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RATE CONSTANTS OF INDIVIDUAL STEPS IN PAPAIN-CATALYSED REACTIONS

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Summary

Rate constants for acylation of papain (EC 3.4.22.2) by specific substrates and its subsequent deacylation are derived from kinetic analysis of the reactions in the presence of aminoacetonitrile and methanol. Methyl and ethyl hippurate and methyl *N*-benzyloxycarbonylglycinate have marginally higher values of rate constants for acylation than for deacylation, while the reverse is true for ethyl *N*-benzoyl-L-argininate. Both acylation and deacylation are rate-determining for these substrates, while only deacylation is rate-determining for methyl-*N*-acetyl-L-phenylalanylglycinate. Deacylation is the only rate-determining step for *p*-nitrophenyl esters of hippuric acid, *N*-benzyloxycarbonylglycine and *N*-acetyl-L-phenylalanylglycine. These results are discussed in relation to those from inactivation of the enzyme by alkylating agent in the presence of substrate.

Introduction

It is now widely accepted (e.g., see ref. 1) that the minimal mechanistic scheme for papain (EC 3.4.22.2)-catalysed reactions is Eqn. 1:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} ES' \overset{k_3'}{\rightarrow} E + P_2 \quad (1)$$
$$\qquad\qquad\qquad\quad +P_1$$

where k_1 and k_{-1} are rate constants for formation and dissociation of enzyme · substrate complex, k_2 the rate constant for acylation of the enzyme and k_3' (or $k_3[H_2O]$) the rate constant for deacylation through hydrolysis: ES is the

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Abbreviations: L-BAEE, ethyl α-*N*-benzoyl-L-argininate; D-BAEE, ethyl α-*N*-benzoyl-D-argininate.

enzyme · substrate complex, ES′ the acyl-enzyme intermediate, P_1 the amine or alcohol formed from the leaving group and P_2 the acid from the acyl portion of the substrate. The similarity of the magnitudes of k_{cat} for both aryl and alkyl esters of hippuric acid, except isopropyl and *n*-butyl hippurate, [2] and of α-*N*-benzyloxycarbonylglycine [3] was interpreted as indicating that for these substrates deacylation is the rate-determining step. For another specific ester substrate, ethyl α-*N*-benzoyl-L-argininate (L-BAEE), kinetic study in conjunction with α-*N*-benzoyl-L-argininamide yielded the conclusion [4] that k_2/k_3' is 3.2. By studying the kinetics of inactivation of papain through alkylation of the essential sulphydryl group by chloroacetic acid, it was however concluded [5] that, while the rate-determining step for hydrolysis of ethyl hippurate is deacylation, for hydrolysis of L-BAEE it is acylation. This conclusion was based on the observation that the rate of inactivation approaches zero at saturating concentration of ethyl hippurate, but is independent of L-BAEE concentration. Ambiguities in this conclusion are raised by the evidence for non-productive binding of L-BAEE [6,7] and of alkylating agent [7], and by the finding that papain and ficin [8] are more reactive towards alkylation with chloroacetamide in the presence of saturating concentration of the competitive inhibitor D-BAEE. Hence, even though deacylation is partially rate-determining for L-BAEE, a decrease in alkylation rate may not be observed if the enzyme · substrate complex has greater reactivity than the enzyme. Conversely, even if deacylation is not rate-determining in the hydrolysis of ethyl hippurate, a decrease in alkylation rate will be observed if the enzyme · substrate complex does not react with the alkylating agent.

In this paper it is shown from kinetic study in the presence of aminoacetonitrile or methanol that, although deacylation is slower than acylation for alkyl esters of hippuric acid and *N*-benzyloxycarbonylglycine and the reverse is true for L-BAEE, the faster step in each case is still kinetically significant. For methyl *N*-acetyl-L-phenylalanylglycinate, a highly specific substrate for papain, however, it is shown that $k_2 >> k_3'$. The apparent discrepancy between the results from kinetic study with nucleophiles and those from study of reactivity of the essential sulphydryl group to alkylation in the presence of ethyl hippurate is suggested to be due to the capacity of bound substrate to protect this group.

Materials and Methods

Methyl and ethyl hippurate were prepared by condensation of the acid with the alcohols in the presence of conc. sulphuric acid; m.p. 80—82°C for methyl ester, 59—60°C for ethyl ester. Methyl *N*-benzyloxycarbonylglycinate was prepared by reaction of methyl glycinate hydrochloride with benzyl chloroformate and purified by silica gel chromatography (petrol ether : ether, 4 : 1). L-BAEE hydrochloride was obtained from Sigma, m.p. 130—132°C. Other substrates and competitive inhibitors were prepared as described previously [9]. Aminoacetonitrile was prepared from its bisulphate or hydrochloride by the method of Cook et al. [10], stored under nitrogen in the cold and discarded when brown colour developed. Chloroacetic acid, obtained from Hopkin and Williams, was of analytical grade and redistilled before use, the distillate being

collected at 182—192°C. Its concentration was determined by titration with standardised sodium hydroxide. Twice-recrystallised papain was obtained from Worthington and was activated before use by 0.01 M L-cysteine or *p*-thiocresol, and 10^{-3} M EDTA.

Kinetic studies were performed in the presence of 0.1—0.3 M NaCl, 10^{-3} M EDTA and 0.01 M L-cysteine. The rates of acid (P_2) production from alkyl esters were followed by titrimetry, and the rates of *p*-nitrophenol (P_1) production from *p*-nitrophenyl esters were followed by spectrophotometry as described previously [9]. Rates of inactivation of papain by chloroacetic acid in the presence of substrate and competitive inhibitor were measured by a method similar to that described by Sluyterman [5]. Enzyme concentrations were determined with methyl hippurate (k_{cat} = 4.0.4 s^{-1} at 35°C, pH 6.0) as standard.

Results and Discussion

Kinetic analysis of papain-catalysed reactions in the presence of other nucleophiles than water can yield information on the rate constants of acylation steps [11—14]. The mechanistic scheme in this case is Eqn. 2:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3'} E + P_2 \quad (2)$$

$$ES' + P_1 \xrightarrow{k_4[N]} E + P_3$$

where k_4 is the rate constant for deacylation with the nucleophile N and P_3 is the acyl derivative formed with N. From steady-state assumption, it can be shown that if P_1 production is followed

$$k_{cat,1} = \frac{k_2(k_3' + k_4[N])}{k_2 + k_3' + k_4[N]} \quad (3)$$

whereas if P_2 production is followed,

$$\frac{1}{k_{cat,2}} = \frac{k_2 + k_3'}{k_2 k_3'} + \frac{k_4}{k_2 k_3'}[N] \quad (4)$$

and

$$\frac{K_m}{k_{cat,2}} = \frac{K_s}{k_2} + \frac{k_4 K_s}{k_2 k_3'}[N] \quad (5)$$

where $K_s = (k_{-1} + k_2)/k_1$. By studying the effect of variation of N on $k_{cat,1}$, $k_{cat,2}$ and K_m, the values for k_2, k_3' and k_4 can be calculated.

Aminoacetonitrile, originally shown to be a good nucleophile for the deacylation of *trans*-cinnamoylpapain, [15] was used as the competing nucleophile in the papain-catalysed reactions of methyl and ethyl hippurate, methyl-*N*-benzyloxycarbonylglycinate, methyl-*N*-acetyl-L-phenylalanylglycinate and L-BAEE. The plots between $1/k_{cat,2}$ and [N], calculated as the unprotonated form assuming pK_a of 5.3 [16], are shown in Fig. 1 and the plots between $K_m/k_{cat,2}$

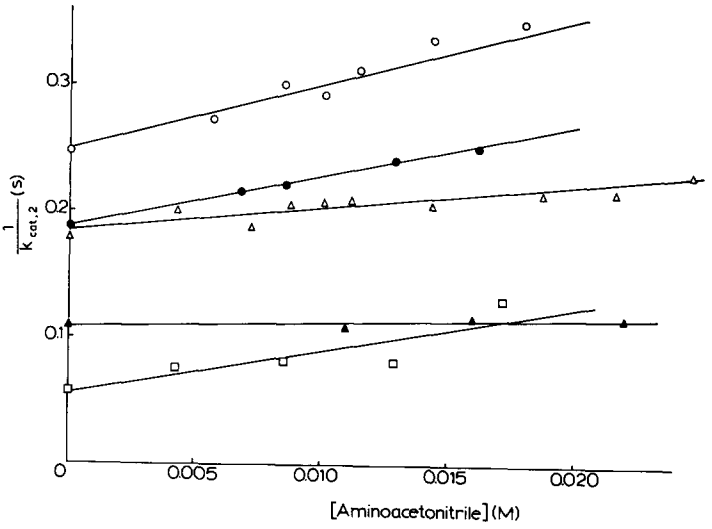

Fig. 1. Plots between $1/k_{cat,2}$ and concentration of aminoacetonitrile for papain-catalysed reactions of methyl hippurate (○), ethyl hippurate (●), methyl *N*-benzyloxycarbonylglycinate (△), methyl *N*-acetyl-L-phenylalanylglycinate (▲) and L-BAEE (□); 35°C, pH 7.0.

and [N] are shown in Fig. 2. The values for k_2, k_3', k_4 and K_s calculated from the slopes and intercepts of these plots are given in Table I, together with the values calculated from similar plots with methanol as the nucleophile. The values of k_2 and k_3' are comparable, although k_4 differs by a very large factor with the two different nucleophiles. It is likely, therefore, that the effect of both added nucleophiles on reaction rates is due mainly to their nucleophilic activity, and not to change in solvent properties.

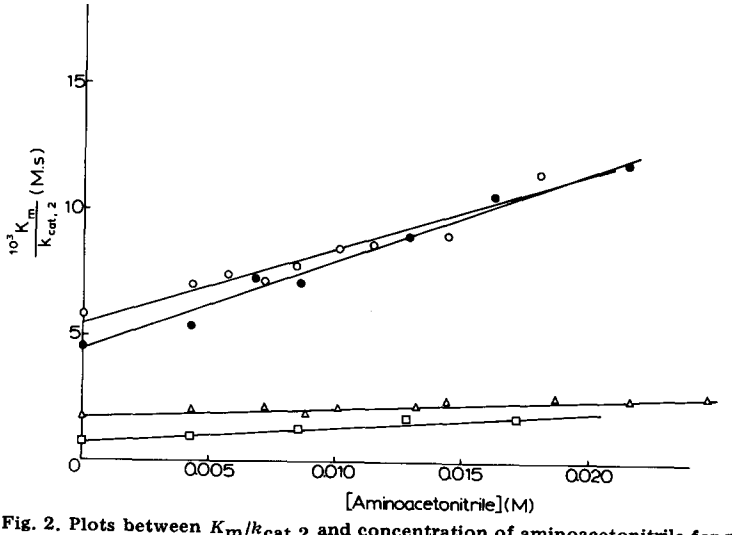

Fig. 2. Plots between $K_m/k_{cat,2}$ and concentration of aminoacetonitrile for papain-catalysed reactions of methyl hippurate (○), ethyl hippurate (●), methyl *N*-benzyloxycarbonylglycinate (△) and L-BAEE (□); 35°C, pH 7.0.

TABLE I

KINETIC CONSTANTS FOR PAPAIN-CATALYSED REACTIONS AS DETERMINED IN THE PRESENCE OF NUCLEOPHILE

The pH was 7.0 for experiments with aminoacetonitrile as nucleophile and pH 6.0 for experiments with methanol as nucleophile. k_{cat} represents $k_{cat,2}$ for alkyl esters, and $k_{cat,1}$ for *p*-nitrophenyl esters. $K_s = (k_{-1} + k_2)/k_1$.

Substrate	Temperature (°C)	k_{cat} (s^{-1})	k_2 (s^{-1})	k'_3 (s^{-1})	k_4 ($M^{-1} \cdot s^{-1}$)	k_4/k_3	K_s (M)
Nucleophile: aminoacetonitrile							
Methyl hippurate	35	4.0	10.9	6.3	315	2800	0.060
Ethyl hippurate	35	5.4	19.7	7.4	580	4300	0.088
Methyl benzyloxycarbonyl glycinate	35	5.4	11.8	10.1	210	1150	0.021
Methyl acetyl-L-phenylalanylglycinate	35	9.2	>>9	9.2	—	—	—
L-BAEE	35	18.0	22	105	7700	4000	0.018
L-BAEE	25	16.7	24	53	4400	4600	0.018
P-Nitrophenyl hippurate	35	3.3	>>30	3.3	229	4000	—
P-Nitrophenyl benzyloxycarbonylglycinate	35	6.7	>>12	6.7	143	1200	—
P-Nitrophenyl acetyl-L-phenylalanylglycinate	35	9.5	>>140	9.5	517	3000	—
Nucleophile: methanol							
L-BAee	25	16.0	18.9	106	25	13	0.016

The data in Table I indicate that k_2 is only marginally larger than k'_3 for methyl and ethyl hippurate and methyl *N*-benzyloxycarbonylglycinate. The kinetic significance of k_2 for alkyl hippurate has been previously implicated in the results of Lowe and Williams [2], who found that k_{cat} for isopropyl and *n*-butyl hippurate are smaller than for other esters of hippuric acid. It was later shown [17] that $k_2 = 1.34\ s^{-1}$ for isopropyl hippurate. It is unlikely, in spite of the structural differences, that k_2 for the methyl and ethyl esters will be higher by a very large factor, especially in view of the fact that papain can accommodate, and even shows preference for, hydrophobic leaving groups [18]. Our results, however, apparently contradict the observation [17,19] that k_{cat} for methyl and ethyl hippurate is independent of pH on the alkaline side up to about pH 8.5, since k_2 is likely to be dependent on an acidic group of $pK_a \approx 8.5$ [17] and k_{cat} should therefore have lower values than optimum if k_2 is partly rate-determining. From the data in Table I, at pH = pK_a, k_{cat} for methyl and ethyl hippurate would be 2.9 s^{-1} and 4.2 s^{-1}, respectively. While we have no explanation for this discrepancy, it may be noted that both the values for k_2, as determined here and the reported values for k_{cat} at high pH [17,19], are subject to large errors. A more sensitive procedure than conventional pH-stat titration may help to clarify this conflict.

For the very specific substrate methyl acetyl-L-phenylalanylglycinate k_2 has a much higher value than k'_3, which is still comparable with other glycine-derived substrates. This is a direct confirmation of the previous observation [9] that kinetic specificity of papain for alkyl esters, as well as for other substrates,

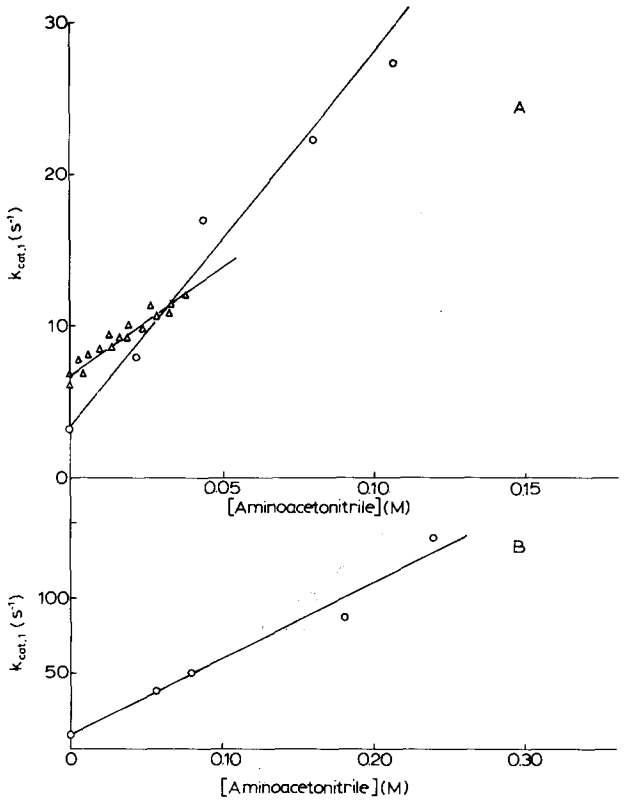

Fig. 3. Plots between $k_{cat,1}$ and concentration of aminoacetonitrile. A. *p*-Nitrophenyl hippurate (○) and *p*-nitrophenyl *N*-benzyloxycarbonylglycinate (△); B. *p*-nitrophenyl *N*-acetyl-L-phenylalanylglycinate. Reactions were carried out in the presence of 5.9% acetonitrile, 2.3% ethanol and 2% dimethylformamide respectively; 35°C, pH 7.0.

is manifested mainly in the acylation step. For L-BAEE, however, k_2 is smaller than k_3', which is the highest observed for any synthetic substrates of papain. This observation does not necessarily imply that the manifestation of kinetic specificity of papain in the acylation step is not generally true, since L-BAEE can bind with papain in non-productive modes [6,7] and the apparent value of k_2 is thereby decreased [20].

The effect of aminoacetonitrile on $k_{cat,1}$ for *p*-nitrophenyl hippurate, *p*-nitrophenyl *N*-benzyloxycarbonylglycinate and *p*-nitrophenyl *N*-acetyl-L-phenylalanylglycinate is shown in Fig. 3. The linearity of the plots indicates that for these substrates k_4 [N] and $k_3 << k_2$ and Eqn. 3 reduces to

$$k_{cat,1} = k_3' + k_4[N] \,. \tag{6}$$

These results are expected since *p*-nitrophenol is a very good leaving group. Only with a highly reactive nucleophile such as tryptophanamide, at high pH values where acylation rate is lower than optimum, was a change in the rate determining step observed with *p*-nitrophenyl esters [14]. The value of k_4/k_3 found from the slopes of the plots of $k_{cat,1}$ vs. [N] (Table I) are similar to the values calculated for the alkyl esters, as would be expected since common acyl-

enzyme intermediates are derived from both series of esters. These results lend further support to the assumption that the effect of added nucleophile is due to nucleophilic activity and not solvent effect. The slightly lower values of k_3' for *p*-nitrophenyl hippurate than those for alkyl hippurate may be due to a kinetically significant step prior to deacylation, possibly the departure of *p*-nitrophenol as suggested by Henry and Kirsch [12].

The conclusion that both k_2 and k_3' for L-BAEE are rate determining does not contradict with the earlier observation [5] that the alkylation rate of papain by chloroacetic acid is not affected by the presence of L-BAEE since, similar to Whitaker's suggestion [8], the alkylation rate of the enzyme · substrate complex may be larger than that of the free enzyme and compensates for the lack of reactivity of the acyl-enzyme intermediate. Another possibility is that L-BAEE bound in the non-productive sites enhances the alkylation rate while the productive species of the enzyme · substrate complex and the acyl-enzyme intermediate are both unreactive to alkylation [6].

The kinetic significance of k_2 for ethyl hippurate needs to be reconciled with the observation that in the presence of saturating concentration of this substrate the enzyme is completely unreactive towards alkylation by chloroacetic acid [5] and chloroacetamide [8]. If k_3' is not entirely rate-determining, such a result can only be possible if the enzyme · substrate complex, as well as the acyl-enzyme, is protected from attack by chloroacetic acid. The susceptibility of the enzyme · substrate complex to alkylation might be inferred from that of enzyme · inhibitor complex, which is more amenable to kinetic analysis, provided the inhibitor is a substrate-analogue, competitive inhibitor. If k_f is the rate constant for alkylation of the free enzyme, k_c for alkylation of the enzyme · substrate complex, and k_{ci} for alkylation of the enzyme · inhibitor complex, the ratio of k, rate constant for alkylation in the absence of inhibitor I, to k', that in its presence is given by Eqn. 7:

$$\frac{k}{k'} = \frac{k_f[E]_1 + k_c[ES]_1}{k_f[E]_2 + k_c[ES]_2 + k_{ci}[EI]_2} \tag{7}$$

where subscripts 1 and 2 indicate the concentration of the various enzyme forms in the absence and presence of I respectively. Since $[E]_1 + [ES]_1 + [ES']_1 = [E]_2 + [ES]_2 + [ES']_2 + [EI]_2$ and since, with L-BAEE as substrate, it has been shown [5,8] that $k_f = k_c \cdot k_3'/(k_2 + k_3')$, if it is assumed that $k_{ci} = 0$, Eqn. 7 reduces to

$$\frac{k}{k'} = 1 + \frac{1}{K_i(1 + [S]/K_m)} \cdot [I] \tag{8}$$

Hence, a plot between k/k' and $[I]$ at a constant value of $[S]$ should yield a straight line with a slope of $1/K_i\,(1 + [S]/K_m)$. But if $k_{ci} \neq 0$ the line will have a slope of $[1 - k_{ci}(k_2 + k_3')/k_c k_3']/K_i\,(1 + [S]/K_m)$ initially, and of progressively smaller values as $[I]$ increases.

Hippuronitrile and *N*-methoxycarbonyl-L-phenylalanylglycine thioamide, both of which are competitive inhibitors of papain [13], were tested for their ability to protect papain from alkylation by chloroacetic acid in the presence of various constant concentrations of L-BAEE. The results in Fig. 4, A and B show that both inhibitors completely protect the essential sulphydryl group of

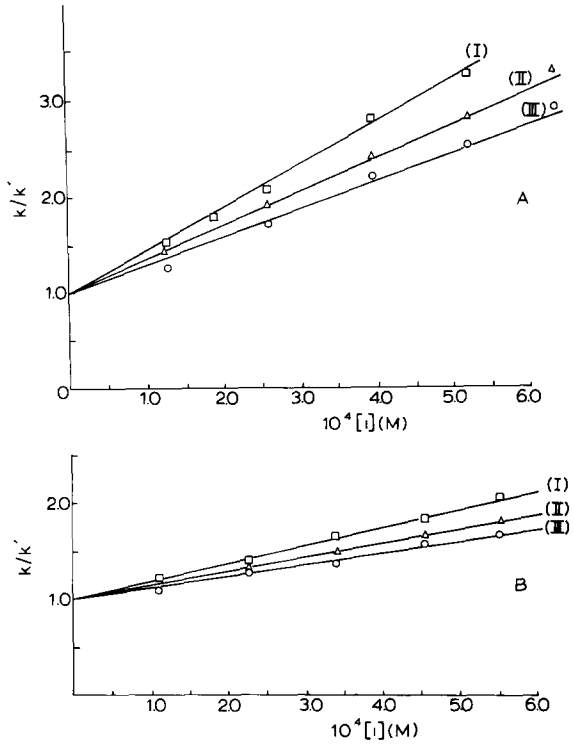

Fig. 4. A. Plots between k/k' and hippuronitrile concentration in the presence of L-BAEE at 10 mM (I), 15.1 mM (II), and 20.1 mM (III); 25°C, pH 6.0. B. Plots between k/k' and *N*-methoxycarbonyl-L-phenylalanylglycine thioamide in the presence of L-BAEE at 9.75 mM (I), 18.2 mM (II) and 27.8 mM (III); 25°C, pH 6.0.

papain from reaction with chloroacetic acid ($k_{ci} = 0$). The plots of k/k' with $[I]$ are linear for all constant values of [S]. For hippuronitrile ($K_i = 0.15$ mM), the experimental values of the slopes at [S] of 10 mM, 15.1 mM and 20.1 mM are 4100 M^{-1}, 3475 M^{-1} and 2950 M^{-1}, respectively, while the calculated values for complete protection are 3920 M^{-1}, 3260 M^{-1} and 2780 M^{-1}. For *N*-methoxycarbonyl-L-phenylalanylglycine thioamide ($K_i = 0.305$ mM) the experimental values of the slopes at [S] of 9.8 mM, 18.2 mM and 27.8 mM are 2179 M^{-1}, 1653 M^{-1} and 1313 M^{-1}, respectively, while the calculated values for complete protection are 2005 M^{-1}, 1483 M^{-1} and 1150 M^{-1}. Since these two competitive inhibitors are expected to bind at the same site as hippuric ester substrates, it is likely, despite the structural differences between the nitrile, thioamide and ester groups, that binding of the latter also protects the enzyme from alkylation. The observation that saturating concentration of ethyl hippurate completely protects the enzyme from alkylation does not therefore necessarily imply that deacylation is the entirely rate-determining step. The differences observed between substrates derived from glycine and L-BAEE, with respect to rate constants of individual steps and capacity to protect the essential sulphydryl group of the enzyme, imply that the side chain of L-arginine must have considerable influence in both binding and subsequent steps.

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References

1 Lowe, G. (1976) Tetrahedron 32, 291—302
2 Lowe, G. and Williams, A. (1965) Biochem. J. 96, 199—204
3 Kirsch, J.F. and Igelström, M. (1966) Biochemistry 5, 783—791
4 Whitaker, J.R. and Bender, M.L. (1965) J. Am. Chem. Soc. 87, 2728—2737
5 Sluyterman, L.A.Æ. (1968) Biochim. Biophys. Acta 151, 178—187
6 Brocklehurst, K., Crook, E.M. and Wharton, C.W. (1968) FEBS Lett. 2, 69—73
7 Glick, B.R. and Brubacher, L.J. (1974) Can. J. Biochem. 52, 877—883
8 Whitaker, J.R. (1969) Biochemistry 8, 4591—4597
9 Lowe, G. and Yuthavong, Y. (1971) Biochemistry 10, 2717—2726
10 Cook, A.H., Heilbron, I. and Levy, A.L. (1948) J. Chem. Soc. 201—206
11 Lake, A.W. and Lowe, G. (1966) Biochem. J. 101, 402—410
12 Henry, A.C. and Kirsch, J.F. (1967) Biochemistry 6, 3536—3544
13 Fink, A.L. and Bender, M.L. (1969) Biochemistry 8, 5109—5118
14 Hinkle, P.M. and Kirsch, J.F. (1971) Biochemistry 10, 2717—2726
15 Brubacher, L.J. and Bender, M.L. (1966) J. Am. Chem. Soc. 88, 5871—5880
16 Perrin, D.D. (1965) Dissociation Constants of Organic Bases in Aqueous Solution, Butterworths, London
17 Lucas, E.C. and Williams, A. (1969) Biochemistry 8, 5125—5135
18 Alecio, M.R., Dann, M.L. and Lowe, G. (1974) Biochem. J. 141, 495—510
19 Sluytermann, L.A.Æ. (1964) Biochim. Biophys. Acta 85, 305—315
20 Jencks, W.P. (1975) Adv. Enzymol. 43, 219—410