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The Reaction of 2-Methyl-1,4-naphthoquinone with Sulfhydryl Compounds

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1. 2-Methyl-1,4-naphthoquinone (K_3) reacted with sulfhydryl compound to form a thioether linkage at 3-position of K_3 with an absorption maximum at 420—430 mμ.

2. For this reaction, molecular oxygen and alkaline metal ions such as Na^+ and K^+ were essential. One half mole of oxygen was consumed for the reaction of one mole of K_3 with one mole of sulfhydryl compound.

3. The reaction product of K_3 with cysteine was unstable and gradually turned to the insoluble amorphous polymer. By methylation of intermediate, it was identified to be methyl 5-methyl-6-methoxy-3H-naphtho[2,1-*b*] [1,4]-thiazine-2-carboxylate.

NADPH oxidase requiring naphthoquinone as a cofactor, such as 2-methyl-1,4-naphthoquinone (K_3), was found in liver microsome.[2] In the previous report,[3] we investigated effect of twenty-two derivatives of naphthoquinone on the oxidation of NADPH by liver microsome and by partially purified preparation of the enzyme, and that the quinones which were found to be effective as cofactor for NADPH oxidation reacted with cysteine to form a yellowish compound and its absorption spectrum has a characteristic peak at 420—430 mμ under the condition for enzyme assay.

On the reaction of K_3 with sulfhydryl compounds, Fieser[4] observed a change in color of K_3 in the presence of cysteine. Burton and David[5] reported that a greenish black solid which obtained from a reaction mixture of K_3 with cysteine after sixty hour's incubation, was S-3-methyl-1,4-naphthoquinon-2-yl-cysteine (I), and Nickerson, *et al.*[6] obtained S-3-methyl-1,4-naphthoquinon-2-yl-glutathione(II) from a reaction mixture of K_3 with glutathione.

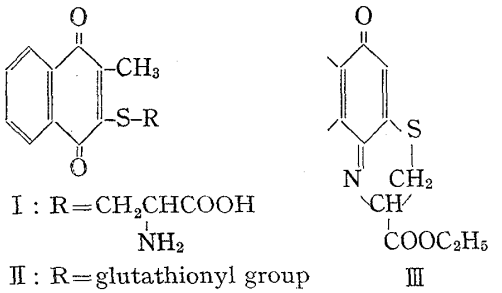


Chart 1

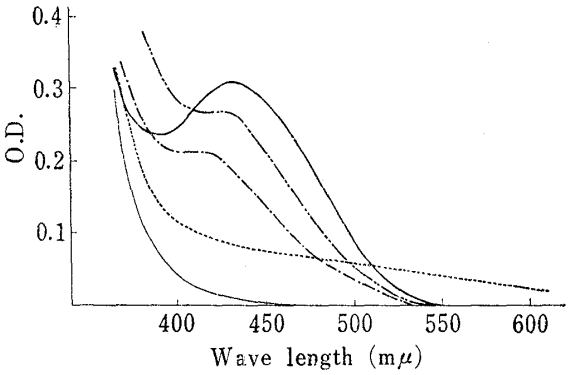


Fig. 1. Absorption Spectra

A mixture consisting of 40 μM K_3, 0.1 M phosphate buffer (pH 6.5) and 40 μM sulfhydryl compound, was allowed to stand at room temperature (about 20°) for 30 min.

——— in the presence of thioglycolate
—·—·— in the presence of glutathione
— · — · — in the presence of cysteine (a)
——— in the absence of sulfhydryl compound
..... (a) after 24 hr

1) Location: *3190, Gofuku, Toyama.*
2) R. Sato, H. Nishibayashi and T. Omura, *Biochim. Biophys. Acta*, **63**, 550 (1962).
3) H. Nishibayashi, N. Nakai and R. Sato, *J. Biochem.*, **62**, 215 (1967).
4) L.F. Fieser, *Ann. Internal Medicine*, **15**, 648 (1941).
5) H. Burton and S.B. David, *J. Chem. Soc.*, **1952**, 2193.
6) W.J. Nickerson, G. Falcone and G. Strauss, *Biochemistry*, **2**, 537 (1963).

On the other hand, Kuhn and Hammer[7] reported that a condensation product, which is ethyl 6-oxo-2,3-dihydro-6H-naphtho[2,1-*b*] [1,4]thiazine-2-carboxylate (III), was obtained from a reaction mixture of 1,4-naphthoquinone and cysteine ethylester.

In this report, we studied on the interaction of K_3 with sulfhydryl compounds in detail and also on the identification of a reaction product of K_3 with cysteine.

Results and Discussions

Change in an Absorption Spectrum

It was previously reported[2] that an absorption spectrum of K_3 was changed by the reaction with cysteine under the condition for enzyme assay.

In absorption spectra, a characteristic peak or shoulder at 420—430 mμ was observed when K_3 was incubated with sulfhydryl compound in 0.1 M phosphate buffer (pH 6.5) (Fig. 1). Either reduced K_3 or sulfhydryl compounds shows no absorption in the same region of wavelength. Absorption spectrum of K_3 incubating with thioglycolate was the same as that of authentic S-3-methyl-1,4-naphthoquinon-2-yl-thioglycolate.[8]

The results show that the thioether linkage at 3-position of K_3 has a characteristic absorption at 420—430 mμ.

A reaction product of K_3 with thioglycolate and with glutathione was stable, while that of K_3 with cysteine seemed to be labil because absorption spectrum of the latter gradually changed during incubation time, as can be seen in Fig. 1.

This finding suggests that K_3 reacted with cysteine to form a thioether linkage at 3-position in an early stage of the reaction as well as with thioglycolate or glutathione and then the product changed to other compound.

Effect of Oxygen

In order to investigate the effect of oxygen, K_3 was incubated with cysteine in an evacuated Thunberg's tube.

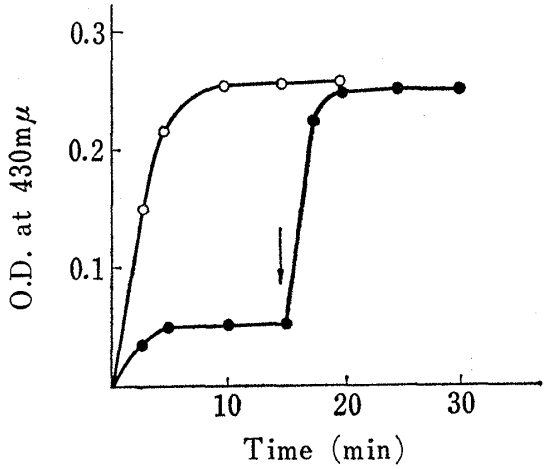


Fig. 2. Effect of Oxygen on the Reaction of K_3 with Cysteine

Each tube contained 0.1mM K_3, 0.1 mM cysteine and 0.1 M phosphate buffer (pH 6.5) in a final volume of 3.0 ml in a Thunberg's tube —○— control; —●— evacuated sample. At the time indicated by an arrow, air was admitted into the tube.

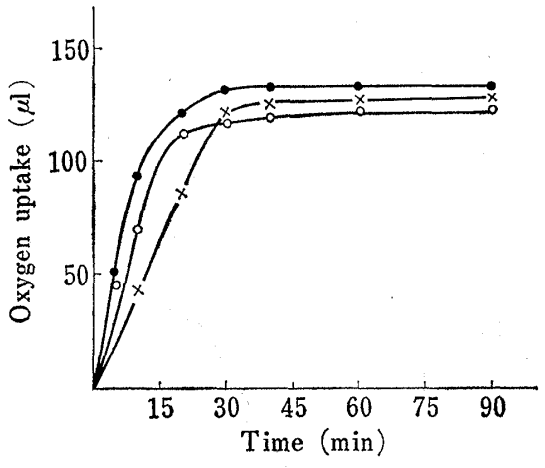


Fig. 3. Oxygen Uptake by the Reaction of K_3 with Sulfhydryl Compounds

Each vessel contained 10 μ moles of K_3, 10 μ moles of sulfhydryl compound and 0.1 M phosphate buffer (pH 6.5) in a final volume of 3.0 ml.

—×— cysteine
—●— thioglycolate
—○— glutathione

7) R. Kuhn and I. Hammer, *Chem. Ber.*, **84**, 91 (1951).
8) R.F. Thomson, *J. Chem. Soc.*, **1951**, 1239.

As is shown in Fig. 2, slight increase in absorbance at 430 mμ was observed in the initial period of this reaction probably because of a trace of oxygen contaminated, and no increase was observed after five min. By admitting air into the tube, absorbance rapidly increased to the value as the control.

This finding suggests that molecular oxygen is essential for the reaction of K_3 with sulfhydryl group and consumption of oxygen may indicate the proceeding of the reaction.

Oxygen uptake was measured by the Warburg's manometric technique using air as the gaseous phase. One half mole of molecular oxygen was consumed per one mole each of K_3 and cysteine, and the reaction was completed within thirty min in 0.1 M phosphate buffer (pH 6.5) at 30° as shown in Fig. 3. Thus, the rate of consumption of molecular oxygen coincides with the rate of the reaction and depends on the concentration of reactants existing in a reaction mixture (Table I). K_3 easily reacted with cysteine in 0.1 M acetate buffer (pH 6.5), but not reacted with it in ethanol, in 0.1 M tris buffer (pH 7.5) or in water (Table II).

Table I. Effect of K_3 and Cysteine Concentration on Oxygen Uptake

K_3 (μmole/vessel)	2	10	10
Cysteine (μmole/vessel)	10	5	10
Oxygen Uptake (μl)	33	61	119

Oxygen uptake was measured at 30° in a Warburg's vessel using air as the gaseous phase. A reaction mixture (final volume, 3.0 ml) consisting of K_3, cysteine and 0.1 M phosphate buffer (pH 6.5), was incubated for 30 min.

Table II. Effect of Solvents on Oxygen Uptake

Solvent	Ethanol	0.1 M Tris[a] buffer, pH 7.5	Water	0.1 M acetate buffer, pH 6.5
Oxygen uptake (μl)	6	0	0	110

a) Tris (hydroxymethyl) aminomethane

Oxygen uptake was measured at 30° in a Warburg's vessel using air as the gaseous phase. A reaction mixture (final volume, 3.0 ml) consisting of K_3 (10 μmoles), cysteine (10 μmoles) and the various solvent indicated in table, was incubated for 30 min.

These results suggest the participation of alkaline metal ion, such as Na^+ or K^+ in the reaction.

Effect of pH on the Consumption of Oxygen

Oxygen uptake owing to the reaction was measured at various pH (pH 5.0 to pH 9.0) of 0.1 M phosphate buffer after 30 min.

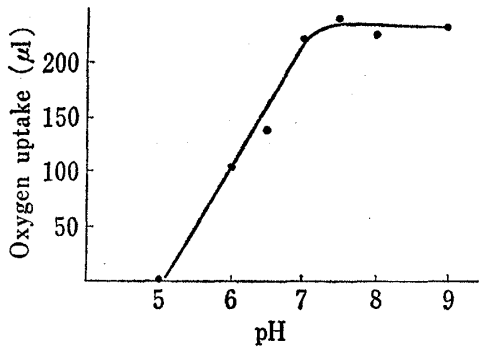


Fig. 4. Effect of pH on the Reaction of K_3 with Cysteine

Each vessel contained 10 μmoles of K_3, 10 μmoles of cysteine and 0.1 M phosphate buffer (pH 5.0 to 9.0) in a final volume of 3.0 ml.

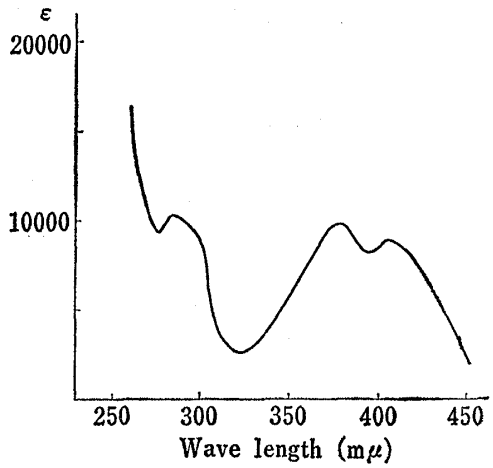
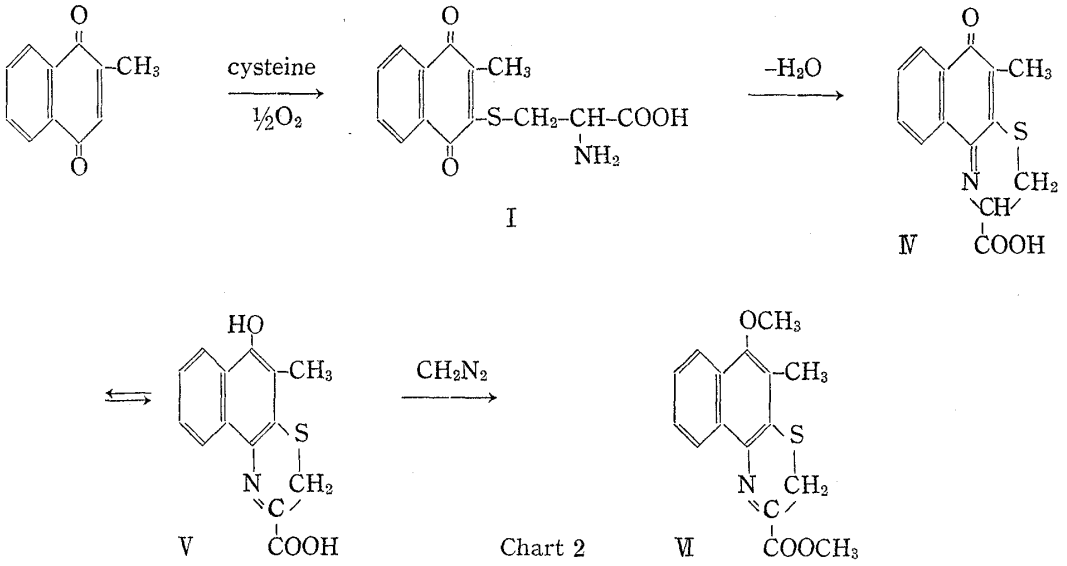


Fig. 5. Absorption Spectrum of VI (in hexane)

Oxygen was not consumed at pH 5.0 at all. However, one equivalent mole of molecular oxygen was consumed at alkaline region (Fig. 4).

As can be seen in absorption spectrum (Fig. 1), the reaction product of K_3 with cysteine seemed to be unstable and changes gradually to other compound *via* secondary reaction, which might be accelerated in alkaline solution.



Identification of a Reaction Product of K_3 with Cysteine

In reference to a reaction product, Burton and David[5] obtained greenish black solid from a reaction mixture of K_3 and cysteine which was incubated for sixty hours at 35° in methanol and provided to be S-3-methyl-1,4-naphthoquinon-2-yl-cysteine (I) for this substance from the result of elemental analysis. However, the substance is assumed to be a polymer of I because it is amorphous and insoluble in water and usual organic solvents. In the reaction of K_3 with cysteine, absorbancy at 420—430 mμ, a characteristic absorption of the thioether linkage at 3-position of K_3, decreased with reaction time, and one equivalent mole of molecular oxygen was consumed in alkaline solution, mentioned above. Therefore, I is unterable for the structure of the substance.

In the present study, we attempted to isolate an intermediate compound from a reaction of K_3 with cysteine.

K_3 was incubated with cysteine in phosphate buffer (pH 6.5). When one half equivalent moles of oxygen was consumed, the reaction was stopped by acidification. The product was extracted with ethyl ether and the ethereal extract was shaken with 5% sodium bicarbonate solution. Then this alkaline solution was made acidic and was again extracted with ethyl ether. This second ethereal extract was evaporated and the residue was crystallized from 50% methanol solution giving yellow needles. The needles were also unstable and gradually change to an orange amorphous compound during drying in vacuum at room temperature.

This compound was positive on the test with ferric chloride and was assumed to be 5-methyl-6-hydroxy-3H-naphtho[2,1-*b*] [1,4]thiazine-2-carboxylic acid (V) from the result of elemental analysis.

It was too difficult to keep this compound stable and methylation with diazomethane was carried. The second ethereal extract from a reaction mixture was methylated by excess addition of diazomethane in ethyl ether. Methylated compound was purified by the method of column chromatography using Florisil. The first fraction which was eluted with benzene, gave an orange compound. This compound was crystallized from ethanol, giving orange needles, mp 128—129°. From the results of elemental analysis, determination of methoxy

groups, IR spectrum and NMR spectrum, this compound is concluded to be methyl 5-methyl-6-methoxy-3H-naphtho[2,1-*b*] [1,4]thiazine-2-carboxylate (VI).

Thus, K_3 reacts with one of cysteine derivatives, of which amino or carboxyl group was substituted, such as glutathione and cysteine ethylester, to produce a stable compound (II)[6] and (III)[7] respectively. By the reaction with cysteine, K_3 forms a thioether linkage at 3-position at first stage of the reaction as well as with other sulfhydryl compounds. However, the product (I) is unstable and intramolecular dehydration occurs in (I) to be form the thiazine (IV or V). Then it probably turns to the insoluble polymer by oxidative polymerization which was obtained by Burton and David.[5]

Experimental[9]

Interaction of K_3 with Sulfhydryl Compounds——For a spectrophotometric study of the interaction of K_3 with sulfhydryl compounds, a mixture consisting of 0.1 mM K_3, 0.1 mM sulfhydryl compound, 10% ethanol (for the aid of dissolving of K_3) and 0.1 M phosphate buffer (pH 6.5) in a final volume of 3.0 ml was allowed to stand for 30 min at room temperature (about 20°) and then absorption spectrum was measured.

The reaction in vacuum was carried in an evacuated Thunberg's tube. The reaction mixture contained 0.1 mM K_3, 0.1 mM cysteine and 0.1 M phosphate buffer (pH 6.5) and absorbance was measured at 430 mμ.

Oxygen uptake in the reaction of K_3 with sulfhydryl compounds was measured at 30° in a Warburg's vessel using air as the gaseous phase.

Isolation of a Reaction Product of K_3 with Cysteine——A mixture consisting of 2 μmoles of K_3 in 40 ml of ethanol and of 2 μmoles of cysteine hydrochloride in 80 ml of 0.2 M phosphate buffer (pH 6.5) was shaken at room temperature for 15—30 min and then was extracted with ethyl ether. The ethereal extract was shaken with aq. 5% $NaHCO_3$ solution and then the alkaline solution was acidified with HCl, and was extracted again with ethyl ether. The second ethereal extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was crystallized from aq. 50% methanol solution to22 give 0 mg of yellow needles. These needles were changed its color to orange by heating at 130° and decomposed at 213—215° and also turned to orange amorphous solid during in vacuum at room temperature. *Anal.* Calcd. for (V), $C_{14}H_{11}O_3NS$: C, 61.54; H, 4.05; N, 5.15. Found: C, 61.61; H, 3.90; N, 4.81. IR cm^{-1}: ν_{O-H} 3480; $\nu_{C=O}$ 1730.

The second ethereal extract which was dried over anhydrous Na_2SO_4, was methylated by the addition of excess diazomethane in ethyl ether solution and the solvent was evaporated. The residue dissolved in benzene was chromatographed on a Florisil column. 180 mg of crystals was obtained from the eluate with benzene and it was recrystallized from ethanol to give orange needles, mp 128—129°. *Anal.* Calcd. for (VI), $C_{16}H_{15}O_3NS$; C, 63.77; H, 5.02; N, 4.65; CH_3O as 2 mole, 20.60. Found: C, 64.07; H, 4.98; N, 4.41; CH_3O (Zeisel), 20.15. IR cm^{-1}: $\nu_{C=O}$ 1695. NMR (10% solution in $CDCl_3$) τ: 7.56 (3H, singlet, C-CH_3), 6.44 (2H, singlet, -CH_2-), 6.09 (3H, singlet, O-CH_3), 6.00 (3H, singlet, O-CH_3), 1.50—2.67 (4H, multiplet, aromatic proton).

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9) Melting points are uncorrected. NMR spectrum was determined at 60 Mcps in $CDCl_3$ solution using Me_4Si as internal standard.