Acta Chemica Scandinavica B 37 (1983) 809–815

The Nucleophilic Reactivity and Tautomerism of the Imidazole Nitrogens of N^2-Acetyl-histidine Methylamide and N^2-Acetyl-histidine

CARL JOHAN CALLEMAN [a] and VERONIQUE POIRIER [b]

[a] Department of Radiobiology, University of Stockholm, S-106 91 Stockholm, Sweden and
[b] Pavillon Pasteur, Institut Curie, 26, Rue d'Ulm, 75231 Paris, France

The second order reaction rate constants for the reactions of N^2-acetyl-histidine methylamide (*1*) and N^2-acetyl-histidine (*2*) with a series of alkylating agents were determined at pH=7.4 and 37 °C. The nucleophilicities, *n*, in the Swain-Scott scale, of *1* and *2* were estimated to 3.35. The $k_N\pi/k_N\tau$-ratio was found to decrease with the constant, *s*, in the Swain-Scott equation and with the molecular size of the alkylating agent. Agents of low molecular size with low *s*-values produced a $k_N\pi/k_N\tau$-ratio higher than one, since the concentration of the N^τ-H-tautomer is higher than that of the N^π-H-tautomer, whereas bulky agents with high *s*-values reacted preferentially with the N^π-H-tautomer which is sterically less hindered and a stronger nucleophile, thus producing $k_N\pi/k_N\tau$-ratios lower than one.

The imidazole ring of histidine is an important group in proteins and enzymes which is implicated to play a role as a general base and acid catalyst in chymotrypsin,[1] ribonuclease[2] and many other enzymes. In the phosphotransferase system histidine residues act as nucleophilic catalysts transferring phosphoryl groups between enzymes.[3] Furthermore, as exemplified by hemoglobin and myoglobin, imidazole nitrogens may be ligands in the coordination of metals. The versatility of histidine residues as catalysts is primarily explained by the fact that they are endowed with p*K*-values in the range of the pH of many important biological fluids.

Unprotonated histidine residues exist in two tautomeric forms (Fig. 1), the N^τ-H-tautomer (a) and the N^π-H-tautomer (c), being named by the nitrogen with a hydrogen atom.* The interconversion between the tautomeric forms is mediated by protonation (b) of the imidazole ring.[4] In ^{13}C-magnetic resonance spectroscopy[5] and Raman scattering[6] studies it has been estimated that the N^τ-H-tautomer of L-histidine is four times more common than the N^π-H-tautomer in basic solutions. In two peptide-bound histidines it was estimated that roughly 70 % existed in the form of the N^τ-H-tautomer.[5]

* IUPAC terminology is used [*J. Biol. Chem. 247* (1972) 977]. The N^π-nitrogen is proximal to the substituted carbon of the imidazole ring, whereas the N^τ-nitrogen is more distant from that carbon.

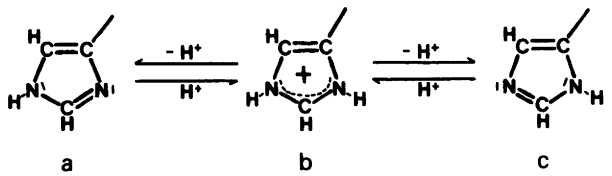
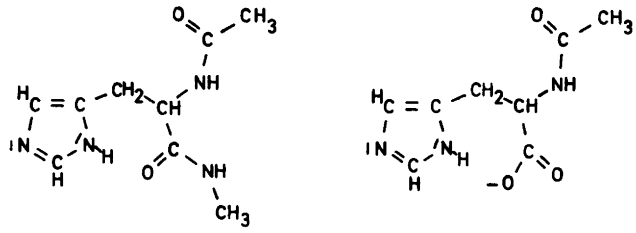


Fig. 1. The tautomerism of histidine residues. (a) N^τ-H-tautomer, (b) protonated histidine, (c) N^π-H-tautomer.

0302-4369/83 $2.50



1 *2*

Fig. 2. N^2-Acetyl-histidine methylamide (*1*) and N^2-acetyl-histidine (*2*).

In seeming contradiction to this, alkylations performed with the purpose of probing the catalytic mechanisms of enzymes have sometimes been shown to yield exclusively the N^{τ}-alkylated isomer of histidine. Thus, for instance, His-57 in the active site of chymotrypsin is alkylated at the N^{τ}-nitrogen both by methylating agents[7] and substrate analogues.[8] Moreover, in a number of synthetic preparations[2,9–13] the yield of N^{τ}-alkylated isomer has been higher than that of the N^{π}-alkylated isomer, and it has been claimed that the N^{τ}-nitrogen of histidine derivatives is the most reactive in nucleophilic substitution reactions.[2,9,12]

In order to come to an understanding of the factors governing the distribution of alkylations between the imidazole nitrogens in histidine derivatives and in order to determine their intrinsic nucleophilicities, *n*, we have reacted N^2-acetyl-histidine methylamide (*1*, Fig. 2), which was chosen as the most suitable model compound for histidine residues in proteins, with a series of alkylating agents at 37 °C and pH=7.4. For comparison, the reactivity of N^2-acetyl-histidine (*2*, Fig. 2) *versus* a few alkylating agents has been included.

Our results are in direct contradiction to claims that nucleophilic substitution reactions at the imidazole nitrogens of histidine derivatives invariably produce higher yields of the N^{τ}- than of the N^{π}-alkylated isomer and it is our aim to show that the distribution of alkylations between the two imidazole nitrogens is a function of both the constant *s* in the Swain-Scott equation and sterical properties of the alkylating agent.

MATERIALS AND METHODS

Chemicals. N^2-Acetyl-L-histidine (*2*) was obtained from Sigma Chemical Co., St. Louis, Mo. N^2-Acetyl-L-histidine methylamide (*1*) was prepared by esterification of *2* with 1.25 M HCl in methanol for 15 h at room temperature. After evaporation to dryness and repeated evaporations with methanol the ester was dissolved in 40 % methylamine (Merck *p.a.*) and left to react at room temperature for 15 h. The reaction product was then applied to a Dowex 1 (20×2 cm, OH^--form) column which was eluted with water and rinsed with HCl. After evaporation of the water fraction, *1* was recrystallized twice from a minimal amount of hot methanol. The compound was chromatographically pure on TLC ($CHCl_3$–CH_3OH–NH_3, 40:40:20, Silica gel) developed with Pauly's reagent and had an m.p. of 260 °C. MS gave |*m/z* (interpr.)|: 210 (M), 180 (M−$HNCH_3$), 167 (M−$COCH_3$), 152 (M−$CONHCH_3$) and 151 (M−H_2NCOCH_3) upon electron impact on a HP 5930A quadrupole instrument. N^{im}-Methyl-L-histidines were from Sigma Chemical Co., S:t Louis, Mo. and N^{τ}- and N^{π}-(2-hydroxyethyl)-L-histidines were synthesized as in *14*. N^{τ}- and N^{π}-ethyl-L-histidines were kind gifts of Dr. M.S.S. Murthy.[13]

[U-^{14}C]Ethylene oxide (414 MB_q/mmol), [^{14}C-methyl]methanesulfonate (2200 MBq/mmol) and [2-^{14}C]iodoacetic acid (1900 MBq/mmol) were from The Radiochemical Centre, Amersham, England. [^{3}H-Methyl]nitrosourea (60 GBq/mmol) and [1-^{14}C-ethyl]nitrosourea (550 MBq/mmol) were from New England Nuclear, Boston, Mass. [^{14}C-dimethyl]-2,2-dichlorovinyl phosphate (DDVP) (4200 MBq/mmol) and [^{3}H-

ethyl]-methanesulfonate were kind gifts of Prof. B. Holmstedt and Dr. S. Osterman-Golkar, respectively.

Reaction conditions. Preheated 0.05 M solutions of *1* or *2* were adjusted to pH=7.4 by the addition of hydrochloric acid and the reactions between the alkylating agents and *1* or *2* were carried out in a thermostated water bath at 37 °C. About 37 kBq of each radiolabeled alkylating agent was used, corresponding to concentrations of less than 0.1 mM. The reaction time was 90 min.

The reactions of *1* were terminated by the addition of an equal volume of 1 M HCl and applied to Dowex 50 (12×1 cm, Na^+-form) columns which were first washed with 100 ml H_2O to remove excess of radiolabeled reagents and then eluted with 30 mM sodium phosphate buffers at pH=6.75. In this separation system, *1* eluted after 120 ml of buffer. The retention volumes of the N^{τ}- and N^{π}-methylated, N^{τ}- and N^{π}-ethylated and N^{τ}- and N^{π}-(2-hydroxyethyl-ated) derivatives of *1* were 100, 180, 135, 215, 60 and 100 ml of buffer, respectively. The identities of the alkylation products with *1* were checked by comparison with the synthetic N^{im}-alkyl-histidines on a Biotronic LC 6000E amino acid analyzer following hydrolysis of the radiolabeled peaks in 2 M HCl at 100 °C for 15 h.

The reactions of *1* with iodoacetate ion and those of *2* were terminated by the addition of an equal volume of 4 M HCl. The reaction mixtures were hydrolyzed for 15 h at 100 °C. The iodoacetate ion reaction mixtures were then freed from excess radiolabeled reagent on a 5×2 cm Dowex 50W×4 exchanger in its NH_4^+-form which was washed with water and eluted with ammonia. The ammoniacal eluates were evaporated and injected into the amino acid analyzer. The positions of the radioactive peaks were compared to those of the common amino acids according to Ref. 2. Following hydrolysis of the acetyl group under the same conditions as for *1*, the reactions of *2* were applied directly to an Aminex A-5 ion exchanger (20×0.9 cm, Na^+-form) which was washed with water and then eluted with a 0.05 M sodium phosphate buffer at pH=7.0. In this separation system histidine, N^{τ}- and N^{π}-methyl-histidine, N^{τ}- and N^{π}-ethyl-histidine, N^{τ}- and N^{π}-(2-hydroxyethyl)histidine eluted with 120, 100, 165, 145, 195, 65, and 100 ml of buffer, respectively.

The fractions taken out from the separations were counted for radioactivity in an Intertechnique SL 30 scintillation spectrometer after mixing with an equal volume of Instagel and counting efficiency was determined by automatic external standardization.

Reaction kinetics. For the reactions of the alkylating agents the second order rate constants, k, were calculated according to the formula:

$$k = \frac{|RY|\ k'}{|Y|_0 |RX|_0 t\ (1-e^{-k't})} \qquad (1)$$

where $|RY|$ is the concentration of product determined from the radioactivity in the corresponding peaks and $|RX|_0$ and $|Y|_0$ the starting concentrations of alkylating agent and nucleophile (*1* or *2*), respectively. The term $k'/(1-e^{-k't})$, where k' is the first-order rate constant for disappearance of the alkylating agent estimated from its rate constant for hydrolysis as given in Ref. 15 and its uncorrected rate of reaction with *1* or *2*, gives a correction for the decay of alkylating agent during the course of the reaction. For methyl methanesulfonate k' was estimated to 35 10^{-6} s^{-1}, whereas for the other alkylating agents it was considerably lower. Due to the excess of *1*

Table 1. Second order reaction rate constants, k±S.D.×10^6($M^{-1}s^{-1}$) for *1* and *2* at 37 °C and pH=7.4.

	1			*2*		
	$k_N\tau$	$k_N\pi$	$k_N\tau+k_N\pi$	$k_N\tau$	$k_N\pi$	$k_N\tau+k_N\pi$
Methyl methanesulfonate	136±7	191±20	327±28(n=4)[a]	150	137	287
Ethyl methanesulfonate	7.8±0.5	6.5±0.1	14.3±0.4(n=4)[a]	9.9	5.6	15.5
Ethylene oxide	27±3	32±4	59±7(n=5)[a]	27	22	49
Iodoacetate	39	18	57			
Dichlorvos	3.3	4.4	7.7			

[a] n is the number of determinations.

Table 2. Nucleophilicity constants, n, for the imidazole group of *1* and *2* calculated from rate data of a few directly alkylating agents at 37 °C.

	1	*2*
Methyl methanesulfonate	3.4	3.4
Ethyl methanesulfonate	2.5	2.6
Ethylene oxide	3.3	3.3
Iodoacetate	4.2	

and *2* in relation to the alkylating agents, the secondary alkylations of the mono-N^{im}-alkylated compounds could be neglected in the kinetic treatment.

RESULTS

The observed second order rate constants of the two imidazole nitrogens of *1* and *2* and the sums of these rate constants for the reactions with a few alkylating agents at pH=7.4 and 37 °C are presented in Table 1.

Except where indicated the rate constants are based on duplicate experiments. The absolute values of the rate constants of *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea were not determined since they are transformed to reactive intermediates after base-catalyzed decompositions, the rates of which under the reaction conditions are unknown.

The sums of the rate constants of the two imidazole nitrogens in Table 1 were divided by the degrees of dissociation of the nucleophiles (calculated from the pK-values given in Refs. 16 and 17) at pH=7.4 to obtain the pH-independent rate constants which were used for the calculation of the nucleophilicity constants, n, of *1* and *2* in the equation of Swain and Scott [18]

$$\log (k_y/k_{H_2O})=sn \qquad (2)$$

where k_y and k_{H_2O} are the second order rate constants for the reactions of an alkylating agent with the nucleophiles Y and water, respectively, and s is a constant expressing the sensitivity of the alkylating agent to the nucleophilic strength, n, of the nucleophile. The values for k_{H_2O} were taken from Ref. 15. The calculated n-values of *1* and *2* are shown in Table 2. No estimate of the nucleophilicity could be based on the reactivity of DDVP since its reactivity with water is not known.

Table 3. The relative reactivities ($k_N\pi/k_N\tau$) of the imidazole nitrogens of a few histidine derivatives *versus* a number of alkylating agents together with their substrate constants. Unless otherwise stated at 37 °C and pH=7.4.

Alkylating agent	*1*	*2*	Ac-His methyl ester	His	*s*
Methyl nitrosourea	1.79				0.32[a]
Methyl methanesulfonate	1.37	0.91			0.89[b]
Dichlorvos	1.33				0.9[c]
Ethyl nitrosourea	1.49				0.0[a]
Ethyl methanesulfonate	0.86	0.53			0.69[b]
Ethylene oxide	1.22	0.81	1.1[d]		0.96[b]
Styrene oxide		very low[e]	very low[e]		0.81[e]
Bromoacetate				0.33[f]	
Iodoacetate	0.48	0.33[g]		0.50[h]	1.33[i]
Methyl iodoacetate			0.26[j]		
Iodoacetonitrile			0.1[k]		
2-Acetylaminoacrylic acid		very low[e]			

[a] Ref. 19. [b] Ref. 15. [c] Ref. 20. [d] Ref. 14, 1.25 M HCl in MeOH, 25 °C. [e] No N^{π}-alkylated isomer was detected, H_2O:MeOH 1:1, Osterman-Golkar, S. personal communication. [f] Ref. 12, Cu^{2+}-His at pH=6.0–6.1, 22 °C. [g] Ref. 2, pH=8.1, 100 °C. [h] Ref. 2, pH=5.5, 25 °C. [i] Ref. 21. [j] Ref. 10, acetone + K_2CO_3, 25 °C. [k] Ref. 10, acetone + K_2CO_3, 25 °C. [l] Ref. 9, N^{π}-alkylated isomer present only as impurity, H_2O, 50 °C.

In Table 3 the ratios between the observed rate constants of the N^{π}- and N^{τ}-nitrogens for a few histidine derivatives are listed. Some values from other sources with different reaction conditions have been included in Table 3. These are sometimes given in the literature as ratios between products, but since it was indicated in these cases that the formation of di-N^{im}-alkylated compounds was small, the ratios between product yields should be close to ratios between rate constants.

Since the conditions were not the same as in our experiments, these ratios are not strictly comparable to those obtained in this study. The *s*-values of the alkylating agents are also included in Table 3.

DISCUSSION

The mean of the *n*-values in Table 2 calculated from the rate constants for their reactions with methyl methanesulfonate and ethylene oxide is 3.35 for both model compounds, *1* and *2*. This value lies close to the *n*-values estimated for imidazole, 3.58 (*22*), pyridine, 3.6 (*18*) and pyrimidine 3.30,[22] all of which are heterocyclic nitrogen compounds with reactive "pyridine" nitrogens.

When comparing the *n*-values of *1* and *2* with that of imidazole, the Brønsted dependence on basicity has to be considered. If the values 0.32 for ethylene oxide[23] and 0.2 for methyl methanesulfonate[15] are used for β, *1*, whose p*K*-value at 25 °C is 6.5[16] compared to 7.0 of imidazole,[17] should display a 0.16 and 0.1 *n*-units lower nucleophilicity constant than imidazole in its reactivity *versus* these agents. The nucleophilicity of *2* (pK=7.1 at 25 °C)[17] should be roughly the same as that of imidazole. Thus, it can be seen that for these two alkylating agents the *n*-values in Table 2 agree well with expectation, meaning that the side chains of *1* and *2* present relatively little steric hindrance against reactions of these agents with the imidazole group.

The *n*-values of *1* and *2*, if calculated from their reactivities towards ethyl methanesulfonate are 0.85 and 0.75 units, respectively, below the above-mentioned mean value. This parallels the low nucleophilicity, n=2.9, of the *N*-7 group of guanosine *versus* the same agent as compared to 3.7 if calculated from its reactivity with methyl methanesulfonate (*24*). (The *N*-7 atom of guanosine is part of an imidazole moiety of the guanosine molecule in which tautomerism has been eliminated due to the sugar bonding of the *N*-9-atom.) Ethyl methanesulfonate may thus be characterized by a lower reactivity *versus* nitrogens than expected from eqn. (2). (See also Ref. 25.)

The *n*-value of *1* calculated from the rate constant with the iodoacetate ion is based on a comparison with a series of rate constants with negatively charged nucleophiles.[21] Since the rate constants of the iodoacetate ion with negatively charged nucleophiles will be lowered by anion–anion repulsion, its reactivity *versus* *1* probably appears higher than it would in a comparison with uncharged nucleophiles.

The nucleophilic reactivity of *1* may be regarded as the intrinsic reactivity of histidine residues in proteins. Differences between the reactivity of a protein histidine residue and *1* should be ascribed either to the microenvironment of the protein histidine residue and/or to the distribution of the alkylating agent within the protein.

It has been directly shown that the N^{τ}-H-tautomer is the predominating form in *2*[5] (*i.e.* that the equilibrium constant of the tautomerism, K_T, is higher than one). There are several indications that this is the case also for *1*; Firstly, NMR-studies in two small histidine-containing peptides have revealed that the proportion of N^{τ}-H-tautomer was about 70 %.[5]

Secondly, N^{π}-alkylated histidines, including a tripeptide,[26] have higher p*K*-values than do their N^{τ}-alkylated isomers[14,27] pointing to the N^{π}-H-tautomer as a stronger base and thus a stronger nucleophile than the N^{τ}-H-tautomer. Assuming the p*K*-difference of 0.65 between the two N^{im}-methylated histidine residues of this tripeptide to be valid also for the p*K*-difference between the two tautomers of *1*, it may be estimated that the proportion of N^{τ}-H-tautomer is about 80 % in *1*. Thirdly, ethylnitrosourea, which has an *s*-value close to zero (*19*), and thus is virtually insensitive to the nucleophilicity of nucleophiles, produces a $k_N\pi/k_N\tau$-ratio higher than one in its reaction with *1*. An approximation of the proportion of N^{τ}-H-tautomer in *1* may be obtained by estimating the ratio between the rate constants of the two tautomers from the Brønsted equation

$$\log \frac{k_{N^{\pi}\text{-H-tautomer}}}{k_{N^{\tau}\text{-H-tautomer}}} = \beta \times (pK_{N^{\pi}\text{-H-tautomer}} - pK_{N^{\tau}\text{-H-tautomer}}) \quad (3)$$

by assuming the same pK-difference as above, 0.65, and using the value 0.32 for β of ethylene oxide.[23] This ratio between rate constants may then be used together with the ratio between oberved rate constants in Table 3 to estimate the equilibrium constant, K_T, according to

$$K_T = \frac{|N^{\tau}\text{-H-tautomer}|}{|N^{\pi}\text{-H-tautomer}|} = \frac{k_{N^{\pi}\text{-H-tautomer}}}{k_{N^{\tau}\text{-H-tautomer}}} \times \frac{k_{N^{\pi}}}{k_{N^{\tau}}} \quad (4)$$

indicating a proportion of about 60 % of N^{τ}-H-tautomer in *1*. Due to the sterical hindrance against reaction of alkylating agents at the N^{π}-nitrogen, the proportion of N^{τ}-H-tautomer is, however, probably slightly underestimated by this method.

Although the above estimates of the proportion of N^{τ}-H-tautomer in *1* are relatively consistent, a discussion in quantitative terms of the difference in nucleophilicity between the two tautomers of *1* and *2* would require values for K_T determined directly by means of physical methods. Thus only a few general remarks will be made about this difference in nucleophilic reactivity.

The influence of the *s*-value on the relative reactivity of the two tautomers and consequently on the observed ratios between rate constants, is evident when different alkylating agents donating the same alkyl group are intercompared (Table 3). Compounds with low substrate constants react relatively more with the predominating N^{τ}-H-tautomer, whereas compounds with higher substrate constants are more sensitive to the nucleophilicity of the nucleophile, thus favouring reaction with the N^{π}-H-tautomer, the stronger nucleophile.

The difference in reactivity between the two tautomers increases with the molecular size of the alkylating agent. This is probably because the side chains on imidazole in *1* and *2* present greater sterical obstacles against reactions of bulky agents with the N^{τ}-H-tautomer than with the N^{π}-H-tautomer. The sterical effect on the reactivity appears to be a major factor determining ratios between observed rate constants ($k_N\pi/k_N\tau$) and consequently between product yields of mono-N^{im}-alkylated histidines in synthetic preparations. An especially clear example of this effect on the product yield is presented by the reaction of styrene oxide with *2*, which despite the fact that the agent has a relatively low substrate constant produces a very low amount of the N^{τ}-alkylated isomer (Osterman-Golkar, S., personal communication). Previous claims that synthetic preparations of mono-N^{im}-alkylhistidines always produce a higher yield of the N^{τ}-alkylated product[9] may be explained by the fact that they were based on reactions with relatively bulky reagents.

The observed $k_N\pi/k_N\tau$-ratios (Table 3) for the compounds studied are about fifty per cent higher for *1* than for *2*. This is presumably explained by a lower equilibrium constant, K_T, in *2* as compared to *1* (this is indicated in the study of Reynolds *et al.*[5] where *2* had a lower K_T-value than glycyl-histidyl-glycine), which in turn may be explained by a stabilization of the N^{π}-H-tautomer in *2* by the formation of a hydrogen bond to the α-carboxylate ion.

High yields of both mono-N^{im}-alkylated histidine isomers are sometimes difficult to obtain in synthetic preparations. This has especially been true when mono-N^{im}-alkylhistidines are synthesized from *2* with bulky, electron-repelling groups like the ethyl or carboxymethyl group where low yields of the N^{π}-alkylated isomer have been obtained.[2,12,13] The reason for this is mainly that the apparent reactivity of an imidazole nitrogen (*i.e.* the rate constant for the formation of di-N^{im}-alkylated imidazolium ions) is increased when the other nitrogen has been alkylated, which if the $k_N\pi/k_N\tau$-ratio is different from one leads to a progressive decrease in the ratio between product yields of the minor and major N^{im}-alkylated isomer as the reaction time is prolonged.

Thus, for instance, in the carboxymethylation of Cu^{2+}-His, Wieghardt and Goren[12] observed a 6–7-fold increase in the reactivity of either imidazole nitrogen when the other nitrogen had been carboxymethylated, and the authors ascribed this to the electron-repelling effect of the carboxymethyl group introduced. (For a kinetic model of extensive alkylation of histidine compounds confer the article quoted above[12]).

In the synthesis of mono-N^{im}-(2-hydroxyethyl)histidines[14], Calleman and Wachtmeister observed, however, a corresponding doubling in the reactivity upon 2-hydroxyethylation of either imidazole nitrogen also with an electron-withdrawing group such as the 2-hydroxyethyl group (unpublished data). This indicates that part of the explanation to the increase in the observed rate constant of an imidazole nitrogen when the other has been alkylated is that tautomerism is eliminated in mono-N^{im}-alkylated histidine derivatives.

The relationships derived in this study may be of value in planning synthetic preparations where a high yield of mono-N^{π}-alkylated histidine product is desired. In such cases *1* rather than *2* should be used as starting material, and when there is a choice between different alkylating agents, the reagent with the smallest size and lowest *s*-value should be used.

Acknowledgements. We are grateful to Dr. Siv Osterman-Golkar and Prof. Lars Ehrenberg for valuable discussions and to Ellinore Richardsson for carrying out the amino acid analyses. This work has been financially supported by the Swedish Cancer Society, the Swedish Natural Science Research Council and by a fellowship to V.P. from the French National Union against Cancer.

REFERENCES

1. Blow, D. M., Birktoft, J. J. and Hartley, B. S. *Nature 221* (1969) 337.
2. Crestfield, A. M., Stein, W. H. and Moore, S. *J. Biol. Chem. 238* (1963) 2413.
3. Doojeward, G., Roossien, F. F. and Robillard, G. T. *Biochemistry 18* (1979) 2996.
4. Hoffmann, K. *Imidazole and its Derivatives,* Interscience, New York 1953, p. 29.
5. Reynolds, W. F., Peat, I. R., Freedman, M. H. and Lyerla, J. R., Jr. *J. Am. Chem. Soc. 95* (1973) 328.
6. Ashikawa, I. and Itoh, K. *Chem. Lett.* (1978) 681.
7. Nakagawa, Y. and Bender, M. L. *Biochemistry 9* (1970) 259.
8. Stevenson, K. J. and Smillie, L. B. *Can. J. Biochem. 46* (1968) 1357.
9. Fujimoto, D., Hirama, M., and Iwashita, T. *Biochem. Biophys. Res. Commun. 104* (1982) 1102.
10. Matthews, H. R. and Rapoport, H. *J. Am. Chem. Soc. 95* (1973) 2297.
11. Tallan, H. H., Stein, W. H. and Moore, S. *J. Biol. Chem. 206* (1954) 825.
12. Wieghardt, T. and Goren, H. J. *Bioorg. Chem. 4* (1975) 30.
13. Murthy, M. S. S. *et al. To be published.*
14. Calleman, C. J. and Wachtmeister, C. A. *Acta Chem. Scand. B 33* (1979) 277.
15. Osterman-Golkar, S. *Thesis,* University of Stockholm, Stockholm 1975.
16. Tanokura, M., Tasami, M. and Miyazawa, T. *Chem. Lett.* (1978) 739.
17. Perrin, D. D. *Dissociation Constants of Organic Bases in Aqueous Solution, IUPAC, Vol. 1 and 2,* Butterworth, London 1972.
18. Swain, C. G. and Scott, C. B. *J. Am. Chem. Soc. 75* (1953) 141.
19. Osterman-Golkar, S. *To be published.*
20. Segerbäck, D. and Ehrenberg, L. *Acta Pharmacol. Toxicol. 49 Suppl. V* (1981) 56.
21. Hawthorne, M. F., Hammond, G. S. and Grayvill, B. M. *J. Am. Chem. Soc. 77* (1955) 486.
22. Koskikallio, J. *Acta Chem. Scand. 23* (1969) 1490.
23. Hudson, R. F. and Loveday, G. *J. Chem. Soc.* (1962) 1068.
24. Walles, S. *Thesis,* University of Stockholm, Stockholm (1971).
25. Poirier, V. and Calleman, C. J. *Acta Chem. Scand.* B 37 (1983) 817.
26. Grant, G., Ling, N., Rivier, J. and Vale, W. *Biochemistry 11* (1972) 3070.
27. Gurd, F. R. N. *Meth. Enzymol. 11* (1967) 532.

Received December 23, 1982.