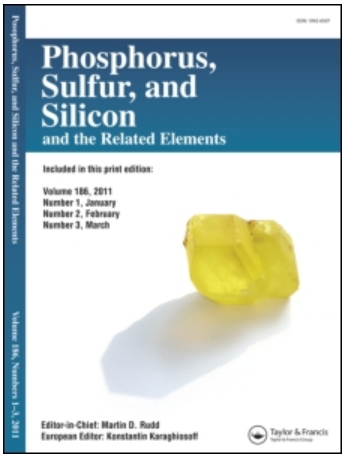
This article was downloaded by:
On: *30 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

KINETICS OF CHOLINESTERASE INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS: DETERMINATION OF DISSOCIATION AND PHOSPHORYLATION CONSTANTS IN THE PRESENCE OF LOW SUBSTRATE CONCENTRATIONS

Jean Debord[a]; Bernard Penicaut[b]; Michel Labadie[c]
[a] Laboratoire d'Histologie, Limoges Cédex, France [b] Laboratoire de Chimie Analytique, Limoges Cédex, France [c] Laboratoire de Biochimie Médicale, Limoges Cédex, France

To cite this Article Debord, Jean , Penicaut, Bernard and Labadie, Michel(1987) 'KINETICS OF CHOLINESTERASE INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS: DETERMINATION OF DISSOCIATION AND PHOSPHORYLATION CONSTANTS IN THE PRESENCE OF LOW SUBSTRATE CONCENTRATIONS', Phosphorus, Sulfur, and Silicon and the Related Elements, 29: 1, 57 — 65
To link to this Article: DOI: 10.1080/03086648708072841
URL: http://dx.doi.org/10.1080/03086648708072841

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Phosphorus and Sulfur, 1986, Vol. 29, pp. 57–65
0308-664X/86/2901-0057/$15.00/0
© 1986 Gordon and Breach, Science Publishers, Inc.
Printed in the United Kingdom

KINETICS OF CHOLINESTERASE INHIBITION BY ORGANOPHOSPHORUS COMPOUNDS: DETERMINATION OF DISSOCIATION AND PHOSPHORYLATION CONSTANTS IN THE PRESENCE OF LOW SUBSTRATE CONCENTRATIONS

JEAN DEBORD*

Laboratoire d'Histologie

BERNARD PENICAUT

Laboratoire de Chimie Analytique

MICHEL LABADIE

Laboratoire de Biochimie Médicale
Faculté de Médecine et de Pharmacie, 2 rue du Docteur-Marcland, 87025 Limoges Cédex (France)

(*Received December 16, 1985*)

Downloaded At: 07:56 30 January 2011

A method has been developed to determine the dissociation constant K_d and the phosphorylation constant k_2 of an irreversible organophosphorus inhibitor of cholinesterase. The method consists in recording the progress curve of the enzymatic hydrolysis of a substrate in the presence of the inhibitor. The use of a very low substrate concentration limits the competitive action of the substrate and renders the method more sensitive to inhibition, particularly to the binding step. The method is more general than the early method of Hart and O'Brien[1] because it is not limited by the extent of substrate hydrolysis. The method has been used to study the anticholinesteratic activity of the anticancer drug triethylenethiophosphoramide (thio-TEPA). The results are discussed with respect to the chemical reactivity of this compound.

INTRODUCTION

Many organophosphorus compounds are able to inhibit cholinesterases in an irreversible manner by binding covalently to a catalytic serine residue of the enzyme. This inhibition proceeds in two steps:

$$E + I \overset{K_d}{\rightleftharpoons} EI \overset{k_2}{\rightarrow} E'I$$

The first step is the reversible binding of the inhibitor I to the catalytic site of the enzyme E, leading to the complex EI. This step is characterized by an equilibrium

*Author to whom the correspondence should be addressed.

dissociation constant K_d. The second step is a progressive phosphorylation of the serine residue, leading to the irreversibly inhibited enzyme E'I. This step is characterized by the phosphorylation rate constant k_2. A proper analysis of the structural factors governing the inhibitory power of the organophosphorus compounds would require a separate determination of the two constants K_d and k_2. Methodological difficulties have however limited these determinations to only a few compounds, so that the inhibitory power of most of these compounds is expressed by the overall rate constant $k_i = k_2/K_d$.

In 1973 Hart and O'Brien introduced an interesting spectrophotometric method for measuring K_d and k_2 by following the progress curve of the enzymatic hydrolysis of a substrate in the presence of an irreversible inhibitor.[1] Their method has been further refined by the use of stop-flow instrumentation and automatic data processing.[2] This method has been applied successfully to the study of organophosphate[1-4] and carbamate[5] inhibitors.

Unfortunately, the kinetic analysis of Hart and O'Brien is limited to the case when the fraction of substrate hydrolysed during the measurement is very low. Obviously, this approximation cannot be satisfied in all experimental designs: for instance if the inhibitor is a slow-acting one (low k_2 value), an appreciable amount of substrate will be consumed before complete inhibition of the enzyme.

Furthermore, there is a competition between substrate and inhibitor for the catalytic site of the enzyme. Therefore, it is advisable to use as low a substrate concentration as possible, in order to render the method more sensitive (one could also use a *high* inhibitor concentration but this is generally impossible because most organophosphorus inhibitors are poorly soluble compounds). Of course, if a low substrate concentration is used, it will be even more difficult to obtain a very low amount of hydrolysis.

These difficulties have prompted us to examine the possibility of designing an extension of the method of Hart and O'Brien, which would be applicable at low substrate concentration, and independent of the amount of substrate hydrolyzed. The present paper describes the principle of this method and its application to the anticancer drug triethylene-thiophosphoramide (thio-TEPA).

Downloaded At: 07:56 30 January 2011

THEORY

If enzyme E, inhibitor I and substrate S are mixed at zero time (with initial concentrations e_0, i_0, s_0 such that $s_0 \gg e_0$ and $i_0 \gg e_0$), the following reactions will occur:

$$E + S \overset{K_m}{\rightleftharpoons} ES \overset{k_{cat}}{\rightarrow} E + P$$

$$E + I \overset{K_d}{\rightleftharpoons} EI \overset{k_2}{\rightarrow} E'I$$

where K_m is the Michaelis constant, k_{cat} the catalytic rate constant, ES the enzyme-substrate complex and P the hydrolysis products. In what follows, the concentration of a chemical will be denoted by the corresponding lower-case letter (e.g. [E] = e) except for the enzyme complexes for which [ES] = u, [EI] = v,

[E′I] = w. The following equations hold:

$$\frac{dp}{dt} = k_{cat}u \tag{1}$$

$$\frac{dw}{dt} = k_2 v \tag{2}$$

$$K_m = \frac{es}{u} \tag{3}$$

$$K_d = \frac{ei}{v} \tag{4}$$

$$e_0 = e + u + v + w \tag{5}$$

$$s_0 = s + u + p \simeq s + p \qquad \text{since } s_0 \gg e_0 \tag{6}$$

$$i_0 = i + v + w \simeq i \qquad \text{since } i_0 \gg e_0 \tag{7}$$

Dividing eq. (2) by eq. (1), one obtains:

$$\frac{dw}{dp} = \frac{k_2}{k_{cat}} \cdot \frac{v}{u} \tag{8}$$

From eq. (3) and (4):

$$\frac{v}{u} = \frac{K_m}{s} \cdot \frac{i}{K_d} \tag{9}$$

hence

$$\frac{dw}{dp} = \frac{k_2 i K_m}{k_{cat} K_d} \times \frac{1}{s} = \frac{b}{k_{cat}} \times \frac{1}{s_0 - p} \tag{10}$$

where $b = k_2 i K_m / K_d \simeq k_2 i_0 K_m / K_d$ is an apparent constant. Integration with the initial conditions ($t = 0$; $p = 0$; $w = 0$) leads to:

$$w = -\frac{b}{k_{cat}} \mathrm{Ln}\left(1 - \frac{p}{s_0}\right) \tag{11}$$

From equations (3) and (4):

$$e = \frac{K_m}{s} \cdot u; \tag{12}$$

$$v = \frac{K_m}{s} \cdot \frac{i}{K_d} \cdot u \tag{13}$$

Substituting eq. (12) and (13) into eq. (5):

$$e_0 = \left[1 + \frac{K_m}{s}\left(1 + \frac{i}{K_d}\right)\right]u + w \tag{14}$$

hence:

$$u = \frac{e_0 - w}{1 + K'_m/s} = \frac{e_0 + (b/k_{cat})\mathrm{Ln}(1 - p/s_0)}{1 + K'_m/(s_0 - p)} \tag{15}$$

where $K'_m = K_m(1 + i/K_d)$.

Downloaded At: 07:56 30 January 2011

Substituting eq. (15) into eq. (1):

$$\frac{dp}{dt} = k_{cat} \cdot u = \frac{k_{cat} \cdot e_0 + b \cdot \mathrm{Ln}(1 - p/s_0)}{1 + K'_m/(s_0 - p)} \tag{16}$$

It is convenient to introduce the maximal velocity of the enzymatic reaction, $V = k_{cat} \cdot e_0$, and the fraction of substrate hydrolyzed at time t, $x = p/s_0$, so that equation (16) becomes:

$$\frac{dx}{dt} = \frac{V + b \cdot \mathrm{Ln}(1 - x)}{s_0 + K'_m/(1 - x)} \tag{17}$$

Equation (17) is *the general differential equation for substrate hydrolysis in the presence of an irreversible inhibitor.* If we use a low substrate concentration ($s_0 \ll K'_m$) equation (17) simplifies to:

$$\frac{dx}{dt} = (1 - x)\left[\frac{V}{K'_m} + \frac{b}{K'_m}\mathrm{Ln}(1 - x)\right] \tag{18}$$

or

$$\frac{1}{1 - x} \cdot \frac{dx}{dt} = \alpha + \beta\,\mathrm{Ln}(1 - x) \tag{19}$$

with:

$$\alpha = \frac{V}{K'_m} = \frac{k_0}{1 + i/K_d} \quad \text{and} \tag{20}$$

$$\beta = \frac{b}{K'_m} = \frac{k_2 \cdot i}{K_d + i} \tag{21}$$

where $k_0 = V/K_m$ is the pseudo-first order rate constant at low substrate concentration in the absence of inhibitor, since in this case the Michaelis-Menten equation, $dp/dt = Vs/(K_m + s)$, simplifies to $dp/dt = (V/K_m)s = k_0 s$.

The simplified differential equation (19) admits the following analytical solution (with $x = 0$ at $t = 0$):

$$x = 1 - \exp\left\{\frac{\alpha}{\beta}[\exp(-\beta t) - 1]\right\} \tag{22}$$

Fitting this equation to an experimental progress curve $x = f(t)$ gives values of α and β from which K_d and k_2 can be determined.

MATERIALS AND METHODS

Chemicals

Enzyme (Butyrylcholinesterase from Horse serum, EC 3.1.1.8, BuChE), substrate (Butyrylthiocholine iodide, BuSCh) and chromogenic reagent (5,5′-dithiobis-2-nitrobenzoate, DTNB) were from Sigma. They were dissolved in a Tris/HCl buffer, pH 7.4 at 37°C. ThioTEPA was a gift from Specia (France). Its purity was checked by

Downloaded At: 07:56 30 January 2011

HPLC on ODS-bonded silica, with MeOH/H_2O 60/40 as mobile phase and 215 nm detection, and by TLC on a silica gel plate developed in ethyl acetate and sprayed with 4-nitrobenzyl pyridine[6] and with a cholinesterase reagent.[7] No impurities were found. ThioTEPA is a very polar compound and was easily dissolved in Tris buffer.

Spectrophotometric recording of progress curves

The enzymatic hydrolysis of BuSCh is followed at 37°C with a Hewlett-Packard HP8450 spectrophotometer linked to a HP9825 computer by means of a RS-232C interface.

1 mL of a solution containing BuSCh ($3 \cdot 10^{-5}$ M), thioTEPA (variable concentration) and DTNB (10^{-4} M) in Tris buffer is placed in a thermostated cell. At zero time 0.05 mL of a solution containing BuChE and DTNB is introduced and the absorbance of this mixture is measured every 5 s during 10 min. The spectrophotometer generates a data file consisting of $(n + 1)$ absorbance values $(A_0, A_1, \ldots A_i, \ldots A_n)$ at times $(t_0, t_1, \ldots t_i, \ldots t_n)$. After completion of the measurement this file is transferred to the computer for data processing. A progress curve without inhibitor is also recorded.

Downloaded At: 07:56 30 January 2011

Data processing in the absence of inhibitor

The progress curve recorded in the absence of inhibitor is described by the pseudo-first order equation:

$$A = A_\infty[1 - \exp(-k_0 t)]$$

Data processing begins by computing the velocity y_i at time t_i using the finite difference approximation:

$$y_i = (A_{i+1} - A_{i-1})/2\,\Delta t \quad \text{for } i = 1, \ldots, n-1$$

where Δt is the time interval between two successive measurements (here $\Delta t = 5$ s).

Approximate values of the parameters A_∞ and k_0 are obtained by applying linear regression to the relation:

$$\text{Ln}\, y_i = \text{Ln}(k_0 A_\infty) - k_0 t$$

Note that, due to experimental errors, negative values of y_i can arise (particularly near the end of the experiment when the velocities are very weak): of course these negative values are discarded from the regression. The approximate values of A_∞ and k_0 are subsequently refined by standard methods of nonlinear regression.[8,9]

Data processing in the presence of inhibitor

–Each absorbance value is divided by A_∞ to obtain the fraction of substrate hydrolysed, x_i

–Approximate values of the derivative (dx/dt) are obtained by the same difference formula as above

–According to eq. (19), approximate values of α and β are determined by linear regression of $(dx/dt)/(1 - x)$ against $\text{Ln}(1 - x)$

–The values of α and β are further refined by nonlinear regression.

Determination of K_d and k_2

K_d is obtained by fitting eq. (20) to the experimental values of α at various inhibitor concentrations. k_0 is treated as an adjustable parameter, using the experimental value of k_0 as a supplementary data point corresponding to $i = 0$. Once K_d is known, k_2 can be determined from eq. (21).

RESULTS AND DISCUSSION

Application of our method to the anticancer drug triethylenethiophosphoramide (thio-TEPA) shows that this compound is an irreversible inhibitor of serum cholinesterase. This property has been mentioned earlier[10] but no kinetic parameters have been reported. The anticholinesterasic activity of some anticancer drugs can explain some of the side effects of these compounds.[10,11]

Figure 1 shows a typical progress curve in the presence of thio-TEPA. The enzymatic reaction produces a chromogenic compound, 5-thio-2-nitro benzoate (TNB), which undergoes a slow discoloration. For this reason, only the points corresponding to the first ten minutes of the reaction (*i.e.* 120 points per curve) were used to compute α and β. The theoretical curve, fitted according to eq. (22) by the nonlinear least-squares method, shows an excellent agreement with the experimental points. This confirms the validity of our kinetic analysis. Eq. (20) and (21) have been fitted to the values of α and β obtained at different thio-TEPA concentrations.

Downloaded At: 07:56 30 January 2011

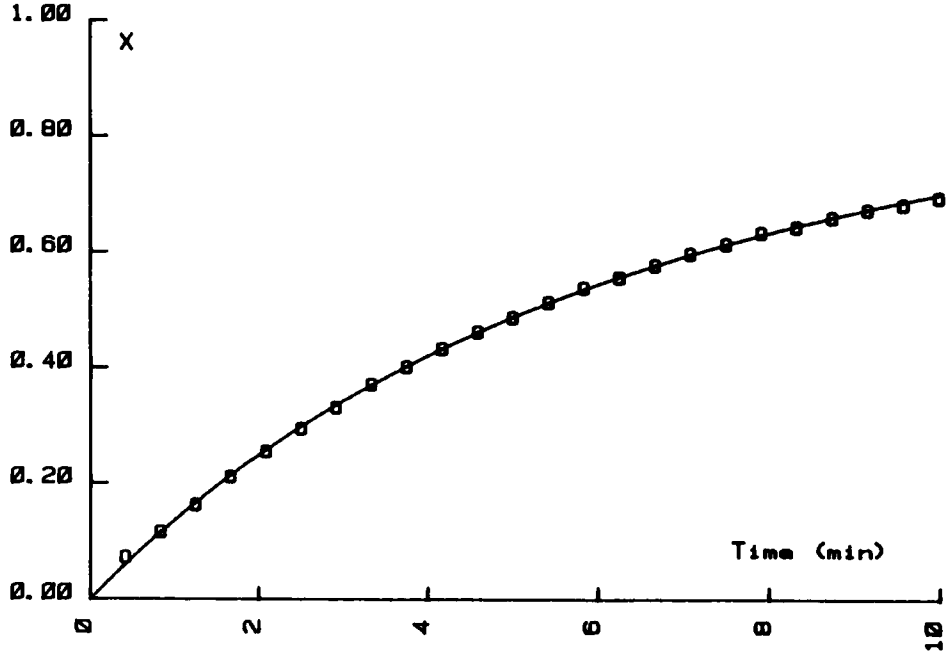


FIGURE 1 Progress curve of butyrylthiocholine hydrolysis in the presence of 25 mM thio-TEPA, at pH 7.4 and 37°C. x = fraction of substrate hydrolysed. Only every fifth data point is presented. The curve corresponds to eq. 22.

Comparison of the experimental points with the fitted curves reveals that α is a more reliable parameter than β (Figures 2 and 3). In fact, thio-TEPA is a slow-acting inhibitor of cholinesterase, resulting in low values of β (< 0.1 min^{-1}): such low values are likely more sensitive to experimental errors. This discrepancy between the reliabilities of α and β is also reflected in the values of K_d and k_2, shown in Table I. The coefficient of variation of k_2 (which is computed from β) is about two times that of K_d (which is computed from α). However, the precision of these determinations of K_d and k_2 compares favourably with that of published values obtained with the method of Hart and O'Brien.[1-4]

On Table I we have also presented literature values for TEPA, the oxygen analog of thio-TEPA.[11] These values have been obtained by a conventional method but in the same conditions than ours, except that the substrate was procaine hydrochloride. Assuming that the substrate has no effect upon the inhibition parameters, we can compare the results of Table I.

According to the overall rate constant k_i, thio-TEPA is a weaker inhibitor than TEPA, but this is due mainly to differences in affinity, as reflected by K_d. Replacement of the oxygen in TEPA by sulfur results in a more than 100-fold decrease of affinity for cholinesterase. The reason for this is not clear. In the enzyme-inhibitor complex the inhibitor is probably "frozen" in a peculiar conformation. Perhaps this conformation is more difficult to adopt for thio-TEPA. The irreversible step of inhibition, reflected by k_2, is about two times faster for thio-TEPA. This step can be viewed as the result of covalent binding of the inhibitor

Downloaded At: 07:56 30 January 2011

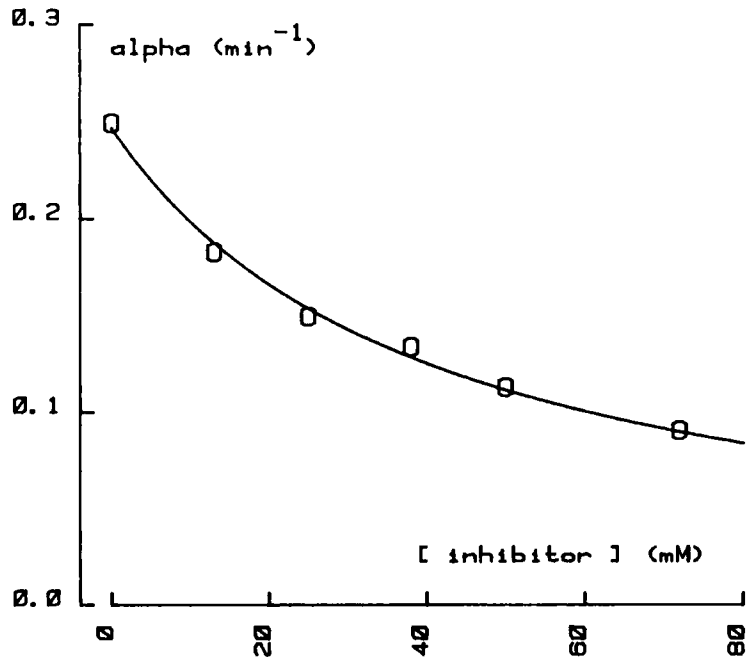


FIGURE 2 Variation of the rate constant α (eq. 22) with thio-TEPA concentration. The curve is fitted according to eq. 20.

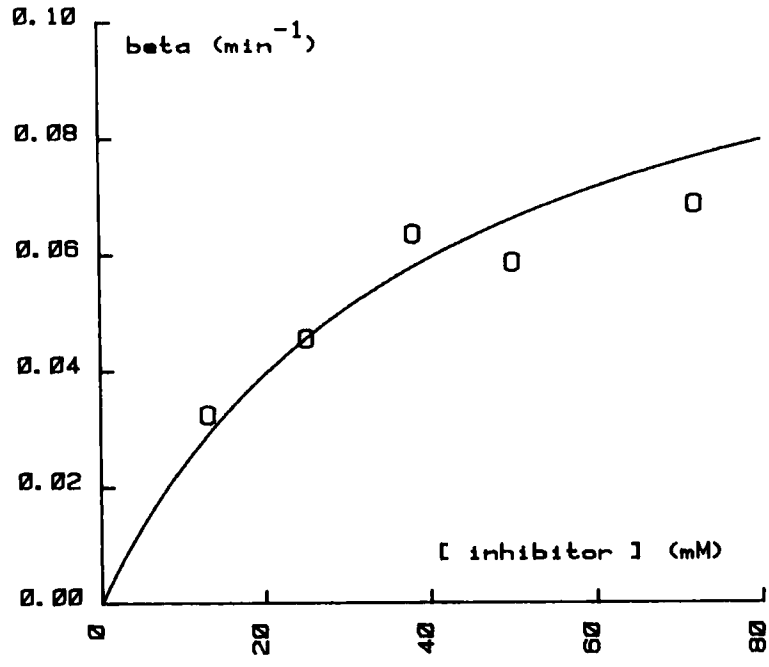


FIGURE 3 Variation of the rate constant β (eq. 22) with thio-TEPA concentration. The curve is fitted according to eq. 21.

TABLE I

Kinetic parameters for inhibition of horse serum cholinesterase by aziridinyl phosphoramides, at pH 7.4 and 37°C

Compound	K_d (mM)	k_2 (min^{-1})	k_i ($min^{-1} \cdot mM^{-1}$)	Ref.
TEPA $OP(N\triangleleft)_3$	0.50	0.052	0.104	11
thio-TEPA $SP(N\triangleleft)_3$	41.2 ± 2.1	0.121 ± 0.013	$(2.9 \pm 0.46)10^{-3}$	this work

to a catalytic hydroxyl group of the enzyme. This binding can occur by phosphorylation (breakage of P—N bond and liberation of aziridine) or by alkylation (opening of the aziridine ring). The second hypothesis is more in agreement with the conventional view of the aziridinyl derivatives as "alkylating agents". However, a recent acid hydrolysis study of thio-TEPA suggests a coexistence of both mechanisms.[12] Furthermore, Modro has shown that the alkaline hydrolysis of the phosphoramidate $OP(OMe)_2N\triangleleft$ proceeds mainly by breakage of the P—N bond, in competition with the breaking of the P—OMe bond.[13]

The kinetics of thio-TEPA inhibition is similar to that of organophosphate esters, for which phosphorylation of the enzyme is clearly established.[14] So, one can accept the hypothesis of P—N bond breakage in thio-TEPA as an unifying concept of cholinesterase inhibition.

Downloaded At: 07:56 30 January 2011

The anticholinesteratic activity of thio-TEPA has probably no clinical significance since it occurs at doses much higher than the plasma concentration of this drug.[15] Our results suggest, however, that enzymes could be possible targets of this drug. It is generally assumed that DNA alkylation is the principal mode of action of the aziridine antitumorals; but there is an increasing bulk of evidence suggesting other sites of action, particularly the plasma membrane and the enzymes it contains.[16] Such enzymes could be inhibited by alkylation and/or phosphorylation, but more work is required to precise these points.

ACKNOWLEDGMENTS

The authors wish to thank the Laboratoires Specia (of the Rhône Poulenc pharmaceutical group) for a generous gift of thioTEPA and Dr. J. C. Bollinger (Fac. Sci., Limoges) for his comments on the manuscript. Part of this work was supported by the Ministère de l'Industrie et de la Recherche (Contract N° 84H0341).

Downloaded At: 07:56 30 January 2011

REFERENCES

1. G. J. Hart and R. D. O'Brien, *Biochemistry*, **12**, 2940 (1973).
2. G. L. Horton, J. R. Lowe and C. N. Lieske, *Anal. Biochem.*, **78**, 213 (1977).
3. G. L. Horton, C. N. Lieske and J. R. Lowe, *Pestic. Sci.*, **9**, 135 (1978).
4. A. Forsberg and G. Puu, *Eur. J. Biochem.*, **140**, 153 (1984).
5. T. Nishioka, K. Kitamura, T. Fujita and M. Nakajima, *Pestic. Biochem. Physiol.*, **6**, 320 (1976).
6. R. F. Struck, M. C. Kirk, M. H. Witt and W. R. Laster, *Biomed. Mass Spectrom.*, **2**, 46 (1975).
7. H. Breuer, *J. Chromatogr.*, **243**, 183 (1982).
8. G. N. Wilkinson, *Biochem. J.*, **80**, 324 (1961).
9. R. G. Duggleby, *Anal. Biochem.*, **110**, 9 (1981).
10. E. K. Zsigmond and G. Robins, *Canad. Anaesth. Soc. J.*, **19**, 75 (1972).
11. D. Lalka and T. J. Bardos, *Biochem. Pharmacol.*, **24**, 455 (1975).
12. B. E. Cohen, M. J. Egorin, M. S. Balachandran Nayar and P. L. Gutierrez, *Cancer Res.*, **44**, 4312 (1984).
13. B. Davidowitz and T. A. Modro, *J. Chem. Soc., Perkin Trans. 2*, 303 (1985)
14. A. R. Main, *Pharmacol. Ther.*, **6**, 579 (1979).
15. M. J. Egorin, S. R. Akman and P. L. Gutierrez, *Cancer Treat. Rep.*, **68**, 1265 (1984).
16. J. A. Hickman, K. J. Scanlon and T. R. Tritton, *Trends Pharmacol. Sci.*, **5**, 15 (1984).