lilley,* and Edwin Haslam*
Department of Chemistry, University of Sheffield, Sheffield S3 7HF

The kinetics of the acid-catalysed decomposition of some procyanidins are determined. The reactions are specific acid-catalysed. The rate-determining step is that of the initial protonation of the procyanidin. The relevance of this degradation to haze formation in certain beverages is discussed. The structure of some polymeric proanthocyanidins is briefly commented upon in the light of this work.

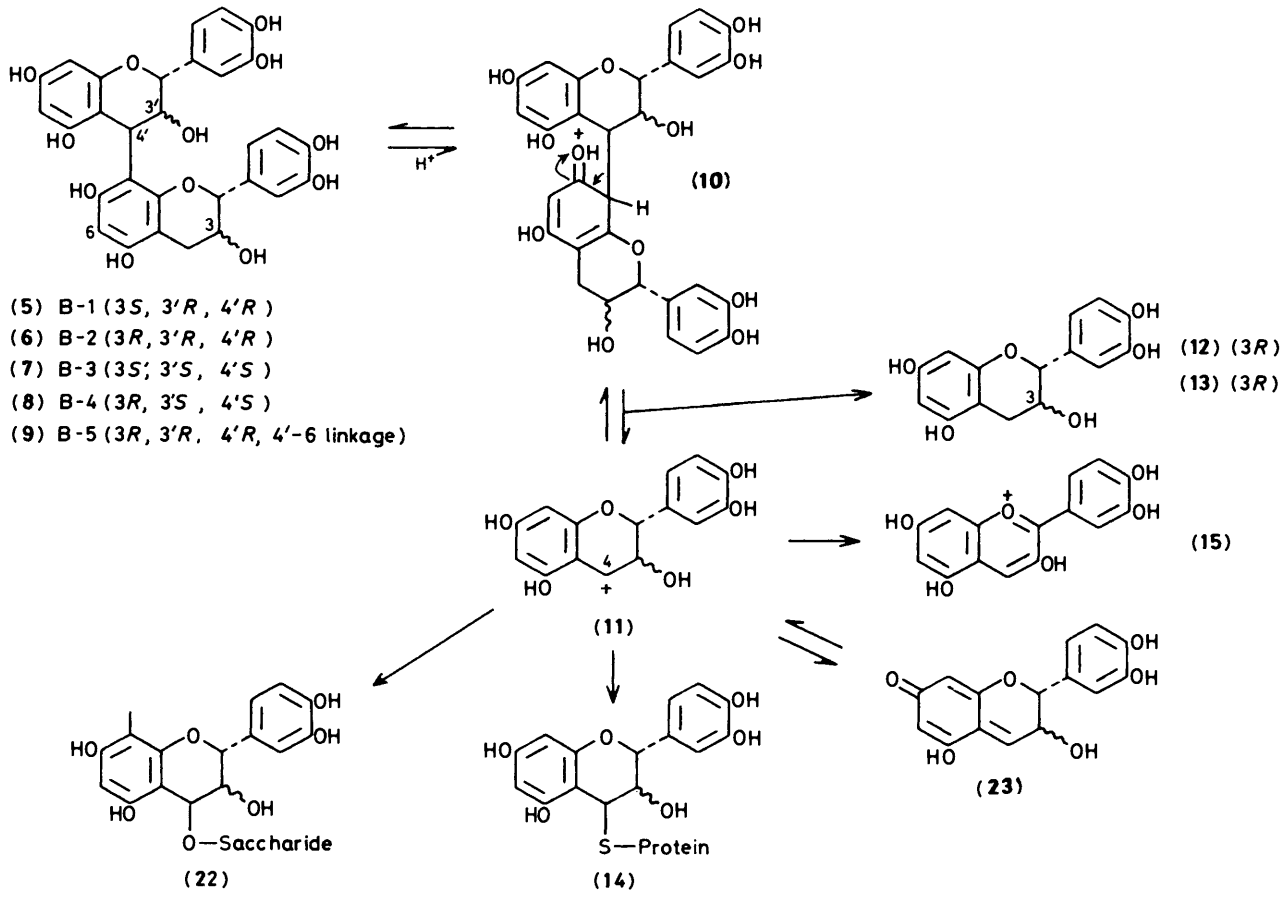
The interaction between polyphenols and cell constituents such as proteins and polysaccharides may be a reversible or an irreversible process. When the complexation is reversible non-covalent forces (hydrogen bonding, van der Waals forces) are usually implicated.[1] Irreversible association takes place *via* the formation of covalent bonds between the protein or polysaccharide and the phenolic substrate. Under oxidative conditions, at or near physiological pH and with or without enzymic catalysis, phenols are readily transformed to quinones which may then react with nucleophilic groups (*e.g.* SH, NH_2) on the protein. Particular examples of this phenomenon are the formation of sclerotins[2] (responsible for the exo-skeleton of insects) and the tanned silks of saturnid moths,[3] where considerable physical and chemical changes in the properties of proteins are produced by reaction with phenol oxidation products. When an insect cuticle is freshly secreted it is flexible and pale; in a matter of hours it hardens and generally darkens. *o*-Dihydroxyphenols, occasionally free or as glyosides secreted with the presclerotin, are enzymically hydrolysed to liberate the aglycone and oxidised to *o*-quinones (**2**) which react with nucleophilic groups on the protein to introduce exogenous cross-links between the polypeptide chains [equation (1)]. Tanning of fibroin and sericin in saturnid silks occurs by similar means and here (**3**) and (**4**) have been identified as phenolic substrates. *o*-Quinones and related species formed during the oxidation of plant phenols by the ubiquitous polyphenol-oxidases can similarly modify both cell constituents and plant pathogens, such as viruses.[4,5]

The importance of polyphenols in brewing has been recognised for a long time. When uninfected bottled beers are stored for any length of time they 'throw' a haze. Before a beer shows such a permanent haze at ambient temperatures it may form a chill haze if cooled. Such hazes redissolve on warming. The major components of hazes[6,7] are proteins, polypeptides, polyphenols, carbohydrates, and traces of inorganic matter. Various theories have been put forward to explain the formation of hazes and the general consensus supports the view that they are produced by the gradual polymerisation of simple phenols, during storage, and the subsequent reaction of these polymers with proteins to give insoluble complexes.[7,8] Much of the published work on haze formation has implicated a group of polyphenols known generically as anthocyanogens,[9] a useful but chemically imprecise term which has led to some ambiguities in interpretation. Proanthocyanidins[10] are the only anthocyanogens which have been shown unequivocally to occur in barley, malt, hops, and beers[11–13] and these have been the main focus of attention in this study. Chief amongst these has been procyanidin B-3 (**7**), (catechin-4α→8-catechin),[10,14] previously isolated from beer.[11,12]

Initially a brief chemical study was made of the haze isolated typically as a black amorphous powder from beer by centrifugation. The presence, in large part, of proteinaceous material was inferred from elemental analysis (Found: N, 7.5%; S, 1.6%) and by decomposition with strong acid (5M-HCl). Chromatographic analysis of the hydrolysate[15] showed a typical 'fingerprint' of protein α-amino acids and traces of carbohydrate material. During the initial stages of this degradation the hydrolysate assumed a deep red colour ($\lambda_{max.}$ *ca.* 535 nm) characterised as due to the pigment cyanidin (**15**) by chromatography in Forestal solvent after extraction into amyl alcohol.[16] The precursor of cyanidin (**15**) was presumably covalently bound to other materials in the haze, probably protein, and could not be removed by solvent extraction (water, acetone, dimethylformamide). Its presence, along with carbohydrates, is probably responsible for the lower than average value (*ca.* 11%) of nitrogen in the precipitated haze.

It was considered at the outset that haze formation probably resulted, in some way, from the familiar acid-catalysed degradation of procyanidins[10] present in beer, since this beverage has a pH typically at *ca.* pH 4.0. The hydrolytic decomposition of various procyanidins, B-2 (**6**) (epicatechin-4β→8-epicatechin); B-3 (**7**); B-4 (**8**), (catechin-4α→8-catechin), and B-5 (**9**) (epicatechin-4β→6-epicatechin), was therefore studied in acetonitrile–water–phosphoric acid mixtures, generally at 40 °C, and over a range of pH values.* Kinetics were monitored by h.p.l.c. analysis. The acid-catalysed solvolysis of the interflavan bond of procyanidin dimers is believed to proceed *via* the formation of the resonance-stabilised benzylic carbocation at C-4 in the 'upper' flavan-3-ol unit (**11**) and of the

* All measurements of pH relate to acetonitrile–water mixtures.



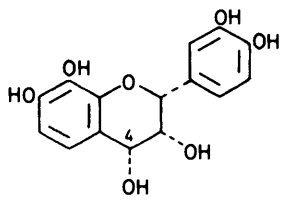
Scheme. Acid-catalysed decomposition of procyanidins

appropriate flavan-3-ol—(−)-epicatechin (**12**) or (+)-catechin (**13**) from the 'lower' flavan-3-ol unit (Scheme). Earlier work by Clark-Lewis and Mortimer[17] with the diastereoisomeric flavan-3,4-diols, melacacidin (**16**) and isomelacacidin (**17**), had indicated that acid-catalysed solvolysis was facilitated in the isomer (**17**) where the hydroxy group at C-4 occupies a quasi-axial position on the heterocyclic ring. This, it was argued, was due to the enhanced π-resonance stabilisation of the incipient C-4 carbocation which is only possible when the 4-hydroxy group assumes a quasi-axial orientation. It was anticipated on the basis of this stereochemical analysis that procyanidins B-2 (**6**) and B-5 (**9**), in which the 'lower' flavan-3-ol unit occupies a quasi-axial position at C-4 on the 'upper' flavan-3-ol unit, would undergo more facile acid-catalysed decomposition than procyanidins B-3 (**7**) and B-4 (**8**) where the 'lower' flavan-3-ol unit occupies a quasi-equatorial position. In the event this anticipation was not borne out in practice. The initial rates of hydrolysis of procyanidins B-2, B-3, and B-4 (**6**)—(**8**) were almost identical, whilst that of the C-4 to C-6 linked dimer procyanidin B-5 (**9**) was much slower (Table). This observation suggests that in the acid-catalysed decomposition of 4-aryl-flavan-3-ols, such as the natural procyanidins, formation of the carbocation (**11**) from the protonated intermediate (**10**) is *not* in this instance the rate-determining step. This conclusion was confirmed using the model substrates (**18**) and (**19**). The measured initial rates of hydrolysis (Table) were almost identical and both were marginally slower than that of procyanidin B-2 (**6**). The doubly bridged proanthocyanidin A-2 (**21**) was resistant to hydrolysis under all the conditions examined and this observation is consistent with the earlier failure to degrade the natural product using toluene-α-thiol and acetic acid.[18] Similarly the thiobenzyl ether (**20**) underwent slow acid-catalysed decomposition in contrast to the parent procyanidins (Table). This confirms the view that the sulphide degradation products derived from the procyanidins[19] are products of kinetic as opposed to thermodynamic control. The effects of variation of pH and temperature on the decomposition

Table. First-order rate constants for the acid-catalysed hydrolysis of procyanidins in acetonitrile–water–phosphoric acid

Compound	Temp. (°C)	pH* meter reading	$10^3k/h^{-1}$
(**6**)	40	0.55	75
(**7**)	40	0.55	71
(**8**)	40	0.55	75
(**9**)	40	0.55	30
(**19**)	40	0.55	65
(**18**)	40	0.55	58
(**20**)	40	0.55	18
(**6**)	25	0.55	10
(**6**)	30	0.55	22
(**6**)	35	0.55	51
(**6**)	45	0.55	169
(**6**)	25	0.95	5.2
(**6**)	35	0.95	17
(**6**)	25	1.49	1.7
(**6**)	35	1.49	7.5
(**6**)	25	0.25	63

* All measurements of pH relate to acetonitrile–water mixtures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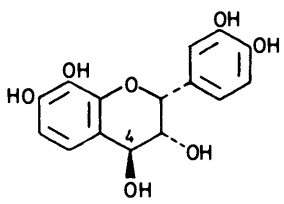
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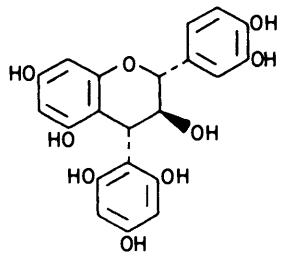
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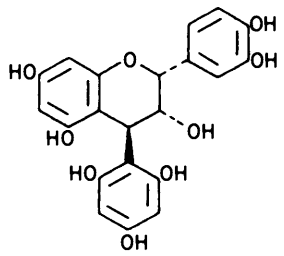
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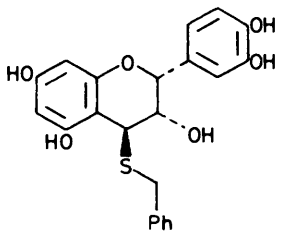
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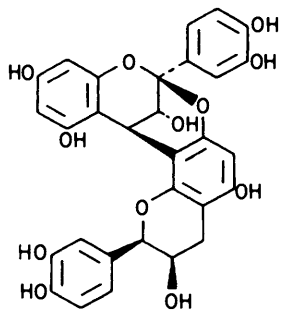
(21)

of procyanidin B-2 (**6**) were also systematically examined (Table). The reaction is clearly dependent on pH; the lower the pH the faster the rate. Plots of the log of the rate constant *versus* pH gave a linear relationship with lines of slope −1.0 indicative of a specific acid-catalysed reaction which is first order in hydrogen ion concentration.[20] The rate of hydrolytic cleavage of procyanidin B-2 (**6**) also increased with increasing temperature and at pH 0.55 the data (Table) gave typical Arrhenius plots. Finally it was noted that in all cases after hydrolysis had proceeded for some time (3—4 days) an equilibrium position corresponding to around 80% decomposition was reached. This is consistent with the known reverse reaction (Scheme) which has been employed synthetically.[21] To substantiate this observation it was noted with procyanidin B-2 (**6**) that addition of molar quantities of (−)-epicatechin (**12**), although it did not affect the initial rate, resulted in a measurable shift in the equilibrium position (*ca.* 60% decomposition, 3 days).

The data presented are thus consistent with a specific acid-catalysed mechanism for the decomposition of procyanidin dimers of the B-type (Scheme). The rate-determining step is clearly the protonation of the 'A' (phloroglucinol type) ring of the 'lower' flavan-3-ol unit and in the cases and conditions examined the effect of stereochemical factors is surprisingly small. Most of the observations made in this study and earlier studies can now be interpreted satisfactorily in terms of this mechanism (Scheme). The clear difference observed in the rate of hydrolysis of dimers of the C-4–C-8 type [*e.g.* (**6**)] and the isomeric C-4–C-6 linked polyphenols [*e.g.* (**9**)] which supports an earlier[22] qualitative observation is, however, not readily explicable. Empirically it seems probable that the presence of a *para*-phenolic hydroxy group to the interflavan bond is more effective in assisting protonation at the inter-flavan bond (and hence decomposition) than an *ortho*-hydroxy group. Likewise the stability of proanthocyanidin A-2 (**21**) supports the conclusion that, under the conditions examined, two phenolic hydroxy groups (*ortho* and *para* or both *ortho* to the interflavan bond) are essential to promote the degradation. Extrapolation of the data obtained for procyanidin B-2 (**6**) (Table) permitted a rough estimate to be made for the hydrolytic rate constant at pH 4.0, such as appertains in beers at 25 °C. The value obtained was $k_{obs,4.0}$ 6 × 10^{-6} h^{-1}.

Model systems were evaluated in the light of this information in relation to the formation of polyphenol–protein hazes. Sterile solutions (acetate buffer, pH 4.1), containing bovine serum albumin (BSA) (300 p.p.m.) with (−)-epicatechin (**12**), procyanidin B-2 (**6**), and a polymeric procyanidin from *Crataegus monogyna* [approximately a hexamer based on (−)-epicatechin chain extension and termination units[23]], all at varying concentrations from 50 to 200 p.p.m., were set up and the turbidity monitored as an index of haze formation. Over a period of 6—8 months, solutions of BSA remained translucent and (−)-epicatechin (**12**) had a negligible effect on turbidity. However, both procyanidin B-2 (**6**) and the polymeric procyanidin from hawthorn caused a steady increase in turbidity (haze) and in the case of the latter substrate a precipitate, similar to haze from beer, was eventually formed. This evidence, although circumstantial, thus strongly suggests that the formation of haze in beers is correlated with the presence of oligomeric procyanidins.

Under the weakly acidic conditions prevailing, slow decomposition occurs with the initial formation of carbocation species such as (**11**) (Scheme). In the presence of protein, it is postulated, these may then be captured by nucleophilic species on the protein such as SH groups (**14**), thus modifying the hydrophilic surface of the protein, resulting in aggregation and finally precipitation. Sulphide adducts such as (**14**), although themselves acid labile, would be expected to have greater stability than the parent procyanidins in the acidic media (Table). It is interesting to note that in acetate buffer solutions at pH 4.1, in the absence of BSA, procyanidin B-2 (**6**) and the oligomer from hawthorn both gave fine brick-red precipitates (phlobaphen[24]) which result presumably from decomposition and polymerisation of the intermediate carbocation (**11**) (Scheme).

Given the mild conditions under which this type of acid-induced decomposition of procyanidins may occur it seems entirely reasonable and predictable that analogous interactions with proteins and other macromolecules to give covalently bound complexes of polyphenols may occur in other situations, for example the microbial decomposition of plant tissues during their incorporation into soils and the acid-mediated breakdown of polyphenol-containing materials in the stomach of ruminants and other animals. Similarly, acid-catalysed decomposition of procyanidin-type polyphenols (such as have been described above) may play a significant part in the covalent binding of polyphenols to collagen which occurs during the traditional protracted forms of vegetable tannage[24,25] practised in earlier times in the conversion of animal hides into leather.

In the light of these observations it is also pertinent finally to comment upon the natural occurrence of plant pro(antho)-cyanidins, since the quinone-methide (**23**) or its protonated derivative the carbocation (**11**) have been implicated by numerous workers as intermediates in their biosynthesis in plants.[26–29] During these and earlier investigations two groups

of pro(antho)cyanidins have been regularly encountered which clearly fall within the category (a) 'those which are insoluble in water and the usual organic solvents' of the original classification of the Robinsons.[30] These are respectively the residual apparently insoluble forms remaining in plant tissues after repeated solvent extraction (*e.g.* methanol→acetone→acetone–water→DMF) and those which are frequently found in plant gums and exudates (*e.g. Butea frondosa*[31]). The former group are, in our experience, indeed characteristic of all pro(antho)-cyanidin tissues such as stem, twig, leaf, and fruit where they invariably predominate over the more freely soluble oligomeric forms, typically by as much as 5 or 10:1 based on the release of anthocyanidin with acid.[19] Present evidence strongly suggests that these pro(antho)cyanidins are covalently bound to a polysaccharide matrix within the plant. Support for this view comes from the analysis of several acetone–water-soluble oligomeric pro(antho)cyanidins, for example that isolated from hawthorn after chromatography on Sephadex LH-20 (with Zhaobang-Shen). Degradation with toluene-α-thiol in acetic acid[19] gave a ratio of (**12**) to (**20**), determined by h.p.l.c., corresponding to an approximately hexameric structure. However, residual material of a carbohydrate character was also detected in the aqueous soluble fraction following this degradation. D-Glucose could be detected (paper chromatography, butan-2-ol–acetic acid–water 14:1:5; aniline–hydrogenphthalate spray, R_F 0.20[32]) after acid-catalysed degradation of the original polymer. The ^{13}C Fourier transform n.m.r. spectrum of the polymer gave a complex pattern of signals and detailed analysis was not possible, although the broad general pattern of absorptions was, by analogy, consistent with a procyanidin polymeric structure.[33] However, signals at δ 102.5—104.5 (CH) and 60.0—63.0 p.p.m. (CH_2) are not readily accommodated within such a structural proposal; they are nevertheless consistent with the presence of pyranose sugar residues[34] within the heterogeneous polymeric structure. The former signals may be attributed to the anomeric carbon atom of glycosides and the latter to C-6 of pyranose sugars. This picture does not change substantially after rechromatography of the procyanidin polymer.

Proanthocyanidins generally occur free although some examples are now recorded of the occurrence of the parent flavan-3-ols in glycosylated forms. Whilst non-covalent forces may be involved in the complexation of polyphenol and carbohydrate[1] it seems reasonable, in the light of the earlier observations, to consider that such pro(antho)cyanidin species may well result from the capture of (**11**) or (**23**) during biosynthesis by hydroxy groups of saccharide structures in the plant cell. Alternatively pro(antho)cyanidin–carbohydrate structures may be derived subsequently (*e.g.* post-mortally in the case of plant gums) by processes analogous to the acid-catalysed degradation described earlier and capture of transient intermediates such as (**11**) by carbohydrate [*e.g.* (**22**)].

The pro(antho)cyanidins thus bear many analogies to the plant lignins; patterns of polymerisation are dictated principally by the inherent chemical reactivity of the monomer precursors and soluble forms of the simple dimers (*e.g.* lignans and procyanidins) are found in many plants. Moreover lignin is found not only in intimate physical contact with hemicelluloses in the cell wall but, like the pro(antho)cyanidins, appears to be bound by covalent linkages to it.[35] Freudenberg in his classical work suggested that lignin becomes bound to the polysaccharides of wood during polymerisation by addition of aliphatic hydroxy groups of the carbohydrate to quinone-methide intermediates such as (**23**) and the formation of acid-labile ether linkages. Bate-Smith[36] in his botanical surveys has moreover consistently drawn attention to the occurrence of proanthocyanidins in plants with a woody habit of growth and this further chemical analogy emphasises the probability that polymeric pro(antho)cyanidins function in a similar manner as structural fragments in the cells of particular plants.

* See Table.

Experimental

Compounds.—All procyanidins and their derivatives were isolated as previously described.[18,19]

Kinetic Measurements.—Solutions of appropriate acidity were prepared by adding orthophosphoric acid (AnalaR grade) to the solvent system acetonitrile (h.p.l.c. grade; 950)–water (glass distilled; 50)–orthophosphoric acid (AnalaR grade; 1) which had a pH* meter reading of 1.49, measured using a Radiometer PHM62 pH meter calibrated using KCl–HCl buffer pH 2.20. Orthophosphoric acid (AnalaR grade; *y* ml) when added to 50 ml of the above solvent system gave solutions of the following pH* values:

y/ml	pH* meter reading
0.1	0.95
0.3	0.55
1.2	0.25

Solutions of procyanidins (*ca.* 0.1 g l^{-1}) were prepared in the appropriate acetonitrile solution which had been filtered and allowed to obtain equilibrium at the appropriate temperature in a thermostat. Kinetic measurements were made by withdrawal of a portion (10 μl) and analysis by h.p.l.c. [Dupont 860, Zorbax-NH_2 column using the isochratic solvent system acetonitrile–water–phosphoric acid (950:50:1)]. Measurements were made in triplicate and analysis followed either the increase in concentration of the flavan-3-ol (**12**, **13**) peak or decrease in the procyanidin concentration.

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