Glutathione. 13. Mechanism of Thiol Oxidation by Diazenedicarboxylic Acid Derivatives

Edward M. Kosower*[1] and Hanna Kanety-Londner[1a]

Contribution from the Department of Chemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel, and the Department of Chemistry, State University of New York, Stony Brook, New York 11794. *Received June 19, 1975*

Abstract: Kinetic, spectroscopic, and product studies on the reaction of the tripeptide thiol, glutathione (GSH), with the diazenedicarboxylic acid bisamides, $X(CH_2CH_2)_2NCON{=}NCON(CH_2CH_2)_2X$, with X = NCH_3 (DIP), X = $N(CH_3)_2^+$ (DIP+2), X = O (MOR), X = CH_2 (PIP), etc., revealed two distinguishable stages: (a) GSH + YN=NY → YN(SG)NHY; (b) GSH + YN(SG)NHY → GSSG + YNHNHY. The rate of disappearance of diazene (stage a) increased greatly as X changed from CH_2 to $N(CH_3)_2^+$; the rate of disappearance of intermediate (stage b) also increased greatly for the same change in substitution but not as much as the rate for stage a. Hydrolysis rates for the diazene derivatives paralleled the rates for thiol oxidation. Hydrolysis of DIP between pH 10.2 and 10.9 generated the monoacyldiazenecarboxylate ion $CH_3N(CH_2CH_2)_2NCON{=}NCOO^-$, which gave rise to *N*-formyl-*N'*-methylpiperazine, *N*-methylpiperazine, N_2, and CO_2 on further hydrolysis. The s-cis conformation is assigned to the diazenedicarboxylic acid bisamides.

Thiol oxidation by a diazene derivative was first carried out by Kosower and co-workers[2] within red blood cells, using methyl phenyldiazenecarboxylate (**1**) (eq 1). Somewhat later,

$$2RSH + \underset{\mathbf{1}}{C_6H_5N{=}NCOOCH_3} \longrightarrow RSSR + C_6H_5NHNHCOOCH_3 \quad (1)$$

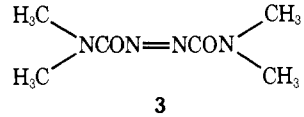
Yoneda utilized diethyl diazenedicarboxylate (**2**) to produce disulfides from thiols,[3] following an old suggestion by Diels and Wulff[4] that the diazene color disappeared slowly in the presence of thiols. Mukaiyama and Takahashi[5] found that thiols reacted with **2** in hexane to yield an adduct, which could then be reacted with a different thiol in a more polar solvent like benzene to afford useful quantities of mixed disulfides. Kosower and co-workers examined the effect of substituent change on **1** (ρ value 2.7) for the reaction with the tripeptide thiol, glutathione (GSH), in aqueous solution and showed that a delay in the formation of final products after the disappearance of the starting materials could be attributed to an intermediate.[6-8]

A thiol oxidation reaction via an intermediate raises two mechanistic questions. The first involves the mechanism of the formation of the intermediate (i.e., the mechanism of addition to the diazene double bond). The second relates to the mechanism of the formation of the final disulfide product through the reaction of the thiol with the intermediate adduct. There is intense interest in the mechanism of reactions at atoms other than carbon, and replacements at sulfur are still not as well defined as they might be, as examination of the works of Cluffarin,[9] Fava,[10] Kice,[11] and Hogg[12] would reveal. These chemical points encouraged a more precise investigation into the mechanism of the thiol oxidation.

Oxidation of glutathione (GSH) to glutathione disulfide (GSSG) by means of diazenedicarboxylic acid derivatives represents a convenient way to change the concentrations of these cellular constituents within biological systems. The ratio of GSH to GSSG within cells can be described as the GSH-GSSG status of the system; a precise understanding of the mechanism of oxidation of thiols by diazenes would aid in their application to biological problems. For example, "azoester" (**1**) and "diamide" (**3**) [diazenedicarboxylic acid bis(*N,N*-dimethylamide)] have been used to stop the initiation of protein synthesis,[13,14] to increase the spontaneous and stimulated release of neurotransmitter,[15,16] and to affect (eye) lens properties,[17] sugar transport,[18] and the viability of red cells.[19] Through relatively specific intracellular oxidation of GSH (a tripeptide thiol, γ-glutamylcysteinylglycine, ubiquitous in biological systems) to GSSG, the GSH-GSSG status of the cells is changed and with that change come many alterations in biological behavior.[20]

The choice of molecule used in our present study was made on two grounds: one, need for a substantial change in electronic effects at the N=N double bond without any great change in the steric arrangements around the reactive N=N bond; two, the need for two similar reagents, one able to penetrate the membranes of cells and one unable to penetrate cell membranes, permitting a distinction between intracellular and extracellular thiols to be made.

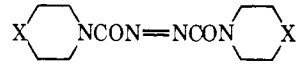
Several of the molecules we have introduced in this work have not only been useful in the study of the detailed mechanism but they have also proven their merit (a) for the purpose for which they were designed in neurophysiological experiments[21] and (b) for measurements of the membrane diffusion coefficients of red blood cells.[22]



3

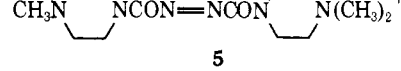
Results

The six primary molecules used in this study are depicted below. Since the molecules are so similar, trivial designations are given and utilized in the text. The five symmetrical molecules are represented by the general formula **4.** The unsymmetrical molecule DIP+1 is shown as **5.** Formal names are written as diazene derivatives.[23]



4

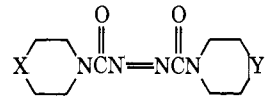
X = NCH_3 (DIP); X = O (MOR); X = CH_2 (PIP);
X = $N(CH_3)_2^+$ (DIP+2); X = $N(CH_3)CH_2CH_3^+$ ($DIPEt^{2+}$)



5

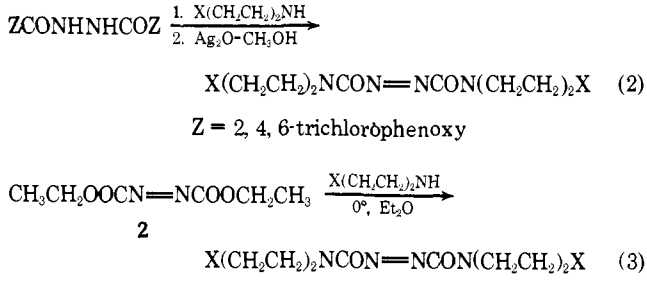
Synthesis. Two syntheses were used as shown in eq 2 and 3. The direct method (Figure 3), from diethyl diazenedicarboxylate (**2**), was used by Bock and Kroner[24] and is superior to the approach using a reactive diazane ester, especially since 2,4,6-trichlorophenol forms rather stable complexes with the esters which are formed.

Table I. Ultraviolet–Visible Spectroscopic Maxima of Diazenecarboxamides



		Uv spectrum					Visible spectrum				
		Acetonitrile		Water		ΔE,	Acetonitrile		Water		ΔE,
X	Y	λ, nm	ϵ^a	λ, nm	ϵ^a	kcal/mol	λ, nm	ϵ^a	λ, nm	ϵ^a	kcal/mol
NCH_3	NCH_3	290.5	1810	299.3	3600	2.89	423	39	442	40	2.90
NCH_3	$^+N(CH_3)_2$	287	1730	293.1	3100	2.07	426	38	*c*		
$^+N(CH_3)_2$	$^+N(CH_3)_2$	284	*b*	291.2	3300	2.49	425	*b*	447	39	3.32
O	O	298	2030	306.4	3450	2.63	427	42	447.5	43	3.06
CH_2	CH_2	292	1760	296.8	3250	1.61	422.5	43	444	44	3.28
$^+N(CH_2CH_3)CH_3$	$^+N(CH_2CH_3)CH_3$	*b*	*b*	291.2	3350		*b*	*b*	442	42	

^{a} ±5%. ^{b} Solubility too low for accurate measurement. ^{c} Not possible to locate peak accurately.



$$ZCONHNHCOZ \xrightarrow[2.\ Ag_2O\text{-}CH_3OH]{1.\ X(CH_2CH_2)_2NH} X(CH_2CH_2)_2NCON{=}NCON(CH_2CH_2)_2X \quad (2)$$

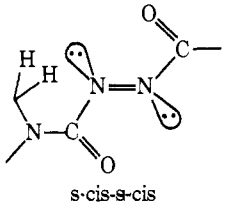
Z = 2, 4, 6-trichlorophenoxy

$$CH_3CH_2OOCN{=}NCOOCH_2CH_3 \xrightarrow[0°,\ Et_2O]{X(CH_2CH_2)_2NH} X(CH_2CH_2)_2NCON{=}NCON(CH_2CH_2)_2X \quad (3)$$

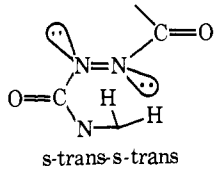
2

Structures were established through NMR, uv, and ir (see the Experimental Section). Purity was established by TLC. DIP+2 and $DIPEt^{2+}$ were prepared through reaction of either methyl iodide or ethyl bromide with DIP in CH_3CN. DIP+1 was prepared by reacting a limited quantity of methyl iodide with DIP. The solubilities of DIP+1 and DIP+2 in CH_3CN were sufficiently different to allow the preparation of pure DIP+1. In addition to the usual criteria, halide titrations gave the predicted equivalent weights for all quaternary salts.

Conformation of Diazenedicarboxamides. Models indicate differences in steric hindrance such that all structures like **4** and **5** have an s-cis-s-cis conformation rather than the s-trans-s-trans conformation which might have been written to maximize the length of the conjugated system. The estimated distance between X groups in **4** is s-trans-s-trans, 9 Å, s-cis-s-cis, 11 Å, and s-trans-s-cis, 7 Å. The s-cis-s-cis conformation clearly minimizes charge repulsion in those molecules containing a charge-carrying X group.



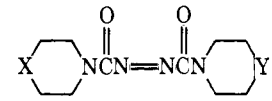
s-cis-s-cis



s-trans-s-trans

Electronic Spectra of Diazenecarboxamides 4 and 5. The n → π* and π → π* transitions are listed in Table I in the solvents acetonitrile and water, together with the transition energy changes corresponding to the solvent change. Several points are of especial interest. 1. The π → π* transition energy decreases for the change $XCH_2 \rightarrow NCH_3 \rightarrow O$ and then increases as a stronger electron-withdrawing group $[N(CH_3)_2^+]$ is introduced. 2. The n → π* transition energy is less sensitive to structure but changes considerably to lower energies in the more polar solvent. The latter fact suggests that solvation of the nonbonding electrons on nitrogen is not significant in the ground state. The characteristics of the π → π* transition were useful in determining the pK_a values for DIP+1 and DIP. The change in maximum is illustrated for DIP in Figure 1. The pK_a for the diprotonated form was confirmed through the finding that DIP+1 gave a curve very similar to that assigned to the diprotonated form of DIP. The diazenecarbonyl group exerts a strong influence on the basicity of the piperazinyl nitrogen, the second pK_a (monoprotonated form) changing from a value estimated to be more than 10 to 7.1.

Table II. Rate Constants for the Hydrolysis of Diazenecarboxamides in Phosphate Buffer, pH 7.2, 25 °C

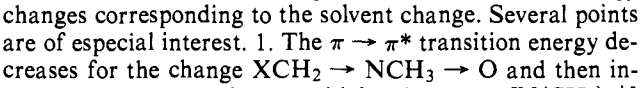


X	Y	$k \times 10^6$, s^{-1} ^{a}
DIP	NCH_3	NCH_3	34.9
DIP+1	NCH_3	$^+N(CH_3)_2$	133.0
DIP+2	$^+N(CH_3)_2$	$^+N(CH_3)_2$	158.0
MOR	O	O	4.0 ^{b}
PIP	CH_2	CH_2	0.17 ^{b}

^{a} ±7%. ^{b} Measured at pH 9.0 and corrected by assuming linearity with pH.

Hydrolysis of Diazenecarboxamides 4 and 5. The absorption spectra of the diazene derivatives **4** and **5** decreased smoothly in aqueous solution at pH 7.2 and 25 °C. Hydrolyses of MOR and PIP were carried out at pH 9.0 because of the low rates of reaction at pH 7.2. Plots of log optical density against time yielded straight lines, from which the rate constants listed in Table II were obtained. The hydrolysis rate for DIP varied with pH as shown in Figure 2, representing hydrolysis rates for differently protonated species. The rate constants vary from 1.7×10^{-7} s^{-1} for PIP to 1.58×10^{-4} s^{-1} for DIP+2 at pH 7.2.

At pH values between 10.2 and 10.9, the spectroscopic changes observed for DIP are different from those at lower pH. The π → π* absorption shifts to shorter wavelenghts, from 290 to 265 nm, as illustrated for pH 10.27 in Figure 3. The n → π* absorption also shifts to shorter wavelengths, from 445 to ~420 nm. These changes are ascribed to the hydrolysis of only one amide linkage and increase in rate with an increase in pH (eq 4). At still higher pH values, e.g., pH 13.0, hydrolysis is very



$$CH_3N(CH_2CH_2)_2NCON{=}NCON(CH_2CH_2)_2NCH_3 \xrightarrow{OH^-} CH_3N(CH_2CH_2)_2NCON{=}NCOO^- + HN(CH_2CH_2)_2NCH_3 \quad (4)$$

fast and yields a moderately stable solution with a weak transition at 403.5 nm, no doubt the dianion of diazenedicarboxylic acid, $^-OOCN{=}NCOO^-$, which has a short wavelength maximum (π → π* transition) near 200 nm (P. K. C. Huang and E. M. Kosower, unpublished results).

Reaction of Diazenecarboxamides 4 and 5 with the Thiol, Glutathione (GSH). The stoichiometry of the reaction was established through the demonstration that 1 mol of diazene disappeared (light absorption decrease) in the reaction with

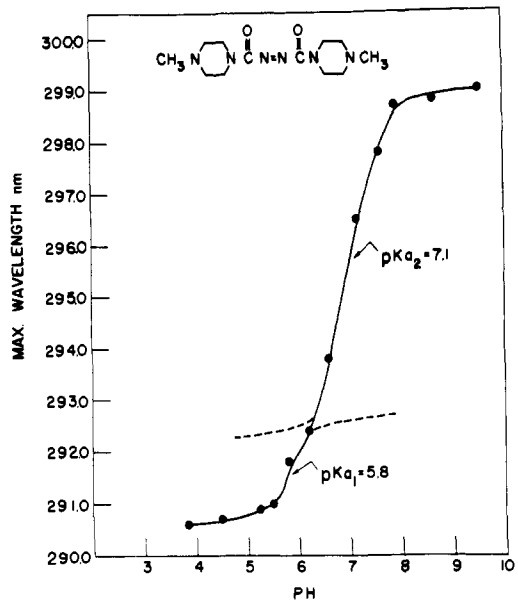


Figure 1. The position of the absorption maximum for the $\pi \rightarrow \pi^*$ transition for DIP (4, X = NCH_3) as a function of pH at 25 °C.

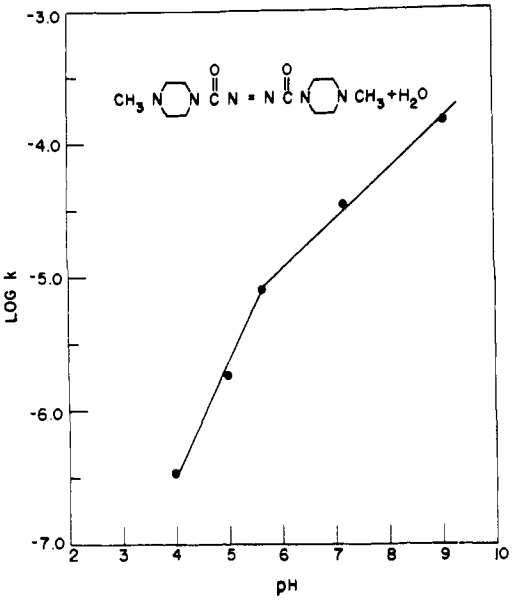


Figure 2. The dependence of log k [rate constant for the hydrolysis of DIP (4, X = NCH_3)] on pH at 25 °C.

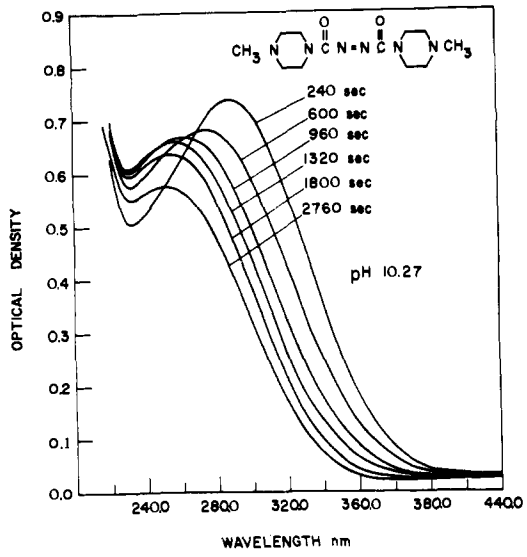


Figure 3. Spectroscopic behavior of a solution of DIP (4, X = NCH_3) (2.9×10^{-4} M) in carbonate buffer, pH 10.27, as a function of time at 25 °C. Note the shift in maximum from that of DIP to a shorter wavelength absorption band assigned to the $\pi \rightarrow \pi^*$ transition of the acyldiazenecarboxylate, ZCON=NCOO$^-$.

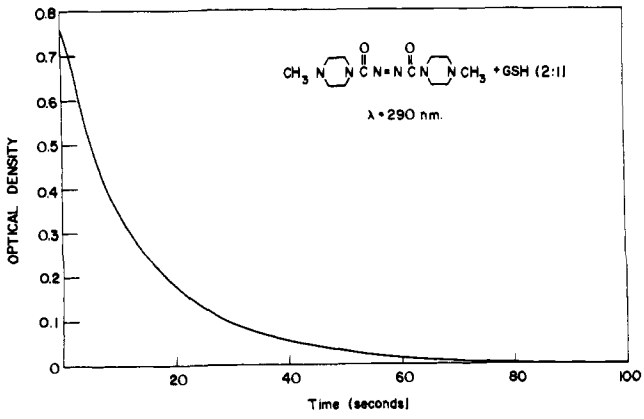


Figure 4. The change in light absorption of DIP (4, X = NCH_3) (initial concentration 2.04×10^{-4} M) as a function of time in the presence of glutathione (4.08×10^{-4} M) in acetate buffer, pH 3.81, at 25 °C.

excess GSH, and 1 mol of the disulfide GSSG appeared, as shown through the change in absorption of NADPH (dihydronicotinamide adenine dinucleotide phosphate) in the presence of the enzyme, glutathione disulfide reductase (eq 5).

$$\text{ZCON=NCOZ} + 2\text{GSH} \longrightarrow \text{ZCONHNHCOZ} + \text{GSSG} \quad (5)$$

$$\text{Z} = \text{CH}_3\text{N(CH}_2\text{CH}_2)_2\text{N}^-$$

The kinetics of the reaction of DIP with 2 equiv of GSH were followed at low pH (Figure 4) and gave good bimolecular constants for a reaction involving one molecule of DIP and one molecule of GSH. The reaction of 1 molar equiv of DIP with 1 molar equiv of GSH led to almost complete disappearance of the diazene absorption, and the kinetic constant for the disappearance was the same as that obtained for the reaction of DIP with larger amounts of GSH. It was thus evident that the first step involved loss of diazene through addition of the GSH to the double bond (eq 6).

$$\text{ZCON=NCOZ} + \text{GSH} \longrightarrow \underset{\mathbf{6}}{\text{ZCON(SG)NHCOZ}} \quad (6)$$

$$\text{Z} = \text{CH}_3\text{N(CH}_2\text{CH}_2)_2\text{N}^-$$

Rapid mixing at low pH was chosen as the method for creating the reaction mixtures with which the disappearance of diazene could be followed, rather than, for example, a stopped-flow method at neutral pH.[25]

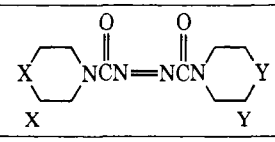
Rate constants were calculated according to eq 7, corresponding to the mechanism in which the first step is considerably faster than the second step in a two-stage mechanism.

$$k_1 = [1/t(B_0 - A_0)] \ln[(B_0 - A_0 + A_t)(A_0)/(A_t \cdot B_0)] \quad (7)$$

$$A = [\text{diazene}];\ B = [\text{GSH}]$$

In two cases, MOR and PIP, reaction with 1 equiv of GSH left 9 and 25% of the original diazene unreacted. In these cases the rate constants calculated according to eq 7 were extrapolated to zero time, a correction which was appreciable only in the case of PIP. The rate constants obtained for the disappearance of diazene are listed in Table III. The activation energy for the first-stage reaction of DIP with GSH was de-

Table III. Rate Constants for the Reaction of Diazenecarboxamides with Glutathione at pH 3.8 and 25 °C



X	Y	k, M^{-1} s^{-1}
CH_2	CH_2	0.14
O	O	3
NCH_3	NCH_3	470
$^+N(CH_3)_2$	NCH_3	590
$^+N(CH_3)_2$	$^+N(CH_3)_2$	720
$^+N(CH_2CH_3)CH_3$	$^+N(CH_2CH_3)CH_3$	700

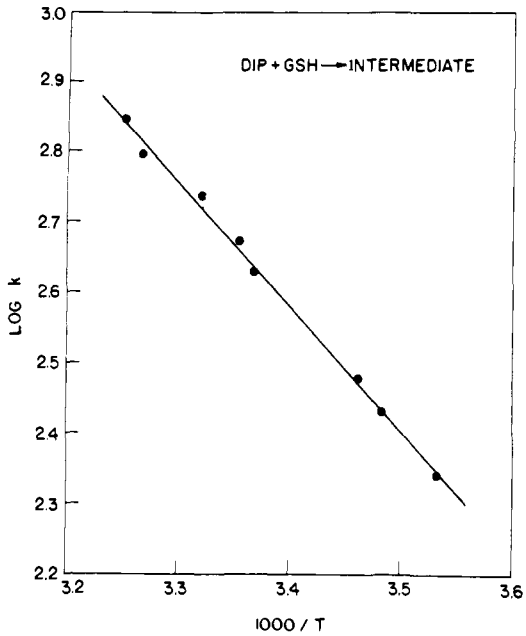


Figure 5. Plot of log k [rate constant for the reaction of DIP (4, X = NCH_3) and glutathione in acetate buffer, pH 3.81] against $1000/T$ (T = temperature in °K).

termined as 8.1 ± 0.3 kcal/mol from a careful study of the temperature effect at pH 3.8 (Figure 5).

Properties of Intermediate 6. The intermediate generated from the reaction of 1 equiv of DIP and 1 equiv of GSH was not stable and disappeared in a two-stage reaction which could be followed spectroscopically (Figure 6) and which did not depend upon the buffer concentration (Figure 7).

It was possible to follow the rate of the reaction of the intermediate with GSH through the change in light absorption at 234 nm. Approximate rate constants were calculated according to eq 8 and are listed for pH 5.22 in Table IV.

$$k_2 = 1/t_{1/2} \cdot C_0 \qquad (8)$$

(C_0 = inital concentration of reactants; $t_{1/2}$, measured half-life)

By examining the 1:1 reaction of MOR and PIP at different pH values, it could be shown that the ratio of the first-stage rate constant to the second-stage rate constant did not vary with pH.

Discussion

The mechanism for the first step can be written as the addition of a thiolate anion to the N=N bond of the diazenecarboxamide, as shown in eq 9. The transition state is illustrated in Figure 8. The reaction is first order in diazene and in glutathione (GSH). The pH dependence of the reaction for MOR and PIP is consistent with the active species being a GS^- anion, the log of the rate constant falling in an approximately linear fashion vs. pH with a slope of 1. In addition, the rate of

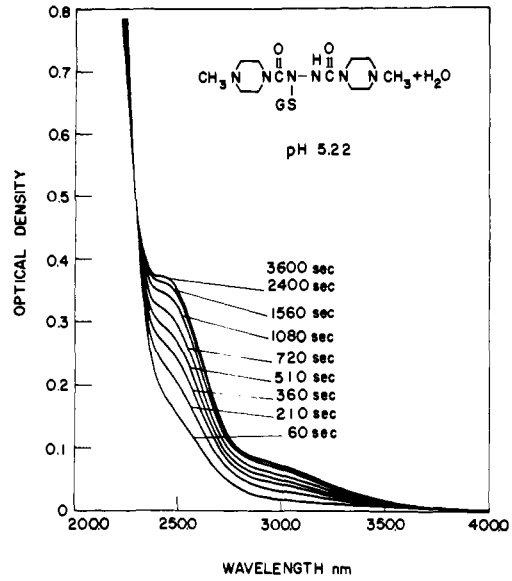


Figure 6. Spectroscopic changes observed for the adduct of glutathione (GSH) and DIP (4, X = NCH_3) (i.e., GSDIP, intermediate 6) (2.7×10^{-4} M) in acetate buffer, pH 5.22, at 25 °C over a 60-min period.

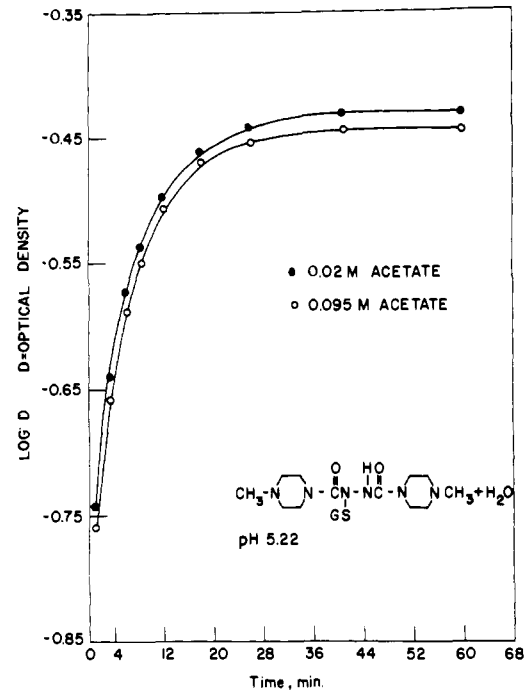
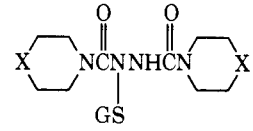


Figure 7. Changes in log D (D = optical density) at 234 nm for GSDIP (see caption, Figure 6) as a function of time in acetate buffers, pH 5.22, which differed in buffer concentration.

Table IV. Rate Constants for the Reaction of Glutathione with the Intermediate Glutathione–Diazenecarboxamide Adduct at pH 5.22 and 25 °C



X		k_2, M^{-1} s^{-1}
O	GSMOR	18.5
^+NHMe	GSDIP	835, 838
$^+N(Me)Et$	$GSDIPEt^{2+}$	1160, 1206

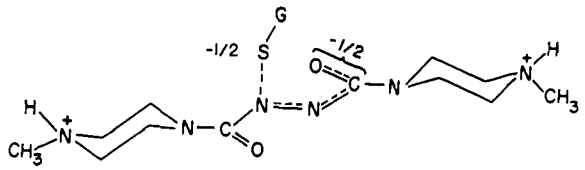


Figure 8. A representation of the transition state for addition of GS^- to the N=N bond of the diazene system (see eq 8).

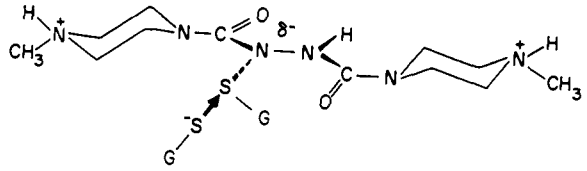
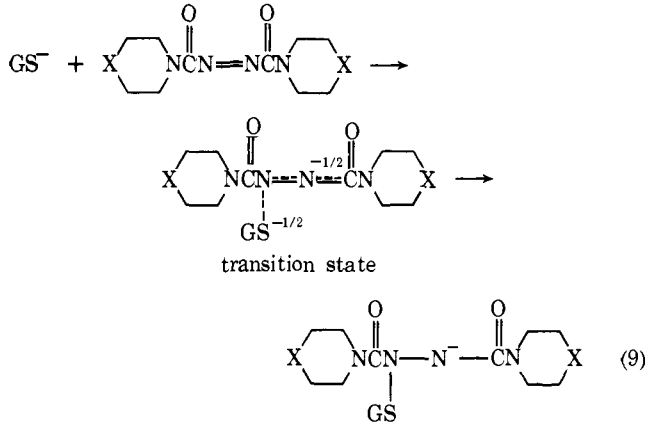


Figure 9. A representation of the transition state for the displacement of diazanyl anion from the sulfenylhydrazine adduct by GS^-. It is probable that some compromise between the equilibrium stereochemistry for the initial state of the sulfenylhydrazine and a geometry which allows accommodation of the developing negative charge in the amide (hydrazide) group must be achieved for the reaction to occur (see eq 9).

reaction of diazene with GSH parallels that of the diazene with OH^- over a wide range of intrinsic reactivity as a comparison of the data in Tables I and II will indicate.

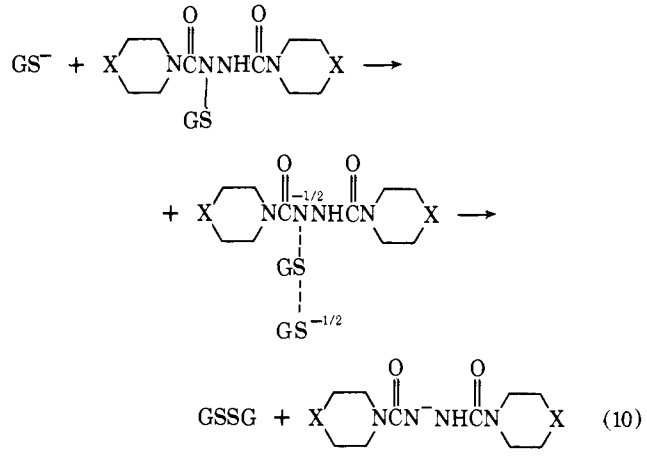
Previous results obtained by Correa[8] for the reaction shown in eq 1 showed that the ρ value for the reaction was 2.67. The moderate ρ value combined with a calculation on the energetics of electron-transfer argues against electron transfer being responsible for the first step of the reaction. The transition state for the addition reaction would be favored by positive charge in the ring opposite to the nitrogen bonded to the diazene group. A major part of the increase in reactivity of the DIP, DIP+1, and DIP+2 group of compounds toward GS^- must be due to the interaction of the negative charge appearing in the diazene and the positive charge already in the ring. (Molecules of the series are fully charged at pH 3.8.)



transition state

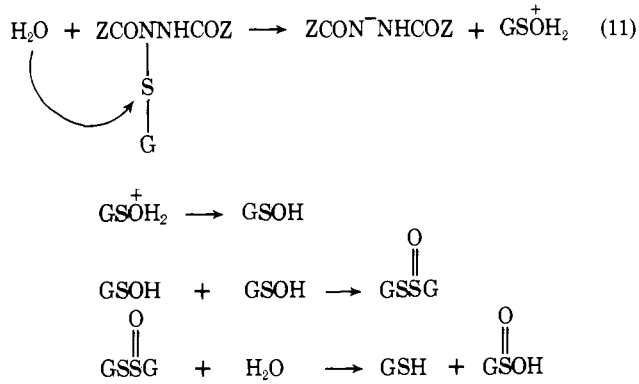
(9)

The second step of the overall oxidation reaction is first order in intermediate and GSH. The pH dependence for the reaction of GSH with GS-DIP (slope of a plot of log k vs. pH, 0.9) shows that the anion, GS^-, reacts with the intermediate. This is consistent with the constant ratio of rate constants for the first and second step at different pH values (eq 10). The transition state is shown in Figure 9. To remove the possibility that simultaneous proton transfer from the protonated nitrogen aided in the reaction, the pH dependence of the rate of reaction of GS-$DIPEt^{2+}$ with GSH was examined and found to be very similar to that of GS-DIP with GSH.



(10)

The disappearance of the intermediate (a sulfenylhydrazine derivative) in the absence of GSH proceeded at a substantial rate in (at least) two stages. By analogy to other sulfenyl compounds the reaction is regarded as a displacement of nitrogen by water, yielding a sulfenic acid.[26] Such acids are known to disproportionate, yielding the thiol and a sulfonic acid (eq 11). This sequence accounts for the two stages and the lack of an effect from a change in the buffer concentration.



(11)

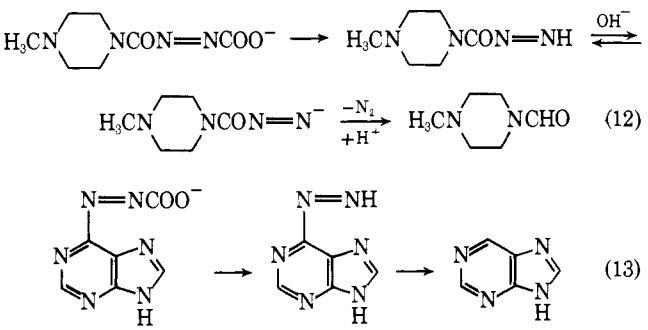
The first and second steps proceed at roughly the same rate within an order of magnitude. The rate constant for the reaction of the most reactive diazene with glutathione (DIP+2) is 5150 times greater than that of the least reactive diazene PIP and 233 times as great as the rate constant for the reaction of GSH with MOR. The corresponding rate ratio for GS-$DIPEt^{2+}$ and GS-MOR in the second-stage reaction of the intermediate with glutathione is 64. We may thus infer that the S-N bond energy in the intermediate is comparable to the energy of the second bond of the N=N double bond in the diazene since the charge displacements between the reactants and the transition state are fairly similar (see Figures 8 and 9). The N=N bond strength would be sensitive to the substitution on the nitrogen in the compound YN=NY. Changing the bond strength is relatively simple and certain studies along this line are planned.

The activation energy for the observed reaction of GSH with DIP at pH 3.8 is relatively low, ca. 8.1 kcal/mol. Since the actual transition state is composed of GS^- anion and the diazene, we estimate the rate of reaction of diprotonated DIP with GS^- as 1.2×10^8 M^{-1} s^{-1} (the pK_a for GSH is 9.2[27]). The entropy of activation derived from this rate constant at 25 °C is +10 eu/mol. The implication is that the transition state for thiolate addition to diazene involves a loss in hydrogen bonding interactions in the initial state sufficient to compensate for arranging the atoms in a way appropriate for the transition state. The reaction probably requires a fairly well specified geometry for the transition state, with π approach of the thiolate anion to the N=N bond. The transition state for the second step, reaction of the thiolate anion with the sulfenylhydrazine, is probably an S_DN reaction[28] with attack of the thiolate anion on the sulfenyl sulfur on the side opposite to the

nitrogen as implied by the sulfite exchange experiments of Fava[29a] (see also discussion of Pryor and Smith[29b]).

The geometric requirements for the reaction, even with a loose transition state, would cause the rate of reaction of large molecule thiols with diazenes to be lower than the rate of reaction of small molecule thiols (e.g., GSH). Thus, protein thiols would react with diazene oxidizing agents less rapidly than with GSH. Experience seems to bear this expectation out, both from the point of view of the stoichiometry observed in most cells (an equivalent of added diazene reacts largely with GSH) and from the direct experiment in which papain was shown to react rather slowly with a diazenecarboxamide.[30]

The monoacyldiazenecarboxylate ion, obtained through hydrolysis of DIP at pH values near 10.5, reacts with protons to lose carbon dioxide. This follows from (a) immediate abolition of the ultraviolet absorption assigned to the $\pi \rightarrow \pi^*$ transition after the addition of sufficient concentrated buffer to change the pH to 5.2 and (b) our extensive previous work on the rates of decarboxylation of diazenecarboxylic acids.[31,32] New ultraviolet absorption could be observed in the pH-shift experiment, and data on the first acyldiazene will be reported elsewhere.[32b] Base-catalyzed decomposition of the acyldiazene leads to *N*-formyl-*N'*-methylpiperazine, a reaction similar to that found for purine-6-diazenecarboxylic acid[30] (eq 12 and 13).



(12)

(13)

Experimental Section

Synthesis. Bis(2,4,6-trichlorophenyl) 1,2-Diazanedicarboxylate (7). 2,4,6-Trichlorophenyl chloroformate was allowed to react with a small excess of 99% hydrazine in dichloromethane (under 10 °C for mixing) in the presence of 1 equiv of sodium carbonate in water. After 1 h, the precipitate was filred, washed with water and dichloromethane, and dried in the oven (91%): mp 260 °C; ir 3250 (NH), 1735, 1795 cm^{-1} (CO).[33]

1,2-Diazanedicarboxylic Acid Bis(*N'*-methylpiperazide) (8). *N*-Methylpiperazine (2 equiv) was refluxed with ester **7** (1 equiv) in acetonitrile for 7 h. Removal of the solvent gave a complex of 2,4,6-trichlorophenol and **8** which was separated by chromatography on silica, the phenol being eluted with chloroform and the diazene with chloroform–methanol: mp 177 °C; ir 3250, 3390 sh (NH), 1635, 1675 cm^{-1} (CO); NMR ($CDCl_3$) δ 7.4 (2 H, s), 3.45 (8 H, t), 2.36 (8 H, t), 2.3 (6 H, s).

1,2-Diazenedicarboxylic Acid Derivatives. Bis(*N'*-methylpiperazide) 4 (X = NCH_3) (DIP). (A) Diazane **8** and 1 equiv of silver oxide in methanol for 15 min yielded an orange solution. The solution was dried with $MgSO_4$ and evaporated, the solid was treated with a small quantity of acetonitrile and filtered, the solution was evaporated, and the solid crystallized as golden needles from ligroin: mp 141 °C (68%); ir 1707 cm^{-1} (C=O); NMR ($CDCl_3$) δ 2.32 (6 H, s), 2.50 (8 H, t), 3.65 (8 H, q); uv, see Table I. (B) Lead tetraacetate in dichloromethane was also used for oxidation. (C) *N*-Methylpiperazine was added slowly to a solution of 0.5 equiv of diethyl diazenedicarboxylate (synthetic or Merck) in petroleum ether–ether (3:1) at 2 °C. The product was filtered and crystallized as golden needles from hexane–benzene (10:2): mp 141–142 °C; yield 65%.

4 [X = $N(CH_3)_2^+$] (DIP+2). This compound was produced from DIP and excess methyl iodide in refluxing acetonitrile as a yellow-orange solid: mp 262 °C; ir 1712 cm^{-1} (C=O); NMR (D_2O) δ 3.50 (12 H, s), 3.88 (8 H, m), 4.35 (8 H, m); uv, see Table I; equiv wt calcd 283.0, found 281.5.

4 [X = NCH_3, X' = $N(CH_3)_2^+$] (DIP+1). DIP (1 equiv) and 1 equiv of methyl iodide were refluxed together in acetonitrile. After filtering off the precipitated DIP+2, the solvent was removed and DIP+1 freed from unreacted DIP by multiple crystallizations from acetonitrile and ethyl acetate to give a yellow solid: mp 180–182 °C; ir 1688, 1712 cm^{-1} (C=O); NMR (D_2O) δ 2.45 (3 H, s), 2.78 (4 H, m), 3.45 (6 H, s), 3.88 (8 H, m), 4.33 (4 H, m); uv, see Table I; equiv wt calcd 424.1, found 420.2.

4 (X = CH_2) (PIP). Procedure C for DIP using piperidine gave, after recrystallization from methanol, a 20% yield of yellow crystals: mp 134–135 °C (lit.[24] mp 130–132 °C, obtained in 20% yield); ir 1702 cm^{-1} (C=O); NMR ($CDCl_3$) δ 1.68 (12 H, t), 3.52 (8 H, m); uv, see Table I.

4 (X = O) (MOR). This compound was prepared from ester **7** and procedure A in 42% yield (mp 141–142 °C) and from procedure C in 27% yield [mp 142 °C (lit.[24] 140–141 °C); ir 1712 cm^{-1} (C=O); NMR (D_2O) δ 3.78 (8 H, s), 3.82 (8 H, s); uv, see Table I].

4 [X = $N(CH_3)CH_2CH_3^+$] ($DIPEt^{2+}$). This compound was prepared from DIP and ethyl bromide, after 3 days in acetonitrile: mp 235–236 °C; ir 1712 cm^{-1} (C=O); NMR (D_2O) δ 1.58 (6 H, t), 3.40 (6 H, s), 3.86 (12 H, m), 4.35 (8 H, m); uv, see Table I; equiv wt calcd 250.0, found 247.9.

Kinetic Procedures. Hydrolysis. Diazenecarboxamide was dissolved in temperature-equilibrated buffer, a sample was transferred to a thermostated quartz cell, and the absorption spectrum was followed as a function of time using a Cary Model 17. The initial concentrations were $1–5 \times 10^{-4}$ M. Kinetic constants were obtained from a plot of log *D* (optical density) against time.

Reaction with Glutathione (GSH).[25] A solution of diazene in the appropriate buffer (acetic acid–sodium acetate in most cases) ($1–2 \times 10^{-4}$ M) (2.9 cc) was placed in a thermostated 1-cm^2 quartz cell containing a tiny Teflon-coated magnetic stirring bar. (A small stirring magnet was installed below the cell holder.) A glutathione solution ($3–6 \times 10^{-3}$ M) (0.1 cc) was introduced with an Eppendorf pipet with the Cary chart running and the wavelength fixed at the desired value (usually the diazene absorption maximum). Addition was carried out under a black cloth to shield the photomultiplier from stray light. The small change in optical density made by dilution could be followed and the mixing time generally ranged between 1 and 2 s. (Correction to zero time was made to take into account the reaction which occurred during the time of mixing.) Stirring was maintained throughout the reaction. Rate constants were obtained from eq 7 with a program for a Hewlett-Packard 9810 calculator to which an X–Y plotter was connected. Using the plot of rate constant vs. time, the small corrections to zero time and initial concentration required by the mixing time were made. The rate of reaction of the intermediate **6** with GSH was followed at 234 nm by generating the intermediate through the addition of 1 equiv of GSH to 1 equiv of diazene. A study of the change in optical density at 234 nm was initiated at the time of addition of a second equivalent of GSH, in the manner described above with an Eppendorf pipet (0.1 cc).

Stoichiometry of Reaction of DIP with GSH. To 1 cc of a solution of DIP (1×10^{-2} M) in buffer, pH 7.2, was added excess GSH (7 mg, $> 2.2 \times 10^{-2}$ M). After 30 min, 0.2 cc of a tenfold diluted reaction solution was added to 2.63 cc of Tris buffer, pH 8.0, 0.1 cc of Na EDTA (10 mg/cc of buffer, pH 7.2), 0.05 cc of a solution of the sodium salt of NADPH (10 mg/cc of buffer, pH 7.2). and 0.02 cc of glutathione disulfide reductase (Sigma) in buffer, pH 7.2. The final volume was 3.0 cc. All buffer concentrations were 0.1 M. pH 7.2 buffer was sodium phosphate. A solution of 1.66×10^{-3} M glutathione disulfide (GSSG) caused a decrease in NADPH absorption of 0.75 optical density units. The unknown caused a change of 0.44 units, corresponding to a GSSG concentration of 0.97×10^{-3} M. The GSSG concentration in the reaction solution was thus 0.97×10^{-2} M which may be compared with the value of 1×10^{-2} M expected.

Products of Hydrolysis. DIP (**4**, X = NCH_3) (55.3 mg, 0.196 mmol) was dissolved in 250 cc of 0.08 N phosphate buffer, pH 7.4, and allowed to stand for 8 days. The pH at the end of the experiment was 7.9. The solution was carefully evaporated at low temperature, and the salt residue was extracted with chloroform. Evaporation of the chloroform yielded 30 mg of a mixture of *N*-formyl-*N'*-methylpiperazine and *N*-methylpiperazine. NMR analysis (using authentic materials as reference) showed a ratio of about 6:1 with the *N*-formyl compound predominating. A blank procedure using only *N*-methyl-

piperazine indicated that only 16.4% of *N*-methylpiperazine could be recovered under these conditions. Hydrolysis of DIP in neutral solution thus leads to *N*-formyl-*N'*-methylpiperazine and *N*-methylpiperazine in stoichiometric and equivalent amounts. Authentic *N*-formyl-*N'*-methylpiperazine was synthesized by the reaction of methyl formate with *N*-methylpiperazine, followed by purification with column chromatography. The liquid obtained in this way gives a single spot on TLC and has an NMR spectrum in complete accord with its assigned structure: NMR ($CDCl_3$) δ 2.32 (3 H, s), 2.45 (4 H, t), 3.50 (4 H, q), 8.05 (1 H, s); ir 1660 cm^{-1} (C=O).

Acknowledgment. Support of this research from the donors of the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged.

References and Notes

(1) (a) Department of Chemistry, Tel-Aviv University, Ramat-Aviv, Tel-Aviv, Israel; (b) Department of Chemistry, State University of New York, Stony Brook, N.Y. 11794.
(2) N. S. Kosower, G. A. Vanderhoff, E. M. Kosower, and P. K. C. Huang, *Biochem. Biophys. Res. Commun.*, **20**, 469 (1965).
(3) F. Yoneda, K. Suzuki, and Y. Nitta, *J. Org. Chem.*, **32**, 729 (1967).
(4) O. Diels and C. Wulff, *Justus Liebigs Ann. Chem.*, **437**, 309 (1924).
(5) T. Mukaiyama and K. Takahashi, *Tetrahedron Lett.*, 5907 (1968).
(6) N. S. Kosower, K. R. Song, E. M. Kosower, and W. Correa, *Biochim. Biophys. Acta*, **192**, 8 (1969).
(7) E. M. Kosower, W. Correa, B. J. Kinon, and N. S. Kosower, *Biochim. Biophys. Acta*, **264**, 39 (1972).
(8) W. Correa, Ph.D. Thesis, State University of New York, Stony Brook, Jan 1970.
(9) L. Senatore, E. Ciuffarin, A. Fava, and G. Levita, *J. Am. Chem. Soc.*, **95**, 2918 (1973).
(10) E. Ciuffarin and A. Fava, *Prog. Phys. Org. Chem.*, **6**, 81 (1968).
(11) J. L. Kice, *Acc. Chem. Res.*, 1, 58 (1968).
(12) D. R. Hogg and J. Stewart, *J. Chem. Soc., Perkin Trans. 2*, 1040 (1974).
(13) N. S. Kosower, G. A. Vanderhoff, and E. M. Kosower, *Biochim. Biophys. Acta*, **272**, 623 (1972).
(14) N. S. Kosower and E. M. Kosower in "Glutathione", L. Flohe et al., Ed., Georg Thieme Verlag, Stuttgart, 1974, pp 276–287.
(15) R. Werman, P. L. Carlen, M. Kushnir, and E. M. Kosower, *Nature (London), New Biol.*, **233**, 120 (1971).
(16) E. M. Kosower and R. Werman, *Nature (London), New Biol.*, **233**, 121 (1971).
(17) D. L. Epstein, *Exp. Eye Res.*, 11, 351 (1971).
(18) M. P. Czech, J. C. Lawrence, Jr., and W. S. Lynn, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4173 (1974).
(19) N. S. Kosower, Y. Marikowsky, B. Wertheim, and D. Danon, *J. Lab. Clin. Med.*, **78**, 533 (1971).
(20) See ref 14, pp 287–302.
(21) P. L. Carlen, E. M. Kosower, and R. Werman, *Brain Res.*, in press.
(22) N. S. Kosower, E. M. Kosower, G. Saltoun, and L. Levi, *Biochem. Biophys. Res. Commun.*, **62**, 98 (1975). See also N. S. Kosower et al., *ibid.*, **65**, 901 (1975).
(23) DIP, 1,2-diazenedicarboxylic acid bis(*N'*-methylpiperazide); MOR, 1,2-diazenedicarboxylic acid bismorpholide; PIP, 1,2-diazenedicarboxylic acid bispiperidide; DIP+2, 1,2-diazenedicarboxylic acid bis(*N',N'*-dimethylpiperazinide) dilodide; $DIPEt^{2+}$, 1,2-diazenedicarboxylic acid bis(*N'*-ethyl-*N'*-methylpiperazinide) dibromide; DIP+1, 1,2-diazenedicarboxylic acid *N'*-methylpiperazide *N',N'*-dimethylpiperazinide iodide.
(24) H. Bock and J. Kroner, *Chem. Ber.*, **99**, 2039 (1966).
(25) The rate constants for hydrolysis of some of the compounds were high enough to make an operation which required storing the solution in a syringe inconvenient; in addition, the formation of CO_2 as a result of the hydrolysis could have created problems in the observation, a difficulty we have encountered in attempts to carry out stopped-flow studies on diazenecarboxylates. Furthermore, it was also safer not to handle low concentrations of glutathione more than necessary.
(26) J. P. Danehy, *Int. J. Sulfur Chem., Part B*, **6**, 103 (1971).
(27) G. Jung, E. Breitmaier, and W. Voelter, *Eur. J. Biochem.*, **24**, 438 (1972).
(28) Nonmenclature described in E. M. Kosower, "An Introduction to Physical Organic Chemistry", Wiley, New York, N.Y., 1968 (utilized for sulfur compounds by E. Ciuffarin in ref 9).
(29) (a) A. Fava and A. Iliceto, *J. Am. Chem. Soc.*, **80**, 3478 (1958); (b) W. A. Pryor and K. Smith, *ibid.*, **92**, 2731 (1970).
(30) E. M. Kosower, unpublished results.
(31) P-k. C. Huang and E. M. Kosower, *J. Am. Chem. Soc.*, **90**, 2354 (1968).
(32) (a) E. M. Kosower, *Acc. Chem. Res.*, **4**, 193 (1971). (b) An acetyldiazenyl anion has been suggested as the structure for an unstable but observable intermediate in the reaction of hydroxide ion with chloroacetyl hydrazide: W. LeNoble and Y.-S. Chang, *J. Am. Chem. Soc.*, **94**, 5402 (1972).
(33) C. M. Kraebel, S. M. Davis, and M. J. Landon, *Spectrochim. Acta, Part A*, **23**, 2541 (1967).

Polyglucosidic Metabolites of Oleaceae. The Chain Sequence of Oleoside Aglucon, Tyrosol, and Glucose Units in Three Metabolites from *Fraxinus americana*

Robert Thomas LaLonde,* Chunfook Wong, and Amy Inn-Mei Tsai

Contribution from the Department of Chemistry, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210. *Received August 21, 1975*

Abstract: Metabolites, previously designated Gl 3, Gl 5, and Gl 6, from embryos of the American ash (*Fraxinus americana*) were examined by spectral methods, degradation achieved by methanolysis and conversion to acetates. Gl 6 is identified as nüzhenide. Acetylation of Gl 3 and Gl 5 gives ^1H NMR determined undecaacetate and octaacetate, respectively, thus providing evidence for the number of free hydroxyl groups and furnishing material suitable for molecular weight determinations by vapor-phase osmometry. Evidence for the identity and sequence of the various units was obtained, among other methods, through methanolysis, Gl 3 giving nüzhenide and oleoside 7-methyl ester and Gl 5 giving ligstroside and oleoside 7-methyl ester. Gl 3 contains one unit of 2-(4-hydroxyphenyl)ethanol (Tyo), three units of glucose [all as 1-β-D-glucopyranosides (β-D-Glc)], and two of oleoside aglucon (β-Olo) in the sequence: β-D-Glcl-1β-Olo7-6β-D-Glcl-1Tyo6-7β-Olol-1β-D-Glc. Gl 5 contains the same components as Gl 3 but one less glucose unit in the sequence β-D-Glcl-1β-Olo7-1Tyo6-7β-Olol-1β-D-Glc. ^{13}C NMR proves to be an especially valuable tool in determining the sequence of units in the intact metabolites.

Sondheimer and co-workers[1] have described the discovery of three abundant glucosides, designated simply Gl 3, Gl 5, and Gl 6, from the seeds of *Fraxinus americana*. Besides the genus *Fraxinus*, the glucosides were detected in the seeds from the genera *Olea* and *Syringa*, also of the family Oleaceae. These workers observed that the levels of metabolites Gl 3 and 6, but not Gl 5, diminished in the course of germination and were regulated by gibberellic and abscisic acids. The nature of Gl 3, Gl 5, and Gl 6 remained obscure despite an earlier investigation of Gl 3.[2] This paper describes our efforts to elucidate the structures of all three metabolites.

The earlier examination[2] of Gl 3 gave evidence for the incorporation of the following structural features. Spectral data indicated the presence of -OCH=C(C)COOR in Gl 3. Acid-promoted hydrolysis gave glucose but the results to establish its presence in a β-glucoside linkage were equivocal.