The specific rotation of reticuline has been found to vary according to its origin between −55° and +132° and this is probably due to different rates of consumption of the two enantiomorphs in the biosynthetic pathways of the various species. Comparison with the value (+132°) found by Kusuda (8) for (+)-reticuline indicates that reticuline from *P. boldus* contains, at most, 10% (−)-reticuline.

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Keyphrases

Alkaloids-*Peumus boldus*
(+)-Reticuline—isolation
Isoboldine—isolation
Countercurrent distribution
Column chromatography—separation
TLC—analysis
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectrometry

Michaelis Constants for Isolated Cholinesterase Systems

By SAMUEL T. CHRISTIAN* and JAMES G. BEASLEY

The enzymatic hydrolysis of acetylcholine chloride by human plasma cholinesterase has been studied in detail. Results suggest that at substrate concentrations below 0.01 *M*, Michaelis-Menten kinetics are followed and enzyme hydrolysis rates are due to a single enzyme component. At higher substrate concentrations, a second enzyme component appears to contribute significantly to the total velocity of the reaction and a Lineweaver-Burk plot yields a hyperbolic-type curve. Apparent K_m values were calculated for the two components. The autohydrolysis rate for acetylcholine chloride at 26° was determined.

EVALUATION OF accurate Michaelis constants (K_m) has long been considered by the enzymologist to be practical only with highly purified isolated enzyme preparations. Nevertheless, enzyme kinetic constants from preparations of varying degrees of purity by investigators with divergent disciplinary backgrounds has led to the publication of a wide range of K_m values for most enzymes. This is particularly true in the case of human plasma (pseudo) cholinesterase (acylcholine acylhydrolase; E.C. 3.1.1.8) where many *apparent* K_m values (1–7) have been reported for the hydrolysis of butyrylcholine and benzoylcholine although comparatively little attention has been given to the hydrolysis of acetylcholine halides by the same enzyme. Acetylcholine is generally the substrate utilized in cholinesterase inhibitor evaluation.

In considering the variety of enzyme preparations (*e.g.*, commercial preparations,[1] Harvard fractions, IV-6-3 through IV-6-4 (8), Kabi fraction IV-6-3 (9), and completely unfractionated human serum (10), used for kinetic and inhibitor studies, it became of interest to ascertain the validity of expressing the Michaelis constants obtained with these preparations over wide ranges of substrate concentrations as valid, reproducible expressions of the true Michaelis constant.

METHOD

Acetylcholine chloride (Sigma Chemical Co.) was used as substrate in these studies; 17 different substrate concentrations covering a range from $3.00 \times 10^{-3}M$ to $1.50 \times 10^{-1}M$ were utilized. Enzyme initial velocity measurements were determined at 26.0° (±0.05°) with a continuous titration method (11) in a 0.15 *M* sodium chloride solution

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[1] Sigma Chemical Co., Worthington Biochemical Corp., Cutter Laboratories, Inc., and A. B. Kabi (Stockholm, Sweden), among others.

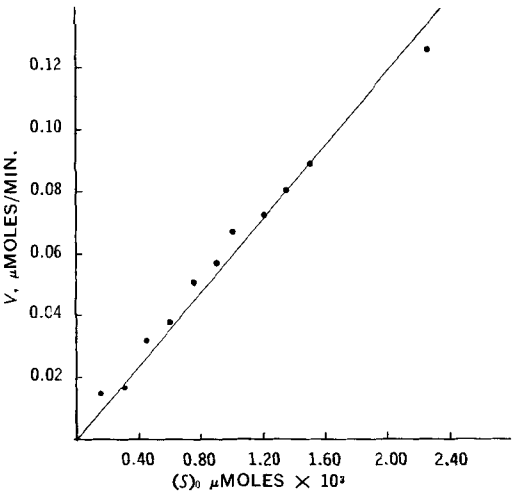

Fig. 1—Autohydrolysis of acetylcholine chloride at 26°. Key: S_0, abscissa, represents the initial micromolar substrate concentration; V, the ordinate, represents the reaction rate in μmoles/min. determined directly from titration curves for each substrate concentration; k = 5.59 × 10^{-5} min.$^{-1}$.

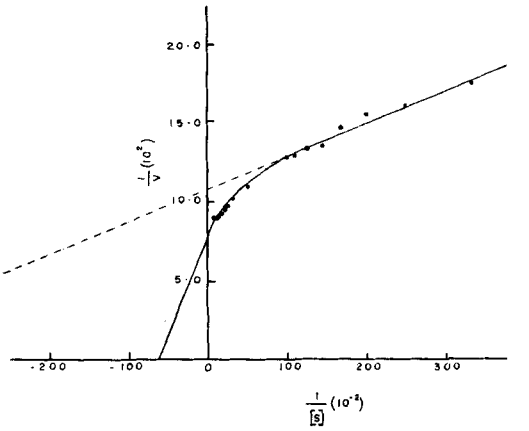

Fig. 2—Hydrolysis of acetylcholine chloride by human plasma cholinesterase at 26°, and pH 7.40 ± 0.05. Reactions were initiated by addition of 0.1 mg. of enzyme. Key: abscissa, reciprocal molar acetylcholine chloride concentration; ordinate, reciprocal enzyme initial velocity expressed in μmoles/min. per mg. of protein.

which was also 0.04 *M* in magnesium chloride. The total reaction volume was 15.0 ml. with each sample containing 0.1 mg. of enzyme (Type II Pseudo cholinesterase from human plasma; E.C. 3.1.1.8, Sigma Chemical Co.). The autohydrolysis of acetylcholine chloride was evaluated under the same conditions used for the enzyme reaction.

RESULTS AND DISCUSSION

The apparent first-order rate constant for the autohydrolysis of acetylcholine chloride as determined from Fig. 1 is 5.59 × 10^{-5} min.$^{-1}$. The effect of autohydrolysis on enzymatic initial velocities is negligible below substrate concentrations of 0.01 *M*; the data have been corrected for autohydrolysis effects at substrate concentrations above 0.01 *M*.

Walter (12), Dixon and Webb (13), and Reiner (14) have discussed the interesting case in which a reaction mixture containing two similar enzymes with different reaction rate constants is allowed to act on a single substrate; theoretically, if $K_{m1} \neq K_{m2}$, a Lineweaver-Burk (15) plot of such a reaction would show a hyperbolic relationship rather than the usual linear one. Reiner (14) has shown, by further theoretical treatment, that in order to distinguish between the two major enzyme components of such a system, the slopes of the two branches of the hyperbola must differ by 50% or more.

The authors have succeeded in demonstrating experimentally with human plasma cholinesterase[2] an actual example of a mixed enzyme system which adheres to this theoretical treatment.

A Lineweaver-Burk transformation constructed from our data for substrate concentrations ranging from 0.15 *M* to 0.003 *M* is presented in Fig. 2 and illustrates the hyperbolic-like relationship.

The results were not unexpected since Hess *et al.* (16) have previously demonstrated, by starch gel electrophoresis studies, the existence of six active butyrylcholinesterase fractions in human serum and Svensmark (17) has observed five fractions using DEAE-cellulose and DEAE-sephadex chromatography. However, from these studies it appears that in the enzyme preparations used, only two components were sufficiently active or present in sufficient concentration to contribute to the observed reaction rates.

A close look at the curve depicted in Fig. 2 shows that bending of the curve occurs at substrate concentrations greater than 0.01 *M*. Obviously, this bend is not caused by inhibition due to high ionic strength (18) but represents accelerated hydrolysis of substrate by a minor, kinetically distinct enzyme component.[3]

For substrate concentrations below 0.01 *M* it is apparent that this arm of the hyperbola is linear, as suggested by Walter (12) and is most probably due to a single, kinetically distinct major enzyme component. The K_m value determined for this component is 2.0 × 10^{-3} *M* (Fig. 2). This value is in good agreement with those previously reported using acetylcholine in low concentrations (<0.01 *M*) as a substrate (4, 20).

The wide range of K_m values previously reported may be due to the failure of some investigators to recognize the hyperbolic nature of this curve and to the inclusion of some high substrate concentration values in their K_m evaluation plots.

The relative importance of these findings should have special significance for investigators using plasma cholinesterase preparations to evaluate kinetically the effectiveness of various cholinesterase inhibitors.

From a practical standpoint, it appears safe to use acetylcholine in concentrations less than 0.01 *M* for evaluating enzyme-inhibitor kinetic constants[4] for

[2] Similar hyperbolic type curves were obtained from human plasma cholinesterase preparations supplied by Sigma Chemical Co. and by Cutter Laboratories, Inc. Berkeley, California.

[3] Humiston and Wright (19) have reported a similar break in the curve when acetylthiocholine iodide was used as a substrate for human plasma pseudocholinesterase.

[4] Dixon and Webb (13), in their treatment of a theoretical two enzyme system, suggest the practicability of this approach.

plasma cholinesterase systems. At these concentrations, the enzyme-substrate reaction follows classical Michaelis-Menten kinetics, suggesting a single enzyme–substrate interaction.[5] Although the apparent K_m value obtained from such plots appears reliable and reproducible, it does not necessarily represent a true K_m value but rather one intermediate between those for the two enzyme components present.

A complete kinetic analysis of pseudo (butyryl) cholinesterases must await separation and purification of each of the active enzyme species.

[5] The above suggestion does not apply to any rigorous kinetic treatment but is offered only as a guide for determining the relative potencies of cholinesterase inhibitors. Although unlikely, it is possible that specific inhibitors may have abnormally high affinities for minor enzyme components in impure cholinesterase preparations. Such a possibility cannot be ruled out completely on the basis of the available data.

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Keyphrases

Cholinesterase systems—human plasma
Michaelis constants—acetylcholine Cl hydrolysis
Acetylcholine Cl—autohydrolysis
Hydrolysis—cholinesterase fractions

Correlation of Ratios of Drug Metabolism by Microsomal Subfractions with Partition Coefficients

By ERIC J. LIEN and CORWIN HANSCH

A strong correlation between ratios of drug metabolism by microsomal subfractions with 1-octanol/water partition coefficient was found. Explanation for the spread of ratios is presented.

A VARIETY OF drug-metabolizing enzymes appear to be distributed between rough and smooth particles derived from the endoplasmic reticulum of liver cells (1–6). Using density gradient centrifugation, Fouts' group has separated the smooth-surfaced particles from the denser, rough-surfaced particles and then studied the metabolism of various drugs by the two types of particles. The rates of metabolism for each type of particle were obtained in terms of micromoles of drug metabolized per hr. per mg. of microsomal nitrogen. The ratios of enzymic activity in the two types of particles differed by almost 10-fold (depending on the drug). Fouts interpreted the different ratios as indicating different concentrations (activities) of enzymes in the two types of tissues. The authors wish to show that an alternative explanation can be advanced from a consideration of the physical properties of the substrates.

Equation 1 was derived *via* the method of least squares from the data of Fouts (2) and Gram *et al.* (6) in Table I. These data were obtained using Rothschild's method of preparation of microsome fractions. In Eq. 1, $R_{(s/r)}$ stands for the ratio of enzyme activity in the two types of particles (smooth/rough) and P is the partition coefficient of the drug in 1-octanol/water (7, 8), n is the number of data points used in deriving the equation, r is the correlation coefficient, s is the standard deviation from regression, and the figures in parentheses are the 90% confidence intervals. Addition of a term in $(\log P)^2$ to Eq. 1 does not result in an improved correlation (9). Three of the $\log P$ values in Table I were calculated (7, 10, 11). The value for benzpyrene was based on $\log P = 3.37$ for naphthalene, codeine was based on 0.76 for morphine, and amphetamine was based on 1.41 for 2-phenylethylamine.

$$\log R_{(r/s)} = \underset{(\pm 0.035)}{-0.101} \log P + \underset{(\pm 0.108)}{0.859}$$

n	r	s	
10	0.885	0.116	(Eq. 1)

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