SYNTHESIS AND STUDY OF GLUTARYL-S-(ω-AMINOALKYL)-L-CYSTEINYLGLYCINES AS INHIBITORS OF GLYOXALASE I

THESIS

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This thesis describes the synthesis and preliminary enzymatic study of glutaryl-S-(8-aminooctyl)-L-cysteinylglycine and glutaryl-S-(10-aminodecyl)-L-cysteinylglycine as inhibitors of glyoxalase I. These analogs of glutathione were prepared as potential ligands for affinity chromatography purification of glyoxalase I.

The compounds were synthesized by a seven-step procedure in overall yields of 24% for the octyl analog and 33% for the decyl analog. Both compounds exhibited mixed type inhibition of the enzyme, with the decyl derivative being more inhibitory than the octyl derivative. The inhibition was nonlinear (parabolic) for both compounds.

Although less inhibitory than the corresponding S-substituted glutathione derivatives, these analogs are promising candidates for affinity chromatography ligands. Such compounds may also be useful in studying the mechanism of glyoxalase I.

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CHAPTER I

INTRODUCTION

The synthesis and study of substrate analogs as inhibitors of specific enzymatic reactions have proven to be important procedures in the investigation of enzymes.[1-3] The glyoxalase enzyme system has been known for many years.[4] It has received considerable attention from various investigators, and numerous compounds have been studied as inhibitors of this enzyme system.[5-11]

The glyoxalase system, which is widely distributed in all forms of life,[12,13] catalyzes the conversion of methylglyoxal (pyruvaldehyde) and other α-ketoaldehydes, such as phenylglyoxal, into the corresponding α-hydroxy acids. Reduced glutathione (GSH) is required as a cofactor in this reaction.[14] The glyoxalase system is composed of two enzymes,[14] glyoxalase I and glyoxalase II, which function in a two-step sequence in the following manner.

methylglyoxal + glutathione $\xrightarrow{\text{glyoxalase I}}$
S- lactoylglutathione $\xrightarrow{\text{glyoxalase II}}$
lactic acid + glutathione

Several mechanisms have been proposed for the action of glyoxalase I. It is well known[5,6] that methylglyoxal and

glutathione rapidly react nonenzymatically to form a hemimercaptal adduct.

$$CH_3COCHO + GSH \rightleftharpoons CH_3COCHOH\text{-}SG$$

The proposed mechanisms for the enzyme include a one-substrate mechanism,[6] in which the hemimercaptal is the substrate, a two-substrate mechanism,[5] where free glutathione and methylglyoxal serve as substrates and an alternative one- or two-substrate branch mechanism.[11] The latter mechanism was found to best describe the action of the enzyme; the one-substrate branch appears to predominate except at very low hemimercaptal and free glutathione concentrations.[11]

It has been shown that α-ketoaldehydes, such as methylglyoxal, are cancerostatic and generally cytotoxic[15] and are converted by glyoxalase into the nontoxic α-hydroxy acids. It has also been noted that a rapid increase in glutathione concentration occurs just prior to cell division.[16] On the basis of these observations, it has been suggested that the glyoxalase system may be involved in regulation of cell growth by maintaining methylglyoxal at the proper concentration.[7,17] The findings that there is a deficiency of methylglyoxal[18] and a high level of lactic acid[19] in cancer cells have further led to the suggestion that cancer cells have lost the ability to maintain the level of methylglyoxal and thus continue to grow at an uncontrolled rate.[7] It has been

proposed that the use of suitable glyoxalase I inhibitors, or a combination of such inhibitors with methylglyoxal, may provide a possible approach to anticancer therapy by retarding cell growth through an increase in the ketoaldehyde level.[7]

Since glutathione is a required cofactor in the glyoxalase reaction, various S-alkyl and S-aryl glutathione derivatives have been prepared and have been found to be potent inhibitors of glyoxalase I.[7,8] Some of these inhibitors have also been found to exhibit cancerostatic activity and to increase the toxicity of methylglyoxal toward leukemia cells. These glutathione derivatives, however, were rendered ineffective when tested *in vivo* because of the rapid hydrolysis of such compounds by glutathionase (γ-glutamyl transpeptidase) and cysteinylglycinase.[9] Therefore, in an effort to obtain inhibitors which would be resistant to the *in vivo* metabolism, glutaryl-S-(*p*-bromobenzyl)-L-cysteinylglycine was prepared.[9] This compound, which lacks the α-amino group of the corresponding glutathione derivative, was found to be an effective inhibitor of glyoxalase I and was not susceptible to hydrolysis by glutathionase.

Although the glyoxalase system is well known, difficulty has been experienced in obtaining a homogeneous preparation of glyoxalase I. The purification and characterization of glyoxalase I from mouse liver has recently been accomplished

by workers in this laboratory.[20] A major step in the purification procedure was the use of affinity chromatography[3] employing the glyoxalase I inhibitor S-(ω-aminodecyl)glutathione(SADG)[10] as the ligand. It was found, however, that this technique could not be satisfactorily utilized during early stages in the purification due to a rapid degradation of the ligand material and could be used only after the enzyme had been partially purified by other procedures. The degradation of the SADG ligand was attributed to hydrolysis by glutathionase and cysteinylglycinase,[9] which may be present in the more impure enzyme preparations.

It was therefore of interest to prepare compounds which would be inhibitors of glyoxalase I, would be capable of serving as affinity chromatography ligands, and would still be resistant to the action of certain degradative enzymes. Such compounds would potentially be useful in allowing the affinity chromatography technique to be employed at an earlier stage in the enzyme purification process and hopefully would shorten and improve the overall isolation procedure.

Vince and coworkers have found that S-benzylglutathione is degraded by glutathionase, whereas glutaryl-S-(p-bromobenzyl)-L-cysteinylglycine is not affected by glutathionase, but does act as an inhibitor of glyoxalase I.[9] In view of these observations it was thought that compounds containing

both the glutaryl- and S-(ω-aminoalkyl)-substituents would be of interest. Thus the synthesis of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines was undertaken. The octyl and decyl derivatives were the compounds of choice since these chain lengths provided the most potent inhibitors among the S-(ω-aminoalkyl) glutathione series.[10] These new compounds were to be investigated for their inhibitory effect on glyoxalase I, with subsequent evaluation as potential affinity chromatography ligands, through linkage to an appropriate support through the ω-amino group. This report describes the synthesis and preliminary enzymatic study of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines.

CHAPTER II

EXPERIMENTAL

General. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. Melting points and yields are reported in Table I. Elemental analyses were performed by Heterocyclic Chemical Corporation and the analytical data are shown in Table II. The enzyme inhibition studies were conducted on a Beckman DBG recording spectrophotometer. Thin layer-chromatography was performed by the ascending technique using commercial Silica Gel G plates with fluorescent indicator (Eastman No. 6060) and cellulose plates (Eastman No. 6064). Solvent systems for TLC development included n-butanol:glacial acetic acid:water (BAW, 4:1:1), 95% ethanol:ammonium hydroxide (EA, 7:3), and methanol. TLC plates were visualized by ultraviolet lamp, ninhydrin spray and iodine vapor.

Organic Syntheses. The general methods of preparation of compounds listed in Table I are given below. The N-(8-bromooctyl)phthalimide and N-(10-bromodecyl)phthalimide were prepared by published procedures[10] and the melting points agreed closely with the literature values.[21] New compounds synthesized are designated by the numbers in parentheses, which correspond to the numbers in Tables I and II and Figure 1.

TABLE I

GLUTARYL-S-(ω-AMINOALKYL)-L-CYSTEINYLGLYCINES AND INTERMEDIATES

Compound	n****	Formula	Mp, °C	Yield, %
1a	8	$C_{19}H_{26}N_2O_4S \cdot H_2O$	185-188 dec	85
1b	10	$C_{21}H_{30}N_2O_4S \cdot 0.5H_2O$	184-188 dec	95
2a	8	$C_{20}H_{26}N_2O_5S$	118-121	77
2b	10	$C_{22}H_{30}N_2O_5S$	118-121	73
3a	8	$C_{23}H_{31}N_3O_6S$	117-119	84
3b	10	$C_{25}H_{35}N_3O_6S$	123-126	68
4a	8	$C_{22}H_{31}N_3O_5S \cdot HCl \cdot 2.5H_2O$	81- 83	92
4b	10	$C_{24}H_{35}N_3O_5S \cdot HCl \cdot H_2O$	84- 86	87
5a	8	$C_{27}H_{37}N_3O_8S$	108-111	74
5b	10	$C_{29}H_{41}N_3O_8S \cdot H_2O$	110-113	91
6a	8	$C_{26}H_{37}N_3O_9S$	*	80
6b	10	$C_{28}H_{41}N_3O_9S \cdot H_2O$	**	92
7a	8	$C_{18}H_{33}N_3O_6S \cdot HCl \cdot 2H_2O$	***	80
7b	10	$C_{20}H_{37}N_3O_6S \cdot HCl \cdot 0.5H_2O$	141-144 dec	94

*Slowly decomposes with bubbling from about 75°-140°. **Slowly decomposes with bubbling from about 90°-150°. ***Hygroscopic; slowly decomposes with bubbling from about 75°-120°. ****n = number of methylene units in the alkyl chain.

TABLE II

ANALYTICAL DATA**

Compound	% Calculated			% Found		
	C	H	N	C	H	N
1a	57.54	7.13	7.07	57.58	7.18	6.85
1b	60.69	7.53	6.74	61.02	7.35	6.58
2a	59.08	6.46	*	58.89	6.56	*
2b	60.80	6.97	*	60.59	6.93	*
3a	57.83	6.56	8.80	57.62	6.48	8.64
3b	59.37	6.99	8.31	59.19	7.25	8.02
4a	49.75	7.04	*	49.49	7.17	*
4b	54.16	7.21	*	54.32	7.36	*
5a	57.52	6.63	7.46	57.70	6.66	7.23
5b	57.11	7.12	6.89	57.25	7.09	6.87
6a	55.00	6.58	7.40	54.81	6.78	7.29
6b	54.79	7.08	6.85	54.29	7.12	6.52
7a	43.93	7.80	8.54	43.79	7.52	8.78
7b	48.71	7.99	8.52	48.98	8.11	8.42

*Nitrogen analyses were not performed on these compounds.

**Analyses were performed by Heterocyclic Chemical Corporation.

S-(ω-phthalimidoalkyl)-L-cysteines (1).--These compounds were prepared in a manner similar to that reported for synthesis of S-(ω-phthalimidoalkyl)glutathiones.[10] L-Cysteine hydrochloride monohydrate (0.07 mol) and 280 ml DMF were added to 140 ml 1.0 N NaOH. N-(ω-bromoalkyl)phthalimide (0.07 mol) dissolved in 140 ml DMF was introduced to the reaction mixture dropwise over a period of two hours. A large amount of white precipitate formed during the addition and the mixture was stirred overnight at room temperature. The product was filtered, washed with EtOH, Me_2CO and Et_2O and dried in a heated vacuum desiccator. TLC (Silica Gel, BAW) indicated one component, which was ninhydrin-positive and UV-positive. Analytical samples were prepared by recrystallization from HOAc-H_2O.

N-formyl-S-(ω-phthalimidoalkyl)-L-cysteines (2).--S-(ω-phthalimidoalkyl)-L-cysteine (0.06-0.07 mol) was dissolved in 140-150 ml 97% HCOOH and cooled in an ice bath. Acetic anhydride (50 ml) was added dropwise to the cold, stirring solution over one hour. After addition was complete, the solution was stirred overnight at room temperature. Cold H_2O (300-350 ml) was added to the solution, causing precipitation of a light tan solid. After cooling in the refrigerator, the mixture was filtered and the solid was washed with cold H_2O. The crude product was recrystallized from EtOH-H_2O and dried in a heated vacuum desiccator over

P_2O_5. TLC (Silica Gel, MeOH and BAW) showed the spot, UV-positive and ninhydrin-negative. Analytical samples were prepared by recrystallization from EtOH-H_2O.

N-Formyl-S-(ω-phthalimidoalkyl)-L-cysteinylglycine methyl esters (3).--N-Formyl-S-(ω-phthalimidoalkyl)-L-cysteine (0.05 mol) dissolved in 120 ml CH_2Cl_2, was added to a solution of 0.06 mol glycine methyl ester hydrochloride and 0.06 mol Et_3N in a mixture of 170 ml DMF and 170 ml CH_2Cl_2. N,N'-Dicyclohexylcarbodiimide (0.05 mol) in 50 ml CH_2Cl_2 was added to the reaction mixture at 0-5°. The reaction mixture was stirred at about 5° for one hour, then at room temperature for two days. The insoluble dicyclohexylurea which formed during this time was filtered and the filtrate was evaporated to low volume in vacuo. $CHCl_3$ (200 ml) was added to the residue, cooled and filtered from some insoluble material. About 100 ml Et_2O was added to the $CHCl_3$ filtrate and the resulting solution was washed successively with 0.5 N HCl, H_2O, 5% $NaHCO_3$, and H_2O. The organic layer was dried over Na_2SO_4. After filtration of the drying agent, the solution was evaporated to low volume in vacuo and redissolved in the minimum amount of $CHCl_3$ by warming. The product was precipitated by addition of Et_2O. After cooling and filtering, the product was dried in a heated vacuum desiccator. Only one spot was shown by TLC (Silica

Gel, MeOH), which was UV-positive and ninhydrin-negative. Analytical samples were prepared by recrystallization from $CHCl_3$-Et_2O.

S-(ω-phthalimidoalkyl)-L-cysteinylglycine methyl ester hydrochlorides (4).--N-Formyl-S-(ω-phthalimidoalkyl)-L-cysteinylglycine methyl ester (0.024-0.031 mol) was dissolved in about 100 ml MeOH. A volume of 1.0 N methanolic HCl (prepared by diluting 4 ml con HCl to 48 ml with MeOH), sufficient to provide about 20-25% molar excess, was added to the dipeptide ester solution. The reaction mixture was stirred overnight at 45° and was then evaporated to low volume *in vacuo*. The residue was dissolved in MeOH and precipitated by addition of Et_2O. The mixture was refrigerated and then filtered. The slightly hygroscopic product was washed with cold Et_2O and dried *in vacuo* over P_2O_5. TLC (Silica Gel, MeOH and BAW) indicated only one spot, UV-positive and ninhydrin-positive. Recrystallization from MeOH-Et_2O provided the analytical samples.

Glutaryl-S-(ω-phthalimidoalkyl)-L-cysteinylglycine monomethyl esters (5).--S-(ω-Phthalimidoalkyl)-L-cysteinylglycine methyl ester hydrochloride (0.023 mol) was added to a solution of NaOAc (0.024 mol) in 150 ml glacial HOAc, followed by addition of glutaric anhydride (0.027 mol). After about 12 hr stirring at room temperature, an additional 0.003

mol of glutaric anhydride was added and stirring was continued for 24 hours at room temperature. After this time, the reaction mixture was evaporated to low volume in vacuo. Water was added to the residue and the mixture was agitated vigorously. After cooling, the light brown, solid material was filtered and washed with cold H_2O. The crude product was reprecipitated from MeOH-H_2O and dried in vacuo over P_2O_5. TLC (Silica Gel, EA, MeOH and BAW) indicated a single spot, UV-positive and ninhydrin-negative. Analytical samples were obtained by another reprecipitation from MeOH-H_2O.

Glutaryl-S-(ω-(o-carboxybenzamido)alkyl)-L-cysteinylglycines (6).--Glutaryl-S-(ω-phthalimidoalkyl)-L-cysteinylglycine monomethyl ester (1.7-2.7 mmol) was dissolved in 15-20 ml abs EtOH. To this solution was added a volume of 1.0 N NaOH sufficient to give three molar equivalents of OH^- per mole of tripeptide ester plus a 10% excess. The resulting solution was stirred at room temperature for about 1.5 hr. The solution was cooled to 0-5°, then acidified to pH 7 with 1.0 N HCl. The solution was then evaporated to low volume in vacuo at room temperature. About 20 ml H_2O was added to the residue and the solution was cooled in an ice bath. The solution was acidified to pH 3-3.5 with 1.0 N HCl in the cold, causing precipitation of a thick, white gummy material, which

solidified upon refrigeration for 2-3 hr. The solid product was filtered and washed several times with cold H_2O, then dried at room temperature in vacuo over P_2O_5.

Glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines (7).--Glutaryl-S-(ω-(o-carboxybenzamido)alkyl)-L-cysteinylglycine (0.8-2.0 mmol) was dissolved in 5-12 ml p-dioxane. To this solution was added one-half volume of H_2O and sufficient 0.5 N HCl to provide a slight (5-7%) excess of the equimolar amount of acid. The solution was heated on a steam bath for 30 min and then cooled in an ice bath. TLC (Silica Gel, BAW and EA) indicated a UV-positive, ninhydrin-negative spot which corresponded to phthalic acid, a major UV-negative, ninhydrin-positive spot and a much weaker UV-negative, ninhydrin-positive spot. The reaction mixture was evaporated to low volume in vacuo. p-Dioxane was added to the residue and the solution again was evaporated in vacuo. Upon further addition of p-dioxane to the residue, an insoluble, oily material formed, which, after standing in the refrigerator several hours, crystallized upon scratching and stirring with a glass rod. The resulting solid product was filtered and dried in vacuo over P_2O_5. TLC (Silica Gel, BAW and EA) of this material indicated that all phthalic acid had been removed and essentially one spot was seen, which was UV-negative and ninhydrin-positive, although in some cases, a small

amount of impurity, UV-negative and ninhydrin-positive, was also detected. This impurity could usually be removed by recrystallization from EtOH-Et_2O. Analytical samples were afforded by recrystallization from EtOH-Et_2O.

Enzyme Inhibition Studies.--The inhibitory effects of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines on glyoxalase I were studied by established procedures.[10,20] The enzyme solutions used in this study were highly purified enzyme preparations obtained from the affinity column in the isolation of glyoxalase I from mouse livers.[20]

Substrate solutions of varying concentrations were prepared by adding reduced glutathione (GSH) and methylglyoxal to an imidazole-HCl buffer solution (100 mM, pH 6.8) containing 16 mM $MgSO_4$. Commercial 40% methylglyoxal solutions were employed after removal of acidic contaminants by passing through AG-1X8 resin (carbonate form). The methylglyoxal solutions were standardized by the method of Friedmann.[22] The concentration of the hemimercaptal ($CH_3COCHOH$-SG) at equilibrium was calculated using the dissociation constant $K=[CH_3COCHO][GSH]/[CH_3COCHOH\text{-}SG]$, assuming K=3.1.[8] The level of free glutathione at equilibrium was maintained constant at 0.3 mM and the concentration of the hemimercaptal was varied from 0.05 mM to 0.40 mM for these studies.

The inhibitor solution was prepared by dissolving the glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycine in H_2O, adding

sufficient 1.5 N KOH (2 molar equivalents) to raise the pH to about 7, and then adjusting the total volume with H_2O to obtain a final concentration of 120 mM.

Each reaction cell and the reference cell contained 2.9 ml of the substrate-buffer solution. In studying the inhibitor effects, varying amounts of inhibitor solution (6 µl to 125 µl) were added to the reaction cell; the reaction was initiated by the addition of a rate-limiting amount of the enzyme preparation. The production of S-lactoylglutathione was followed by measuring the increase in absorbance at 240 nm at 25° on a double beam recording spectrophotometer. The initial rate of the reaction was determined by measuring the slope of the linear portion of the plot.

CHAPTER III

RESULTS AND DISCUSSION

The reaction sequence employed for the synthesis of the glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines is shown in Figure 1. The Gabriel reaction was utilized for introduction of the ω-amino group on the alkyl chain. The phthalimidoalkyl chain was attached to the sulfhydryl group of the cysteine moiety early in the sequence with subsequent attachment of the glycine and glutaric acid groups.

The formyl group was chosen to protect the amino group of S-(ω-phthalimidoalkyl)-L-cysteine. The formation and subsequent cleavage of the N-formyl derivative was accomplished by the method of Sheehan and Yang.[23,24] The use of the formyl protective group combined with the subsequent use of N,N'-dicyclohexylcarbodiimide (DCC)[25,26] as a coupling reagent for peptide bond formation has been shown to give little or no racemization when optically active amino acid residues are involved.[24] The introduction of the glycine residue by the DCC method provided good yields of the fully protected dipeptide. The glutaric acid moiety was attached in good yield by reaction of compounds 4a and 4b with glutaric anhydride.[9]

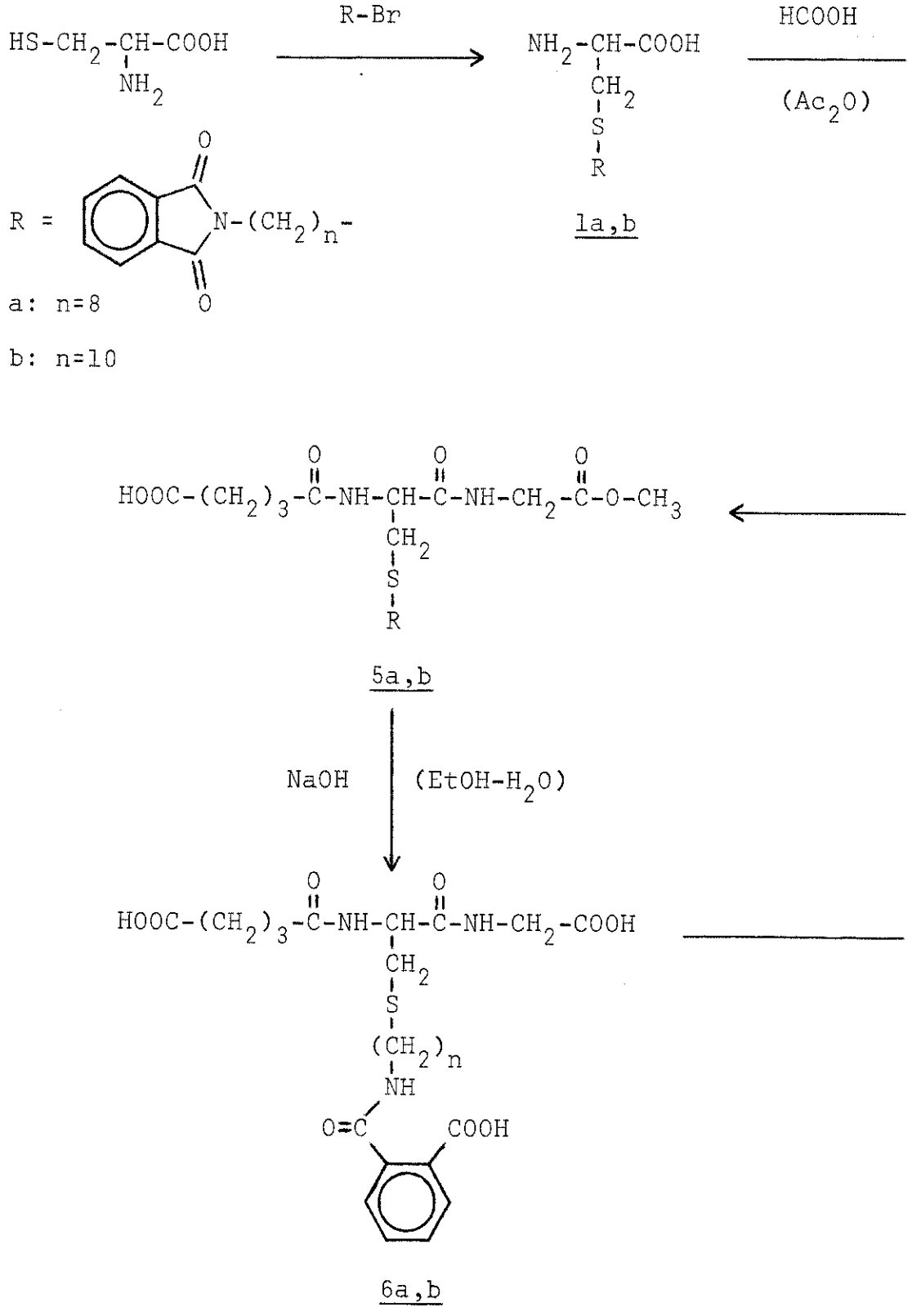
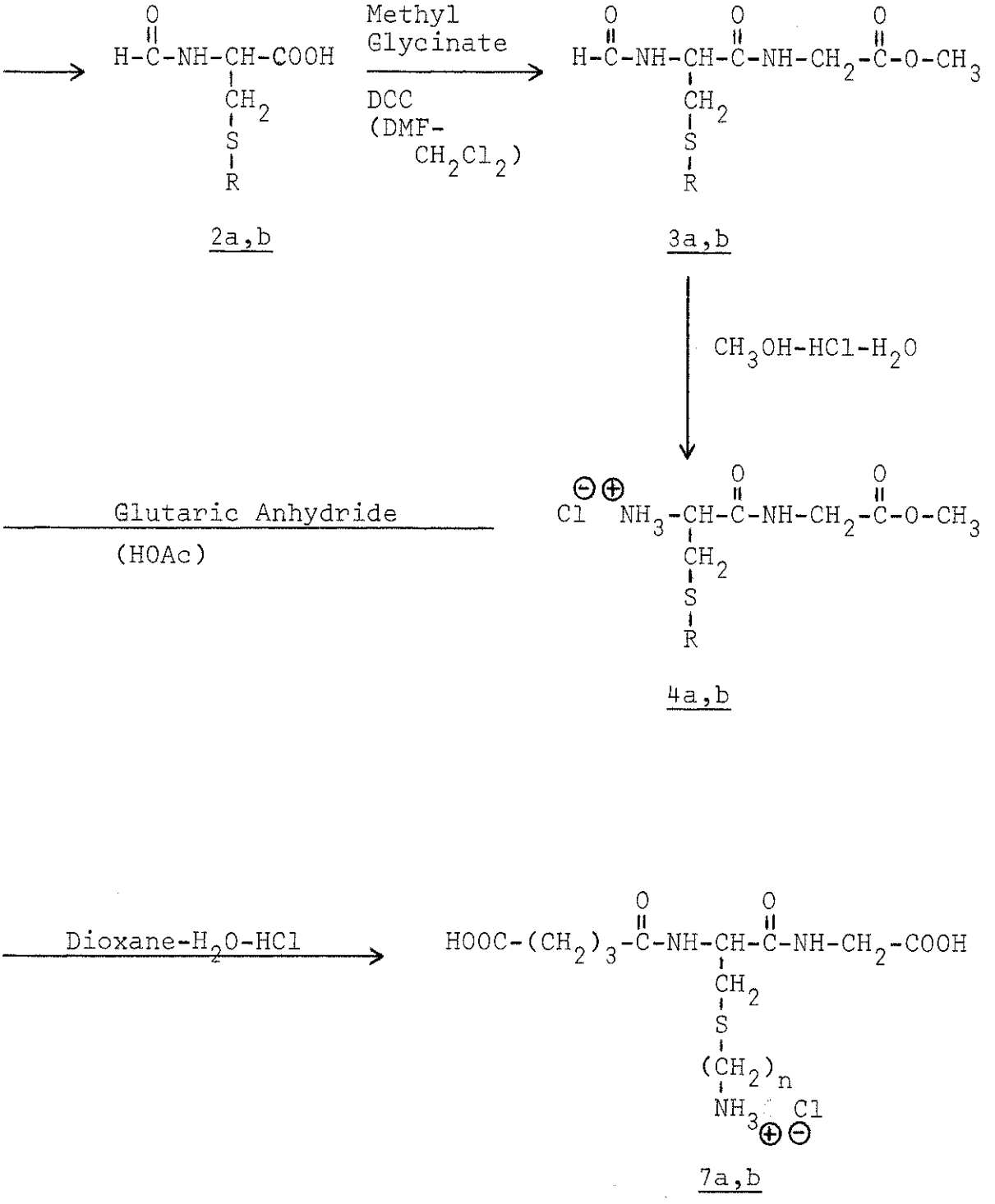


Figure 1--Reaction sequence for preparation of



glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycines.

Considerable difficulty was encountered in the removal of the phthalimido and methyl ester protective groups from compounds 5a and 5b. Attempts at selective hydrazinolysis[27] of the phthalimido group with a variety of solvents (ethanol,[28,29] methanol,[30,31] and DMF-H_2O[10]), selective mild acidic hydrolysis[32-34] of the ester and simultaneous removal[35] of both groups were all unsuccessful. Thin layer chromatography (TLC) showed multiple spots in all cases. Removal of both groups was accomplished by two-stage hydrolysis.[27] Mild basic hydrolysis of the ester was performed with the simultaneous rupture of the phthalimido group to form an N-substituted phthalamic acid. The latter compound was then degraded to phthalic acid and the aminoalkyl peptide hydrochloride by mild acidic hydrolysis in dioxane-water. The final hydrolysis proceeded only very slowly at room temperature, as shown by TLC, but was complete after 30 minutes on a steam bath. Lower temperatures or even shorter reaction times may suffice for this reaction, since under the conditions used, a small amount of impurity was obtained as evidenced by a minor ninhydrin-positive spot on TLC. This small amount of impurity could generally be removed by recrystallization from ethanol-ether.

Other synthetic routes to the desired compounds were initially attempted using different protective groups for the amino and sulfhydryl groups of cysteine, such as the

benzyloxycarbonyl[25,36] and thiazolidine[23] groups. Low yields of intermediates were generally obtained and the intermediate compounds, particularly with the benzyloxycarbonyl protective agent, were often isolated only as oils or were quite resistant to crystallization. The sequence eventually employed appeared to be superior to others attempted for these particular compounds, even though a procedure using an S-protective group would be more amenable to introduction of a variety of S-substituents by replacement of the protective group at a late stage in the sequence.

Compounds 1, 4, 5, 6, and 7 were isolated with varying amounts of water of hydration. Compounds 4 and 7 were hygroscopic, with the octyl derivative being noticeably more so than the decyl counterpart. Compounds 1, 5, and 6, while not perceptibly hygroscopic, were all isolated from aqueous solutions. The reaction scheme used provided overall yields of 24% for the octyl compound and 33% for the decyl compound from cysteine, based on the individual yields of the intermediate products.

In the present inhibition study, the enzymatic reaction rates were measured at several different fixed levels of the hemimercaptal substrate with varying concentrations of glutaryl-S-(ω-aminoalkyl)-L-cysteinylglycine (7a,b). Due to its known inhibitory effect on glyoxalase I,[6] the concentration of free glutathione was held at a relatively low constant

level where this inhibitory effect is minimal.

It has been reported[9] that replacement of the α-amino group of S-substituted glutathiones by a hydrogen results in compounds which are inhibitors of glyoxalase I, but are less potent than the glutathione counterparts. Consistent with this report, compounds 7a and 7b were found to be inhibitors of glyoxalase I, but are less effective than the corresponding S-(ω-aminoalkyl)glutathiones. The results of the studies with the two glutaryl compounds prepared were similar, but the decyl derivative (7b) was found to be a more potent inhibitor than was the octyl derivative (7a), which is in keeping with the previous results for the glutathione series.[10]

A double reciprocal plot (Figure 2) obtained from the study of 7b indicates a mixed type of inhibition.[1] Plots of [I]α/1-α vs substrate concentration (where α = velocity of inhibited reaction/velocity of uninhibited reaction)[1,5] and of percent inhibition vs substrate concentration at various levels of inhibitor (7b) indicate that the inhibition is more competitive at substrate levels less than 0.2 mM and more noncompetitive at substrate levels greater than 0.2 mM. This change in inhibitory mode is particularly pronounced at inhibitor levels greater than 2 mM, while at levels less than 2 mM, the inhibition appears more consistently competitive. However, under the same reaction conditions which were used in this study, SADG gave more nearly competitive inhibition.[20]

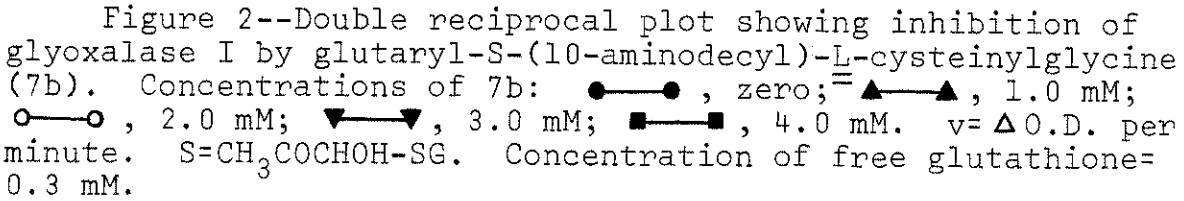
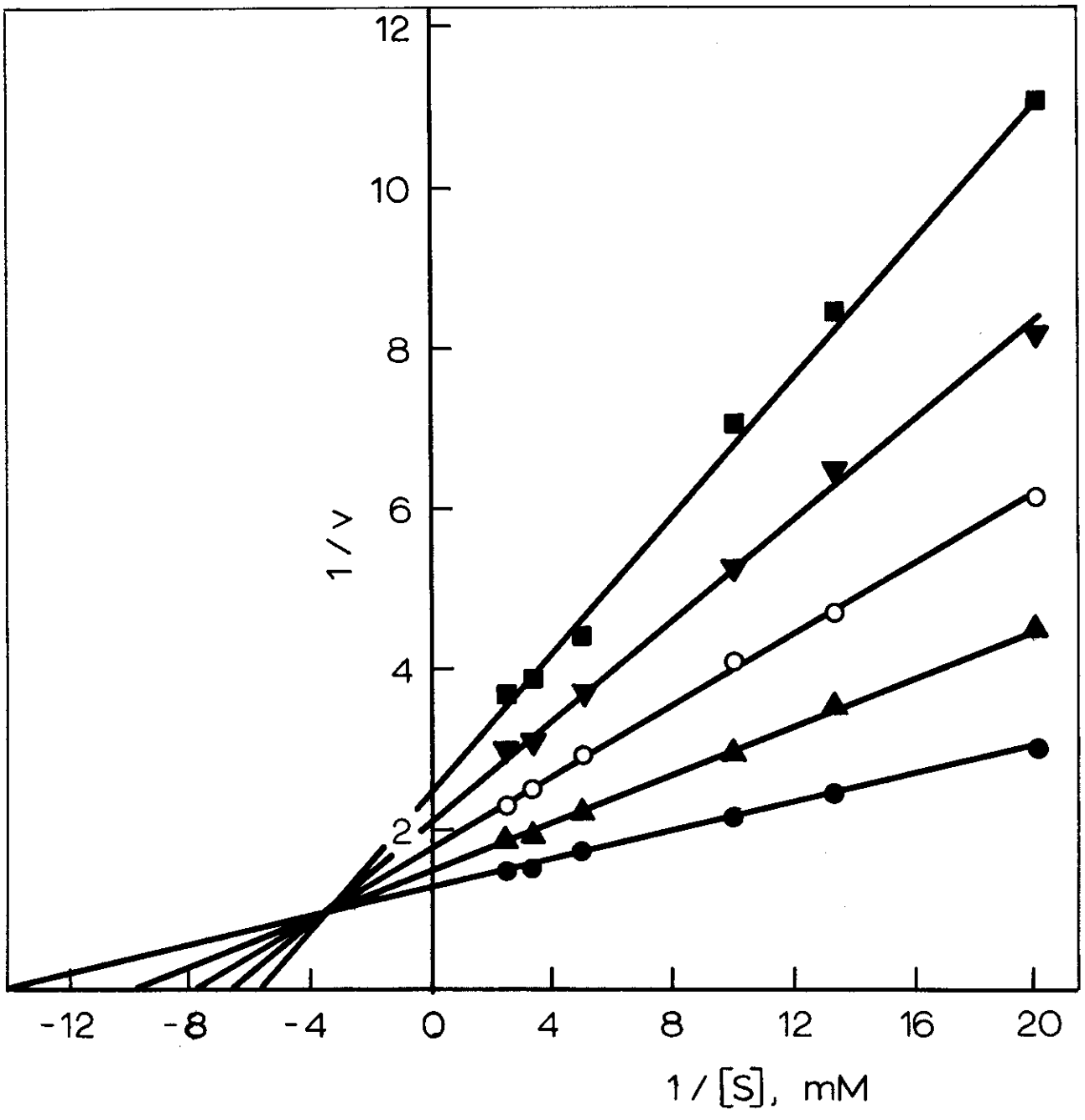


Figure 2--Double reciprocal plot showing inhibition of glyoxalase I by glutaryl-S-(10-aminodecyl)-L-cysteinylglycine (7b). Concentrations of 7b: ●—● , zero; ▲—▲ , 1.0 mM; ○—○ , 2.0 mM; ▼—▼ , 3.0 mM; ■—■ , 4.0 mM. v= Δ O.D. per minute. S=$CH_3COCHOH$-SG. Concentration of free glutathione= 0.3 mM.


12
10
8
6
4
2
1 / v
-12
-8
-4
0
4
8
12
16
20
1 / [S], mM

A Dixon plot[1] (Figure 3) for 7b indicates nonlinear (parabolic) inhibition, as do replots of slopes and of intercepts (from the double reciprocal plot) vs inhibitor concentration. Such parabolic inhibition curves indicate the possibility of involvement of two molecules of inhibitor to produce an I^2 term in the rate equation.[37] Nonlinear inhibition was not observed for SADG.[10,20] An apparent K_i of approximately 1.4 mM was determined for 7b from the intersection of lines in the Dixon plot while a value of about 4.3 mM was obtained for 7a. As the substrate concentration was increased from 0.05 mM to 0.4 mM in these studies, the concentration of 7b required for 50% inhibition ranged from approximately 2 mM to 3 mM and the value of [I]/[S] for 50% inhibition decreased from 41.0 to 7.2.

The observations that the α-amino group enhances binding[9] of glutathione analogs to the enzyme and that more nonpolar substituents attached to the sulfhydryl group of glutathione also increase the binding ability of such inhibitors[8,10] imply that at least two binding regions, one hydrophilic and one hydrophobic, are available near the active site of the enzyme. The data obtained in this study may suggest that the enzyme is able to simultaneously accommodate, in these two regions, two molecules of inhibitor or one molecule of inhibitor and one of substrate. This condition obviously would be more likely to arise at higher levels of inhibitor.

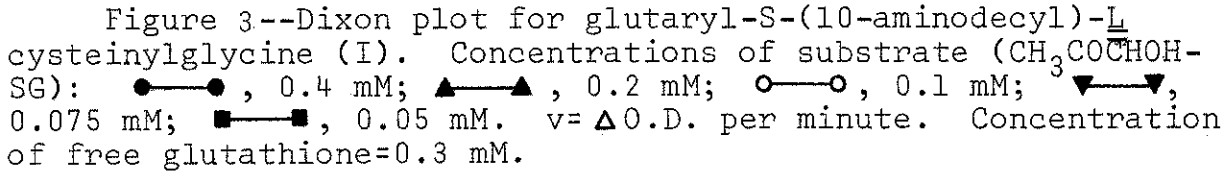
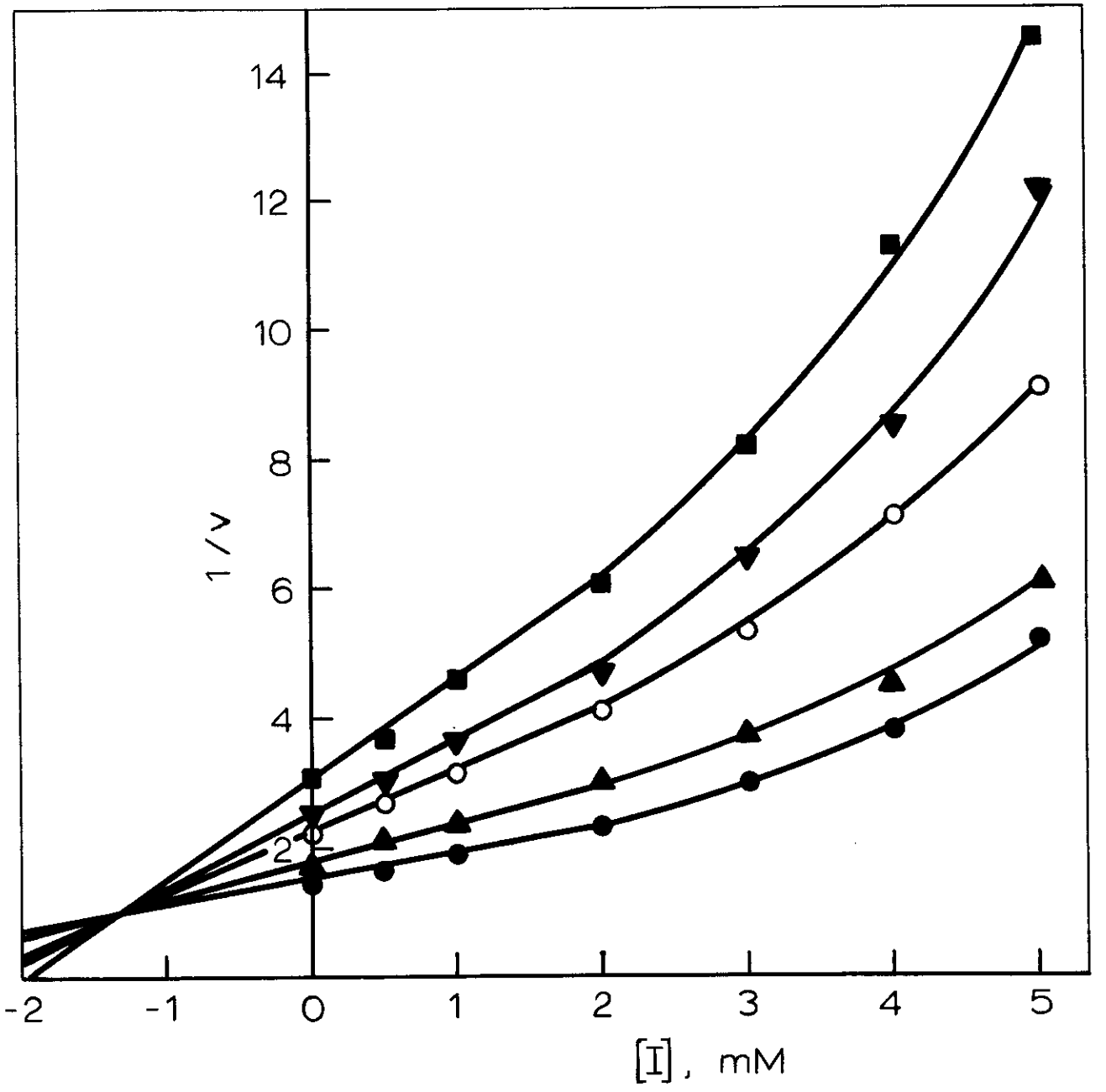


Figure 3--Dixon plot for glutaryl-S-(10-aminodecyl)-L cysteinylglycine (I). Concentrations of substrate ($CH_3COCHOH$-SG): ●—● , 0.4 mM; ▲—▲ , 0.2 mM; o—o , 0.1 mM; ▼—▼, 0.075 mM; ■—■ , 0.05 mM. v= Δ O.D. per minute. Concentration of free glutathione=0.3 mM.


14
12
10
8
6
4
2
1/v
-2
-1
0
1
2
3
4
5
[I], mM

If such multiple binding with the inhibitor exists, it may be due to the lack of an α-amino substituent in 7b which could result in weaker binding in the hydrophilic region than if an α-amino group were present. This could allow a second molecule of inhibitor or a molecule of substrate to occupy that site, while the first molecule of inhibitor remains bound at the hydrophobic region by virtue of the long alkyl chain. Thus, at low concentrations of inhibitor, the substrate may be competing with only one molecule of inhibitor, while at higher inhibitor levels it may be competing with two molecules of inhibitor in order to completely occupy the active site and be able to form product. The fact that SADG does not give rise to nonlinear kinetics may be due in part to the fact that the more inhibitory glutathione derivative was tested at concentrations approximately tenfold lower than was the glutaryl analog. The lower levels of inhibitor present in the system and the stronger binding afforded by the α-amino group in SADG could so reduce the probability of binding of a second molecule of inhibitor to the enzyme that the effects seen with 7b would not be observed. More detailed investigations must be conducted, however, to determine whether multiple binding of the glutaryl analog, or of analog and substrate with the enzyme can in fact occur and what effect free glutathione has if the two-substrate pathway is involved.

While compounds 7a and 7b are lower in inhibitory quality than the corresponding glutathione analogs,[10] they do appear to be promising for evaluation as ligands for the affinity chromatography purification of glyoxalase I, and analogs such as these may further prove to be of aid as probes in studying the mechanism of the enzyme.

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