

THE CONVERSION OF PROCYANIDINS AND PRODELPHINIDS TO CYANIDIN AND DELPHINIDIN*

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Abstract—The hydrolysis of proanthocyanidins to anthocyanidins in *n*-BuOH-HCl (95:5) has been shown to be an autooxidation, the yield of anthocyanidin being critically dependent on trace metal-ion impurities. Reproducible yields of anthocyanidin may be achieved if iron (III) salts are added to the reaction medium, and a standard method of analysis of proanthocyanidins based on use of an *n*-BuOH-HCl-Fe^{III} mixture is given. The ratio of absorbance maxima of the cyanidin (550 nm) produced to that near 280 nm for the original procyanidin polymer solution was ~ 3.5.

INTRODUCTION

The presence of proanthocyanidins (i.e. condensed tannins) in plant tissue has been traditionally determined by their conversion to anthocyanidins in hot mineral acid solution—either on ground-up tissue or suitable extracts [1]. The early history of this reaction has been summarized by Haslam [2], and it is sufficient for our purposes to note that it has become almost universally the practice among chemists and biologists to use a method employing heating the substrate in *n*-butanol-conc. hydrochloric acid in the proportions of 95:5 v/v to estimate proanthocyanidins. This procedure was originally due to Swain and Hillis [3], and Bate-Smith [1, 4] later did much to increase its popularity [4]. The reaction is thought [5] to proceed by the steps in Scheme 1, which will be considered in more detail later.

It has been noted by many workers, since the method was first developed, that this procedure suffers from a number of drawbacks. One was noted by Riberau-Gayon [6]: that it was difficult to relate the yield of cyanidin (3), say, from the plant extract to the original concentration of procyanidin (PC). The second, noted many times, and leading to a number of interesting modifications to the original method, was the lack of reproducibility of yield of cyanidin from the reaction [6].

Because of the fact that biologists are largely interested in the quantitative aspects of the reaction, whereas chemists utilize it for qualitative purposes, there has to date been no systematic study of this reaction or its possible mechanism, even though suitable proanthocyanidin (PA) standards are now available. At the suggestion of Professor Helen Stafford we have undertaken such a study with the purpose of understanding the reaction

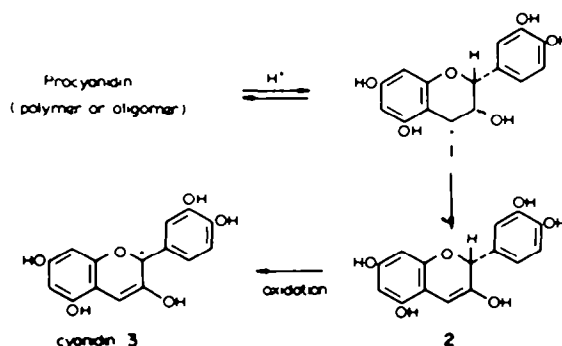
more fully and developing a reproducible method of analysis based on it.

At the outset we decided to confine our attention to study the reaction in the same medium as employed by Swain and Hillis [3], rather than exploring other more exotic media, in the belief that this solvent combination has gained such wide acceptance that any drastic modification would create confusion. In the event it shall be seen that one minor modification must be made to assure a reproducible analytical method.

RESULTS AND DISCUSSION

The method used to carry out the reaction was designed to approximate fairly closely the original Swain and Hillis [3] method. The hydrolyses were performed in thick-walled, 8 ml capacity, screw-topped glass vials with teflon-lined caps. These were immersed at a reproducible level in a constant-level water-bath maintained at 95°.

The first experiments used the simple BH solvent mixture and were aimed at establishing the rate of the



Scheme 1.

*This paper is dedicated to Dr. E. C. Bate-Smith and Prof. T. Swain.

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reaction for various substrates and the dependence of yield on concentration. Under the above conditions 0.002–0.003% solutions of (2*R*,3*S*,4*R*)-leucocyanidin (4) and epicatechin-(4*β* → 8)-catechin (5) had half-lives of 2.4 and 5.0 min respectively for the reactions, which were complete within 40 min. The PC polymers had half-lives which were only slightly greater than the dimer (5) and also had produced a maximum yield of pigment within 40 min. Therefore the reaction is relatively fast.

Figure 1 shows a plot of cyanidin yield versus PC polymer concentration. This shows that the curve is biphasic and consists of two straight lines. This feature of the reaction had been noted previously, first by McFarlane [7] and later by Stafford and Lester [8]. It creates the problem [8] that a Beer-type relationship between yield of cyanidin (absorbance at 550 nm) and concentration is only applicable in the lower concentration range, up to $A = 0.2$. Swain and Hillis [3] and later Bate-Smith [4] utilized concentrations in the upper part of the curve, and the former authors alluded to this problem in the statement: "... but an absolute straight-line relationship between concentration and absorptivity was not obtained."

Later work with the BH solvent showed that further problems arose from its use. After carrying out a series of experiments with one batch of BH, a fresh batch gave a different yield of cyanidin for the same concentration of PC under the same conditions. A further batch of BH solvent made up from a different batch of *n*-butanol and a freshly-opened bottle of conc. HCl gave yet another absorbance. The three A_{550} values obtained were 0.57, 0.31 and 0.20, all using a 0.002% solution of *Chaenomeles chinensis* PC polymer. Two conclusions were drawn: (a) that the successive batches of BH solvent must contain trace impurities which have a possible catalytic, or less probably, inhibitory effect on the reaction, and (b) the yield may in fact be under kinetic control so that the ultimate yield is determined by the availability of oxidant.

It has generally been assumed that the final oxidation step (Scheme 1), formally abstraction of hydride ion from C-2 of the flav-3-ene (presumed) intermediate (2) to form

cyanidin (3) involves molecular oxygen, and therefore may be classified as an autoxidation [9]. The only evidence for this was Swain's [10] observation that carrying the reaction out in nitrogen lowered the yield of cyanidin produced from synthetic leucocyanidin. There are various earlier reports which purport to establish the necessity, or otherwise, of the presence of air for the conversion to cyanidin to take place (these are summarized both by Bancroft and Rutzler [11] and Swain and Bate-Smith [12]) but it is difficult to judge their relevance in the present context because they do not deal with defined chemical species.

Three experiments were carried out on a PC polymer (isolated from *Chaenomeles speciosa* 'Japonica' fruit) all at 0.0012% concentration of substrate in BH solvent. Firstly the stoppered tubes containing the reaction mixture were simply heated and gave a maximum A_{550} value of 0.18. A second sample was continuously purged with argon for 30 min before, and during, heating. This gave an A_{550} value of 0.12. A third sample was purged continuously with pure oxygen and gave a maximum A_{550} value of 0.47. These results confirm Swain's [10] observations that the yield is dependent on the oxygen partial pressure, which is typical of an autoxidation [9], and that some cyanidin is formed, even in the absence of oxygen. The result also implies that the final yield of cyanidin is dependent on the rate of oxidation, i.e. the rate of conversion of 2 to 3, and therefore the supply of oxidant.

Jurd [5] observed that the yield of 5,7,3',4'-tetramethylcyanidin (6) from 5,7,3',4'-tetramethyl-(2*R*,3*S*,4*S*)-leucocyanidin (7) could be increased from 20–30% in BH solvent to 75–80% by the addition of small amounts of *p*-benzoquinone (PBQ). We therefore treated (2*R*,3*S*,4*R*)-leucocyanidin (4) with increasing amounts of PBQ which resulted in the yield of cyanidin being increased dramatically. For instance a 0.0015% solution

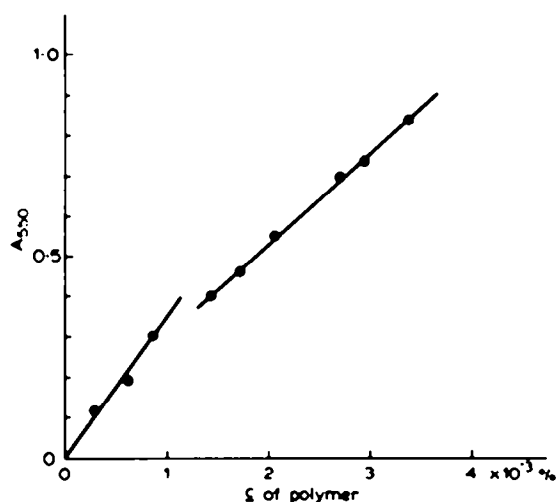
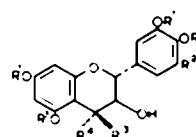
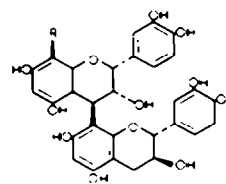


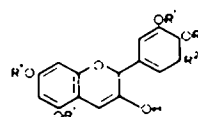
Fig. 1. Dependence of cyanidin (expressed as absorbance at 550 nm) yield on procyanidin polymer (ex. *Chaenomeles chinensis*) concentration in BH solvent.



- 4 $H^1 = R^1 = R^3 = H$, $R^2 = OH$
 7 $R = Me$, $R^2 = R^3 = R^4 = OH$
 13 $R = H^1 = H^2 = H$, $R^3 = 8$ catechin
 17 $H = R^1 = R^3 = H$, $R^2 = 2,4,6$ trihydroxyphenyl
 18 $R = R^1 = H$, $H^2 = CH$, $R^3 = 2,4,6$ -trihydroxyphenyl



- 5 $H = H$
 14 $H = \text{epicatechin} \cdot 4\beta$



- 6 $H = Me$, $H^2 = H$
 20 $R = H$, $R^2 = CH$

of 4 gave a maximum absorbance of 0.31 in BH solvent, but this increased to 0.62 in the presence of 0.0033% PBQ and thereafter the yield slowly fell off. These results show that although PBQ may be used to increase the yield of cyanidin, excess PBQ must react further with the intermediate species causing a drop in yield. It should be noted that addition of PBQ to a PC polymer solution is just as effective in increasing yield.

The reaction in acid solution normally proceeds by way of prior protonation of PBQ followed by hydride-ion abstraction from the oxidized species (Scheme 2), although in many cases it occurs by a two-step free radical mechanism [13]. PBQ is a relatively powerful oxidant with an oxidation potential of 0.7 V [13]. Replacement of PBQ with the milder oxidant 1,4-naphthoquinone (E_0 0.48 V) resulted in the same enhancement of yield as PBQ. Next a quinone with an even lower E_0 (0.16 V), 9,10-anthraquinone was used (which is normally not considered to be an oxidizing agent). The yield of cyanidin was still enhanced. The actual values obtained, on a $2.4 \times 10^{-3}\%$ solution of *Chaenomeles chinensis* PC polymers were: BH alone, $E_{350}^{1\%}(E) = 180$; in the presence of PBQ or 1,4-naphthoquinone, $E = 385$; in the presence of 9,10-anthraquinone, $E = 298$.

It may be concluded, therefore, that marked increases in cyanidin yield may be achieved by the addition of oxidant, and that the oxidative abstraction of hydride ion is an extraordinarily facile process.

Addition of transition-metal ions

The fact that PBQ or 1,4-naphthoquinone (or quinhydrone, which has the same ability to enhance the cyanidin yield) were only effective over a critical range of concentration, meant that they were not useful for the development of a convenient analytical method. Both McFarlane [7] and Govindarajan and Mathew [14] had previously suggested that iron salts were effective in enhancing the amount of pigment obtained in the reaction. It is well-known that autoxidations are greatly accelerated by transition-metal ions having two readily accessible oxidation states differing by one unit [9]. The metal-ion catalyses the formation of free radicals, normally via peroxy-species, by both oxidation and reduction steps [9]. Small amounts of the transition-metal ions Co^{II} , Cu^{II} , Mn^{II} , Fe^{II} and Fe^{III} were added to a $1.3 \times 10^{-3}\%$ solution of *C. chinensis* PC polymer in BH solvent, and all enhanced the yield of cyanidin (Table 1). The most

Table 1. Effect of adding transition metal-ions on the yield of cyanidin from a procyanidin polymer from *Chaenomeles chinensis* fruits (polymer concentration $1.3 \times 10^{-3}\%$ w/v in all cases)

Metal-ion added	Amount* (μl)	A_{550} (1 cm cell)
None	—	0.18
Co^{II}	10	0.23
	100	0.29
Cu^{II}	10	0.36
	100	0.02†
Fe^{III}	10	0.29
	100	0.54
Mn^{II}	10	0.49
	100	0.36†

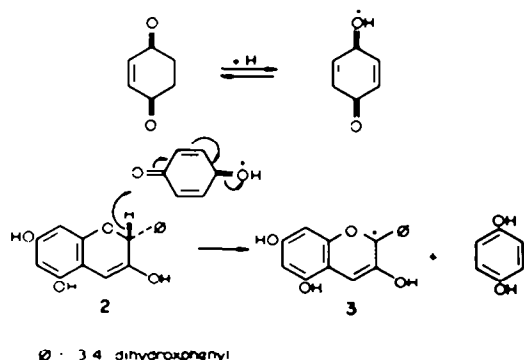
* Volume of a 1% solution in water of $\text{Co}^{\text{II}}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\text{Cu}^{\text{II}}\text{SO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Fe}^{\text{III}}_2(\text{SO}_4)_3 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24\text{H}_2\text{O}$ and $\text{Mn}^{\text{II}}\text{Cl}_2 \cdot 4\text{H}_2\text{O}$ respectively.

† Causes decomposition of the cyanidin.

effective catalysts were the Fe^{II} and Fe^{III} salts, which both gave the same yield of cyanidin. Although quite effective as catalysts, the Mn^{II} and Cu^{II} salts led to decomposition of the PC at higher concentrations.

On the basis of these observations it was resolved to develop a method of analysis based on Fe^{III} salts. The Fe^{III} salts were chosen, rather than Fe^{II} , because the latter are unstable in acid solution over a long period and gradually oxidize to Fe^{III} [15].

Addition of increasing concentrations of Fe^{III} (as ferric



Scheme 2.

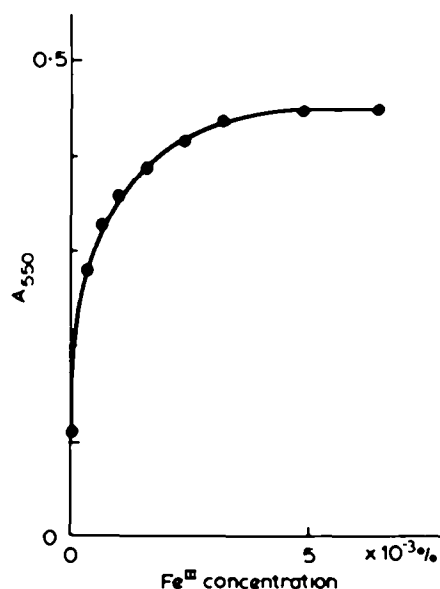


Fig. 2. Dependence of cyanidin yield (expressed as absorbance at 550 nm) on ferric ion concentration for a constant concentration ($0.87 \times 10^{-3}\%$) of procyanidin polymer (ex. *Chaenomeles speciosa*).

ammonium sulphate) to a $2.2 \times 10^{-3}\%$ solution of *C. chinensis* PC polymer gave the A_{550} values, for the maximum cyanidin concentration formed at each Fe^{III} concentration, shown in Fig. 2. It may be seen that the yield reaches a maximum at an Fe^{III} concentration of $\sim 4.5 \times 10^{-3}\%$ ferric ion (or $\sim 7 \times 10^{-4}$ molar in Fe^{III}), beyond which the yield remains constant. Therefore Fe^{III} salts may be used as a catalyst for the reaction so long as they are present in excess concentration.

Figure 3 shows a plot of A_{550} versus time for the hydrolysis of a typical PC polymer in BH solvent plus Fe^{III} . This shows that the rate of cyanidin is relatively fast in the presence of Fe^{III} , with a half-life of approximately 4 min, the reaction being complete in 30–40 min. Therefore the actual rate of formation of cyanidin is about the same as in the absence of Fe^{III} , but the yield is approximately three times higher. A similar result was obtained for the PBQ catalysed reaction.

Figure 4 shows plots of A_{550} versus PC concentration for the hydrolysis of some typical oligomers and polymers in BH + Fe^{III} solvent. These show that a Beer type relationship is obeyed to much higher absorbances (~ 0.6 – 0.7) than the uncatalysed reaction; but that the yield still tails off at higher substrate concentration. However, it may be seen that addition of iron (III) salts increases the sensitivity of the reaction three-fold, and replicate studies both by ourselves and in Professor Stafford's laboratory have shown that the method is perfectly reproducible.

The yield of cyanidin obtained by addition of Fe^{III} is limiting as addition of PBQ together with iron salts to the reaction mixture makes no difference to the yield. However, if O_2 is bubbled continuously through the Fe^{III} containing solution, the yield of cyanidin increases still further. Table 2 summarizes the influence various factors have on the yield of cyanidin from a solution of a typical PC polymer. The yields from these factors are divided out in Table 2, which establishes that the final yield of cyanidin cannot be explained on the basis of a single reaction pathway.

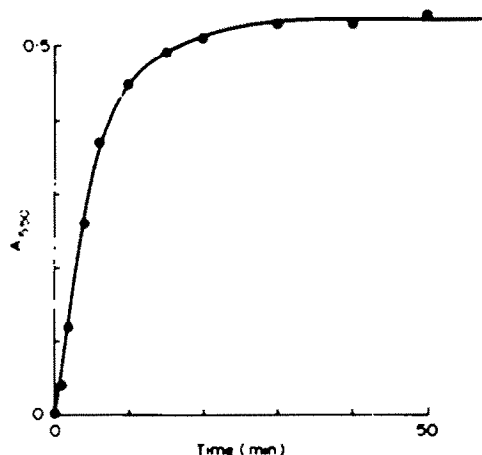


Fig. 3. Dependence of cyanidin yield (expressed as absorbance at 550 nm) on time at a constant procyanidin polymer (ex. *Chaenomeles speciosa*, $c = 0.87 \times 10^{-3}\%$) and ferric ion (0.1 ml of 2% ammonium ferric sulphate solution in 7.1 ml) concentration.

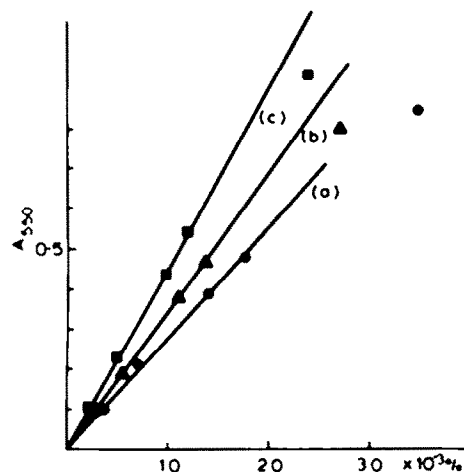


Fig. 4. Dependence of cyanidin yield (expressed as absorbance at 550 nm) on procyanidin concentration for a series of different substrates, all analysed after 40 min reaction in the BH + Fe^{III} solvent. Curve (a) epicatechin-(4 β $\rightarrow$ 8)-catechin (5); curve (b) epicatechin-(4 β $\rightarrow$ 8)-epicatechin-(4 β $\rightarrow$ 8)-catechin (13); curve (c) *Chaenomeles speciosa* procyanidin polymer.

Effect of light

It was suggested by Swain and Hillis [3], apparently on the basis of the work of Nordstrom [16], that it was necessary to take precautions against light causing fading of cyanidin. We accordingly carried out a series of hydrolyses on a PC polymer in BH solvent, with and without added Fe^{III} , with the tubes in normal laboratory fluorescent lighting and also completely wrapped in Al foil. We could find no difference in the yield of cyanidin, and moreover detected no change in absorbance of solutions standing in laboratory light for several hours after reaction.

'Pinking' reaction

There has been some debate over whether or not procyanidins are capable of producing cyanidin while standing in solution at room temperature. There has also been some discussion on the role of this reaction in the 'pinking' of grape juice or other fruit products [16]. As pointed out by Watterson and Butler [17] a pink colour does slowly develop in BH solvent. We found that if a solution of PC polymer was stored in BH solvent for 48 hr

Table 2. Yields (in units of absorbance at 550 nm) for the oxidative hydrolysis of a $1.2 \times 10^{-3}\%$ w/v solution of a procyanidin polymer from *Chaenomeles speciosa* (Japonica)

Reaction conditions	Yield
1. In BH solvent, purged with Argon	0.12
2. In BH solvent alone	0.18
3. In BH solvent, purged with O_2	0.47
4. With Fe^{III} added	0.54
5. With Fe^{III} added, purged with Argon	0.35
6. With Fe^{III} added, purged with O_2	0.67

the yield of cyanidin was the same as for the reaction if it had been heated. However, if Fe^{III} was added, there was no increase in yield. This experiment showed that the only function of heating in the uncatalysed reaction was to increase the rate of the reaction.

Effect of water

The yield of cyanidin is also affected by small amounts of water. Figure 5 shows a plot of absorbance (yield) versus volume percentage of water and it may be seen that the maximum yield of pigment is achieved at $\sim 6\%$ v/v water in the solvent, or 0.4–0.5 ml total in 7 ml. This is achieved by our standard method, when allowance is made for the volume of water in the concentrated HCl. The yield for zero water content was achieved by replacing HCl by H_2SO_4 . The unfortunate aspect of this feature of the reaction is that if there is water in the tannin extract the yield of cyanidin will drop. However, this problem may be overcome by evaporating the tannin extract to dryness, and dissolution in methanol, or else application of a suitable calibration factor to allow for the effect of water.

Mechanism of the reaction

Table 2 summarizes the contribution of various factors to the yield of cyanidin from the oxidative hydrolysis of a PC polymer. McFarlane [7] originally proposed that the function of Fe^{III} was to augment the intensity of colour through complex formation. However, Govindarajan and Mathew [14] correctly deduced that its function is catalytic as the absorbance at 550 nm remains unchanged compared with the uncatalysed reaction, whereas a bathochromic shift would be expected if Fe^{III} complexed with the product [18]. It is also known that a catechol-type complex would be present in very low concentrations at such a pH value [19].

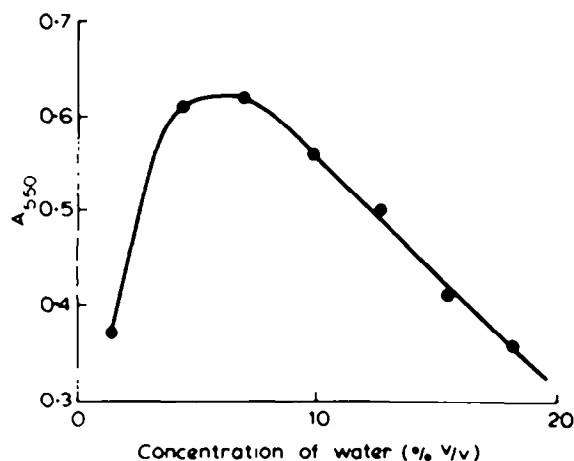
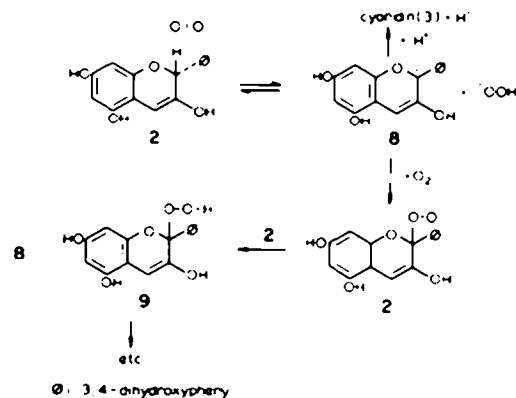


Fig. 5. Dependence of cyanidin yield (expressed as absorbance at 550 nm) on the concentration of water (% v/v). The solutions were held at constant procyanidin polymer (*C. speciosum*) concentration ($1.4 \times 10^{-3}\%$), Fe^{III} concentration ($6.4 \times 10^{-3}\%$), volume (7.2 ml), but varying water concentration. The low water concentration value was obtained by using $n\text{-BuOH-H}_2\text{SO}_4$ (95:5) instead of the normal BH solvent.

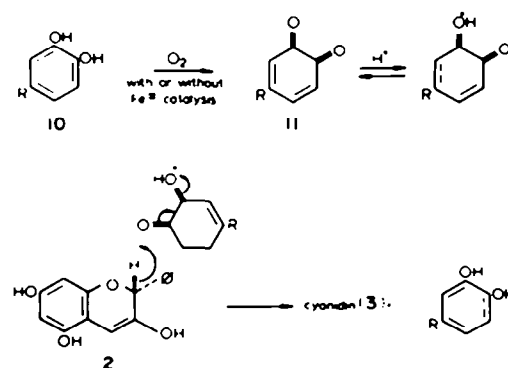
As stated earlier the dependence of the reaction on oxygen partial pressure and the ability of transition metal-ions to act as catalysts shows that the reaction, at least partly, is an autoxidation. Also the fact that H-2 is attached to a tertiary benzylic carbon means that the reaction would be expected to be especially facile [9]. This is confirmed by the facts that 9,10-anthraquinone may act as a catalyst, and also that it takes place in the presence of phenolic molecules (i.e. the substrate itself) which are normally inhibitors of autoxidations [9]. By analogy therefore with other autoxidations [9] the conversion of 2 to 3 would be expected to proceed by the chain reaction shown in Scheme 3. The chain-reaction will be terminated by the formation of cyanidin, or dimerization of species such as 4, or the formation of other products by acid catalysis from the starting material (2).

Another autoxidation pathway which must contribute is shown in Scheme 4, and involves the oxidation of the B-ring (10) to an *ortho*-quinone (by metal ion catalysed or uncatalysed radical oxidation by O_2). The *ortho*-quinone (11) will then abstract hydride ion by either an ionic or free radical pathway. The pH is such that there will be a low, but sufficient level of Fe^{II} -B-ring complexation (obligatory for catalysis) for the metal-ion catalysed pathway to take place.

The oxidation to cyanidin must compete with other (irreversible) reaction pathways involving PC substrate. The maximum yield of cyanidin from a PC polymer (in



Scheme 3.



R: chroman moiety from any flavonoid species (A-C rings)
O: 3,4-dihydroxyphenyl

Scheme 4.

the presence of Fe^{III} and O_2 , Table 2) was 58% (with allowance for the average chain length of the polymer of 9.4 units and using the value $\epsilon = 35000$ for the molar extinction coefficient of cyanidin at 550 nm [20]).

The reverse of step 1 in Scheme 3 ($4 \rightarrow 2$) has been measured polarographically by Harper [20] who obtained a half-wave potential of -0.41 V for the addition of an electron by a pH independent process at pH < 2 in methanol-water (1:1, v/v). It is interesting to note that PBQ, 1,4-naphthoquinone, and the $\text{Fe}^{\text{II}}/\text{Fe}^{\text{III}}$ couple in acid solution (0.46 V) all have oxidation potentials greater than this, and all yield the same amount of cyanidin, whereas 9,10-anthraquinone, with a lower oxidation potential, yields less product. It is possible, therefore, that the former three systems are reacting partly by a direct oxidation process, whereas 9,10-anthraquinone is acting only as a free-radical promoter.

The formation of cyanidin in the absence of O_2 may be explained if it is assumed that small amounts of the C-2 peroxy species (9) and/or the *ortho*-quinone (11) are preformed in the PC polymer chains during isolation and purification (the *ortho*-quinone could also be formed enzymatically during isolation). The cyanidin formation is therefore partly auto-catalytic. If this proposal is correct, then the yield from the O_2 -free reaction will depend on the history of the sample. This was supported by the observation that the ratio of yields from the reaction in the presence or absence of O_2 were inconsistent, and varied from one polymer to another.

Table 3 summarizes the three reactions which contribute to the final yield of cyanidin on the oxidative hydrolysis of a procyanidin. Support for the fact that a

direct oxidation (discussed above) occurs in the presence of PBQ or Fe^{III} , in addition to the autoxidation, comes from the fact that the yields from the uncatalysed reactions, and that in the presence of Fe^{III} , but absence of O_2 (see Table 2), almost exactly equal the yield in the presence of Fe^{III} and O_2 at atmospheric pressure (i.e. Table 2, no. 4). The yields from the uncatalysed reaction purged with O_2 (Table 2, no. 3) and the Fe^{III} reaction in the absence of O_2 (Table 2, no. 5) when combined exceed that of the reaction with Fe^{III} purged with O_2 . This probably means that the autoxidation and Fe^{III} oxidation become competitive at higher O_2 concentrations. The results do not exclude the very likely possibility that there is a fourth reaction in which the autoxidation is catalysed by PBQ or Fe^{III} (i.e. the free radical, Scheme 3, and direct hydride ion abstraction, Scheme 2, processes may both be operational).

Dependence of yield on proanthocyanidin chain length

In a typical PC dimer, such as 5, only the upper unit may yield a carbocation (Scheme 1, structure 1) and therefore be capable of producing cyanidin. Oligomer and polymer chains are built up by addition of further 'upper' units [21] and therefore the yield of cyanidin should increase as the chain length increases. Table 4 summarizes the yields, in units of $E_{550}^{1\%}$, obtained from plots such as shown in Fig. 4.

Considering the lower M , compounds first, two features of interest emerge. Firstly, if the yield of cyanidin (3), or delphinidin (20), is converted to that based on the mole fraction of PA units in the molecule ($E/\text{m.f.}$, final column Table 4), it may be seen that the yield of anthocyanidin is, as expected, fairly consistent and in the range 440–560 for all the simple proanthocyanidins. Also, the yield of cyanidin is significantly higher for a 4 $\rightarrow$ 8 linked (5), compared with a 4 $\rightarrow$ 6 linked (12), dimer [these latter include the phloroglucinol derivatives (15, 17–19) which approximate to this class of PA dimers]. A possible explanation of this is that the 4 $\rightarrow$ 6 bond is cleaved at a

Table 3. Reactions contributing to the final yield of cyanidin

1. Autocatalytic: no O_2 or catalyst required.
2. Autoxidation: O_2 required (Schemes 3 and 4).
3. Direct oxidation: Fe^{III} or PBQ required (Scheme 2).

Table 4. Yields of cyanidin and/or delphinidin from proanthocyanidins on oxidative hydrolysis in BH Fe^{III} solvent

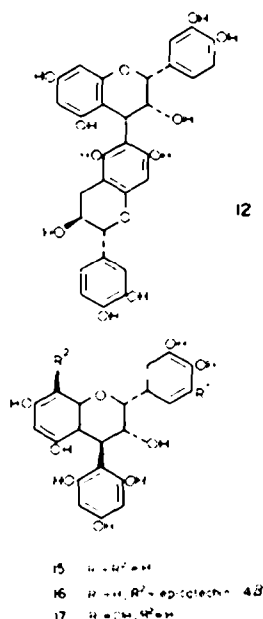
Proanthocyanidin	Product*	Chain length†	$E_{550}^{1\%}$	$E_{280}^{1\%}$ ‡	E_{550}/E_{280}	$E_{550} \text{ §/m.f.}$
(2R,3S,4R)-Leucocyanidin (4)	C	1	460	113	4.1	546
Epicatechin-(4 β $\rightarrow$ 8)-catechin (5)	C	2	270	132	2.0	540
Epicatechin-(4 β $\rightarrow$ 6)-catechin (12)	C	2	225	140	1.6	450
Catechin-(4 α $\rightarrow$ 8)-catechin (13)	C	2	280	137	2.0	560
Epicatechin-(4 β $\rightarrow$ 8)-epicatechin-(4 β $\rightarrow$ 8)-catechin (14)	C	3	345	132	2.6	515
Epicatechin-(4 β $\rightarrow$ 2)-phloroglucinol (15)	C	1	330	118	—	485
Epicatechin-(4 β $\rightarrow$ 8)-epicatechin-(4 β $\rightarrow$ 2)-phloroglucinol (16)	C	2	400	136	—	488
Catechin-(4 α $\rightarrow$ 2)-phloroglucinol (17)	C	1	333	121	—	475
Gallocatechin-(4 α $\rightarrow$ 2)-phloroglucinol (18)	D	1	315	63	—	443
Epigallocatechin-(4 β $\rightarrow$ 2)-phloroglucinol (19)	D	1	330	68	—	465
<i>Chaenomeles speciosa</i> fruit PC polymer	C	9.4	450	138	3.3	505
<i>Chaenomeles chinensis</i> fruit PC polymer	C	12.5	490	134	3.6	532
<i>Gossypium hirsutum</i> flower bud PA polymer	C + D	9.4	465	84	5.5	522

*C = cyanidin, D = delphinidin.

†Average chain length of polymers = no. average degree of polymerization, $\bar{P}_n$, obtained by CMR or VPO.

‡Measured in MeOH.

§m.f. = $(\bar{P}_n - 1)/\bar{P}_n$.



slower rate than the 4 $\rightarrow$ 8 bond [21], and less anthocyanidin is formed, as some of the starting material is diverted to other acid rearrangement products before this can occur.

The second interesting observation is that the procyanidins catechin-(4 α $\rightarrow$ 2)-phloroglucinol (17) and epicatechin-(4 β $\rightarrow$ 2)-phloroglucinol (15) give the same yield of pigment as their prodelphinidin (PD) analogues, gallocatechin-(4 α $\rightarrow$ 2)-phloroglucinol (18) and epigallocatechin-(4 β $\rightarrow$ 2)-phloroglucinol (19). This corroborates our earlier assumption [22], supported by ^{13}C NMR and thiolysis measurements, that PC:PD ratios could be estimated from the spectrophotometrically obtained ratio of the cyanidin (3) and delphinidin (20) produced on hydrolysis. This is in spite of the fact that the reported value for ϵ_{560} for delphinidin (20) is some 30% greater than that for cyanidin [5]. This former estimate may therefore be in error.

It may also be seen, from the similar yields (Table 4) of cyanidin from the 4 $\rightarrow$ 8 linked dimers (5 and 13), and the phloroglucinol derivatives (15 and 17), that the stereochemistry of the C-ring of the PA unit has no influence on the reaction. This would be expected from the proposed reaction pathway (Scheme 1).

The yields from three freshly prepared PA polymers are also given in Table 4. It may be seen that the yields are all in the range $E^{1\%} = 450\text{--}490$. The yield of the mixed PC-PD polymer from *Gossypium hirsutum* flowers (PC:PD = 40:60) is, as expected, about the same as the PC polymers.

Some variation in yield would be expected as different samples of PA polymers exhibit variable average chain length [22–24]. The number-average degree of polymerization, P_n (i.e. average chain length) for each of the polymers is given in Table 4. If the $E^{1\%}$ values are converted to yield per mole fraction of PA unit (Table 4) then the yields are now in the range 500–530, or very similar to the simple model compounds 4, 5 and 13. This observation may be interpreted as implying that these polymers contain largely 4 $\rightarrow$ 8 interflavanoid linkages.

It is particularly important to use freshly prepared samples of polymer to estimate $E^{1\%}$. It was observed that the yield of cyanidin or delphinidin steadily dropped (as did methanol solubility) with storage time. For example, a sample of *C. chinensis* PC polymer isolated in 1982 had an $E_{550}^{1\%}$ value of 360, and a 1983 sample of 420, compared with a 1985 sample of 490.

The ratio E_{550}/E_{280} is commonly used by biologists to estimate the relative degree of polymerization of procyanidin extracts [4, 8]. It may be seen (Table 4) that this ratio varies from 2 for 4 $\rightarrow$ 8 linked PC dimers to 3.3–3.7 for PC polymers. As would be expected from the dimer results, the ratio is 4 for the leucocyanidin monomer.

The ratio is much higher for a PD polymer as E_{274} is much smaller than the equivalent value, E_{280} , for a PC polymer [23]. If we take the value [25] of $E_{274} = 62$ for a PD polymer, and assume $E_{560} \sim 460$ then the corresponding ratio $E_{560}/E_{62} \sim 7.4$. Mixed PD-PC polymers will have values between 3.5 and 7.4, such as observed for the *G. hirsutum* polymer (Table 4).

CONCLUSION

The hydrolysis of proanthocyanidins to anthocyanidins has been shown to occur by an autooxidation following acid-catalysed cleavage of the interflavanoid bonds. The yield of anthocyanidin is critically affected by trace impurities of transition metal ions, and consistent yields of anthocyanidins may be obtained if iron salts are added to the reaction mixture. The yield of anthocyanidin is also dependent on the water concentration in the reaction mixture.

The yield of anthocyanidin from PC or PD polymers is not only dependent on the mole fraction of PA units in the chains, but also on the age of the polymer preparation. For freshly prepared polymers the ratio $E_{550}/E_{280} \sim 3.5$ for a PC polymer, whereas the corresponding ratio E_{560}/E_{274} is estimated to be ~ 7.4 for a PD polymer.

EXPERIMENTAL

The proanthocyanidins (compounds 4, 5, 11–18) were synthesized and purified as described elsewhere [25–27]. All compounds were purified by HPLC except leucocyanidin (4) which was the crystalline dihydrate [27]. The proanthocyanidin polymers were freeze-dried powders isolated and purified as described previously [22]. The quinones were all Fluka samples recrystallized before use. The hydrolyses were carried out using A.R. grade reagents and solvents. The iron reagent was a 2% (w/v) soln of $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 2 M HCl. The hydrolyses were carried out in 8 ml capacity thick-walled, screw-top, glass tubes, sealed with a teflon-lined screw cap. The standard method of hydrolysis was as follows: To each tube is added 1 ml of MeOH soln containing the proanthocyanidin, 6 ml of a soln of *n*-BuOH-conc. HCl (95:5, v/v) and 0.2 ml of a 2% (w/v) soln of the ferric reagent. The solns were capped and thoroughly mixed and suspended in a constant-level water bath run at $95.0 \pm 0.2^\circ$ and heated for 40 min. The solns were cooled and the visible spectrum recorded between $\lambda = 520$ and 580 nm in a 10 mm path-length glass cell.

Normally five pairs of solns were hydrolysed with the highest concn of proanthocyanidin in the MeOH soln of $\sim 0.02\%$ (w/v), and this soln was serially diluted by 5:10, 4:10, 3:10 and 2:10. The final concns of proanthocyanidin in the hydrolysis solns were therefore in the approximate range $0.6\text{--}3 \times 10^{-3}\%$ w/v. The absorbance maxima were then plotted versus proanthocyanidin

concn (on the hydrolysis soln) and the yield of anthocyanidin in terms of the $E^{1\%}$ value calculated from the slope of this curve (Δ absorbance/ Δ proanthocyanidin concn).

The expts involving purging with oxygen or argon were performed in the same way as above, but the cap was replaced with one of annular design lined with a teflon septum. The glass inlet and outlet were syringe needles covered with teflon sleeves to prevent contact between the solvent and metal. The inlet tube was positioned near the bottom of the tube, and the outlet above the surface of the liquid. The soln was purged with a slow stream of gas for 30 min before heating started. The gas flow was such that solvent loss was negligible.

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