The Specificity of the Interaction of Bovine Pancreatic Ribonuclease A with Natural and Halogenated Purine Nucleotides

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The interaction between bovine pancreatic ribonuclease A (EC 3.1.4.22) and the purine nucleotides AMP, GMP, 6-chloropurine 5'-ribonucleotide and 8-bromoadenosine 5'-monophosphate was studied by u.v. difference spectroscopy. The stoichiometry of the binding of the halogenated nucleotides to the enzyme shows a 1:1 ratio, as for the natural ones. The binding constants, K_a , for all four nucleotides at pH 5.5 were determined. They are within the same order of magnitude, though the nucleotides with a 6-amino group show a stronger interaction. The magnitude of the binding shows a reciprocal dependence on the ionic strength, which indicates an electrostatic interaction between ligand and enzyme. Finally, solvent-perturbation experiments show that all four nucleotides bind to the enzyme in a partially hydrophobic region. It is concluded that both halogenated and natural purine ribonucleotides interact in a similar manner with the enzyme molecule. The special synthesis and identification of 6-chloropurine 5'-ribonucleotide are discussed extensively. It is concluded that both halogenated and natural purine ribonucleotides interact in a similar manner with the enzyme molecule and thus the halogenated analogues are potential reagents for the affinity labelling of the purine-binding site.

Witzel & Barnard (1962) found that when RNAase A acted on dinucleoside monophosphates as substrate the rate of the first step of the reaction was dependent on the type of nucleoside attached to the 5'-position of the phosphodiester linkage, the order being $A > G > C > U$. On the other hand a strong interaction between the enzyme and the natural purine nucleotides, different from that obtained with pyrimidine nucleotides, was demonstrated by Deavin *et al.* (1966*a,b*, 1968). On the basis of these results the existence of a secondary binding site in the enzyme that would prefer purines was postulated (Rabin *et al.*, 1970). The interaction between this site and the purine base would favour the correct positioning of the reacting groups and hence the velocity of the reaction. X-ray-diffraction studies on the three-dimensional structure of ribonuclease S and its complexes with several substrate analogues (Richards & Wyckoff, 1971) also support this hypothesis, as the purine base in the dinucleoside phosphonate analogue UpcA occupies a specific position. However, the mechanism of binding of purines as well as the amino

acids involved in this interaction are still unknown, although attempts to label this region by using 6-chloropurine have been carried out (Rabin *et al.*, 1970). This reaction was not very useful because the number of derivatives was too high, although it was demonstrated that all derivatives were formed by reaction of 6-chloropurine with lysine amino groups, as deduced from spectrophotometric data (Rabin *et al.*, 1970; Cuchillo, 1974). Haffner & Wang (1973) have shown that AMP binds to the enzyme by means of a strong interaction mainly due to the phosphate group in a site of the enzyme that is identical with the one that binds the pcA moiety of UpcA. For this reason and because the binding of nucleotides to RNAase A is much stronger than that of free bases or nucleosides, it was thought that the use of a halogenated nucleotide would be much more specific for the binding and therefore for eventually labelling the secondary purine-binding site of ribonuclease. However, as the presence of the bulky halogen atom could hinder the interaction with the enzyme, several tests were carried out to show that both the strength and the site of interaction of 6-ClPuMP and 8-BrAMP are of the same nature as that for natural purine nucleotides, a prerequisite for affinity labelling. To this end the stoichiometry, the binding constants and the nature of the interaction were measured by u.v. difference spectroscopy. This technique is very useful, as it has been shown (Irie & Sawada, 1967; Sawada & Ishii,

Abbreviations used: 6-ClPuMP, 6-chloropurine 9- β -D-ribofuranosyl 5'-monophosphate; 8-BrAMP, 8-bromoadenosine 5'-monophosphate; RNAase A, bovine pancreatic ribonuclease A; UpcA, the phosphonate analogue of UpA in which the oxygen atom of the phosphoester bond between the phosphate and the adenosine moiety is replaced by a $-CH_2-$ group.

1968; Deavin *et al.*, 1968) that the changes in absorbance found on interaction between the enzyme and nucleotides are due only to changes in the nucleotide chromophore and not in the protein. Therefore changes in the nucleotide environment which mimic the difference spectra found on interaction with the enzyme give an idea about the nature of the binding site. Because of the novel conditions used for the synthesis and purification of 6-ClPuMP, special attention is given throughout the present paper to the identification procedures used to confirm its chemical structure unambiguously.

Experimental

Synthesis of 6-ClPuMP

The method of Yoshikawa *et al.* (1967, 1969) described for the phosphorylation of natural nucleosides was chosen for the synthesis of 6-ClPuMP. In comparison with other methods (Hampton & Maguire, 1961; Brox & Hampton, 1968; Shaw *et al.*, 1968) this method has the important advantage that it does not require the initial protection of the nucleoside and, suitably modified, has proved satisfactory for the phosphorylation of 6-chloropurine riboside. The method consists of the phosphorylation of nucleosides with POCl_3 as reagent in the presence of a trialkyl phosphate as solvent.

In the case of the phosphorylation of 6-chloropurine riboside the reagent concentration, the temperature and the reaction time were increased in comparison with the conditions used by Yoshikawa *et al.* (1969). The best yield was obtained at 4°C, 16 h and a trimethyl phosphate: POCl_3 molar ratio of 2:1, in contrast with the conditions used by Yoshikawa *et al.* (1969) for natural nucleosides (0°C, 4 h and a molar ratio of 5.3:1). Higher temperatures and more prolonged times of reaction led to a decrease in the yield of 6-ClPuMP. The purification procedure of Yoshikawa *et al.* (1969) for natural nucleotides was not applicable to 6-ClPuMP. The use of ion-exchange resins such as IRA-400 or Dowex 1 (X10) with formic acid as eluent did not separate the nucleotide from P_i . This may be due to the very low pK of the halogenated base (0.45 in the free form and 0.2 when methylated at position N-9; Barlin & Chapman, 1965), and thus the protonation of the nucleotide that differentiates it from P_i occurs at too low a pH. Moreover, some hydrolysis of the nucleosidic bond was detected under these conditions. For these reasons the purification procedure based on the fractional precipitation and removal of contaminants according to the different solubilities was used.

A typical experiment was as follows. To 0.8 mmol of 6-chloropurine riboside (Cambrian Chemicals, Croydon, Surrey, U.K.) suspended in 8.5 mmol of trimethyl phosphate (Fluka, Buchs, Switzerland) and

cooled to -15°C , 4.2 mmol of phosphoryl chloride (Fluka) was added. The solution was stirred at 6°C for 15 h. To stop the reaction the solution was cooled to -15°C and 20 ml of water was added. This was followed by the addition of 12.3 ml of 1 M-LiOH, which stoichiometrically neutralized the resulting HCl, and P_i was precipitated by the addition of 10.5 ml of 1 M-barium acetate. The mixture was left at 0°C for 1 h and centrifuged at 2000g for 10 min. The precipitate was discarded and the nucleotide was then precipitated by the addition of 10 vol. of ethanol. The unchanged nucleoside, trimethyl phosphate and LiCl remained in the supernatant. The precipitate was separated by centrifugation at 2000g for 10 min and redissolved sequentially with three 3 ml portions of water, the portions were combined and the nucleotide was reprecipitated by the addition of 2 vol. of ethanol. These operations were based on the fact that the contaminating salts had lower solubility in water than the nucleotide, and thus repeated dissolution in small portions of water increased the purity of the nucleotide until a practically pure preparation was obtained. The barium salt of 6-ClPuMP was finally washed twice with ethanol, twice with diethyl ether and dried at room temperature (15 – 20°C): 0.4 mmol of 6-ClPuMP (barium salt) was obtained, corresponding to a yield of 50%. This product showed a loss of drying (110°C for 24 h) of 6.25%. This dried material gave a molar absorption coefficient of $8570 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 264 nm, in contrast with the reported values of 8400 (Hampton & Maguire, 1961) and 8260 (Brox & Hampton, 1968). The spectral characteristics in 0.05 M-sodium phosphate buffer, pH 7.0, were: $\lambda_{\text{max.}} = 264 \text{ nm}$; $\lambda_{\text{min.}} = 226.5 \text{ nm}$; ratio $A_{264}/A_{226.5} = 4.27$; $A_{250}/A_{260} = 0.73$; $A_{270}/A_{260} = 0.92$; $A_{280}/A_{260} = 0.19$. These ratios are very similar to those found by Brox & Hampton (1968) and did not change over the pH range 1.5–12.

The purity and identification of the product was checked by t.l.c. and also by g.l.c.–mass spectrometry. Details of the g.l.c. and mass-spectrometry procedures are described in Gelpi *et al.* (1976). The nucleotide shows a single and well-defined spot when assayed by t.l.c. on cellulose plates (E. Merck, Darmstadt, Germany) with all the solvent systems shown in Table 1. A Uvatom 70 dual-wavelength u.v. lamp (Atom, Barcelona, Spain) was used to locate spots on the chromatograms. By comparison with other purine nucleosides and nucleotides the R_F values are in the range of what would be expected for the nucleotide. The gas chromatograms also yielded a single peak showing a longer retention time than that of the corresponding nucleoside (Gelpi *et al.*, 1976).

The specificity of the reaction for the 5'-hydroxy group was explained by Yoshikawa *et al.* (1969) as being due to the formation of an ionic complex between the POCl_3 and the trialkyl phosphate. However, Taylor *et al.* (1973), using n.m.r. spectro-

Table 1. R_F values for several ribonucleosides and ribonucleotides on thin-layer cellulose plates in four different solvent systems

Solvents: A, propan-2-ol/1% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (2:1, v/v); B, butan-1-ol/acetone/acetic acid/5% (v/v) NH_3 /water (9:3:2:2:4, by vol.); C, butan-1-ol/acetic acid/water (5:2:3, by vol.); D, propan-1-ol/aq. NH_3 (d 0.91)/water (20:20:3, by vol.).

Compound	Solvent ...	R_F values			
		A	B	C	D
Adenosine		0.60	0.50	0.55	0.64
AMP		0.57	0.25	0.22	0.42
6-Chloropurine					
riboside		0.77	0.65	0.72	0.80
6-ClPuMP		0.54	0.47	0.48	0.56
8-Bromoadenosine		0.63	0.65	0.70	0.71
8-BrAMP		0.49	0.33	0.35	0.49

Fig. 1. Proposed intermediate penta-co-ordinate complex between a ribonucleoside and trimethyl phosphate

scopy, did not detect this postulated complex. An alternative explanation for the directionality of the reaction could be the formation of a penta-co-ordinate compound between the trialkyl phosphate and the nucleoside, which would be subsequently hydrolysed (Fig. 1). The presence of a phosphoester bond in the 5'-position was checked by enzymic hydrolysis with 5'-nucleotidase from *Crotalus adamanteus* (BDH, Poole, Dorset, U.K.). For this 2mg of the enzyme was dissolved in 2ml of 0.01M-Tris/HCl buffer, pH8.5, and 0.1ml of this mixture was added to 1ml of the same buffer containing 1mg of nucleotide. This final mixture was incubated at 37°C for 30min and the products of the reaction were identified by t.l.c. A single spot was found, with an R_F value corresponding to that of the nucleoside. The specificity of the enzyme preparation was checked by incubating a mixture of cytidine 2'- and 3'-monophosphates (Cambrian) in the same conditions. After 16h incubation no free cytidine was detected in the chromatogram, indicating the lack of contaminating 2'- and 3'-nucleotidase activities. The sensitivity of the t.l.c. technique was studied by using several

samples of known concentration, and from these experiments it can be concluded that the amount of 2'- and 3'-isomers plus nucleoside diphosphate was less than 4%.

Neither the chromatographic nor the mass-spectrometric experiments gave any indication of the presence of nucleoside diphosphates. This fact can be explained on the basis of the purification procedure, which would selectively remove nucleotides with a higher degree of substitution because of the lower solubility in water of their barium salts.

Synthesis of 8-BrAMP

The phosphorylation of 8-bromoadenosine by the method of Yoshikawa *et al.* (1967, 1969) gave a yield of 8-BrAMP of only 4%. The lack of efficiency of the method is probably due to the known lability of many 8-substituted purine nucleosides at low pH values. For this reason 8-BrAMP was synthesized by direct bromination of AMP (Merck) by the procedure of Ikehara & Uesugi (1969) and Ikehara *et al.* (1969). The final yield was 35% and the 8-BrAMP obtained had 92% purity, based on a molar absorption coefficient of $15000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 263nm, pH 7.0, as reported by Ikehara *et al.* (1969), and contained 7.9% water.

Ribonuclease A

Bovine pancreatic ribonuclease (five times crystallized) was from Calbiochem (Los Angeles, CA, U.S.A.). This material was purified by the method of Taborsky (1959) to obtain the ribonuclease A fraction.

U.v. spectrometry

Spectrophotometric measurements were carried out on a Beckman Acta MVI double-beam recording spectrophotometer. The difference spectra were recorded directly by using split-compartment cells as described by Deavin *et al.* (1968). Results of experiments in which the base-line was not recovered were discarded. Binding constants (K_a) were determined by measuring the difference spectra by the methods of Hammes & Schimmel (1965) and of Anderson *et al.* (1968). The method of Deranleau (1969) for the calculation of K_a was also used. The K_a values were obtained in sodium acetate acetic acid buffers of different ionic strength, pH 5.5, at $25 \pm 0.5^\circ\text{C}$. For the determination of each K_a value ten points were measured. The concentration of the enzyme was kept constant at 0.1mM (before mixing), calculated on the basis of a molar absorption coefficient of $9800 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 277nm (Sela & Anfinsen, 1957). The ligand concentrations ranged from 0.05 to 1.05mM (also before mixing). The wavelengths used

were: 273 nm for AMP, 289 nm for GMP, 262 nm for 6-ClPuMP and 252 nm for 8-BrAMP. The slit width was 1 mm, except for the highest concentration measurements, for which it was set at 2 mm.

The stoichiometry of the reaction was determined by the continuous variation method of Anderson *et al.* (1968), by using nine points of different nucleotide:enzyme concentration ratios. Solvent-perturbation experiments were also carried out by u.v.-difference-spectrometry measurements. For the change of the dielectric constant, by the addition of dioxan (C. Erba, Milano, Italy; spectroscopy grade), the initial concentration of nucleotide was always 0.02 mM in 0.02 M-sodium acetate/acetic acid, pH 5.5. The initial dioxan concentration was 40% (v/v) (20% after mixing) in 0.024 M-sodium acetate/acetic acid, pH 5.5, or 20% (v/v) (10% after mixing) in 0.022 M-sodium acetate/acetic acid, pH 5.5 (this was to compensate for the decrease in ionic strength due to the addition of dioxan).

The experiments involving pH changes were carried out only with 6-ClPuMP and 8-BrAMP. The results for AMP and GMP had been published by Deavin *et al.* (1968). The initial concentration of nucleotide was 0.1 mM and in the second cell compartment there was 0.02 M-HCl, except for one experiment with 6-ClPuMP in which the initial HCl concentration was 2 M.

Results and Discussion

Interaction between RNAase A and purine nucleotides

Fig. 2 shows that the strongest interaction between the halogenated purine nucleotides and RNAase A is obtained when enzyme and ligand are present in equimolar amounts, as deduced from the magnitude of the difference spectra. The fact that the tangents at the origin of both curves intersect at a value on the abscissa of 0.5 also lends support to the 1:1 ratio for the interaction. These results show that the stoichiometry of the binding of halogenated purine nucleotides is the same as that for the natural ones (Deavin *et al.*, 1968). This is also the case for pyrimidine nucleotides (Anderson *et al.*, 1968).

The interaction between RNAase A and the purine nucleotides, both natural and halogenated, can then be described in terms of the equilibrium:

where P represents the enzyme and L the ligand. Thus the association, or binding, constant, K_a , is given by the expression:

$$K_a = \frac{[PL]}{[P][L]} \quad (2)$$

[PL], the concentration of enzyme-ligand complex,

can be related to the change in absorbance (ΔA) by the equation:

$$[PL] = \frac{\Delta A}{\Delta \epsilon} \quad (3)$$

where $\Delta \epsilon$ is the difference molar absorption coefficient, i.e. the difference in absorbance found when 1 mol of the enzyme is completely saturated by ligand.

[P] and [L], the free concentrations of enzyme and nucleotide, can be found from the initial concentration $[P_0]$ and $[L_0]$ and the [PL] value. The value of K_a is finally given by the equation:

$$K_a = \frac{\frac{\Delta A}{\Delta \epsilon}}{\left([P_0] - \frac{\Delta A}{\Delta \epsilon}\right)\left([L_0] - \frac{\Delta A}{\Delta \epsilon}\right)} \quad (4)$$

The best value of $\Delta \epsilon$ was found by the method of Hammes & Schimmel (1965) and Anderson *et al.* (1968). Values of K_a were calculated with different

Fig. 2. Change in absorbance as a function of the relative concentrations of ribonuclease A and halogenated purine nucleotides

ΔA was measured at 252 nm for 8-BrAMP (○) and at 262 nm for 6-ClPuMP (Δ). Experimental conditions were 0.02 M-sodium acetate/acetic acid buffer, pH 5.5, at 25°C. The total concentration of enzyme plus ligand was 50 μ M in the experiment with 8-BrAMP and 88.5 μ M with 6-ClPuMP.

values of $\Delta\epsilon$ around the approximate value of $\Delta\epsilon$ found from the direct plot of ΔA versus $[L_0]$. The value of $\Delta\epsilon$ that minimized the relative standard deviation for K_a was taken as the correct value. Table 2 shows the values of $\Delta\epsilon$ and K_a for the four nucleotides obtained by this method and by the method of Deranleau (1969). It should be stressed that all nucleotides gave a clear minimum for the relative standard deviation, and therefore the value of $\Delta\epsilon$ is highly reliable. The values of K_a are not in the range of what could be considered as weak interacting ligands, the basis of the method of Deranleau (1969). It is not surprising, then, to find discrepancies between both methods, except for some of the lower values (Tables 2 and 3).

The data shown in Table 2 indicate that the strength of the interaction is within the same order of magnitude for those nucleotides with an amino group in the 6-position, 8-BrAMP and AMP, or a polar group, GMP. The weakest interaction is with 6-CIPuMP, which has no polar group in that position. The remarkably strong interaction of 8-BrAMP could be due to the fact that this compound adopts the syn conformation instead of the anti conformation found with natural purine nucleotides (Ts'o, 1974*a,b*). The published values for the binding of purine nucleotides (Rabin *et al.*, 1970; Haffner & Wang, 1973) differ considerably from those reported here. The discrepancies are very likely to be due to the different methods used for their calculation.

The data in Table 3 indicate that the strength of the binding is also dependent on the ionic strength showing a reciprocal relationship, which means that the interaction between enzyme and ligand is essentially of an electrostatic nature. This is in agreement with the well-established fact that the main interaction between RNAase A and nucleotides takes place through the negatively charged phosphate group of the nucleotide molecule and the positively charged active site of the enzyme (Anderson *et al.*, 1968; Meadows *et al.*, 1969; Haffner & Wang, 1973).

Fig. 3 shows the difference spectra found on interaction of the enzyme with the different nucleotides as well as the difference spectra found on perturbation of the solvent. The similarities between the difference

spectra found when the hydrophobicity of the nucleotides environment is changed and those found on interaction of the same nucleotides with RNAase A are to be noticed. Thus, in the case of GMP, the wavelength that shows a positive maximum is the same in both experiments (solvent-perturbation and interaction with the enzyme) and it is similar in the cases of AMP and 8-BrAMP. The shape of the curves in the region 240–270 nm is very similar in both cases for all nucleotides when a final concentration of 10% dioxan is used (results not shown). When 20% dioxan is used a closer agreement is found, though there is a lack of definition below 250 nm. There are also many similarities in the region 270–300 nm, although some differences can also be found. Thus the split peak found in the binding of AMP and 8-BrAMP to the enzyme is not well defined in the difference spectra obtained by changing the polarity of the solvent. In the spectrum for 6-CIPuMP this region has the same shape, but it is shifted 3–5 nm. The difference spectra for change in pH show some similarities to those for enzyme binding for AMP and GMP, but not for 8-BrAMP and 6-CIPuMP. In this latter case there was no difference spectrum when the pH was lowered to 1.5–1.7. To find a difference spectrum it was necessary to go down to pH 0.0. This was probably due, as it has been stated above, to the very low pK of the base (Barlin & Chapman, 1965). Even in that case the difference spectrum was very different from that found on binding to the enzyme. The similarities

Table 2. $\Delta\epsilon$ and K_a values at pH 5.5 found on interaction of RNAase A with several purine nucleotides

(1) Values obtained by the method of Anderson *et al.* (1968). (2) Values obtained by the method of Deranleau (1969). Experimental conditions were: sodium acetate/acetic acid buffer, pH 5.5, I 0.2, at 25°C.

Nucleotide	$\Delta\epsilon$ (litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$)	$10^{-4} \times K_a$ (M $^{-1}$)	
		(1)	(2)
AMP	1625	0.53 ± 0.02	0.4
GMP	950	0.25 ± 0.03	0.19
8-BrAMP	1450	1.65 ± 0.15	0.95
6-CIPuMP	825	0.12 ± 0.05	0.11

Table 3. Variation of the association constant K_a between purine nucleotides and RNAase A as a function of the ionic strength. Values in parentheses are the values obtained by the method of Deranleau (1969). All experiments were carried out in sodium acetate/acetic acid buffer, pH 5.5, at 25°C.

Nucleotide	$I \dots$	$10^{-4} \times K_a$ (M $^{-1}$)			
		0.004	0.02	0.04	0.2
AMP		2.66 ± 0.41 (1.14)	2.10 ± 0.24 (1.02)	1.57 ± 0.2 (0.86)	0.53 ± 0.02 (0.4)
6-CIPuMP		0.48 ± 0.06 (0.52)	0.37 ± 0.13 (0.443)	0.28 ± 0.017 (0.23)	0.12 ± 0.005 (0.11)

Fig. 3. Solvent perturbation and RNAase A interaction difference spectra for several purine nucleotides (a) 50 μM -AMP + 50 μM -RNAase A (—); 66 μM -AMP from neutral to pH 1.82 (.....); 100 μM -AMP from aqueous to 20% dioxan (----). (b) 50 μM -GMP + 50 μM -RNAase A (—); 44 μM -GMP from neutral to pH 1.8 (.....); 100 μM -GMP from aqueous to 20% dioxan (----). (c) 50 μM -6-ClPuMP + 50 μM -RNAase A (—); 50 μM -6-ClPuMP from neutral to pH 0.0 (.....); 100 μM -6-ClPuMP from aqueous to 20% dioxan (----). (d) 50 μM -8-BrAMP + 50 μM -RNAase A (—); 50 μM -8-BrAMP from neutral to pH 1.2 (.....); 100 μM -8-BrAMP from aqueous to 20% dioxan (----). The data for the change in pH with AMP and GMP are taken from Deavin *et al.* (1968). Other experimental details are given in the text.

found with AMP and GMP were attributed by Deavin *et al.* (1968) as being due to a protonation of the base when the nucleotides bind to the enzyme. Such a protonation would take place through the formation of a hydrogen bond between N-7 atom of the base and an acid group of the enzyme. Richards & Wyckoff (1971) have suggested that an interaction with a basic group, capable of increasing the pK of the base, would result in the same effect.

The results reported in the present paper indicate that the difference spectra found on interaction of the enzyme with purine nucleotides are more closely reproduced by increasing the hydrophobicity of the ligand environment than by lowering the pH, suggesting that the enzyme region which interacts with the purine nucleotides has a partially hydrophobic character. However, the discrepancies between both sets of curves indicate that interactions other than the hydrophobic ones can also take place. The results cannot be taken as conclusive proof for a unique well-defined site in the enzyme specific for purine nucleotides, as it is known that small differences in the base cause a change in the place of binding, e.g. the bases in 3'-AMP and in 8-oxo-adenosine 3'-monophosphate interact with slightly different regions of the enzyme (Haar *et al.*, 1974).

In summary, the stoichiometry of the interaction is in all four cases 1:1, the values of the binding constants are similarly, within an order of magnitude, showing a reciprocal relationship to the ionic strength, and the environment of the binding region is partially hydrophobic. It can then be concluded that the halogenated purine nucleotides 6-ClPuMP and 8-BrAMP interact with RNAase A in a very similar manner to the natural purine nucleotides AMP and GMP. For these reasons and because of the enhanced reactivity due to the presence of the halogen atom they meet all the requirements to act as reagents for the affinity labelling of amino acids present in the purine-binding site of the enzyme.

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References

- Anderson, D. G., Hammes, G. G. & Walz, F. G., Jr. (1968) *Biochemistry* **7**, 1637-1645
- Barlin, G. B. & Chapman, N. B. (1965) *J. Am. Chem. Soc.* **87**, 3017-3022
- Brox, L. W. & Hampton, A. (1968) *Biochemistry* **7**, 398-405
- Cuchillo, C. M. (1974) Ph.D. Thesis, University of London
- Deavin, A., Mathias, A. P. & Rabin, B. R. (1966a) *Nature (London)* **211**, 252-255
- Deavin, A., Mathias, A. P. & Rabin, B. R. (1966b) *Biochem. J.* **101**, 14C-16C
- Deavin, A., Fisher, R., Kemp, C. M., Mathias, A. P. & Rabin, B. R. (1968) *Eur. J. Biochem.* **7**, 21-26
- Deranleau, D. A. (1969) *J. Am. Chem. Soc.* **91**, 4044-4049
- Gelpi, E., Parés, X. & Cuchillo, C. M. (1976) in *Advances in Mass Spectrometry in Biochemistry and Medicine* (Frigerio, A. & Castagnoli, N., eds.), vol. 1, pp. 215-230, Spectrum Publications, New York
- Haar, W., Maurer, W. & Rüterjans, H. (1974) *Eur. J. Biochem.* **44**, 201-211
- Haffner, P. H. & Wang, J. H. (1973) *Biochemistry* **12**, 1608-1618
- Hammes, G. G. & Schimmel, P. R. (1965) *J. Am. Chem. Soc.* **87**, 4665-4669
- Hampton, A. & Maguire, M. H. (1961) *J. Am. Chem. Soc.* **83**, 150-157
- Ikehara, M. & Uesugi, S. (1969) *Chem. Pharm. Bull.* **17**, 348-354
- Ikehara, M., Tazawa, T. & Fukui, T. (1969) *Chem. Pharm. Bull.* **17**, 1019-1024
- Irie, M. & Sawada, F. (1967) *J. Biochem. (Tokyo)* **62**, 282-284
- Meadows, D. H., Roberts, G. C. K. & Jardetzky, O. (1969) *J. Mol. Biol.* **45**, 491-511
- Rabin, B. R., Cuchillo, C. M., Deavin, A., Kemp, C. M. & Mathias, A. P. (1970) in *Metabolic Regulation and Enzyme Action* (Sols, A. & Grisolia, S. eds.), pp. 203-218, Academic Press, New York and London
- Richards, F. M. & Wyckoff, H. W. (1971) *Enzymes 3rd Ed.* **4**, 647-806
- Sawada, F. & Ishii, F. (1968) *J. Biochem.* **64**, 161-165
- Sela, M. & Anfinsen, C. B. (1957) *Biochim. Biophys. Acta* **24**, 229-235
- Shaw, G., Smallwood, B. M. & Wilson, D. V. (1968) *J. Chem. Soc.* 1916-1919
- Taborsky, G. (1959) *J. Biol. Chem.* **234**, 2652-2656
- Taylor, M. J., Kohn, B. D., Taylor, W. G. & Kohn, P. (1973) *Carbohydr. Res.* **30**, 133-142
- Ts'o, P. O. P. (1974a) in *Basic Principles in Nucleic Acid Research* (Ts'o, P. O. P., ed.), vol. 1, pp. 453-584, Academic Press, New York
- Ts'o, P. O. P. (1974b) in *Basic Principles in Nucleic Acid Research* (Ts'o, P. O. P., ed.), vol. 2, pp. 305-409, Academic Press, New York
- Witzel, H. & Barnard, E. A. (1962) *Biochem. Biophys. Res. Commun.* **7**, 295-299
- Yoshikawa, M., Kato, T. & Takenishi, T. (1967) *Tetrahedron Lett.* 5065-5068
- Yoshikawa, M., Kato, T. & Takenishi, T. (1969) *Bull. Chem. Soc. Jpn* **42**, 3505-3508