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SEQUENTIAL ENZYMIC ACTIVATION AS A MEANS OF INCREASING THERAPEUTIC EFFICIENCY OF NITROGEN MUSTARDS

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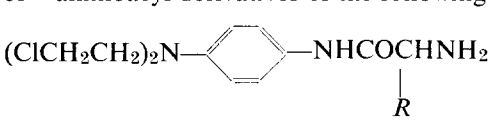
Abstract—Chemical and biological activities of a series of derivatives of N,N-bis(2-chloroethyl)-N′-glycyl-*p*-phenylenediamine (A) have been compared with N,N-bis(2-chloroethyl)-*p*-phenylenediamine (B). The glycyl–mustard (A) and derivatives with N-acyl substituents in the glycine moiety had similar chemical reactivities which were considerably less than that of (B). The low values suggested that only borderline activity would be expected in antitumor tests unless enzymic activation occurred. A wide range of toxicity values and therapeutic indices was observed in a Walker carcinosarcoma-256 test system. There was a direct correlation between LD_{50} values and the rates of hydrolysis of the glycyl–mustard bond of (A) and of its N-acetyl and N-methylcarbamyl derivatives. (A) was rapidly hydrolyzed by rat tissue homogenates: the ratio of tumor/liver enzyme activities was small and the therapeutic index was small. N-Acetyl derivative of (A) was hydrolyzed at a moderate rate: tumor/liver enzyme activity was high and the therapeutic index was high. Similar relationships held when enzyme activity value of tumor was compared with that of ileum, spleen, or kidney. Evidence has been obtained which suggests that, with derivatives of (A), deacylation of the substituted amino acid moiety is the rate-limiting step for the subsequent enzymic release of (B).

PREVIOUS studies[1, 2] have shown that the selectivity of action of nitrogen mustards for tumor cells may be increased by making the drug dependent for activity upon the action of constitutive enzymes in the tumor. Acylation of the free amino group of the extremely reactive but nonselective compound, N,N-bis(2-chloroethyl)-*p*-phenylenediamine, decreased the chemical reactivity, i.e. the ease of formation of the alkylating species. However, with some derivatives, an increased selectivity of action against the Walker carcinosarcoma was observed. Antitumor action was correlated with the ability of the tumor cells to remove the inactivating group enzymatically, thus releasing the active parent mustard intracellularly.

A continuation of the design of drugs based on the principle of enzymic activation led to the synthesis[3] of α-aminoacyl derivatives of the following type:



where *R* is the acyl group of the amino acid. Phenylalanyl and glycyl analogs were tested against the Walker tumor, but were not found to be consistently effective. From these results the authors concluded that deacylation to *p*-phenylenediamine mustard

probably did not occur. The latter problem has been studied in this laboratory by means of the glycyl analog. In addition, antitumor activity and ease of hydrolysis of the glycyl–phenylenediamine mustard bond of some N-acylated derivatives of this compound have been studied. The rationale was that the glycyl-mustard bond could be hydrolyzed by an aminopeptidase. Substitution into the amino group of the amino acid moiety would prevent hydrolysis by this enzyme, which requires an unsubstituted amino group in the α-position relative to the peptide bond being hydrolyzed.[4] Such N-substituted derivatives would require for activation an acylase and an aminopeptidase acting in an obligatory sequence. Evidence to this effect has been obtained and is reported here.

MATERIALS AND METHODS

Drugs. Compounds were prepared by Drs. D. J. Triggle, L. N. Owen, and B. J. Johnson according to previously described methods.[3, 5]

Animals. Male Holtzman rats, 160 to 180 g, were fed pelleted Purina laboratory chow and tap water *ad libitum* and were housed in galvanized wire-mesh cages at a temperature of 74°–76°F.

Tumor. Walker carcinosarcoma 256 was implanted subcutaneously in the right flank of the rat by means of a trochar and cannula inserted through a small incision in the skin.

Antitumor assay

Carcinostatic effects of the drugs were assessed by administering a single intraperitoneal injection of various dose levels on the day after tumor implantation. The rats were killed on the tenth day after implantation, and the tumors were dissected out and weighed. The ratio of the mean weight of treated tumors to the mean weight of control tumors (T/C) was determined and plotted against the log dose. The therapeutic index was obtained from the ratio LD_{50}/ED_{90}, where ED_{90} is the dose corresponding to a T/C ratio of 0·1. Approximate LD_{50} values were estimated graphically from percentage mortality/log dose plots by the method of Litchfield and Wilcoxon.[6] Six rats were used at each dose level. This combined toxicity–therapeutic activity assay technique, similar to that described by Connors *et al.*,[7] used four logarithmically spaced doses of a drug in preliminary experiments. The range of doses was extended if a toxic or therapeutically ineffective dose was not encountered. With compounds which had little or no therapeutic activity even at near toxic doses, LD_{50} and ED_{90} values were estimated from data obtained from four dose levels only.

Enzyme assay

Hydrolysis of the glycyl–mustard derivatives was studied by measuring the rate of release of *p*-phenylenediamine mustard from these compounds. The free amino group of *p*-phenylenediamine mustard reacts with 8-hydroxyquinoline in the presence of oxidizing agents to form a colored indoaniline compound,[8] which is estimated colorimetrically.

The animals were killed by cervical fracture, immediately desanguinated, and the fresh tissue placed in a petri dish on ice. The tissue was weighed, minced, and homogenized in 50 mM Tris buffer at pH 7·36 to give a final concentration of 100 mg tissue/3 ml homogenate. The incubation mixtures contained 100 mg homogenized tissue,

0·95 mM drug, and mM KCN in a final volume of 4·2 ml. This contained 4% acetone as solvent for the drugs. KCN was added to prevent enzymic oxidation of the released diamine. Incubation of duplicate samples was carried out for varying periods of time at 37°. N,N-bis(2-chloroethyl)-*p*-phenylenediamine was estimated as follows: 1 ml of 15% (w/v) trichloracetic acid was added to the homogenate which was centrifuged at 2,000 *g* for 5 min. The supernatant was decanted into a glass-stoppered test tube; the residue was resuspended in 2 ml 0·1 N hydrochloric acid, again centrifuged at 2,000 *g* for 5 min, and the supernatant added to the contents of the glass-stoppered tube. Sodium hydroxide (1·0 ml, N) was added, followed by 2 ml of 5% (w/v) sodium bicarbonate solution, 1·0 ml of 0·9% 8-hydroxyquinoline in absolute ethanol, and 0·5 ml of 1·0% (w/v) aqueous potassium ferricyanide. These additions were well dispersed at each stage of the operation by means of a Vortex junior mixer. The green-colored product was extracted with 10 ml of n-butanol, in which it formed a blue solution, which was centrifuged to clear. The extinction of the butanol solution at 595 mμ was determined by using matched 5-ml test tubes in a Bausch and Lomb Spectronic 20 colorimeter.

Chemical activity determinations

The hydrolysis rates of the mustards were determined by the method of Ross[9] and the alkylation rate studies by the method of Bardos *et al.*[10] The latter is a determination of the rate constant, under standard conditions, for the alkylation of the nucleophile, 4-(*p*-nitrobenzyl)pyridine (NBP).

RESULTS

The data for compound III presented in Fig. 1 were pooled from three combined toxicity–therapeutic activity assays. In spite of the wide range of control tumor weights, mean values from the three experiments were in reasonable agreement (mean values ± S.D. were 48 ± 9, 39 ± 15, and 44 ± 6). Figure 1 shows that the therapeutic dose (ED_{90}) was considerably below the toxic dose range. ED_{90} and LD_{50} values, expressed on a molar basis, are presented in Table 1.

TABLE 1. COMPARATIVE CHEMICAL AND BIOLOGICAL DATA OF N,N-DI-(2-CHLOROETHYL)-*p*-PHENYLENEDIAMINE AND SUBSTITUTED GLYCYL DERIVATIVES

$(ClCH_2CH_2)_2N$—C$_6$H$_4$—$NH{\cdot}R$ R	LD_{50} (μmole/kg)*	ED_{90} (μmole/kg)	$\frac{LD_{50}}{ED_{90}}$	Hydrolysis (%)†	Alkylating activity ($k^1_{80} \times 10^3$)‡
I. H·HCl	15 (12–19)	20	<1·0	100	42
II. $COCH_2NH_2{\cdot}HCl$	34 (30–39)	34	1·0		10·9
III. $COCH_2NHCOCH_3$	75 (60–103)	16	4·5	20	12·3
IV. $COCH_2NHCOOCH_3$	230 (195–276)	140	1·7	21	12·3
V. $COCH_2NHCOCHCl_2$	>1,000				12·3

* Confidence limits, 95%

† In 50% aqueous acetone at 66°.[9]

‡ Rate of alkylation of 4-(*p*-nitrobenzyl)pyridine at 80°.

Table 1 shows that *p*-phenylenediamine mustard, I, and its glycyl derivative, II, were both very toxic and had poor antitumor activity (therapeutic indices were not greater than 1·0). This confirms the original report by Bergel and Stock[3] for compound

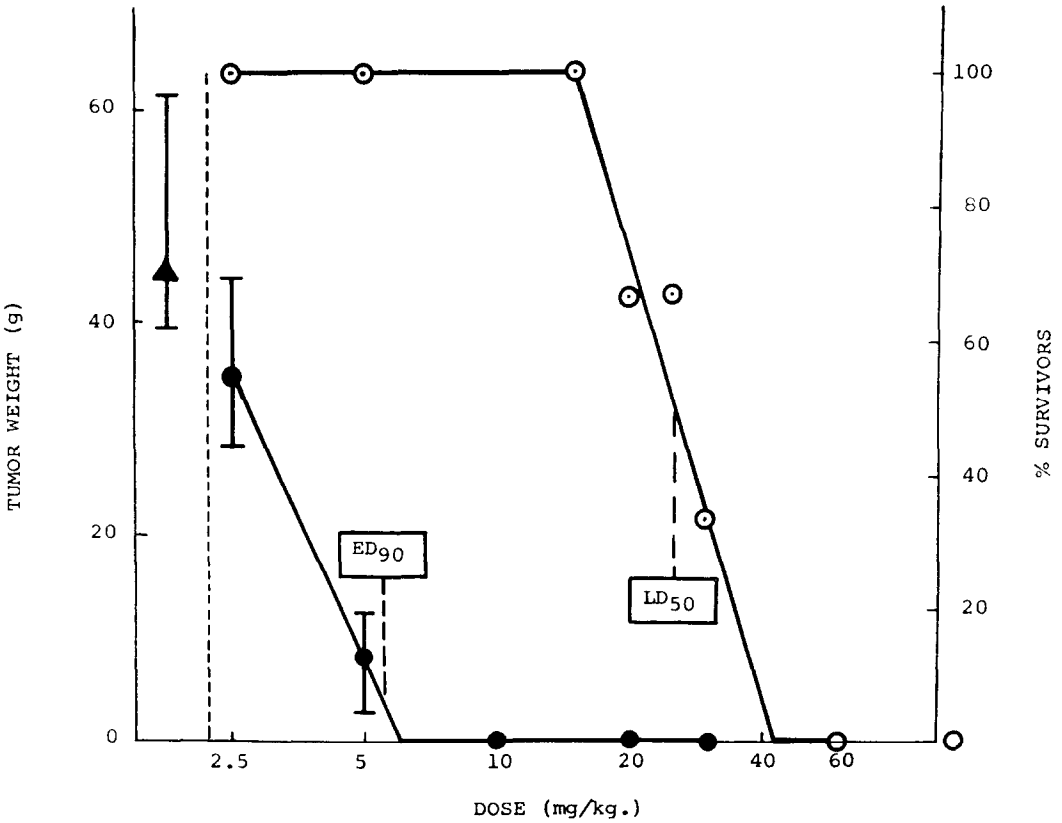


FIG. 1. Combined toxicity–therapeutic activity assay of compound III in a Walker carcinosarcoma-256 test system. ▲: Mean and range of tumor weights of control rats. ●: Mean and range of tumor weights of treated rats. O: Percentage survival of treated rats.

II. In contrast, the marked increase in therapeutic index (to 4·5) in the case of III, the N-acetyl derivative of II, suggests a considerable improvement in selective action on the tumor. The LD_{50} of III was greater than that of I or II, but the tumor-inhibitory dose was less than that of I or II, calculated on a molar basis. The N-methylcarbamate derivative, IV, was six times less toxic than II. Also the therapeutic index (1·7) showed a moderate increase over II and was considered to be significantly different from II (the ED_{90} value lies outside the 95% confidence limits for the LD_{50} value). The N-dichloracetyl derivative, V, was relatively nontoxic and had only a moderate growth-inhibitory effect at the dose tested. The therapeutic index was not determined, but these limited data are included for comparison with the other derivatives in the enzyme-activation studies.

Symptoms of toxicity produced by compounds were essentially similar at equitoxic doses. For example, at doses approximating the $LD_{50} \times 2$, animals died 2 to 5 days after dosing, with body weight loss (30–40%), diarrhea, leucopenia, and porphyrin-derived pigmentation of the eye and nostril region.

The relative chemical reactivities of the compounds are presented in Table 1. The figure for percentage hydrolysis and the rate of alkylation of NBP both indicate

that the reactivity of *p*-phenylenediamine mustard, I, was considerably reduced by substituting glycine into the molecule. The N-acylglycyl derivatives did not differ significantly from II.

The rates of hydrolysis of the glycyl–mustard bond of the compounds by various rat tissues are shown in Table 2. No spontaneous hydrolysis occurred upon incubation

TABLE 2. RATES OF HYDROLYSIS OF GLYCYL–MUSTARD DERIVATIVES BY RAT TISSUE HOMOGENATES

Tissue	Rate of formation of I* from			
	II	III	IV	V
Liver	66·0	6·4	0·58	0·40
Walker 256	18·5	4·3	0·18	0·32
Kidney	508·0	8·0		
Spleen	83·7	7·8		
Ileum	114·3	6·0		

* expressed as μmoles/g tissue/hr at 37° at pH 7·36, substrate concentration, 0·95 mM.

I: M·NH_2·HCl. II: M·$NHCOCH_2NH_2$·HCl.
III: M·$NHCOCH_2NHCOCH_3$. IV: M·$NHCOCH_2NHCOOCH_3$.
V: M·$NHCOCH_2NHCOCHCl_2$.
(M = $(ClCH_2CH_2)_2N$—  —.)

Rates are mean values from two experiments with data obtained from three different times of incubation for each substrate. Individual values differed from the mean values by no more than 15 %.

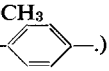
at 37° in buffer solution or with boiled liver homogenate. The pH optimum for enzymic hydrolysis was 7·36. The rates of hydrolysis of compounds III, IV, and V were linear with time for up to 2-hr incubation. Hydrolysis of II was very rapid and was linear with time only during the first 5 min of incubation. Thereafter the rate of hydrolysis declined. The data in Table 2 were calculated from the amount of product formed after 5-min incubation of compound II. It can be seen that the rates of hydrolysis of these compounds varied enormously. The amine was released from the glycyl–mustard, II, at a very rapid rate. Actylation of the α-amino group slowed this reaction considerably, and the rates were very slow in the case of the methylcarbamate and dichloroacetyl derivatives.

In order to support the initial working hypothesis, it was necessary to demonstrate hydrolysis of these compounds in tumor homogenates. This was found to occur, and the rates of hydrolysis are shown in Table 2. The distribution of the enzyme system responsible for hydrolysis was investigated more extensively in the case of compounds II and III, for which the rates of hydrolysis were determined for liver, tumor, kidney, spleen, and ileum homogenates. With both compounds the hydrolysis rate was found to be less in the tumor than in other tissues. However, the relative rates of hydrolysis by the tumor and other tissues differed considerably for II and III. Thus II was hydrolyzed 270 times more rapidly by kidney than by tumor homogenate. Relative rates for spleen and ileum were respectively 4·5 and 6·2 times the tumor value; III was

also hydrolyzed most rapidly by the kidney, but at a rate which was only 1·9 times that of the tumor. Relative rates of hydrolysis by the spleen, ileum, and liver were less than the latter value.

TABLE 3. EFFECT OF N-ACETYLMETHIONINE ON HYDROLYSIS OF THE GLYCYL–MUSTARD DERIVATIVES

Substrate*	Time of incubation (min)	Substrate hydrolysed† Control	Substrate hydrolysed† + Inhibitor (μmoles/100 mg liver)	Inhibition (%)	Mean inhibition (%)
II	3	0·192	0·182	5·3	
	2	0·136	0·118	13·3	13·4
	2	0·102	0·080	21·7	
III	30	0·360	0·030	92·0	
	20	0·304	0·012	96·1	94·4
	20	0·268	0·012	95·2	
IV	90	0·066	0·004	88·0	88·0

* II: M·$NHCOCH_2NH_2$·HCl. III: M·$NHCOCH_2NHCOCH_3$
IV: M·$NHCOCH_2NHCOOCH_3$. (M = $(ClCH_2CH_2)_2N$—  —.)

† Means values of four replicates.

The effect of N-acetylated amino acids on the hydrolysis of compounds II, III, and IV was studied. N-Acetylglycine dissolved in 5% (w/v) sodium bicarbonate solution was added to incubation mixtures containing liver homogenate; 17% inhibition of hydrolysis of III was observed in the presence of 40 mM N-acetylglycine and, at a concentration of 100 mM, 34% inhibition occurred. Concentrations higher than this could not be studied because the limit of solubility had been reached. The inhibitory effect of 40 mM N-acetylmethionine on the hydrolysis reaction are presented in Table 3. At this concentration, no interference with the estimation of *p*-phenylenediamine mustard occurred. N-acetylmethionine had an inconsistent effect on the hydrolysis rate of the glycyl–mustard, II; the mean inhibition from three determinations was 13·4%. The hydrolysis of N-acetylglycyl–mustard, III, however, was almost completely inhibited, as was that of the methylcarbamate derivative, IV: the percentage inhibitions were 94·4 and 88·0 respectively.

DISCUSSION

Ross[11] has presented data which correlated chemical reactivity with biological activity. In general, compounds which had a "percentage hydrolysis" figure of less than 20 were inactive as antitumor agents. Bardos *et al.*[10] have presented more quantitative data indicating a direct relationship between the percentage hydrolysis figure and the molar antitumor activity value (ED_{90} value). The percentage hydrolysis figures for the glycyl–mustard derivatives were such that only borderline activity was expected in the biological test system. However, a wide range of therapeutic indices was observed. Consequently, the variations in toxicity values of the compounds which had almost identical chemical reactivities suggested that a mechanism was affecting the biological activity of the compounds *in vivo*.

An explanation of the variation in toxicity and the high therapeutic index of compound III was obtained from a study of the enzymic hydrolysis of these compounds in tissue homogenates. All the compounds were found to be hydrolyzed at the glycyl–mustard linkage, but the rates of hydrolysis varied enormously. A significant observa-

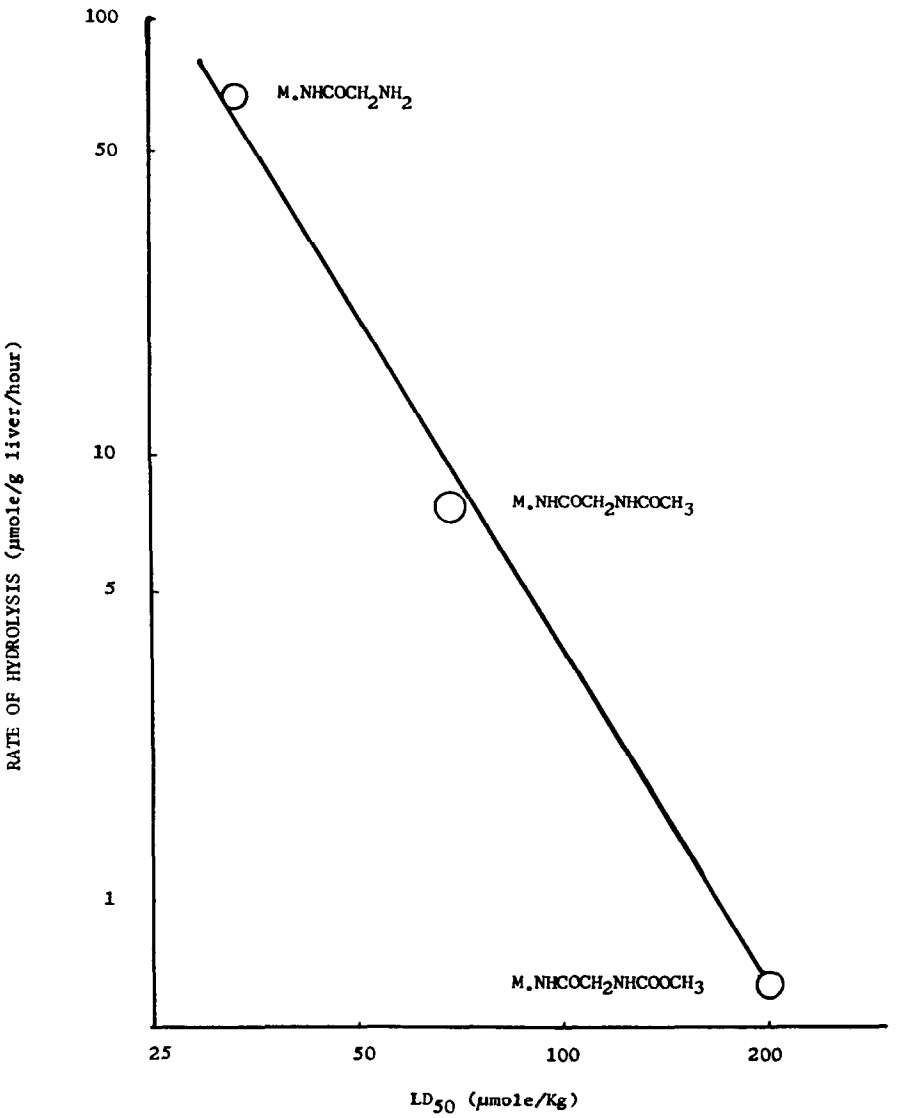


FIG. 2. Correlation of toxicity and rate of hydrolysis of glycyl-mustard bond.

M = $(ClCH_2CH_2)_2N$—⟨⟩—.

tion, however, was that a decrease in the rate of hydrolysis was paralleled by a decrease in the toxicity of the compounds (Fig. 2). The rate of release of the highly reactive phenylenediamine mustard, I, from the glycyl-mustard derivative, II, was very

rapid, and the toxicity of this compound was close to that of I. This could be attributed to the very rapid liberation of the latter substance in all tissues. Hydrolysis of the N-acetyl-substituted compound was at least 10 times less rapid and was accompanied by a decrease in toxicity and a considerable increase in the therapeutic index. It is interesting to note that the ratio of tumor/liver enzyme activity value approaches unity in the case of III but is less than 0·35 in the case of II and IV. In addition, the ratios of tumor/spleen and tumor/ileum enzyme activity values are 0·22 and 0·16 respectively for II, and 0·56 and 0·71 for III.

These differences in relative enzyme activity values suggest that the proportion of II and III activated by hydrolysis in the tumor compared with other tissues would be relatively greater for the latter compound. Furthermore, it may be anticipated that for equivalent toxicity to other tissues (e.g. ileum, spleen), III would have a greater toxic effect on the tumor than II. The rate of hydrolysis of IV (and V) may be too low to allow an amount of the compound to be hydrolyzed to the reactive species at a sufficiently rapid rate to produce a cytotoxic effect on the tumor. Excretory processes may remove a large amount of the compounds still in the inactive form.

It can be stated unequivocally that acylation of the glycine moiety slowed the hydrolysis of the "peptide" bond linking that amino acid to the aromatic nitrogen mustard and also improved the antitumor properties of the molecule. It cannot be stated definitely how acetylation of the α-amino group affects the hydrolysis of the glycyl–mustard bond but, if hydrolysis of the latter is dependent upon the action of an aminopeptidase, this result is not unexpected. Evidence is presented which suggests that the amino acid moiety is first deacylated. An acetyl group combined in amide linkage to aniline has been shown to be readily hydrolyzable in tissue homogenates,[2, 12, 13] and N-acylated amino acids have been shown to be deacylated.[14–19] Deacylation of these molecules appears to take place quite readily in animal tissues; thus it might be anticipated that deacylation would still occur when the glycine is bound to a nitrogen mustard molecule. Evidence in favor of deacylation of glycine as the rate-limiting step in the activation of these drugs was obtained with N-acetylated amino acids as competitive inhibitors.

Mounter *et al.*[19] have shown that N-acetylmethionine had a much greater affinity for hog kidney amino acylase than other acetylated amino acids studied, including N-acetylglycine. Moreover, substrate inhibition due to N-acetylmethionine at 40 mM was considerably greater than that due to N-acetylglycine at 40 mM. N-Acetylglycine, 100 mM, was moderately effective in inhibiting the hydrolysis of III. However, 40 mM N-acetylmethionine almost completely inhibited the hydrolysis of two of the acylated glycyl–mustards tested, while hydrolysis of the parent glycyl compound was little affected. It is reasoned that N-acetylmethionine competed with N-acetylglycyl–mustard for adsorption onto the active site of an acylase, which resulted in a considerable reduction in the rate at which the latter compound was deacylated to compound II.

Hydrolysis of the carbamate bond appears to take place in the case of compound IV, but the slowness of this hydrolysis adds little selectivity to the action of the drug because there is only a slight increase in the therapeutic index over that of the parent glycyl compound. Methylcarbamate per se has been reported to be inactive against the Walker tumor;[20] however, in the case of compound IV, active transport for amino acid uptake may be operating in favor of the drug molecule being taken up by

the tumor cells. Furthermore, localized in an intracellular position, the methylcarbamate moiety may have some antitumor effect in combination with an alkylating agent.

An anomaly that does exist is the lack of activity of the dichloroacetylglycyl–mustard, compound V. The rate of hydrolysis of the glycyl–mustard bond of this molecule was very slow. This would be expected if deacylation of the amino acid did not take place or took place extremely slowly. The evidence seems to indicate that the latter is in fact the explanation. Dichloroacetanilide has been shown to be readily hydrolyzed;[21] however, the halogenoacetyl group appears to have different properties when substituted into amino acids. Reports on the deacylation of chloroacetyl amino acids are conflicting. Otani[18] found many N-chloroacetyl amino acids to be readily hydrolyzed and Birnbaum *et al.*[17] found N-chloroacetyl-DL-alanine to be asymmetrically hydrolyzed by crude kidney preparations. Price and Greenstein[15] found N-chloroacetylglycine was not hydrolyzed by partially purified kidney and liver amino acid acylase. The dichloroacetyl group had a profound influence in the present series; hydrolysis of the glycyl–mustard linkage was very slow, and the toxicity of the compound was also very low. It is not easy to offer an explanation of the lack of activity of this grouping; it is possible that the size of the two chlorine atoms, being greater in size than the hydrogen atoms they replace, sterically prevent the effective binding of the molecule to the active site of the enzyme responsible for deacylation.

One of the main features in the design of the compounds investigated was the incorporation of normal metabolites (amino acids) into the drug molecule. In the cell membrane, active transport processes exist for the uptake of essential metabolites A high rate of metabolism results in tumor cells competing favorably with other cells of the body for these metabolites. Thus it might be expected that tumor cells will concentrate essential metabolites and drug molecules which have metabolites incorporated into their structure. This may be an explanation for the moderate antitumor activity of compound IV for which little activation by enzymic hydrolysis could be demonstrated.

Studies are in progress with partially purified enzyme systems to study further the kinetics of the enzyme reactions involved.

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