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ALKYLATION OF ADENOSINE DEAMINASE BY BENZYLBROMOACETATE AND 9-(*p*-BROMOACETAMIDOBENZYL)ADENINE

G. RONCA, M. F. SAETTONE* AND A. LUCACCHINI
Istituto di Chimica Biologica, University of Pisa, Pisa (Italy)

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SUMMARY

1. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa is not affected at pH 8 by haloacetate and haloacetamide, while benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine inactivate the enzyme. Substrate analogs protect against inactivation by these reagents.

2. One mole of reagent is bound per mole of inactivated enzyme. The alkylated amino acid has been identified as lysine.

3. The reaction of alkylation consists of two stages: binding of the alkylating agent (fast reaction) and alkylation of the ε-amino group of lysine (slow reaction).

4. Bromoacetate, which does not inactivate the enzyme, does not alkylate the reactive lysine.

5. The difference in reactivity is explained on the basis of a selectivity of the alkylation site on the enzyme towards the alkylating reagents.

INTRODUCTION

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic breakdown of adenosine to inosine and free ammonia. The enzyme is distributed widely in animal tissues and in microorganisms and has been obtained in highly purified form from calf intestinal mucosa, calf spleen and *Aspergillus oryzae*[1–3].

The enzyme from bovine intestinal mucosa is relatively specific in deaminating, in addition to adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 2-fluoroadenosine and 2-aminoadenosine and also converts 6-chloropurine riboside to inosine[4,5].

The enzyme is inactivated by some mercurials and a stoichiometric relationship exists between the equivalents of mercaptide formed and the inactivation of enzyme; substrate analogs protect the enzyme against mercurial inactivation[6]. The enzyme is also inactivated by acetic anhydride and formaldehyde *plus* acetamide even if the sulphydryl groups are protected by *p*-chloromercuribenzoate (PCMB)[7]. At alkaline

Abbreviations: PCMB, *p*-chloromercuribenzoate; FDNB, 1-fluoro-2,4-dinitrobenzene; CM-, carboxymethyl-.

* Istituto di Chimica Farmaceutica, University of Pisa, Pisa, Italy.

pH the enzyme is rapidly inactivated by 1-fluoro-2,4-dinitrobenzene (FDNB) with the formation of 2 moles of ε-*N*-DNP-lysine per mole of inactivated enzyme and of small amounts of *O*-DNP-tyrosine and *S*-DNP-cysteine[8].

Between pH 6 and 8 the common alkylating agents, iodoacetate, iodoacetamide and *N*-ethylmaleimide, do not affect enzyme activity and do not react with the sulphydryl groups of adenosine deaminase[6]. Iodoacetate at pH 9 slowly inactivates the enzyme and 5 equivalents of radioactive reagent are incorporated per mole of inactivated adenosine deaminase; 2.9, 0.7 and 0.55 moles of ε-CM-lysine, ε,ε-di-CM-lysine and *S*-CM-cysteine, respectively, were found per mole of iodoacetate-inactivated enzyme[9].

SCHAEFFER AND ODIN[10] have synthesized two substrate analogs, 9-(*p*- or *m*-bromoacetamidobenzyl)adenine which at pH 7.5 inactivate the enzyme. They have also suggested that the reaction proceeds through the formation of a reversible adenosine deaminase–reagent complex. In this paper the alkylation of adenosine deaminase has been further considered. It has been shown that one equivalent of 9-(*p*-bromoacetamidobenzyl)adenine reacts per mole of enzyme; the alkylated amino acid has been identified as lysine and the kinetic rate constants of the alkylation reaction have also been determined.

Benzylbromoacetate, which is not a substrate analog, inactivates the enzyme, while bromoacetate, which does not inactivate the enzyme, does not react with this lysine.

MATERIALS

Adenosine deaminase was purified from calf intestinal mucosa[1]. The final enzyme preparation was devoid of any phosphatase activity and showed a specific activity of 450 units per mg of protein. The enzyme was eluted from Sephadex G-75 and DEAE-cellulose columns as a single symmetric peak with constant specific activity[11]. However, electrophoresis on starch gel revealed five active bands. The electrophoresis was performed as described by BRADY AND O'CONNELL[1].

9-(*p*-Bromoacetamidobenzyl)adenine (systematic name: 6-amino-9-[*p*-(2-bromoacetamido)benzyl]purine; structural formula I) and 9-(*p*-acetamidobenzyl)adenine (systematic name: 6-amino-9-(*p*-acetamidobenzyl)purine) were synthesized according to SCHAEFFER AND ODIN[10].

The CM-derivatives of lysine, histidine and methionine were prepared as described by GUNDLACH *et al.*[12,13]. *S*-CM-Cysteine, poly-L-lysine hydrobromide (mol. wt. 10 000–20 000) and benzylbromoacetate were purchased from Mann, Cyclo Chemical Co. and Schuchardt, respectively. All other chemicals were of analytical reagent grade and were used as obtained.

METHODS

The concentration of adenosine deaminase was measured by the microbiuret method using lyophilized enzyme as a standard, or from its absorbance at 280 nm; the determination of ε at 280 nm based on dry weight gives a value of $31.8 \pm 0.7 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The molecular weight of adenosine deaminase was considered to be 35 000 (refs. 6, 14). The concentration of poly-L-lysine was determined by ninhydrin reaction

after acid hydrolysis. The enzymatic activity was determined with adenosine as substrate by following the decrease in absorbance at 265 nm, according to the method of KALCKAR[15] in 0.1 M sodium phosphate buffer (pH 7.5). One enzyme unit is the amount of enzyme which will convert 1 μmole of adenosine to inosine per min at pH 7 and at 37°. All activities given are the average of at least two assays.

The reactivity of the alkylating reagents with 4-(*p*-nitrobenzyl)pyridine was determined as described by SCHAEFFER AND ODIN[10]. Adenosine deaminase (0.5–200 μM) was treated at 37° in the dark and in 0.2 M sodium phosphate buffer (pH 8) containing 10% (v/v) dimethylsulphoxide, with bromoacetate, benzylbromoacetate or 9-(*p*-bromoacetamidobenzyl)adenine (0.01–5 mM). Benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine were added as solutions in dimethylsulphoxide. When necessary the pH during reaction was kept constant by the addition of 1 M NaOH. At intervals 5–10-μl samples were removed, diluted in 0.1 M sodium phosphate buffer (pH 7.5) and assayed for adenosine deaminase activity.

The enzyme treated with 9-(*p*-bromoacetamidobenzyl)adenine was passed through a column (2.5 cm × 10 cm) of Sephadex G-25 medium equilibrated with 0.1 M NH_4HCO_3; the major part of unreacted 9-(*p*-bromoacetamidobenzyl)adenine precipitated on the column; alternatively, before column separation the reaction mixture was kept at 0° for 60–120 min: during this period no further inactivation of enzyme was observed and a great part of unreacted 9-(*p*-bromoacetamidobenzyl)adenine precipitated. The equivalents of reagent bound to protein were estimated spectrophotometrically as described under RESULTS. The spectra were determined with a Zeiss PMQ II spectrophotometer and the kinetic measurements were made with the same apparatus equipped with a Zeiss T-E converter and a Varian G-2000 recorder. For the measurement of difference spectra the experimental procedure suggested by HAMMES AND SCHIMMEL[16] was followed. Rectangular tandem cells having a 4.4-mm path length for each compartment were used. The difference spectra were determined directly. One set of tandem cells contained adenosine deaminase and 9-(*p*-acetamidobenzyl)adenine in separate compartments while the other set contained the same concentrations of adenosine deaminase and 9-(*p*-acetamidobenzyl)adenine in one compartment and buffer in the other. The concentrations of adenosine deaminase used ranged from 20 to 40 μM while those of 9-(*p*-acetamidobenzyl)adenine from 40 to 120 μM. The experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.5) at 25°. Protein hydrolysis was carried out in evacuated sealed vials according to the method of MOORE AND STEIN[17] for the highest recovery of *S*-CM-cysteine and tyrosine.

Amino acid analysis was performed on an Unichrom amino acid analyzer equipped with 60-cm and 7-cm columns[18]. In the case of alkylated adenosine deaminase hydrolysates a second analysis was made under modified conditions (in pH 3.19; ref. 19) for the resolution of CM-histidines from other peaks; in order to separate ε-CM-lysine from methionine the following conditions were used: first buffer pH 3.25, second buffer pH 3.80, buffer change after 120 min; the analysis was started at 42° and immediately after starting the temperature was raised to 60°: this temperature was reached with our apparatus and with a room temperature of 20–22°, in about 1 h. In these conditions the ε-CM-lysine peak is observed well separated between methionine and isoleucine.

RESULTS

Inactivation of adenosine deaminase by benzylbromoacetate and 9-(p-bromoacetamidobenzyl)adenine

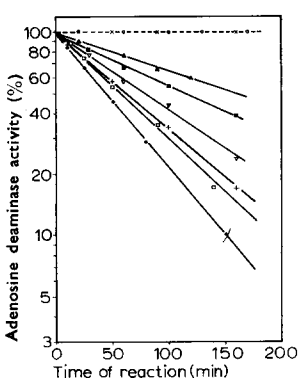
When adenosine deaminase was treated with 5 mM bromoacetate at pH 8.0 no activity was lost as may be seen in Fig. 1. However, when the enzyme is treated with benzylbromoacetate or with 9-(*p*-bromoacetamidobenzyl)adenine activity is lost (Fig. 1). Since the two reagents are in excess, straight lines are obtained when the log (percentage of adenosine deaminase activity remaining) is plotted against time. The slopes of these lines, however, are not proportional to the concentration of alkylating reagent. The pseudo-first-order rate constants calculated from these slopes are given in the legend of Fig. 1. The lack of proportionality of the pseudo-first-order rate constants may be explained assuming formation of a reversible enzyme–alkylating reagent complex (*EI*), as proposed by SCHAEFFER AND ODIN[10]:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} EA$$

where *EA* is the alkylated enzyme.

If $E \ll I$ and the formation of *EA* is slow with respect to *EI* formation, then the integrated rate equation for the enzyme alkylation is:

$$k = k_2 \frac{[I]}{K_i + [I]} = \frac{1}{t} \ln \frac{[E_0]}{[E_0] - [EA]} \qquad (1)$$



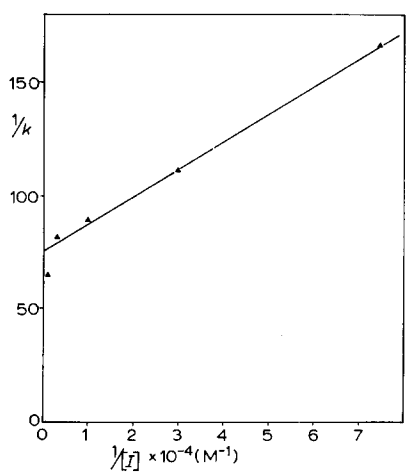
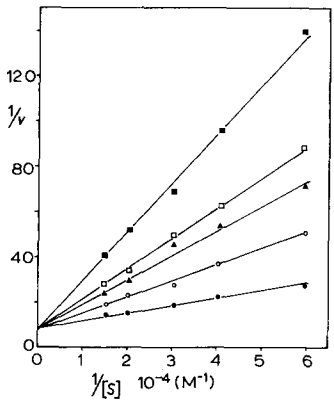


Fig. 1. Inactivation of adenosine deaminase by benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine. Temperature, 37°; 0.5 μM adenosine deaminase in 0.2 M sodium phosphate (pH 8.0) containing 10% (v/v) dimethylsulphoxide. Control without alkylating reagents (○); 5 mM bromoacetate (×); 1 mM benzylbromoacetate (▲); 0.0133 mM (■), 0.033 mM (▽), 0.1 mM (+), 0.33 mM (□) and 1 mM (●) 9-(*p*-bromoacetamidobenzyl)adenine. Owing to its low solubility benzylbromoacetate was in a fine emulsion. The pseudo-first-order rate constants calculated from the slopes of the lines reported in the figure are $4.35 \cdot 10^{-3}$ (▲), $6.0 \cdot 10^{-3}$ (■), $8.9 \cdot 10^{-3}$ (▽), $11.2 \cdot 10^{-3}$ (+), $12.2 \cdot 10^{-3}$ (□) and $15.4 \cdot 10^{-3}$ (●) min^{-1}.

Fig. 2. Dependence of the pseudo-first-order rate constants on 9-(*p*-bromoacetamidobenzyl)adenine concentration. The reciprocal value of the pseudo-first-order rate constants given in Fig. 1 is reported as a function of the reciprocal value of the alkylating reagent concentration. Further details are given under RESULTS.



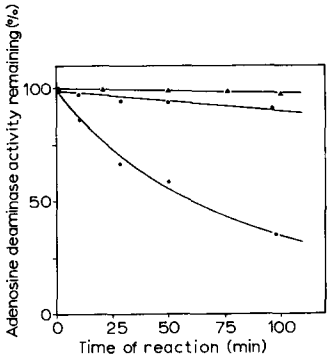


Fig. 3. Competitive inhibition of adenosine deaminase by 9-(*p*-bromoacetamidobenzyl)adenine and 9-(*p*-acetamidobenzyl)adenine. The reaction was carried out in 0.1 M sodium phosphate buffer (pH 7.5) at 25°. Velocity (v) is expressed as absorbance change per min at 265 nm. ●, no inhibitor; ○, 10 μM 9-(*p*-bromoacetamidobenzyl)adenine (K_i = 9.3 μM); □, 25 μM 9-(*p*-bromoacetamidobenzyl)adenine (K_i = 8.7 μM); ▲, 10 μM 9-(*p*-acetamidobenzyl)adenine (K_i = 4.6 μM); ■, 25 μM 9-(*p*-acetamidobenzyl)adenine (K_i = 4.8 μM).

Fig. 4. Protection of purine riboside against adenosine deaminase inactivation by benzylbromoacetate. Temperature, 37°; 2 μM adenosine deaminase in 0.2 M sodium phosphate (pH 8.0) containing 10% (v/v) dimethylsulphoxide. Control without benzylbromoacetate or purine riboside (▲); 1 mM benzylbromoacetate added (●); 1 mM benzylbromoacetate and 10 μM purine riboside added (○).

where K_i is the dissociation constant of EI to E and I, (E_0) is the total enzyme concentration and k the experimental rate constant reported in the legend of Fig. 1. From Eqn. 1 it appears that when $I \ll K_i$, k is proportional to I while when $I \gg K_i$, k is independent from I. By plotting $1/k$ against $1/[I]$, k_2 and K_i can be calculated. The values of k_2 and K_i, as calculated from Fig. 2, are $1.3 \cdot 10^{-2}$ min^{-1} and 16 μM, respectively.

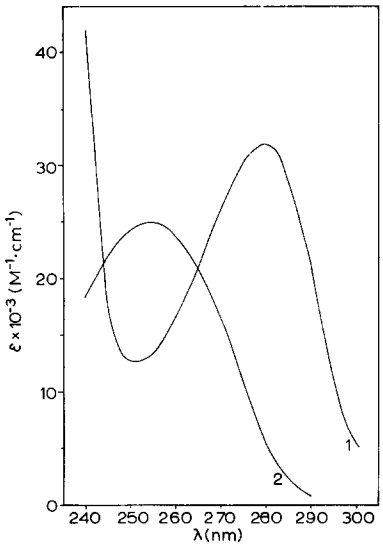
From Fig. 3 it appears that 9-(*p*-bromoacetamidobenzyl)adenine is also a competitive inhibitor with respect to adenosine with a K_i value of 9 μM.

The inactivation of adenosine deaminase at different benzylbromoacetate concentrations, and the behavior of this reagent as enzyme inhibitor were not investigated owing to its low solubility.

It was not possible to use adenosine, owing to its rapid deamination, for the study of the protection by substrate against benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine inactivation. We have used purine riboside, which is a competitive inhibitor of adenosine deaminase (K_i = 7 μM; ref. 20). Fig. 4 shows that the presence of purine riboside strongly protects the enzyme against benzylbromoacetate inactivation. Protection by purine riboside against 9-(*p*-bromoacetamidobenzyl)adenine has been also observed.

Stoichiometry of the reaction of adenosine deaminase with 9-(p-bromoacetamidobenzyl)-adenine

The equivalents of 9-(*p*-bromoacetamidobenzyl)adenine reacted with the enzyme were calculated spectrophotometrically after separation of the excess of reagent by gel filtration. The calculation is based on the assumption that the spectrum of the



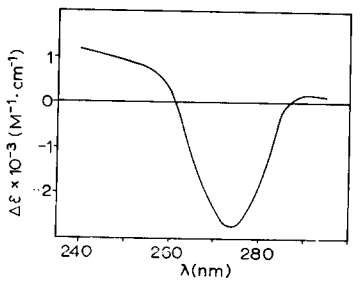


Fig. 5. Ultraviolet spectra of adenosine deaminase and 9-(*p*-acetamidobenzyl)adenine. Adenosine deaminase spectrum was determined in 0.1 M NH_4HCO_3 (Curve 1) while that of the N-9-substituted adenine in 5% (v/v) ethanol (Curve 2). Temperature, 25°.

Fig. 6. Ultraviolet difference spectrum resulting from the adenosine deaminase–9-(*p*-acetamidobenzyl)adenine binding. Temperature, 25°. Further details are given in the text.

alkylated enzyme results from the addition of adenosine deaminase and 9-(*p*-acetamidobenzyl)adenine spectra reported in Fig. 5. However, when the enzyme is mixed with 9-(*p*-acetamidobenzyl)adenine a difference spectrum may be observed; nevertheless the differences are small in comparison with the absorption of the enzyme and the reagent and have been disregarded. 9-(*p*-Acetamidobenzyl)adenine is a competitive inhibitor with respect to adenosine with a K_i of 5 μM at 25° (Fig. 3). In Fig. 6 the molar difference spectrum resulting from the enzyme-9-(*p*-acetamidobenzyl)adenine binding is reported; the molar difference coefficient ($\Delta\varepsilon$) is equal to ($\varepsilon_E + \varepsilon_I - \varepsilon_{EI}$) which is the sum of the molar extinction coefficients of enzyme, reagent and enzyme–reagent complex, respectively.

From Fig. 5 it appears that the spectra have two isosbestic points at 244 nm (ε = 21 100) and at 265 nm (ε = 21 000); from the absorption of the enzyme eluted from Sephadex G-25 column at these two wavelengths it is possible to calculate the sum of the molar concentrations of the protein and the reagent bound to the enzyme. The results obtained at both wavelengths, which agree within a range of ± 2%, have been averaged and used to calculate the equivalents of reagent bound, by the same method used by GOODWIN AND MORTON[21] to calculate tyrosine and tryptophan in protein. The following equation was used:

$$y = \frac{A_{255\text{ nm}} - x\varepsilon_2}{\varepsilon_1 - \varepsilon_2}$$

where x is total molar concentration (reagent bound *plus* adenosine deaminase) calculated from the isosbestic points; y, adenosine deaminase concentration; $A_{255\text{ nm}}$, experimental absorption at 255 nm of the 9-(*p*-bromoacetamidobenzyl)adenine-treated

TABLE I

RELATIONSHIP BETWEEN 9-(*p*-BROMOACETAMIDOBENZYL)ADENINE FIXED TO ADENOSINE DEAMINASE AND LOSS OF ENZYMATIC ACTIVITY

The experimental procedure is given under METHODS.

Expt. No.	*Inactivation before Sephadex column (%)*	*Specific activity after Sephadex column (units/mg)*	*Reagent fixed* (equivalents)*
1	0	442	0
2	15	374	0.15
3	39	275	0.36
4	70	132	0.68
5	80	88	0.81
6	99	—	0.97

* Moles of reagent fixed per mole of adenosine deaminase (see RESULTS for calculation).

enzyme after Sephadex column separation; ε_1 and ε_2 the molar extinction coefficients at 255 nm of adenosine deaminase and 9-(*p*-acetamidobenzyl)adenine, respectively. The concentration of reagent bound (z) was calculated from the following equation:

$$x = y + z$$

The absorption at 255 nm (ε of reagent and protein 25 000 and 13 400, respectively) was used to calculate the equivalents of reagent bound, although other wavelengths may be used.

In Table I the percent inactivation, the specific activity after gel filtration and the equivalents of reagent bound, calculated as described, are reported. It must be pointed out that no variation of the specific activity and of the spectrum of the alkylated enzyme can be detected several hours after elution from Sephadex column.

A good relationship exists between inactivation and equivalents of reagent bound (Table I). Fig. 7 reports the spectra of both 39 and 70% inactivated enzyme (Expts. 3 and 4 of Table I).

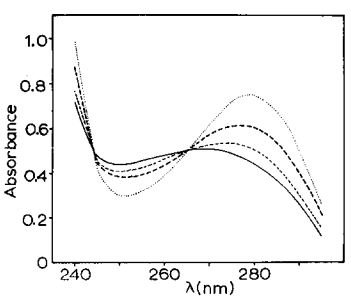


Fig. 7. Ultraviolet spectra of adenosine deaminase and 9-(*p*-bromoacetamidobenzyl)adenine inactivated adenosine deaminase; . . . . , adenosine deaminase spectrum; ———, theoretical spectrum resulting from the addition of the spectra of adenosine deaminase and 9-(*p*-acetamidobenzyl)-adenine; — — —, experimental spectrum of 39% inactivated adenosine deaminase (Expt. 3, Table I); - - - - - -, experimental spectrum of 70% inactivated adenosine deaminase (Expt. 4, Table I). The spectra were normalized using the 265 nm isosbestic point. Temperature, 25°.

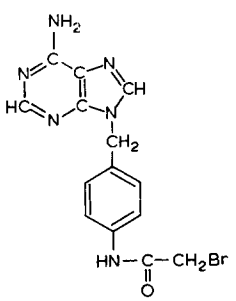
Inactivation of bromoacetate-treated enzyme by benzylbromoacetate and 9-(p-bromoacetamidobenzyl)adenine

In order to determine if bromoacetate reacts at the same site of benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine without loss of adenosine deaminase activity, the enzyme was treated with 5 mM bromoacetate at 37° in 0.2 M sodium phosphate buffer (pH 8.0) containing 10% dimethylsulphoxide. After 210 min the enzyme was passed through a Sephadex G-25 column; no loss of enzyme activity was detected. The bromoacetate-treated enzyme and the control (untreated enzyme) were allowed to react with benzylbromoacetate: no difference in the rate of inactivation was observed; furthermore a complete inactivation was reached.

Similar results have been obtained with 9-(*p*-bromoacetamidobenzyl)adenine.

Identification of alkylated amino acid residues

In order to determine the nature of the amino acid residues alkylated by 9-(*p*-bromoacetamidobenzyl)adenine, the alkylated enzyme has been hydrolysed in 5.7 M HCl. From the structure of the 9-(*p*-bromoacetamidobenzyl)adenine-modified enzyme it appears that upon acid hydrolysis CM-amino acids and 9-(*p*-aminobenzyl)adenine will be expected (see Scheme II, where the broken line represents the hydrolysable bond).



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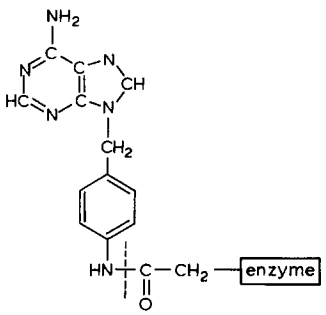

II

To check this hypothesis and to evaluate the best conditions of hydrolysis poly-L-lysine was treated with 9-(*p*-bromoacetamidobenzyl)adenine and passed through a Sephadex G-25 column; the amount of reagent fixed (8.5 residues of reagent per 100 residues of lysine) was determined spectrophotometrically at 255 nm. The alkylated poly-L-lysine was hydrolysed for 24, 48 and 72 h and analysed for amino acid composition. Only three peaks are present on the chromatogram namely, α-CM-lysine, ε-CM-lysine and lysine. From Table II it appears that the highest recovery of α- and ε-CM-lysine was obtained with the 48-h hydrolysate; a longer hydrolysis (72 h) gives lower values, while after 24 h hydrolysis seems to be incomplete. The 48- and 72-h values for ε- and α-CM-lysine extrapolated to zero time give 5.4 and 3.4 moles per 100 moles of lysine, respectively, and account for the 104% of the expected values on the basis of the spectrophotometric determination.

The amino acid composition of adenosine deaminase and of 80% inactivated enzyme (Expt. 5, Table I) is reported in Table III. From the inactivation and from the equivalents of reagent bound, 0.8 residues of CM-amino acids per mole of adenosine deaminase would be expected. ε-CM-lysine (0.68 residues per molecule) accounts for

TABLE II

α- AND ε-CM-LYSINE RECOVERIES FROM ACID HYDROLYSATES OF 9-(*p*-BROMOACETAMIDOBENZYL)-ADENINE-TREATED POLY-L-LYSINE

10 mg of poly-L-lysine (mol. wt. 10 000–20 000) were allowed to react with a limiting amount of 9-(*p*-bromoacetamidobenzyl)adenine (1 mole of reagent per 10 moles of lysine residues) at pH 10 and at 37° overnight and passed through a Sephadex G-25 column. The reagent fixed (8.5 residues per 100 residues of lysine) has been calculated spectrophotometrically from the absorption at 255 nm without correction for the very small poly-L-lysine absorption, while poly-L-lysine concentration was determined by ninhydrin after acid hydrolysis. Duplicate samples have been hydrolysed for 24, 48 and 72 h and analysed for amino acid composition.

Hydrolysis time (h)	*ε-CM-lysine* (μmoles per 100 μmoles of lysine)*	*α-CM-lysine* (μmoles per 100 μmoles of lysine)*
24	3.9	1.6
48	4.2	3.0
72	3.6	2.8

* The 48- and 72-h values for ε- and α-CM-lysine extrapolated to zero time give 5.4 and 3.4 μmoles of ε- and α-CM-lysine per 100 μmoles of lysine, respectively.

85% of the expected CM-amino acids. This value is not corrected for possible destruction during hydrolysis, as demonstrated by the recovery of ε-CM-lysine from the alkylated poly-L-lysine.

A trace of *S*-CM-cysteine is also present.

Similar results (0.87 moles of ε-CM-lysine per mole) have been obtained with the 99% inactivated enzyme (Expt. 5, Table I).

DISCUSSION

Adenosine deaminase is not inactivated by the more usual reagents for the alkylation of enzymes (haloacetate and its amide) while other alkylating reagents as benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine inactivate the enzyme. The different behavior of these compounds towards adenosine deaminase cannot be explained on the basis of the different reactivity with model compounds or of their different size. In fact, benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine are only 4 and 2.5 times more reactive than bromoacetate towards 4-(*p*-nitrobenzyl)-pyridine. It is also conceivable that haloacetate and its amide react at the same place as benzylbromoacetate and 9-(*p*-bromoacetamidobenzyl)adenine, *e.g.* near the active site, without determining loss of adenosine deaminase activity owing to their low steric hindrance. This hypothesis, however, may be ruled out, since bromoacetate-treated enzyme is inactivated by benzylbromoacetate and 9-(*p*-bromoacetamido-benzyl)adenine.

The difference in reactivity may be well explained assuming a selectivity of the reaction site on the enzyme molecule towards the alkylating reagents used. HEINRIKSON *et al.*[23] who have studied the reactivity of the histidines in the active site of ribonuclease towards a number of haloacids of different structure, demonstrated that the site (histidine 12 or 119) and the rate of reaction depend on the structural characteristics of the reagent. These authors suggest that the over-all reaction of

TABLE III

AMINO ACID COMPOSITION OF ADENOSINE DEAMINASE AND 9-(*p*-BROMOACETAMIDOBENZYL)ADENINE-TREATED ENZYME

Duplicate analyses of adenosine deaminase by ion-exchange chromatography were performed on 24-, 48- and 72-h hydrolysates while duplicate analyses of 80% inactivated enzyme by 9-(*p*-bromoacetamidobenzyl)adenine (Expt. 4, Table I) were performed on 48-h hydrolysates. Replicate analyses agreed to ± 2.5%. A molecular weight of 35 000 was used to calculate the residues per molecule.

Residue	*Adenosine deaminase*		*Alkylated adenosine deaminase (residues per molecule)*
	Residues per molecule	*Nearest integral number of residues per molecule*	
Lys	22.2	22	21.1
His	10.0	10	10.2
Arg	12.9	13	13.0
Asp	27.6	28	27.7
Thr*	17.7	18	17.9
Ser*	18.1	18	17.7
Glu	38.1	38	37.4
Pro	17.6	18	18.0
Gly	20.2	20	20.1
Ala	23.3	23	23.2
Cys**	6.8	7	6.7
Val***	21.9	22	21.7
Met**	5.8	6	5.8
Ile***	11.0	11	10.9
Leu	26.9	27	26.7
Tyr*	12.0	12	12.1
Phe	9.9	10	10.0
Trp†	3	3	†††
ε-CM-lysine††	0	0	0.68
S-CM-cysteine	0	0	traces

* The average 24-, 48- and 72-h values for threonine, serine and tyrosine were extrapolated to zero time to correct for destruction during hydrolysis.

** Half-cystine and methionine were determined in duplicate as cysteic acid and methionine sulfone on performic acid-oxidized protein[22]. For the alkylated enzyme the values of these two amino acids, determined as half-cystine and methionine, were corrected for destruction during hydrolysis.

*** The average 72-h values were taken for isoleucine and valine. For the alkylated enzyme the average 48-h values are reported.

† A molecular ratio of 4.0 was determined for tyrosine to tryptophan by the method of GOODWIN AND MORTON[21].

†† A value of 0.8 is expected on the basis of the equivalents of reagent bound and of the inactivation.

††† Not determined.

alkylation consists of two stages: the binding of the alkylating reagent and the alkylation of the imidazole ring; in their case these two stages cannot be distinguished. In the alkylation of adenosine deaminase by 9-(*p*-bromoacetamidobenzyl)adenine two reaction steps are distinguishable. In fact, owing to the high affinity of the reagent for the enzyme and to the slowness of the alkylation, a rate-saturation effect is observed with increasing reagent concentration and it is possible to calculate both the dissociation constant of the enzyme–alkylating reagent complex and the first order rate constant of the alkylation of the amino acid residues.

Although the reaction of adenosine deaminase with benzylbromoacetate was not studied in detail it must be pointed out that the esterification of the carboxyl group of bromoacetate with a benzyl alcohol greatly increases the reactivity of the compound towards the enzyme, while the amidation of haloacetate does not change the reactivity. It must be also underlined that benzylbromoacetate, contrary to 9-(*p*-bromoacetamidobenzyl)adenine, is not a substrate analog. Previous results obtained with *N*-alkyl-substituted ureas, which are competitive inhibitors with respect to adenosine, indicated that the affinity of these compounds for the enzyme increases with increasing chain length of the substituent[24]. Similar results were obtained with aliphatic alcohols and 9-alkyladenines, and it was suggested that an apolar region exists near the active site[25,26]. It is likely that both the benzylbromoacetate and bromoacetamidobenzyl moiety of 9-(*p*-bromoacetamidobenzyl)adenine bind at this region and in the case of the last reagent the presence of the purine moiety further increases the affinity.

ε-CM-lysine, which accounts for 85–90% of the CM-amino acids expected, is the sole measurable CM-amino acid present in the chromatogram. This value is not corrected for the possible destruction during acid hydrolysis as demonstrated from the recovery of ε- and α-CM-lysine in the 9-(*p*-bromoacetamidobenzyl)adenine treated poly-L-lysine. It is possible, however, that a small amount (not measurable in amino acid analysis) of other CM-amino acids is formed. In the case of the alkylation of ribonuclease with [^{14}C]bromoacetate, it was shown that several CM-amino acids or their degradation products were detected by means of flow-cell scintillation counting, while in the amino acid chromatogram they did not appear[27].

Previous experiments with mercurials indicate that a sulphydryl group located in or near the active site of adenosine deaminase is essential for the activity. This sulphydryl, however, does not react with iodoacetate, iodoacetamide and *N*-ethylmaleimide. To explain these results it was suggested that the sulphydryl group is contained in a hydrophobic region[6]. It does not react with 9-(*p*-bromoacetamidobenzyl)adenine; only traces of *S*-CM-cysteine are detectable in the acid hydrolysate of the alkylated enzyme. The lack of reactivity of the sulphydryl with this reagent may be explained in at least two ways: for steric reasons the sulphydryl group is not accessible to the bromoacetamido moiety of the reagent; if both the sulphydryl and the ε-amino group (which are supposed to be accessible to the reagent in a similar way) are contained in a hydrophobic environment, the pK_a of the ε-amino group decreases while that of the sulphydryl group increases[28]; the reactivity of these groups also will change since alkylating compounds react preferentially with the deprotonated form of the sulphydryl and ε-amino groups[29].

Although the substrate analogs protect against inactivation by 9-(*p*-bromoacetamidobenzyl)adenine and only 1 mole of ε-CM-lysine is formed per mole of inactivated enzyme, it is not possible on the basis of these results to speculate on the possible role of this lysine either in the binding of the substrate at the active site or in the catalytic process. In fact there is no evidence that this lysine really participates in the formation of the active site. It has been shown that the uncharged forms of substrate analogs and amidine derivatives are competitive inhibitors of adenosine deaminase, while their positively charged forms do not bind at the active site[20,24]. It may be supposed that lysine, possibly together with other positively-charged groups distributed around and/or in the active site, determines a positively-charged

environment, preventing the binding of positively-charged compounds and pushing out of the active site the NH_4^+ formed in the hydrolytic breakdown of adenosine.

MURPHY *et al.*[14] showed that adenosine deaminase purified from bovine intestinal mucosa contains multiple forms (5 or 6) which are isolable both by ion-exchange chromatography and by zone electrophoresis. These forms have some fundamental common characteristics: (a) they are formed by a single polypeptide chain of 35 000 mol. wt.; (b) are inactivated by mercurials; (c) the N-terminal group is in some way substituted; and (d) after tryptic digestion of the isolated isoenzymes no significant difference is observed in the number and type of peptides released.

Although the enzyme preparation used in this study contained five multiple forms, a complete inactivation by benzylbromoacetate or 9-(*p*-bromoacetamidobenzyl)adenine was obtained. This indicates that all the forms present in the preparation are sensible to these reagents. From the amino acid analysis of the alkylated enzyme it also appears that at least 85–90% of the molecules of the enzyme preparation used in this study contain the reactive lysine.

Studies are in progress to isolate the peptide containing the alkylated lysine after tryptic digestion.

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