NON-STOICHIOMETRIC SULFHYDRYL LOSS WITH VITAMIN K_5 *

Charles R. Heisler+ and H. Y. Yang

Department of Food Science and Technology
Oregon State University
Corvallis, Oregon

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A water soluble form of vitamin K, 4-amino-2-methyl-1-naphthol · HCl (designated K_5), has been shown to diminish the number of titratable sulfhydryl groups of solutions of cysteine and glutathione (Kitamikado and Mori, 1954) and suspension of bacteria (Kitamikado, 1956 and Rasulpuri et al., 1965). These workers have suggested that an attack of vitamin K_5 upon vital and biologically significant sulfhydryl compounds may account for the bactericidal effects upon a large number of microorganisms. Rasulpuri et al. (1965) demonstrated the loss of -SH groups of purified alcohol dehydrogenase when treated with vitamin K_5 but did not relate this to enzymatic activity. This communication presents evidence for the loss of sulfhydryl groups in the presence of vitamin K_5, but only if O_2 is available, and a complete lack of stoichiometry between K_5 and the sulfhydryl group loss.

MATERIALS AND METHODS

Crystalline yeast ADH[1], alpha-amylase (Type II from Bacillus subtilis), crystalline catalase (beef liver), GSH, thiolated gelatin (MW 100,000, Bloom No. 250, Type II) and beta-nicotinamide adenine dinucleotide (98% pure) were obtained from the Sigma Chemical Company. L(+)-cysteine·HCl and mercaptoacetic

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+ Present Address: Department of Biochemistry, University of Nevada, Reno, Nevada.

1 Abbreviations: ADH, alcohol dehydrogenase; GSH, reduced glutathione; K_5, vitamin K_5.

acid (80% in water) were obtained from Eastman Organic Chemicals and vitamin K_3 from Heterochemical Corporation and Nutritional Biochemicals Corporation. Vitamin K_5 was synthesized according to the procedure of Oneta and Suh, (1949).

The enzymatic activities were measure according to the methods described previously: ADH (Racker, 1950), catalase (Beers and Sizer, 1952) and alpha-amylase (Bernfeld, 1955). The reactions were started by adding the enzyme, which had been preincubated with vitamin K_5, to the reaction mixture. The procedure used for determining the number of -SH groups and the enzymatic activity are as follows: approximately 10 μ moles of the simple -SH compounds (or up to 10 μ-moles of -SH groups in the case of the enzymes) were added to the Tris-HNO_3 buffered titration mixture of Benesch et al., (1955). A very small aliquot was withdrawn for the determination of the enzymatic activity and the remainder used for the amperimetric sulfhydryl determination (Benesch et al., 1955).

The anaerobic incubation of GSH with vitamin K_5 was done in Thunberg tubes by placing the dry freshly weighed GSH and K_5 in the side arm and 5 ml of 0.1M Tris buffer, plus sufficient KOH to bring the final reaction mixture to a pH of 7.0, in the test tube proper. The buffer was frozen and the Thunberg tube evacuated to 0.10 mm of Hg pressure. The buffer was melted and used to dissolve the GSH and vitamin K_5. The tubes were placed in a desiccator, flushed with N_2 gas and the reaction mixture allowed to incubate at room temperature in the dark for 17 hours at which time aliquots were taken for sulfhydryl determination while the remainder was allowed to react another 17 hours in air.

RESULTS AND DISCUSSION

The reactivity of vitamin K_5 with the -SH groups of yeast ADH is shown in Figure 1. The enzyme control at pH 7.6 in 0.01M potassium phosphate buffer gave titration values which changed little in 48 hours. The number of -SH groups per molecule of ADH varied from 21-27 between experiments and lot numbers of ADH

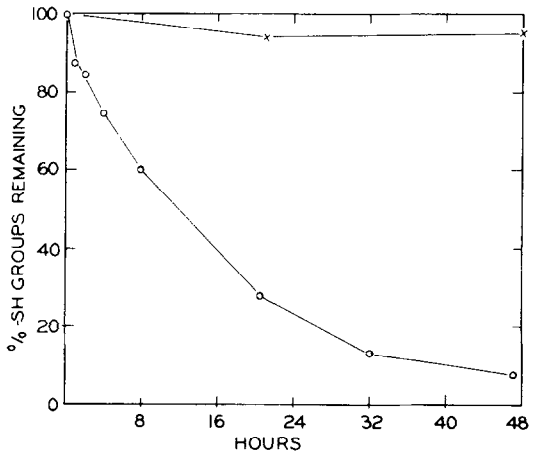

FIGURE 1: Reactivity of ADH-sulfhydryls and vitamin K_5. ADH and K_5 (0—0) were incubated in 0.01 M potassium phosphate buffer, pH 7.6, at 0°. Aliquots containing 0.146 μmoles of ADH and 5.00 μmoles of K_5 were titrated for -SH groups at the times indicated. X——X, ADH control.

preparations. The addition of K_5 in a K_5/ADH sulfhydryl molar ratio of approximately 1/1 brought about a rapid loss of sulfhydryl groups during the first 4-8 hours, and tapered off to 10% remaining at 48 hours. The enzymatic activity was reduced to 10% within 2 hours.

TABLE I: Percent of Sulfhydryl Groups and Enzymatic Activity Remaining after 17 Hours Incubation with Vitamin K_5

Sulfhydryl Compounds	Molar Ratio of K_5/-SH Group	Percent Remaining	
		Sulfhydryl	Activity
Cysteine ·HCl	1/10	20	--
Mercaptoacetic Acid	1/10	53	--
Glutathione	1/10	36	--
Thiolated Gelatin	2/7	50	--
Alcohol Dehydrogenase	1/9	54	25
	1/27	77	64
Catalase	1/10	64	46
α-amylase	1/1	--	100

Every attempt at establishing a stoichiometric relationship between the -SH group and K_5 failed. The variability of K_5 in initiating sulfhydryl loss is illustrated in Table I which also compares the molar ratios of K_5 to -SH compound with the reduction of enzyme activity.

ADH and catalase show losses in the number of -SH groups and enzymatic activity but without correlation to the K_5/-SH group ratio. Bacillus subtilis alpha-amylase reported to contain no sulfhydryl or disulfide groups (Junge et al., 1959) is unaffected by amounts of K_5 which very effectively reduce the activity of the sulfhydryl enzymes.

Table II, in comparing -SH loss of GSH with added K_5 in molar ratios of 1/10 and 1/100 under aerobic and anerobic conditions, shows that the absence of air disallows any loss of the titratable sulfhydryl groups of GSH.

TABLE II. Effect of Vitamin K_5 upon the Sulfhydryls of Glutathione in the Presence and Absence of Air[a]

Conditions	µmoles of Sulfhydryl	Percent Sulfhydryl Remaining
Control	5.00	100
Anaerobic, 17 hours		
GSH	5.04	100
GSH+0.05 µmole K_5	5.01	100
GSH+0.5 µmole K_5	5.45	100
Anerobic, 17 hours		
GSH	4.18	83
GSH+0.05 µmole K_5	1.23	25
GSH+0.5 µmole K_5	0.00	0

[a]Glutathione, 5 µmoles, and vitamin K_5 as indicated were incubated at pH 7.0 in vacuo 23° for 17 hours followed by 17 hours in air. Sulfhydryls were determined at the end of each period.

Vitamin K_5 undergoes oxidation in water solution, quite rapidly at neutral pHs, first to a pink and later to a purple compound which, on standing, precipitates. Knobloch (1949) has identified this purple insoluble precipitate as (4-oxy-3-methyl-naphthylimine)-2-methyl-1,4-naphthoquinone, which is a condensation product of K_5 and vitamin K_3 resulting from air oxidation and deamination of K_5.

The rate of air oxidation of the sulfhydryl group is very much slower. Yet the presence of 1/100 of a molar equivalent of K_5 brings about a many fold increase in this rate. When K_5 and -SH are incubated anaerobically no pink coloration appears until air is admitted. The oxidation of K_5 appears to be a prerequisite for the speedier disappearance of the -SH groups. This idea is strengthened by the experiments of Merrifield and Yang (1965) who showed that microorganisms grown anaerobically with K_5 had a much greater viability than those grown aerobically.

The failure to show any stoichiometry between the sulfhydryl group and K_5, plus the fact that many -SH groups disappear per molecule of K_5 strongly suggests that the effect of this vitamin derivative upon the -SH group is secondary. The present evidence points to an increased rate of air oxidation of the sulfhydryl groups which is brought about by vitamin K_5 or some oxidized product of K_5 acting as a redox-agent between the sulfhydryl and oxygen. The sulfhydryl may be thought of as a reducing agent which protects to some extent the rapid air oxidation of the K_5. However, the net result is the same and it suggests a mechanism for the bactericidal activity of K_5 whereby vital sulfhydryl compounds, including enzymes, are oxidized.

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