# 9-HYDRAZINO-1,2,3,4-TETRAHYDROACRIDINE, A COMPETITIVE INHIBITOR OF BUTYRYLCHOLINESTERASE

J.PATOČKA, J.BAJGAR and J.FUSEK

*J. E. Purkyně Medical Research Institute, 502 60 Hradec Králové*

Received August 10th, 1976

The kinetics of inhibition of horse plasma butyrylcholinesterase by 9-hydrazino-1,2,3,4-tetrahydroacridine was investigated *in vitro*. The compound was found to behave as a competitive reversible inhibitor of butyrylcholinesterase with a $K_i$ of 0·08 μM and to be equally powerful as 9-amino-1,2,3,4-tetrahydroacridine. Both inhibitors are bound to the same active site of butyrylcholinesterase but the stability of the binding is different.

9-Hydrazino-1,2,3,4-tetrahydroacridine was first prepared by Bielavský[1]. It resembles structurally 9-amino-1,2,3,4-tetrahydroacridine, a compound with a potent anticholinesterase activity[2-4] and it was hence assumed that the compound might behave as an inhibitor of cholinesterases. The present communication deals with the inhibition kinetics of horse plasma butyrylcholinesterase (acylcholine-acylhydrolase E.C. 3.1.1.8) by 9-hydrazino-1,2,3,4-tetrahydroacridine *in vitro*.

## EXPERIMENTAL

*Reagents.* 9-Hydrazino-1,2,3,4-tetrahydroacridine hydrochloride was prepared here[1] as a slightly yellowish crystalline substance melting at 260–261°C (decomp.). The compound was characterized on the basis of elementary analysis and of UV, IR and fluorescence spectra. 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride was prepared as described before[1,4]. Butyrylcholine iodide was a product of Lachema, Brno, just as all other common reagents of analytical purity.

*Enzyme.* The source of butyrylcholinesterase was a purified preparation of the enzyme from horse serum prepared according to Strelitz[5]. A freeze dried preparation had a specific activity of 6·6 μmol hydrolyzed butyrylcholine $min^{-1}$ $mg^{-1}$ (110 nkat) at 25°C and pH 8·0.

The activity of butyrylcholinesterase was estimated by titration[6] as the initial rate of hydrolysis of butyrylcholine ($v$) in a Radiometer pH-stat at 25°C and pH 8·0. Titration was done with 0·05M-NaOH.

Inhibition experiments were done under the same conditions of pH and temperature in 150 mM-NaCl. The inhibition was analyzed by graphical methods, the curves were fitted by regression analysis on a Hewlett–Packard 9830A computer.

## RESULTS

When butyrylcholinesterase was incubated with 9-hydrazino-1,2,3,4-tetrahydroacridine the inhibition was not progressive, *i.e.* the degree of enzyme inhibition did not

depend on the duration of its incubation with the inhibitor. The dependence of the percentage of inhibition on inhibitor concentration showed that the value of $I_{50}$, *i.e.* concentration of inhibitor resulting in a 50% decrease of enzyme activity, depends on the substrate concentration used. It amounts to 0·14 μM for 2 mM butyrylcholine and to 0·30 μM for 10 mM butyrylcholine. However, the shape of the inhibition curves does not depend on substrate concentration and the Hill coefficient[7] calculated from the slope of the dependence of $\log \{v_i/(v_0 - v_i)\}$ on $\log [I]$ where $v_0$ is the rate of the enzyme reaction without and $v_i$ the rate with inhibitor, is equal to 1 for both concentrations of butyrylcholine.

As indicated by the dependence of reciprocal fractional inhibition $(1/i)$ $(i = 1 - (v_i/v_0))$ on reciprocal values of concentration of 9-hydrazino-1,2,3,4-tetrahydroacridine $(1/[I])$ (Fig. 1) the inhibition of butyrylcholinesterase by this inhibitor is full since both straight lines, the slope of which depends only on the inhibitor concentration used, intersect the $1/i$ axis at the value of 1. At the same time, it is

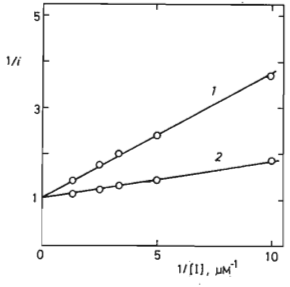


FIG. 1

Inhibition of Butyrylcholinesterase by 9--Hydrazino-1,2,3,4-tetrahydroacridine Plotted according to Webb[8]

Dependence of reciprocal values of fractional inhibition ($i$) on reciprocal concentration of inhibitor ([I]). Enzyme activity was measured at 25°C and pH 8·0 with butyrylcholine as substrate, either 10 mM *1* or 2 mM *2*. Competitive type of inhibition yielding a $K_i$ of 0·075 μM (for 10 mM substrate) or 0·054 μM (for 2 mM substrate).

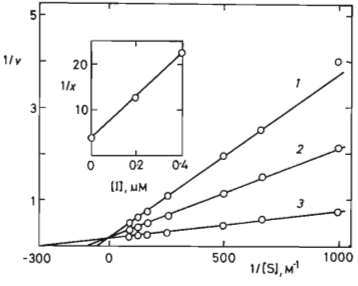


FIG. 2

Inhibition of Butyrylcholinesterase by 9--Hydrazino-1,2,3,4-tetrahydroacridine Plotted according to Lineweaver and Burk[9]

Dependence of reciprocal rate of hydrolysis of butyrylcholine ($v$) on its reciprocal concentration ([S]). Enzyme activity was measured at 25°C and pH 8·0. Inhibitor concentration: *1* 0·4 μM, *2* 0·2 μM, *3* no inhibitor. The inset shows the dependence of reciprocal intercepts of the straight lines with the $1/[S]$ axis ($x$) on the inhibitor concentration used ([I]). A purely competitive inhibition with $K_i$ of 0·08 μM.

clear that we are dealing here with a competitive type of inhibition so that the dependence may be described by[8]

$$1/i = 1 + K_i/[I](1 + [S]/K_m) . \qquad (1)$$

This describes a straight line with slope equal to $K_i(1 + [S]/K_m)$. Since the value of $K_m$ for butyrylcholine as substrate is 3·70 mM, the value of $K_i$ can be derived, its mean value being 0·065 μM. For $[S] = 2$ mM the $K_i$ was 0·054 μM, for $[S] =$ = 10 mM it was 0·075 μM.

The double-reciprocal plot according to Lineweaver and Burk[9] (Fig. 2) indicates a competitive character of inhibition of butyrylcholinesterase with 9-hydrazino--1,2,3,4-tetrahydroacridine since all the straight lines intersect in a single point $(1/V)$ on the $1/v$ axis. From the inset of the dependence of reciprocal values of intercepts $(1/x)$ with the $1/[S]$ axis on the concentration of 9-hydrazino-1,2,3,4-tetrahydroacridine ($[I]$) one can judge on a competitive character of the inhibition, the dependence being linear. The value of $K_i$ computed from this plot is 0·080 μM.

9-Hydrazino-1,2,3,4-tetrahydroacridine is a reversible inhibitor of butyrylcholinesterase because dilution of the mixture of enzyme with inhibitor results in a decrease of enzyme inhibition. If the degree of dilution is designated with $r = [I_0]/[I_f]$, where $[I_0]$ is the inhibitor concentration before dilution and $[I_f]$ after dilution, fractional inhibition expressions $i_0$ and $i_f$ for a competitive type of inhibition are given by[10]

$$i_0 = \frac{[I_0]/K_i}{[I_0]/K_i + [S]/K_m + 1} \qquad (2)$$

and

$$i_f = \frac{[I_0]/K_i}{[I_0]/K_i + [S]/K_m + r} \qquad (3)$$

Eq. (3) can be converted to a straight-line equation of the form

$$1/i_f = 1 + [S]\,K_i/[I_0]\,K_m + rK_i/[I_0] . \qquad (4)$$

Hence if one plots $1/i_f$ against $r$ a straight line should be obtained, the slope of which is equal to $K_i/[I_0]$. The straight line intersects the $1/i_f$ axis at A and the $r$ axis at B, the values of A and B being defined by

$$A = 1 + [S]\,K_i/[I_0]\,K_m \qquad (5)$$

and

$$B = 1 + \frac{([S]\,K_i)/([I_0]\,K_m)}{K_i/[I_0]} . \qquad (6)$$

The dependence of $1/i_f$ on $r$ for butyrylcholinesterase inhibition by 9-hydrazino-1,2,3,4-tetrahydroacridine is shown in Fig. 3. The experimental points lie on a straight line defined by Eq. (4) (correlation coefficient 0·98); the $K_i$ value derived from the slope of the straight line is 0·085 μM.

A combined inhibitory effect of 9-hydrazino-1,2,3,4-tetrahydroacridine and 9-amino-1,2,3,4-tetrahydroacridine on butyrylcholinesterase was tested as described by Yonetani and Theorell[11], measuring the rate of enzyme hydrolysis of butyrylcholine ($v$) under varying concentrations of the two inhibitors. Plotting values of $1/v$ against the concentration of 9-amino-1,2,3,4-tetrahydroacridine ([I]) at different concentrations of 9-hydrazino-1,2,3,4-tetrahydroacridine (0, 0·6, 1 and 2 μM) we obtained a set of parallel lines (Fig. 4). This fact indicates a mutual competitive action of both inhibitors which probably bind to the same active site of butyrylcholinesterase.

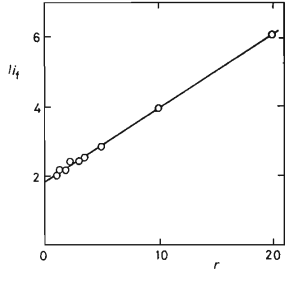


FIG. 3

Inhibition of Butyrylcholinesterase by 9-Hydrazino-1,2,3,4-tetrahydroacridine

Dependence of reciprocal values of final fractional inhibition ($i_f$) on the degree of dilution ($r$) of a mixture of enzyme with inhibitor. $r = [I_0]/[I_f]$ with $[I_0] = 0{\cdot}4$ μM. Measured at 25°C and pH 8·0. Competitive type of inhibition with $K_i$ equal to 0·085 μM.

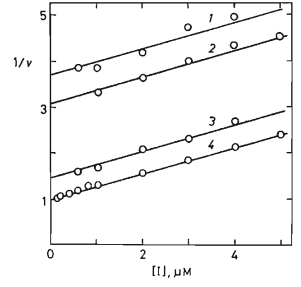


FIG. 4.

Inhibition of Butyrylcholinesterase by a Mixture of 9-Hydrazino-1,2,3,4-tetrahydroacridine and 9-Amino-1,2,3,4-tetrahydroacridine

Dependence of reciprocal rate of hydrolysis of butyrylcholine ($v$) (initial concentration 10 mM) on the concentration of 9-amino-1,2,3,4-tetrahydroacridine ([I]). Rate of hydrolysis was measured at 25°C and pH 8·0 in the presence of 2 μM *1*, 1 μM 2, 0·6 *3* or 0 μM *4* 9-hydrazino-1,2,3,4-tetrahydroacridine.

Mutual antagonism of inhibitors competing for the same binding site was confirmed by comparing calculated and observed values of fractional inhibition (Table I). Values of $i_{1,2}$ calculated according to[12]

$$i_{1,2} = i_1 + i_2 - i_1 i_2 \qquad (7)$$

are seen to be greater than $i_{1,2}$ values measured for a mixture of inhibitors.

## DISCUSSION

9-Hydrazino-1,2,3,4-tetrahydroacridine was found to be a powerful inhibitor of butyrylcholinesterase *in vitro*. Interaction of this inhibitor with butyrylcholinesterase is purely competitive and is characterized by a $K_i$ of 0·08 μM. The enzyme forms a reversible complex with the inhibitor in a 1 : 1 ratio, the complex exhibiting no enzyme activity.

TABLE I

Combined Inhibitory Effect of 9-Amino-1,2,3,4-tetrahydroacridine (ATA) and 9-Hydrazino-1,2,3,4-tetrahydroacridine (HTA) on the Activity of Horse Plasma Butyrylcholinesterase

The calculated value of fractional inhibition $i_{1,2}$ was obtained from values of fractional inhibition for the individual inhibitors $i_1$ and $i_2$, using the formula[12] $i_{1,2} = i_1 + i_2 - i_1 i_2$. The observed value of $i_{1,2}$ was obtained by measuring inhibition in the presence of a mixture of the two inhibitors.

| [ATA] μM | [HTA] μM | $i_{1,2}$ | |
|---|---|---|---|
| | | calculated | found |
| 0·01 | 0·04 | 0·68 | 0·73 |
| 0·02 | 0·04 | 0·75 | 0·79 |
| 0·04 | 0·04 | 0·83 | 0·85 |
| 0·01 | 0·06 | 0·70 | 0·75 |
| 0·02 | 0·06 | 0·77 | 0·82 |
| 0·04 | 0·06 | 0·84 | 0·87 |
| 0·01 | 0·08 | 0·72 | 0·77 |
| 0·02 | 0·08 | 0·78 | 0·82 |
| 0·04 | 0·08 | 0·85 | 0·88 |
| 0·01 | 0·10 | 0·74 | 0·79 |
| 0·02 | 0·10 | 0·79 | 0·83 |
| 0·04 | 0·10 | 0·86 | 0·89 |
| 0·01 | 9·20 | 0·75 | 0·80 |
| 0·02 | 0·20 | 0·80 | 0·85 |
| 0·04 | 0·20 | 0·87 | 0·89 |

In comparison with 9-amino-1,2,3,4-tetrahydroacridine which is a mixed competitive-noncompetitive inhibitor of butyrylcholinesterase with $K_i$ (comp.) of 0·17 and $K_i$ (noncomp.) of 0·07 μM[4], the enzyme–inhibitor complex of the hydrazine derivative is less stable[2,4] because it can be broken down by mere dilution. Both inhibitors are bound to the same active site of butyrylcholinesterase as indicated by experiments with a combined action of the enzyme. The binding site lies probably on the anionic side of the active centre[13]. According to Bergmann[13] this anionic side is formed by a free carboxyl group of glutamic or aspartic acid and therefore it reacts with cation-active compounds but electrostatic forces participating in the complex formation are not the only ones and certainly are not decisive[14–16].

It appears that the binding of 9-hydrazino- as well as of 9-amino-1,2,3,4-tetrahydroacridine to the active centre of butyrylcholinesterase is determined by hydrophobic interactions which play a role in the inhibition of acetylcholinesterase by 9-amino-1,2,3,4-tetrahydroacridine[4,17] and by some of its analogues and derivatives[17]. A similar situation obtains in the case of binding of some other types of inhibitors on butyrylcholinesterase[18,19].

On the basis of existing experiments it cannot be decided whether the site of hydrophobic interaction is the anionic site of the active centre[20,21] or whether an independent site is involved[16].

The hydrophobic interaction is made possible by the fact that 9-hydrazino-1,2,3,4-tetrahydroacridine is highly lipophilic in spite of its ionic character. Its distribution coefficient for the system of cyclohexanol and water is 100 (ref.[1]) which is apparently due to delocalization of the positive charge of the mono-cation by a mesomeric effect, similarly to the case of the 9-amino analogue (distribution coefficient for the same system is 3·2; ref.[4]).

*The authors are indebted to Dr J. Bielavský for a kind gift of 9-hydrazino- and 9-amino-1,2,3,4-tetrahydroacridine, to Mr O. Ochrymovič for technical assistance and to Mrs V. Pacovská for doing the computer calculations.*

REFERENCES

1. Bielavský J.: This Journal, in press.
2. Heilbronn E.: Acta Chem. Scand. *15*, 1386 (1961).
3. Rosić N., Milošević M. P.: Yugoslav. Physiol. Pharmacol. Acta *3*, 43 (1967).
4. Patočka J., Bajgar J., Bielavský J., Fusek J.: This Journal *41*, 916 (1976).
5. Strelitz F.: Biochem. J. *38*, 86 (1944).
6. Patočka J., Bajgar J.: This Journal *38*, 3940 (1973).
7. Monod J., Wyman J., Changeux J.-P.: J. Mol. Biol. *12*, 88(1965).
8. Webb J. L.: *Enzyme and Metabolic Inhibitors*, Vol. I., p. 153. Academic Press, New York and London 1963.
9. Lineweaver H., Burk D.: J. Amer. Chem. Soc. *56*, 658 (1934).
10. Ref. 8, p. 610.

11. Yonetani T., Theorell H.: Arch. Biochem. Biophys. *106*, 243 (1964).
12. Ref. 8, p. 507.
13. Bergmann F.: Advan. Catal. Relat. Subj. *10*, 130 (1958).
14. Wilson I. B.: J. Biol. Chem. *197*, 215 (1952).
15. Bernhard S. A.: J. Amer. Chem. Soc. *77*, 1966 (1955).
16. Augustinsson K.-B.: Biochim. Biophys. Acta *128*, 351 (1966).
17. Steinberg G. M., Mednick M. L., Maddox J., Rice R.: J. Med. Chem. *18*, 1956 (1975).
18. Purcell W. P., Beasley J. G.: Mol. Pharmacol. *4*, 402 (1968).
19. Millner O. E. jr, Stanley J. W., Purcell W. P.: J. Med. Chem. *17*, 13 (1974).
20. Augustinsson K.-B., Eriksson H.: Croat. Chem. Acta *47*, 277 (1975).
21. Kamarić L.: Croat. Chem. Acta *47*, 287 (1975).

Translated by A. Kotyk.