increasing absorption of the compound, which is then excreted in milk and urine.

We have also observed a correlation between the concentration of Ia (measured fluorimetrically) and delay in the acidification of milk due to lactic bacteria (Bertoni, 1978). However, no specific bacteriostatic activity was found in vitro for Ia against lactic bacteria. The hypothesis that Ia could be decarboxylated to 1-methyl-β-carboline (harman), which is known to inhibit the growth of molds, and which has been detected in fermentation products such as sake, beer, and wine (Takase et al., 1967), was ruled out as no trace of harman could be found in urine and milk of animals (TLC in comparison with a synthetic sample). Therefore it might be possible that other active substance(s), present in small amounts, and produced by processes correlated with those leading to an unusual amount of Ia, could be responsible for this effect.

LITERATURE CITED

Battistotti, B., Bertoni, G., *Scienza Tec. Latt. Casearia*, **25**, 7 (1974).
Bertoni G., unpublished results, 1978.
Budzikiewicz, H., Djerassi, C., Williams, D. H., "Structure Elucidation of Natural Products by Mass Spectroscopy", Vol. 1, Alkaloids, Holden-Day, San Francisco, 1964, p 85.
Hesse, M., "Indolalkaloide in Tabellen", Springer Verlag, Heidelberg, 1964, p 95.
Scott, A. I., "Interpretation of the Ultraviolet Spectra of Natural Products", Pergamon, Oxford, 1964.
Snyder, H. R., Hansch, C. H., Katz, L., Parmeter, S. M., Spaeth E. C., *J. Am. Chem. Soc.* **70**, 219 (1948).
Takase, S., Sakai, T., Sato, N., Murakami, H., *Nippon Jozo Kyokai Zasshi* **62**, 1415 (1967); *Chem. Abstr.* **68**, 67724.

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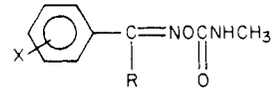
Inhibition of Acetylcholinesterase by *O*-(Methylcarbamoyl)oximes. Structure–Activity Relationships

Since the anticholinesterase activity and the mechanism of alkaline hydrolysis of *O*-(methylcarbamoyl)benzaldoximes and -acetophenoximes are analogous to those of phenyl *N*-methylcarbamates, these two groups of derivatives were compared by means of structure–activity relationships. The correlations with the electronic substituent parameter σ showed that the mechanism of inhibition of acetylcholinesterase by *O*-(methylcarbamoyl)oximes is the same as that observed for phenyl *N*-methylcarbamates bearing strongly electron-withdrawing substituents. The correlations with the bimolecular rate constant k_{OH} suggest that the mechanism of the alkaline hydrolysis of oxime carbamates may closely parallel their mechanism of interaction with acetylcholinesterase at the serine hydroxyl.

Fukuto et al. (1969), as well as Jones et al. (1972), showed that *O*-(methylcarbamoyl)oximes of substituted acetophenones and benzaldehydes have a poor insecticidal activity against species such as the housefly (*Musca domestica* L.) in spite of their good anticholinesterase activity as measured on fly heads by the I_{50} parameter (i.e., the molar concentration of carbamate necessary to cause 50% inhibition of cholinesterase activity). In contrast to phenyl *N*-methylcarbamates (Hansch and Deutsch, 1966; Jones et al., 1969), lipophilic bonding plays almost no role in the inhibition of cholinesterase by *O*-(methylcarbamoyl)-oximes. The fact that anticholinesterase activity is dependent only on the field (*F*) and resonance (*R*) constants of Swain and Lupton suggests that cholinesterase inhibition by *O*-(methylcarbamoyl) oximes is determined by the reactivity of the carbamoyl moiety, analogous to the hydrolysis of these esters.

The kinetic data reported earlier for the alkaline hydrolysis of *O*-(methylcarbamoyl)oximes (Mrlina and Calmon, 1980) can be discussed in terms of substituent effects on biological activity and in relation to the mechanism of inhibition of acetylcholinesterase. Two kinds of correlations, calling upon the electronic substituent constant σ or the bimolecular rate constant k_{OH}, were considered so as to check whether the mechanism of hydrolysis can account for the mechanism of inhibition of acetylcholinesterase. Since the bimolecular rate constant k_{OH} is closely related to σ and can be measured easily, it could be used in structure–activity relationships when σ values are unknown or when the transmission of the substituent effect is complex. The k_{OH} parameter is, as a matter of fact, liable to reflect the overall substituent effects.

Table I. Kinetic (log k_{OH}), Electronic (σ), and Biological (log $1/I_{50}$) Parameters Used in Structure–Activity Relationships for Oxime Carbamates $XC_6H_4C(R)$:NOCONHCH$_3$



R	X	log (1/I_{50})[a]	σ	log k_{OH}
H	H	4.34	0	−1.054
H	*p*-iPr	4.43	−0.150	−1.258
H	*p*-Br	4.42	0.232	−0.896
H	*m*-NO_2	5.08	0.710	−0.462
H	*p*-NO_2	4.85	0.780	−0.108
Me	H	4.15	0	−2.096
Me	*p*-Me	4.05	−0.170	−2.194
Me	*m*-MeO	3.82	0.115	−1.939
Me	*p*-Br	4.41	0.232	−1.730
Me	*m*-NO_2	5.35	0.710	−1.424
Me	*p*-NO_2	5.09	0.780	−1.250

[a] Fukuto et al. (1969); Jones et al. (1972).

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MATERIALS AND METHODS

Preparation and physical properties of carbamates used in this work were previously described (Mrlina and Calmon, 1980).

I_{50} values for the inhibition of the fly cholinesterase by the individual compounds were determined by Fukuto et al. (1969) and Jones et al. (1972) and are listed in Table I.

The bimolecular rate constants $k_{OH} = k_{obsd}/[OH^-]$ were determined graphically from the plots of the observed pseudo-first-order rate constant k_{obsd} against hydroxide ion concentration for the alkaline hydrolyses of *O*-(methylcarbamoyl)benzaldoximes and -acetophenoximes (Mrlina and Calmon, 1980).

The structure–activity correlations were carried out using the method developed by Hansch and Fujita (1964). Regression analysis was performed using a Hewlett-Packard HP 2921. The σ values used are those of McDaniel and Brown (1958).

RESULTS AND DISCUSSION

The anticholinesterase activity of the *O*-(methylcarbamoyl)oximes investigated is accounted for by the electronic effects as estimated by σ. Regression analysis gave the following equations which best fit the data.

O-(Methylcarbamoyl)benzaldoximes:

$$\log (1/I_{50}) = \underset{(0.189)}{0.681\sigma} + 4.405$$

$$n = 5,\ s = 0.158,\ r = 0.901$$

O-(Methylcarbamoyl)acetophenoximes:

$$\log (1/I_{50}) = \underset{(0.319)}{1.458\sigma} + 4.076$$

$$n = 6,\ s = 0.274,\ r = 0.916$$

In the above equations, n is the number of points used in deriving the constants for the equations, s is the standard deviation, and r is the correlation coefficient. The figures in parentheses are the 95% confidence intervals for the respective coefficients.

A noteworthy point in these equations is the positive values for the coefficients of σ. This is in direct contrast to the values obtained by Hansch and Deutsch (1966) for phenyl *N*-methylcarbamates ($-2.05 < \rho < -1.30$ depending on the position of the substituents).

For bovine erythrocyte acetylcholinesterase inhibition by substituted phenyl *N*-methylcarbamates, Nishioka et al. (1977) could show that the plot of the association equilibrium constant $1/K_d$ against σ° was biphasic. Strongly electron-withdrawing substituents at the meta or para position ($\sigma^\circ > 0.5$) led to a ρ value of 1.66, whereas lower σ° constants gave rise to a ρ value of −1.78. This change of sign was interpreted in terms of a change-over in reaction mechanism. The positive ρ electronic effect was assigned to the nucleophilic attack of the enzymic serine OH on the carbonyl carbon of the ester moiety.

The following scheme for acetylcholinesterase inhibition by carbamates was put forward by Wilson et al. (1960) and used by Nishioka et al. (1977):

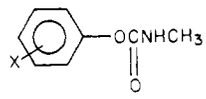
$$\mathrm{EOH} + \mathrm{ArOCONHCH_3} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{reversible complex} \xrightarrow{k_2}$$

$$\mathrm{EOCONHCH_3} + \mathrm{ArO^-} \xrightarrow{k_3} \mathrm{EOH}$$

The generalized mechanism of inhibition can be written more simply as

$$\mathrm{EOH} + \mathrm{I} \underset{K_d}{\rightleftharpoons} \mathrm{E{\cdot}I} \xrightarrow{k_2} \mathrm{E'} \xrightarrow{k_3} \mathrm{E}$$

Table II. Effect of Substituents on the Biological Response for Phenyl *N*-Methylcarbamates $XC_6H_4OCONHCH_3$



	$\log (1/K_d)^a$			$\log (1/I_{50})^b$	
	ρ			ρ	
	$\sigma^\circ > 0.5$	$\sigma^\circ < 0.5$	r		r
para substituted	1.385 (0.443)	−1.186 (0.676)	0.898	−1.032 (0.879)	0.404
meta substituted	0.827 (0.507)	−2.393 (0.441)	0.984	−2.052 (1.109)	0.511

[a] Nishioka et al. (1977). [b] Hansch and Deutsch (1966).

where EOH is the active enzyme, E′ the carbamyl or inactive enzyme, and E·I is a reversible complex between the enzyme and the inhibitor. This reaction scheme leads to the following equations (Wilson et al., 1960):

$$d(E')/d_t = k_2(E{\cdot}I) - k_3(E')$$

and

$$(E^\circ) = (E) + (E{\cdot}I) + (E') = (E') + (\epsilon)$$

where (E°) is the total enzyme and (ϵ) the amount of enzyme which is measured as a function of time. Assuming a steady state is reached, these equations have the solution:

$$(\epsilon)/(E') = k_3[1 + K_d/(I)]/k_2$$

When $(\epsilon) = (E')$, $I_{50} = K_d/(k_2/k_3 - 1)$. The substituent effects on K_d and I_{50} are analogous. As a matter of fact, k_3 does not depend upon the nature and position of the substituent and the value of k_2 is not sensitive to the structural variation (Nishioka et al., 1977).

This analogy in the substituent effects can be observed when the data of Hansch and Deutsch (1966) are compared to those of Nishioka et al. (1977) for meta- and para-substituted phenyl *N*-methylcarbamates (see Table II), although the acetylcholinesterase used was different.

The attenuation ratio (τ 0.4), which was previously defined (Mrlina and Calmon, 1980), characterizes the difference in the transmission of substituent effects between phenols and benzaldoximes. Therefore, the ρ value of 1.66 found by Nishioka et al. (1977) would then actually be 0.66 for *O*-(methylcarbamoyl)benzaldoximes. Such a value is very close to the observed value of 0.68. The acetylcholinesterase inhibition mechanism put forward by Nishioka et al. when ρ is positive is therefore likely to be involved in the present case.

The difference in the ρ values observed for the benzaldoxime and acetophenoxime derivatives can result from a change-over in reaction mechanism. The inhibition mechanism suggested by Nishioka et al. (1977)—nucleophilic attack of the enzymic serine OH on the carbonyl carbon of the ester moiety—is close to that of the alkaline hydrolysis via a $B_{Ac}2$ pathway. The higher ρ value (1.46) observed for *O*-(methylcarbamoyl)acetophenoximes reflects their higher susceptibility to substituent effects like the one which can be observed in alkaline hydrolyses involving isocyanate formation (E1cB pathway). *O*-(Methylcarbamoyl)acetophenoximes are therefore better carbamoylating agents than benzaldoxime carbamates.

An alternative explanation for the difference between *O*-(methylcarbamoyl)benzaldoximes and *O*-(methylcarbamoyl)acetophenoximes might be the tendency for the

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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LITERATURE CITED

Crawford, R. J., Woo, C., *Can. J. Chem.* **43**, 1534 (1965).
Fukuto, T. R., Metcalf, R. L., Jones, R. L., Myers, R. O., *J. Agric. Food Chem.* **17**, 923 (1969).
Hansch, C., Deutsch, E. W., *Biochim. Biophys. Acta* **126**, 117 (1966).
Hansch, C., Fujita, T., *J. Am. Chem. Soc.* **86**, 1616 (1964).
Jones, R. L., Metcalf, R. L., Fukuto, T. R., *J. Econ. Entomol.* **62**, 801 (1969).
Jones, R. L., Fukuto, T. R., Metcalf, R. L., *J. Econ. Entomol.* **65**, 28 (1972).
McDaniel, D. H., Brown, H. C., *J. Org. Chem.* **23**, 420 (1958).
Mrlina, G., Calmon, J. P., *J. Agric. Food Chem.* **28**, 605 (1980).
Nishioka, T., Fujita, T., Kamoshita, K., Nakajima, M., *Pestic. Biochem. Physiol.* **7**, 107 (1977).
Payne, L. K., Jr., Stansbury, H. A., Jr., Weiden, M. H. J., *J. Agric. Food Chem.* **14**, 356 (1966).
Wilson, I. B., Hatch, M. A., Ginsburg, S., *J. Biol. Chem.* **235**, 2312 (1960).

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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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