Studies on the Inhibition of Glyoxalase I by S-Substituted Glutathiones[1]

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S-Substituted glutathiones were prepared and investigated as inhibitors of the enzyme, glyoxalase I. A nonpolar region exists on the enzyme and plays an important role in the formation of an enzyme–inhibitor complex. The amount of inhibition of glyoxalase increases as the alkyl chain is lengthened from methyl to *n*-octyl. Several *S*-arylglutathiones were also prepared in order to take advantage of this nonpolar region on the enzyme. For example, *S*-(*p*-bromobenzyl)glutathione (**14**) gave about 920-fold better binding than the previously reported *S*-methylglutathione (**1**).

It has recently been shown that *S*-alkylglutathiones are potent competitive inhibitors of glyoxalase.[3] The glyoxalase system (glyoxalase I and glyoxalase II) is present in plant and animal cells and converts methylglyoxal into lactic acid. Since methylglyoxal and other α-ketoaldehydes are known to be carcinostatic agents,[4] it was suggested that inhibitors of the glyoxalase system may create a build-up of methylglyoxal in cells and thus inhibit cellular growth.[3] Also, coadministration of a glyoxalase inhibitor and methylglyoxal may potentiate the carcinostatic action of the ketoaldehyde. The rationale for the potential carcinostatic activity of glyoxalase inhibitors has been presented in a previous paper.[5]

Enzyme Kinetics.—Upon addition of glyoxalase I to a solution of methylglyoxal and reduced glutathione (GSH), *S*-lactoylglutathione is produced. It is known[6,7] that methylglyoxal and GSH react nonenzymatically to form a hemimercaptal according to the equation

$$\underset{(M)}{CH_3COCHO} + \underset{(G)}{GSH} \underset{K}{\rightleftharpoons} \underset{(GM)}{CH_3COCH(OH)SG}$$

and it has been demonstrated that the hemimercaptal is the substrate for the enzyme.[7,8] The hemimercaptal (GM) forms immediately upon mixing the methylglyoxal and GSH (pH 6.6, 30°), and equilibrium is reached in less than 5 min. A spectrophotometric determination of the dissociation constant, K, was made from the absorbances at 240 mμ of equilibrated mixtures of methylglyoxal and GSH. Since the amount of GM formed depends upon the initial concentration of methylglyoxal and GSH, K may be obtained from eq 1, and (GM) may be obtained spectrophotometrically, since

$$K = \frac{(M)(G)}{(GM)} = \frac{[(M_0) - (GM)][(G_0) - (GM)]}{(GM)} \quad (1)$$

$$A_{240} = \epsilon_G(G) + \epsilon_M(M) + \epsilon_{GM}(GM) \quad (2)$$

or

$$(GM) = \frac{A_{240} - \epsilon_G(G_0) - \epsilon_M(M_0)}{\epsilon_{GM} - \epsilon_M - \epsilon_G} \quad (3)$$

where A_{240} = total absorbance at 240 mμ; (M) = concentration of methylgloxal; (G) = concentration of glutathione; (GM) = concentration of hemimercaptal adduct; (M_0), (G_0) = initial concentrations; and ϵ_M, ϵ_G, ϵ_{GM} = molar extinction coefficients. The value of K at 30°, calculated with ϵ_M 3.15 M^{-1} cm^{-1}, ϵ_{GM} 395 M^{-1} cm^{-1}, and values of ϵ_G taken from Figure 1 by

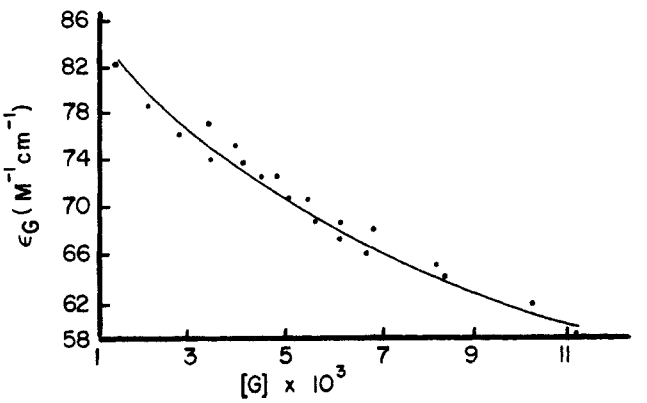

Figure 1.—Plot of molar extinction coefficient *vs.* concentration for GSH at pH 6.6.

successive approximation, as described in the Experimental Section, was 3.1 ± 0.2 mM. Cliffe and Waley[7] reported a value of 2 mM for K, but no details were given concerning concentrations or purity of the solutions used. We have found that the extinction coefficient of GSH in the range of 1–10 mM varied considerably, and the appropriate extinction coefficient for a particular concentration of GSH was obtained from the experimentally determined plot (see Figure 1). Thus, the value of 3.1 mM represents a more accurate value for K.

In an enzymatic determination of K, the rate of product formation was measured in two experiments, the first determined at concentrations of methylglyoxal and GSH given by (M_1) and (G_1), and the second at concentrations (M_2) and (G_2). If the rate is the same in both experiments, then the hemimercaptal (GM) concentrations must be equal, thus (M_1 − GM) (G_1 − GM) = (M_2 − GM) (G_2 − GM) or (GM) = (G_2M_2 − G_1M_1)/(G_2 + M_2 − G_1 − M_1). Values of K ranging from 3.21 to 3.54 mM were obtained by this method. These values are in good agreement with K obtained by

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(2) Supported as a postdoctoral trainee on U. S. Public Health Service Training Grant GM-01769 during the period 2-1-69 to 1-31-70.

(3) R. Vince and W. B. Wadd, *Biochem. Biophys. Res. Commun.*, **35**, 593 (1969).

(4) (a) F. A. French and B. L. Freedlander, *Cancer Res.*, **18**, 172 (1958). (b) F. E. Knock, *Lancet*, **1**, 824 (1966). (c) M. A. Apple and D. M. Greenberg, *Cancer Chemother. Rep.*, **51**, 455 (1967). (d) L. G. Egyud and A. Szent-Gyorgyi, *Proc. Nat. Acad. Sci. U. S.*, **55**, 388 (1966).

(5) R. Vince and S. Daluge, *J. Med. Chem.*, **14**, 35 (1971).

(6) W. O. Kermack and N. A. Matheson, *Biochem. J.*, **65**, 48 (1957).

(7) E. E. Cliffe and S. G. Waley, *ibid.*, **79**, 475 (1960).

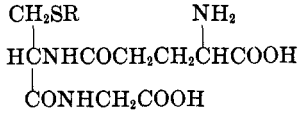
(8) K. A. Davis and G. R. Williams, *Can. J. Biochem.*, **47**, 553 (1969).

the spectrophotometric method. However, high concentrations of GSH relative to methylglyoxal must be avoided in these experiments due to inhibition of glyoxalase by free GSH.

Enzyme Inhibition Studies.—The *S*-alkyl- and *S*-arylglutathiones prepared for possible inhibition of glyoxalase I are listed in Table I. An examination of the

TABLE I

INHIBITION OF GLYOXALASE BY S-SUBSTITUTED GLUTATHIONES



R	No.	mM concentration for 50% inhibition
CH_3	1	8.30 ± 0.50
C_2H_5	2	0.964 ± 0.046
$(CH_2)_2CH_3$	3	0.187 ± 0.017
$(CH_2)_3CH_3$	4	0.057 ± 0.002
$(CH_2)_4CH_3$	5	0.055 ± 0.003
$(CH_2)_5CH_3$	6	0.032 ± 0.002
$(CH_2)_6CH_3$	7	0.021 ± 0.002
$(CH_2)_7CH_3$	8	0.020 ± 0.002
$CH_2C_6H_5$	9	0.185 ± 0.004
$CH_2C_6H_4OCH_3$-*p*	10	0.098 ± 0.008
$CH_2C_6H_4CH_3$-*p*	11	0.065 ± 0.000
$CH_2C_6H_4F$-*p*	12	0.061 ± 0.003
$CH_2C_6H_4Cl$-*p*	13	0.016 ± 0.001
$CH_2C_6H_4Br$-*p*	14	0.009 ± 0.000
$CH_2C_6H_4NO_2$-*p*	15	0.033 ± 0.001
$CH_2C_6H_4CN$-*p*	16	0.023 ± 0.002
$CH_2C_6H_4CH_3$-*m*	17	0.147 ± 0.015
$CH_2C_6H_4Cl$-*m*	18	0.042 ± 0.001
$CH_2C_6H_4NO_2$-*m*	19	0.111 ± 0.021
$CH_2C_6H_4Cl$-*o*	20	0.134 ± 0.005
$CH_2C_6H_4NO_2$-*o*	21	0.510 ± 0.017
$(CH_2)_2C_6H_5$	22	0.198 ± 0.004
$(CH_2)_3C_6H_5$	23	0.018 ± 0.002
$(CH_2)_4C_6H_5$	24	0.080 ± 0.008
$(CH_2)_5C_6H_5$	25	0.015 ± 0.001
$(CH_2)_2C_6H_4NO_2$-*p*	26	0.133 ± 0.009
$CH_2COC_6H_5$	27	0.394 ± 0.042
$CH_2COC_6H_4NH_2$-*p*	28	0.873 ± 0.008
$CH_2COC_6H_4OH$-*p*	29	0.393 ± 0.042
$CH_2COC_6H_4Cl$-*p*	30	0.102 ± 0.003
$CH_2COC_6H_4Br$-*p*	31	0.074 ± 0.002
$CH_2COC_6H_4NO_2$-*m*	32	0.145 ± 0.002
$CH_2COC_6H_3$-3-NO_2-4-Br	33	0.129 ± 0.012
$(CH_2)_2COC_6H_5$	34	0.077 ± 0.006
$(CH_2)_5CH_3$ (*N*-Ac)	35	0.227 ± 0.019
$(CH_2)_2CH_3$ (*N*-Ac)	36	1.000 ± 0.045
$CH_2C_6H_4Br$-*p* (*N*-Ac)	37	0.075 ± 0.010
C_6H_4Br-*p*	38	0.068 ± 0.001
C_6H_3-2,4-$(NO_2)_2$	39	0.766 ± 0.067
C_6H_2-2,4,6-$(NO_2)_3$	40	Too weak to measure

50% inhibition concentrations for the *S*-alkylglutathiones (compds **1–8**) reveals that glyoxalase exhibits a significant hydrophobic region extending out from the *S*-alkyl chain of glutathione. The greatest amount of inhibition occurs as the alkyl group is lengthened from Me to Et to Pr. The large increase in binding of the *S*-Et derivative **2** with respect to the *S*-Me derivative **1** may be due to the Me group occupying a hydrophilic region normally occupied by the OH of the hemimercaptal. The addition of one more CH_2 unit may allow the alkyl chain of **2** to reach a hydrophobic region on the enzyme and enhance the binding within the enzyme–inhibitor complex. By taking advantage of the nonpolar character of glyoxalase, we have been able to increase binding by 430 times over that of *S*-methylglutathione by extending the chain to 8 carbons.

A series of 2,3- and 4-substituted *S*-benzylglutathiones is represented by **9–21** in Table I. Both electron-withdrawing and electron-donating substituents on the 4 position of the benzyl group increased binding over **9**. Only fair correlation was obtained for **9–16** in regression studies using π and σ constants and steric parameters, E_s.[9] These substituents may influence the conformation of the tripeptide structure which may account for the variation in binding to the enzyme. Substitution in the 3 position decreased activity by a factor of 2 to 3. For example, **17** binds 2.3 times less than **11**. Comparison of **20** and **21** to **13** and **15** indicates a significant reduction in binding of the ortho- over the para-substituted derivatives.

Hydrophobic bonding[10] with *S*-aralkylglutathiones was also investigated. The *S*-phenethyl derivative **22** complexed to about the same extent as the *S*-benzyl **9**. However, the phenylpropyl **23** gave a significant increase in binding over the benzyl derivative. The anomolous behavior of **24** relative to **23** and **25** is difficult to assess and may reflect conformational changes induced in the enzyme or inhibitor by the presence of these large hydrophobic groups. The placement of a *p*-NO_2 (**26**) on the phenethyl derivative did not significantly increase binding to the enzyme.

A series of substituted acetophenone derivatives (**27–33**) showed that hydrophobic electron-withdrawing para substituents (**30**, **31**) gave significant increase in binding while a hydrophilic electron-donating substituent did not contribute to binding (**29**) or even decreased activity (**28**) when compared with **27**. Hydrophobic contribution of the para substituent seems to be more significant than electron withdrawal, since Br and Cl both have σ 2.3 while π is 0.86 and 0.71, respectively. The greater binding of **31** over **30** must be a reflection of the hydrophobic character of Br. The propiophenone derivative **34** also gave better activity than **27**. N-acetylation of the glutathione derivatives (**35–37**) gave a five- to eightfold decrease in binding indicating that the free amine is not essential for activity, but does contribute to binding with the enzyme.

The *S*-phenyl derivatives (**38–40**) did not show any advantage over the *S*-benzyl compounds as far as binding to glyoxalase was concerned. However, they did exhibit interesting growth inhibitory activity on cell cultures.[5] All of the glyoxalase inhibitors are being screened for antitumor activity. Some preliminary results on cytotoxicity have been reported.[5]

Experimental Section[11]

Determination of K.—All absorbance measurements were made in 0.05 m*M* phosphate buffer at pH 6.6 and at 30°. The extinction coefficient of freshly prepared and standardized methylglyoxal soln was found to be 3.15 ± 0.12 M^{-1} cm^{-1} in the concn range of 10–50 m*M*. The extinction coefficient of GSH in the

(9) E. Kutter and C. Hansch, *J. Med. Chem.*, **12**, 647 (1969).

(10) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967.

(11) All enzymatic analyses were performed on a Gilford Model 2400 spectrophotometer. Melting points were determined on a Mel-Temp and are uncorrected. Elemental analyses for C, H, and N were obtained for all compounds in Table II and were within 0.4% of the theoretical value.

range of 1–10 mM varied considerably, and the appropriate ϵ_G for a particular concentration of GSH was obtained from the experimentally determined plot of ϵ_G *vs.* (G) (see Figure 1). A first approximation to the appropriate ϵ_G for a given mixt of methylglyoxal and GSH was taken to be that of a soln of concn (G_0). This estimate of ϵ_G allowed calcn of an approximate value of (GM), which in turn allowed a closer approximation of (G), and thus of ϵ_G. The refined value of ϵ_G was then used to calc a closer approximation to (GM) for this mixt. This procedure was repeated until no further change was made in the value of (GM) upon refinement of the value of ϵ_G, usually requiring only two recalcns.

The extinction coefficient, ϵ_{GM}, of the hemimercaptal adduct was calcd from absorbance measurements of mixts of methylglyoxal and GSH in which the ratio of (M_0) to (G_0) was at least 100:1. In such mixts, since the order of magnitude of K is $2\text{–}5 \times 10^{-3}$ M[6,7] it may be shown that (GM) $\cong$ (G_0). For this case, eq 3 may be written as $\epsilon_{GM} = [A_{240} - \epsilon_M(M_0) + \epsilon_M(G_0)]/(G_0)$, for ($M_0$) $\gg$ (G_0), and ϵ_{GM} calcd. The extinction coefficient of the hemimercaptal adduct calcd in this way was 391 ± 14 M^{-1} cm^{-1} for the concn range of 1–3 mM. Recently, Davis and Williams,[8] using reaction velocities at high enzyme concns, calcd 2 possible values of 395 or 198 M^{-1} cm^{-1} for the extinction coefficient of the hemimercaptal, depending upon whether glyoxalase reacts with both or only one of the enantiomorphs. Our value is in agreement with the mechanism involving reaction of both hemimercaptal enantiomorphs with the enzyme.

The value of K at 30° calcd with ϵ_M 3.15 M^{-1} cm^{-1}, ϵ_{GM} 395 M^{-1} cm^{-1}, and values of ϵ_G taken from Figure 1 by successive approximations was $(3.1 \pm 0.2) \times 10^{-3}$ M. All readings were taken from duplicate experiments of 16 different combinations of methylglyoxal and GSH concns.

Enzyme Assay.—A commercial 40% methylglyoxal solution was distd to remove polymerization products and dild with distd H_2O. The acidic materials were removed by passing the dild dist through Dowex 1-X8 (carbonate form) resin, and the soln was standardized by the method of Friedemann.[12] Glyoxalase I was obtained from Sigma Chemical Company and was dild to a concn of 20 μg/ml with 30% glycerol contg 0.1% bovine serum albumin. All enzymatic reactions were performed at 30° in 0.05 mM phosphate buffer at pH 6.6. A fresh GSH solution was prepared daily using distd H_2O. For each assay the cell contained a total vol of 3.0 ml which was 5.0 mM with respect to methylglyoxal and 0.87 mM with respect to GSH. Sufficient amounts of glyoxalase were employed to give an easily measurable initial rate which was followed by increase in absorption at 240 mμ. Methylglyoxal, GSH, inhibitor, and buffer were added to the cell and allowed to equilibrate at 30° for 6 min (to allow formation of hemimercaptal) before addition of the enzyme. In order to determine the concn of inhibitor required for 50% inhibition, a plot of V_0/V_i *vs.* (I) was made where V_0 = initial velocity of the uninhibited enzymatic reaction and V_i = initial velocity of the inhibited reaction at various inhibitor concns.

Syntheses.—The procedures for the preparation of the compounds listed in Table II are illustrated by the general methods below.

Method A.—Reduced glutathione (6.14 mg, 2.00 mmoles) was dissolved in H_2O (2.0 ml) and 2 N NaOH (2.0 ml, 4.0 mmoles) with stirring at room temp. EtOH (12–15 ml) was added to the cloud point. An equimolar amount of RX was then added, either all at once, or in portions over about 30 min if solubility problems were encountered. Additional EtOH was also added if solid appeared in the reaction mixt. Vigorous stirring was contd for about 3 hr after addn was complete. In many cases the reaction mixts were allowed to stir overnight without affecting the yield. Solid had often pptd at this point. The pH of the mixt was adjusted to 3.5 by dropwise addn of 47% HI, and the mixt was chilled. The solid was removed by filtration and washed with H_2O (20 ml). Anal. samples were prepd by two recrystns from H_2O–EtOH.

Method B.—For cases in which RX had very low solubility in aq EtOH, the method of Kermack and Matheson,[6] in which liq NH_3 is the solvent, was found to give better yields than method A.

(12) T. E. Friedmann, *J. Biol. Chem.*, **73**, 331 (1927).

TABLE II

S-SUBSTITUTED GLUTATHIONES[a]

GSH + RX ⟶ GSR

Compd	RX	Method	% yield	Mp, °C	Formula
9	$ClCH_2C_6H_5$	A	67	155–160	$C_{17}H_{23}N_3O_6S$
10	$ClCH_2C_6H_4OCH_3$-*p*	B[b]	70	192–193	$C_{18}H_{25}N_3O_7S$
11	$BrCH_2C_6H_4CH_3$-*p*	A	82	204–205	$C_{18}H_{25}N_3O_6S$
12	$ClCH_2C_6H_4F$-*p*	A	62	196–197	$C_{17}H_{22}N_3SF$
13	$ClCH_2C_6H_4Cl$-*p*	A	50	198–199	$C_{17}H_{22}N_3O_6SCl$
14	$BrCH_2C_6H_4Br$-*p*	A	74	206–207	$C_{17}H_{22}N_3O_6SBr$
15	$BrCH_2C_6H_4NO_2$-*p*	A	74	199–200	$C_{17}H_{22}N_4O_8S$
16	$BrCH_2C_6H_4CN$-*p*	A	38	198–199	$C_{18}H_{22}N_4O_6S$
17	$ClCH_2C_6H_4CH_3$-*m*	A	74	204–205	$C_{18}H_{25}N_3O_6S$
18	$ClCH_2C_6H_4Cl$-*m*	A	54	201–202	$C_{17}H_{22}N_3O_6SCl$
19	$BrCH_2C_6H_4NO_2$-*m*	A	73	202–203	$C_{17}H_{22}N_4O_8S$
20	$ClCH_2C_6H_4Cl$-*o*	A	32	205–206	$C_{17}H_{22}N_3O_6SCl$
21	$ClCH_2C_6H_4NO_2$-*o*	A	65	196–197	$C_{17}H_{22}N_4O_8S$
22	$Br(CH_2)_2C_6H_5$	B	87	199–200	$C_{18}H_{25}N_3O_6S$
23	$Br(CH_2)_3C_6H_5$	A[c]	38	200–201	$C_{19}H_{27}N_3O_6S$
24	$Br(CH_2)_4C_6H_5$	B[d]	69	203–204	$C_{20}H_{29}N_3O_6S$
25	$Br(CH_2)_5C_6H_5$	B[c]	53	204–205	$C_{21}H_{31}N_3O_6S$
26	$Br(CH_2)_2C_6H_4NO_2$-*p*	A	62	201–202	$C_{18}H_{24}N_4O_8S$
27	$ClCH_2COC_6H_5$	A	72	190–191	$C_{18}H_{23}N_3O_7S$
28	$ClCH_2COC_6H_4NH_2$-*p*	A	84	207–208	$C_{18}H_{24}N_4O_7S$
29	$ClCH_2COC_6H_4OH$-*p*	A	58	193–194	$C_{18}H_{23}N_3O_8S$
30	$ClCH_2COC_6H_4Cl$-*p*	A	81	184–185	$C_{18}H_{22}N_3O_7SCl$
31	$BrCH_2COC_6H_4Br$-*p*	A	52	191–192	$C_{18}H_{22}N_3O_7SBr$
32	$ClCH_2COC_6H_4NO_2$-*m*	A	44	194–195	$C_{18}H_{22}N_4O_8S$
33	$BrCH_2COC_6H_3$-3-NO_2-4-Br	C[e]	26	198–200	$C_{18}H_{21}N_4O_9SBr$
34	$Cl(CH_2)_2COC_6H_5$	A[f]	58	201–202	$C_{19}H_{25}N_3O_7S$
35	$Cl(CH_2)_5CH_3$(*N*-Ac)	A[g]	20	145–150	$C_{18}H_{31}N_3O_7S$
36	$Br(CH_2)_5CH_3$(*N*-Ac)	A	67	196–197	$C_{15}H_{25}N_3O_7S$
37	$BrCH_2C_6H_4Br$-*p*(*N*-Ac)	A	26	128–130	$C_{19}H_{24}N_3O_7SBr$
38	N_3-C_6H_4Br-*p*	D	26	213–214	$C_{16}H_{20}N_3O_6SBr$
39	ClC_6H_3-2,4-$(NO_2)_2$	C	94	194–195	$C_{16}H_{14}N_5O_{10}S$
40	ClC_6H_2-2,4,6-$(NO_2)_3$	C	89	198–199	$C_{16}H_{13}N_6O_{12}S$

[a] Prepn of the *S*-alkylglutathiones (**1–8**) has been previously reported [R. Vince and W. B. Wadd, *Biochem. Biophys. Res. Commun.*, **35**, 593 (1969)]. [b] 4-Methoxybenzyl chloride was prepd according to K. Rorig, J. D. Johnston, R. W. Hamilton, and T. J. Telinski, "Organic Syntheses," Collect. Vol. 4, Wiley, New York, N. Y., 1963, p 576. [c] RX not sol in H_2O–EtOH; additional EtOH added and vigorous shaking over 24 hr still left considerable RX undissolved. [d] Prepd by modification of procedure of S. Oae and C. A. Van der Werf, *J. Amer. Chem. Soc.*, **75**, 5037 (1953). [e] α-Chloroacetophenone was nitrated by the procedure of J. R. Catch, D. F. Elliott, D. H. Hey, and E. R. H. Jones, *J. Chem. Soc.*, 552 (1949). [f] α,*p*-Dibromoacetophenone was nitrated by the same procedure described in footnote *e*; mp 101–102°. [g] The S-substituted glutathione was acetylated.

Method C.—Method A was modified by using Na_2CO_3 (0.160 g, 1.00 mmole) instead of NaOH.

Method D. *S*-(*p*-Bromophenyl)glutathione (38).—*p*-Bromoaniline (0.602 g, 3.50 mmoles) was diazotized in 1 N H_2SO_4 (12.5 ml) and condensed with reduced glutathione (1.00 g, 3.25 mmoles) according to the procedure of Booth, *et al.*[13] The product was collected as a tan ppt (0.459 g) and recrystd from H_2O–EtOH; yield, 0.355 g (24%), mp 213–214°. *Anal.* ($C_{16}H_{20}BrN_3O_6S$) C, H, N.

Acetylation Procedure. *N*-Acetyl-*S*-(*p*-bromobenzyl)glutathione (37).—*S*-(*p*-bromobenzyl)glutathione (0.953 g, 2.00 mmoles) was dissolved in 10% NaOH (2.4 ml, 6.0 mmoles) and H_2O (1.6 ml). The soln was chilled, and Ac_2O (0.5 ml, 5.0 mmoles) was added and stirring was contd for 15 min. The mixt was adjusted to pH 2 with 2 N HCl, EtOH (10 ml) was added, and the soln was warmed (steam bath) for 5 min. The volatile materials were removed *in vacuo*, and the residue was extd with three 30-ml portions of hot EtOAc–EtOH (9:1). Addition of petr ether (bp 30–60°) to the combined extracts resulted in the formation of a gummy solid; yield, 0.788 g, mp *ca.* 130°. The crude material was crystd from EtOAc–petr ether and gave a white powder; yield, 0.271 g (26%), mp 128–130°. *Anal.* ($C_{19}H_{24}BrN_3O_7S$) C, H, N.

(13) J. Booth, E. Boyland, and P. Sims, *Biochem. J.*, **74**, 117 (1960).