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STRUCTURE–ACTIVITY RELATIONSHIPS OF NORMEPERIDINE CONGENERS ON CHOLINESTERASE SYSTEMS *IN VITRO* AND ANALGESIA *IN VIVO*

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Abstract—The relative importance of hydrophobic or Van der Waals forces has been evaluated in a quantitative manner with regard to the binding of a series of *N*-alkyl substituted normeperidine homologs to both acetyl- and butyrylcholinesterases. The normeperidine compounds gave mixed inhibition with both enzymes when acetylcholine was used as substrate. However, the mixed inhibition changed to pure noncompetitive with acetylcholinesterase when the hexyl through the decyl substituted normeperidines were used. Both the competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants were determined for the enzyme systems. With butyrylcholinesterase, simple linear relationships were observed when alkyl chain length, R_m values, and molecular parachors were plotted as a function of the logarithm of the inhibitor dissociation constants. Linear relationships were not observed, however, with acetylcholinesterase. The suggestion is put forth that many of the differences between the two enzyme systems may be explained on the basis of a difference in the physical characteristics of the nonpolar region in the vicinity of their active sites. Binding energies were determined with the inhibitor series for both systems and carbon–carbon distances were calculated between enzyme and inhibitor molecules from these data. The analgesic potencies of the normeperidine congeners were determined by the hot-plate method and, when plotted as a function of alkyl chain length, showed similarities to the acetylcholinesterase kinetic data and to other physical–chemical parameters. These observations may indicate similarities in the nature of the acetylcholinesterase active center and the analgesic receptor. Calculations also indicated that a change in binding energy of only 0·3 kcal/mole is reflected as a change in the ED_{50} of 50 per cent in going from the butyl to the pentyl normeperidine derivatives.

VARIOUS analgesics have been studied with respect to their inhibitory properties toward both acetyl- and butyrylcholinesterase.[1,2] To date, little emphasis has been placed on a quantitative determination of the relative importance of nonpolar binding forces in the association of analgesics with their receptors or the association of these compounds with cholinesterases. Therefore, in the present investigation this parameter was evaluated by utilizing a homologous series of normeperidine compounds which display a gradual increase in lipophilic character with only minor changes in structure. In this regard, a series of ten *N*-alkyl substituted normeperidines (1-alkyl-4-phenyl-4-carbethoxypiperidines) were utilized as inhibitors of both acetyl- and butyrylcholinesterase. In addition to their inhibitory properties, the relative analgesic potencies of these compounds were investigated in order to determine if any correlation exists between analgesia and the interaction of these compounds with the enzyme systems. Therefore, this communication represents an attempt to determine the role of hydrophobic bonding at the various levels of molecular organization.

MATERIALS AND METHODS

Materials. All reagents and materials used in this study were of analytically pure grade or equivalent. The *N*-ethyl and *N*-butyl congeners of normeperidine as the hydrochloride salts were kindly supplied by Dr. H. Wick of C. H. Boehringer Sohn, Ingelheim, Germany and the propyl and amyl derivatives from Dr. E. L. May of the National Institutes of Health, Bethesda, Md. The remaining *N*-alkyl derivatives (i.e. methyl and hexyl through decyl) were obtained from Dr. Sidney Archer, Sterling-Winthrop Research Institute, Rensselaer, N. Y. All ten compounds were shown to be chromatographically pure on activated silica gel G and cellulose thin-layer chromatographic plates. The solvent systems used were acetonitrile–water (50:50, v/v) and ethanol–water (40:60, v/v). Visualization of the compounds was accomplished with a modified iodoplatinate spray[3] reagent.

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) type V from electric eel and acetylcholine chloride were obtained from the Sigma Chemical Company, St. Louis, Mo. Butyrylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.8) from horse serum was obtained from the Worthington Biochemical Corp., Freehold, N. J.

Swiss-albino mice weighing between 18 and 21 g were obtained from the Maxfield Animal Supply Company of Cincinnati, Ohio.

Enzyme kinetic measurement. Enzyme rate measurements were carried out potentiometrically with both butyrylcholinesterase and acetylcholinesterase using a Radiometer automatic titrator (type TTT 1c) equipped with a recorder (SBR2c) and syringe buret unit (SBUla). Reactions were recorded at pH 7·40 $\pm$ 0·05 in a 25-ml thermostated vessel at 26·0 $\pm$ 0·10°. All reactions were run under a nitrogen atmosphere using a combination glass–calomel electrode (GK2026c), mechanical stirring and with 0·01 N sodium hydroxide as a titrant. The titration procedure is essentially the method developed by Stein and Laidler[4] in their studies on the kinetics of α-chymotrypsin. The enzyme reaction mixtures were 0·04 M in magnesium chloride and 0·10 M in sodium chloride. In the case of butyrylcholinesterase each reaction mixture contained 0·11 mg enzyme, and with acetylcholinesterase each reaction mixture contained 0·01 mg enzyme. The reaction mixtures, both control and with inhibitor, were preincubated for a period of 5 min at 26° prior to the initiation of the reaction by the addition of substrate. Control studies showed that a preincubation period of up to 20 min did not affect the initial rate measurements.

In the case of butyrylcholinesterase, at least six substrate concentrations ranging from $7{\cdot}7 \times 10^{-3}$ to $2{\cdot}2 \times 10^{-3}$ M were used. With acetylcholinesterase, the acetylcholine concentration varied from $6{\cdot}6 \times 10^{-4}$ to $1{\cdot}65 \times 10^{-4}$ M. The total reaction volume was 15 ml. At least two or more inhibitor concentrations were used and the data were plotted according to the method of Lineweaver and Burk,[5] and in some cases, as an independent check, according to the method of Dixon.[6] Where the method of Dixon was utilized, at least three different substrate concentrations were employed. Data on the inhibitor dissociation constants were calculated from the Lineweaver–Burk plots by the method described by Krupka.[7,8] The inhibitor dissociation constants were calculated from Dixon plots according to a method described by Webb.[9]

Analgesic measurements. A slight modification of the hot-plate method of Eddy and Leimbach[10] was used to determine the analgesic potency of the normeperidine con-

geners. Instead of the customary ethyl formate azeotrope, a ternary azeotrope consisting of chloroform–ethanol–water (92·5:4·0:3·5, v/v) was utilized to maintain the hot-plate at 55·5°. Drugs were given intravenously in a constant volume of 0·1 ml isotonic saline to Swiss-albino mice weighing between 18 and 21 g. A minimum of 13 animals was used for each dose level and five different dose levels were used to determine the ED_{50} of each of the normeperidines. Each mouse was utilized for only one dose of drug and the animals were not reused at a different dose level. Probit curves were constructed[11] and data analysis was programmed on the Wang 370 electronic calculator.

Hemolytic studies. Studies of the ability of the various normeperidine compounds to bring about the hemolysis of human erythrocytes were carried out by a modification of the method of Chen and Dallam.[12] Fresh human citrated whole blood was centrifuged for 10 min at 2500 *g* and the sedimented red cells were washed six times with 0·163 M sodium chloride. The washed red cells were finally resuspended in the same medium. A suspension of cells was prepared such that 1·5 ml of the suspension gave an optical density at 400 mμ of 1·20 after complete hemolysis. Complete hemolysis was obtained by adding 1·5 ml of distilled water to the packed red cells from 1·5 ml of the suspension. Thirty to 60 μl of an aqueous solution of the normeperidine compound as the hydrochloride salt was added to 1·5 ml of the red cell suspension in a 10-ml conical centrifuge tube. After a 5-min incubation period at 25°, the suspension was centrifuged for 5 min at 2300 *g* in a clinical centrifuge. The optical density of the supernatant fluid was then measured at 400 mμ. The extent of hemolysis caused by 30 or 60 μl of distilled water was determined and subtracted as a control value from that observed on the addition of the normeperidine solutions. The hemolytic activity of each congener was determined at two different concentrations.

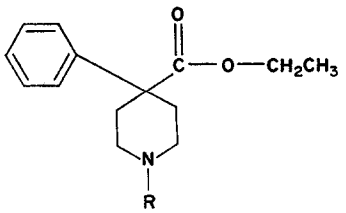
Determination of other physical parameters. The R_m value, as defined by Boyce and Milborrow,[13] is a simple constant derived from thin-layer chromatographic data relating the migratory properties of a compound on a thin-layer plate to its partition coefficient. The R_m values for the *N*-propyl through the *N*-decyl derivatives were calculated from the R_f values obtained on cellulose thin-layer plates with the ethanol–water solvent system (40:60, v/v). Plates were 250 μ thick and approximately 30 μg of sample in methanol was applied.

Molecular parachors were calculated using tables of atomic parachors.[14] For example, the molecular parachor for *N*-methyl normeperidine (meperidine) is calculated from the sum of the atomic parachors minus 19 cc for each covalent bond in the molecule [i.e. $C_{14}H_{21}O_2N$; 14 C(47·6 cc/C) + 21 H (24·7 cc/H) + 2 O(36·7 cc/O) + N(41·9 cc/N) − 40 covalent bonds (19 cc/bond) = 539·4 cc].

RESULTS

The alkyl substituted normeperidine compounds were studied as inhibitors of the hydrolysis of acetylcholine by both horse plasma butyrylcholinesterase and electric eel acetylcholinesterase. Data obtained from studies with butyrylcholinesterase, when plotted according to the method of Lineweaver and Burk,[5] demonstrated that all of the normeperidine derivatives from methyl through decyl function as mixed inhibitors with respect to acetylcholine in these reactions. The mixed inhibition seen with the pentyl derivative is shown in Fig. 1. The competitive (K_I) and noncompetitive (K_I')

TABLE 1. INHIBITION OF HORSE SERUM BUTYRYLCHOLINESTERASE BY *N*-ALKYL SUBSTITUTED NORMEPERIDINES



R	Inhibitor dissociation constants* (M)	
	Competitive (K_I)	Noncompetitive (K_I')
Methyl (meperidine)	$1{\cdot}45 \times 10^{-4}$	$1{\cdot}53 \times 10^{-3}$
Ethyl	$1{\cdot}14 \times 10^{-4}$	$1{\cdot}15 \times 10^{-3}$
Propyl	$7{\cdot}11 \times 10^{-5}$	$6{\cdot}95 \times 10^{-4}$
Butyl	$4{\cdot}91 \times 10^{-5}$	$4{\cdot}00 \times 10^{-4}$
Pentyl	$2{\cdot}86 \times 10^{-5}$	$2{\cdot}18 \times 10^{-4}$
Hexyl	$2{\cdot}29 \times 10^{-5}$	$1{\cdot}44 \times 10^{-4}$
Heptyl	$1{\cdot}25 \times 10^{-5}$	$1{\cdot}09 \times 10^{-4}$
Octyl	$9{\cdot}86 \times 10^{-6}$	$6{\cdot}96 \times 10^{-5}$
Nonyl	$5{\cdot}25 \times 10^{-6}$	$3{\cdot}38 \times 10^{-5}$
Decyl	$2{\cdot}76 \times 10^{-6}$	$2{\cdot}39 \times 10^{-5}$

* Average of duplicate determinations.

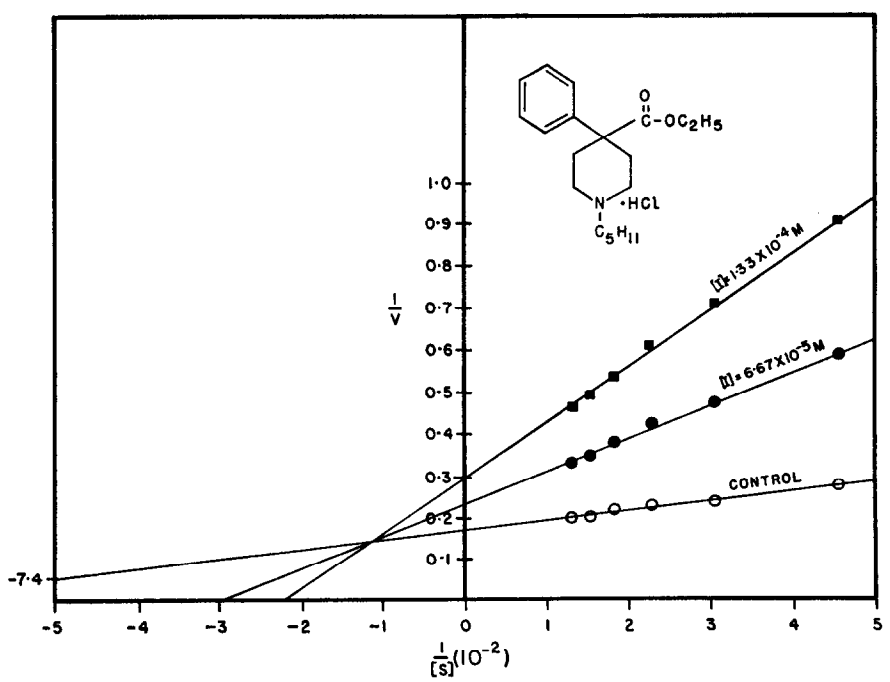


FIG. 1. Inhibition of butyrylcholinesterase by two different concentrations of the pentyl normeperidine derivative indicating mixed inhibition. The velocity is expressed on the vertical axis as micro-moles acetylcholine hydrolyzed per minute per milligram of protein. The acetylcholine concentration is expressed on the horizontal axis as the molar concentration. (The average K_m from 20 determinations was found to be $1{\cdot}37 \pm 0{\cdot}08$ S.D. $\times 10^{-3}$ M.)

inhibitor dissociation constants were determined for each of the normeperidine compounds and are given in Table 1. It is evident from these data that as the alkyl chain increases, the inhibitory properties of these compounds likewise increase. From this observation the logarithms of the reciprocal inhibitor constants K_I and K_I' were plotted as a function of the alkyl chain length (Fig. 2) and demonstrate the linear relationship observed in the binding of these compounds to the enzyme. In order to obtain a more quantitative estimate of this binding, the change in the free energy of binding per methylene group ($\Delta\Delta F$) may be calculated from the equation

$$\Delta\Delta F = 2{\cdot}303\ RT\ \Delta pK_I \tag{1}$$

where ΔpK_I (or $\Delta pK_I'$) is the change in the logarithm of $1/K_I$ per methylene group, R is the universal gas constant, and T is the temperature in degrees Kelvin.[15] From this relationship a $\Delta\Delta F$ value of 275 cal/mole is obtained from K_I and a value of 115 cal/mole from K_I'.

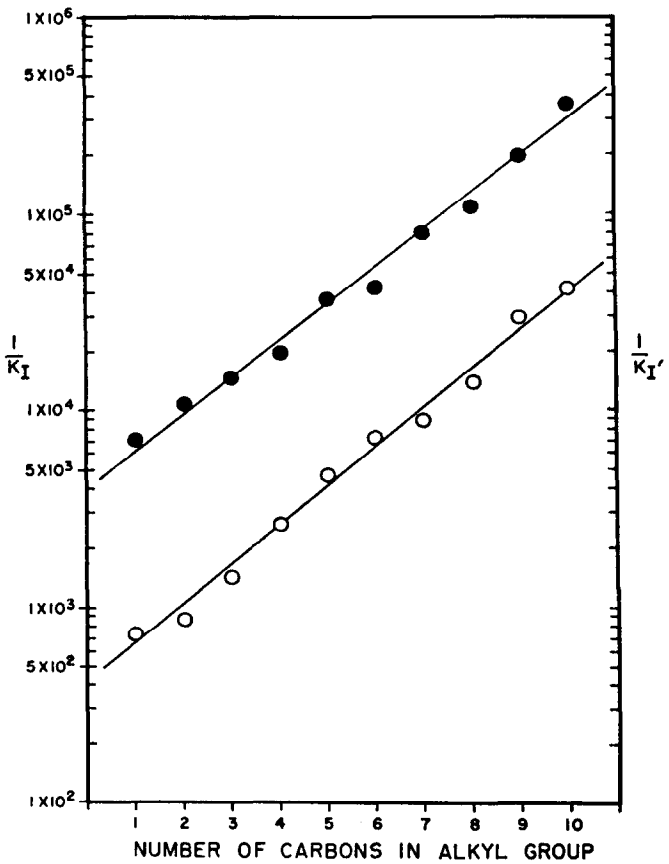


FIG. 2. Effect of increasing the inhibitor alkyl chain length on the competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants found with butyrylcholinesterase. The $1/K_I$ values are represented by ●——● and the $1/K_I'$ values are represented by ○——○.

TABLE 2. INHIBITION OF ELECTRIC EEL ACETYLCHOLINESTERASE BY *N*-ALKYL SUBSTITUTED NORMEPERIDINES

R	Inhibitor dissociation constants* (M)	
	Competitive (K_I)	Noncompetitive (K_I')
Methyl (meperidine)	$4{\cdot}35 \times 10^{-3}$	$1{\cdot}14 \times 10^{-2}$
Ethyl	$4{\cdot}28 \times 10^{-3}$	$9{\cdot}98 \times 10^{-3}$
Propyl	$2{\cdot}68 \times 10^{-3}$	$5{\cdot}55 \times 10^{-3}$
Butyl	$1{\cdot}73 \times 10^{-3}$	$2{\cdot}95 \times 10^{-3}$
Pentyl	$8{\cdot}83 \times 10^{-4}$	$1{\cdot}88 \times 10^{-3}$
Hexyl	none†	$3{\cdot}18 \times 10^{-4}$
Heptyl	none†	$2{\cdot}05 \times 10^{-4}$
Octyl	none†	$1{\cdot}28 \times 10^{-4}$
Nonyl	‡	
Decyl		

* Average of duplicate determinations.

† The disappearance of the competitive inhibition component with the hexyl and heptyl derivatives was confirmed by Dixon plots. From these plots, a K_I' of $4{\cdot}01 \times 10^{-4}$ M was found for the hexyl derivative and a K_I' of $1{\cdot}86 \times 10^{-4}$ M for the heptyl derivative.

‡ The nonyl and decyl compounds were not run, since the amount needed to produce a significant inhibition was not soluble in the standard reaction volume.

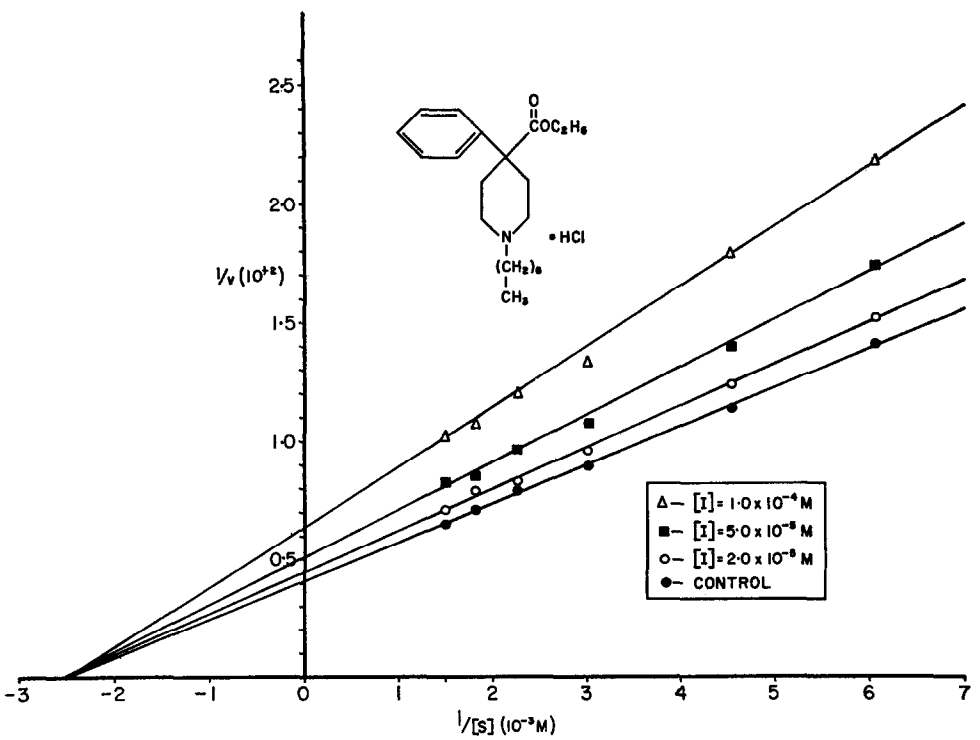


FIG. 3. Inhibition of acetylcholinesterase by three different concentrations of the heptyl normeperidine derivative indicating noncompetitive inhibition. The velocity is expressed as micromoles acetylcholine hydrolyzed per minute per milligram of protein. The substrate concentration is expressed as the molar concentration. (The average K_m for acetylcholinesterase under these conditions was $4{\cdot}33 \pm 0{\cdot}34$ S.D. $\times 10^{-4}$ M.)

The inhibitory effects of these compounds on acetylcholinesterase were also studied and the K_I and K_I' values were determined (Table 2). Figure 3 shows a double reciprocal plot for acetylcholinesterase with the heptyl substituted normeperidine congener as the inhibitor. These data indicate a complete noncompetitive mode of inhibition. A plot of log $1/K_I$ and $1/K_I'$ versus the number of carbons in the alkyl group (Fig. 4) for acetylcholinesterase clearly indicate a difference in the way the two enzyme systems interact with these inhibitors. As opposed to the simple linear relationship seen with butyrylcholinesterase (Fig. 2), acetylcholinesterase interacts with the normeperidines in a complex multiphasic manner. It should also be noted that the type

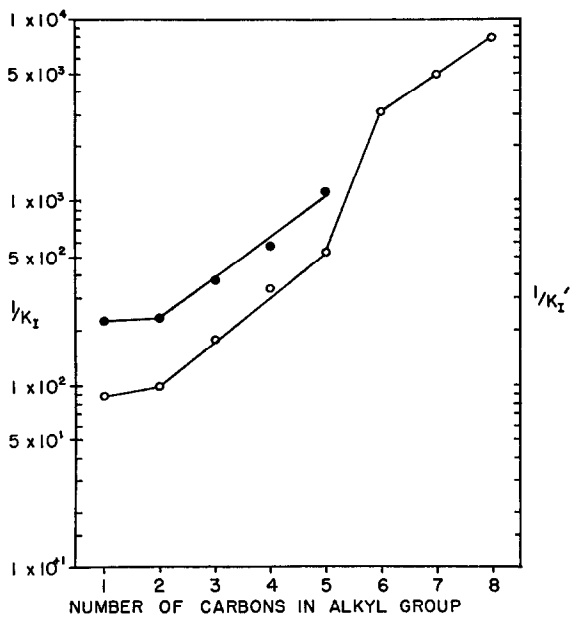


FIG. 4. Effect of increasing the inhibitor alkyl chain length on the competitive (K_I) and noncompetitive (K_I') inhibitor dissociation constants found with acetylcholinesterase. The $1/K_I$ values are represented by ●——● an the $1/K_I'$ values are represented by ○——○.

of inhibition changes at C_6 (Fig. 4) from mixed to noncompetitive. Since the substituted normeperidines were not as effective as inhibitors against acetylcholinesterase, the lack of solubility of the nonyl and decyl compounds precludes their use as inhibitors in this system. The calculations of $\Delta\Delta F$ per methylene group from the linear portions of Fig. 4 yield values of 334 cal/mole from K_I (from C_2 to C_5), 331 cal/mole from K_I' (C_2 to C_5) and 270 cal/mole from K_I' (C_6 to C_8).

In order to validate the unusual occurrence of a change in the type of inhibition from mixed (competitive plus noncompetitive) to a complete noncompetitive mode with the C_6 derivative, Dixon plots [9]were constructed for the hexyl and heptyl inhibitors. As an example, the plot for the hexyl derivative is reproduced in Fig. 5. These data confirm the conclusions drawn from the double reciprocal plots.

The analgesic activity of these compounds was also studied in mice using the hot-plate method. A graphical presentation of the ED_{50} values as a function of the number of methylene carbons in the "tail" of the normeperidine molecule is given in Fig. 6.

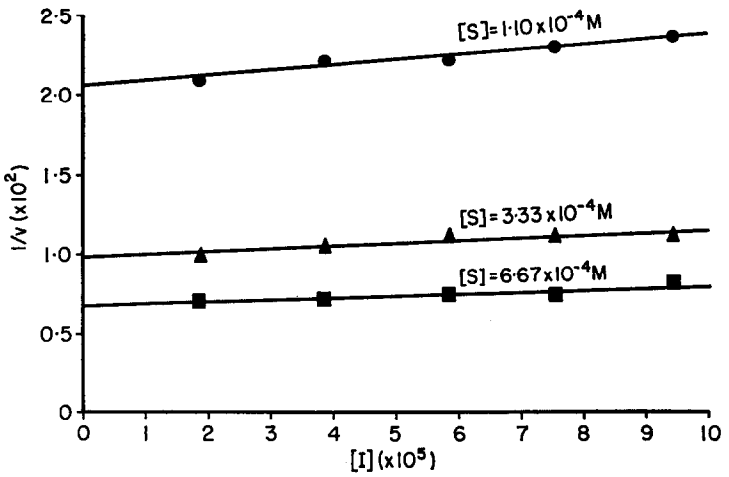


FIG. 5. Inhibition of acetylcholinesterase by the heptyl normeperidine derivative. Dixon plot with three different substrate concentrations. Molar inhibitor concentrations are indicated on the horizontal axis and enzyme velocity is expressed on the vertical axis as in Fig. 3.

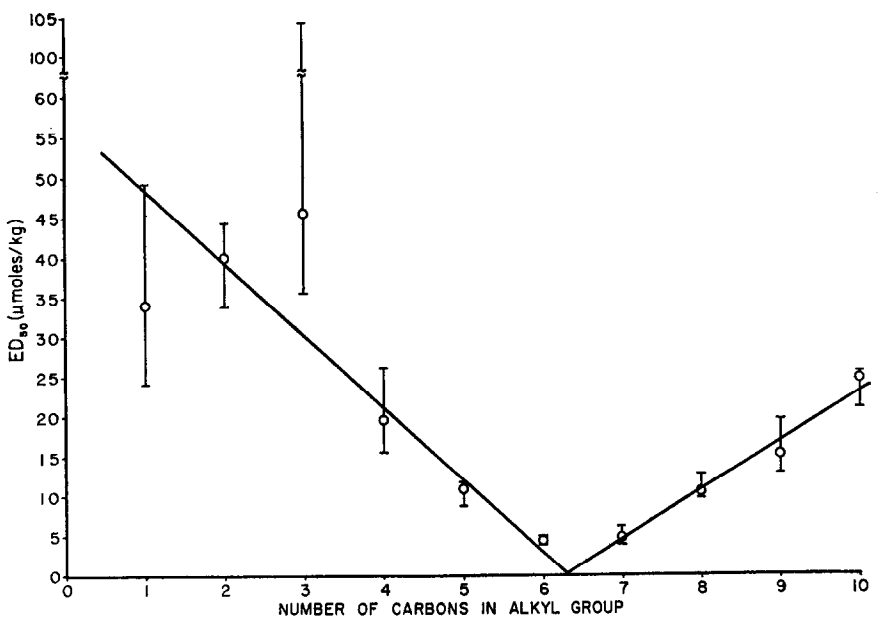


FIG. 6. The ED_{50} of the alkyl substituted normeperidine compounds as determined by the hot-plate method in micromoles per kilogram versus the number of carbons in the alkyl group. The vertical lines represent the 95 per cent confidence intervals calculated for each compound.

The vertical lines represent the 95 per cent confidence intervals. The slopes of both linear segments were determined by linear regression analysis and were significant at the $P = 0{\cdot}05$ level. These data indicate an overall decrease in ED_{50} as a function of chain length until the sixth or seventh carbon is reached. After this point, an increase in ED_{50} is apparent. Although the data on the methyl, ethyl and propyl compounds are somewhat variable, a definite trend can be seen.

Having obtained data from both the enzyme systems *in vitro* and the analgesic potency measurements *in vivo*, it was of interest to determine whether or not various chemical–physical parameters associated with the normeperidine compounds would aid in correlating their activity. In this regard, the hemolytic activity of each compound was determined on human erythrocytes in order to gain information on the order of cell membrane penetration as a function of alkyl chain length. This experiment indicated a gradual increase in permeability until the hexyl or heptyl derivative (Fig. 7)

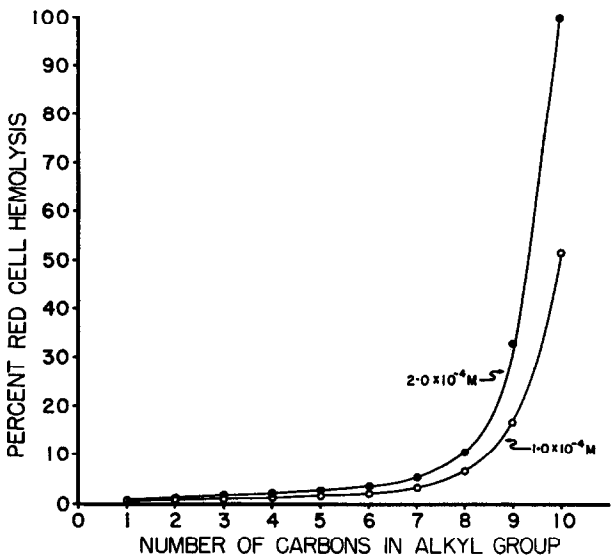


FIG. 7. Per cent red cell hemolysis versus the number of carbons in the alkyl chain of the normeperidine inhibitors. Curves were run at two different inhibitor concentrations as indicated.

was reached. At this point, a greater amount of hemolysis was seen with each increasing methylene carbon. This would seem to indicate an increase in the rate of molecular penetration. This concept is based on the assumption that the red cell hemolysis brought about by these compounds at the concentrations specified is a function of permeability and not some unknown mechanism based on surface activity. However, in the light of a recent review by Cornwell *et al.*[16] on the red cell as a model membrane system, the latter concept seems unlikely.

Since the permeability of cell membranes has been shown, at least in part, to depend on the partition coefficient of the penetrating molecule,[16] a determination of the partition coefficient of these compounds was in order. In lieu of the actual partition

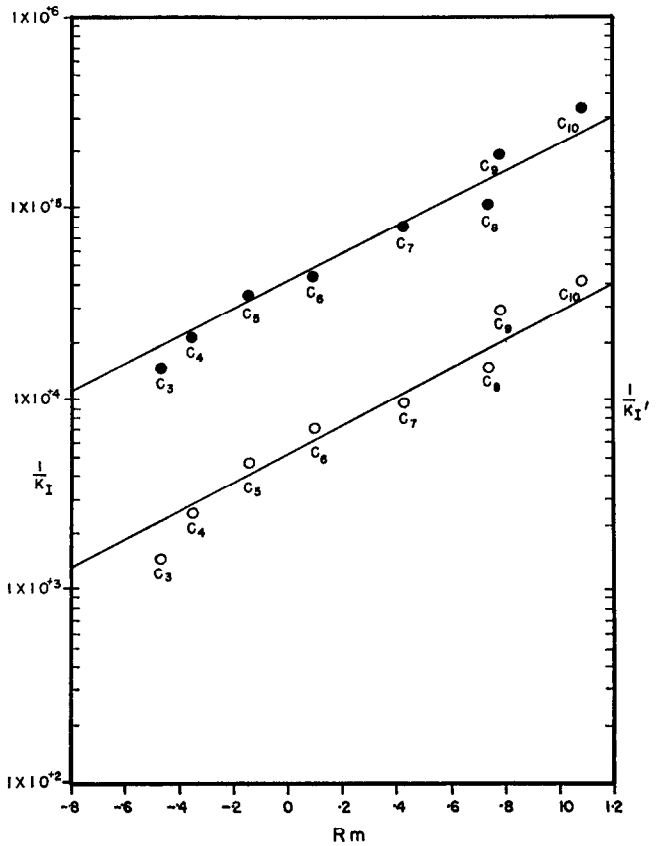


FIG. 8. Correlation between the inhibitor dissociation constants for butyrylcholinesterase and the R_m value determined from thin-layer chromatography. The number of carbons in the alkyl chain is indicated in the figure and the inhibitor constants are represented as in Fig. 4.

coefficient, the R_m value for each compound was determined by thin-layer chromatography. The R_m value is defined as being equal to log $(1/R_f - 1)$. The R_f value is obtained by dividing the distance the compound migrates by the distance the solvent front moves on the thin-layer plate. Boyce and Milborrow[13] have shown a direct relationship between the R_m value of a compound and its partition coefficient. Although we were not able to find a simple solvent system that would separate all ten of the normeperidine derivatives on the same plate, the ethanol–water system gave an excellent linear separation of the C_3 through C_{10} compounds.

The relationship between the R_m value and the inhibitor dissociation constants found with butyrylcholinesterase is indicated in Fig. 8. From these data it can be concluded that an increase in the lipophilic character of each compound is related in a linear manner to its ability to inhibit butyrylcholinesterase.

Molecular parachors were also calculated for these compounds from tables of atomic parachors[14] and the results of these calculations are shown in Fig. 9 as a function of the inhibitor dissociation constants. The inhibitor constants used are again for butyrylcholinesterase. Since the molecular parachor is in essence a direct reflection

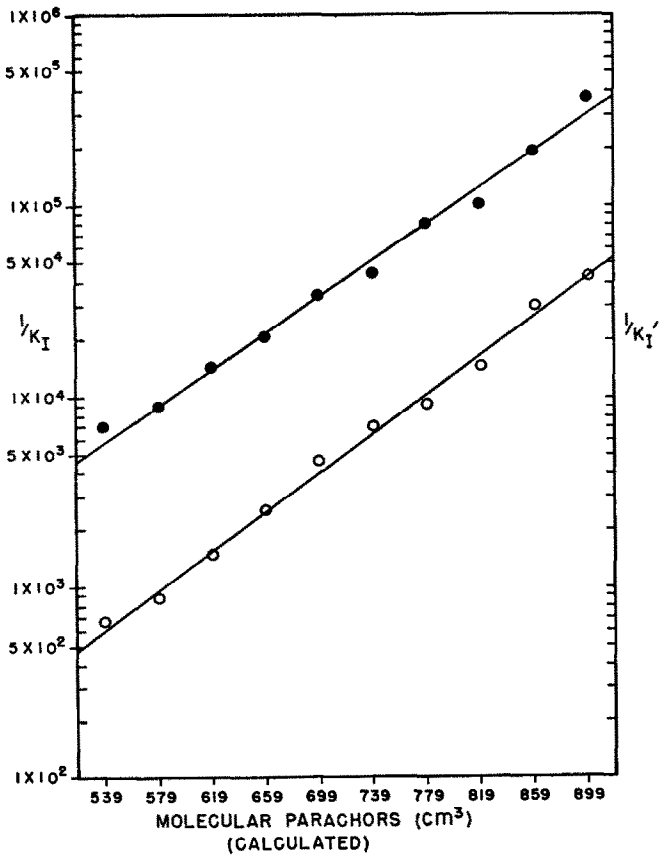


FIG. 9. Correlation between the inhibitor dissociation constants for butyrylcholinesterase and the calculated molecular parachors. Values of the competitive (K_I) and noncompetitive (K_I') dissociation constants are plotted as the logarithm of the reciprocals versus the calculated molecular parachors for each of the normeperidine derivatives. The $1/K_I$ values are represented by ●——● and $1/K_I'$ values are represented by ○——○.

of the molecular volume, it was of interest to determine the relationship between the degree of inhibition and the volume occupied by the inhibitor. It is evident from these results that in the case of butyrylcholinesterase a direct relationship between these two parameters exists.

DISCUSSION

The compounds chosen for this study are homologs of the analgesic meperidine. The pK_a of the piperidine nitrogen in meperidine (*N*-methylnormeperidine) as reported by Farmilo *et al.*[17] is about 8·72. Beckett[18] has shown that the addition of sequential methylene carbons to *N*-methylpiperidines does not alter the pK_a of these compounds to any appreciable degree. Therefore, the *N*-alkyl derivatives of normeperidine should all exist to the extent of about 95 per cent in their cationic form at pH 7·4. Since the pK_a values of these compounds are nearly constant, any change in their inhibitory properties toward either enzyme should therefore be related directly to a change in their relative lipophilic character.

A mixed type of inhibition is seen (Fig. 1) with the alkylnormeperidines, indicating that these inhibitors bind to butyrylcholinesterase and prevent the breakdown of the active enzyme–substrate complex. However, since mixed inhibition is observed, the normeperidines must also be interfering in a competitive manner with the binding of acetylcholine.

The decrease in K_I and K_I' seen with butyrylcholinesterase, which accompanies the increase in alkyl chain length of the inhibitor (Fig. 2 and Table 1), is indicative of a more favorable binding of inhibitor to enzyme. These results are in agreement with the findings of Beasley and Christian[19] in that an increase in the nonpolar nature of a series of *N*-alkyl substituted nipecotamide homologs also increased the ability of these compounds to act as inhibitors of butyrylcholinesterase.

Since the change in free energy calculated per methylene group with these inhibitors on butyrylcholinesterase ($\Delta\Delta F = 275$ cal/mole from K_I and $\Delta\Delta F = 115$ cal/mole from K_I') is somewhat lower than that proposed by Webb[20] for interactions through dispersion forces (i.e. 0·36 to 0·95 kcal/mole), a determination of the "closeness of fit" between enzyme and inhibitor should disclose whether or not the forces involved are of the Van der Waals type. These values were determined from the $\Delta\Delta F/CH_2$ values calculated for both K_I and K_I' by a method developed by Pauling and Pressman[21] from their work on hapten–antibody interactions. If the binding of inhibitor to enzyme is in the energy range of Van der Waals interactions, the following equation will describe this condition.

$$\Delta W = (4 \times 10^5)\,(R_A - R_H)/r^6 \qquad (2)$$

ΔW is equal to the change in Van der Waals energy and 4×10^5 is a constant for the aqueous system. A change in ΔW is brought about by the substitution of any group A, for hydrogen. In the equation, R_A and R_H are the molar refractions of group A and hydrogen, and r is equal to the distance between like atoms on inhibitor and enzyme. Substituting the free energy of binding per methylene group for ΔW, the molar refraction of a methyl group, 5·71 cm^3, [22] for R_A and the molar refraction of hydrogen, 1·10 cm^3,[22] for R_H, a distance of 4·3 Å is obtained from K_I and a distance of 5·0 Å is obtained from K_I'. These values are very close to the Van der Waals distance that would be expected from a carbon–carbon association, since the Van der Waals radius of carbon is 2·0 Å.[23] These values are in agreement with those determined recently by Purcell and Beasley[24] with a different series of inhibitors on this enzyme.

Based on the preceding findings, indications were that any parameter based on a simple, linear, free energy relationship would correlate with the inhibitor dissociation constants determined from the butyrylcholinesterase kinetic data. In order to further illustrate this premise, the R_m values for the inhibitors were determined and plotted as a function of the inhibitor dissociation constants found with butyrylcholinesterase (Fig. 8). Boyce and Milborrow[13] have shown that the R_m value determined from thin-layer chromatographic R_f data is directly related to the partition coefficient of the compounds. Consequently, a change in the value of R_m for a substituent $(\Delta R_m)/CH_2$ is a free energy based constant. The value of this constant for the alkyl substituted normeperidines is approximately $0{\cdot}29/CH_2$ when determined in the ethanol–water solvent system on cellulose thin-layer plates.

Molecular parachors were also calculated for the normeperidines. The results of these calculations, when plotted as a function of the inhibitor constants (Fig. 9), again

indicate a simple linear relationship derived from the molecular volume of the inhibitors. These findings coupled with the kinetic data suggest the existence of a distinct nonpolar region in the immediate vicinity of the substrate binding site in butyrylcholinesterase.

The interaction of these inhibitors with acetylcholinesterase was found to be of a more complex nature than their interaction with butyrylcholinesterase. However, a calculation of the interaction distance between a carbon atom in the inhibitors and a like atom on the enzyme still gave values indicative of Van der Waals interactions. For example, a distance of 4·2 Å is calculated using both K_I and K_I' from C_2 and C_5, and the distance from K_I calculated from the $\Delta\Delta F$ value for C_6 to C_8 is 4·35 Å. From the kinetics of acetylcholinesterase inhibition when plotted as a function of chain length (Fig. 4), it is evident that there is a change in the type of inhibition at the C_6 derivative; this suggests a limited capacity for the enzyme to bind the inhibitor at the substrate binding site when a critical chain length of about six methylene carbons is reached. This phenomenon might occur through one of two mechanisms. If the hexyl derivative is of sufficient size, it may be excluded from the acetylcholine binding site and may therefore bind at some peripheral hydrophobic site. On the other hand, the binding of the C_6 derivative (and longer chain derivatives) could induce a specific conformational change in the enzyme structure. This change in structure might then account for a change in the mode of inhibition. The idea of conformational changes when substrate or inhibitors bind to enzymes has been proposed by Koshland[25] and applied to cholinesterases by Hein and Powell.[26] These two mechanisms, however, need not be mutually exclusive.

The comparative kinetic data from both enzyme systems seem particularly significant in the light of data presented by Folds *et al.*[27] These investigators determined that the distance between the "anionic" and "esteratic" sites in both butyryl- and acetylcholinesterase are approximately equal, with the value being about 5·0 Å. Therefore, an optimal inhibitor might be expected to incorporate a like distance between its esteratic and cationic moieties. The distances between the piperidine nitrogen and the carbonyl carbon in the normeperidine inhibitors was measured as 4·2 Å with Dreiding stereo models. Measurements were made with the piperidine ring in the chair conformation and with the carbonyl group in the equatorial position.

All the data thus far presented support the concept that a definitive and important difference exists with regard to the relative lipophilic character of the environment of the active sites of these two enzymes. Augustinsson[28] suggested that the differences in catalytic activity between acetylcholinesterase and butyrylcholinesterase could be explained by the presence of a second "nonesteratic" site in butyrylcholinesterase. It was suggested that the dominant type of force involved in the binding of compounds (substrate or inhibitors) to this site was of the Van der Waals class as opposed to the coulombic forces involved at the "anionic" site of acetylcholinesterase. Our investigations do not disprove this hypothesis but do, in fact, suggest that many of the differences seen in the binding of compounds to the two enzymes may be explained entirely on the basis of differences in the geometry of the nonpolar regions, which in part define the catalytic sites of both enzymes.

Since the differential activity of the *N*-alkyl substituted normeperidines has been compared on both butyryl- and acetylcholinesterase, an investigation of whether or not the activity of these compounds at the enzyme level might in some manner correlate

with their activity as analgesics at the analgesic receptor level seemed advisable. Although this idea is perhaps not new,[29] it was felt that an approach to the problem through a differential analysis of both enzyme systems and the analgesic receptor system might offer new evidence. In fact, in a recent study by Waser[30] the existence of at least two types of cholinesterases at the endplates of mouse diaphragm was confirmed by autoradiographic techniques. Along these same lines, Martin[31] recently suggested that there may be at least two distinct analgesic receptors. Therefore, a study of both types of cholinesterases and a subsequent comparison of these data to the analgesic data may prove useful in further understanding the nature of the analgesic receptors. In order to obtain data for this comparison, the analgesic properties of each of the normeperidines were determined by the "hot-plate" method.[10] The results of these determinations are shown in Fig. 6 as a plot of ED_{50} (μmoles/kg) versus the number of carbons in the alkyl chain of the substituted normeperidines. When the analgesic data are analyzed as a function of alkyl chain length, a general trend toward greater analgesic potency is noted with increasing chain length until six or seven methylene carbons are reached. After this point, a decrease in the analgesic potency is seen with a further extension of the alkyl chain up through the decyl derivative. Portoghese[32] in analyzing the structure–activity relationships of analgesics has concluded that an analgesiophore binds to its receptor through hydrophobic bonds formed at nonpolar sites on the receptor and through coulombic interactions at an "anionic" site. In the case of the normeperidines, it should again be emphasized that any binding due to the "head" (i.e. phenylpiperidine ring system) of the molecule should remain constant and changes in binding energies should reflect increased nonpolar interactions brought about by the alkyl chain. Therefore, excluding any interfering events, an increase in the relative lipophilic character of an analgesiophore should be accompanied by a concomitant increase in the analgesic potency of the molecule. The data presented in Fig. 6 are in agreement with this premise up through the hexyl derivative. The decrease in analgesic potency with the longer alkyl chain lengths from hexyl to decyl, however, may be brought about by several factors. For example, the decrease in potency may represent a decrease in the binding constant of the drug–receptor complex due to some spatial limitations inherent in the receptor itself or to a decrease in the concentration of the drug at the receptor site. A decreased concentration at the receptor could in turn be caused by an increase in the binding of drug to so-called "silent receptors" or by other mechanisms dealing with the transport or metabolism (or both) of the drug. However, if the hemolytic activity of these compounds (Fig. 7) represents an accurate measure of their ability to diffuse through membranes and if, indeed, the R_m data (Fig. 8) also represent a measure of this characteristic, then one might expect the hexyl through decyl compounds to retain their ability to reach the receptor. If this is the case, then the idea that the analgesic receptor has definitive structural limitations is again borne out.

The unusually high variability in the analgesic activity of the propyl substituted compound is difficult to explain. This variability was also noted in an earlier review on meperidine-like analgesics by Janssen and Van der Eycken.[33] At present, we have no plausible explanation for this phenomenon.

In comparing the analgesic potencies of these compounds with their enzyme inhibitory properties, it is interesting to note that no break in log $1/K_I$, $1/K_I'$ versus alkyl chain length curve occurs with butyrylcholinesterase (Fig. 2), while with ace-

tylcholinesterase (Fig. 4) a break in the log $1/K_I$, $1/K_I'$ versus alkyl chain length curve is seen between the hexyl and heptyl derivatives with a concurrent abolition of the competitive inhibition component. Therefore, the inhibition curve derived from the acetylcholinesterase kinetic data resembles the ED_{50} versus carbon chain length curve (Fig. 6) generated from the analgesic data *in vivo* to within one methylene carbon unit. If a further examination is made of the relative ability of these compounds to penetrate cell membranes as a function of the number of carbon atoms in the alkyl group of the normeperidine molecules, a change in the amount of hemolysis per methylene carbon unit is also noted at about seven carbon units (Fig. 7). These facts when taken together may indicate a similarity in the mechanism of action of these compounds both at the enzyme level and at the receptor level due to a similarity in the physical–chemical make up of the enzyme and the analgesic receptor.

Therefore, in terms of drug–receptor interactions, a study of the kinetic inhibitory characteristics of analgesiophores on acetylcholinesterase may prove to be a convenient model system for the determination of the relative importance of various nonpolar molecular modifications. The importance of nonpolar or Van der Waals interaction has been systematically evaluated during the course of this investigation at both receptor and enzyme levels. It is, in fact, quite remarkable that a very small change in binding energy brought about by an increase in only one methylene carbon can in turn bring about an appreciable rise in the analgesic potency of the normeperidines. For example, in going from the butyl to the pentyl derivative, only about one-half as much drug is required to produce the same analgesic effect (Fig. 6). In terms of the change in binding energy found with either acetyl- or butyrylcholinesterase, this represents an average change in energy of only 0·3 kcal/mole as calculated from $\Delta\Delta F$ values.

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REFERENCES

1. D. Nachmansohn, *Biochim. biophys. Acta* **26**, 1 (1957).
2. H. Busch, P. V. Nair, M. Frank and W. R. Martin, *J. Pharmac. exp. Ther.* **123**, 48 (1957).
3. E. Stahl, *Thin-Layer Chromatography*, p. 493. Academic Press, New York (1965).
4. B. R. Stein and K. J. Laidler, *Can. J. Chem.* **37**, 1272 (1959).
5. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).
6. M. Dixon, *Biochem. J.* **55**, 170 (1953).
7. R. M. Krupka, *Biochemistry, N.Y.* **3**, 1749 (1964).
8. R. M. Krupka, *Biochemistry, N.Y.* **5**, 1983 (1966).
9. J. L. Webb, *Enzymes and Metabolic Inhibitors* Vol. 1, p. 162. Academic Press, New York (1963).
10. N. B. Eddy and D. Leimbach, *J. Pharmac. exp. Ther.* **197**, 385 (1953).
11. N. Goldstein, *Biostatistics, An Introductory Text*, p. 49. Macmillan, San Diego (1964).
12. L. H. Chen and R. D. Dallam, *Archs Biochem. Biophys.* **111**, 104 (1965).
13. C. B. C. Boyce and B. V. Milborrow, *Nature, Lond.* **208**, 537 (1965).
14. J. D. McGowan, *Chemy Ind.* **495**, May 31 (1952).
15. B. M. Anderson, M. L. Reynolds and C. D. Anderson, *Biochim. biophys. Acta* **99**, 53 (1965).
16. D. G. Cornwell, R. E. Heikkila, R. S. Bar and G. L. Biago, *J. Am. Oil Chem. Soc.* **45**, 5 (1968).
17. C. G. Farmilo, P. M. Oestreicher and L. Levi, *Bull. Narcot.* **6**, 7 (1954).
18. A. H. Beckett, *J. Pharm. Pharmac.* **8**, 848 (1956).

19. J. G. BEASLEY and S. T. CHRISTIAN, *Nineteenth Southeastern Regional Meeting of the American Chemical Society*, Atlanta, Ga. (November 1967).
20. J. L. WEBB, *Enzymes and Metabolic Inhibitors*, Vol. 1, pp. 58–300. Academic Press, New York (1963).
21. L. PAULING and D. PRESSMAN, *J. Am. chem. Soc.* **67**, 1003 (1945).
22. C. D. SMYTH, *Dielectric Behavior and Structure*, p. 409. McGraw-Hill, New York (1955).
23. R. C. WEAST (Ed.), *Handbook of Chemistry and Physics*, 49th edn, p. D-107. The Chemical Rubber Co., Cleveland (1968).
24. W. P. PURCELL and J. G. BEASLEY, *Molec. Pharmac.* **4**, 404 (1968).
25. D. E. KOSHLAND, *Proc. natn. Acad. Sci. U.S.A.* **44**, 98 (1959).
26. G. E. HEIN and K. POWELL, *Biochem. Pharmac.* **16**, 567 (1967).
27. F. F. FOLDS, G. VAN HEES, D. L. DAVIS and S. P. SHANOR, *J. Pharmac. exp. Ther.* **122**, 457 (1958).
28. K-B. AUGUSTINSSON, *Biochim. biophys. Acta* **128**, 351 (1966).
29. F. BERNHEIM and M. L. C. BERNHEIM, *J. Pharmac. exp. Ther.* **57**, 427 (1936).
30. P. G. WASER, *Progress in Drug Research*, Vol. 3, p. 81. Academic Press, New York (1966).
31. W. R. MARTIN, *Pharmac. Rev.* **19**, 464 (1967).
32. P. S. PORTOGHESE, *J. med. Chem.* **8**, 609 (1965).
33. A. J. JANSSEN and C. A. M. VAN DER EYCKEN, in *Drugs Affecting the Central Nervous System*, Vol. II, pp. 25–44. Marcel Dekker, New York (1968).