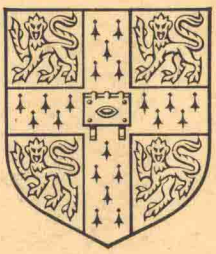
BIOCHEMICAL SOCIETY SYMPOSIA

No. 17

GLUTATHIONE



CAMBRIDGE
AT THE UNIVERSITY PRESS
1959

Price 15*s. net*

GLUTATHIONE

Biochemical Society Symposium No. 17
held at Senate House, University of London
on 15 February 1958

ORGANIZED AND EDITED
BY
E. M. CROOK

CAMBRIDGE
AT THE UNIVERSITY PRESS
1959

PUBLISHED BY
THE SYNDICS OF THE CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Printed in Great Britain at the University Press, Cambridge
(Brooke Crutchley, University Printer)

BIOCHEMICAL SOCIETY SYMPOSIA

NO. 17

GLUTATHIONE

CONTENTS

INTRODUCTION

By R. H. S. THOMPSON

Guy's Hospital Medical School, London, S.E. 1
Chairman of Morning Session

It is a great privilege to be asked to take the Chair at this morning's session of this Symposium, and I understand that it is my duty as Chairman to say a few words by way of giving a brief introduction to our subject.

My own interest in glutathione dates from just before the war when, in Sir Rudolph Peters's Laboratory at Oxford, we began to test glutathione and other simple mono- and di-thiols as possible antidotes against the vesicant and toxic actions of the arsenical war gases. This, of course, was only one narrow application of the biochemistry of this compound, and today we are going to hear about the extensive developments that have taken place in recent years in our knowledge of its chemistry and functioning.

I am not going to take up your time in recounting the early history of glutathione. Its isolation and crystallization from yeast by Hopkins in 1921 is well known, and I have no doubt that the other speakers today will be dealing, among other things, with the early work of the Cambridge school, which did so much to lay the foundations of our knowledge about glutathione.

The importance of glutathione and its implications in different fields of biological research are far-reaching, and, because of the diverse range of interests into which this compound has penetrated, a symposium on glutathione, such as this present meeting is concerned with, is particularly valuable.

The various fields of investigation that have at one time or another concerned themselves with the biological significance of this compound, and which indeed are still concerning themselves with it, make up an impressive list.

To give a few examples, first, in the field of enzymology, its role as an activator of glyoxalase and also, in the bound form, as a coenzyme of the triose phosphate dehydrogenase has been extensively studied, and the first of these functions, demonstrated as long ago as 1932 by Lohmann, has provided us with a specific method of assay of glutathione.

Another problem into which a study of glutathione and other thiol compounds has penetrated has been the question of the mechanisms underlying growth and cell division. In this connexion the early work of Hammett and of Rapkine deserves a special mention.

While speaking of Rapkine, I would like also to mention another

French scientist whose work in the field of sulphur biochemistry is well known, and that is the late Professor Fromageot, whose recent death members of the Society will have heard of with regret.

Again, in the field of toxicology, Voegtlin and his colleagues in America had early on suggested that the toxic action of the therapeutic organic arsenicals involved a reaction with thiol groups in living cells, a view first put forward by Ehrlich in 1909, and they had shown that their trypanocidal activity, for example, can be counteracted by the presence of glutathione or other simple thiol.

More recently, glutathione has been studied extensively in connexion with the problems of diabetes mellitus. It is of interest here to recall that, not only has this work concerned itself with alloxan diabetes, but also that Houssay and his colleagues, and others, have produced evidence indicating that the glutathione content of the tissues may be of importance in determining the rate of onset and the progression of diabetes in partially pancreatectomized animals. This work has naturally been extended to studies involving the adrenal cortex, although here perhaps in particular the results of experimental observations have been conflicting.

Then, as we shall hear this afternoon, glutathione has also figured prominently in the study of the biochemistry of the lens, and in particular of the processes concerned with cataract formation.

Turning to still more topical applications, glutathione and other sulphur compounds have been investigated in connexion with radiation injury. An interesting extension of this work has been the demonstration that simple monothiols can also protect against oxygen poisoning.

Lastly, and here perhaps we are looking more towards the future, work on the glutathione metabolism in the brain, and on the influence of mental disease on tissue levels of this compound, is now going ahead. Of particular interest in this connexion is the recent claim that the injection of extracts of the pineal gland causes a significant rise in the blood-glutathione level in psychotic patients.

This morning Dr Isherwood is going to open the Symposium with an account of some aspects of the biochemistry and biosynthesis of glutathione. Dr Thomson and Miss Martin are then going to speak about the techniques for its determination. I am particularly glad that we are to have a paper dealing with this, since much of the earlier work, and also some of the more recent work, on the levels of glutathione in blood and other tissues is rendered difficult to interpret owing to the lack of specificity of the methods used. Next, Dr Mapson will deal with the enzyme systems concerned with the oxidation and reduction of glutathione in plant tissues, and Dr Jocelyn with its metabolism in animal tissues, while this afternoon we shall turn to some of the applications that I have briefly mentioned.

CHEMISTRY AND BIOCHEMISTRY OF GLUTATHIONE

By F. A. ISHERWOOD

Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

The generally accepted formula for glutathione is γ-glutamylcysteinylglycine. This is shown in the straight-chain form in Fig. 1.

Glutathione in a crude form was first isolated by De Rey-Pailhade (1888) from yeast, but Hopkins (1921) was the first to obtain a crystalline preparation. The exact formula was suggested in 1929 by Pirie & Pinhey (1929) based on a physicochemical study of the tripeptide. Electrometric titration gave four different pK_a values which were correlated with –SH, $-NH_3^+$ and two $-CO_2H$ groups.

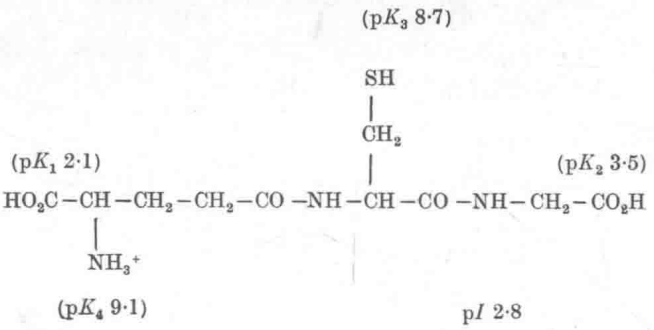


Fig. 1. Formula for glutathione.

The figures actually given in Fig. 1 against the appropriate groups are based on those reported by Cohn & Edsall (1943) with an inversion of the figures for the $-NH_3^+$ group and the –SH group. Earlier workers suggested that the $-NH_3^+$ group was more acidic than the –SH group, but recently doubt has been cast on this assumption. Study of simple mercaptans indicates that the –SH group has a pK_a of about 10, but that in the presence of a β-amino group (β-mercaptoethylamine) it is strengthened by the presence of a positive charge in the β position, so that its pK_a value is about 8·6, whereas the effect of the negative charge on the sulphur atom raises the pK_a of the $-NH_3^+$ group to 10·75 (an ordinary alkylamine is about 10). In compounds such as glutathione the greater distance between the –SH and $-NH_3^+$ groups

diminishes their effect on each other and the distinction between them will be less marked. In Table 1 pK_a values are given for a number of compounds which have similarities to glutathione.

Comparison of the pK_a values of the γ-ethyl ester of glutamic acid

Table 1. *Acid dissociation constants of compounds analogous to glutathione*

Compound	$-\log K_a$ (*Note* 1)	$-\log K_a$ (*Note* 2)
$HO—CH_2—CH_2—SH$	9·5	—
$CH_2—SH$	8·6	8·35
$CH_2—NH_3^+$	10·75	—
COO^-	4·3	—
$[CH_2]_2$	—	—
$HC—NH_3^+$	9·96	—
COO^-	2·16	—
$CO—NH_2$	—	—
$[CH_2]_2$	—	—
$HC—NH_3^+$	9·13	—
COO^-	2·17	—
$COOC_2H_5$	—	—
$[CH_2]_2$	—	—
$HC—NH_3^+$	9·19	—
COO^-	2·15	—
$CO—NH—CH_2—COO^-$	3·5	—
$CH—CH_2—SH$	8·7	9·2
NH		
CO		
$[CH_2]_2$		
$HC—NH_3^+$	9·12	
COO^-	2·12	

Note 1: Calvin (1954); Cohn & Edsall (1943). E.m.f. measurements on cells with liquid junction at 25°.

Note 2: Benesch & Benesch (1955). Ultraviolet absorption of mercaptide ions at 23°.

and of glutamine with glutathione suggests that the $-NH_3^+$ group is slightly less acidic than the –SH group and that the correct assignment is as shown in Fig. 1. The difference in the pK_a values is very small and it is probable that the proton affinities of the sulphur and nitrogen groups in glutathione are about equal. This is confirmed by the ultra-

violet-absorption measurements of Benesch & Benesch (1955). They found that the absorption of the mercaptide ion in amino thiols shifts from about 236 to 230 mμ with decreasing pH and that the shift was due to the fact that the $^{-}S \cdot R \cdot NH_3^{+}$ form has a maximum absorption at somewhat shorter wavelengths than the $^{-}S \cdot R \cdot NH_2$ form. Using this observation they determined the proton affinities of the sulphur and nitrogen functions in glutathione and several other amino thiols. They confirmed that the –SH group in β-mercaptoethylamine was more acidic than the $-NH_3^{+}$, but that in glutathione the contributions of the –SH group and the $-NH_3^{+}$ group to the ionization of each proton were about equal.

The assignment of a lower pK_a for the –SH group is important in any reaction occurring at a physiological pH which is known to proceed through the mercaptide ion, e.g. the reduction of disulphide bonds by –SH groups (Calvin, 1954).

ISOLATION AND OCCURRENCE

Glutathione is found in all living cells. Some figures for the amounts present in different tissues are given in Table 2 (Waelsch, 1952).

Table 2. *Concentration of glutathione in various tissues (glyoxalase method)*

Material	GSH(mg./100 g. fresh wt.)
Rat	
Blood plasma	0
Whole blood	60
Liver	175, 177, 164
Spleen	76, 118
Heart	67, 64
Muscle (leg)	26
Brain	32
Yeast	128
Potato	10·8
Apple	0
Corn sprouts	12·6
Soaked peas	50–70

Most of these figures were measured by the glyoxalase method. Glutathione is a specific activator of the enzyme glyoxalase, which converts methylglyoxal into lactic acid, and, under conditions in which the glutathione is the limiting factor, the rate of conversion is determined by the glutathione.

Yeast is generally used for the isolation of glutathione and the method of isolation used by Hopkins (1929) is shown briefly in Fig. 2.

SYNTHESIS

Five methods have been published (Harington & Mead, 1935; du Vigneaud & Miller, 1936; Hegedüs, 1948; Rudinger & Šorm, 1951; and Goldschmidt & Jutz, 1953). These differ mainly in the way the various reactive groups have been 'protected' during the formation of peptide bonds and in the chemical reactions used to form the peptide bonds. A simplified summary is given in Fig. 3.

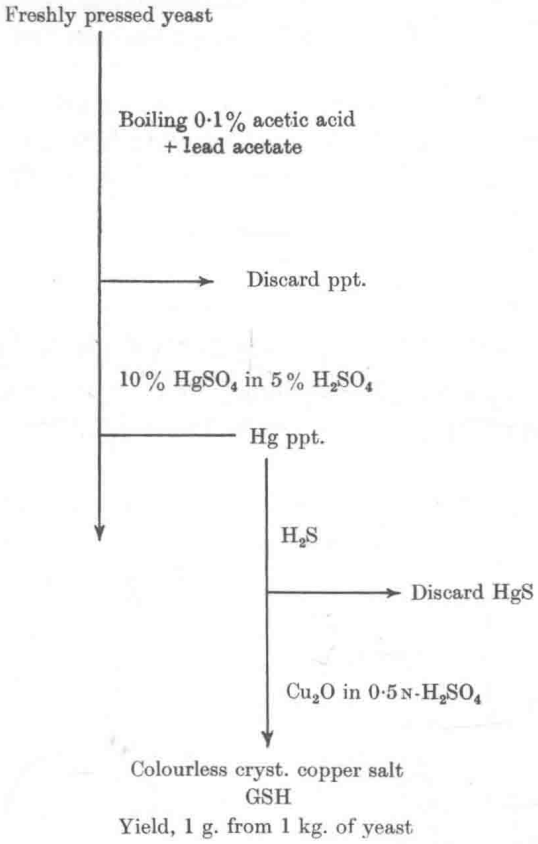


Fig. 2. Isolation of glutathione from yeast (Hopkins, 1929).

To effect the combination of the various amino acids in the desired order, all possible reactive groups, other than the ones designated for the reaction, must be 'protected' by chemical groups which can be easily removed at the end of the synthesis without disturbing the peptide bonds. Those shown in Fig. 3 can be removed either by gentle alkaline hydrolysis (ester) or by hydrogenation with sodium in liquid ammonia or phosphonium iodide (benzyloxycarbonyl, –S-benzyl, and –S–S–). Various methods of forming the peptide bond are shown; the

acyl chloride method was used by Harington & Mead in the first synthesis of glutathione.

The route taken in these syntheses contrasts with that for the biological synthesis (Snoke & Bloch, 1952) which proceeds:

Glutamic acid + cysteine → γ-glutamylcysteine; γ-glutamylcysteine + glycine → glutathione.

Route

Glycine + cysteine ⟶ cysteinylglycine

Cysteinylglycine + glutamic acid ⟶ γ-Glutamylcysteinylglycine

Protection of SH group	$-S-CH_2-C_6H_5$
SH group	$-S-S-$
CO_2H group	$-CO-O-CH_2-CH_3$
NH_2 group	$-NH-CO-O-CH_2-C_6H_5$

Reaction to give peptide link

$-CO_2H + -N{=}P-NH-$

$-CO_2-N_3 + -NH_2$

$-COCl + -NH_2$

$-HC(-NH)-CO + -NH_2$ (OC–O)

→ $-NH-CO-$

Fig. 3. Chemical synthesis of glutathione.

PHYSICAL PROPERTIES

Colourless crystalline compound, m.p. 195°, $[\alpha]^{17} - 21°$; disulphide, $[\alpha]^{20} - 108$ in water. Soluble in water, liquid ammonia and dimethylformamide.

CHEMICAL PROPERTIES

These can be considered under four headings:

(1) *α-Aminocarboxylic acid.* Evolution of 1 mole of CO_2 and of NH_3 takes place on treatment with ninhydrin. The tripeptide reacts acid; isoelectric point pI, 2·83.

(2) *Peptide nature.* Glutathione gives a positive biuret reaction and, of course, can be hydrolysed to its constituent amino acids.

(3) *Glutathione as γ-glutamyl peptide.* Both the –SH compound and the disulphide can be easily hydrolysed. Thus the linkage between the glutamic acid and the cysteine is broken by heating in water at 62° within 5 days or in 1·2 N-HCl at 94° for 1 hr. The moving force behind

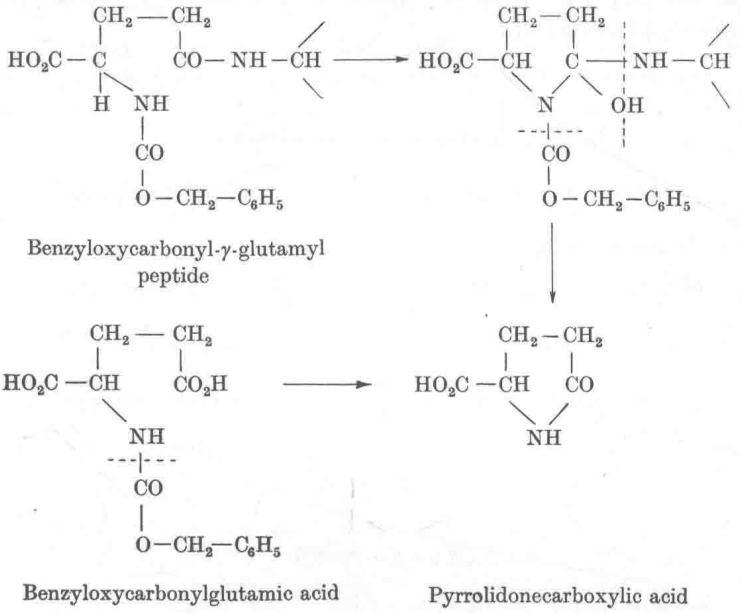
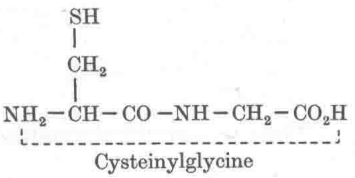


Fig. 4. Autohydrolysis of γ-glutamyl peptides.



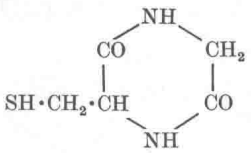


Fig. 5.

this hydrolysis is the tendency of the glutamyl residue to cyclize in order to form pyrrolidonecarboxylic acid. This compound is the main product of the process of autohydrolysis of γ-glutamyl peptides and glutamine. In Fig. 4 the process is illustrated for the *N*-benzyloxycarbonyl derivatives, which also suffer hydrolysis to give pyrrolidone-

carboxylic acid. Even the free benzyloxycarbonylglutamic acid gives rise to pyrrolidonecarboxylic acid under the same conditions. The benzyloxycarbonylpyrrolidonecarboxylic acid readily loses the benzyloxycarbonyl residue (Wessely, Schlögl & Wawersich, 1953).

The other product of glutatione autohydrolysis, cysteinylglycine, undergoes cyclization either when heated or on standing at room temperature, and the diketopiperazine is formed (Fig. 5). The disulphide of this is crystalline.

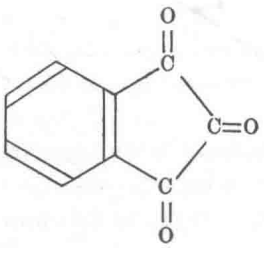
(4) *Reaction of the* –SH *group*. Thiols are very reactive substances. They can be readily oxidized, form compounds with heavy metals, form –S-alkyl derivatives, react with carbonyl compounds and form –S-acyl derivatives and the sulphur can be removed by boiling with alkali. These will be considered separately.

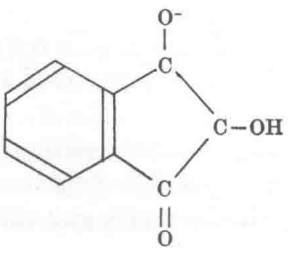
OXIDATION

A list of representative oxidizing agents is given in Table 3 in a simplified form.

Table 3. *Oxidation of glutathione with various compounds*

Reagent	Products		Comments
	1	2	
I_2	GSSG		Stoicheiometric
$IO \cdot C_6H_4 \cdot CO_2H$	GSSG	$I \cdot C_6H_4 \cdot CO_2H$	Stoicheiometric
O_2	GSSG		Very slow reaction
O_2 + Cu or Fe	GSSG		Fast reaction inhibited by HCN
$O_2 + HS \cdot R$	GSSR		Mixed disulphides
S + alcohol	GSSG	H_2S	H_2S converted into ZnS for analysis
$Fe(CN)_6^{3-}$	GSSG	$Fe(CN)_6^{4-}$	Stoicheiometric
H_2O_2 + Cu pH 2–5	GSSG		
H_2O_2 + Mo or V	G-SO_3H		
Alloxan	Uncertain		Absorbs at 305 mμ Used for analysis





Red enolate of dihydroninhydrin

Oxidation of glutathione is usually accomplished by iodine in the presence of a large amount of acetic acid (Kuhn, Birkofer & Quackenbush, 1939). *o*-Iodosobenzoic acid also reacts in neutral solution and ferricyanide is reduced to ferrocyanide at pH 6. These methods have been used to form the basis of quantitative methods for the analysis of glutathione.

Oxygen alone reacts slowly with metal-free glutathione, but in the presence of traces of Cu and Fe the reaction is greatly accelerated. Cyanide inhibits this heavy-metal catalysis.

Hydrogen peroxide oxidizes glutathione, and heavy metals again act as catalysts. In the presence of V or Mo salts the oxidation leads to the sulphonic acid.

Alloxan behaves both as an oxidizing agent and also couples with the glutathione. The product has a characteristic absorption at 305 mμ and is used for the analysis of glutathione.

Ninhydrin is readily reduced in weakly alkaline solution to the red enolate of dihydroninhydrin.

If glutathione is oxidized in the presence of other thiols, mixed disulphides are formed. This can complicate the isolation of coenzymes containing sulphydryl groups such as coenzyme A.

The disulphide of glutathione can be reduced by various agents such as H_2S, other mercaptans in excess, nascent hydrogen and Na or Li in liquid ammonia.

MERCAPTIDES

Glutathione forms compounds with many heavy metals. A list of some is given in Table 4.

The first two, Cu_2O in 5N-H_2SO_4 and $HgSO_4$ in N-H_2SO_4, have already been mentioned in connexion with the isolation of glutathione from yeast. The copper salt is preferred for the final isolation because the mercury salt contains sulphate, which is liberated as H_2SO_4 on decomposition with H_2S (Voegtlin, Johnson & Rosenthal, 1931).

Silver nitrate in neutral solution (tris being used as 'complexing' reagent for Ag^+ ions) gives an insoluble compound. The reaction forms the basis of an amperometric-titration method for glutathione (Benesch, Lardy & Benesch, 1955).

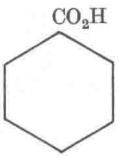
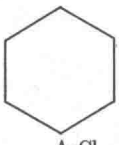
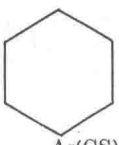
p-Chloromercuribenzoic acid couples with the –SH group in a stoicheiometric manner. The reaction can be reversed by the addition of other thiols.

Non-metals can also combine with the –SH groups, but the compounds are mostly unstable and are hydrolysed in aqueous solution.

Lewisite (the war gas) combines with glutathione, but the compound

formed is easily hydrolysed. 1:2-Dithiols such as British anti-lewisite (BAL) form a ring compound which is very much more stable (Stocken & Thompson, 1946).

Table 4. *Mercaptides of glutathione*

Reagent	Products	Comments
Cu_2O in 0·5N-H_2SO_4	Cu(GS)	Insoluble
$HgSO_4$ in N-H_2SO_4	(GS)HgOH,$HgSO_4$	Insoluble
$AgNO_3$	Ag(GS)	Insoluble (amperometric) Analytical method
		Stoicheiometric
HgCl	Hg(GS)	
As_2O_3 in CH_3·OH	$As(GS)_3$	Relatively unstable (cf. 1:2-dithiols)
		
$AsCl_2$	$As(GS)_2$	
$CHCl:CH·AsCl_2$ (lewisite)	$(GS)_2As·CH:CHCl$	Precipitated by ethanol from aqueous solution. Decomposed by alkali to GSSG and Se
H_2SeO_3	$Se(GS)_4$	

FORMATION OF *S*-ALKYL AND ACYL GROUPS

A representative list of these is shown in Table 5.

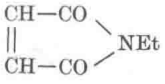
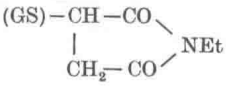
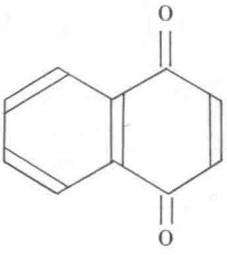
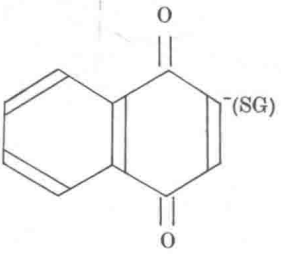
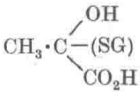
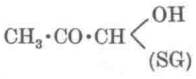
The inhibiting effect of iodoacetate on a number of enzymes is the result of an alkylation taking place at the –SH groups. The reaction in the case of glutathione is analogous. Other compounds of the same type are iodoacetamide, iodoethanol, chloroacetophenone, chloroacetone and mustard gas, and they all form appropriate alkyl derivatives.

A different type of reaction occurs with $\alpha\beta$-unsaturated compounds such as maleic acid. The –SH group adds on to the unsaturated bond to give an alkyl derivative (Friedmann, Marrian & Simon-Reuss, 1949). *N*-Ethylmaleimide (Hanes, Hird & Isherwood, 1951) is an important example of this type of compound because it forms stable compounds

with glutathione and other thiols which can be used on paper chromatograms.

The addition compound isolated from the reaction with naphthaquinone is unusual, but is the result of the intermediate compound being oxidized by the excess of quinone present initially (Friedmann, Marrian & Simon-Reuss, 1948).

Table 5. *Alkyl and acyl compounds of glutathione*

Reagent	Product	Comments
$CH_2I \cdot CO_2H$	$(GS)CH_2 \cdot CO_2H$	Enzyme inhibitor
$CH_2Cl \cdot CO \cdot Ph$	$(GS)CH_2 \cdot CO \cdot Ph$	
$CH_2Cl \cdot COCH_3$	$(GS)CH_2 \cdot CO \cdot CH_3$	
$(CH_2Cl \cdot CH_2)_2S$	$[(GS)CH_2 \cdot CH_2]_2S$	
		Mitotic inhibitor
		Mitotic inhibitor
$CH_3 \cdot CO \cdot CO_2H$		Reversible
$CH_3 \cdot CO \cdot CHO$		
$(CH_3 \cdot CO)_2O$	$(GS)CO \cdot CH_3$	
$Ph \cdot S-Acyl$ + pyridine	(GS)Acyl	

All these compounds are mitotic inhibitors, presumably because they react with –SH groups on the proteins.

Another type of reaction occurs with carbonyl compounds: semimercaptals are formed. They are relatively unstable compounds, being easily hydrolysed. The compound with methylglyoxal is of considerable interest because there is some uncertainty whether the glutathione is attached to the aldehyde group or to the keto group. In Table 1, I have represented it in the usual hemimercaptal form.