Phytochemistry, Vol. 22, No. 10, pp. 2183–2184, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

OXIDATION OF 2-MERCAPTOETHANOL IN THE PRESENCE OF TRIS BUFFER

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(*Revised received* 18 *February* 1983)

Key Word Index—2-Mercaptoethanol; oxidation; Tris buffer.

Abstract—2-Mercaptoethanol is oxidized by molecular oxygen, especially at high pH. This reaction is markedly increased by primary amines such as asparagine, glutamine and their corresponding amino acids and by the buffer tris(hydroxymethyl)aminomethane. Thus, the use of 2-mercaptoethanol in Tris buffer may present several complications: an uptake of oxygen which could be confused with metabolic activity; destruction of the protective mercaptoethanol; and by inference, quite possibly an enhanced oxidation of the protein sulphydryls in need of protection.

INTRODUCTION

Thiol compounds are commonly included in extraction and assay buffers for retarding oxidation of native –SH groups of isolated enzymes. These protectants aid in maintaining such proteins in their biologically functional states. The thiols generally employed for this purpose include cysteine, reduced glutathione, 2-mercaptoethanol and dithiothreitol. Although these have been used in a variety of buffer systems [1–3], the basis of their choice in a particular buffer system is seldom indicated. While determining ribulose-1,5-bisphosphate oxygenase activity by the polarographic method [4], we found that the aberrant behaviour of some of the enzymic preparations was attributable to non-enzymic oxidation of 2-mercaptoethanol in the assay buffer. The present investigations were therefore undertaken to examine the suitability of using 2-mercaptoethanol in different buffers which are commonly used in assaying plant enzymes.

RESULTS AND DISCUSSION

2-Mercaptoethanol was oxidized rapidly when incubated at 25° with Tris. Thus at pH 7.8, 0.105 μmol O_2 per min was consumed when 10 mM 2-mercaptoethanol was added to 50 mM Tris. It is known that in air 2-mercaptoethanol undergoes a gradual auto-oxidation [5] and the uptake of O_2 is attributable to the oxidation of the thiol groups. Rates of O_2 uptake increased progressively with increasing Tris concentrations. Maximum uptake of O_2 was obtained at 50 mM. In all the subsequent experiments, 50 mM Tris was used. Appreciable rates of oxidation were also observed when the incubation was performed at 4° instead of at room temperature.

To ascertain whether oxidation of 2-mercaptoethanol was enhanced in other buffers as well, the reaction was carried out in glycine, phosphate or HEPES buffer, and also in the presence of glutamine. The rate of oxidation of 10 mM 2-mercaptoethanol in Tris, glycine, phosphate and HEPES buffer (50 mM, pH 8.3) were, respectively, 0.105, 0.078, 0.032 and 0.022 μmol O_2/min. In all the buffers, 2-mercaptoethanol-dependent O_2 uptake was not detectable at pHs below 7.0 (Fig. 1), suggesting that the reduced form of this reagent is stable under neutral and acidic conditions. The rate of oxidation was, however, always markedly enhanced when the pH was elevated from 7.5 to 9.0. A high rate of oxidation between pH 7.5 and 9.0 was also obtained when 2-mercaptoethanol was incubated with a 50 mM solution of glutamine, comparable with that found with Tris in Fig. 1. Almost identical results were obtained with asparagine, aspartate and glutamate.

Since many oxidations of organic substances are cata-

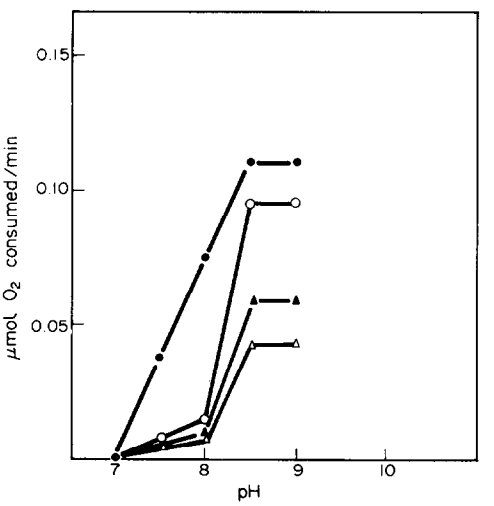

Fig. 1. Effect of pH on the rate of oxidation of 10 mM 2-mercaptoethanol in different buffers. Tris (●——●), glycine (○——○), phosphate (▲——▲) and HEPES (△——△). All buffers were 0.1 M.

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Table 1. Nitrate reductase activity in cell-free extracts of maize leaves

Buffer	Sulphydryl reagent (5 mM)	μmol NO_2^- produced/g tissue in 20 min	% Activity*
0.1 M Tris, pH 7.8	Cysteine	1.40	100
	2-Mercaptoethanol	0.85	61
0.1 M phosphate, pH 7.8	Cysteine	1.33	95
	2-Mercaptoethanol	1.10	78

Leaves (1 g) of field-grown maize were macerated in 0.1 M of either Tris–HCl or phosphate buffer (pH 7.8) containing either cysteine or 2-mercaptoethanol. Homogenate was centrifuged at 10000 *g* for 20 min and nitrate reductase activity was determined in the supernatant according to ref. [6].

*Activity as % of that obtained in Tris–HCl buffer containing 5 mM cysteine.

lysed by heavy metals or heavy metal complexes, it was of interest to know whether the oxidation with the other amines was affected by metallic cations. The oxidation of 2-mercaptoethanol in 50 mM Tris–HCl (pH 8.3) was not affected either by EDTA (1–10 mM) or by heavy metals like Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+} between 1 μM and 1 mM.

In order to ascertain to what extent the enhanced rate of oxidation of sulphydryl groups of 2-mercaptoethanol could affect enzymic activities, nitrate reductase (EC 1.6.6.1) was extracted from leaves of maize seedlings in either Tris–HCl or phosphate buffer containing 5 mM cysteine or 5 mM mercaptoethanol [6]. This enzyme is known to lose its activity rapidly unless adequate precautions are taken to maintain its –SH groups in their reduced state [7]. As shown in Table 1, similar activities were obtained on macerating the tissue in the presence of cysteine either in Tris–HCl or phosphate buffer. Replacement of cysteine with 2-mercaptoethanol in the extraction media, however, resulted in a much lower recovery of the enzyme in Tris–HCl than in phosphate buffer. As has been shown earlier, 2-mercaptoethanol is oxidized much faster in Tris–HCl than in phosphate buffer. Thus the rapid oxidation in Tris–HCl of the protectant 2-mercaptoethanol may have been responsible for this relatively low recovery of nitrate reductase. Of course, there is a very real possibility that the protein sulphydryls themselves are also more rapidly oxidized by molecular oxygen in the presence of Tris by a reaction analogous to the Tris-stimulated mercaptoethanol oxidation.

Thiols, including 2-mercaptoethanol, are known to be oxidized to their disulphide forms, particularly under alkaline conditions [5]. The results of the present investigation confirm that 2-mercaptoethanol is susceptible to auto-oxidation in an alkaline media. It is, however, important to note that the extent of its oxidation is not exclusively determined by the pH, but also by the nature of other constituents in the medium. Oxidation of 2-mercaptoethanol was more marked in the presence of the primary amines (Tris and amino acids) than in phosphate or in the tertiary amine (HEPES). It is thus likely that a free primary amino group is responsible for enhanced oxidation of this thiol reagent. More comprehensive studies need to be undertaken to examine the stability of thiol reagents in a variety of buffer systems. Nonetheless, it is evident that the enhanced uptake of O_2 by 2-mercaptoethanol would seriously interfere with enzyme assays which monitor the changes in oxygen concentration. The results of the present investigation suggest that the use of 2-mercaptoethanol along with Tris at a pH higher than 7.5 should be avoided. However, other –SH thiol compounds, for example DTT and cysteine, which are more stable in Tris even under mild alkaline conditions, can be employed without these complications.

EXPERIMENTAL

Chemicals. Two different batches of each of Tris and 2-mercaptoethanol used in the present investigation were from Sigma and Merck. The amino acids, amines and HEPES buffer were from Sigma. The other chemicals were of analar quality from B.D.H. Double-distilled H_2O was used for the preparation of all solns.

Oxidation of 2-mercaptoethanol. The rate of oxidation was determined from the uptake of O_2 at 25° using a Clark electrode equipped with a Gilson Oxygraph. The standard reaction mixture in a final vol. of 1.5 ml contained 50 mM Tris (pH 8.3) and 10 mM 2-mercaptoethanol. The reaction was started by the addition of 0.1 ml of 0.15 M soln of 2-mercaptoethanol. When examining the effect of different buffers on the rate of oxidation of 2-mercaptoethanol, Tris was replaced by an appropriate buffer after adjusting its pH to the required value either with 1 M HCl or NaOH. The amount of O_2 uptake was calculated by assuming that 0.25 μmol of O_2 was dissolved per ml of the initial air-saturated medium at room temp.

Acknowledgement—We are grateful to Prof. Norman E. Good, Michigan State University for discussions and for his valuable suggestions in preparing the manuscript.

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