Acetylcholinesterase Substrates: β-Methylcholine Esters

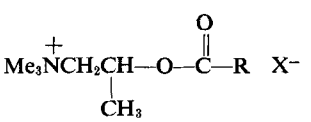
G. M. STEINBERG, M. L. MEDNICK, R. E. RICE, and J. P. MADDOX

Abstract □ *β-Methylcholine propionate* (Compound II) and isobutyrate (Compound III) are "good" substrates for eel acetylcholinesterase. Enzymatic hydrolysis of the corresponding pivalyl ester is barely detectable. For study of acetylcholinesterase under conditions where the "blank" hydrolysis rate of substrate must be reduced to a minimum, Compounds II and III can be used advantageously. Like acetylcholine and β-methylcholine acetate, Compound II shows substrate inhibition. The isobutyryl ester (Compound III) gives no significant substrate inhibition. Kinetic constants for both enzymatic and nonenzymatic hydrolysis are reported for the group of esters. Evidence is presented which suggests that the rate of acylation of the enzyme parallels reactivity with hydroxide ion for acetylcholine and the acetyl and propionyl esters of β-methylcholine. With the isobutyryl ester of β-methylcholine, there is a relative decrease in enzymatic acylation rate.

Keyphrases □ Acetylcholinesterase substrates—β-methylcholine esters □ β-Methylcholine esters—synthesis □ Hydrolysis rates, enzymatic, nonenzymatic—β-methylcholine esters □ Michaelis constants—β-methylcholine esters

For studies (to be published) on oxime reactivation of phosphonylated acetylcholinesterase (AChE) (acetylcholine acetylhydrolase, EC 3.1.1.7), which the authors wished to perform by the continuous monitoring procedure of Kitz *et al.* (1) using an autotitrator, it was important to reduce the "blank" hydrolysis rate of the substrate to a minimum. In the region of pH 9, acetylcholine (ACh), its normal substrate, gives undesirably high "blank" values.

Like most enzymes, AChE has a moderately wide range in its specificity and is capable of hydrolyzing a considerable variety of esters other than ACh (2). It is well known that the rates of aqueous (alkaline) hydrolysis within a family of esters can be decreased by insertion of bulking groups near the ester linkage (3). Recently, Gunter (4) observed that *ortho*-substitution in phenyl acetate, which decreases the alkaline hydrolysis rate, does not interfere with enzymatic hydrolysis by AChE. Hence, it seemed appropriate to examine several homologs of acetyl β-methylcholine with increasing bulk on the acetyl methyl group. The compounds are listed in general structure I. Two of the compounds, Compounds II and III, satisfied the requirements.



Compound	R	X^-
I	$-CH_3$	Br^-
II	$-C_2H_5$	I^-
III	$-CH(CH_3)_2$	I^-
IV	$-C(CH_3)_3$	I^-

β-methylcholine esters

I

Since these compounds may have general utility for studies with AChE, particularly where the "blank" hydrolysis of substrate becomes a problem, the authors explored their properties. In this paper, they report the nonenzymatic and enzymatic hydrolysis rates and Michaelis constants (at pH 7.4).

EXPERIMENTAL

Acetylcholine bromide (ACh) and acetyl β-methylcholine bromide (Compound I) were obtained commercially. The AChE (Worthington Biochemicals) was purified, stable, dry powder from electric eel, Code ECHP, 1000 units/mg. Twenty milligrams of enzyme was dissolved in 1.6 ml. of a previously boiled aqueous solution, pH approximately 7.4, containing KCl (0.225 *M*) and 0.25% gelatin (KCl/gel) to give enzyme concentrate E_w. The concentration of active sites in E_w is estimated to be 2.5×10^{-5} *M*.[1]

Both enzymatic and solvolytic rates were determined from the rate of addition of standard alkali needed to maintain constant pH using a Radiometer TTTl autotitrator, fitted with an ABUl buret, a SBR2 recorder, and a PHA 630 scale expander. The titrator was fitted with the 0.25-ml. buret (0.25 ml. = 100 scale divisions of chart displacement), and the 6-ml. jacketed titration vessel was covered and maintained at 25°. Nitrogen was slowly passed over the reaction solution to minimize CO_2 absorption. The titrant was 0.00353 *M* carbonate-free sodium hydroxide.

Enzymatic Hydrolysis—All measurements were made at pH 7.4, 25°. To 3.0 ml. of 0.225 *M* KCl, there were added 2.0 ml. of aqueous substrate and 15–500 μl. of appropriately diluted (in KCl/gel) enzyme concentrate, E_w. Dilutions ranged from 1/1000 to 1/10,000 (except for Compound IV, in which case undiluted E_w was used). Records were made of initial hydrolysis rates. The enzymatic hydrolysis data are presented in Lineweaver-Burk reciprocal plots (Figs. 1 and 2). For convenience of comparison, the results have been normalized by conversion of the observed rates to those calculated for concentrated enzyme solution, E_w, assuming a direct linear relationship between enzyme concentration and v, the velocity in moles per liter of ester hydrolyzed per minute. The values of $k_{cat.}$ and $K_{m(app.)}$ were computed from the slope and y-intercept using Eqs. 2 and 2a.

Nonenzymatic Hydrolysis—Reactions were run at 25° at the appropriate pH values, which were maintained constant during each run. The 5.0 ml. reaction mixture contained 0.135 *M* KCl and 0.1 *M* ester. Measurements were made of initial hydrolysis rates under conditions where less than 0.1% of the ester was hydrolyzed. The continuous records of alkali delivered (to maintain constant pH) as a function of time were linear over the period of measurement. The values reported have been corrected for the CO_2 blank. At the pH values employed the water component of the observed hydrolytic reaction rate constant, k_{obs}, is negligible (5). Hence the reaction, $RCO_2R' + OH^- \rightarrow RCO_2^- + R'OH$, is first order in ester and in hydroxide ion. Because the autotitrator adds alkali automatically to maintain a constant pH, the rate of addition of alkali is equivalent to the rate of formation of acid.

Synthesis—Compounds II, III, and IV were prepared as follows.

2-Hydroxypropyldimethylamine—To a cooled (8°) stirred solution of dimethylamine (54 g., 1.2 moles) in absolute methanol (300 ml.), 1,2-propylene oxide (58 g., 1.0 mole) was added in small portions over 25 min. while maintaining the temperature below 8°. After this addition, the temperature was permitted to rise to 30° and was held there for 20 min. After removal of the cooling bath, the solution was left to stand 1.5 hr. (maximum temperature 40°). Distillation through a simple head followed by redistillation through a 15 × 1-cm. vacuum-jacketed Vigreux column gave 46 g. (45%) of 2-hydroxypropyldimethylamine, b.p. 126° (uncorrected).

Compounds II, III, and IV—The propionyl and isobutyryl esters were prepared by treating 2-hydroxypropyldimethylamine with a

[1] Calculated from turnover number = 6.7×10^5 min.$^{-1}$, pH 7.4, 0.33 *M* KCl, 0.0073 *M* AChE, 0.25% gelatin, 25°, $K_{m(app.)} = 2.8 \times 10^{-4}$ *M* (H. Michel, private communication).

Table I—Esters II, III, and IV

Compound	Tertiary Aminoester, b.p.[a]	Quaternary Aminoester, m.p.[a]	Formula	Anal., % Calcd.	Found
II	171–171.5°	165–167°	$C_9H_{20}INO_2$	C, 35.89 H, 6.69 N, 4.67 O, 10.62	C, 35.8 H, 6.6 N, 4.7 O, 10.8
III	66.5–67° (10 mm.)	195.5–196.5°	$C_{10}H_{22}INO_2$	C, 38.11 H, 7.04 I, 40.26 N, 4.44 O, 10.15	C, 38.1 H, 6.9 I, 40.2 N, 4.5 O, 10.3
IV	70.5–71.5° (11 mm.)	241–242°	$C_{11}H_{24}INO_2$	C, 40.13 H, 7.35 I, 38.55 N, 4.25 O, 9.72	C, 40.0 H, 7.3 I, 38.4 N, 4.2 O, 10.0

[a] Uncorrected.

small excess of the corresponding acyl anhydride. For the pivalate, the reaction was run in benzene using equivalent quantities of aminoalcohol, pivalyl chloride, and pyridine. The tertiary aminoesters were quaternized by treatment with a small excess of methyl iodide in acetone and recrystallized from acetone or acetone–methanol. The physical properties of the tertiary aminoesters and the quaternary products, together with elemental analyses of the latter, are given in Table I.

RESULTS AND DISCUSSION

Nonenzymatic Hydrolysis—Table II contains the observed rates of reaction with the aqueous solvent. Measurements on each compound were made at two pH values. The essential identity in the pairs of computed k_{OH} values (within the range of experimental error) indicates that for each ester the "water" reaction is negligible. Thus, one can compute the rate of the "blank" hydrolysis reaction, $k_{obs.}$, at any pH above those reported[2] from the relationship $k_{obs.} = k_{OH}[OH^-]$. For convenience in comparison, the actual observed "blank" hydrolysis rates are also given for each of the esters (Table II). It is noteworthy that the effect of increased bulking on going from ACh to Compound III reduces the hydrolysis rate approximately 20×, so that the "blank" for Compound III at pH 9.5 is hardly greater than that of ACh at pH 8.0.

Enzymatic Hydrolysis—In Fig. 1, Lineweaver-Burk reciprocal plots are given for ACh and Compounds I and II. Like ACh, Compounds I and II are good substrates for AChE and each shows marked substrate inhibition at high concentration.[3] Compound III (Fig. 2) does not show substrate inhibition even at the highest concentration of substrate that was used, 0.1 *M*.

Table III contains data on enzymatic hydrolysis at pH 7.4 and substrate concentrations of 5×10^{-3} and 5×10^{-2} *M*, together with the computed ratios of the rates of the enzymatic to nonenzymatic hydrolyses. It also lists the values of $K_{m(app.)}$, computed from the Lineweaver-Burk plots in Figs. 1 and 2. Enzymatic studies were performed at pH 7.4 rather than at another higher pH because it provides data which can be more readily collated with those of other AChE substrate studies, the bulk of which have been performed in the pH 7–7.5 range. It may be assumed that changes in the Michaelis constants due to variation in pH will be substantially the same for the entire group of compounds, so the relative value of v/k_{OH} for the group can be applied at higher pH values.

For the selection of the best substrate for a particular study using an automatic titrating device such as the Radiometer TTTl, one must bear in mind that the conveniently useful range in delivery rate of the buret is perhaps only 20×, *i.e.*, from approximately 1–2 div./min. of chart paper to 20–30 div./min. At pH values above 9, where the "CO_2" blank becomes significant, the range becomes even

Table II—Nonenzymatic Hydrolysis of Substrates

Substrate	pH	Solvolysis: Rate Observed,[a] div./min.	Solvolysis: k_{OH},[b] M^{-1} min.$^{-1}$
Acetylcholine	8.0	5.08	89.6[c]
	8.5	16.56	91.0[c]
β-Methylcholine esters			
(I) Acetyl	9.0	9.35	16.5
	9.5	31.25	17.4
(II) Propionyl	9.0	7.21	12.7
	9.5	22.0	12.3
(III) Isobutyryl	9.0	1.89	3.2
	9.5	7.87	4.4
(IV) Pivalyl	10.0	1.93	0.34
	10.5	7.7	0.43

[a] Div. (on radiometer) = 2.5×10^{-3} ml.; 5.0 ml. of 0.1 *M* ester was present in titration vessel. Titrant, 0.00353 *M* NaOH. [b] $k_{obs.} = k_{OH}[OH^-]$. [c] Reported: 72 and 80.4 (*Reference 5*).

smaller. By increasing the concentration of titrant, the "blank" delivery rate for a given substrate concentration can be reduced. However, by doing so, sensitivity is reduced and, therefore, more of the substrate must be hydrolyzed to obtain measurable hydrolysis records.

Under conditions where the enzyme concentration is the limiting factor, Compound II is best. Its values of v/k_{OH}, the ratio of the enzymatic to the "blank" hydrolysis rates, are equal to or greater than the others. Its lower "blank" value makes it more useful than Compound I. Where the enzyme is nonlimiting, *i.e.*, its concentration can be made high enough to give maximum delivery of titrant, Compound III is preferred because of its lower "blank." The enzymatic hydrolysis of Compound IV is so slow that one may question whether it is hydrolyzed by AChE at all. It is certainly possible that the slight activity is produced by some impurity in the enzyme preparation. However, if it is truly a substrate for AChE, it might be used for monitoring the concentrated enzyme, if and when it becomes available in reasonably large quantities.

β-Methylcholine exists as a DL-pair. It is known that with the acetate ester, only the L(+)-isomer acts as an AChE substrate, while the D(−)-isomer is an inhibitor[4] (10, 11). In these studies, the authors have observed that enzymatic hydrolysis of Compounds II and III give only approximately one-half of the theoretical

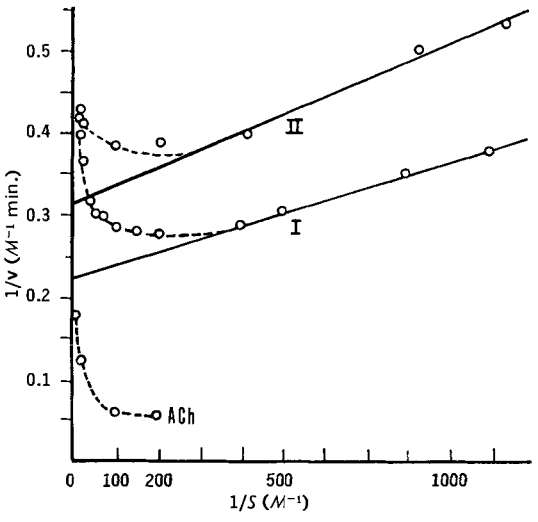


Figure 1—*Lineweaver-Burk reciprocal plots of velocity of substrate hydrolysis as a function of substrate concentration, pH 7.4, 25°. Substrates: ACh and Compounds I and II.* S *represents concentration of racemic mixture.*

[2] Extrapolation probably can be carried to lower pH also. An estimated lower limit for extrapolation is approximately pH 5 (6).

[3] Mounter and Ellin (7) report no substrate inhibition by Compound I. Their studies appear to have been made without addition of supporting electrolyte. If this is so, the ionic strength of the medium changed with each change in concentration of substrate. Since the properties of eel AChE vary significantly with changes in ionic strength (see, for example, *References 8 and 9*), this may be the cause of the difference.

[4] A very rough estimate of the value of K_I for the D(−)-isomer is 10^{-3} *M*, calculated from data in *Reference 11*.

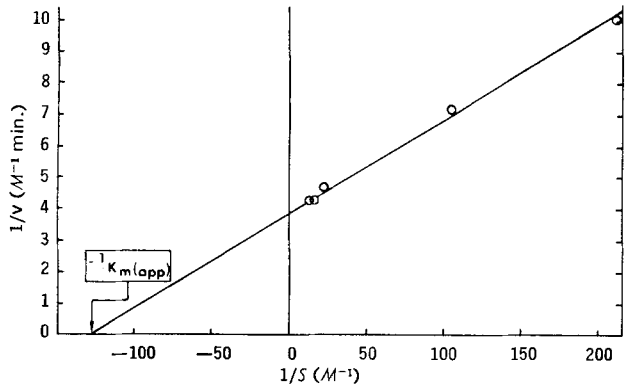


Figure 2—*Lineweaver-Burk plot for Compound III.* S *represents concentration of racemic mixture.*

quantity of acid. Here, too, only one member of each DL-pair acts as substrate. Hence, by using the pure enzymatically active isomers, one should be able to increase the v/OH ratios by at least two times and probably even more, since the inactive isomers may inhibit the enzymatic reaction.

For both the Lineweaver-Burk plots (Figs. 1 and 2) and the computation of $K_{m(app.)}$ (Table III), use was made of total ester concentration and not actual concentration of active isomer.

Observations on Enzyme Kinetics—Since the purposes of this study were pragmatic, the choice of substrates was made entirely on that basis and without regard to theoretical implications of the kinetic results. However, analysis of the data that were obtained points to probable relationships between substrate structure and enzyme kinetic constants which may warrant further investigation.

AChE functions kinetically in a three-step reaction (Eq. 1) (12). Enzymatic hydrolysis follows Michaelis-Menten kinetics (at concentrations below those which cause substrate inhibition) (Eq. 2); however, the kinetic constants are complex functions (Eqs. 3–5) (13).

$$E + \text{ester} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot\text{ester} \xrightarrow{k_2} \text{E-acyl} \xrightarrow{k_3} E + \text{acid} \quad \text{(Eq. 1)}$$

(E-acyl + alcohol)

$$v = \frac{V_{\max.}S}{S + K_{m(app.)}}; \quad \text{where } V_{\max.} = k_{cat.}E_0 \quad \text{(Eq. 2)}$$

$$\frac{1}{v} = \frac{1}{V_{\max.}} + \left(\frac{K_{m(app.)}}{V_{\max.}}\right)\frac{1}{S} \quad \text{(Eq. 2a)}$$

$$k_{cat.} = \frac{k_2k_3}{k_2 + k_3} \quad \text{(Eq. 3)}$$

$$K_{m(app.)} = \frac{K_sk_2}{k_2 + k_3}; \quad K_s = \frac{k_2 + k_{-1}}{k_1} \quad \text{(Eq. 4)}$$

$$\frac{k_{cat.}}{K_{m(app.)}} = \frac{k_2}{K_s} \quad \text{(Eq. 5)}$$

where k_1, k_{-1}, k_2, and k_3 are rate constants; v is the observed rate of product formation; and E_0 and S are, respectively, the concentrations of enzyme and substrate.

Inhibitors can bind to E or E-acyl or to both (Eq. 6).

$$\begin{array}{ccccc} E + \text{ester} & \rightleftharpoons & E\cdot\text{ester} & \rightarrow & \text{E-acyl} \rightarrow E \\ + & & + & & \\ \text{Inh} & & \text{Inh} & & \\ \Big\Updownarrow K_I & & \Big\Updownarrow K_\alpha & & \\ E\text{Inh} & & E\text{Inh-acyl} & \nrightarrow & E \end{array} \quad \text{(Eq. 6)}$$

Binding to E results in competitive inhibition while binding to E-acyl results in noncompetitive inhibition. Inhibition by excess substrate is believed to result from its binding to E-acyl and consequent interference with the deacylation step (14). Binding and interference in this fashion will be observed kinetically only if the relevant kinetic step is rate limiting [with ACh, deacylation is rate limiting; $k_2 = 6$–$10 \times k_3$ (15)]. Thus, the failure of Compound III to give substrate inhibition may be due to its failure to bind to the acyl enzyme, *i.e.*, a marked increase in K_α, or alternatively may be the result of a change in the rate-limiting step, *i.e.*, $k_3 > k_2$.

Table III—Enzymatic Hydrolysis by Eel AChE[a]

Substrate	v, M/min., S_0 = 5×10^{-3} M	v, M/min., S_0 = 5×10^{-2} M	v/k_{OH}, S_0 = 5×10^{-3} M	v/k_{OH}, S_0 = 5×10^{-2} M	$K_{m(app.)}$[b], M
ACh	16.8	7.9	0.185	0.087	2.8×10^{-4}[d]
I	3.5	3.1	0.206	0.182	6.9×10^{-4}[e]
II	2.55	2.4	0.204	0.192	6.9×10^{-4}
III	0.1	0.22	0.0264	0.058	7.6×10^{-3}
IV	—	3×10^{-5}	—	7.7×10^{-5}	—

[a] pH 7.4, 25°; v, moles/l. min. of substrate hydrolyzed, calculated for enzyme concentration E_0. [b] Based upon total ester concentration. [c] S_0, initial substrate concentration. [d] H. Michel (see *Footnote 1*). [e] Reported, 8×10^{-4} M (7).

Table IV—Kinetic Constants for Enzymatic Hydrolysis

Substrate	$k_{cat.}$, min.$^{-1}$	k_2/K_s,[a] M^{-1} min.$^{-1}$	$k_2/K_s\,k_{OH}$
ACh	7×10^5[b]	2.5×10^9[b]	2.8×10^7
I	1.74×10^5	5.0×10^8	3×10^7
II	1.27×10^5	3.7×10^8	3×10^7
III	1.03×10^4	2.7×10^6	7×10^5

[a] Equation 5. Computed from concentration of active ester (one-half total ester concentration) for I, II, and III. [b] See *Footnote 1*.

Table IV contains the values of $k_{cat.}$ computed (Eq. 2a) from the linear portions of the Lineweaver-Burk plots in Figs. 1 and 2 together with the computed values of k_2/K_s (Eq. 5)[5] for each substrate. For the latter calculation, the actual concentration of active isomer was used in computing $K_{m(app.)}$.

It can be seen that for ACh and Compounds I and II, the values of k_2/K_s, the rate constant for acylation of the enzyme divided by the dissociation constant of the enzyme substrate complex, closely parallel the corresponding values of k_{OH}, the rate constant for acylation of the hydroxide ion; *i.e.*, the value of k_2/K_sk_{OH} (Table IV) is constant. If one makes the not unreasonable assumption that the value of K_s does not vary greatly among this group of substrates,[6] then the results suggest that over the range in structural variation represented by ACh and Compounds I and II, the enzyme displays little kinetic selectivity, the differences in reaction rate paralleling the reactivity of the ester toward hydroxide ion.

In the case of Compound III, there is a marked fall in $k_{cat.}$ and in the value of k_2/K_s. Also, as noted earlier, Compound III does not show substrate inhibition. These results suggest that the principal cause of the reduction in the rate of hydrolysis of Compound III by AChE is due to reduction in the rate constant of the acylation step, k_2. Further, the $k_{cat.}$ values for the hydrolysis by eel AChE of the acetyl and isobutyryl esters of phenol are substantially the same.[7] For phenyl acetate, $k_2 \gg k_3$ (17). Therefore, the values of k_3 for the acetate and isobutyrate (and probably the propionate) are closely similar.[8] Hence, the fall in $k_{cat.}$ for Compound III represents a fall in the value of k_2.

The rate of reaction of Compound III with hydroxide is less than that of the esters I and II (Table II). The reduction in its "normalized" reaction rate with enzyme, k_2/K_sk_{OH} (Table IV), is appreciably greater. Thus, with the increase in molecular size or com-

[5] The values of both $k_{cat.}$ and $K_{m(app.)}$ are reduced by the presence of the inhibitory nonsubstrate isomer. However, it can be shown that both are affected to approximately an equal extent, so that the value of k_2/K_s is not appreciably affected.

[6] With alkyl trimethylammonium salts, K_I values vary by no more than 2–3 × over a several carbon atom range in the chain length of the alkyl group. See *Reference 16*.

[7] The $k_{cat.}$ value for phenyl acetate is approximately 3 × greater than that for phenyl isobutyrate (G. M. Steinberg and J. Maddox, to be published).

[8] An inversion in the rate-determining step for the phenyl isobutyrate hydrolysis by AChE is highly unlikely in view of the extremely high rate of the reaction. However, if this was the case, it would mean that k_3 would be greater than $k_{cat.}$ and, hence, would not invalidate the conclusions.

plexity on passing from Compound II to III, one observes the beginning of kinetic selectivity by the enzyme.

It is noteworthy that if one sets $k_2 = k_{OH}[OH^-]$ for ACh, to achieve the enzymatic acylation rate one would have to use a hydroxide-ion concentration of 6×10^4 *M*.

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Carotid Sinus Pressure Reflex Bioassay for *Veratrum viride*

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Abstract □ From a total of 238 anesthetized dogs, a series of regression analyses was performed examining the relationship between the variables (sex of the animal, date on which the experiment was performed, effect of the three veratrum alkaloidal drug preparations studied, and the number of days the dogs were stored in the animal house) on the arterial pressure pressor amplitudes of the predrug and postdrug carotid occlusion response and on the blood pressure-lowering effects of the drug treatments. The seasonal date of the experiment and the drug treatments significantly influenced the arterial pressure responses, while sex of the animal or days stored prior to use did not consistently alter the slope constants or arterial pressure responses for the drug subgroups.

Keyphrases □ *Veratrum viride* alkaloids—bioassay □ Carotid sinus pressure reflex—*V. viride* bioassay □ Sex, time of year effects—blood pressure lowering, drugs □ Regression analysis—factors affecting blood pressure lowering

The method presently used for the quantitative assessment of extracts of *Veratrum viride* is based on the progressive loss of the pressor response induced by bilateral occlusion of the common carotid arteries in the anesthetized dogs as reported by O'Dell (1). The potency of the test material is expressed in terms of carotid sinus reflex (CSR) units defined as follows: "One CSR unit represents the amount of intravenously administered hypotensive agent per kilogram of body weight which just abolishes the pressor response to the carotid sinus reflex in dogs" (1).

In a study on anesthetized dogs, Prochnik *et al.* (2) concluded that minimal pressor response to bilateral carotid occlusion is to be expected when the mean arterial pressure is below 88 mm. Hg. Since the regression line approaches zero response at the basal mean arterial pressure level of 60 mm. Hg, it was suggested that minimum variability would be obtained if the carotid occlusion pressor responses were expressed as: (mm. Hg rise due to occlusion × 100)/(mean arterial pressure [mm. Hg] − 60).

Rubin and Burke (3) reported that since the changes in carotid pressor reflex response and in the existent mean arterial pressure are highly correlated, the steeper dose–response curve exhibited by the carotid pressor reflex response would be a more sensitive measure. They also concluded that the *V. viride* hypotensive principles examined did not act *via* adrenergic, sympathetic, or ganglionic blocking actions. However, more recently, Jandhyala and Buckley (4) reported that the effects of cryptenamine, an alkaloidal preparation from *V. viride*, were inhibited by reserpine, α-methyldopa, and adrenalectomy and therefore did act through a mechanism of action involving catecholamines and the adrenal medulla.

The authors have used the method as described by O'Dell (1) for the bioassay of several alkaloidal preparations from *V. viride*. The studies referred to previously alluded to the high correlation between the basal arterial pressure and the pressor response amplitude to bilateral carotid occlusion. The authors have also made unpublished observations on other possible influencing variables, such as time of the year the study was performed and length of time the dog was maintained in the animal quarter. To understand more fully the relationships between these variables, a series of regression analyses was performed on the data obtained over the last few years. This report is concerned with the findings obtained in this study.