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THE POLYMERIC NATURE OF WINE PIGMENTS

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Abstract—During conservation and ageing of red wines, the grape anthocyanins initially responsible for wine colour are displaced, progressively and irreversibly, by more stable polymeric pigments, which account for up to 50 per cent of colour density within the first year. These new pigment forms are much less sensitive to changes in pH than are the anthocyanins, and are quite resistant to decolorization by sulphur dioxide. Evidence is given in support of the hypothesis that the polymers contain quinonoid anhydrobase chromophores derived from the anthocyanins, and which have been stabilized by substitution with reactive flavans. Some possible structures of these tannin pigment materials are discussed.

INTRODUCTION

COLOUR in red wines is at the same time the most appreciated and yet the most ill defined aspect of wine quality. Whereas precise analytical data are available for all other involatile constituents, as well as for numerous volatiles, the chemistry of wine phenolics present at levels ranging from 1–5 g/l. and which are entirely responsible for wine colour and tannin character, is still largely a matter for speculation. As the subtle alterations in colour, astringency and taste which occur during conservation and ageing are due to progressive chemical changes in these materials, the fate of grape phenolics after extraction presents a major challenge in oenological research.

The anthocyanin pigments of wine grapes have been well documented in recent years,[1,2] and the greater complexity of red wine colour, in relation to that of fresh grape extract, has been evident from paper chromatography. The content of anthocyanins, initially present at levels up to 1 g/l., decreases steadily during wine ageing, and the existence of polymeric pigment forms, related to the anthocyanins and responsible for the relative stability of wine colour, has been demonstrated.[3] Gel filtration in acidified aqueous acetone permits separate measurement of polymeric and monomeric pigments, and the pigment profiles obtained effectively represent the 'chemical age' of the wine.[4] The formation in wines of polymeric pigments, considered to be synonymous with the wine tannins, is evidently a vital aspect of the ageing process.

However, an unsatisfactory feature of the gel filtration method, having regard to the extremely labile character of some known tannin precursors, is the need to use acid solvent media ($\mathrm{pH} < 2$), because of the dependence of anthocyanin colour on pH. Thus there has been uncertainty about the origins of the polymeric pigments isolated from wines and,

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[1] P. RIBÉREAU-GAYON, *Les Composés Phénoliques du Raisin et du Vin* (Institut National de la Recherche Agronomique, Paris (1964).

[2] V. L. SINGLETON and P. ESAU, *Phenolic Substances in Grapes and Wine, and their Significance*, p. 31, Academic Press, New York (1969).

[3] T. C. SOMERS, *Nature* **209**, 368 (1966).

[4] T. C. SOMERS, *Vitis* 7, 303 (1968).

although their presence in untreated wines had been confirmed here by dialysis, their structures might yet be modified at low pHs. In any case, the tannin pigments isolated on gel columns in the presence of HCl, because of their cationic character, can not be in the same condition as in wines, in which tartrate is the major anion. This paper reports new observations which provide unequivocal evidence for the predominant role of tannin pigments in aged wine.

RESULTS AND DISCUSSION

Pigment Distribution

Two independent methods give a direct measure of the contribution of polymeric pigments to wine colour, and one of these gives a visual presentation of the actual distribution between polymeric and monomeric forms at the pHs of wine (3·4–4·2). This method is based on the simple finding that the anthocyanins in their various equilibrium forms are completely extractible from wines by iso-amyl alcohol, whereas the polymeric pigments remain in wine solution. Equilibration of this solvent with 10% aqueous ethanol saturated with potassium hydrogen tartrate enables the original wine volume and pH to be maintained during several extractions. A single extraction of a red wine (2 ml) with this solvent mixture (10 ml) removes about 70 per cent of the available anthocyanins and removal is 98 per cent complete after four such extractions. After brief centrifugation to separate phases, the content of polymeric pigments is then evident and measurable at wine pH. In view of the fact that distribution of anthocyanins between iso-amyl alcohol and aqueous acid was introduced many years ago to distinguish glycoside types,[5,6] and that iso-amyl alcohol is routinely used for extraction of anthocyanidins from pigment hydrolysates, it seems remarkable that the utility of this simple technique has not been previously recognized in oenology.

Anthocyanins and other monomeric phenolics, notably cinnamic acids and flavanols, were recovered from the iso-amyl alcohol extract, and identified with known grape phenolics by cellulose TLC. In contrast to the anthocyanins, the polymeric pigments remaining in the aqueous phase are not dialysable, and, though accompanied by discrete colourless phenolics, were seen to be chromatographically similar to the materials previously isolated from wines by gel filtration;[4] they are diffuse to R_f 0·5, but largely immobile in *n*-butanol–acetic acid–water (4:1:5), and are immobile in 2% aqueous acetic acid.

By such analysis of many dry red wines aged to 10 yr, it has been established that the polymeric pigment forms are normally responsible for the major portion of colour in aged wines, accounting for up to 50 per cent within the first year, and reaching an average level of 85 per cent in the oldest wines examined (Fig. 1). Furthermore, the intimate association of colour with total wine phenolics rather than the monomeric anthocyanin fraction is evident from the fact that analyses of residual wine solutions for total phenolics by the Folin Denis reagent showed a similar trend during ageing; phenolics remaining after exhaustive iso-amyl alcohol extraction increased from around 30 per cent in current vintage wines to over 90 per cent in aged wines.

Thus, although colour in young wines is largely due to that small portion of total anthocyanins existing in the coloured forms at wine pH, this situation does not persist much beyond the current vintage, there being continuous shift towards more stable pigment structures. With increasing wine age, minor chromatographically mobile, but indiscrete

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[5] R. WILLSTÄTTER and E. H. ZOLLINGER, *Ann.* **412**, 208 (1916).

[6] G. M. ROBINSON and R. ROBINSON, *Biochem. J.* **26**, 1647 (1932).

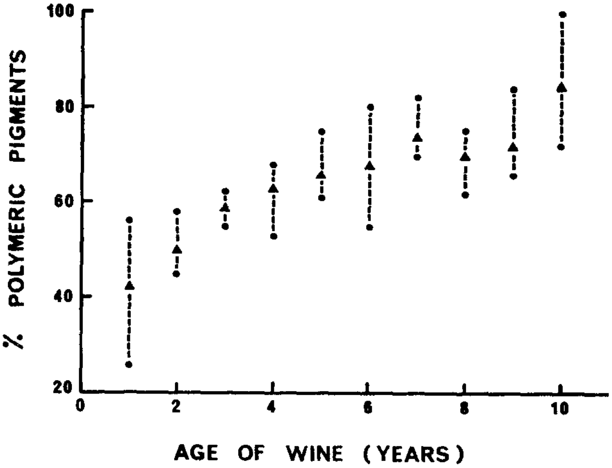

FIG. 1. CONTRIBUTION OF POLYMERIC PIGMENTS TO WINE COLOUR AGAINST AGE OF WINE, DETERMINED BY EXTRACTION PROCEDURE.

Average values (▲) in each year are plotted, and the extremes (●) of each group are shown. The first value, at 1 yr, is the mean of 40 different analyses, and the others are means of four or more different wines from each year.

pigments begin to appear in the iso-amyl fraction in addition to residual anthocyanins, but at all times the visible spectrum of the polymeric pigment fraction generally parallels that of the total wine, with coincident λ_{max} decreasing from 530 nm in young wines to 515 nm over 10 yr.

The colour composition in these terms of a 5-yr-old dry red wine is shown in Fig. 2. The considerable response of the polymeric pigment fraction to lowered pH, in relation to that of the total wine is a measure of the anthocyanin-like character of these pigments;

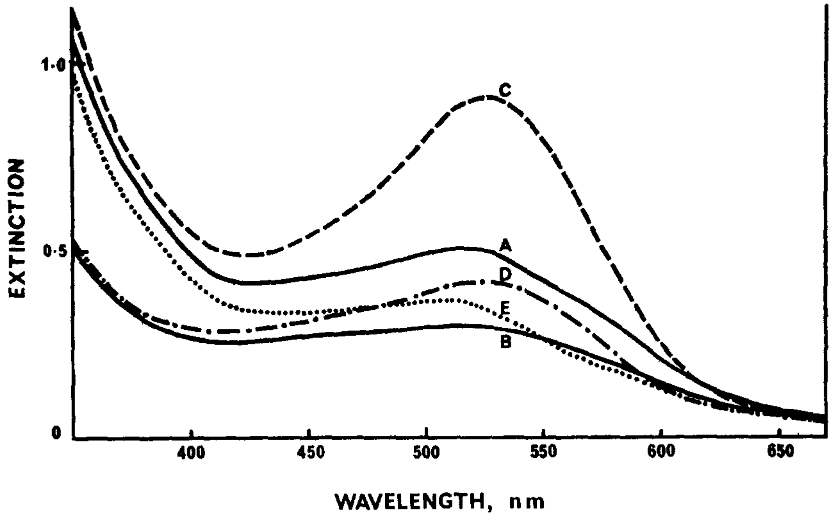

FIG. 2. SPECTRA SHOWING COLOUR COMPOSITION OF A 5-YR-OLD RED WINE, AND RESPONSES TO CHANGE IN pH AND BISULPHITE TREATMENT.

(A) whole wine, pH 3·70; (B) residual pigments after solvent extraction, pH 3·70; (C) whole wine pH 0·70; (D) residual pigments, pH 0·70; (E) whole wine after bisulphite treatment.

although the pH response of this fraction does decrease steadily during wine ageing, it is still as much as 25 per cent in 10-yr-old wines. Thus, analyses for anthocyanins which have been based on such treatment of intact wines[7] must always give higher than the true values.

A further significant feature in which these polymeric pigments differ markedly from the accompanying grape anthocyanins is their resistance to decolorization by sulphur dioxide, and this is the basis of the second method for their detection in wines. Anthocyanins in aqueous alcoholic buffers at wine pH are immediately decolorized by an excess of sodium bisulphite, whereas the percentage of colour remaining in a red wine after such treatment is seen to be a measure of the content of polymeric pigment forms which approximates to that remaining after exhaustive extraction with iso-amyl alcohol (Fig. 2). This test enables an almost immediate estimate of the 'chemical age' of a dry red wine.

Origins of the Polymeric Pigments

The progressive nature of the contribution by the polymeric pigments to wine colour, which was so clearly evident in these analyses (Fig. 1), led to re-examination of their origins. The bulk of extractible grape phenolics have been described as tannins,[1] and tannin pigments similar to the polymeric pigments of wines have been isolated from grape extracts by gel filtration.[8] However, the use of HCl (0·1% methanolic and 0·1% with aqueous acetone) during extraction and treatment of pigment solutions, which has become standard practice since the flavylium salt character of anthocyanins was first recognized by Willstätter,[9] suggested that these tannin pigments might be artifacts of extraction, especially in view of the acid sensitivity of accompanying proanthocyanidins and catechins.[10] This supposition has been verified by various new examinations of grape extracts and young wines.

Whereas immobile red brown tannins invariably accompany the bright anthocyanins on chromatograms of grape skin extracts prepared with methanolic 0·1% HCl, only slight to zero quantities of such materials have been found on chromatograms made immediately after extraction with cold methanol or 50% aqueous methanol. In the later cases, however, the presence of colourless phenolics at the origin and at low R_f was revealed by spray reagents. Thus, it is apparent that the use of extraction media of low pH gives rise to tannin pigments as artifacts from wine grapes, and the occurrence of similar compounds in red wines is now seen to be the natural consequence of reaction processes which most likely begin with crushing of the berry and mixing of plant components in the pH range 3·4–4·2.

Direct evidence in support of the above view was gained from the following experiments. A 'model wine' was prepared by solution of pigment residues from a cold methanolic extract of fresh grape skins (100) in 10% aqueous alcohol saturated with potassium hydrogen tartrate (200 ml, pH 3·7)—a few drops of chloroform was added to prevent bacterial spoilage. At intervals over a period of 90 days, the visible spectrum was recorded (1 mm cell) and also that of residual pigments after treatment with excess bi-sulphite (10 mm cell). The initial contribution of resistant pigments to colour of the solution was 4 per cent, but continuous increase occurred at room temperature, reaching 41 per cent after 90 days (Fig. 3), during which a 45 per cent loss in absorbance of the solution also occurred. During this period, the portion of total pigments remaining after exhaustive extraction with the iso-amyl alcohol

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[7] P. RIBÉREAU-GAYON and E. STONESTREET, *Bull Soc. Chim Biol.* **47**, 2649 (1965).

[8] T. C. SOMERS, *J. Sci. Food Agric.* **17**, 215 (1967).

[9] R. WILLSTATTER and A. E. EVEREST, *Ann.* **401**, 189 (1913).

[10] K. FREUDENBERG and K. WEINGES, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 197, Pergamon Press (1962).

mixture increased from 0 to 40 per cent, with corresponding changes in the pigment profiles obtained by gel filtration.[4]

Shifts in the visible λ_{max} during ageing of the 'model wine' solution, from 532 nm to 539 nm after 15 days, and decreasing thereafter to 530 nm at 90 days, were apparently due to the increasing level of bisulphite resistant pigments, the λ_{max} of the latter being in the range 540–556 nm for the first 15 days, with gradual decrease to 530 nm at 90 days. These spectral changes correlated well with the loss of purple tints which normally characterize a new wine.

In a parallel experiment, skins from fresh grapes were put directly into boiling methanol, and maintained at this temperature for 10 min prior to solution of pigments as before in the tartrate medium. Spectral observations, and the increase with time in residual pigments, were closely similar to those described above for the other 'model wine'. Such pretreatment would prevent enzymic influences and although oxidases may be also involved in pigment changes during actual vinification, the major factor in these reactions appears to be the pH of the medium.

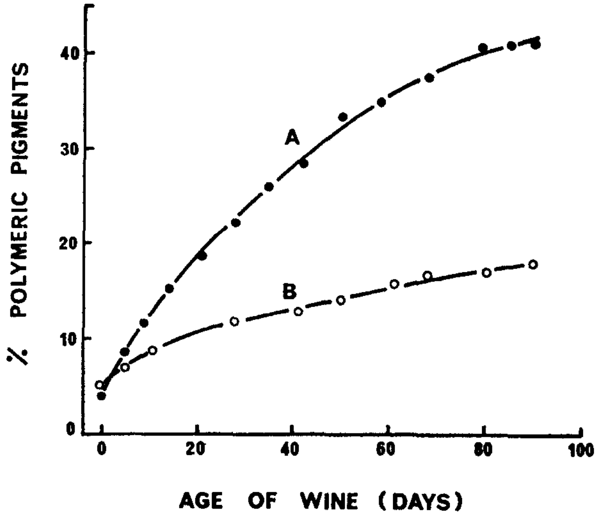

FIG. 3. PROGRESSIVE INCREASE IN COLOUR CONTRIBUTION OF POLYMERIC PIGMENTS IN (A) 'MODEL WINE' SOLUTION, AND (B) NEW WINE FROM END OF FERMENTATION, DETERMINED BY BISULPHITE PROCEDURE.

Similar analyses were also made on a 1970 Shiraz dry red wine for a 90-day period from conclusion of the primary fermentation. In this wine the portion of total pigments resistant to decolorization by bisulphite increased from 5 to 18 per cent (Fig. 3) with, however, less marked spectral changes than in the model solution, in which much more rapid changes in pigment distribution occurred.

Pigment Stability and likely Structures

Colour in acid solutions of anthocyanins[11,12] and also in young wines[1] has generally been attributed to the flavylium cation (I) in equilibrium with the colourless chromenol

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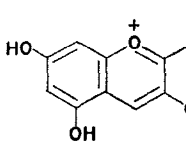
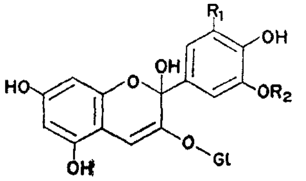
[11] E. SONDHEIMER, *J. Amer. Chem. Soc.* 75, 1507 (1953).

[12] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, p. 388, Butterworth, London (1963).

pseudo-base (II). The violet quinonoid anhydrobase (III) is seen at pH 4·5–6, but is transient, the anhydrobase rapidly changing to the pseudobase (II) in this range.



I



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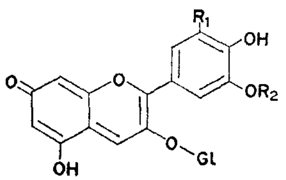

III

Gl = glucose, R_1 = H, OH,OCH_3; R_2 = H,CH_3 in *Vitis vinifera* anthocyanins, some of which may be acylated with hyroxycinnamic acids or acetic acid.

The existence of colourless forms in equilibrium with the polymeric pigments of wines is indicated by the colour dependence of the latter on pH (Fig. 2), which suggests the presence of flavylium chromophores in the polymer structure. However, the equilibria are more complex than those in solutions of anthocyanins and other flavylium salts, the spectra of which show *iso*sbestic points and yield constant pK values in acid solutions.[13,14] Thus the

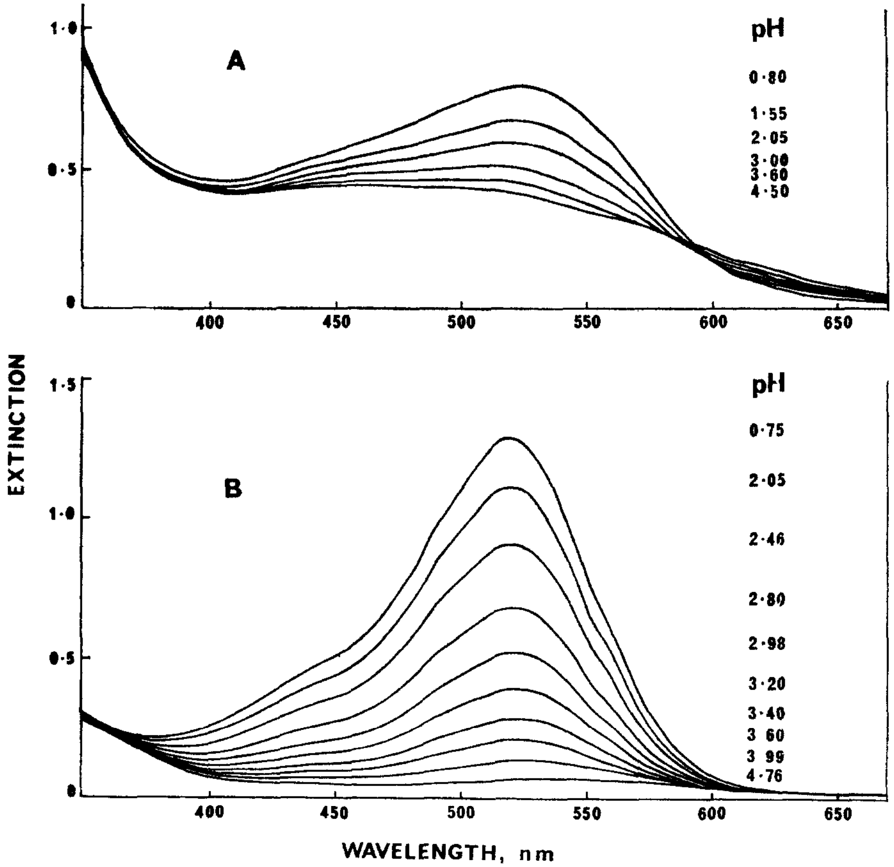

Fig. 4. Spectral responses to pH of (A) polymeric pigment fraction from a 5-yr-old red wine, and (B) total grape anthocyanins; in aqueous buffers, each series measured at 24 hr.

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[13] L. Jurd, *J. Org. Chem.* **28**, 987 (1963).

[14] C. F. Timberlake and P. Bridle, *J. Sci. Food Agric.* **18**, 473 (1967).

effect of pH on the polymeric pigments (Fig. 4A) is contrasted with the much greater colour dependence of total grape anthocyanins on pH (Fig. 4B). The greater colour stability of the polymeric pigments at all pH levels and the resistance of these solutions to decolorization by bisulphite were other notable points of difference from the anthocyanins solutions.

Percentages of the coloured forms and the calculated pK values for a simple pH dependent equilibrium[11] between such forms as (I) and (II) are presented in Table 1. Judging by the constancy of pK values, such a relationship appears to hold true for total anthocyanins up to pH 3·2 at least, and the higher pK values above this pH are attributed to the increasing contributions from anhydrobases (III), as has been reported for cyanidin 3-glucoside.[14] This is supported also by shifts in λ_{max} of the anthocyanins solution (Table 1) and especially by the striking colour difference between strongly acid solutions (scarlet below pH 2·5) and solutions in the pH range 3·0–4·5 (bluish pink to purple); similar spectral and colour observations were made for pure malvidin 3-glucoside, the major *V. vinifera* pigment (pK 2·76 ± 0·03 up to pH 3·50). Thus it is clear that, throughout the wine pH range, the quinonoid forms (III) are present in addition to the cationic forms (I), in equilibrium with the colourless forms (II), and that less than 20 per cent of total anthocyanins in wines would be expected to exist in these coloured forms. In this regard, it is intriguing to note that because of the considerably lower pK values (1·9–2·1) of anthocyanidin 3,5 diglucosides,[14] these pigments can make only slight contribution *per se* to the colour of wines or juices from such species as *V. rotundifolia* and *V. rupestris* in which diglucosides are the major grape pigments—such colour must be dependent upon a pronounced displacement of the natural equilibria, and stabilization of pH sensitive chromophores.

These observations assist in interpretation of the remarkable colour stability of the polymeric wine pigments, which retain more than 50 per cent of maximum colour in the wine pH range (Table 1) and are largely responsible for total wine colour (Figs. 1 and 2). The rapidly increasing pK values found for the latter above pH 1·5 are considered to be due to anhydrobase formation, just as occurs in anthocyanin solutions at much higher pH.

TABLE 1. IONIZATION DATA—TOTAL GRAPE ANTHOCYANINS AND TOTAL POLYMERIC WINE PIGMENTS

Total anthocyanins				Total polymeric pigments			
pH	λ_{max} (nm)	Coloured forms (%)	pK*	pH	λ_{max} (nm)	Coloured forms (%)	pK*
0·75	519	100	—	0·80	524	100	—
				1·20	523	92·5	2·29
1·70	519	93·4	2·85	1·55	522	85·0	2·30
2·05	520	85·3	2·81	2·05	519	75·0	2·53
2·46	520	69·5	2·82				
2·80	520	51·7	2·83	2·70	516	66·8	3·00
2·98	522	40·5	2·81	3·00	514	65·0	3·27
3·20	522	30·5	2·84				
3·40	523	22·4	2·86				
3·60	523	16·6	2·90	3·60	508	58·1	3·74
3·99	523	11·2	3·09	4·00	508	56·9	4·12
4·18	523	8·9	3·17				
4·76	526	6·2	3·58	4·50	500	55·0	4·59

* Calculated from the supposed relationship pK = log (coloured/colourless forms) + pH.

It is therefore proposed that the chromophores of the polymeric pigments are such quinonoid structures stabilized by substitution against the pH and bisulphite responses characteristic of the monomeric pigments.

The above conclusion is well supported by the close spectral similarities, in λ_{max} and absorbance in the 550–700 nm region, between the polymeric pigment fraction (purple) of the 'model wine' solution at pH 3·7 and that of malvidin 3-glucoside at pH 5·4; at this pH the violet anhydrobase (III) fades within a few minutes (Fig. 5). Although the colour of this fraction is greatly modified during further ageing, with shifts in λ_{max} towards 500 nm in aged wines, the general similarities of the very stable polymeric pigments with the transient monomeric anhydrobases seen at higher pH are retained throughout the wine pH range (see Fig. 4A).

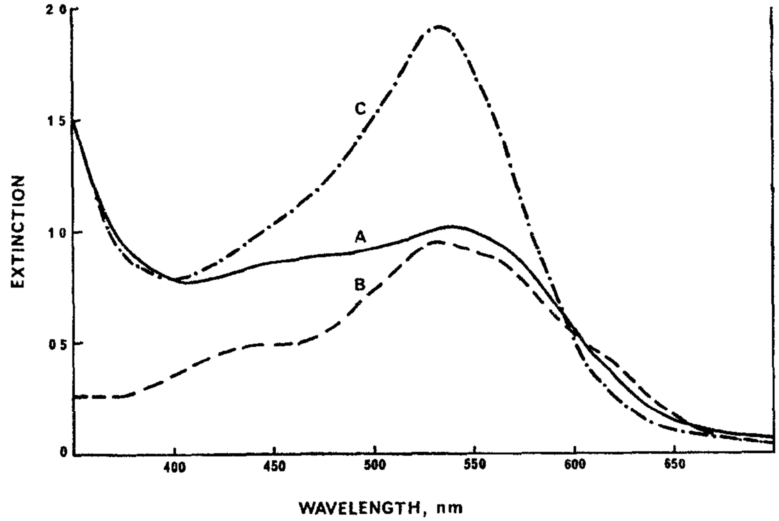
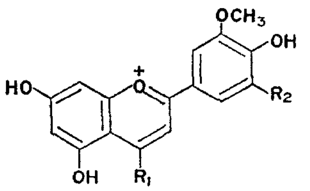


Fig. 5. Spectral comparison of stable polymeric forms with a transient monomeric anhydro base.

(A) Polymeric pigment fraction from 'model wine' experiment at 15 days, pH 3·75; (B) malvidin 3-glucoside at pH 5·4, measured immediately, (C) shows response of sample (a) to lower pH, 1·5.

The nature of reactions leading to stable pigment forms in wine is suggested by recent reports on the effect of 4-substitution in simple flavylium salts. Thus introduction of CH_3 or Ph at this position, as in such compounds as (IV) and (V), confers extreme resistance to decolorization by sulphur dioxide.[15] Our spectral studies of (IV) and (V) in 50% aqueous methanolic buffers through the pH range 1–6 have revealed that they also have remarkable



IV $R_1 = CH_3$, $R_2 = H$
V $R_1 = Ph$, $R_2 = OCH_3$

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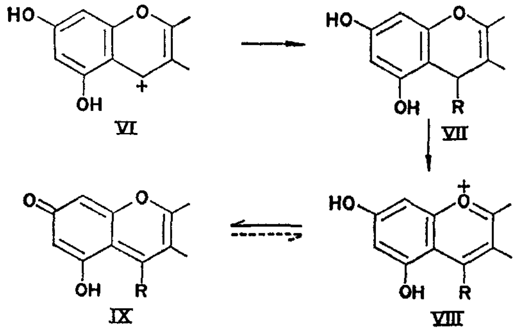
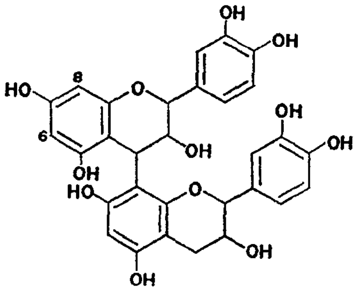
[15] C. F. Timberlake and P. Bridle, *Chem. & Indust.* 1489 (1968).

colour stability to higher pH, retaining respectively 95 and 56 per cent of maximum colour at pH 4·3. Furthermore, the anhydrobase forms are quite stable even at pH 6, both solutions retaining 40–50 per cent of maximum colour for more than a week.

Although similar structural stabilization of anthocyanins or natural flavylium salts has not yet been proven, their susceptibility to attack by nucleophilic reagents such as are present in wines has been well recognized.[12] Jurd and Waiss have demonstrated condensation products from synthetic flavylium salts with various phenolics in aqueous solutions at pH 3–5[16] and, by reaction with catechin, have obtained a dimeric flavylium-flavan pigment.[17] All these reactions involve substitition of the 4-position of the heterocyclic ring, and demonstrate the feasibility of similar condensations of anthocyanins in wines.[18]





X

Reactions by which the colour of young wines is altered and stabilized during ageing are thus considered to follow the sequence shown. Electrophilic substitution into a wine component RH by the carbonium ion (VI) yields the labile flavene (VII), which is oxidized by air or by involvement in redox systems to the condensed anthocyanin species (VIII). The more stable quinonoid structure (IX) assigned to the chromophores of the polymeric pigments is then formed by deprotonation.

The substituent R is most likely to be a dimeric proanthocyanidin (X) linked at the 6 or 8 positions. Several such dimers have been isolated from plant sources, and Weinges *et al.* have recently presented evidence for their biosynthesis directly from catechins,[19] and not, as previously assumed, by acid catalysed condensation of a flavan-3,4-diol with a catechin; this is supported by the work of Haslam.[20] The presence of flavan-3,4-diols, which have been freely implicated in speculation about fruit and wine tannins, has never been proven in fruit or grape extracts, the proanthocyanidins being accompanied principally by catechins.[19]

However, a larger degree of condensation in the tannin pigment complex is indicated by the UV–visible spectra,[3] and the above may serve merely as a starting point. Numerous structural possibilities exist for the complexities of wine colour and tannin character during ageing, quite apart from the stereochemical aspects consequent upon the presence of many asymmetric centres. Slow increase in mean molecular size of the polymers could proceed via the species (XI) by further electrophilic substitution into (IX) or (X), leading to structures like (XII); such mechanisms for the acid catalysed condensation of catechins have been

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[16] L. Jurd and A. C. Waiss, *Tetrahedron* **21**, 1471 (1965).

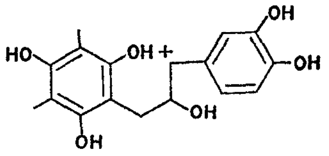
[17] L. Jurd, *Tetrahedron* **23**, 1057 (1967).

[18] L. Jurd, *Amer. J. Enol. Vit.* **20**, 191 (1969).

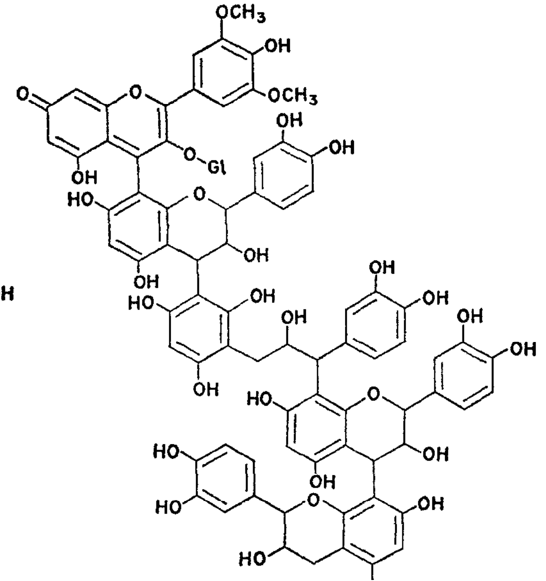
[19] K. Weinges, R. Wild and W. Kaltenhäuser, *Z. Lebensmittel Untersuch u. Forsch.* **140**, 129 (1969).

[20] E. Haslam, *J. Chem. Soc.* (C), 1824 (1969).

favoured by Freudenberg and other workers.[21] The presence of additional quinone structures, arising from oxidation of catechol and phloroglucinol residues in the polymers, is also very likely, and would account for the browner tints of aged wines. In support of this, it is known that oxidative polymerization of catechins, whether by autoxidation or polyphenoloxidase, yields tannins having high absorbance in the 400–500 nm region, and this is attributed to the presence of repeated quinone units.[22] Further condensations and cross-linkages would then be possible by quinone polymerization mechanisms.[21]



XI



XII

Another condensation mechanism which may be very significant in wine ageing is a Baeyer reaction by which $CH(CH_3)$ bridges between phloroglucinol residues could be formed from acetaldehyde; observations on effects of the latter on wine colour have been reported over many years.[23] Preliminary experiments here, in which pigment analyses have been based upon the solvent extraction procedure, have shown that the intensification of colour which results from addition of acetaldehyde to red wines is due to increase in the level of polymeric pigments, and that there is an accompanying decrease in anthocyanin content.

There are, therefore, several likely mechanisms by which tannin pigment materials may be elaborated from the flavylium species (I) during wine ageing. I have proposed that the stabilized chromophores are essentially quinonoid, and that they are based upon the anhydro base structures (III) substituted with flavan units as in (XII), with further colour contribution from quinone moieties produced by slow oxidative changes. Plant pigments of related structure are known, and quinonoid forms such as (III) may prove to be the natural state

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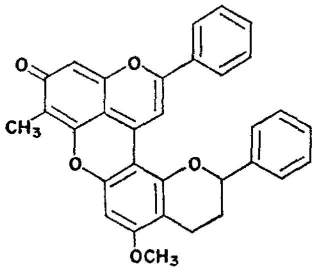
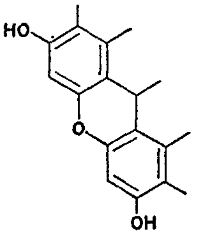
[21] E. Haslam, *Chemistry of Vegetable Tannins*, p. 66, Academic Press, London (1966).

[22] D E. Hathway and J. W. T. Seakins, *Biochem. J.* 67, 239 (1957).

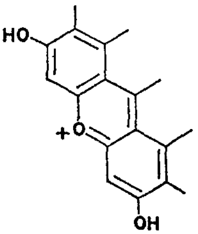
[23] V. L. Singleton and P. Esau, *Phenolic Substances in Grapes and Wine and their Significance*, p. 197, Academic Press, New York (1969).



XIII



XIV



XV

of anthocyanins in some flowers tissues.[12,24] The structural relationship between a portion of the structure (XII) and the red anhydrobase pigment dracorubin (XIII) is noteworthy, as is the similarity to xanthene (XIV) and xanthylium structures (XV). Evidence for the presence of xanthene nuclei in condensed tannins has recently been reported,[25] and on this basis xanthylium chromophores (yellow) may also contribute to the high absorbance of wine tannin pigments in the 400–500 nm region.[18]

Although the structural problems presented here are very formidable, it is expected that model experiments with natural pigments may at least enable elucidation of the simpler polymeric pigments such as are formed in young wines. However, it is evident, from the distribution behaviour of wine pigments, that the real state of the polymers is even more complex than already indicated. The very high aqueous affinity of the polymeric pigment forms, relative to that of the anthocyanins, suggest that the former are strongly associated with hydrophilic molecules in wine solution. In their natural state, therefore, these materials may conceivably exist as molecular aggregates, in which quinonoid carbonyl groups are in combination with such compounds as ethanol, glycerol and tartaric acid to form sugar-like hemiacetal structures of high aqueous solubility.

EXPERIMENTAL

Materials

With the exception of some of the current vintage wines, which were mostly of commercial origin, all wines and grape extracts were *Vitis vinifera*, cv. Shiraz (syn. Petite Syrah, Hermitage). The grapes were used fresh from a local vineyard, and all aged wines were from the Experimental Winery cellars of this Institute. Pure anthocyanins were isolated from grape extracts by paper chromatography and finally gel filtration on Sephadex G-25 in 50% aq. acetone (HCl)[4]. Grape pigment extracts were freshly made by several macerations of the washed skins with methanol; concentrates were prepared, by removal of solvent < 30°, within 1 hr before use.

Measurement of Polymeric Pigment Content

Extraction proceduer. Iso-amyl alcohol (500 ml) was equilibrated with a saturated solution (pH 3·7) of potassium hydrogen tartrate in 10% aq. EtOH by shaking with 4 × 50 ml vol. of this solution. The wine or pigment solution (2·0 ml) was then extracted with 4 × 10 ml aliquots of this mixture in a small centrifuge tube. Phases were separated by brief centrifugation and the lower layer removed between each extraction. Colour density ($A_{420} + A_{520\ nm}$) of the aqueous phase was then measured in a 1 or 2 mm cell and expressed as a percentage of the original colour density. Total anthocyanins and other phenolics were recovered from the combined extracts by addition of a large volume of light petroleum and transfer into 1% aq. HCl (1–2 ml).

[24] K. Hayashi, in *The Chemistry of Flavonoid Compounds* (edited by T. A. Geissman), p. 248, Pergamon Press, Oxford (1962).

[25] L. Jurd and T. C. Somers, *Phytochem.* 9, 419 (1970).

Bisulphite Procedure. The wine or pigment solution (5·0 ml) was treated with 20% aq. sodium metabisulphite (0·2 ml) and, after 15 min, the visible spectrum was compared with that of the original solution, which had been similarly diluted with water. Residual colour was expressed in the same way as above.

Spectral Responses of Pigment Fractions to Varying pH

The polymeric pigment fraction was prepared by exhaustive extraction of a 5-yr-old wine (5 ml) with the equilibrated iso-amyl alcohol mixture (5 × 25 ml), checking the progressive removal of anthocyanins by acidification of each extract. A fresh aq. sol. of total grape anthocyanins was obtained via methanol extract of grape skins; it was slightly acidified with HCl prior to preparation of the test solutions.

The two solution series were prepared by dilution of aliquots with HCl, formate and acetate buffers to give solutions of equal concentrations over the pH range 1–5. The spectra were recorded, after 24 hr, on a Unicam S.P. 800 spectrometer in 1 cm cells. Solutions were not protected from light, but no spectral changes were noted between readings at 2 and 24 hr.

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