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Effect of Molybdenum on 4-Hydroxyaminoquinoline 1-Oxide Reductase Activity in the Liver of Mice[1]

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Effect of molybdenum on 4-hydroxyaminoquinoline 1-oxide (4-HAQO) reductase activity was examined in the liver of mice. 4-Nitroquinoline 1-oxide reductase in the mouse liver hardly showed decrease in the activity by the administration of tungsten. 4-HAQO reductase activity showed 40—50% decrease of the control by the administration of tungsten to mice as in xanthine oxidase and sulfite oxidase, molybdenum-containing enzymes. The 4-HAQO reductase activity decreased by the administration of tungsten was recovered by the administration of molybdenum.

Keywords——molybdenum; tungsten; 4-nitroquinoline 1-oxide; 4-nitroquinoline 1-oxide reductase; 4-hydroxyaminoquinoline 1-oxide; 4-hydroxyaminoquinoline 1-oxide reductase; molybdenum-containing enzyme; mouse liver

4-Nitroquinoline 1-oxide (4-NQO) is a chemical carcinogen that induces tumor in the lung and skin of mice and rats.[3,4] It is known that 4-NQO is metabolically reduced to its proximate carcinogen, 4-hydroxyaminoquinoline 1-oxide (4-HAQO), by various microorganisms[5] and by tissues and organs of mammals,[6] and this 4-HAQO is further reduced to the noncarcinogenic 4-aminoquinoline 1-oxide (4-AQO) and the 4-aminoquinoline (4-AQ). Thus, it seems of significance to follow the activity of enzymes that reduces 4-NQO to 4-HAQO and reduces 4-HAQO to 4-AQO in various organs.

There are reports[7,8] on the reductase responsible for these reduction. 4-NQO reductase is inhibited by a low concentration (10^{-8} M) of dicoumarol, and Sugimura and others[9] reported this reductase to be DT-diaphorase. Although there have been a few reports on the 4-HAQO reductase, there still leaves some problems on its purification and method of its measurement, and its properties have not been revealed other than the enzyme contains a metal.[10]

In order to establish a method for the measurement of 4-HAQO reductase, we have developed a method for direct determination of various metabolites by the fluorescence analysis.[11,12] On the other hand, by examining the effect of various metals on the chemical reduction of 4-NQO and 4-HAQO to 4-AQO and 4-AQ, it was found that only molybdenum in the metals promoted the reduction of 4-HAQO to 4-AQO.[13] Therefore, we started to study the effect of molybdenum on 4-HAQO reductase activity in animals.

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In the present series of work, effect of molybdenum on 4-HAQO reductase activity was undertaken to study in livers of tungsten-treated mice, molybdenum-deficient mice.

Materials and Methods

Animals——Female mice of ddy strain weighing about 20 g were used for the experiments. These mice were fed in the commercial diet, CE-2 (CLEA Japan, Inc.) as a basal diet.

Experimental Group and Methods of Administration——Mice were divided into the following 7 groups.

Group 1: Group given CE-2 as a basal diet.

Group 2: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed CE-2.

Group 3: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed CE-2+$Na_2MoO_4 \cdot 2H_2O$ 0.5 mg/ml for 3 days.

Group 4: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed CE-2+$Na_2MoO_4 \cdot 2H_2O$ 0.25 mg/ml for 3 days.

Group 5: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed sucrose for 3 days.

Group 6: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed sucrose+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 3 days.

Group 7: Group given CE-2+$Na_2WO_4 \cdot 2H_2O$ 2 mg/ml for 21 days and changed sucrose+$Na_2MoO_4 \cdot 2H_2O$ 0.1 mg/ml for 3 days.

A solution of tungsten or molybdenum dissolved in the drinking water was given freely.

4-NQO, 4-HAQO and 4-AQO——These compounds were synthesized from quinoline according to the method of Ochiai.[14] 4-NQO; mp 146—150, 4-HAQO·HCl; mp 195—199, 4-AQO; mp 268—273.

Thin-Layer Chromatography of 4-NQO Metabolites——Enzymatic reduction products of 4-NQO were determined on Kieselgel G thin-layer chromatography comparing the *Rf* value of those of authentic standards. Using the solvent system sec-butanol/ethyl acetate/water (2: 1: 1), 4-HAQO was found to have an *Rf* 0.60 and 4-AQO was found to have an *Rf* 0.45.

Enzyme Solution——The 600 *g* supernatant of mouse liver homogenate using 1.15% KCl was prepared as enzyme solution for enzyme assay.

Measurement of 4-NQO Reductase Activity——Enzyme solution was pre-incubated at 37° for 10 min in a total volume of 1.3 ml of 0.2 M tris-HCl buffer (pH 7.6) with 28.8 μmol of NADH. Then the reaction was started by adding 0.5 ml of 1 μmol of 4-NQO/ml and the reaction mixture was incubated at 37° for 5 min. 4-NQO reductase activity was assayed by measuring the amount of residue 4-NQO as previously described.[11,12]

Measurement of 4-HAQO Reductase Activity——The reaction mixture containing enzyme solution was incubated at 37° for 50 min in a total volume of 1.9 ml of 0.2 M tris-HCl buffer (pH 7.6) with 28.8 μmol of NADH and 0.5 μmol of 4-HAQO as a substrate. Enzyme activity was assayed by measuring the amount of 4-AQO formed from 4-HAQO as previously described.[11,12]

Measurement of Xanthine Oxidase Activity and Sulfite Oxidase Activity——Xanthine oxidase activity was assayed by measuring the amount of residue xanthine according to the method of Schardinger.[15] Sulfite oxidase activity was assayed by measuring the amount of disappeared ferricyanide according to the method of Cohen and others.[16]

Determinations of Molybdenum and Tungsten in Tissues——The biological sample contained molybdenum was digested by heating it in Kjerdahl frasks of 25 ml vol in the presence of an appropriate amount of 36 N H_2SO_4 and 14 N HNO_3 on an electric burner. After the original material was completely digested, 28% NH_4OH was added to adjust at pH 2.0—2.4. After the sample was added 1.0% ascorbic acid and shaken, 5 ml of 1.0% oxine-MIBK solution was added and shaken. The MIBK layer was carefully separated, and the solution was measured by the atomic absorption spectrophotometer.[17]

After the destruction of organic compounds in the original materials, the amount of tungsten was determined by tungsten-dithiol absorption spectrophotometry using citrate as a masking agents[18]

Results

Tissue Level of Molybdenum in the Liver of Mice After Molybdenum Administration

In order to find the additional capacity of accumulation of molybdenum in mice, tissue level of molybdenum in the liver was examined after administration of molybdenum by

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various routes. Oral administration was made by forced intubation of an aqueous solution with a stomach prove, and subcutaneous injection with aqueous solution, lecithin solution, or a suspension in propylene glycol was made on the back. As shown in Fig. 1, absorption and excretion of molybdenum were found to be quite rapid in mice.

Comparison of various routes of administration showed that, in the liver, concentration of molybdenum at 30 min after the administration was lower when given by oral route than by subcutaneous route and that, in subcutaneous administration, use of aqueous solution showed residual molybdenum to be nil in the liver after 3 hr. In the case of propylene glycol suspension and lecithin solution, amount of molybdenum remained in the liver was 2.16 μg/g liver 24 hr after their subcutaneous injection, and its value was about twice that of the control (1.01 μg/g liver). However, preparation of the suspension in propylene glycol and administration of such a suspension are attended with an experimental error. Therefore, administration of molybdenum in subsequent enzyme experiments was made as a lecithin solution.

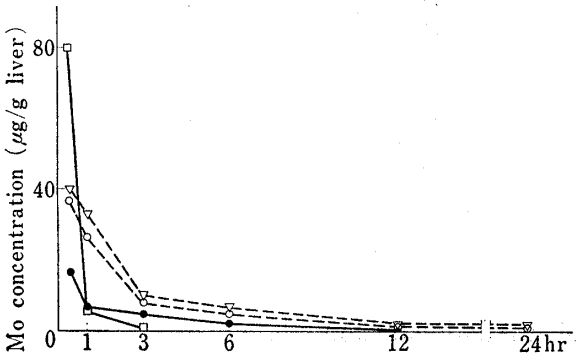

Fig. 1. Time-course of Molybdenum Concentration in the Liver of Mice after Molybdenum Administration in Various Routes

Molybdenum was administered to each mouse by using 5 mg of $Na_2MoO_4 \cdot 2H_2O$ dissolved in 0.1 ml of solution. Each point is the mean of 3 individual animals.
---○---, lecithin solution (*s.c.*).
—●—, water (oral).
---△---, propylene glycol suspension (*s.c.*).
—□—, water (*s.c.*).

Table I. Effect of Molybdenum Administration on 4-HAQO Reductase Activity in the Liver of Mice

Molybdenum administration[a)]	Total activity[b)]	Specific activity[c)]
Experiment-1		
Control	16.45	0.091
5 mg for 4 days	18.45	0.084
Experiment-2		
Control	14.00	0.053
3 mg for 6 days	17.60	0.065

Three mice were used for each group and each value is expressed as the mean.
a) *s.c.* in 0.1 ml lecithin solution.
b) 4-AQO formed in μg/whole organ/min.
c) 4-AQO formed in μg/mg protein/min.

Deviation of 4-HAQO Reductase Activity by Molybdenum Administration

As shown in Table I, total activity of 4-HAQO reductase increased slightly by the molybdenum administration, but there was hardly difference in the specific activity.

Since it is known that molybdenum is present in the liver of untreated mice, and the diet CE-2 also contains molybdenum *ca.* 1 μg/g diet, there is a possibility that, when activation of 4-HAQO reductase can be made by a small amount of molybdenum. it would be difficult to observe the stimulation of 4-HAQO reductase activity by excessive molybdenum addition to the diet CE-2. For this reason, removal of molybdenum from the animal body was attempted.

Physiological Removal of Molybdenum

For the removal of molybdenum from animal body, fasting and administration of tungsten, a molybdenum antagonist *in vivo*, were attempted. As shown in Table II, 1.97 μg of molybdenum present per gram liver was reduced to 0.73 μg by fasting for 2 days but it was impossible to make further reduction by fasting. Forced oral administration of 25 or 50 mg of tungsten, once a day for 1 week, while feeding the mice with normal diet (CE-2), showed that administration of 25 mg of tungsten resulted in the reduction of molydbenum in the mouse liver to 0.12 μg/g liver, approximately 1/15 of the control value, and molybdenum was reduced

under the limitation of determination by means of 50 mg of tungsten. Thus, molybdenum in the animal was found to be reduced by the administration of tungsten but this method of administration was found to have a toxic effect on mice and resulted in the fall of their body weight. Therefore, a longterm administration was attended by dissolving a small amount of tungsten in the drinking water and it was found that a solution of $Na_2WO_4 \cdot 2H_2O$ dissolved in drinking water at the rate of 2 mg/ml, given freely for 3 weeks, resulted in the reduction of molybdenum *ca.* 0.1 μg/g liver, without a decrease in body weight (Table II).

Table II. Effect of Tungsten on Molybdenum Concentration in the Liver of Mice

Group	Molybdenum concentration	
	μg/whole organ	μg/g liver
Control	1.79	1.97
Starvation for 2 days	0.64	0.73
W administration[a] (25 mg for 7 days)	0.18	0.12
W administration[a] (50 mg for 7 days)	0.10	0.10
W administration[b] (2 mg/ml for 21 days)	0.12	0.10

Each value is the mean of duplicated experiments and the combined tissue of three mice was used for each group in one experiment.

a) Each concentration of Na_2WO_4 $2H_2O$ was dissolved in the drinking water and given forced oral administration.

b) A solution of Na_2WO_4 $2H_2O$ was dissolved in the drinking water at the rate of 2 mg/ml, and it was given freely.

Variation of 4-HAQO Reductase Activity by Tungsten Administration

Since the long-term administration of tungsten was able to reduce the amount of molybdenum *in vivo*, without giving toxic symptoms, 4-HAQO reductase activity in the mouse liver under such molybdenum deficiency was measured. As marker enzymes, xanthine oxidase and sulfite oxidase containing molybdenum, and 4-NQO reductase not to contain molybdenum, were also used for the measurement for comparative examination of the activity. As shown in Table III, 4-NQO reductase in the mouse liver hardly showed decrease in the activity, but those of xanthine oxidase and sulfite oxidase were reduced to approximately 20—30% of the control value. 4-HAQO reductase activity was reduced to 40—50% of the control by the administration of tungsten, as in other molybdenum-containing enzymes.

Table III. Variation of Enzyme Activities in the Mouse Liver by Tungsten Administration

Enzyme	Control	W administration[a]
4-HAQO reductase	46.7[b] (100)	21.2(45.4)[c]
4-NQO reductase	19.9(100)	17.6(88.4)
Xanthine oxidase	28.5(100)	7.0(24.6)
Sulfite oxidase	37.1(100)	7.8(21.0)

Each value is the mean of duplicated experiment and the combined tissue of three mice was used for each group in one experiment.

a) A solution of $Na_2WO_4 \cdot 2H_2O$ dissolved in the drinking water at the rate of 2 mg/ml was given freely for 3 weeks.

b) The values were specific activity: 4-HAQO reductase activity was expressed as 4-AQO formed in μg/mg protein/50 min, 4-NQO reductase was 4-NQO decreased in μg/mg protein/10 min, xanthine oxidase was xanthine decreased in μg/mg protein/50 min, and sulfite oxidase was activity/mg protein.

c) The values were the ratio % of the control values.

Effect of Molybdenum Administration on Molybdenum-deficient Mice

Since the activity of 4-HAQO reductase was found to decrease by the administration of tungsten, same as other molybdenum-containing enzymes, recovery of 4-HAQO reductase

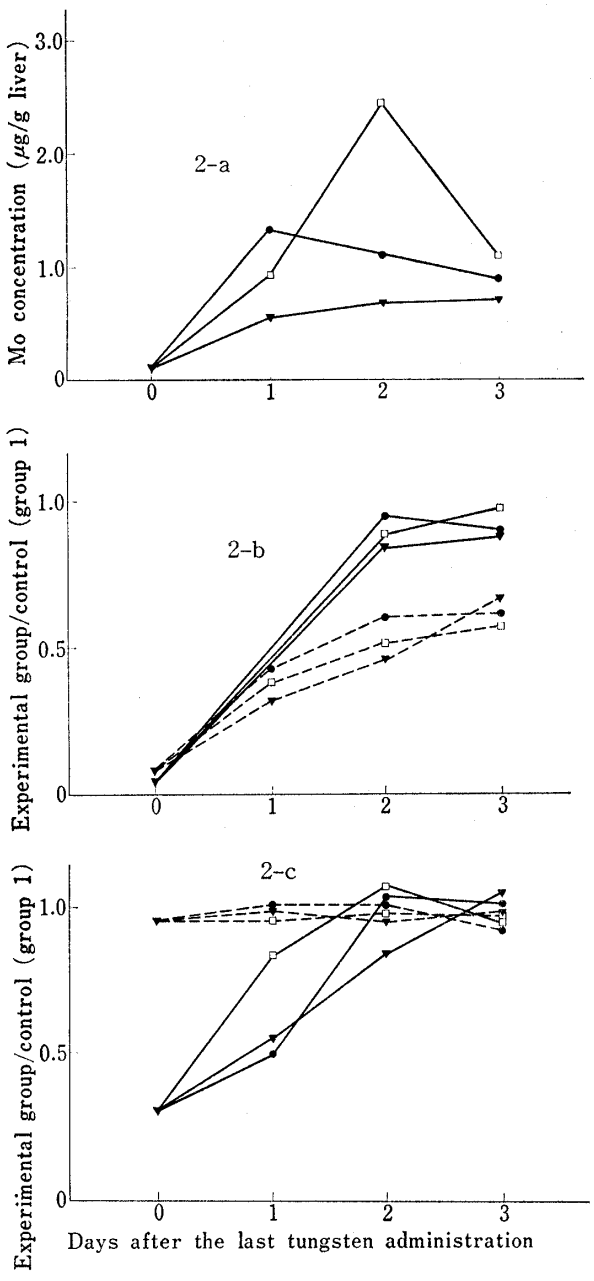

Fig. 2. Effect of CE-2–Molybdenum Administration on the Liver of Mice Administered Tungsten

Four mice were used for each group and each point is expressed as the mean value.

—▼— ---▼--- CE-2 (group 2).

—□— ---□--- high concentration molybdenum (group 3).

—●— ---●--- low concentration molybdenum (group 4).

Fig. 2-a.

Fig. 2-b. ——, xanthine oxidase activity; ------, sulfite oxidase activity.

Fig. 2-c. ——, 4-NQO reductase activity; ------, 4-HAQO reductase activity.

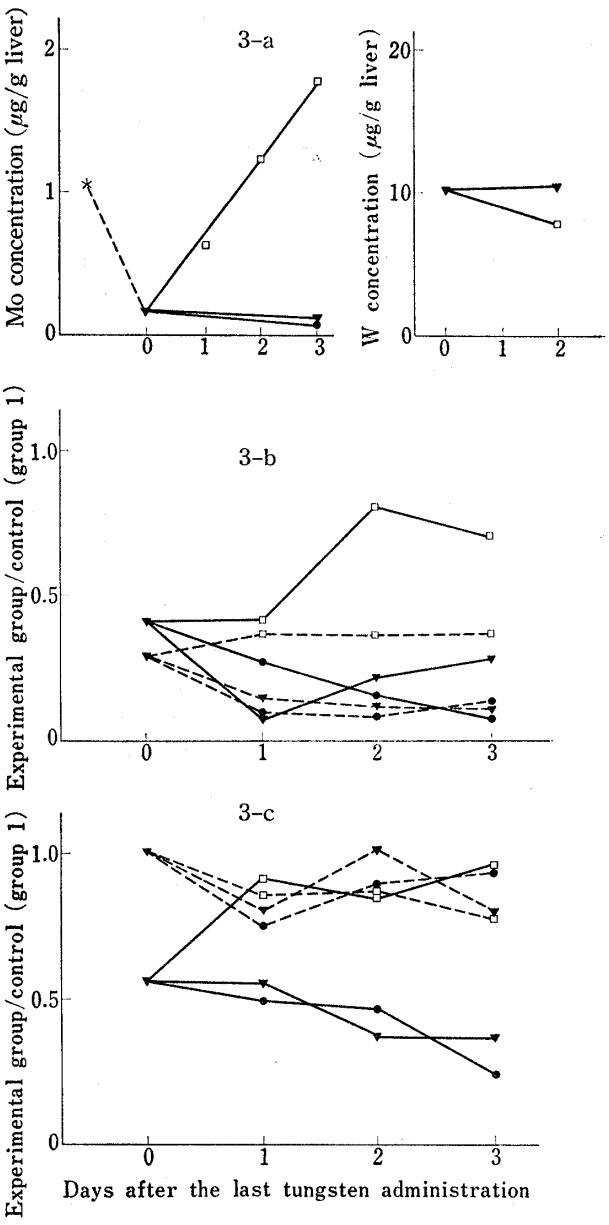

Fig. 3. Effect of Sucrose–Molybdenum Administration on the Liver of Mice Administered Tungsten

—▼— ---▼--- sucrose (group 5).

—●— ---●--- sucrose tungsten (group 6).

—□— ---□--- sucrose-molybdenum (group 7).

Fig. 3-a. The combined tissue of five mice for the measurement of molybdenum was used for each point and the combined tissue of ten mice for the measurement of tungsten was used for each group.
*: no treatment (group 1)

Fig. 3-b. Four mice were used for each group and each point is expressed as the mean value.
——, xanthine oxidase activity;
------, sulfite oxidase activity.

Fig. 3-c. Four mice were used for each group and each point is expressed as the mean value.
------, 4-NQO reductase activity;
——, 4-HAQO reductase activity.

activity was examined by the administration of molybdenum dissolving in the drinking water. Xanthine oxidase, sulfite oxidase and 4-NQO reductase in the liver were used as marker enzymes, as in the foregoing experiment, and variation in the liver molybdenum and tungsten levels was also examined. These results are illustrated in Fig. 2 and 3. As shown in Fig. 2-c, there was no difference in the 4-NQO reductase activity among various groups after administration of tungsten. The activities of xanthine oxidase and sulfite oxidase slightly increased in the groups of molybdenum administration for 2 days (in groups 3 and 4) than in the CE-2 group (group 1), as shown in Fig. 2-b. The difference was no longer apparent on the 3rd day. As shown in Fig. 2-c, 4-HAQO reductase activity also increased by molybdenum administration for 2 days ($p<0.05$), but the difference from CE-2 group became nil after 3 days. These activities were expressed arbitrarily, taking the value of control as 1.0. Thus, 4-HAQO reductase showed the same tendency as other molybdenum-containing enzymes. As will be apparent from Fig. 2-a, showing the amount of molybdenum in the liver, increased activity of various enzymes is considered to be due to the accumulation of molybdenum contained in CE-2 diet in the mouse liver.

Mice were then fed a diet of sucrose, which contains molybdenum below the limit of identification, in stead of molybdenum-containing diet (CE-2) after administration of tungsten, and it was found that the amounts of molybdenum and tungsten (Fig. 3-a) showed a significant difference from sucrose-diet group (group 5) and sucrose-tungsten group (group 6) by the administration of molybdenum (group 7). The amount of tungsten in the group given molybdenum showed approximately 20% decrease of that of sucrose-diet group. By the administration of molybdenum the activities of xanthine oxidase and sulfite oxidase (Fig. 3-b) showed a significant difference from sucrose-diet group and sucrose-tungsten group ($p<0.01$). However, as will be apparent from Fig. 3-c, 4-NQO reductase activity showed no difference among these three groups. The activity of 4-HAQO reductase increased in the group given molybdenum ($p<0.01$) and decreased in the group given sucrose-diet and that given sucrose and tungsten, as shown in Fig. 3-c.

These results supported the possibility that molybdenum might be a requisite for the activity of 4-HAQO reductase *in vivo*.

Discussion

There have been many reports on the carcinogenesis of 4-NQO, and Tada and Tada[19,20] have recently reported the requirement of L-serine for enzymic activation of carcinogen, 4-HAQO. Their reports suggest that the ultimate form of 4-HAQO may be a complex of serine and 4-HAQO. Kawazoe and others[21] studied the metabolism of 4-NQO *in vivo*, by using its labeled compound, found that 4-HAQO is metabolically reduced in a shorter time in the liver than in other organs, and surmised that the reason for 4-NQO not inducing cancer in the liver must be correlated to its rapid metabolism in the liver.

As stated above, level of 4-HAQO in organs might be significant on carcinogenesis by 4-NQO and both 4-NQO reductase activity and 4-HAQO reductase activity affecting the level of 4-HAQO should be investigated further.

Molybdenum is an essential metal for living organism,[22] is contained in xanthine oxidase,[23] sulfite oxidase,[24] and nitrate reductase,[25] and plays an important role in the oxidation or

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reduction of various substrate. Westerfeld and others[26] proved that molybdenum is a requisite for the reduction of organic nitro group by experiments with molybdenum-deficient rats, and Otsuka and others[27] reported that only molybdenum increases, though to a small extent, the reductase activity for the nitro group in *p*-nitrophenol.

It is very important that, as shown in Fig. 2-c and 3-c, 4-NQO reductase activity was not affected by the administration of tungsten, molybdenum-deficiency, while 4-HAQO reductase activity decreased by the administration of tungsten and then recovered by the administration of molybdenum. The fact suggests the possibility that 4-HAQO might be metabolically reduced in a longer time in the molybdenum-deficient liver.

In the previous paper we showed the specificity of molybdenum in the chemical reduction of 4-NQO and 4-HAQO.[13] Stiefel and others[28] suggested the strong affinity between the substrate and molybdenum as the role of molybdenum in the molybdenum-containing enzymes like xanthine oxidase. It was suggested that the accumulation of tungsten in the liver might decrease the activity of 4-HAQO reductase. Therefore, we are studying on the enzyme purification and the role of molybdenum in the enzyme in comparison with those of molybdenum-containing enzymes.

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