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KINETIC STUDY OF IRREVERSIBLE INHIBITION OF AN ENZYME CONSUMED IN THE REACTION IT CATALYSES. APPLICATION TO THE INHIBITION OF THE PUROMYCIN REACTION BY SPIRAMYCIN AND HYDROXYLAMINE

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A systematic procedure for the kinetic study of irreversible inhibition when the enzyme is consumed in the reaction which it catalyses, has been developed and analysed. Whereas in most reactions the enzymes are regenerated after each catalytic event and serve as reusable transacting effectors, in the consumed enzymes each catalytic center participates only once and there is no enzyme turnover. A systematic kinetic analysis of irreversible inhibition of these enzyme reactions is presented. Based on the algebraic criteria proposed in this work, it should be possible to evaluate either the mechanism of inhibition (complexing or non-complexing), or the type of inhibition (competitive, non-competitive, uncompetitive, mixed non-competitive). In addition, all kinetic constants involved in each case could be calculated. An experimental application of this analysis is also presented, concerning peptide bond formation *in vitro*. Using the puromycin reaction, which is a model reaction for the study of peptide bond formation *in vitro* and which follows the same kinetic law as the enzymes under study, we have found that: (i) the antibiotic spiramycin inhibits the puromycin reaction as a competitive irreversible inhibitor in a one step mechanism with an association rate constant equal to $1.3 \times 10^4\,M^{-1}s^{-1}$ and, (ii) hydroxylamine inhibits the same reaction as an irreversible non-competitive inhibitor also in a one step mechanism with a rate constant equal to $1.6 \times 10^{-3}\,M^{-1}s^{-1}$.

Keywords: Irreversible inhibition; Consumed enzymes; Puromycin reaction; Spiramycin; Protein biosynthesis; Hydroxylamine

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INTRODUCTION

Enzyme inhibition has always been an important field of study, not only because of its usefulness in providing valuable information on fundamental aspects of enzymatic catalysis and metabolic pathways, but also for its implications in pharmacology and toxicology. Compared to reversible inhibition, the kinetics of irreversible inhibition have received relatively little attention.[1-4] However, it is well established that irreversible modification of enzyme activity is important for studies on the nature of functional groups essential to enzymatic catalysis; such studies cannot be conducted with reversible inhibitors.[5-7]

The kinetics of irreversible enzyme modification in the presence of substrate have been extensively studied[8-12] and experimental studies have produced useful results both for inhibition,[11,13-16] or for activation kinetics.[17]

The purpose of this paper is to extend the study of irreversible inhibition to another type of reaction which follows the kinetic scheme of equation (1).

$$E + X \overset{K_x}{\rightleftharpoons} EX \xrightarrow{k_3} P + E^* \tag{1}$$

This reaction has been used extensively to describe: (i) enzyme reactions in which the enzyme is not regenerated after each catalytic event, but is consumed in the reaction it catalyzes as in the cases of RNA splicing[18] and puromycin reaction;[19] (ii) inactivation of enzymes by an irreversible inhibitor in the absence of substrate[20,21] and, (iii) affinity and photoaffinity labeling of enzymes.[22] In case (i) the reaction shown in (1) takes the form of equation (2)

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_3} P + E^* \tag{2}$$

while for cases (ii) and (iii) it takes the form of equation (3),

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k} E^*I \tag{3}$$

where S is the substrate, P is the product, I the irreversible inhibitor or alternatively the affinity or photoaffinity label and E^* the consumed or inactivated enzyme. These reactions will be analyzed kinetically in the presence of an irreversible inhibitor and a practical application of this analysis will be presented. The application concerns the puromycin reaction which

is a useful model system for studying the synthesis of individual peptide bonds *in vitro*. It is now generally accepted that puromycin acts as an analogue to the 3′ terminus of the aminoacyl-tRNA bound in the ribosomal A site.[23] The peptide bond is formed between Ac-Phe-tRNA and the free amino group of puromycin in the presence of 70S ribosomes, initiations factors, Poly (U), GTP, magnesium and ammonium ions, according to the scheme.

$$Ac\text{-}Phe\text{-}tRNA + Puromycin \xrightarrow{(70S\ Ribs,\ Poly(U),\ GTP,\ Ifs,\ Mg^{2+},\ NH_4^+)} Ac\text{-}Phe\text{-}Puromycin + tRNA$$

This complex reaction can be simplified by separating the first step (binding step), from the second step (peptide bond formation). In the binding step, the initiation ternary complex C [Ac-Phe-tRNA-70S Ribosome-Poly(U)] is formed, which is then isolated free of unbound donor Ac-Phe-tRNA. In the second step, the isolated complex C reacts with excess of puromycin (S) to give Ac-Phe-Puromycin (P), according to the reaction of equation (2). The enzyme (E) of reaction (2) has been replaced now by complex C and E^* by C^*. The form C^* represents the consumed enzyme after peptide bond formation. This enzyme species is not exactly inactivated, but, under the experimental conditions employed, it cannot be converted again to the active form C. Hence, the puromycin reaction follows the kinetic scheme of consumed enzymes depicted by equation (2) and its progress curve either in the absence or in the presence of an irreversible inhibitor should be described by the integrated rate law as will be shown in the next paragraph. In this work, we examine the puromycin reaction either in the presence of spiramycin or in the presence of hydroxylamine both of which act like irreversible inhibitors.

THEORY

It is known that the reaction (1) in the presence of excess X follows saturation kinetics[20,21] with an integrated rate law given by equation (4)

$$\ln \frac{E_0}{E_0 - E^*} = k_{obs} t \tag{4}$$

where the apparent rate constant k_{obs} is given by equation (5) and

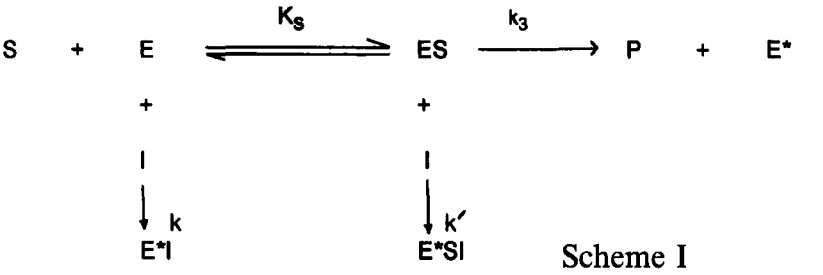
equation (6) for the reactions (2) and (3), respectively.

$$k_{obs} = \frac{k_3 S}{K_s + S} \tag{5}$$

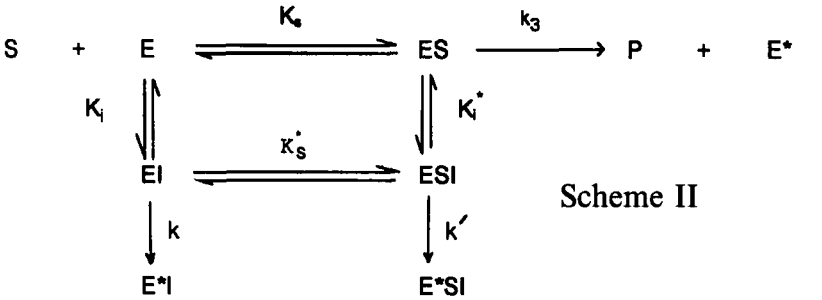
$$k_{obs} = \frac{k \cdot I}{K_i + I} \tag{6}$$

Both reactions (2) and (3), follow the same kinetic law and in the algebraic integrated equations only the symbols change. Our application concerns reaction (2) and therefore it's symbols are used in this study.

In the case where reaction (2) takes place in the presence of an irreversible inhibitor (I), Schemes I and II, depict the simplest models for one-step, and two-step mechanism of inhibition, respectively.



Scheme I



Scheme II

In Scheme I, the following types of inhibition exist: competitive $k' = 0$, non-competitive $k = k'$, mixed non-competitive $k \neq k'$ and uncompetitive $k = 0$. In Scheme II: competitive $K_i^* = \infty$, $K_s^* = \infty$; uncompetitive $K_i = \infty$, $K_s^* = 0$; non-competitive: $K_i = K_i^*$, $K_s = K_s^*$, $k = k'$ and mixed non-competitive $K_i \neq K_i^*, K_s \neq K_s^*$, and $k \neq k'$. For the above classification, we

have followed the general rules as they have been established for classical reversible[1-3] and irreversible inhibitors.[11,24] That is: in competitive inhibition, substrate binding prevents inhibitor binding; in non-competitive inhibition, substrate binding does not affect inhibitor binding, while in mixed non-competitive it does. For uncompetitive inhibition, substrate binding promotes inhibitor binding.

For both schemes, the integrated rate law is given by equation (7) (see Appendix)

$$\ln(P_\infty - P) = \ln P_\infty - A\,t \tag{7}$$

where P is the product at time t, P_∞ the product at infinite time and A is the apparent rate constant of enzyme (E) consumption. It is useful to notice here that when reaction (2) takes place in the absence of an irreversible inhibitor, P_∞ is equal to the total enzyme concentration (E_0) because all enzyme is converted to product. In this case the apparent rate constant A is equal to k_{obs} since the enzyme (E) is consumed only by S. In the presence of an irreversible inhibitor (I), P_∞ is not equal to E_0, since part of E_0 is inactivated by I. In this case, P_∞ is lower than E_0 and equal to the ratio B/A (see Appendix), where B is the apparent rate constant of product (P) formation multiplied by E_0. Table I shows the expressions for A, B and P_∞ for competitive, non-competitive, mixed non-competitive and uncompetitive inhibition for a one-step mechanism, as they have been derived in Appendix A. Similar calculations have been derived in Appendix B for a two-step mechanism and values for A, B and P_∞ are presented in Table II. From the plot $\ln P_\infty/(P_\infty - P)$ versus time according equation (7), the apparent rate constant A can be calculated as a slope and subsequently from P_∞ and A the apparent rate constant B can be calculated. For the

TABLE I Expressions for the apparent rate constants A and B and for the product formed at infinite time (P_∞), for one step mechanism (non-complexing type)

Type of inhibition	*A*	*B*	P_∞
competitive	$\frac{k_3(S) + kK_s(I)}{K_s + (S)}$	$\frac{k_3(S)(E_0)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{k_3(S) + kK_s(I)}$
non-competitive	$\frac{kK_s(I) + k(I)(S) + k_3(S)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{k_3(S) + kK_s(I) + k(I)(S)}$
mixed non-competitive	$\frac{kK_s(I) + k'(I)(S) + k_3(S)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{k_3(S) + kK_s(I) + k'(I)(S)}$
uncompetitive	$\frac{k_3(S) + k'(I)(S)}{K_s + (S)}$	$\frac{k_3(E_0)(S)}{K_s + (S)}$	$\frac{k_3(E_0)}{k_3 + k'(I)}$

TABLE II Expressions for the apparent rate constants A and B and for the product formed at infinite time (P_∞), for the two-step mechanism (complexing type)

Type of inhibition	*A*	*B*	P_∞
competitive	$\frac{k_3(S)}{K_s[1+((I)/K_i)]+(S)}+\frac{k(I)}{K_i[1+((S)/K_s)]+(I)}$	$\frac{k_3(E_0)(S)}{K_s[1+((I)/K_i)]+(S)}$	B\A
non-competitive	$\frac{k_3(S)}{(K_s+S)(1+I/K_i)}+\frac{k(I)}{(K_i+I)(1+S/K_s)}+\frac{k(I)(S)}{(K_s+S)(K_i+I)}$	$\frac{(k_3/(1+I/K_i))(E_0)(S)}{K_s+S}$	B/A
mixed non-competitive	$\frac{k_3(S)}{K_s[1+((I)/K_i)]+(S)[1+((I)/K_i^*)]}+\frac{k(I)}{K_i[1+((S)/K_s)]+(I)[1+((S)/K_s^*)]}+\frac{k'(S)(I)}{K_s^*[K_i+(I)]+(S)[K_i^*+(I)]}$	$\frac{k_3(E_0)(S)}{K_s[1+((I)/K_i)]+(S)[1+((I)/K_i^*)]}$	B\A
uncompetitive	$\frac{k_3K_i^*+k'(I)}{K_i^*[1+(K_s/(S))]+(I)}$	$\frac{k_3(E_0)(S)}{K_s+(S)[1+((I)/K_i^*)]}$	B/A

one-step mechanism (non-complexing type), B is independent of inhibitor (I) concentration, while for the two-step mechanism B is a function of (I). Thus, the plot of B versus (I) is capable of discriminating between the two mechanisms.

The evaluation of the type of inhibition for the one-step mechanism, comes from the plot of $1/P_\infty$ versus 1/S. When the inhibition is competitive, the plot is linear and intercepts the $1/P_\infty$ axis at the same point for all concentrations of I, while the slope varies, depending on the concentration of I. When the inhibition is non-competitive, both the slope and the intercept change as a function of I. When the inhibition is uncompetitive, the plot is a line parallel to the 1/S axis, because P_∞ is independent of S. In the case of the two-step mechanism, the evaluation of the type of inhibition comes from the plot of 1/B versus 1/S rather than the plot $1/P_\infty$ versus 1/S. For competitive inhibition, the plots are straight lines with constant intercept on the axis 1/B and slopes varying as a function of I. For non-competitive inhibition, the plots are straight lines with constant intercept on the axis 1/S and varying intercepts on the axis 1/B. For mixed non-competitive, the plots have variable intercepts on the axis 1/B and varying slopes; and for uncompetitive inhibition, the plots have a variable intercept while the slope remains constant.

MATERIALS AND METHODS

Materials

L-Phenylalanine, poly(U) GTP (disodium salt), ATP (disodium salt), puromycin dihydrochloride, and transfer ribonucleic acid and *Escherichia coli* strain W, were purchased from Sigma Chemical Co (U.K.). [^{3}H]-L-Phenylalanine was purchased from Amersham (U.K.). Zwittergent 3–12 (ZW) detergent (*N*-dodecyl-*N*, *N*-dimethyl-3-ammonium-1-propanesulfonate) was obtained from Calbiochem AG. Cellulose nitrate filterdisks (type HA, 24 mm diameter, 0.45 μm pore size) were purchased from Millipore Corporation.

Methods

Salt washed ribosomes (0.5 M NH_4Cl) and factors washable from ribosomes were prepared from frozen *E. coli* B cells.[19] Complex C was prepared and isolated on a cellulose nitrate disk as described elsewhere.[19] When required, complex C was desorbed[25] into a solution (zw-extract),

containing the detergent Zwittergent 3–12. The isolated complex C, either on a disk or in solution, was allowed to react with excess of puromycin for pre-determined time intervals and the reaction was terminated by adding a solution of NaOH. The product (Ac-Phe-Puromycin) was recovered via extraction in ethyl acetate,[19] and was expressed as a percentage of the isolated complex C on the filter disk (C_0).

The puromycin reaction was also performed in the presence of the afore-mentioned inhibitors, spiramycin or hydroxylamine. The procedure followed was exactly the same as that in the absence of inhibitor. It is known,[26] that both spiramycin and hydroxylamine inhibit the reaction almost irreversibly. All data presented in the figures denote the mean of values obtained from four experiments. The standard error of the mean (S.E.) was calculated according to Daniel.[27]

RESULTS

Figure 1 depicts the time course of the reaction between complex C isolated on a cellulose nitrate disk and 4×10^{-4} M puromycin in the absence and in the presence of increasing concentrations of spiramycin. In the absence of inhibitor, a straight line is obtained from the plot of $\ln C_0/(C_0 - P)$ versus time, until all complex C has been converted to product, Ac-Phe-Puromycin. It appears that the reaction follows pseudo-first order kinetics and reaches its end point after seven half lives. In the presence of spiramycin the rate of product formation is lower and finally it becomes zero. Finally, the reaction stops, although not all of C_0 has been converted to product. From the plateau of product formed at infinite time, P_∞ can be calculated. For each progress curve P_∞ is lower than C_0, depending on the concentrations of puromycin and spiramycin. The results of Figure 1 indicate that spiramycin inhibits product formation rather irreversibly. Similar behavior has been observed,[26] for the case in which complex C was in solution (zw-extract) and not on cellulose nitrate disks.

In order to investigate the reversibility of the inhibition, a systematic analysis of the puromycin reaction was followed. First, the data of Figure 1 were replotted in the form of equation (7), where P_∞ is not equal to C_0, since part of it has been inactivated by spiramycin. This P_∞ was calculated from the plateau of each progress curve as mentioned above. The resulting Figure 2 shows that the new plots are linear, meaning that there is a good fitting of the present data in the kinetic law of equation (7). The apparent rate constant A, at each concentration of puromycin (S) and spiramycin (I), is determined as a slope of the corresponding straight line of Figure 2.

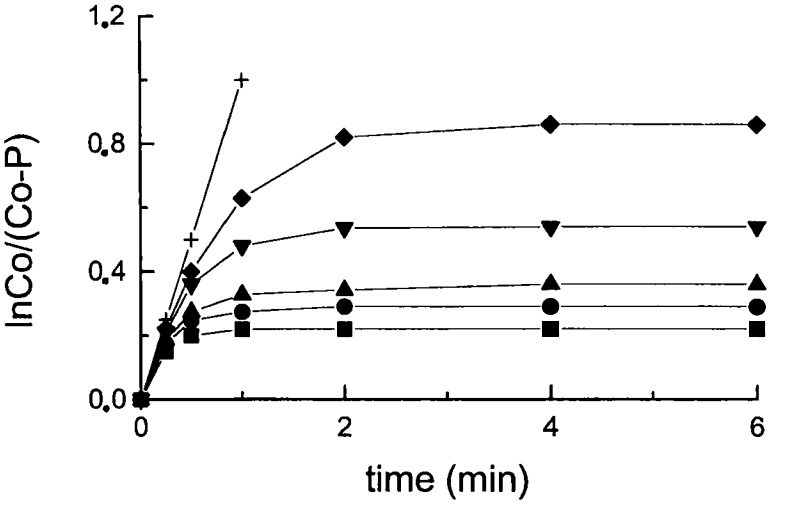


FIGURE 1 First order time plots for Ac-Phe-Puromycin formation. Complex C isolated on a cellulose nitrate disc reacts with (+) 4×10^{-4} M puromycin in the absence (control) and in the presence of spiramycin at the following concentrations: (◆) 2×10^{-6} M; (▼) 4×10^{-6} M; (▲) 6×10^{-6} M; (●) 8×10^{-6} M; (■) 1×10^{-5} M.

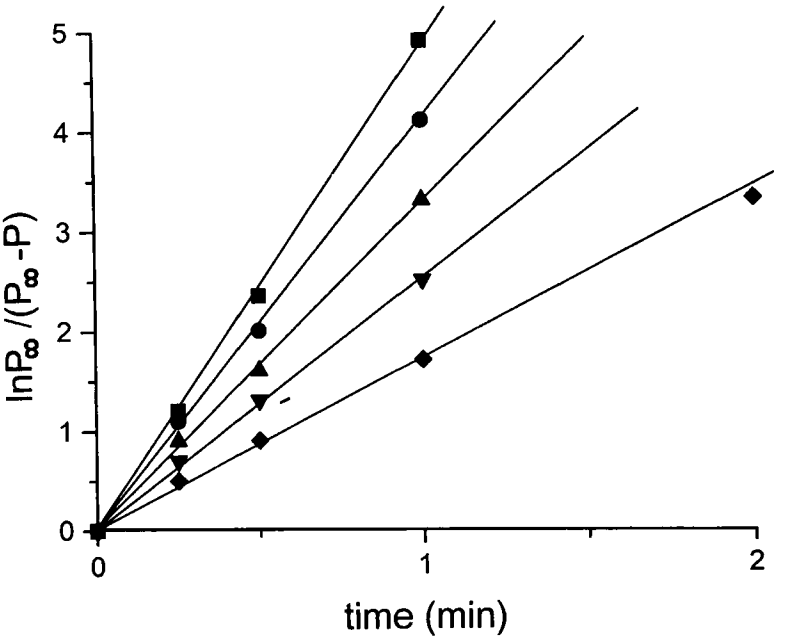


FIGURE 2 Plot of $\ln P_\infty/(P_\infty - P)$ versus time. In each case P_∞ is lower than C_0 and is calculated from the data of Figure 1.

The conformity of the data to the kinetic law of equation (7) may be taken as an indication that the reaction is irreversible. To further elucidate the mechanism of inhibition, calculation of the apparent rate constant B is needed. Having determined the values of P_∞ and A at each combination of S and I the apparent rate constant B can be obtained (see Appendix). A plot of B versus I, shows that B is independent of I (Figure 3). This is an indication that a one-step (non-complexing) mechanism is most probable. From the plot $1/P_\infty$ versus 1/S (Figure 4) it is evident that the functions are straight lines with constant intercept on the axis $1/P_\infty$ and slopes which depend on the concentration of I. In accordance with the previous discriminative rules, it is ascertained that the inhibition is competitive. Figure 4 also, allows the calculation of the second order association constant for C*I formation which is equal to $1.3 \pm 0.1 \times 10^4\,M^{-1}s^{-1}$. This value is somewhat lower than the corresponding value when complex C is in solution (ZW-extract).[26]

The present study was applied to the puromycin reaction in the presence of hydroxylamine. It is known that hydroxylamine is a chemical reagent which reacts with activated esters to give hydroxamic acids.[28] Hydroxylamine reacts also with Ac-Phe-tRNA either free or bound to ribosomes. This reaction follows the one-step mechanism with a second order rate constant equal[26] to $1.3 \times 10^{-3}\,M^{-1}s^{-1}$. Hydroxylamine inhibits the puromycin

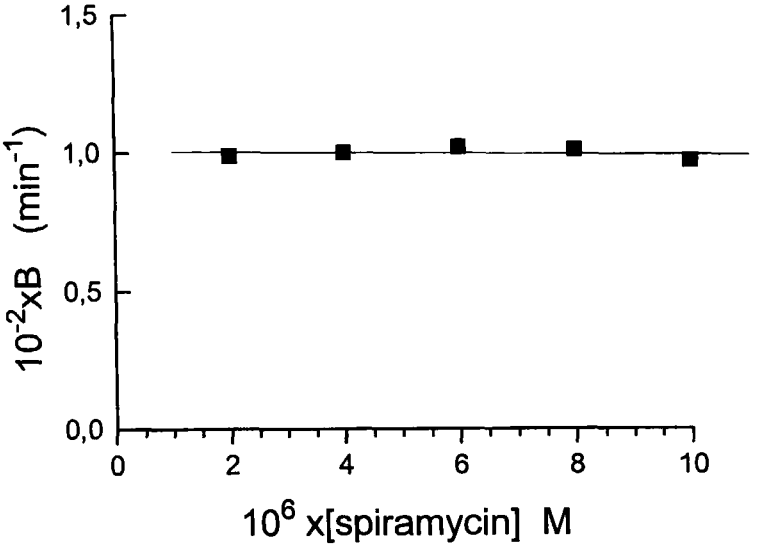


FIGURE 3 Variation of the apparent rate constant B as a function of inhibitor (I) concentration. The concentration of puromycin is 4×10^{-4} M.

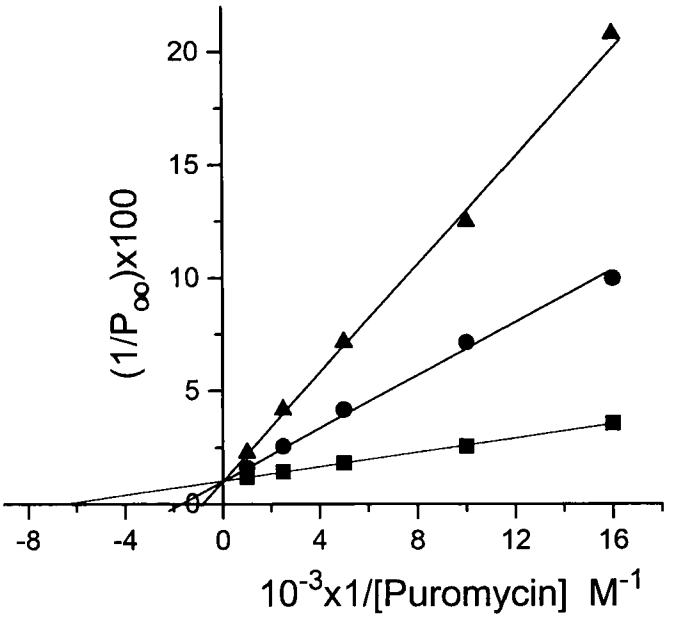


FIGURE 4 Dependence of the reciprocal of P_∞ versus the reciprocal of puromycin concentration. The concentration of spiramycin is; (■) 1×10^{-6} M; (●) 4×10^{-6} M; (▲) 8×10^{-6} M.

reaction because it does not inhibit the catalytic centre of the enzyme but it decomposes the bound substrate (Ac-Phe-tRNA). Therefore, we considered it as an irreversible inhibitor and consequently the puromycin reaction was examined in its presence, according to the analysis which has been already described.

Figure 5 presents the experimental data of the course of the reaction between complex C in solution (zw-extract) and 3×10^{-5} M puromycin in the absence and in the presence of increasing concentrations of hydroxylamine. It is seen that hydroxylamine inhibits product formation in a similar way to spiramycin. Thus, complex C is consumed by two different and parallel irreversible reactions. We have previously shown,[26] that hydroxylamine acts as a chemical reagent in a one-step mechanism, without formation of a reversible complex CI prior to C consumption. Therefore, the reactions are described by the one-step mechanism of Scheme I, although we don't know the type of inhibition. This conclusion concerning the mechanism is also confirmed by the analysis of the present study: the results of Figure 5 are replotted in the form of $\ln(P_\infty - P)$ versus time (data not shown). From the slope of this linear plot the apparent rate

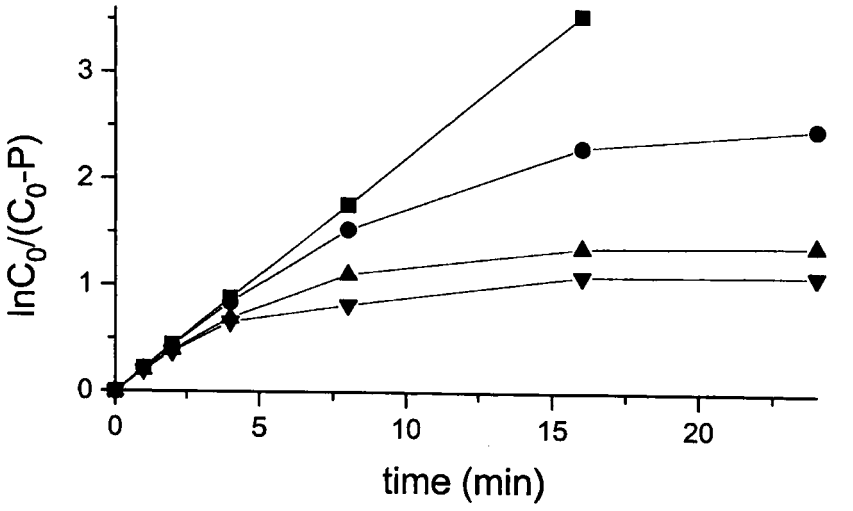


FIGURE 5 First order time plots for Ac-Phe-Puromycin formation in the absence (■) and in the presence of hydroxylamine at the following concentrations: (●) 0.2 M; (▲) 0.6 M; and (▼) 0.8 M. Complex C reacts in solution (zw-extract) and the concentration of puromycin is 3×10^{-5} M.

constant A according to equation (7) is obtained. From P_∞ and A the apparent rate constant B is calculated. Plotting B versus hydroxylamine concentration (data not shown), results in a linear plot parallel to the axis of I, which according to the analysis proposed above means that a one-step mechanism of inhibition is most probable.

Because hydroxylamine reacts with complex C,[26] the inhibition type could be either competitive or non-competitive. According to Table I, the discrimination comes from the plot $1/P_\infty$ versus 1/S, rearranged as in equation (8)

$$\frac{1}{P_\infty} = \frac{k(I) + k_3}{k_3 C_0} + \frac{kK_s(I)}{k_3 C_0}\frac{1}{(S)}. \tag{8}$$

Figure 6 shows that both, intercept and slope, vary with the concentration of I. Therefore, the inhibition is non-competitive. Plotting the intercept of axis $1/P_\infty$ of Figure 6 versus inhibitor concentration (I), results in the linear relation shown in Figure 7. From this slope of the linear relationship according to the equation (9)

$$\text{intercept} = \frac{1}{C_0} + \frac{k}{k_3 C_0}(I) \tag{9}$$

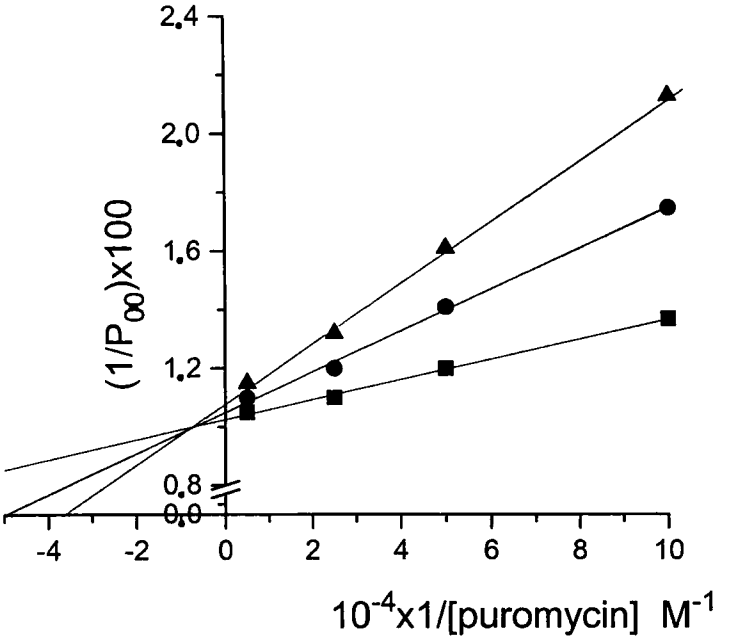


FIGURE 6 Double reciprocal plot ($1/P_\infty$ versus 1/S) at the following concentrations of hydroxylamine: (▲) 0.3 M; (●) 0.6 M; and (■) 0.9 M.

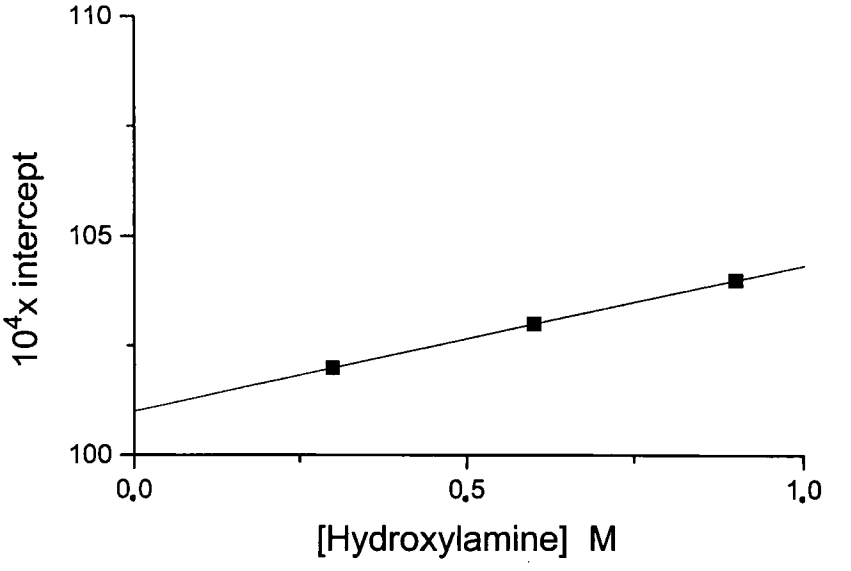


FIGURE 7 Replot of the double reciprocal plot of Figure 6. The $1/P_\infty$ axis intercepts of Figure 6 are replotted versus the concentration of hydroxylamine.

a value for the second order rate constant is calculated as $1.6 \pm 0.1 \times 10^{-3}\,M^{-1}s^{-1}$, almost the same as that calculated in the absence of puromycin.[26] The fact that the slope and the intercept according to equation 8 result in the same value for the rate constant, supports the argument that the reaction is of a simple non-competitive and not of a mixed non-competitive type. We have to point out, that the same results could be concluded with computer fitting that is more robust and relatively easy.

DISCUSSION

Two methods may be employed for the determination of the rate constants for the irreversible inhibition of enzyme activity, one in the presence and the other in the absence of substrate. In the absence of substrate, the conventional method, the examination is carried out as follows: from an enzyme-inhibitor incubation mixture, aliquots are taken at definite time intervals and assayed for the remaining enzyme activity. This method not only is laborious but also too slow to be applied to fast reactions.[29] In contrast, the method in the presence of substrate is easier to perform, and the apparent rate constant can be obtained in one single experiment. These advantages of the study of irreversible inhibition in the presence of substrate, were extended in the case of consumed enzymes during the enzyme reaction. These enzymes follow different kinetics and have been studied using Tsou's approximations.[12] The present study of inhibition of the puromycin reaction by spiramycin and hydroxylamine obtained the values for the rate constants of interaction which are useful for the study of ribosomes and protein biosynthesis.

It is known that the rates of formation and dissociation of the inhibitor-enzyme complex may be examined either indirectly from the inhibitor's effect on the time course of enzyme turnover of substrate, or directly by monitoring the time course of the spectroscopic signal generated by complex formation.[30] The direct method,[31] gives for free ribosomes and spiramycin an association rate constant equal to $2.3 \times 10^4\,M^{-1}s^{-1}$. The indirect method as applied here, gives a k association equal to $1.3 \times 10^4\,M^{-1}s^{-1}$, which is comparable to the direct value obtained. The fact that the two values are close validates our method. In addition, this value is in agreement with that obtained by the discontinuous method (data not shown). This indirect approach is generally successful, when the association rate constants for the inhibitor is significantly smaller than that of the substrate.[30] Here, the value of the calculated rate constant $1.3 \times 10^4\,M^{-1}s^{-1}$

being substantially less than that for the substrates, means that this condition is fulfilled. With ternary complex C in solution, the association rate constant for spiramycin has been calculated[26] as $3.3 \times 10^4\,M^{-1}s^{-1}$. The difference in the value of the constant measured for the two cases, namely the cellulose nitrate disk and zw-extract, could be explained by the difference in the experimental conditions. In the present experiments the ternary complex C is isolated on a cellulose nitrate disk and probably interface problems may exist. It is also known that with immobilized enzymes there is a difference in the kinetic behavior from when, the enzyme is in solution.[32] This difference has also been observed in parallel studies of reversible inhibitors of puromycin reaction, with complex C either on disk or in solution.[33] For all inhibitors tested under these two different conditions[33] the type of inhibition remained the same although kinetic constants varied a little.

A point of interest from the present results is the low value of the calculated association rate constant of spiramycin. In the case of inhibitors of peptidyl-transferase, it is known that all rate constants calculated either directly[31,34] or indirectly[26,35-36] are lower than the lower limit of diffusion control, by at least two or three orders of magnitude. This surprising fact that is also ascertained in this work needs explanation. Do all these inhibitors act as slow binding inhibitors or perhaps as reaction intermediates or is there always a rapid equilibrium with formation of an initial weak complex, which is kinetically insignificant and its detection is too difficult? It is also known[37] that, while association rate constants for natural ribosome substrates are in the range of diffusion control, these constants become lower than the expected diffusion control limited values when the substrates lose their natural form. For example: Phe-tRNA as a complex with GTP and EF-Tu has a k association for programmed ribosome equal to $1 \times 10^7\,M^{-1}s^{-1}$, while Phe-tRNA without GTP and EF-Tu as a complex has a k association equal[37] to $1 \times 10^4\,M^{-1}s^{-1}$. Thus, the unexpectedly found low values for association constants of ribosomes-inhibitors complexes, may not be caused by inhibitors of low molecular weight. This might have to do with the ability of the ribosome to prefer or to select the bound ligands via lowering the association constants, something which may contribute to the ribosome translation machine. Such a low k association could also exist in the case of puromycin which in fact is not a natural substrate, but actually a peptidyl-transferase inhibitor. This possibility perhaps could render invalid the kinetic treatment of the puromycin reaction. However, in the range of puromycin concentrations used in the presents experiments, it appears that the kinetic treatment is valid because with the

exception of the linear double reciprocal plots, the reaction follows the integrated rate law up to more than 95% depletion.[19]

The validity of the kinetic method is also obvious in the case of hydroxylamine. The second order rate constant k_{on} in the presence of puromycin was found to be almost equal to that measured in the absence of puromycin.[26] Moreover, the type of inhibition was clarified, which would have been impossible without the present kinetic models. In addition, the fact that not a competitive but another type of inhibition was concluded, confirms the analysis presented here. These two inhibitors, spiramycin and hydroxylamine could also have been classified in another way, according to their mode of action: i.e. hydroxylamine as a group specific chemical reagent and spiramycin as a slow binding, slowly reversible inhibitor. The almost irreversible action of spiramycin was also recently confirmed[38] by studies of the binding of radioactive dihydrospiramycin to ribosomal proteins and RNA. The application of the kinetic scheme presented here has many advantages and can be used in many cases such as: (i) in affinity and photoaffinity labelling of enzymes; (ii) in inactivation of enzymes either from suicide substrates or k_{cat} inhibitors; (iii) in enzyme reactions of the form $E_A + S \rightleftharpoons E_AS \rightarrow P + E$ where E_A is the complex of enzyme with (A), the first of the two ordered substrates and S is the second and, (iv) in enzyme reactions of consumed enzymes. Here, the enzyme activity is not necessarily destroyed in the consumed form but this form does not participate in more than one cycle over the substrate. In the latter case the following enzymes are included: type (i) restriction endonuclease;[39] poly (ADP-ribose) synthetase;[40] transmethylase for O^6-methylguanine,[41] plus RNA[18] and protein[42] splicing.

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APPENDIX

A. One-step mechanism, mixed non-competitive inhibition (Scheme I)

Assumptions: $S \gg E_0$, $I \gg E_0$ and at zero time there is a rapid equilibrium between E, S and ES. Let E_T be the total concentration of non-inactivated

enzyme and E_0 the total enzyme concentration then,

$$(E_T) = (E) + (ES) \tag{A.1}$$

$$(E_0) = (E_T) + (E^*I) + (E^*SI) \tag{A.2}$$

From the rapid equilibrium, we also have,

$$K_s = \frac{(E)(S)}{(ES)} \tag{A.3}$$

$$(ES) = \frac{(E_T)(S)}{K_s + (S)} \tag{A.4}$$

$$(E) = \frac{K_s(E_T)}{K_s + (S)} \tag{A.5}$$

The differential of non-inactivated enzyme is,

$$-\frac{d(E_T)}{dt} = k(E)(I) + (ES)[k'(I) + k_3]. \tag{A.6}$$

By replacing E and ES and integrating for time t then,

$$(E_T) = (E_0)e^{-At} \tag{A.7}$$

where,

$$A = \frac{kK_s(I) + k'(S)(I) + k_3(S)}{K_s + (S)}. \tag{A.8}$$

The differential of product formation is given by:

$$\frac{d(P)}{dt} = k_3(ES) = Be^{-At} \tag{A.9}$$

where,

$$B = \frac{k_3(E_0)(S)}{K_s + (S)} \tag{A.10}$$

and by integration for time t,

$$[P] = \frac{B}{A}(1 - e^{-At}) \tag{A.11}$$

When t approaches infinity, the product concentration is given by,

$$[P_\infty] = B/A \tag{A.12}$$

By combining equations (A.11) and (A.12) we obtain,

$$\ln(P_\infty - P) = \ln P_\infty - At \tag{A.13}$$

Treating similarly Scheme I in the case of competitive and uncompetitive inhibition, we obtain the corresponding integrated rate law (as equation (A.13)) and the expressions of A, B and P_∞ (Table I). Alternatively, the competitive, non-competitive and uncompetitive forms may be obtained by substituting the appropriate values for k and k′ in the formulae for the mixed non-competitive case, which is the generalized case.

B. Two-step mechanism, mixed non-competitive inhibition (Scheme II)

We assume that $S \gg E_0$ and $I \gg E_0$, and that at zero time there is a rapid equilibrium among E, S, I, ES, EI and ESI. So,

$$K_s = \frac{(E)(S)}{(ES)} \tag{B.1}$$

$$K_i = \frac{(E)(I)}{(EI)} \tag{B.2}$$

$$K_i^* = \frac{(ES)(I)}{(ESI)} \tag{B.3}$$

$$K_s^* = \frac{(EI)(S)}{(ESI)} \tag{B.4}$$

where E_T is the total concentration of non-inactivated enzyme (E) and E_0 the total concentration of enzyme. Then,

$$(E_T) = (E) + (ES) + (EI) + (ESI) \tag{B.5}$$

$$(E_0) = (E_T) + (P) + (E^*I) + (E^*SI) \tag{B.6}$$

Combining equations (B.1)–(B.6),

$$(ES) = \frac{(S)(E_T)}{K_S(1 + I/K_i) + (S)(1 + (I/K_i^*))} \tag{B.7}$$

$$(EI) = \frac{(I)(E_T)}{K_i(1 + S/K_s) + (I)(1 + (S/K_s^*))} \tag{B.8}$$

$$(ESI) = \frac{(S)(I)(E_T)}{K_i^*(K_s + S) + (I)(K_s^* + S)} \tag{B.9}$$

The differential of non-inactivated enzyme (E_T) is given by the equation:

$$-\frac{d(E_T)}{dt} = k_3(ES) + k(EI) + k'(ESI) = A[E_T] \tag{B.10}$$

where,

$$A = \frac{k_3(S)}{K_s(1 + (I/K_i)) + (S)(1 + (I/K_i^*))} + \frac{k(I)}{K_i(1 + (S/K_s)) + (I)(1 + (S/K_s^*))} + \frac{k'(S)(I)}{K_s^*(K_i + I) + (S)(K_i^* + I)} \tag{B.11}$$

Integration of equation (B.10) leads to equation (B.12) which gives the non-inactivated enzyme concentration at any time t,

$$(E_T) = (E_0)e^{-At} \tag{B.12}$$

since at zero time, E_T is equal to E_0. The differential of product formation is given by

$$\frac{d(P)}{dt} = k_3(ES) = Be^{-At} \tag{B.13}$$

where B is given by,

$$B = \frac{k_3(S)(E_0)}{K_s(1 + (I/K_i)) + (S)(1 + (I/K_i^*))} \tag{B.14}$$

By integrating equation (B.13) for time t, then

$$[P] = \frac{B}{A}(1 - e^{-At}) \tag{B.15}$$

When t approaches infinity, the product concentration P_∞ is given by,

$$[P_\infty] = B/A \tag{B.16}$$

By combining equations (B.15) and (B.16) we obtain

$$\ln(P_\infty - P) = \ln P_\infty - At \tag{B.17}$$

For competitive, non-competitive and uncompetitive inhibition, $K_i^* = \infty$, $K_s^* = \infty$; $K_i = K_i^*$, $K_s = K_s^*$, $k = k'$; and $K_i = \infty$, $K_s^* = 0$ in equations (B.11) and (B.14) respectively, and the individuals expressions are given in Table II.

References

[1] Laidler, K.J. and Bunting, P.S. (1973) *The Chemical Kinetics of Enzymes Action*, 2nd edition. Clavendon Press; Oxford.
[2] Segel, I.H. (1975) *Enzymes Kinetics*. Wiley-Interscience; New York.
[3] Cornish-Bowden, A. (1979) *Fundamentals of Enzymes Kinetics*. Butterworths; London.
[4] Wong, J.T.F. (1975) *Kinetics of Enzyme Mechanism*. Academic Press; London.
[5] Singer, S.J. (1967) *Adv. Protein Chem.*, **22**, 1–54.
[6] Shaw, E. (1970) *The Enzymes* (Boyer, P.D., ed)., Vol. 1, pp. 91–146 3rd edition. Academic Press; New York
[7] Wold, F. (1977) *Meth. Enzymol.*, **46**, 3–14.
[8] Tsou, C.L. (1965a) *Acta Biochim. Biophys. Sin.*, **5**, 398–408.
[9] Tsou, C.L. (1965b) *Acta Biochim. Biophys. Sin.*, **5**, 409–417.
[10] Rakitzis, E. (1984) *Biochem. J.*, **217**, 341–351.
[11] Tian, W.X. and Tsou, C.L. (1982) *Biochemistry*, **21**, 1028–1032.
[12] Tsou, C.L. (1988) *Adv. Enzym. Relat. Areas Mol. Biol.*, **61**, 381–436.
[13] Bieth, J.G. (1984) *Biochem. Med.*, **32**, 387–397.
[14] Harper, J.W. and Powers, J.C. (1984) *J. Am. Chem. Soc.*, **106**, 7618–7619.
[15] Leytus, S.P., Toledo, D.L. and Mangel, W.F. (1984) *Biochim. Biophys. Acta*, **788**, 74–86.
[16] Mason, R.W., Green, G.D.J. and Barrett, A.J. (1985) *Biochem. J.*, **226**, 223–241.
[17] Liu, W., Zhao, K.Y. and Tsou, C.L. (1985) *Eur. J. Biochem.*, **151**, 525–529.
[18] Bass, B.L. and Cech, T.R. (1984) *Nature (Lond.)*, **308**, 820–826.
[19] Synetos, D. and Coutsogeorgopoulos, C. (1987) *Biochim. Biophys. Acta*, **923**, 275–285.
[20] Kitz, R. and Wilson, I.B. (1962) *J. Biol. Chem.*, **237**, 3245–3249.
[21] Malkom, A.D.B. and Radda, G.K. (1970) *Eur. J. Biochem.*, **15**, 555–561.
[22] Schloss, J.V. (1989) In: *Target Sites of Herbicide Action* (Boger, B., and Sandmann, G. eds) pp. 165–245. CRC Press Inc.; Boca Raton, Florida.
[23] Gale, E.F., Cundlifee, E., Reynolds, P.E., Richmond, M.H. and Waring, M.J. (1981) *The Molecular basis of Antibiotic Action*, pp. 410–412 2nd ed. John Wiley and Sons; New York.
[24] Teruel, J.A., Tudela, J., Belda, F.F., Carmona, F.G., Fernandez, J.C.G. and Canovas, G. (1987) *Biochim. Biophys. Acta*, **911**, 256–260.

[25] Theocharis, D. and Coutsogeorgopoulos, C. (1989) *Anal. Biochem.*, **176**, 278–283.
[26] Dinos, G., Synetos, D. and Coutsogeorgopoulos, C. (1993) *Biochemistry*, **31**, 10638–10647.
[27] Daniel, W.W. (1988) *Biostatistics: A Foundation for Analysis in the Health Sciences*, pp. 98–106, 2nd ed. John Wiley & Sons; New York.
[28] Eigner, E.A. and Loftfield, R.B. (1981) In: *RNA and Protein Synthesis*, (Moldave, K., ed.) pp. 256–274. Academic Press; New York.
[29] Wang, Z.-X., Preiss, B. and Tsou, C.L. (1988) *Biochemistry*, **27**, 5095–5100.
[30] Kurz, L.C., Moix, L., Riley, M.C. and Frieden, C. (1992) *Biochemistry*, **31**, 39–48.
[31] DiGiambattista, M., Engelborghs, Y., Nyssen, E. and Cocito, C. (1987) *J. Biol. Chem.*, **262**, 8591–8597.
[32] Goldstein, L. (1976) *Meth. Enzymol.*, **64**, 397–450.
[33] Theocharis, D., Synetos, D., Kalpaxis, D., Drainas, D. and Coutsogeorgopoulos, C. (1992) *Arch. Biochem. Biophys.*, **292**, 266–272.
[34] Langlois, R., Cantor, C.R., Vince, R. and Pestka, S. (1977) *Biochemistry*, **16**, 2349–2356.
[35] Kallia-Raftopoulos, S., Kalpaxis, D. and Coutsogeorgopoulos, C. (1992) *Arch. Biochem. Biophys.*, **298**, 332–339.
[36] Theocharis, D. and Coutsogeorgopoulos, C. (1992) *Biochemistry*, **31**, 5861–5868.
[37] Pingoud, A., Gast, F.-U. and Peters, F. (1990) *Biochim. Biophys. Acta*, **1050**, 252–258.
[38] Bischof, O., Urlaub, H., Kruft, V. and Wittmann-Liebold, B. (1995) *J. Biol. Chem.*, **270**, 23060–23064.
[39] Eskin, B. and Linn, S. (1972) *J. Biol. Chem.*, **247**, 6183–6191.
[40] Jump, D.B. and Smulson, M. (1980) *Biochemistry*, **19**, 1024–1030.
[41] Robins, P. and Cairns, J. (1977) *Nature (Lond.)*, **280**, 74–76.
[42] Cooper, A.A. and Stevens, T.H. (1995) *Trends Biochem. Sci.*, **20**, 351–356.