of NAD-linked aldehyde dehydrogenase activity.

Cyclophosphamide Potentiation Studies. In a manner similar to that described by Hill et al.,[2] groups of six BDF_1 female mice weighing 18–20 g were implanted ip with 10^5 L1210 ascites cells. After 24 h (day 1), one group was administered an equal volume of vehicle (5% Tween 80 in normal saline, pH 4) ip to serve as a control; other groups were similarly injected with solutions or suspensions once only with drug or drug and cyclophosphamide on day 1. The acetals **2a**, **2c**, **3a**, **3c**, and **3e** were heated at 80 °C for 2 h just prior to injection. The groups were observed daily and any deaths recorded. Any animals surviving 30 days from day 1 were considered to be "cures" and increase in life span over controls as a percent (% ILS) were calculated.

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Mechanism of Decomposition of *N*-Hydroxyacetaminophen, a Postulated Toxic Metabolite of Acetaminophen

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The decomposition of *N*-hydroxyacetaminophen (*N*-acetyl-*N*-hydroxy-*p*-aminophenol, **2**), a postulated toxic metabolite of acetaminophen (*N*-acetyl-*p*-aminophenol, **1**) in aqueous solution is quantitatively accounted for by the appearance of equimolar amounts of *p*-nitrosophenol and acetaminophen. The rate of decomposition depends on initial concentration and varies with pH. Antioxidants decrease the rate of decomposition and change the products. In the presence of cysteine, *N*-acetyl-3-(*S*-cysteine)-*p*-aminophenol, an in vivo metabolite of acetaminophen, is a product of decomposition.

The toxicity of acetaminophen (**1**, *N*-acetyl-*p*-aminophenol), a widely employed mild analgesic, has been attributed to the formation of the reactive metabolite *N*-hydroxyacetaminophen (**2**, *N*-acetyl-*N*-hydroxy-*p*-aminophenol).[1] Studies on its toxicity have been prompted by the severe liver damage associated with excessively large acute doses, as well as by the possibility that a similar mechanism may be involved in the analgesic-related nephropathy which occurs in man with chronic abuse.[2,3] Neither the hepatic nor renal toxicity can be attributed to the parent compound (**1**) or to its major urinary metabolites, namely, the glucuronide and sulfate conjugates.

The mechanism of toxicity which has been proposed is that a small portion of the administered dose of **1** is oxidized to **2** by the mixed function oxidase system, principally in the liver but also in the kidney. As the result of the formation of **2**, intracellular stores of glutathione become depleted and **2** then becomes covalently bound to tissue proteins, presumably by the formation of thiol adducts. This is then followed by tissue necrosis.[4] This mechanism is supported by the urinary excretion of the cysteine and *N*-acetylcysteine 3 adducts of acetaminophen, which are presumed to have been derived from the prior formation of the glutathione 3 adduct.[5] It has been postulated that **2** decomposes to *N*-acetyl-*p*-benzoquinone imine (**4**), which is the proximate reactant with tissue thiols.[6]

Table I. Product Distribution as a Function of pH[a]

pH	*n*	concn, mM: 1	concn, mM: 9	unidentified peak ht, mm
6.8	2	0.69	0.50	8.3
7.2	3	0.72	0.59	6.7
7.6	3	0.70	0.66	4.4
8.2	1	0.72	0.72	1.3
9.6	1	0.74	0.74	0.0
10.0	1	0.74	0.73	0.0
10.5	1	0.75	0.73	0.0

[a] Initial conditions: 1.5 mM **2**, 200 mM PO_4, 37 °C. Incubation for 3 h or until the decomposition of **2** was complete. Unidentified peak had a retention time of 7.4 min. Standard deviation of replicate analyses was ± 0.02 mM.

The above mechanism is based primarily on correlations observed in experimental animals in vivo. To date, **2** has not been detected in any biological fluid. When **2** became available through a synthetic route,[7,8] it was of pharmacological interest to determine its mechanism of decomposition in vitro with particular emphasis on the factors which might be operative in vivo.

Kinetic Studies. In measuring the disappearance of **2**, we obtained the same results with the ferric chloride assay as with high-performance liquid chromatography.

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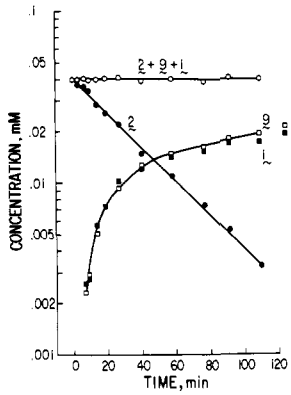


Figure 1. Semilogarithmic plot of the decomposition of 0.040 mM 2 with the appearance of 1 and 9.

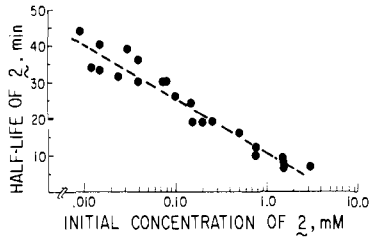


Figure 2. Half-life of 2 at different initial concentrations.

The latter method has two advantages: first, its far greater sensitivity permits the measurements of lower concentrations of 2 and, second, it allows one to monitor the appearance of the products of the decomposition of 2. As indicated in Figure 1, the disappearance of 2 followed first-order kinetics as judged by the linear slope of the semilogarithmic plot. With incubation at pH 9.6 or higher, acetaminophen (1) and *p*-nitrosophenol (9) were formed in equimolar amounts and together accounted for the disappearance of 2 (Table I). At lower pH, the yield of 9 decreased. With standard solutions of a mixture of comparable concentrations of 1 and 9, 9 did not decompose at the lower pH's tested in Table I. Furthermore, by chromatographic analysis the decreased yield of 9 was not due to the accumulation of 6. At the lower pH's an unidentified product was observed.

In all experiments the decomposition of 2 appeared to follow first-order kinetics in that the semilogarithmic plot of its concentration was linear over 3–4 half-lives (i.e., Figure 1). However, the reaction rate (Figure 2) showed a slight dependence on the initial concentration of 2. The decomposition did not follow second-order kinetics in that there was not a linear relationship between the reciprocal of the concentration of 2 plotted against time. The same concentration dependence of the half-life of 2 was observed with samples of 2 synthesized by two different methods.[7,8] At initial concentrations of 1 from 0.075 to 1.5 mM, there was no change in the relative amounts of 1 and 9 as the products of decomposition.

The half-life of 2 was pH dependent with a minimum value at pH 9 (Figure 3). At higher or lower pH's the half-life increased approximately 10-fold for each pH unit. At all pH's, the decomposition of 2 followed first-order kinetics.

The effect of additives on the decomposition of 2 was examined with a standard test system containing 1.5 mM 2 and 20 mM additive. The compounds included ascorbic acid, various thiols, disulfides, amino acids, phenols, and aspirin (Table II). In all cases, the rate of decomposition of 2 followed first-order kinetics. Aspirin, salicylate, and ethanol had no effect; the other compounds all increased

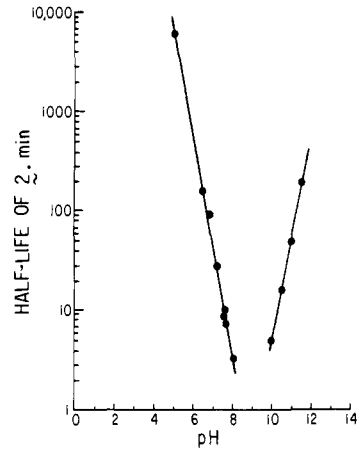


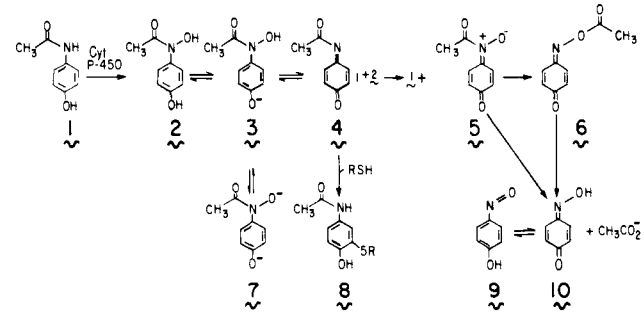
Figure 3. Half-life of 1.5 mM 2 as a function of pH.

Table II. Effect of Various Additives on the Half-life of 2

	$t_{1/2}$ of 2,[a] min	products, % of control		
		1	9	other[b]
control	6.5–9.4	100	100	0
ethanol[e]	6.7			
ascorbic acid	76	200	0	0
cysteine	78	99	0	+[c]
cysteamine	72	96	0[d]	+
N-acetylcysteine	68	69	0	+
thiosulfate	31	103	30	+
cystamine	43	134	25	0
dithionite	24	157	0	+
methionine	31	143	65	0
glycine	24	121	63	0
lysine	23	121	69	0
phenol	14			
p-nitrosophenol (9)	17			
salicylate	6.5			
pyrogallol	60.0			
acetaminophen (1)	33			
aspirin	6.4			

[a] Initial conditions: 1.5 mM 2, 200 mM PO_4 at pH 7.6, 37 °C. [b] Control contained a minor unidentified product (see Table I). Other products indicated below refer to additional unidentified peaks. [c] The additional product had the same chromatographic characteristics as an authentic sample of 3-cysteineacetaminophen. [d] Chromatographic peak had same retention time as 9, but the incubation mixture was colorless in contrast to solutions containing 9. [e] Ethanol concentration was 20% (v/v).

Scheme I



the half life of 2. The greatest effect was obtained with ascorbic acid, pyrogallol, and the thiols.

Discussion

Scheme I depicts the reactions involved in the decomposition. Potter et al.[6] proposed that acetaminophen (1) is oxidized in vivo to *N*-hydroxyacetaminophen (2), which

dehydrates to *N*-acetyl-*p*-benzoquinone imine **4**, the proximate reactant. From in vitro studies, both Gemborys et al.[7] and Healey et al.[8] proposed that **4** is generated by the irreversible loss of hydroxide from the phenolate anion (**3**) and that the fully protonated form (**2**) and the dianionic form (**7**) are stable. This would be consistent with the relationship of pH to the stability of **2**. Calder et al.[9] further proposed that **4** is reduced to acetaminophen (**1**) simultaneously with the oxidation of **2** to the nitrone (**5**). The nitrone is hydrolyzed to *p*-nitrosophenol (**9**) either directly or via the *N*-acetoxy-*p*-benzoquinone imine intermediate **6**. According to this scheme, the decomposition of *N*-hydroxyacetaminophen (**2**) would yield 0.5 equiv of acetaminophen (**1**) and 0.5 equiv of *p*-nitrosophenol (**9**).

In the absence of additives, the decomposition of **2** yields equal parts of **1** and **9**, thus confirming the suggestion of Calder et al.[9] Since the concentration of these products remains essentially constant from pH 7.2 to 10.5, the reaction mechanism does not change over this pH range. However, the rate of decomposition is pH dependent (Figure 3). In agreement with the mechanism previously postulated,[7] the rate of decomposition of **2** increases from pH 4 to 8 due to the increased concentration of phenolate anion (**3**), which is the precursor of **4**. By extrapolation of the data in Figure 3, the maximal rate for the decomposition of **2** would occur at pH 9, which is approximately the same as the probable pK_a of the phenolic proton of **2**. [The pK_a of acetaminophen (**1**) is 9.5.]

The observed first-order kinetics are difficult to interpret in view of the overall complexity of the reaction mechanism and may result from an algebraic fortuity rather than a single rate-determining step. The slight dependence of the rate of decomposition of **2** on its initial concentration remains unexplained.

The observation that thiols decrease the rate of disappearance of *N*-hydroxyacetaminophen by 10-fold suggests that **3** and **4** are in equilibrium. If the conversion of **3** to **4** were irreversible, thiols would have no affect upon the rate of disappearance unless they reduced the nitrone (**5**) to *N*-hydroxyacetaminophen. This would maximally decrease the rate twofold. If **3** and **4** are in equilibrium, the rate of reaction between **4** and **2** is equivalent to a second-order reaction involving the phenol (**2**) and the phenolate (**3**). The product of the concentrations of these two reactants is maximal at the pK_a. Thus, the alkaline stability of **2** may be due to diminished concentration of the fully protonated species (**2**). Hinson et al.[10] detected *N*-hydroxyacetaminophen as a microsomal metabolite of *N*-hydroxyphenacetin but not as a microsomal metabolite of acetaminophen. If **3** and **4** are in equilibrium, the decomposition of *N*-hydroxyphenacetin to the quinone imine **4** could account for the generation of *N*-hydroxyacetaminophen (**2**).

All of the additives which increased the half-life of *N*-hydroxyacetaminophen (Table II) are free-radical scavengers.[11-13] An explanation for their affect is that a free-radical intermediate is involved in the reaction of **3** and **4** to **1** and **9**, analogous to the radical pathway proposed for oxidations by high-potential quinones[14] and for the reduction of *N*,*N*-dialkylquinone diimines.[15] The additives also affected the reaction pathway, since they changed the distribution of the products. In the presence of thiols, the 3-thio adducts and acetaminophen are formed.[16]

It is of note that the 3-(thiomethyl) adduct was not found with methionine since, in an analagous study, Calder and Creek[17] observed the formation of the 3-(thiomethyl) adduct after the addition of **4** to a solution of *N*-acetyl-*S*-methylcysteine. Mulder et al.[18] studied the in vitro decomposition of *N*,*O*-glucuronide and *N*,*O*-sulfate conjugates of *N*-hydroxyphenacetin, which decompose via the quinone imine **4**. They observed an increased yield of acetaminophen in the presence of ascorbic acid and the formation of the 3-glutathione adduct and acetaminophen in the presence of glutathione.

Unlike certain other *N*-arylhydroxylamines which are of biological interest [*N*-hydroxy-2-(acetylamino)fluorene,[19] *N*-hydroxy-*N*-methyl-4-aminoazobenzene,[19] *N*-hydroxyphenacetin,[17] and *N*-hydroxy-*p*-chloroacetanilide],[20] *N*-hydroxyacetaminophen (**2**) has in the para position an electron-donating group with an acidic proton which confers two unique features. First, the acidic decomposition of **2** (at pH <2) generates the nitrenium ion,[21] which in turn generates the neutral quinone imine **4** by the facile loss of a proton. The other *N*-arylhydroxylamines cannot form quinone imines by proton loss. Second, the decomposition of **2** to reactive intermediates also readily occurs at physiological pH, at which other *N*-arylhydroxylamines are relatively stable. Their most reactive form is an N-conjugated derivative; i.e., *N*-hydroxy-2-(acetylamino)-fluorene is N-conjugated to reactive sulfate or glucuronide ester.[19]

Our findings are consistent with the previous suggestion that the toxicity of acetaminophen (**1**) may be mediated by its metabolic conversion to *N*-hydroxyacetaminophen (**2**). It has been observed that thiols react nonenzymatically with a metabolite of acetaminophen (**1**) generated in vitro in a microsomal system.[1] Although antioxidants as antidotes provide some protection against experimental acetaminophen toxicity, the correlation between protection and antioxidant activity has been inconstant.[22] The present experiments provide evidence for free-radical formation during the decomposition of **2**. However, it is uncertain to what extent they are directly applicable to in vivo conditions. Nevertheless, the possibility must be kept in mind that free radicals derived from **2** may be directly cytotoxic. This mechanism may be additive to

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that related to covalent bonding of the reactive metabolites to macromolecules and may provide an explanation for the discrepancies reported between covalent bonding and cytotoxicity.[23,24]

Experimental Section

Melting points were determined with a Thomas-Hoover capillary apparatus and are uncorrected. A Gilford 1300-N spectrophotometer was used for spectral measurements.

The $FeCl_3$ was USP grade. Evaporations were performed under reduced pressure at room temperature unless otherwise stated. Liquid chromatography was performed as follows. Degassed solvent was pumped with a peristaltic pump through a three-way valve attached to a column with flow adapters and to a syringe pump for sample injection. The column effluent was monitored at 254 nm and then collected with a fraction collector.

The high-performance liquid chromatograph was a Waters Associates Model ALC/GPC 202 liquid chromatograph with two Model 6000 pumps and a Waters Associates Model 660 solvent programmer and a Model U6K injector. The columns were Waters Associates μBondapak C_{18}, 0.4 × 30 cm. Chromatography was done at ambient temperature. The complete mobile phase was prepared and then filtered through a 0.2-μm filter. Columns were equilibrated with the mobile phase prior to sample injection—at least 60 column volumes for mobile phases with paired ions, otherwise 20 column volumes. The following mobile phases were used: system A, 1% 1-propanol–5.0 mM *n*-butanesulfonic acid, sodium salt–25 mM phosphoric acid; system B, 15% methanol–0.5% ethyl acetate–25 mM phosphoric acid; system C, system B–20 mM *n*-pentanesulfonic acid, sodium salt; system D, system B–5.0 mM *n*-heptanesulfonic acid, sodium salt.

Quantitation was determined by peak height. With standard solutions of **1**, **2**, and **12**, the peak height was linearly related to concentration. Calibration curves were prepared daily when product concentrations were determined. For experiments on the rate of decomposition of **2**, peak heights were used to determine the relative amounts of **2** for a fixed amount of sample injected and for a given absorbance range; however, since absolute concentrations were not determined, daily calibration curves were not prepared. Compounds were identified chromatographically by comparing their retention time to the retention time of authentic samples.

***N*-Hydroxyacetaminophen (2).** This was prepared by the synthetic route previously described,[7] with the following modifications which increased the yield of the intermediate sulfate from 25–45% to 54%, increased the yield of **2** from 87 to 97%, and decreased the time required for synthesis. To a vigorously stirred solution of potassium *p*-nitrophenyl sulfate (22.0 g, 80 mmol) and NH_4Cl (5.4 g, 100 mmol) in 175 mL of H_2O at 4 °C was added Zn dust (10.5 g, 160 mmol) in very small portions over the course of 30 min. The mixture was then vigorously stirred for an additional 30 min. The reaction is exothermic. The temperature of the mixture rose to 65 °C and was then maintained at 65 °C. The mixture was filtered and the precipitate washed with 50 mL of H_2O (65 °C). The yellow filtrate was lyophilyzed to a yellow powder, which was added to a flask containing 250 mL of ether and a slurry of $KHCO_3$ (20 g, 200 mmol)–H_2O (70 mL) at 0 °C. The mixture was vigorously stirred as AcCl (6.91 g, 88 mmol) in 100 mL of anhydrous ether was added dropwise over 90 min. Additional H_2O (250 mL) was added, and the mixture was left overnight at 4 °C, filtered, and lyophilyzed to a powder. The crude product was partially purified by the procedures previously described—the water elutions were omitted. The crude product was found to be 43% (w/w) potassium *N*-acetyl-*N*-hydroxy-*p*-aminophenyl sulfate, by a ferric chloride assay. A stirred solution of crude product (3.5 g) in 250 mL of H_2O at 33 °C was adjusted to pH 5 with 1.0 M HCl. Sulfatase, Sigma Type H2 (20000 units), was added and the pH monitored. The pH was maintained between 4.9 and 5.0 by the addition of equal amounts of 1.0 M HCl and 1.0 M $BaCl_2$. To this were made successive additions of 10-mL aliquots of a solution of 10.5 g crude product in 50 mL of H_2O (adjusted to pH 5.0 with 1 M HCl) when the pH remained constant. After the last addition of crude sulfate and when the pH was constant, the solution was cooled to 25 °C, centrifuged, decanted, saturated with NaCl (s), and extracted with ethyl acetate (4 × 400 mL). The combined extracts were washed with 100 mL of 0.1 M phosphate buffer (pH 5) and then evaporated to 3.4 g (97%) of **2** as a tan powder, which was recrystallized from THF–AcOEt (1:3, v/v), mp 125–127 °C (lit.[7] mp 125–127 °C).

***N*-Acetoxy-*p*-benzoquinone imine (6)** was prepared by the method of Norris and Sternbell[25] and recrystallized from petroleum ether to give yellow crystals, mp 105.5–106.5 °C; yield 44% (lit.[25] 109 °C; 60%).

Decomposition of *N*-Hydroxyacetaminophen (2). The standard conditions for the determination of the decomposition of **2** were incubation at 37 °C in 0.2 M sodium phosphate buffer at pH 7.6 with **2** at the initial concentration of 1.5 mM. Each of these variables were studied separately. All deviations from these standard conditions are noted in the text. The pH at the end of each experiment was within 0.1 pH unit of the initial pH. For kinetic studies, the start of the reaction was taken as the time when 5.0 mL of **2** in 2 mM HCl at 37 °C was added to 20.0 mL of 250 mM phosphate at the same temperature. For the termination of the reaction, aliquots of the incubation medium were periodically removed and added to an acidic analytical reagent.

Ferric Chloride Assay.[26] This method measures *N*-hydroxylacetylamines. Aliquots (1.0 mL) of the incubation mixture were added to 2.0 mL of 10% $FeCl_3$ in 0.9 M HCl. The assay solutions were immediately vortexed, and the absorbance was determined at 540 nm. The absorbance of 540 nm due to **2** was determined by subtracting the absorbance of an aliquot withdrawn after the completion of the decomposition from the absorbance of the sample. The maximum intrinsic absorbance of the sample at 540 nm was found to be less than 1% of the initial $FeCl_3$ absorbance, and no correction was made.

The absorbance at 540 nm due to **2** was time dependent. Stock solutions of **2** were added to 2.0 mL of 10% $FeCl_3$ in 0.9 M HCl, and the absorbance at 540 nm was determined at various time intervals after sample mixing. The change in absorbance per minute was linear and dependent on the concentration of **2**. The changes in absorbance were: 0.05 (3.0 mM), 0.025 (1.5 mM), and 0.014 OD/min (0.75 mM). No corrections were made for the time-dependent absorbance change other than to keep the time for analysis approximately the same.

Assay by High-Performance Liquid Chromatography. Aliquots of the incubation mixture were withdrawn and added to a fixed volume of an aqueous solution of acetic acid at 0 °C, vortexed, and stored at 0 °C until assayed. The concentration of acetic acid was from 0.4 to 0.8 M and depended on the size of the aliquot sampled, so that the final pH was approximately 3. For each experiment, a constant volume was injected into the chromatograph and the absorbance sensitivity was kept constant.

Two controls were used to check the validity of the assay. First, the rate for the disappearance of 1.5 mM **2** obtained from the ferric chloride assay was the same as that obtained by chromatographic analysis. Second, samples taken after the completion of the decomposition did not contain a peak for **2**. No decomposition of **2** was detected after quenching. After storage at 0 °C for several hours, the initial samples were reanalyzed and no changes in the amount of **2** or other products were observed.

Effect of pH. Sodium phosphate buffers (0.2 M) were used throughout. The data in Figure 3 were obtained with the ferric chloride assay.

Effect of Initial Concentration of 2. Reaction rates were determined with high-performance liquid chromatography using solvent system A (Figure 1).

Effect of Additives. All compounds (Table II) were added to the incubation mixture prior to the addition of **2**. Both the ferric chloride assay and the chromatographic assay (solvent system A) were employed.

Products of Decomposition of 2. The concentration of products was determined by high-pressure liquid chromatography

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immediately after the completion of the decomposition of **2**. For this purpose, solvent system B was employed in the absence of additives, solvent system D when cysteine had been added, and solvent system C with other additives.

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Structure-Taste Relationship of Perillartine and Nitro- and Cyanoaniline Derivatives

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The relationship between structure and taste potency of perillartine and its analogues was investigated quantitatively by physicochemical parameters and regression analysis. The results indicated that the hydrophobicity estimated from the 1-octanol/water partition coefficient and the molecular widths from the bond axis connecting the oxime carbon and alicyclic ring are important, regardless of whether the taste is sweet or bitter, so far as the taste potency is concerned. The SAR for the sweet/bitter ratio was not established quantitatively, but the molecular width and thickness and the position-specific electronic effect seem to delineate the ratio qualitatively; i.e., in principle, the wider and/or the thicker the molecule, the more bitter the taste. Comparatively, the QSAR of 5-nitro- and 5-cyanoaniline sweetners was formulated to show the insignificance of the hydrophobicity within the compounds investigated but the importance of the steric dimensions in determining the activity.

Among the sweet compounds hitherto known,[1] the perillartines are a class in which the activity has been estimated quantitatively in terms of both the taste potency relative to a standard sucrose solution and the sweet/bitter ratio.[2] Thus, the data promote attempts to correlate the relationship between the structure and the sweet/bitter ratio, as well as that between the structure and taste intensity. Whether the sweet materials bind to a common receptor on the tongue or there are different receptor sites for different groups of sweetners has been the subject of extensive studies, as well as the problem whether the receptors for the sweet and bitter taste are common or multiple.

The quantitative structure–activity analysis has been shown in certain cases to lead to a better understanding of the mode of interaction of biologically active molecules with their receptor, as well as the nature of the receptor site.[3] To my knowledge, however, the 2-substituted 5-nitroanilines are the only class of the sweetners whose potency has been analyzed in this sense.[4,5] In the plant hormone area, Iwamura et al. have correlated the variations in the activities of cytokinins and anticytokinins with the hydrophobic parameter derived from the 1-octanol/water partition coefficient and the steric parameter for the maximum width of a substituent from the bond axis.[6-8] Through these studies, the new steric parameters, the STERIMOL parameters recently developed by Verloop et al.,[9] have played a key role for the correlations. These results and those reported by Verloop et al.[9] thus prompted me to analyze the taste characteristics of sweetners in a manner similar to that used for the analyses of cytokinin and anticytokinin activities. In this article are reported the results on two classes of sweetners, perillartines and 2-substituted 5-nitro- and 5-cyanoanilines. Even if different classes of sweetners exert their effect at different receptors, it is more meaningful for designing new tastants to try to seek for the common peculiarities between different classes of compounds than to restrict the analyses within a congeneric series.

Methods

Activity data were taken from literature reported by Acton et al.[2] for perillartines and those reported by Blanksma et al.[10] and Verkade et al.[11] for aniline derivatives.

The STERIMOL parameters[9] were used to evaluate the steric dimensions of compounds. The L parameter expresses the length of the substituent R_1 along the bond axis which connects R_1 and the oxime carbon in perillartines. R_1 is the rest of the molecule from which the common oxime end is subtracted. In aniline derivatives, it is the length of the whole substituted benzene moiety along the bond axis between C_5 and the N atom of nitro or C atom of the cyano group. The W_l, W_r, W_u, and W_d are the molecular width in the directions perpendicular to the L axis and rectangular to each other. W_l in the perillartine series is taken as the width in the direction to which the 4 substituent extends in the fully extended (staggered) conformation, and the $C_1{=}C_2$ double bond is assumed to exist in this direction. In the nitro- and cyanoaniline series, it directs to the same side as the amino

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