former to be converted to the corresponding benzonitriles. The investigation of the alkaline hydrolysis of O-(methylcarbamoyl)-*syn*-benzaldoximes gave no evidence for the involvement of such a reaction (Mrlina and Calmon, 1980). However, such a conversion was reported by Payne et al. (1966) as a side reaction in the alkaline hydrolysis of aldicarb and might therefore occur at the level of the catalytic site of acetylcholinesterase. Furthermore, Crawford and Woo (1965) found a highly negative ρ value (−0.77) for the conversion of *syn*-benzaldoxime arenesulfonates to nitriles. The ρ value observed for benzaldoxime carbamates might therefore be accounted for by such a reaction which cannot be considered in the case of acetophenoxime carbamates.

Since the bimolecular rate constant k_{OH} is susceptible to electronic substituent effects (Mrlina and Calmon, 1980), it seemed interesting to use it in structure–activity relationships:

O-(Methylcarbamoyl)benzaldoximes:

$$\log (1/I_{50}) = \underset{(0.319)}{0.570} \log k_{OH} + 5.055$$

$$n = 5,\ s = 0.217,\ r = 0.816$$

O-(Methylcarbamoyl)acetophenoximes:

$$\log (1/I_{50}) = \underset{(0.375)}{1.450} \log k_{OH} + 7.049$$

$$n = 6,\ s = 0.315,\ r = 0.888$$

It can be pointed out that the correlation coefficients are not as high as those obtained for the plots of log k_{OH} against σ (Mrlina and Calmon, 1980). However, their order of magnitude is significant enough and does not preclude the possible use of k_{OH} instead of σ. The relatively high value of the correlation coefficient may suggest that the behavior of the serine hydroxyl of acetylcholinesterase toward O-(methylcarbamoyl)oximes is, in a first step, analogous to that of the hydroxide ion in the alkaline hydrolysis. This is not inconsistent with the scheme of Nishioka since the in situ formation of isocyanate would lead to the carbamoylation of serine in a second step. The oxime moiety is therefore no more than a nonspecific carrier of a reactive carbamoylating species.

CONCLUSIONS

The results from the limited amount of data used in this investigation indicate that the anticholinesterase activity of substituted O-(methylcarbamoyl)oximes depends largely on the reactivity of the molecule as estimated by the bimolecular rate constant k_{OH} and the free-energy parameter σ. The occurrence of the imine bond between the ester oxygen atom and the phenyl ring does not result in a change-over in the mechanism of alkaline hydrolysis although the Hammett ρ value is different from those usually observed for phenyl carbamates (Mrlina and Calmon, 1980). The kinetic investigation of the alkaline hydrolysis of O-(methylcarbamoyl)oximes therefore allowed the ρ values found in structure–activity relationships (log $1/I_{50}$ vs. σ) to be better understood. These values suggest that oxime carbamates inhibit acetylcholinesterase in the same way as the phenyl N-methylcarbamates which bear strongly electron-withdrawing substituents ($\rho > 0$). Moreover, the difference in the ρ values observed for the benzaldoxime and acetophenoxime derivatives underlines the role of the disubstitution at the carbon atom of the imine bond, although it is not possible to distinguish between a better carbamoylating ability toward the esteratic site and a possible parallel reaction for *syn*-benzaldoxime carbamates.

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Bitterness and Astringency of Phenolic Fractions in Wine

The astringency and bitterness of four fractions of grape seed phenolics were rated in wine by sensory evaluation. All fractions were found to be astringent and bitter. Significant differences in astringency as the phenolic concentration was increased were found for the two intermediate molecular weight anthocyanogen fractions: (II) dimeric anthocyanogens and (III) trimeric and tetrameric anthocyanogens. Bitterness increased significantly only in fraction III. The condensed tannin fraction (IV) on a ppm basis was the most intensely bitter and astringent. Relative astringency expressed in this fashion increased with increasing molecular weight from I to IV ($p < 0.001$). Catechins (I) and condensed tannins (IV) had significantly higher ratios of bitterness to astringency ($p < 0.001$) than fractions II and III.

In wine, beer, cider, and many other fruits, important taste attributes of astringency and bitterness are contributed by phenolics. Often confused with bitterness, which is the sensation perceived at the back of the tongue, as-

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tringency is the "dry-mouth" feeling thought to be produced by the interaction of polyphenols with the proteins of the mouth (Singleton and Esau, 1969). The astringent flavonoid phenolics with a molecular weight of 500 or higher are generally referred to as "tannins" because of their ability to precipitate proteins by cross-linking or hydrogen bonding with them as in the tanning of leather.

Chemically, the relative astringency of flavonoids, defined as their ability to precipitate proteins, has been reported to increase with molecular weight (Bate-Smith, 1973). However, in ripening fruit, astringency is reported to decrease as the flavonoids polymerize to tasteless compounds (Joslyn and Goldstein, 1964). Similarly, in aged red wines, the loss of astringency is thought to be the result of polymerization and precipitation of the astringent flavonoids (Singleton and Noble, 1976).

Although little research on the astringency and bitterness of phenolics has been reported, it is readily apparent that different fractions of phenolic material have different taste properties. Rossi and Singleton (1966) separated the flavonoid phenols of grape seeds into three fractions for chemical and sensory evaluation. The monomeric flavan-3-ol fraction, later shown to contain (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-gallate was reported to have a bitter taste in a wine base (Su and Singleton, 1969). Even at concentrations beyond the levels at which they would be expected to occur in wines, the catechins were not astringent. The fraction containing dimeric anthocyanogens produced both bitter and astringent taste sensations. The anthocyanogens of higher molecular weight in the "condensed tannin" fraction were also both bitter and astringent; at any concentration, they were more astringent than the smaller anthocyanogens in the second fraction.

Lea and Timberlake (1974) evaluated the taste effects of fractionated cider phenolics in a cider phase. Most of the bitterness and astringency of the cider phenolics was associated with two unidentified fractions which were thought to be anthocyanogen trimers or higher polymers. Catechin, chlorogenic acid, and dimeric anthocyanogens were reported to make only small contributions to the bitterness and astringency of the cider. Recently, Lea and Arnold (1978) found no one anthocyanogen (procyanidin) isolated from bittersweet cider could be uniquely identified with bitterness or astringency. However, bitterness was most intense in the tetrameric epicatechin, whereas astringency reached a maximum in the higher anthocyanogen polymers.

In wines, the flavonoid phenolic content [expressed as gallic acid equivalents (GAE)] ranges from 0 to 200 mg/L in white wines to 800 to 1600 mg/L in commercial reds. Since the flavonoid phenolics are found primarily in grape seeds, skins, and stems, only with appreciable pomace contact prior to pressing will flavonoids be extracted into wine (Singleton et al., 1975). In white wines, the overall quality has been reported to decrease with increased phenolic content, produced generally by a delay between crushing and pressing the grapes (Ough, 1969). Singleton et al. (1975) reported for several white grape varieties, that astringency increased significantly with increased pomace contact time from 1 to 5 days, while quality ratings were significantly lower with higher phenolic contents and longer pomace contact times. Bitterness was not reported to increase significantly with the phenolic content increase.

To evaluate the specific sensory contributions of certain characterized phenolic fractions to wine taste and mouthfeel, in this study, the astringency and bitterness of four relatively defined flavonoid phenolic fractions have been determined.

Table I. Analyses[a] of Base Wines

	wine 1	wine 2
titratable acidity, g of tartaric acid/100 mL	0.67	0.70
pH	3.40	3.14
ethanol, v/v	12.20	12.80
phenolics		
nonflavonoid, mg/L of GAE[b]	170.0	190.0
flavonoid, mg/L of GAE[b]	0	0

[a] Determined as described in the text. [b] GAE, gallic acid equivalent.

Table II. Mean Intensity Ratings[a] and Phenolic Content of Samples Evaluated

fraction[b]	phenolics,[c] mg/L of GAE	mean astringency rating[d]	mean bitterness rating[d]
	160	3.95[a]	6.81[b]
I	235	3.81[a]	7.03[b]
	300	3.47[a]	6.92[b]
	109	4.46[c]	7.72[e]
II	170	4.47[c]	7.29[e]
	235	5.46[d]	7.27[e]
III	40	4.32[f]	7.14[h]
	80	4.78[f]	6.91[h]
	120	5.56[g]	7.83[i]
	5	4.05[j]	7.37[k]
IV	10	3.86[j]	7.61[k]
	15	4.02[j]	7.50[k]

[a] Ratings determined in triplicate by 11 judges. [b] Fractions described in the text. [c] Determined by the method of Singleton and Rossi (1964) and expressed as gallic acid equivalents (GAE). [d] Means with the same superscript letter not significantly different at significance levels shown in Table III.

EXPERIMENTAL SECTION

Phenolic Fractions. Grenache grape seeds were separated from grapes, rinsed, and ground. Phenolics were extracted with 75% ethanol (v/v), and the extract was freeze-dried. Four fractions were isolated by elution from a diatomaceous earth column (Celite 545) using a solvent program of ether, ethyl acetate, butanol, and ethanol. The fractions soluble in each mobile solvent were freeze-dried and were found to be chromatographically pure with respect to each other. The phenolic composition of each fraction was checked by paper chromatography as described by Singleton et al. (1966). By these procedures and recent results of Lea et al. (1979), the fractions were shown to contain, respectively, catechins (I), small and polar phenols, primarily dimeric anthocyanogens (II), intermediate (mainly trimeric and tetrameric) anthocyanogens (III), and phlobaphenes and condensed tannins, large anthocyanogen polymers of more than 5 or 6 units (IV). Nonphenolic material in the fractions was largely carbohydrate and present at low concentrations since the gallic acid equivalent (GAE) per unit weight approached theoretical values.

Sample Preparation. Two white wines of clean, neutral character (made in the university winery) were selected as base wines. Their nonflavonoid phenolic content, determined as described by Kramling and Singleton (1969), together with ethanol, pH, and titratable acidity assayed with procedures of Amerine and Ough (1974) are shown in Table I. Neither wine contained any flavonoid phenolics. Fractions I, II, and III were evaluted in wine 1, a blend of Thompson Seedless and Chenin blanc wines, and fraction IV in wine 2, a Chardonnay. The phenolic material was added to the wine and stirred until dissolved. Samples were flushed with nitrogen and held overnight at

Table III. Calculated Mean Square (MS) for Astringency and Bitterness for Four Phenolic Fractions

		fraction I		fraction II		fraction III		fraction IV	
source of variation	df[a]	astringency MS[b]	bitterness MS[b]	astringency MS[b]	bitterness MS[b]	astringency MS[b]	bitterness MS[b]	astringency MS[b]	bitterness MS[b]
phenolic concentration (P)	2	2.01	0.40	10.90**	2.08	12.92**	7.52*	0.49	0.33
judge (J)	10	18.10***	15.37***	19.39***	19.11	19.08***	9.27***	7.57*	10.58***
replication (R)	2	11.00**	2.48	3.44	15.02	4.31	4.22	1.51	0.32
P × J	20	1.30	3.48	1.72	6.30	2.78	2.85	2.30	1.57
P × R	4	1.28	2.87	2.97	13.61	0.38	1.37	3.16	1.08
J × R	20	6.15***	6.59**	9.57***	7.49	2.27	3.59*	3.44	1.90
error	40	1.37	2.16	1.78	14.40	1.87	1.86	2.90	1.47

[a] Degrees of freedom. [b] Significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

10 °C. The reference "bitter" standard was prepared in the same way with 300 mg/L of added caffeine. The total phenolic content of each solution was determined at the time of tasting.

Sensory Evaluation. Sensory judges were 11 male students and workers of the Department of Enology and Viticulture, who had participated previously in a study of astringency and bitterness of total phenolic extract in a model system (Arnold and Noble, 1978). Using the same tasting conditions and procedures as described previously, each fraction was tested four times, with the results from the first session discarded. At each test, three different concentrations of a given fraction were presented for evaluation in a random order, in blue glasses to mask color differences. The "bitter" reference sample, containing caffeine, was assigned a bitterness intensity rating at 7.5 cm and astringency 3.0 cm on the otherwise unstructured 13-cm scale. The scale was anchored at the end points by labels of "none" and "extreme". The scores for intensity of bitterness and astringency were quantified by measuring the distance in centimeters from the left end point on the scale to the rating mark and analyzed by analysis of variance.

RESULTS AND DISCUSSION

All fractions were astringent and bitter. Mean ratings for astringency and bitterness together with phenolic content are shown for each sample in Table II. In Table III, the analyses of variance are shown for astringency and bitterness ratings of each fraction. In all fractions but the condensed tannins (IV), the astringency changed as the phenolic concentration was raised. In the catechin fraction (I), it decreased, although not significantly, as the concentration increased, while in fractions II and III, intensity of astringency increased as the phenolic concentrations were raised ($p < 0.01$). Significant differences in bitterness as the concentrations were increased were only found in the intermediate anthocyanogen fraction (III). The highest concentration of III was rated as the most bitter ($p < 0.05$). Lack of significant differences in intensity of either attribute in fractions I, II, and IV is probably due to the selection of too small increments of change in phenolic concentration.

To compare the relative astringency and relative bitterness of the different fractions, the intensity ratings on a unit weight basis were calculated. Mean intensity ratings per milligram of phenolics are listed in Table IV and their analyses of variance are given in Table V. The relative astringency was highest in the condensed tannin fraction (IV) consistent with the results of Lea and Arnold (1978) in cider. In agreement with the chemically defined astringency pattern reported by Bate-Smith (1973), relative astringency increased with increase in molecular weight from fraction I to IV. It is interesting to note, that catechins (I), which do not have the ability to precipitate proteins and thus are chemically neither astringents nor tanning agents, elicited astringent sensations. Unlike Lea and Arnold (1978), the most bitter fraction on a constant weight basis was the condensed tannin (IV).

To compare the relative intensity of bitterness and astringency for each fraction, the ratios of the bitterness rating to that for astringency, calculated for each fraction, are listed in Table IV. By analysis of variance (Table V), fractions I and IV were shown to have significantly higher bitterness/astringency ratios than fraction II and III ($p < 0.001$). Since relative astringency increases from fraction I to IV, this suggests that fraction I is relatively more bitter

Table IV. Relative Astringency and Bitterness of Each Phenolic Fraction: Mean Intensity Ratings[a] per Milligram of Phenolics for Bitterness and Astringency and Mean Ratios of Bitterness to Astringency for Four Fractions

fraction	astringency rating per mg[b]	bitterness rating per mg[b]	bitter/astring[b]
I	0.018[a]	0.032[d]	2.38[i]
II	0.033[ab]	0.050[de]	1.89[gh]
III	0.070[b]	0.110[e]	1.79[g]
IV	0.452[c]	0.913[f]	2.18[hi]

[a] Mean ratings of three concentrations of each fraction by triplicate estimations of 11 judges ($n = 99$). [b] Means with same letter superscript not significantly different at significance level indicated on Table V.

Table V. Analysis of Variance of Intensity Ratings Across All Fractions: Calculated Mean Squares (MS) for Astringency Rating per Milligram of Phenolics, Bitterness Rating per Milligram of Phenolics and Ratio of Intensities of Bitterness to Astringency

source of variation	df[a]	astringency/mg MS[b]	bitterness/mg MS[b]	bitterness / astringency MS[b]
fraction (F)	3	422.58***	1792.77***	7.11***
judges (J)	10	8.09***	4.89**	9.15***
concentrations (C)	2	77.92***	293.27***	1.07
replications (R)	2	0.26	1.05	1.58
F × J	30	6.89***	3.23**	1.45*
F × C	6	48.42***	184.63***	2.53**
J × C	20	1.72**	1.80	1.32*
F × J × C	60	1.61**	1.58	0.82
F × R	6	0.20	0.92	2.11*
J × R	20	1.14	1.53	2.03**
F × J × R	60	1.03	1.64	1.64***
C × R	4	0.22	1.55	1.94*
F × C × R	12	0.20	1.45	0.76
J × C × R	40	0.97	1.60	0.72
error	120	0.98	1.55	0.79

[a] Degrees of freedom. [b] Significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

and less astringent than the other fractions. In all cases, including the most astringent fraction (IV), the fractions were more bitter than astringent.

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Analysis of Malted Barley for *N*-Nitrosodimethylamine

Four procedures for isolation of *N*-nitrosodimethylamine (NDMA) from direct-fired kiln dried malted barley were compared: Soxhlet extraction with dichloromethane, direct aqueous extraction, steam distillation, and vacuum distillation. A gas chromatograph coupled to a thermal energy analyzer was used to estimate levels of NDMA. All the isolation procedures gave similar results with the exception of the Soxhlet extraction which gave one-fourth the NDMA content of the other three methods. Grinding the malt did not significantly affect the level of NDMA. It was also determined that, without addition of approximately 70% of the sample weight in water, the NDMA concentration found by vacuum distillation was significantly reduced.

European beer has been reported to contain *N*-nitrosodimethylamine (NDMA) at an average concentration of 2.7 ppb (Spiegelhalder et al., 1979). More recently, Goff and Fine (1979) and Scanlan et al. (1980) reported similar levels of NDMA in beer produced in the U.S.

Recent work in our laboratory indicates that malted barley produced by direct-fired kilning is the major source of NDMA in beer. Since initial analyses of malted barley produced confounding data, we undertook work to determine which methods of NDMA isolation produced the best estimate of the NDMA level in malted barley.

REAGENTS

N-Nitrosodipropylamine (NDPA) and NDMA standards were made gravimetrically in hexanes. Dichloromethane (DCM) was all-glass distilled, and hexanes were nanograde. All other reagents were analytical reagent or better and blanks were run on each new reagent lot.

PROCEDURE

Three kilograms of direct-fired kiln dried malted barley was obtained from a commerical maltster. The malt was screened and throughly mixed to insure homogeneity. The NDMA was isolated from the malt by direct solvent extraction, direct aqueous extraction (Congress Wort), steam distillation, and vacuum distillation. Additionally, the sample was analyzed whole kernel after 30 s of grinding in ca. 250 mL of liquid nitrogen (LN_2) in a commercial blender or after grinding in a laboratory malt mill (Table I).

The direct solvent extraction was carried out on 25-g samples in a Soxhlet apparatus (AOAC, 1975) using DCM as the extracting solvent. Whole kernel, blender ground (LN_2), and blender ground (LN_2) with 10 g of anhydrous sodium sulfate samples were extracted for 16 h. The resulting DCM extract was washed with 3 N hydrochloric acid (25 mL), 1.5 N sodium hydroxide (25 mL), dried by passing through anhydrous sodium sulfate, and concentrated as outlined below.

Laboratory mill ground malt was prepared by the fine grinding procedure and was analyzed after preparing a direct aqueous extract (American Society of Brewing Chemists, 1976). A 100-g aliquot of the resulting direct aqueous extract was extracted with DCM (1 × 100 mL followed by 2 × 50 mL); the DCM was dried by passing

Table I. *N*-Nitrosodimethylamine Content of a Commercially Malted Barley Analyzed by Various Procedures

method	concn[b]
I. Soxhlet extraction	
A. whole malt	22
B. LN_2, blender ground	27
C. LN_2, blender ground (Na_2SO_4)	30
II. Direct aqueous extraction	
A. malt mill ground	115
III. steam distillation	
A. LN_2, blender ground	118
IV. vacuum distillation	
A. LN_2, blender ground	114
B. dry, blender ground	117
C. malt mill ground	99
D. unground malt	105

[a] Values are the average of two-three independent assays. [b] Parts per billion.

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