STUDIORUM PROGRESSUS

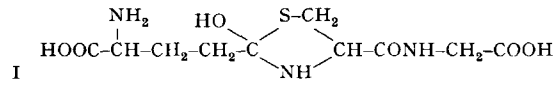
The Oxidation of Glutathione with Formic Acid and Hydrogen Peroxide

There is no certain knowledge of the steps in the metabolism (biological) of peptide-bound cysteine. It has been assumed for example that cysteine sulfenic acid, CySOH, could represent a very labile intermediate oxidation state[1-3] which dismutates rapidly to the lower and higher states, cysteine and cysteine sulfinic acid respectively. (Although cysteine sulfenic acid has not been isolated, the biological oxidation of cysteine to taurine has to pass through an oxidation state equivalent to that of the sulfenic acid.) Recently an investigation by one of us[4] of the *in vitro* oxidation of cystine has indeed led to the isolation of several cystine sulfoxides which were also[5] hydrolyzable to cysteine sulfinic acid. However, all of these cystine sulfoxides have very low solubilities and accordingly their participation in a metabolic pathway seems quite unlikely.

The extensive literature on cysteine metabolism[6-8] seems to imply that knowledge of cysteine metabolism is tantamount to knowledge of the biological metabolism of peptide-bound cysteine as it occurs in enzymic reactions. However, attention is called to the improbability of the occurrence of free cysteine in biological systems and the fact that peptide-bound cysteine is more nearly glutathione-like.

With this thought in mind the present work was initiated – the examination of the *in vitro* oxidation of a simple cysteine-containing peptide, glutathione. By analogy to the cysteine sulfenic acid[4], a glutathione sulfoxide, if it were to exist, would be important as a significant intermediate oxidation state between the lower thiol on the one hand and the higher sulfinic acid state on the other.

It has long been known, that in a mixture of cysteine and glutathione the cysteine is preferentially oxidized[9]. This preference is not explicable by the difference in the redox potentials. An explanation has been sought in the direction of various possible associated structures of glutathione such as have been postulated and summarized by ISHERWOOD[10]. For example, in the covalently associated thiazolidine structure, I, there is no free SH group available for the direct oxidation to the disulfide.



I

The mechanism of association, especially covalent association as postulated in glutathione, I, should be equally relevant to all cysteine-containing peptides and proteins, as long as the cysteine amino group is present as an amide group. Accordingly, *in vitro* oxidation of glutathione presents a more useful model than cysteine with which to assess the biological metabolism of peptide-bound cysteine. We wish to report here experimental evidence that such covalent structures indeed exist. This may be shown by comparing appropriate physical and chemical properties of the following commercial compounds: Glutathione (reagent grade), 'oxidized' glutathione (purportedly glutathione disulfide) and S-acetyl glutathione.

In addition some new derivatives have been prepared and isolated: Glutathione dihydrosulfoxide, glutathione sulfoxide, N-acetyl-glutathione and a compound which is identified as an intra- and intermolecularly associated glutathione.

The reduction of the sulfoxide in a desalter and the behavior against 2,6-dichlorophenolindophenol and the nitroprusside reaction have helped to establish the proof of the identity and property of the new compounds.

The IR-spectra (Figure) of the compounds have been particularly helpful in confirmation of the new compounds not previously isolated such as the glutathione dihydrosulfoxide containing the characteristic 9.6 μ sulfoxide band. The IR-spectra have also been useful in distinguishing the existence of the subtle covalent association postulated by ISHERWOOD[10] of the type I by the presence or disappearance of the sharp and unequivocal S–H band near 4 μ and the concomitant appearance of the C–S linkage indicated by the appearance of the C–S band near 15 μ 11)[11].

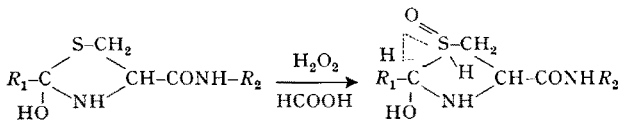
Glutathione dihydrosulfoxide II. Compound II was obtained by oxidation of the reduced form of glutathione (0.60 g, 2 mMoles) in 0.5 ml of 30% H_2O_2 in 20 ml of 98% formic acid. It was isolated under essentially non-aqueous conditions by concentrating the formic acid solution in a 1 mm Hg vacuum to a small volume. N, N-dimethylformamide was then repeatedly added (10 ml at a time) and distilled off again until the solution turned turbid, whereupon it crystallized rapidly. Once crystallized it was no longer soluble in N, N-dimethylformamide. The dihydrosulfoxide was soluble in alcohol and water, in both of which it deteriorated within 10 min. The pink color given by the reduced glutathione in the nitroprusside reaction could not be obtained during that time. Neither could II be reduced rapidly enough to give the thiol form.

In addition to the method of synthesis, an IR band at 9.6 μ which is characteristic of sulfoxides[12], including methionine sulfoxide, identifies the presence of the sulfoxide moiety (Figure a). The compound gave a faint violet spot on paper with nitroprusside as does methionine sulfoxide. However, the dihydrosulfoxide reduced dichlorophenol-indophenol surprisingly rapidly in the cold within a few minutes. Also combustion analysis repeatedly indicated two more hydrogens than anticipated[13]. A hydrate of glutathione is ruled out by appearance of the sulfoxide and modification of the SH band. The titration curve lacks the equivalent for the thiol group and shows instead an equivalent with a pK near 3.8 not present in glutathione.

A conventional structure compatible with all of the above described properties of this dihydrosulfoxide appears to present some difficulties. As stated, it cannot be simply a hydrate of glutathione. It must be concluded

[1] N. W. PIRIE, Biochem. J. *28*, 305 (1934); *27*, 1181 (1933).
[2] G. MEDES and N. FLOYD, Biochem. J. *36*, 359 (1942).
[3] C. FROMAGEOT, F. CHATAGNER, and B. BERGERET, Biochim. biophys. Acta *2*, 294 (1948).
[4] G. E. UTZINGER, Exper. *17*, 374 (1961).
[5] M. EMILIOZZI and L. PICHAT, Bull. Soc. Chim. France *1959/2*, 1887.
[6] L. YOUNG and G. A. MAW, *The Metabolism of Sulfur Compounds* (Methuen & Co., London, and John Wiley & Sons, Inc., New York 1958), p. 29.
[7] N. KHARASCH, *Organic Sulfur Compounds* (Pergamon Press, London 1961), vol. I, p. 97 and p. 399.
[8] Colloque sur la Biochimie du Soufre (Editions du Centre National de la Recherche Scientifique, Paris 1956).
[9] D. B. HOPE, in E. M. CROOK, *Glutathione*, Biochem. Soc. Symposia, No. *17* (Cambridge Univ. Press 1959), p. 97.
[10] F. A. ISHERWOOD, *Chemistry and Biochemistry of Glutathione*, in [9], p. 13.
[11] The UV-spectra of the compounds and their ninhydrin reaction products were measured in pressed KBr discs similarly as for the IR-procedure because these forms were not stable in customary solvents.
[12] L. J. BELLAMY, *The Infrared Spectra of Organo Sulfur Compounds*, in [7], vol. I, p. 53.
[13] GLDHSO II $C_{10}H_{19}N_3O_7S$ (325.34) m.p. 165–168°, calc. C 37.26, H 5.89, N 12.93%; found C 37.47 37.52 37.68, H 5.64 5.84 5.73, N 13.18% (Kjeldahl).

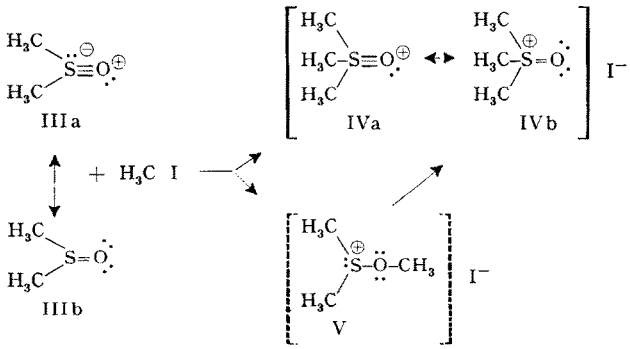
that the unexpected additional two hydrogens are associated with the sulfur or sulfoxide function. This can be the case if the *d* orbitals of sulfur are involved and, through an expanded valence sulfur shell, give a stable *d*-hydridized sulfoxide. Thus it is conceivable that, in addition to the sulfoxide oxygen, two hydrogens attach to give a hexavalent sulfur in the oxidation of glutathione as a covalent thiazolidine structure I, yielding:



R_1CO = γ-glutamyl $R_2\overset{/}{N}H$ = N-substituted glycine

There is a growing literature on the subtle bonding and chemical properties of sulfur arising from the ready electron promotion to the available third orbitals with a rearrangement of charge distribution accompanying the ensuing expanded sulfur outer shell[14]. The hexavalent structure, III, could arise from approximately sp^3d^2-hydrization leading to octahedral or distorted octahedral geometry as regards orbital directions[15].

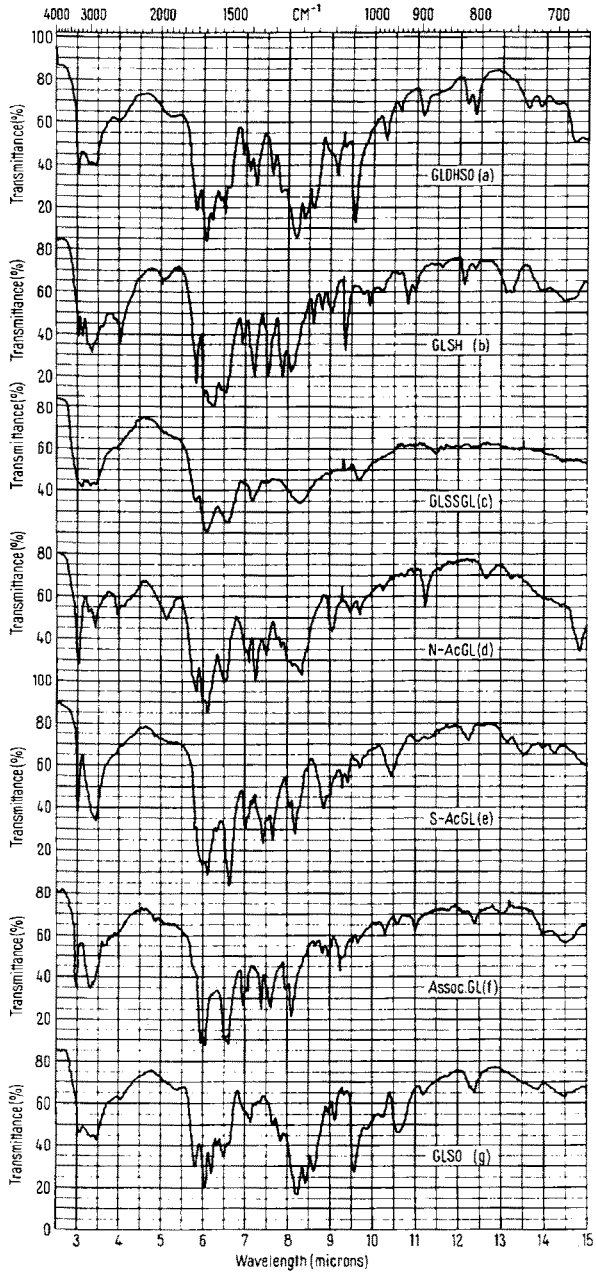
Recent structural formulae advanced to explain the unexpected behavior of certain sulfoxides include resonance contributions which effect a depletion of electronic charge on the oxygen competing with a resonance structure in which sulfur loses electronic charge to assume a positive charge. Thus MAJOR and HESS[16] obtained from dimethyl sulfoxide, III, and methyl iodide a trimethyl sulfoxonium iodide, IV, rather than the anticipated S, S-dimethyl-S-methoxy sulfonium iodide, V:



This reaction, which was corroborated by KUHN and TRISCHMANN[17] and by SMITH and WINSTEIN[18] supports the necessity for structural contributions with positive charge on the oxygen relative to the sulfur such as IIIa and IVa. Such structures may significantly contribute because the associated approximately octahedral geometry arising from the sulfur shell expansion permits use of the unhybridized *p*-orbitals of oxygen with little resulting strain in the S-O bond, and the increased electronegativity of the neighboring atom relative to the sulfur is known to stabilize *d*-hybridization structure[14,15].

It is attractive to postulate for this relatively unstable glutathione dihydrosulfoxide that analogous duodecet structures make significant contributions to II.

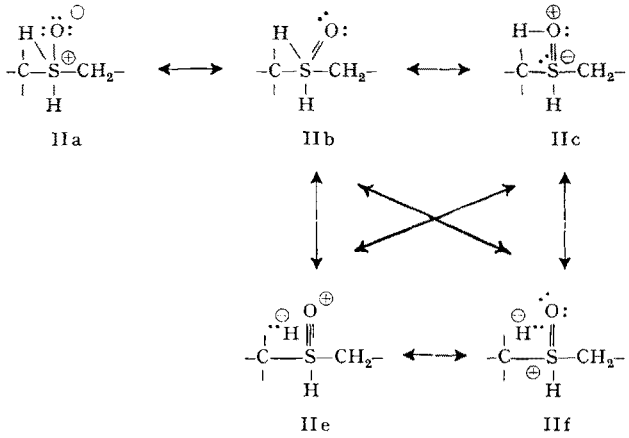
The no-bond resonance structures imply that the hydrogen is alternately involved in a sulfur sp^3d^2 orbital and a *p*-orbital on the oxygen. Octahedral orbital geometry requires but little if any distortion for close approach of these orbitals. It may also be considered that these are a series of structures equivalent to a hydrogen bridge arising from the overlap of the hydrogen, the sulfur sp^3d^2, and one lobe of an oxygen *p*-orbital. A second hydrogen could occupy a similar position at the opposite apex of the sulfur octahedron. However, it cannot be equivalent with respect to the oxygen because of the relative phase of the orbitals. The hybridization requires that the two opposing



IR-spectra of glutathione dihydrosulfoxide GLDHSO (a), reduced glutathione GLSH (b), glutathione disulfide (commercial 'oxidized') GLSSGL (c), N-acetyl glutathione N-AcGL (d), S-acetyl glutathione S-AcGL (e), associated glutathione Assoc. GL (f) and glutathione sulfoxide GLSO (g), in KBr 1:400 measured on a Perkin Elmer Spectrophotometer, Model 21. The spectral measurements done by M. K. HRENOFF are greatly appreciated. NMR-spectra and additional experimental data will be published in the J. Amer. chem. Soc.

[14] G. CILENTO, Chem. Rev. *60*, 147 (1960).
[15] H. EYRING, J. WALTER, and G. E. KIMBALL, *Quantum Chemistry* (J. Wiley & Sons Inc., New York-London 1960), p. 230.
[16] R. T. MAJOR and H. J. HESS, J. org. Chem. *23*, 1563 (1958).
[17] R. KUHN and H. TRISCHMANN, Liebigs Ann. *611*, 117 (1958).
[18] S. G. SMITH and S. WINSTEIN, Tetrahedron *3*, 317 (1958).

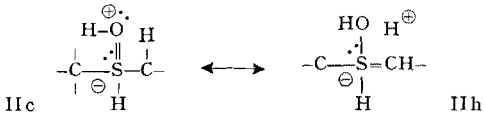
sp^3d^2 orbitals be of identical phase. The oxygen orbital involved, however, is a single *p*-orbital whose lobes are of opposite phase, with the result that the no-bond resonance form is permitted only for one hydrogen at a time.



IIa IIb IIc

IIe IIf

Further stabilization may be anticipated by hyperconjugation of the kind described by McDANIEL[19] involving the methylene group adjoining the sulfoxide. II h is one of the ten possible resonance forms of this kind.



IIc IIh

These particular no-bond resonance forms are additionally of importance because they offer an explanation of the reactivity of the α-hydrogen and carbanion.

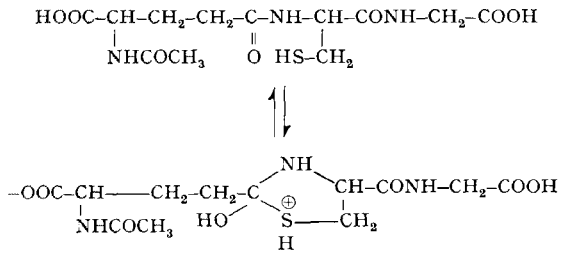
The *d*-hybridized structures are compatible with the observed properties and behavior of the dihydro sulfoxide, especially the reducing property associated with the involvement of a hydride ion resonance contribution. Further, the characteristics of such a structure leads, as described later, to some interesting speculations about certain biological electron transfer reactions.

'Oxidized' glutathione. This term has been applied, conventionally, in the literature to the disulfide oxidation state (inferring the absence of other oxidation states).

It is not surprising that commercial 'oxidized' glutathione is not a single compound but contains as by-products at least other stages of oxidation such as the sulfoxide. The IR-spectrum of commercial 'oxidized' glutathione bears this out (Figure c). It may be seen to have a diffuse spectrum in the fingerprint region in contrast with sharp spectra of glutathione and its other derivatives and traces of the sulfoxide band at 9.6 μ are clearly evident.

N-Acetyl-glutathione V. Although S-acetyl-glutathione has been described by WIELAND and BOKELMANN[20], N-monoacetyl-glutathione, to our knowledge has not yet been reported. It has now been obtained by acetylation of glutathione in 90% aqueous formic acid solution with acetic anhydride. The formic acid was removed from the reaction mixture by vacuum distillation, while N,N-dimethylformamide was repeatedly added in 5 ml portions until the odor of formic acid was no longer perceptible in the reaction mixture. The viscous residue was finally diluted with *n*-butyl acetate until it turned turbid and then it was allowed to stand and crystallize. As anticipated the N-acetyl derivative is readily soluble in methanol, ethanol and water. It is soluble in N,N-dimethylformamide but insoluble in *n*-butyl acetate. It reacts immediately with potassium nitroprusside in alkaline ammonia solution. It reduces dichlorophenolindophenol in the cold immediately. The IR-spectrum shows both a free SH group (near 4 mμ) and a significant peak characteristic of a sulfonium group (near 5 μ). Zwitterion formation is likely to occur between the carboxyl and a thioether as in the thetins, if the NH_2 group is absent or blocked by acetylation. The IR-spectrum shows no congruence with that of S-acetyl glutathione at the wave lengths pertinent to the sulfur function. Although the compound is cleanly crystallized and the spectrum very sharp, it shows with slow heating a double melting point 74°/93° with interconversion between the melting points.

Some of this seemingly contradictory behavior may be explained by the following equilibrium:



The relatively unique band at 5 μ may be attributed to the sulfonium ion. (It appears in the same region for ammonium or oxonium salts.) The new peak, which appears at 675 cm^{-1} (near 15 μ) may be taken as supportive evidence of existence of the well known C–S stretching frequency[21] and agrees with the formation of the covalent C–S bond between thiol and amide carbonyl. The extinction of the thiol peak at 4.0 μ is reduced in comparison to the sulfonium peak indicating that the compound is to a large degree in the sulfonium zwitterion form.

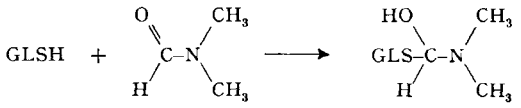
In acyl ethanolamines and acyl propanolamines as well as in the corresponding acyl mercaptoalkylamines the acyl group is usually intramolecularly transferable from S or O to N, the N derivatives being the more stable ones at neutral pH[22-24].

Associated glutathiones. We have isolated several associated glutathiones (intra- and intermolecularly) from formic acid solution with almost identical IR-spectrum, but whose melting points range from 150–190°. The characteristic IR-spectrum of these compounds (Figure f) is very little different one from the other but are clearly distinguished from that of the IR-spectra of the known glutathione derivatives. The SH peak is absent and the amide bands are markedly modified. In spite of the absence of the SH peak these products gave an instant nitroprusside reaction when dissolved in water. This is taken to mean that any covalent association across an amide carbonyl group is readily reversed on addition of water.

These intermolecularly associated glutathiones are interestingly found to be insoluble in cold N,N-dimethylformamide. This may indicate that the solubility in N,N-dimethylformamide of glutathione (the solution converts

[19] D. H. McDANIEL, Science *125*, 545 (1957).
[20] T. WIELAND and E. BOKELMANN, Angew. Chem. *64*, 59 (1952).
[21] L. J. BELLAMY in [7], p. 47.
[22] H. BRETSCHNEIDER, K. BIERMANN, W. KOLLER, and W. SACHSENMAIER, Monatsh. *81*, 31 (1950).
[23] T. WIELAND, E. BOKELMANN, L. BAUER, H. U. LANG, and H. LAU, Liebigs Ann. *583*, 129 (1953).
[24] T. WIELAND, H. U. LANG, and D. LIEBSCH, Liebigs Ann. *597*, 227 (1955).

quickly to a jelly) is due to an addition of the SH group across the carbonyl group of the N,N-dimethyl-formamide using dimethyl formamide as an aldehyde for semi thio-acetal formation as a first step as follows:



A similar structure has been proposed by WIELAND et al.[20] for a postulated intermediate in transacetylations with acetylthiophenol[25].

Glutathione sulfoxide VI. GLSO was obtained by dehydrogenation of GLDHSO II with DMSO (dimethyl sulfoxide). 500 mg II were dissolved in 5 ml DMSO. The DMSO was removed by vacuum destillation (1 mm Hg) repeated twice with addition of 5 ml N,N-dimethylformamide. To the somewhat fluid residue 3 ml of a one-to-one mixture of N,N-dimethylformamide and n-butylacetate was added. It crystallized slowly, m.p. 158–161°. The resulting sulfoxide gives no color reaction with nitroprusside. It does not reduce dichlorophenol-indophenol in dry methanol. The sulfoxide is however slightly hygroscopic. In the presence of humidity it reduces dichlorophenol-indophenol slowly. The reduction becomes visible on paper after more than 10 min. The GLSO obtained in this way is soluble in DMSO, N,N-dimethylformamide, water and slightly in alcohol.

Zusammenfassung. Durch Oxydation von Glutathion mit Wasserstoffsuperoxid in Ameisensäure wurde Glutathion-dihydrosulfoxid erhalten. Glutathion-sulfoxid, N-Acetylglutathion und assoziiertes (reversibel polymerisiertes) Glutathion wurden als Vergleichssubstanzen erstmalig hergestellt.

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Department of Pharmaceutical Chemistry and Spectrographic Laboratory, The University of California, School of Pharmacy, San Francisco (California, U.S.A.),
November 26 1962.

[25] The significance of covalent intermolecular association lies in the creation of a weak C-S bond which might compete with hydrogen bonding[26] in the connection of proteins to strands. It is better understandable, that the associated bond is opened by the frequently used carbonyl-containing reagents like urea[9] and CO than that a thiol could be hindered from a reaction by a hydrogen bond. It may, further turn out to be part of the *inter*molecular sulfur bonds now attributed to the disulfide bonds.

[26] M. LASKOVSKI and H. A. SCHERAGA, J. Amer. chem. Soc. *76*, 6305 (1954).

[27] This work was supported in part by funds of the U.S.P.H.S.

Biological and Pharmacological Significance of the Expanded S-Outer Shell in Electron Transfer Reactions

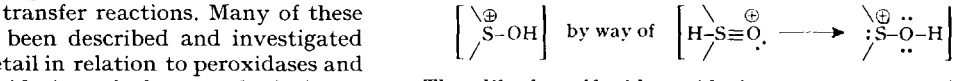
A peptide dihydrosulfoxide[1] having in the same molecule oxidizing and reducing properties such as those present in hydrogen peroxide is given added significance in that it provides, in retrospect, a possible mechanism such as has been sought in the photosynthesis and in anabolic and metabolic electron transfer reactions. Many of these reactions have already been described and investigated in various degrees of detail in relation to peroxidases and catalases[2–4] and the oxidation of photosynthetic intermediates[5].

Further the presence of a carbanion in the α-position of a dihydrosulfoxide provides a 'handle' for the enzymic addition of that carbanion to polar groups like carbonyl groups of substrates or intermediates. Electron transfer within this addition compound appears as an intramolecular rearrangement which may then be followed by dissociation or transfer of the intermediate substrate in a changed oxidation state.

Anaerobic metabolism is, according to WARBURG[6], the effective means by which cancer cells supplement their energy needs. WARBURG[7] has shown that anaerobic metabolism may be inhibited equivalently either by irradiation or by action of H_2O_2. An oxidized cystine has been tested on mice as a cancer growth-retarding agent with partially successful results[8]. It is of further interest in this connection that TOENNIES[9] performed the oxidative conversion of casein in hydrogen peroxide-formic acid mixtures into a protein free of methionine and modified in cystine content.

There are many glutathione oxidizing enzymes reported[10] in the literature which, however, invariably refer to the disulfide oxidation state, which seems to be the predominant form obtained by oxidation in aqueous solution. The usefulness of such enzymes in accomplishing conversion to the dihydrosulfoxide in the semisolid state should be investigated.

A satisfactory simple oxidizing agent has been long sought in photosynthesis, for the oxidation of, e.g., a glycolyl fragment to glycolate. The properties of a dihydrosulfoxide can be shown to satisfy a need to obtain by fission from an energy rich intermediate an oxidizing and a reducing agent, because it can react as hydride ion and



The dihydrosulfoxide oxidation state may satisfy a model for an effect occurring at the sulfur function in the cytochrome of chloroplasts if one postulates that the *in vitro* effect of H_2O_2 in formic acid may mimic photolysis of H_2O. The photo excitation would serve the equivalent of promoting an electron from a *p*-orbital in the sulfur

[1] G. E. UTZINGER, L. A. STRAIT, and L. D. TUCK, Exper. *19*, 324 (1963).

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[3] B. CHANCE, Arch. Biochem. *22*, 224 (1949); Science *109*, 204 (1949).

[4] B. CHANCE and R. R. FERGUSSON, in W. D. MCELROY and B. GLASS, *Mechanism of Enzyme Action* (Johns Hopkins Press, Baltimore 1954). – P. GEORGE, in D. E. GREEN, *Currents in Biochemical Research* (Interscience Publishers, New York 1956).

[5] W. VISHNIAC, W. HORECKER, B. L. and S. OCHOA, Adv. Enzymol. *19*, 1 (1957).

[6] O. H. WARBURG, *New Methods of Cell Physiology* (Interscience Publ. New York; G. Thieme Verlag, Stuttgart 1962).

[7] O. H. WARBURG, W. SCHRÖDER, H. S. GEWITZ, and W. VÖLKER, Z. Naturforsch. *13b*, 581 (1958).

[8] Staff of the Lankenau Hospital Research Institute, Amer. J. Cancer *26*, 554 (1936).

[9] G. TOENNIES, J. biol. Chem. *145*, 167 (1942). – See also G. TOENNIES and R. P. ROMILLER, Amer. chem. Soc. *64*, 3054 (1942).

[10] D. B. HOPE, in *Glutathione*, Biochemical Society Symposium 1959 (University Press, Cambridge), No. *17*, p. 97.