J. Enzyme Inhibition, 1990, Vol. 4, pp. 27–34
Reprints available directly from the publisher
Photocopying permitted by license only

DIETHYLBARBITURATE POTENTIATION OF 2,4,6-TRINITROBENZENESULPHONATE-INDUCED RHODANESE INACTIVATION

THALIA B. MALLIOPOULOU, EMMANUEL T. RAKITZIS* and VASILIKI A. MALLIOPOULOU

Department of Biological Chemistry, University of Athens Medical School, Athens, 115 27 Greece

(Received 20 August 1989)

The rate of rhodanese inactivation by 2,4,6-trinitrobenzenesulphonate is increased in the presence of diethylbarbiturate in the reaction medium. A "rate saturation effect" indicates the formation of a rhodanese-diethylbarbiturate complex, prior to modification-induced enzyme inactivation. The dissociation constant of this complex is 19.0 mM. Diethylbarbiturate has no effect on the trinitrophenylation rate of the free amino groups of rhodanese. When rhodanese modification, in the presence of diethylbarbiturate in the reaction medium, is carried out by the use of a 2,4,6-trinitrobenzenesulphonate concentration much lower than the concentration of rhodanese modifiable amino groups, reaction stoichiometry indicates that 3 to 5 moles of rhodanese are rendered inactive for each mole of 2,4,6-trinitrobenzenesulphonate utilized. This finding indicates the existence of a chain-reaction type mechanism of rhodanese inactivation.

KEY WORDS: Rhodanese, 2,4,6-trinitrobenzenesulphonate, diethylbarbiturate, modification-induced enzyme inactivation.

INTRODUCTION

Trinitrophenylation of the free amino groups of rhodanese (thiosulphate sulphurtransferase, EC 2.8.1.1) results in the loss of enzyme catalytic function.[1,2] The finding is reported here that, when rhodanese modification by 2,4,6-trinitrobenzenesulphonate is carried out in the presence of diethylbarbiturate in the reaction medium, modification-induced enzyme inactivation is increased. The mechanism of this potentiation of enzyme inactivation should be of interest since diethylbarbiturate brings about an increase in both the rate, and also the stoichiometry of modification-induced enzyme inactivation; the stoichiometry exceeds the 1:1 ratio (moles of enzyme inactivated per mole of modifying agent reacted), a finding which indicates the existence of self-regenerating reaction intermediates. In this communication a kinetic analysis of the effect of diethylbarbiturate on rhodanese modification, and inactivation, by 2,4,6-trinitrobenzenesulphonate is presented. A preliminary report of these studies has been published in abstract form.[3]

MATERIALS

Rhodanese, purified from bovine liver, 2,4,6-trinitrobenzenesulphonic acid, 1-

*Correspondence.

methylimidazole-2-thiol, 1,1-diphenyl-2-picryl hydrazyl, and catalase (from bovine liver) were obtained from Sigma Chemical Co., St. Louis, MO., USA. Sodium diethylbarbiturate was a product of Merck, Darmstadt, and barbituric acid a product of Serva, Heidelberg, Germany.

METHODS

The determination of rhodanese activity was carried out, with thiosulphate and cyanide as substrates, as described previously.[2] Studies of the modification, and/or inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonate were carried out under two different sets of conditions: (i) At a high modifying agent/enzyme protein ratio. The enzyme/modifying agent reaction mixture consisted of: sodium phosphate buffer 0.05 M, pH 8.00, sodium thiosulphate 0.01 M, rhodanese 9.12 μM, 2,4,6,-trinitrobenzenesulphonic acid 0.25 mM, and, when required, barbiturate in the range of 0.50 to 25.0 mM. (ii) At comparable modifying agent and enzyme protein concentrations. In this case, the enzyme/modifying agent reaction mixture consisted of: sodium phosphate buffer, 0.05 M, pH 9.00, sodium thiosulphate 0.01 M, rhodanese 30 μM, 2,4,6-trinitrobenzenesulphonic acid in the range of 4 to 30 μM, and, when required, sodium diethylbarbiturate 25.0 mM. After incubation of the enzyme/modifying agent reaction mixture, in a water bath at 25°C, for the time periods required, aliquots were withdrawn and used for the determination of rhodanese activity. The extent of reaction of 2,4,6-trinitrobenzenesulphonate with rhodanese was monitored by taking absorbance readings at 345 nm.[4,5] Enzyme inactivation, and protein reactive groups modification, rate constants were determined by graphical analysis procedures, as described previously.[2] The extent of the reaction of 2,4,6-trinitrobenzenesulphonate with rhodanese free amino groups was determined graphically.[6,7] The fractional concentration of unreacted amino groups was determined from the reaction end-point, and from the value of the reaction product at each particular time. The number, n, of modifiable amino groups, per protein molecule, was determined from the absorbance value at the end-point of the reaction, by the use of the extinction coefficient for trinitrophenylated rhodanese amino group, i.e., 11,500 $M^{-1}cm^{-1}$ at 345 nm, and of the molecular weight of rhodanese (32,900).[8] Graphical analysis of protein modification data was carried out by fitting such data to the equation:[9]

$$((n[\mathrm{Pr}]_{(0)} - [\mathrm{Pr}]_{\mathrm{mod}})/n[\mathrm{Pr}]_{(0)}) = \Sigma_i c_i e^{-k_i t} \quad (1)$$

where $[\mathrm{Pr}]_{\mathrm{mod}}$ is the concentration of modified protein groups, t, is reaction time, $[\mathrm{Pr}]_{(0)}$ is unmodified protein concentration at $t = 0$, k_i are the constants, and c_i the coefficients of the protein modification equation.

To determine the stoichiometry of rhodanese modification-induced inactivation, rhodanese was reacted with 2,4,6-trinitrobenzenesulphonate, at concentrations of this compound equal to, or lower than, the rhodanese concentration used, and also in the presence of diethylbarbiturate in the reaction medium. Since, at the pH of the reaction, i.e., at pH 9.00, the number of modifiable amino groups per rhodanese molecule is 10.6, 2,4,6-trinitrobenzenesulphonate was, in effect, reacted with a large excess of amino groups.[2] The stoichiometry of modification-induced enzyme inactivation is obtained by plotting $\ln(E_{(0)}/E_{\infty})$ *v.* $[M]_{(0)}/[\mathrm{Pr}]_{(0)}$, where $E_{(0)}$ is initial enzyme activity, E_{∞} is enzyme activity at infinite reaction time, $[M]_0$ is initial modifying agent concentration, and $[\mathrm{Pr}]_{(0)}$ is initial enzyme protein concentration. The slope of this plot

is equal to the fractional concentration of enzyme protein reactive groups, or group reactivities, essential for enzyme catalytic function.[11]

At a constant modifying agent concentration, the barbiturate-induced rate enhancement of rhodanese inactivation by 2,4,6-trinitrobenzenesulphonate may be assumed to take place by the following mechanism:[10]

$$E + M \xrightarrow{k} E_{\text{inact.}} \tag{2}$$

$$E + A \underset{}{\overset{K_{\text{diss.}}}{\rightleftharpoons}} EA \tag{3}$$

$$EA + M \xrightarrow{k_A} E_{\text{inact.}} \tag{4}$$

where E is active enzyme, M is modifying agent, $E_{\text{inact.}}$ is inactive enzyme, A is the sensitizing ligand (accelerator), EA is the reversible enzyme-accelerator complex, the dissociation constant of which is $K_{\text{diss.}}$ and where k and k_A are the relevant second-order reaction rate constants. Enzyme inactivation is described by the equation:

$$\ln\{([E]_{(0)} - [E_{\text{inact.}}])/[E]_{(0)}\} = -k_{\text{obs.}}t \tag{5}$$

where:

$$k_{\text{obs.}} = \left(k + [k_A - k]\frac{[A]}{K_{\text{diss.}} + [A]}\right)[M] \tag{6}$$

A plot of $[A]/(k_{\text{obs.}} - k)[M])$ *v.* $[A]$ yields a straight line relationship, with a slope of $1/(k_A - k)[M]$, and an intercept of $-K_{\text{diss.}}$ on the $[A]$ axis.

The possible effect of free radical traps on the reaction system under study, *viz.*, on diethylbarbiturate-potentiated inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonate, was tested by adding to the enzyme-modifying agent-barbiturate reaction mixture the free radical trap employed, and determining the (pseudo)first-order rate constant of rhodanese inactivation. 1-methylimidazole-2-thiol was tested at a concentration of 10 mM, and at a 50 mM concentration of diethylbarbiturate, while 1,1-diphenyl-2-picryl hydrazyl was tested at a concentration of 50 μM, and at a diethylbarbiturate concentration of 10 mM. In both cases, the rest of the conditions were those of reaction at a high modifying agent concentration ratio. 1,1-diphenyl-2-picryl hydrazyl was tested, in addition to its possible effect on the rate of rhodanese inactivation, for the possible change in absorbance at 535 nm, consequent upon its reaction with free radicals. Catalase was tested for its possible effect on the rate of rhodanese inactivation at a concentration of 0.1 mg/ml reaction mixture, the rest of the conditions being those of a high modifying agent/protein ratio.

RESULTS AND DISCUSSION

Rhodanese inactivation, at high modifying agent/protein ratios, was investigated by a study of the time-dependence of enzyme activity loss. Plots of log fractional rhodanese activity v. time, at different diethylbarbiturate concentrations, while the 2,4,6-trinitrobenzenesulphonate concentration is kept constant, are shown in Figure 1. It will be seen from Figure 1 that all of these plots are rectilinear, and also that they pass through the origin of the graph, indicating that rhodanese inactivation is described by a single exponential function of reaction time.

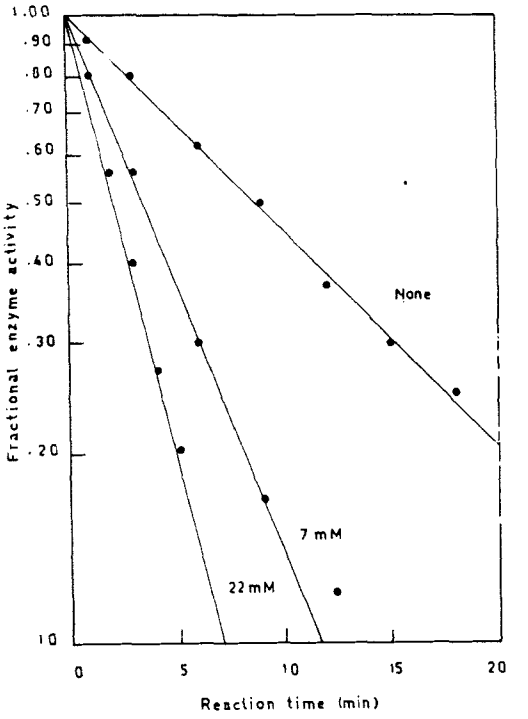

FIGURE 1 Effect of diethylbarbiturate on 2,4,6-trinitrobenzenesulphonate-induced rhodanese inactivation. Rhodanese, 9.12 μM, and 2,4,6-trinitrobenzenesulphonate, 0.25 mM, were incubated, for the time periods shown, in 0.05 M sodium phosphate buffer, pH 8.00, containing sodium thiosulphate 0.01 M. Concentrations of diethylbarbiturate included in the incubation mixtures are shown on the graph. At the time periods shown, aliquots were removed and assayed for rhodanese activity.

A plot of $[A]/(k_{obs.} - k)[M]$ *v.* $[A]$, for the $k_{obs.}$ values obtained from experiments, some of which are shown in Figure 1, is shown in Figure 2. It will be seen from Figure 2 that the value obtained for $K_{diss.}$ is 19 mM, while the value for k_A is 34.1 h^{-1}. The "rate saturation effect" of diethylbarbiturate on rhodanese inactivation indicates that the formation of a diethylbarbiturate-rhodanese complex is a necessary condition for the acceleration of enzyme catalytic activity loss.

Rhodanese inactivation, at comparable protein-modifying agent concentrations, was investigated by employing a reaction time long enough to ensure completion of the reaction of protein and modifying agent: utilization of 2,4,6-trinitrobenzenesulphonate was followed by taking the absorbance of the preparation at 345 nm. The inactivation to modification reaction stoichiometry is studied in two different ways: (a): By a comparison of the number of moles of rhodanese inactivated for every mole of modifying agent reacted (Figure 3). (b): By a kinetic analysis of the data, as was indicated in the "Methods" section. A plot of the moles of rhodanese inactivated per mole of 2,4,6-trinitrobenzenesulphonate reacted is shown in Figure 3, while a plot of $\ln([E]_{(0)}/[E_\infty])$ *v.* $[M]_{(0)}/[\mathrm{Pr}]_{(0)}$ is shown in Figure 4. It will be seen from Figure 3 that, at the end-point of the reaction of rhodanese with 2,4,6-trinitrobenzenesulphonate, the stoichiometry of rhodanese modification-induced inactivation approximately is 2 moles of rhodanese rendered inactive for each mole of modifying agent utilized. On

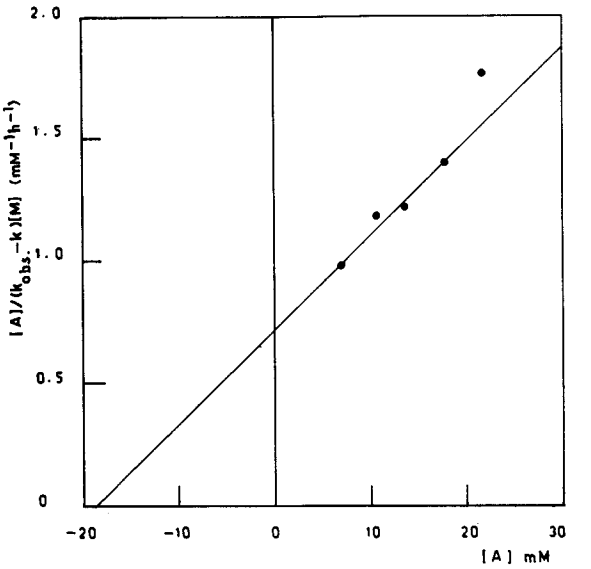

FIGURE 2 Determination of binding and reactivity constants for the potentiation by diethylbarbiturate of 2,4,6-trinitrobenzenesulphonate-induced rhodanese inactivation. Rhodanese inactivation experiments (some of which are those shown in Figure 1), are plotted in accordance with equation (6). The symbols used are: k, the second-order rate constant for rhodanese inactivation, $k_{obs.}$, the same constant in the presence of diethylbarbiturate in the reaction medium, and $[A]$, the concentration of diethylbarbiturate used.

the other hand, the straight-line relationship of the data shown in Figure 4 exhibits a slope of 4.5, indicating the number of moles, at initial reaction conditions, of rhodanese rendered inactive for every mole of 2,4,6-trinitrobenzenesulphonate utilized. However, it was found that under experimental conditions identical to those of Figure 4, markedly different values were obtained of rhodanese inactivation to 2,4,6-trinitrobenzenesulphonate utilization stoichiometry. These results indicate the formation of highly reactive reaction intermediates, such as free radicals. When rhodanese was modified with 2,4,6-trinitrobenzenesulphonate, under the same experimental conditions but in the absence of diethylbarbiturate from the reaction medium, the value for the fraction of enzyme reactive groups, or group reactivities, essential for catalytic function obtained, was 0.881 (Figure 4).

The fraction of enzyme protein groups, or group reactivities, essential for catalytic function may also be determined at a high modifying agent/enzyme protein ratio, if the first derivative, at $t = 0$, of the equation describing the enzyme inactivation reaction be divided by the first derivative, at $t = 0$, of the equation describing the protein modification reaction.[11] By an application of this procedure to the results shown in Figure 1, and also those given in Table I (pH 8.00 values), the fraction of enzyme groups, or group reactivities, essential for catalysis was found to be 1.06 for the enzyme preparation without diethylbarbiturate, and 3.35 with 10 mM diethylbarbiturate in the reaction medium. It may accordingly be seen that, in the presence of diethylbarbiturate in the reaction medium, rhodanese inactivation to modification stoichiometry is higher than unity when enzyme modification is carried out at a high modifying agent/enzyme protein ratio, as well as at comparable modifying agent and enzyme protein concentrations.

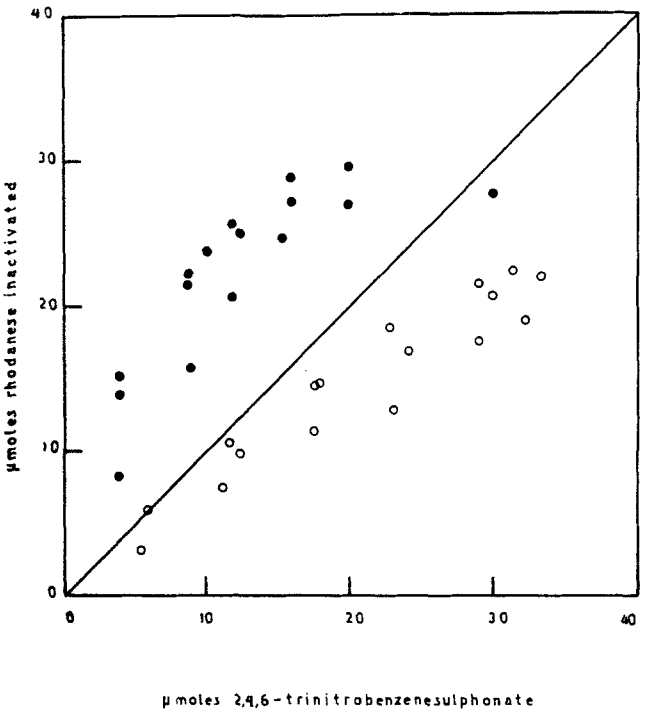

FIGURE 3 Stoichiometry of 2,4,6-trinitrobenzenesulphonate-induced rhodanese inactivation in the presence of diethylbarbiturate in the reaction medium. Rhodanese, 30 μM and 2,4,6-trinitrobenzenesulphonate, as shown, were incubated in 0.05 M sodium phosphate buffer, pH 9.00, containing sodium thiosulphate, 0.01 M, and sodium diethylbarbiturate, 25.0 mM. After incubation, at 25°C, for 60 min (by which time reaction of rhodanese amino groups and of 2,4,6-trinitrobenzenesulphonate was virtually complete), samples were used for the determination of rhodanese activity. Samples without (○), and with (●), diethylbarbiturate in the reaction medium. The solid diagonal line indicates the 1:1 stoichiometry of moles of rhodanese inactivated per mole of 2,4,6-trinitrobenzenesulphonate reacted.

No effect of barbituric acid on the 2,4,6-trinitrobenzenesulphonate-induced rhodanese inactivation could be demonstrated by inclusion of this compound, at a concentration of 10 mM in the reaction mixture (the rest of the conditions being those of a high modifying agent/enzyme protein ratio experiments).

The possible effect of diethylbarbiturate on the rate of rhodanese modification was investigated by the inclusion of this compound in the reaction mixture, the rest of the conditions being those of a high modifying agent/enzyme protein ratio experiment. The reaction was followed by taking absorbance readings at 345 nm, as well as at 420 nm. Protein modification data were analysed graphically, as mentioned above. Results are given in summary form in Table I. It will be noted that diethylbarbiturate exhibits no appreciable effect on rhodanese modification by 2,4,6-trinitrobenzenesulphonate, when this is studied at the wavelength of maximum absorption for free amino groups trinitrophenylation (i.e., at 345 nm), or at the wavelength of sulphite-trinitrophenylated-amino groups-adduct-formation (i.e., at 420 nm).[4,5] Apparently, the mechanism of diethylbarbiturate potentiation of rhodanese inactivation by 2,4,6-

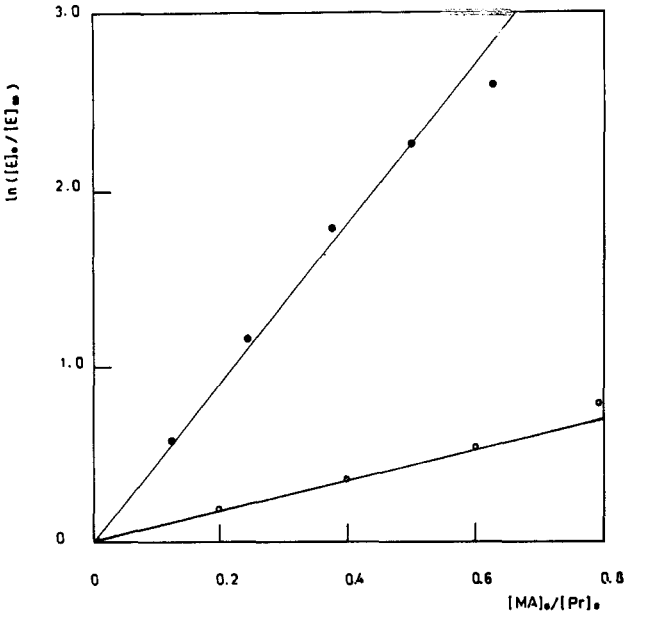

FIGURE 4 The same data as in Figure 3, are plotted according to Rakitzis and Malliopoulou[1] (see also the "Methods" section). Samples without (○) and with (●) diethylbarbiturate in the reaction medium.

trinitrobenzenesulphonate is to be sought at a stage subsequent to that of rhodanese free amino groups modification.

A higher than unity value of rhodanese inactivation to modification stoichiometry indicates the existence of self-regenerating reaction intermediates, such as free radicals. However, the inclusion in the reaction mixture of free radical traps, such as 1-methylimidazole-2-thiol and 1,1-diphenyl-2-picryl hydrazyl, under the conditions

TABLE 1

Kinetic analysis of rhodanese modification by 2,4,6-trinitrobenzene-sulphonate in the presence of 10 mM diethylbarbiturate in the reaction medium. Reaction conditions were those used at a high modifying agent/enzyme protein ratio. Rhodanese modification data were analysed graphically to determine n, the number of modifiable amino groups per protien molecule, and also the constants, k_i, and coefficients, c_i, of the protein modification equation (equation (1)). Modification (pseudo)first-order rate constants are given in h^{-1}. For details of the experimental, as well as the analytical procedures, used, see the text.

Reaction components	n	c_1	k_1	c_2	k_2
At 345 nm, pH 8.00					
Rhodanese	6.1	0.05	5.40	0.95	0.50
Rhodanese plus diethylbarbiturate	6.2	0.04	5.20	0.96	0.48
At 345 nm, pH 9.00					
Rhodanese	10.6	0.24	5.74	0.76	0.681
Rhodanese plus diethylbarbiturate	11.4	0.26	5.19	0.74	0.660
At 420 nm, pH 9.00					
Rhodanese	—	0.20	5.20	0.80	0.742
Rhodanese plus diethylbarbiturate	—	0.26	4.61	0.74	0.659

described in the "Methods" section, was without effect on the rate of diethylbarbiturate-potentiated rhodanese inactivation by 2,4,6-trinitrobenzenesulphonate. Similarly, the rate of the spontaneous decolourization of 1,1-diphenyl-2-picryl hydrazyl was not affected by the inclusion of 10 mM diethylbarbiturate in a reaction mixture containing sodium phosphate buffer 0.05 M, pH 8.00, sodium thiosulphate 0.01 M, rhodanese 33.7 μM, and 2,4,6-trinitrobenzenesulphonate 30 μM. Inclusion of catalase in the reaction mixture also was without effect on the diethylbarbiturate-potentiated rhodanese inactivation by 2,4,6-trinitrobenzenesulphonate. In this connection it is of interest that catalase has been found to prevent rhodanese inactivation by intermediates of oxygen reduction, generated by the reaction of this enzyme with phenylglyoxal and cyanide ions.[12] It should be noted that, while these results render highly unlikely the possible involvement of oxygen free radicals in the diethylbarbiturate-potentiation of trinitrophenylation-induced rhodanese inactivation, they nevertheless do not rule out the possible involvement of other types of free radical species.[13,14] Elucidation of the mechanism of the potentiation by diethylbarbiturate of 2,4,6-trinitrobenzenesulphonate-induced rhodanese inactivation, may prove to be of value in the understanding of the pharmacologic mechanism of action of diethylbarbiturate.

Acknowledgements

The technical assistance of Miss Irene Zosima is acknowledged.

References

1. Rakitzis, E.T. and Malliopoulou, T. (1985) *Biochem. J.*, **230,** 89–93.
2. Malliopoulou, T.B. and Rakitzis, E.T. (1988) *J. Enz. Inhib.*, **2,** 99–115.
3. Malliopoulou, V.A., Malliopoulou, T.B. and Rakitzis, E.T. (1988) *Anticancer Res.*, **8,** 1063–1064.
4. Goldfarb, A.R. (1966) *Biochemistry*, **5,** 2570–2574.
5. Kurono, Y., Ichioka, K., Mori, S. and Ikeda, K. (1981) *Pharmacol. Sci.*, **70,** 1297–1299.
6. Kezdy, F.S., Jas, J. and Bruillants, A. (1958) *Bull. Soc. Chim. Belg.*, **67,** 687–706.
7. Swinbourne, E.R. (1980) *J. Chem. Soc.*, 2371–2372.
8. Russell, J., Weng, L., Keim, P.S. and Heinrikson, R.L. (1978) *J. Biol. Chem.*, **253,** 8102–8108.
9. Frost, A.A. and Pearson, R.G. (1960) *Kinetics and Mechanism*, pp. 162–169, Wiley, New York.
10. Pavlic, M.R. and Zorko, M. (1978) *Biochim. Biophys. Acta*, **524,** 340–348.
11. Rakitzis, E.T. and Mallioupoulou, T.B. (1986) *Biochem. J.*, **237,** 589–591.
12. Canella, R., Berni, R. and D'Ottavio, P. (1983) *FEBS Letters*, **162,** 180–184.
13. Taylor, J.J., Wilson, R.L. and Kendall-Taylor, (1984) *FEBS Letters*, **176,** 337.
14. Halliwell, B. and Gutteridge, M.C. (1985) *Free Radicals in Biology and Medicine*, pp. 37–45, Clarendon Press, Oxford.