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Quantitative Analysis of Degradation Products in Pilocarpine Hydrochloride Ophthalmic Formulations

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Abstract ☐ The presence of isopilocarpine, an epimer of pilocarpine, and of pilocarpinic acid, a hydrolytic degradation product of pilocarpine, was established and all three substances were assayed in various commercial ophthalmic formulations of pilocarpine hydrochloride by ^{13}C-Fourier transform spectroscopy. Assay was based upon integrated intensities of selected resonances from any formulation calibrated against the intensity of tetramethylammonium bromide, used as a common external reference. The normalized intensities were then related to those of a reference solution of pilocarpine hydrochloride, thereby eliminating any factor arising from variability of ^{13}C-relaxation times. The ^{13}C-resonance for the N-methyl group, being common to all products, provides a convenient basis for the assay of the total alkaloid content whereas the C-8 resonances are best suited for assaying residual pilocarpine and its degradation products. This procedure, estimated as accurate to ±5%, constitutes the first comprehensive analytical method to differentiate between pilocarpine and its degradation products.

Keyphrases ☐ Pilocarpine hydrochloride—and degradation products, ^{13}C-NMR analysis, commercial ophthalmic formulations ☐ Isopilocarpine—^{13}C-NMR analysis in commercial ophthalmic formulations of pilocarpine hydrochloride ☐ Pilocarpinic acid—^{13}C-NMR analysis in commercial ophthalmic formulations of pilocarpine hydrochloride ☐ ^{13}C-NMR spectroscopy—analysis, pilocarpine hydrochloride and degradation products, commercial ophthalmic formulations ☐ Ophthalmic formulations, commercial—^{13}C-NMR analysis of pilocarpine hydrochloride and degradation products

No satisfactory methodology[1] is available for the analysis of the degradation products, isopilocarpine (II) and pilocarpinic acid[2] (III), in aqueous formulations of pilocarpine (I). The analysis of such impurities has received little attention because of: (*a*) their apparent relatively low concentrations in pilocarpine formulations, (*b*) the great difficulty in effecting any separation, and (*c*) the difficulty in realizing unequivocal characterization. However, numerous procedures for the determination of pilocarpine, including colorimetric, volumetric, polarimetric, and polarographic (3, 4), have been developed.

All commonly used procedures for the assay of pilocarpine have drawbacks (5). A common deficiency is the inability to distinguish between the parent alkaloid and its degradation products. For example, various colorimetric methods use the ferric hydroxamate reaction in aqueous (4, 6) or alcoholic (7) media, the sodium nitroprusside reaction (8), the hydrogen peroxide dichromate reaction (9), and the phosphomolybdic acid reaction (10). Although none of these colorimetric procedures excludes the contribution from degradation products from the total assay,

I

II

III

[1] The simultaneous determination of pilocarpine and isopilocarpine in pharmaceutical preparations (1) was published after this paper was submitted. The same method had been used to analyze isopilocarpine and pilocarpine in the synthesis of d-pilocarpine-N-$^{14}CH_3$ (2).

[2] Pilocarpinic acid has also been called pilocarpic acid (T. A. Henry, in "The Plant Alkaloids," 4th ed., Blakiston, Philadelphia, Pa., 1949) and pilocarpoic acid (R. H. F. Manske, in "The Alkaloids," vols. 3 and 5, Academic, New York, N.Y., 1953 and 1955).

Table I—Measurements and Calculated Parameters

Pilocarpine Hydrochloride Formulation (%)[a]	Density of Concentrated Formulation[b]	Relative Peak Intensities[c]					Calculated Parameters		
				C-8					
		IV	C-14	Pilocarpine	Isopilocarpine	Pilocarpinic Acid	C_t	F	C_o
A (4)	1.12	55	55	94	—	—	25.5	7.5	3.4
A (3)	1.12	60	42	84	5	5	18.1	6.7	2.7
A (2)	1.15	65	51	95	—	—	19.0	10	1.9
A (1)	1.13	84	75	41	—	—	22.0	20	1.1
B (6)	1.03	97	72	85	6	6	20.5	3.3	6.2
B (4)	1.18	121	59	81	—	5	12.0	4.0	3.0
C (4)	0.92	82	40	80	—	4	15.0	3.75	4.0
D (2)	1.12	73	43	81	4	4	15.0	7.5	2.0
E (4)	1.11	114	24	23	—	—	5.4	1.7	3.2

[a] Percent concentration as read from labeled formulation. [b] Density of concentrated (freeze-dried) sample of formulation in deuterium oxide. [c] To measure the intensities of the small peaks due to isopilocarpine and pilocarpinic acid, the C-8 peak intensities were integrated separately on one intensity scale; integration of the C-14 and IV peak intensities was performed on a different, common scale.

new, similar colorimetric procedures with the same limitations continue to appear.

A recently reported procedure based on the oxidative cleavage of pilocarpine by benzoyl peroxide and colorimetric estimations of the formaldehyde formed after additions of chromotropic acid (11) is alleged to hold some advantage in obviating interferences present in the USP hydroxylamine hydrochloride method for pilocarpine formulations. However, this new procedure probably will account for every substance having the *N*-methyl group (degradation products included) in a pilocarpine solution.

Dissatisfaction with traditional methods prompted the development of rather sophisticated chemical procedures for the indirect assay of pilocarpine and associated impurities. One such procedure employs phase solubility analysis of slightly soluble picrate salts in aqueous buffered solutions (12), but it is assumed that the reference pilocarpine samples are pure and that no chemical alteration occurs upon dissolution. A kinetic method for analyzing microgram quantities of pilocarpine in aqueous solution is based on the catalytic activity of the imidazole portion of the alkaloids on the hydrolysis of 2,4-dinitrophenyl acetate (12). The catalytic constants obtained for pilocarpine, pilocarpinic acid, isopilocarpinic acid, *etc.*, like the rate constants for the same substances obtained in related kinetic studies of the hydrolysis of pilocarpine (13, 14), may be based upon false assumptions regarding the constitution of pilocarpine reference solutions.

From a detailed study of the hydrolysis and epimerization of pilocarpine in aqueous solutions with changing pH using ^{13}C-NMR spectroscopy (15), an unequivocal basis for the characterization of aqueous solutions of pilocarpine and isopilocarpine was reported. The purpose of this paper is to discuss the application of ^{13}C-NMR spectroscopy to the assay of pilocarpine formulations for the content of pilocarpine and its degradation products.

EXPERIMENTAL

Procedure—^{13}C-Fourier transform NMR spectra were obtained[3] from various concentrated formulations of pilocarpine using 10-mm o.d. tubes and operating at 30°. The samples were scanned over 4000 Hz, and chemical shifts were measured in parts per million downfield from a tetramethylsilane external reference. The noise–signal ratio was kept at a constant minimum for each sample. Comparisons of the spectra obtained from commercial formulations with those of reference solutions of pilocarpine and isopilocarpine with varying pH (15) allowed the determination of the ratio of pilocarpine, isopilocarpine, and the corresponding hydroxy acids in the samples.

The spectra used for the calculations were obtained by using 4K data points, an acquisition time of 0.5 sec, a pulse angle of 30°, and no pulse delay. To check the accuracy of the observed intensities, spectra of two samples, 4% A and 4% E, were obtained using 8K data points within a 2000-Hz spectral width, an acquisition time of 2 sec, and a pulse delay of 1 sec. These conditions eliminated any error due to insufficient data points and to differences in relaxation times in the spectra. The results from these spectra were in good agreement (±5% of the measured values) with the results obtained by the more rapid method.

A known pipetted volume of each formulation was reduced to dryness by freeze drying. The residue was dissolved in deuterium oxide and carefully transferred, together with washings, to a volumetric flask (3.0 or 5.0 ml). The final concentration of pilocarpine was usually kept close to 20%; however, more dilution was sometimes necessary to dissolve the residue completely due to the presence of buffering agents and other preservatives. Table I lists the original and prepared pilocarpine concentrations for the various formulations.

For the quantitation of components in the pilocarpine formulations, a solution (2.04 *M*) of tetramethylammonium bromide in deuterium oxide was used for calibration. First, ^{13}C-NMR spectra of a solution (reference standard) of pilocarpine hydrochloride (0.159 *M*) in deuterium oxide were obtained in an NMR tube in which a coaxial inner tube containing the calibration solution of tetramethylammonium bromide had been inserted (Fig. 1). From the resulting proton-decoupled spectra, correlations were made between the peak intensities of the tetramethylammonium carbon resonances and the peak intensities for selected carbon resonances of pilocarpine and their respective solution concentrations. The same coaxial tube of tetramethylammonium bromide solution was then inserted in each formulation analyzed to calibrate them in terms of a common standard. A typical spectrum is shown in Fig. 2.

The following ophthalmic formulations of pilocarpine hydrochloride were examined: 4% A (expiration date December 1972), 3% A (expiration date February 1973), 2% A (expiration date September 1973), 1% A (expiration date December 1972), 6% B (expiration date February 1974), 4% B (expiration date June 1974), 4% C (no expiration date), 2% D (no expiration date), and 4% E (expiration date December 1975). Pilocarpine hydrochloride[4] (mp 204–205°) and tetramethylammonium bromide[5] were used for calibration.

Formulations and Calculations—The integrated intensity of any ^{13}C-NMR resonance from the reference solution of pilocarpine hydrochloride = P_n (where n refers to the carbon numbering position); the intensity of the ^{13}C-peak for tetramethylammonium bromide (IV) calibration solution (2.04 *M*) observed in the reference solution = S; the ratio of any pilocarpine peak intensity to peak IV intensity = P_n/S; the concentration of reference pilocarpine solution, P_R, per unit concentration

[3] Varian CFT-20 spectrometer.

[4] BDH.

[5] Eastman, White Label.

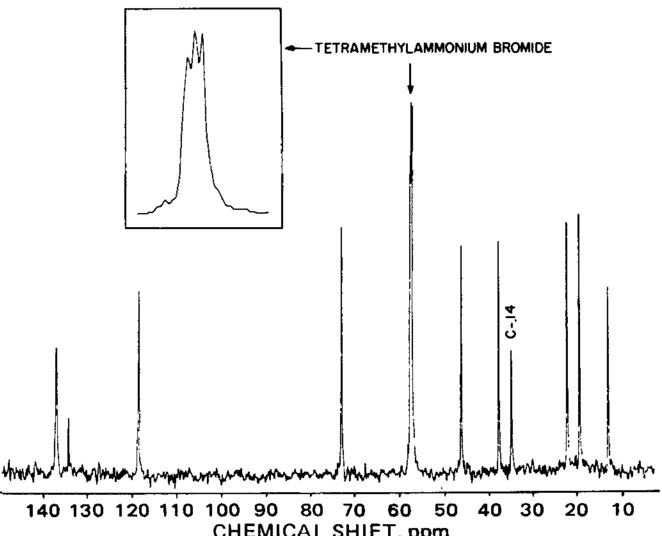

Figure 1—^{13}*C-NMR spectrum of pilocarpine hydrochloride (0.159* M) *in deuterium oxide with tetramethylammonium bromide (2.04* M) *in deuterium oxide as the external standard.*

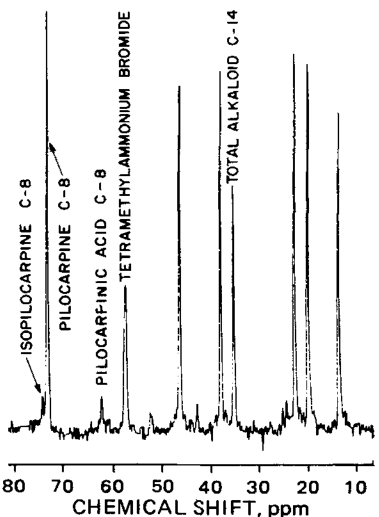

Figure 2—^{13}*C-NMR spectrum of freeze-dried sample from 6% B with tetramethylammonium bromide (2.04* M) *in deuterium oxide as the external standard.*

of IV calibration solution = $[P_R]/[\text{IV}]$ = 0.159/2.04 = 0.078; and the normalized concentration of reference pilocarpine solution per normalized unit intensity, N, of any pilocarpine resonance peak = $[P_R]/[\text{IV}] \times S/P_n$. Thus, for any pilocarpine formulation, the total molar concentration, M_t, of all components having the N-methyl group may be expressed as follows:

$$M_t = \frac{\text{intensity of C-14 peak}}{\text{intensity of internal peak IV}} \times [\text{IV}] \times N \text{ (moles/liter)} \quad \text{(Eq. 1)}$$

The percent concentration (% total alkaloid) is:

$$C_t = \frac{M_t \times \text{mol. wt. of pilocarpine hydrochloride}}{1000 \text{ (ml)} \times \text{solution density (g/ml)}} \times 100 \quad \text{(Eq. 2)}$$

The calculated original concentration C_0, of pilocarpine hydrochloride in any formulation is C_t/F, where the concentration factor is:

$$F = \frac{\text{volume of labeled formulation pipetted}}{\text{final volume of concentrated formulation}} \quad \text{(Eq. 3)}$$

For a particular sample, 3% A, the total concentration of the species that contains the imidazole ring was calculated using Eqs. 1–3. From the known concentrations of the standard solutions of pilocarpine hydrochloride (0.159 M) and IV (2.04 M) and their peak intensities [16 and 117, respectively (Table I)], N = 0.57, M_t = 0.81 mole/liter, C_t = 17.8 moles/liter, F = 6.7, and C_0 = 2.7%.

From the intensities of the C-8 peaks for pilocarpine, isopilocarpine, and the corresponding hydroxy acids in any particular sample, the relative quantities of the different species were calculated as follows:

$$\text{total intensity of C-8 resonances} = I_t = I_1 + I_2 + I_3 \ldots + I_n \quad \text{(Eq. 4)}$$

where $I_n = I_1, I_2$, *etc.*, C-8 intensities for pilocarpine, isopilocarpine, *etc.*

The ratio of any C-8 resonance to total C-8 intensity = I_n/I_t, and the percent species n in solution = $I_n/I_t \times C_0$. Therefore, for 3% A (Table I), pilocarpine = 2.4%, isopilocarpine = 0.1%, and pilocarpinic acid = 0.1%. Similar calculations were performed for each formulation (Table I).

RESULTS AND DISCUSSION

The results of ^{13}C-NMR analysis of pilocarpine formulations are summarized in Table II. Little change in pH resulted from the concentration of any formulation, and the small changes are ascribed to effects arising from the concentration of other formulating agents such as hydroxypropyl methylcellulose (0.33%), benzalkonium chloride (0.01%), edetate disodium (0.01%), potassium chloride (0.25%), boric acid (0.413%), and sodium carbonate (0.006%) present in a 2% pilocarpine hydrochloride formulation. The estimated amount of pilocarpine hydrochloride (percent) after concentration (Table I) was calculated from known changes in volume; usually, 20 ml reduced to 3.0 ml. However, in some instances the final volume had to be increased to keep all components of a formulation in solution. Formulations D and E were particularly troublesome for reconstitution of their components in deuterium oxide; consequently, their analyses were restricted to less concentrated solutions.

Baeschlin *et al.* (16) first utilized proton NMR spectroscopy to differentiate between pilocarpine hydrochloride in deuterium oxide and the lactone-cleaved sodium pilocarpinate in 2 N NaOD. Knowledge of these spectral features was then used to interpret the spectral changes observed when a formulation of pilocarpine hydrochloride (0.14 g) in deuterium oxide (2 ml) at pH 6.1, containing monobasic potassium phosphate (0.056 g) and dibasic sodium phosphate dihydrate (0.04 g), was heated at 70° for 240 hr. The gradual diminution of a multiplet at 0.4 ppm, attributed to the γ-methylene protons of the lactone ring, with the simultaneous development of a more complex multiplet at 1.1 ppm (not assigned) during prolonged heating of the formulations was attributed to hydrolysis (lactone ring cleavage) of pilocarpine. From comparison of the integrals from these multiplets, it was reported that 70% pilocarpine remained intact and that 28% of an "open form" was generated after 240 hr at 70°.

From inspection of the published spectra (16), most of the spectral change can be accounted for by isomerization to isopilocarpine, because obvious development of an additional methyl triplet can be seen on the high field side of the 3.7-ppm triplet for the methyl group of the ethyl substituent of pilocarpine. The recognition that the spectra reveal evidence for appreciable isomerization of pilocarpine also discounts the value of the colorimetric method designed by Brochmann-Hanssen *et al.* (17), with which the proton NMR method was compared because it also cannot distinguish between isopilocarpine and lactone-cleaved products.

Nunes and Brochmann-Hanssen (14) were the first to report that when pilocarpine and isopilocarpine are hydrolyzed in alkaline solution, the NMR triplets of the terminal methyl group of the ethyl substituents are shifted upfield to 1.31 ppm for both epimers and that the chemical shifts differ by 0.1 ppm for the two epimers. Nunes and Brochmann-Hanssen considered that integration of the methyl triplets due to pilocarpinate and isopilocarpinate, recorded with a 10-fold scale expansion and a 1.0-ppm offset, provided the best means of quantitating epimerization. For the best utilization of this method at 100 MHz, it is necessary to use a curve resolver for the expanded spectra (14). In addition, the assay-based integration of methyl triplets is limited to alkaline solutions and cannot be utilized under acidic or nearly neutral conditions where the chemical shifts for the methyl triplets of isopilocarpine and pilocarpine coincide.

Recently, a liquid chromatographic procedure was published for the simultaneous determination of pilocarpine and isopilocarpine in pharmaceutical preparations (1). This procedure involves separation of the isomers on an ion-exchange column followed by detection by UV absorption in the 217-nm region. The conditions used (1), *i.e.*, the buffered mobile phase was adjusted to pH 9, might lead to isomerization and hydrolysis of pilocarpine, as indicated by ^{13}C-NMR spectroscopic studies of the behavior of pilocarpine with varying pH (15). This concern for possible analytical modification of the sample by the chromatographic

Table II—Analysis of Pilocarpine Formulations[a]

Formulation	Pilocarpine Hydrochloride (from Label), %	pH	Estimated Concentration of Pilocarpine Hydrochloride (after Freeze Drying), %	pD after Freeze Drying	Total[b] Alkaloid	Calculated Concentration, %[c]		
						Pilocarpine	Isopilocarpine	Pilocarpinic Acid
A	4	3.85	30	4.60	3.4	3.4	n.d.[d]	n.d.
A	3	4.00	20	4.50	2.7	2.4	0.1	0.1
A	2	3.75	20	4.15	1.9	1.9	n.d.	n.d.
A	1	4.10	20	4.95	1.1	1.1	n.d.	n.d.
B	6	4.20	20	4.65	6.2	5.4	0.4	0.4
B	4	4.20	16	4.50	3.0	2.8	n.d.	0.2
C	4	4.10	15	4.55	4.0	3.8	n.d.	0.2
D	2	4.25	15	4.60	2.0	1.8	0.1	0.1
E	4	3.90	6.8	4.35	3.2	3.2	n.d.	n.d.

[a] Estimated accuracy of intensity measurement = ±5%; precision = ±3%. [b] Calculated on the basis of the C-14 ^{13}C-NMR resonance and expressed as if total intensity arose from pilocarpine hydrochloride. [c] Contents less than 0.1% could not be detected by the method of analysis employed. [d] Not detected.

procedure is especially pertinent when long retention times are involved.

While Urbànyi *et al.* (1) did not describe the determination of pilocarpinic acid, Weber[6] chromatographically determined this acid by treating a second aliquot of the ophthalmic solution with acid (pH <1) to convert pilocarpinic acid to pilocarpine. In contrast, the NMR spectroscopic technique permits pilocarpine, isopilocarpine, and pilocarpinic acid to be characterized and assayed simultaneously in the presence of one another and in the presence of excipients commonly found in commercial pilocarpine solutions.

In a detailed ^{13}C-NMR study of the hydrolysis and epimerization of pilocarpine in aqueous solution of varying pH (15), it was shown that the C-8 resonances[7] afford a particularly good spectral basis for distinguishing among pilocarpine, isopilocarpine, and pilocarpinic acid. In this study, this understanding was applied in the development of an assay procedure based on the relative intensities of the C-8 resonances for these compounds calibrated against the intensity of the ^{13}C-resonance from a known concentration of tetramethylammonium bromide.

Tetramethylammonium bromide (IV) was selected because its ^{13}C-resonance is well removed from any resonance of pilocarpine and the other compounds (Fig. 1). The insert in Fig. 1 reveals the triplet character for the resonance of IV due to quadrupole coupling by nitrogen; thus, the intensity of the resonance of IV was taken as the sum of the three integrated component intensities. Figure 2 illustrates the spectral features of a pilocarpine formulation, showing the C-8 resonances for pilocarpine, isopilocarpine, pilocarpinic acid, and IV.

Because the C-14 resonance for the *N*-methyl moiety has a common frequency for all components of a pilocarpine solution at any pH, its intensity can be used as a measure of the total alkaloid content of any pilocarpine formulation. The intensities of the C-8 resonances, however, cannot be compared directly with the C-14 resonances, because the ^{13}C-relaxation time for the C-14 is markedly different from that for C-8, as may be seen from inspection of the relative intensities in Fig. 2.

To calculate either the percentage composition or the total alkaloid content of any formulation, it is necessary to circumvent the difference in relaxation characteristics by relating any particular resonance intensity to the intensity of the common calibration resonance, IV. The ratio of intensities so obtained is then multiplied by a normalizing factor, N, calculated to relate the intensity of reference IV in terms of the intensity of any resonance of a reference solution of pilocarpine hydrochloride of known concentration. The details of these formulations together with sample calculations are outlined under *Experimental.*

The results of the analysis of a range of pilocarpine formulations having various labeled concentrations of drug and expiration dates are summarized in Table II. The samples were procured from various pharmacies in 1972 and were then stored at room temperature and protected from light. In most instances, no isopilocarpine was detected; where it was detected, it generally did not exceed 5% of the labeled pilocarpine content. In one instance, B (6%) showed degradation to 7% isopilocarpine and 7% pilocarpinic acid which was surprising in view of its more recent expiration date compared to most samples. However, this formulation apparently was the only one containing phenylmercuric nitrate as well as benzalkonium chloride as preservatives. One formulation, 4% E, showed a new resonance at 70.25 ppm, attributed to a ring carbon of povidone, an ingredient not found in the other formulations.

The calculated total alkaloid content agreed well with the labeled content of pilocarpine hydrochloride in each instance. Low results obtained by a colorimetric method for the determination of pilocarpine in ophthalmic solutions were attributed (18) to pilocarpine–methylcellulose interaction. In no case was there any evidence of interference with the ^{13}C-NMR method by methylcellulose, hydroxypropyl methylcellulose, or hydroxyethylcellulose.

In summary, this ^{13}C-NMR spectroscopic assay constitutes the first reported unequivocal and comprehensive method for analyzing degradation products of pilocarpine, isopilocarpine, and pilocarpinic acid, as well as unchanged pilocarpine, in pilocarpine formulations. This method provides for the differentiation and characterization *in situ* of products as well as their convenient assay utilizing resonance intensities treated in such a way as to avoid the limitation imposed upon ^{13}C-NMR spectroscopy for quantitation due to marked variability in relaxation times. The assay, as described, provides the percentage composition within ±5%.

[6] J. D. Weber, Food and Drug Administration, Washington, D.C., personal communication.
[7] By definition, the C-8 resonances arise from the γ-methylene carbon atom of the γ-lactone ring of pilocarpine, isopilocarpine, *etc.*

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Structure–Activity Studies Using Valence Molecular Connectivity

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Abstract □ The extension of the molecular connectivity concept to the treatment of heteroatom molecules affords an opportunity to examine structure–activity relationships in a wide variety of molecule series that possess biological activity. Four series are described in this report. The correlations found indicate that molecular connectivity is an extremely useful descriptor of structure in studying drug molecule structure–activity relationships.

Keyphrases □ Molecular connectivity index—related to structure–activity relationships, various types of molecules □ Structure–activity relationships—various types of molecules, related to molecular connectivity index □ Topological indexes—molecular connectivity index, related to structure–activity relationships, various types of molecules

Recently (1, 2), the extension of molecular connectivity to the treatment of heteroatom molecules was described. By considering the valence electrons, whether bonded or nonbonded, an atom connectivity term, δ^v, for multiply bonded carbon atoms or heteroatoms was assigned.

The work presented in this paper tests the ability of δ^v to describe the atomic connectivity in heteroatoms so that the molecular connectivity indexes bear some relationship to structure influencing biological properties.

DISCUSSION

The simple prescription for assigning δ^v is $Z^v - h_i$, where Z^v is the number of valence electrons and h_i is the number of attached hydrogens. For alcohols, ethers, primary amines, and pyridine, the δ^v values for the heteroatoms are 5, 6, 3, and 5, respectively. The δ^v values for heteroatoms are shown in Table I.

Since halogen atoms have an identical number of valence electrons, this prescription yields identical values of δ^v. It is necessary to derive empirical values of δ^v for the halogens by calibrating them to a physical property. The molar refraction was chosen for this assignment (1, 2) (Table I).

Table I—Valence Delta, δ^v, Values for Heteroatoms

Atom	δ^v	Atom	δ^v
$—NH_2$	3	—OH	5
—NH—	4	—O—	6
>N—	5	—C=O	6
—C≡N	5	Furan O	6
—C=NH	4	O=N—O	6
Pyridine N	5	H_2O	4
Nitro N	6	H_3O^+	3
NH_3	2	F	(−)20
NH_4^+	1	Cl	0.690
>N⁺<	6	Br	0.254
$=NH_2^+$	3	I	0.085

The use of δ^v thus permits the calculation of a valence chi term of the first order, ${}^1\chi^v$, by the expression:

$$ {}^1\chi^v = \sum (\delta_i{}^v \delta_j{}^v)^{-1/2} \quad \text{(Eq. 1)}$$

The ${}^1\chi^v$ terms provide a further structural description of heteroatom molecules, eliminating the redundancies found when simple connectivity values are used. The refinement permits the close correlation of chi terms with physical properties such as boiling point, solubility, and molar refraction (1, 2).

Cytochrome Conversion by Phenols—The conversion of cytochrome P-450 to P-420 in the rabbit liver by a series of phenols was reported (3). An analysis of these molecules using ${}^1\chi^v$ reveals the following relationship to the minimum active concentration ($-\log c$), pC:

$$pC = 0.816\ (\pm 0.105)\,{}^1\chi^v - 0.789\ (\pm 0.329) \quad \text{(Eq. 2)}$$

$$r = 0.914 \quad s = 0.291 \quad n = 14$$

The calculated and observed values are listed in Table II.

The inclusion of the Hammett sigma value for each substituent in a multiple regression with ${}^1\chi^v$ does not improve the relationship. The re-