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Reduction of Benzydamine N-Oxide by Rat Liver Xanthine Oxidase[1)]

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Evidence is presented that a purified xanthine oxidase preparation, like milk xanthine oxidase, is responsible for the xanthine-dependent reduction of benzydamine N-oxide.

Rat liver preparations contain enzymes which catalyze the anaerobic reduction of benzydamine N-oxide by either reduced nicotinamide adenine dinucleotide (phosphate) NAD(P)H or xanthine. In crude enzyme preparations, the enzymatic reduction seems to involve xanthine oxidase and/or cytochrome P-450 because allopurinol, an inhibitor of xanthine oxidase, and *n*-octylamine, an inhibitor of aliphatic tertiary amine N-oxide reductase (cytochrome P-450) block the reduction of benzydamine N-oxide in crude enzyme preparations of rat liver.

Moreover, the enzymatic system exhibited dual pH optima of 7.4 and 9.0 for reductions dependent on NADPH and xanthine, respectively.

An enzyme preparation which did not contain cytochrome P-450 was isolated from rat liver and purified 186-fold in terms of xanthine oxidase activity. Xanthine oxidase activity and xanthine-dependent benzydamine N-oxide reduction activity were copurified in parallel.

Keywords——rat liver xanthine oxidase; milk xanthine oxidase; cytochrome P-450; NADPH-dependent reduction; dual pH optima; *n*-octylamine; allopurinol; xanthine-dependent reduction

Benzydamine hydrochloride (1-benzyl-3-(3-dimethylaminopropoxy)-1H-indazole hydrochloride, BZY·HCl) is a nonsteroidal analgesic and anti-inflammatory agent. BZY·HCl is metabolized *in vivo* by rats or rabbits to an aliphatic tertiary amine N-oxide (benzydamine N-oxide, BZY–NO), a phenol derivative (1-(*p*-hydroxybenzyl)-3-(3-dimethylaminopropoxy)-1H-indazole) and other compounds.[3a)] BZY and BZY–NO were found to be biotransformed into each other in *in vitro* metabolism studies of BZY·HCl and BZY–NO maleate using rat liver preparations.[3b)] It is generally known that aliphatic tertiary amines undergo N-oxygenation and oxidative N-dealkylation and that N-oxygenation product, an N-oxide, can be converted to the parent tertiary amine.[4)] Heteroaromatic amine N-oxides are known to be reduced anaerobically by milk or mammalian liver xanthine oxidase (EC 1.2.3.2).[5,6)] Sugiura *et al.*[7)] showed that the aliphatic amine N-oxides of imipramine, tiaramide and N,N-dimethylaniline were reduced by microsomal cytochrome P-450, but not by xanthine oxidase (XOD) in rat liver. In the present paper, we report several lines of evidence indicating that

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2) Location: *1–14, Bunkyo-machi, Nagasaki, 852, Japan.*
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BZY–NO may be reduced by XOD in crude and purified preparations of rat liver as well as by milk XOD.

Materials and Methods

Chemicals——BZY·HCl was kindly provided by Yoshitomi Pharmaceutical Co., Ltd. and Daiichi Pharmaceutical Co., Ltd. BZY-NO hydrogen maleate (BZY-NO maleate) was synthesized according to the method given in the previous paper.[3] Milk XOD (12.6 units/ml) was purchased from Sigma Chemical Co., Ltd. Glucose 6-phosphate dehydrogenase (G6PDH) was obtained from Boehringer Mannhiem. All chemical used were of analytical grade.

Tissue Preparations——Male Wistar-strain rats weighing about 120 g were killed by cervical fracture and exsanguinated. The livers were removed after perfusion with 0.9% NaCl, and homogenized with a Teflon-glass homogenizer in three volumes of 25 mM Tris–1.15% KCl (pH 7.4); H_2O was used for estimation of the optimal pH in the xanthine-dependent reduction of BZY-NO. For subcellular fractionation of liver homogenates, the homogenates were centrifuged at 4° in a Sakuma SMC 45CFS centrifuge at $600 \times g$ for 10 min. The sediment is referred to as the nuclear fraction, and the supernatant phase was centrifuged at $9000 \times g$ for 10 min to give a mitochondrial fraction. The postmitochondrial supernatant phase was further centrifuged at $105000 \times g$ for 60 min in a Hitachi 40P ultracentrifuge. The sediment and the supernatant phase were referred to as microsomal and soluble fractions, respectively. All fractions were made up to the same volume as the original whole homogenate with 25 mM Tris–1.15% KCl (pH 7.4).

For the purification of liver XOD, livers were homogenized with four volumes of 0.25 M sucrose containing 10 μM ethylenediaminetetraacetic acid (EDTA), and the homogenate was centrifuged at $27000 \times g$ for 30 min. The resulting supernatant fraction was used as a starting material.

Protein content was determined according to the method of Lowry *et al.*[8]

Purification of Liver XOD——XOD was purified from rat liver essentially by the method of Waud and Rajagopalan[9] as follows: step 1 ($27000 \times g$ supernatant fraction), step 3 (hydroxylapatite eluate), step 4 (43% acetone precipitate) and step 5 (pooled DEAE-cellulose eluate). The ammonium sulfate fraction (step 2) was prepared by the method of Della Corte *et al.*[10] (Table VI).

Thin-Layer Chromatography——Thin-layer chromatography (TLC) was carried out on 0.20 mm Silicagel 60 F_{254} pre-coated aluminum sheet (E. Merck) using benzene–chloroform–methanol–ethanol–ammonia (Sp. gr. 0.9) (15: 15: 10: 5: 0.5) as a developing solvent.

Determination of Reduction of BZY-NO——The standard incubation mixture (3 ml) contained 50 mM Tris–HCl buffer, pH 7.4, 1.0 mM BZY-NO, 0.5 ml of enzyme solution (equivalent to 125 mg of liver), and an reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of 0.5 mM nicotinamide adenine dinucleotide phosphate (NADP), 2 mM glucose 6-phosphate, 1.4 units of G6PDH and 10 mM $MgCl_2$. The incubation mixture was transferred to a reaction tube of 15 ml volume which was chilled in ice-water. Nitrogen gas flushing of the reaction tube was carried out for 3 min before and after the addition of BZY-NO solution, and the reaction tube was sealed with a tight glass cap. The incubation was started by placing the reaction tube in a metabolic shaker at 37°, and was stopped 15 min later by immersing the reaction tube in an ice-water bath. Variations of the reaction conditions and additions to the incubation mixture are given in the legends to tables and figures. BZY-NO metabolites were extracted twice with two volumes of ethyl acetate after the addition of 0.2 ml of 1 N NaOH. The combined extracts were dried over sodium sulfate, and concentrated under reduced pressure. The residue was dissolved in 0.5 ml methanol, and 50 μl of the solution was applied to a TLC plate. After development, BZY was detected as a blue spot under ultraviolet light. BZY on the aluminum plate was extracted with 4 ml of methanol. The fluorescence intensity of BZY was measured at 355 nm (emission wavelength) and 305 nm (activation wavelength). The overall recovery of BZY (3 μmol) added to heat-inactivated liver fractions was 69.8±4.7% (mean ±S.D.).

Assays of XOD and Cytochrome P-450——The XOD activity was measured at room temperature in 3 ml of 0.1 M Tris–HCl buffer, pH 8.1, containing 0.2 mM xanthine. An extinction coefficient difference at 295 nm between xanthine and uric acid of $9.6 \times 10^3\ M^{-1}$ was used to convert the observed absorbance change into nmol of uric acid formed per min.[11] The enzyme activities were corrected by subtracting a blank rate obtained in the absence of xanthine.

Cytochrome P-450 was determined by the method of Omura and Sato.[12] These measurements were performed with a Hitachi 323 recording spectrometer.

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Results and Discussion

Effect of Cofactors on the Anaerobic Reduction of BZY-NO

The reducing activity appeared to be greater under anaerobic conditions than under aerobic conditions, and was considerably reduced by heating the enzyme preparation before incubation (Table I). In addition, reduced pyridine nucleotides and/or flavin mononucleotide (FMN) enhanced the reduction of BZY–NO (Tables I and II). Furthermore, as shown in Table II, NADPH was active, as well as NADH, in the reduction of BZY–NO, but the co-addition of NADH and NADPH showed only an additive effect on the reduction of the N-oxide. On the other hand, when FMN was added with NADPH or NADH, the reduction of BZY–NO increased synergistically. The above results indicate clearly that the reduction of BZY–NO involved enzyme systems requiring reduced pyridine nucleotides and or FMN as cofactors.

Table I. Effect of NADPH on the Reduction of Benzydamine N-Oxide by Rat Liver Whole Homogenate under Air or N_2[a)]

Atmosphere	Pretreatment	NADPH	BZY formed[b)] (nmol/g liver/min)
Air	—	−	24.1±3.2
Air	100°	−	5.9±0.3
N_2	—	−	36.6±3.4
N_2	—	+	67.4±2.5

a) Preparation from the liver of one rat. The reactions were carried out at 37° for 30 min.
b) The activity is presented as the mean±S.E. of triplicate values.

Table II. Cofactor Requirement for the Reduction of Benzydamine N-Oxide in Rat Liver Whole Homogenate[a)]

NADH	NADPH	FMN	BZY formed[b)] (nmol/g liver/min)
−	−	−	31.5± 1.2
+	−	−	86.5± 1.3
−	+	−	89.0± 4.4
−	−	+	162.4± 6.4
+	+	−	123.1± 1.7
+	−	+	341.0±19.2
−	+	+	531.3± 7.0
+	+	+	595.0± 0.8

a) Preparation from the pooled livers of two rats. The incubation mixture contained three cofactors, an NADH-, NADPH-generating system and FMN (0.5 mM final conc.). The NADH-generating system was the same as the NADPH-generating system except for the use of NAD instead of NADP.
b) The activity is presented as the mean±S.E. of triplicate values.

Time Course and Optimal pH in NADPH-Dependent Reduction of BZY-NO

Since the effect of NADPH was almost the same as that of NADH on the reduction of BZY-NO, NADPH was used as a cofactor for the reduction of the N-oxide. The NADPH-dependent reductase activity appeared to be almost linear with time for at least the first 20 minutes in the presence of FMN (Fig. 1), and was shown to have an optimal pH in the range from 7.0 to 7.5 with incubation mixtures omitting FMN (Fig. 2). The NADPH-dependent reduction of BZY–NO was therefore carried out at pH 7.4 throughout this work.

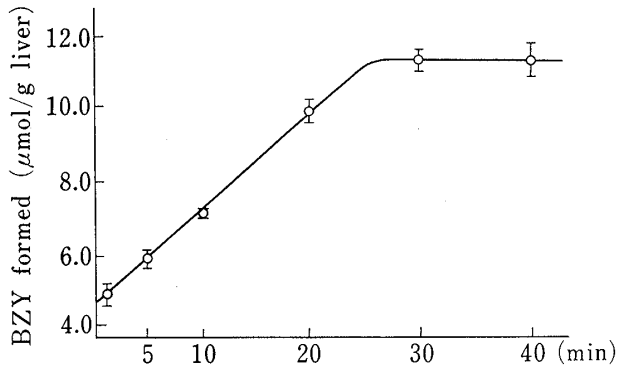

Fig. 1. Effect of Incubation Time on the Reduction of Benzydamine N-Oxide in Rat Liver Whole Homogenate[a]

a) Preparation from the pooled livers of two rats. The incubations were carried out with 125 mg of liver, an NADPH-generating system and FMN (0.5 mM final conc.) at 37° in N_2. The vertical lines indicate mean ±S.D. of triplicate values.

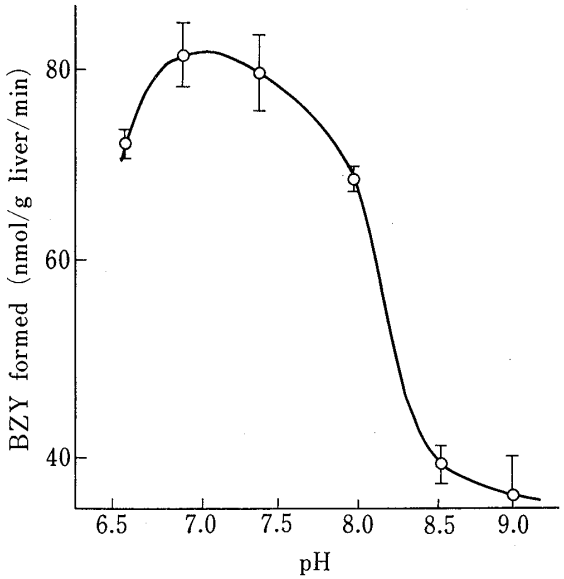

Fig. 2. Effect of pH on the NADPH-Dependent Reduction of Benzydamine N-Oxide under N_2[a]

a) Reaction mixtures consisting of an NADPH-generating system and liver whole homogenate corresponding to 125 mg of liver were incubated under N_2 for 30 min at 37°. The vertical lines indicate mean ±S.D. of triplicate values.

Subcellular Distribution of BZY-NO Reductase Activity

The subcellular distribution of the NADPH-dependent BZY–NO reductase in rat liver and the effect of 2-mercaptoethanol were examined. The data in Table III show that the reduction of BZY–NO was enhanced by 2-mercaptoethanol in all subfractions, but not in whole homogenates. Furthermore, BZY–NO reductase activity was distributed in all fractions regardless of the presence of the 2-mercaptoethanol. In particular, it is of interest to have found a considerable reducing activity in the soluble fraction in addition to the microsomal fraction. Similarly, no clearcut localization has been reported for the reduction of (—)-nicotine-1′-N-oxide[13] or imipramine-N-oxide[14] in rat liver subfractions. These observations suggest that XOD, by donating an electron to heteroaromatic amine-N-oxides such as nicotinamide-[5] and purine-N-oxide,[6] might serve as a reducing enzyme for BZY–NO.

Effect of pH on the Xanthine-Dependent Reduction of BZY-NO

Because of the possibility that XOD may participate in the reduction of BZY–NO in the subfractions of rat liver, the xanthine-dependent reduction of BZY–NO was first examined at various pHs. As shown in Fig. 3, the optimal pH in the xantine-dependent reduction of BZY–NO was 9.0, and was different from the optimal pH of the NADPH-dependent reduction. Such dual optimal pH values have already been reported for the NADH-dependent and hypoxanthine-dependent reductions of nicotinamide-N-oxide[5] and purine-N-oxide.[6]

According to the literature,[5,6] NADH and NADPH serve as electron donors for XOD, and the former functions more effectively than the latter. In contrast to these reports, Dajani *et al.*[13] reported that NADPH was more effective than NADH in the anaerobic reduction of (—)-nicotine-1′-N-oxide with various subfractions of rat liver and small intestine. The discrepancy with respect to the cofactor requirements seems to have arisen because the

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Table III. Subcellular Distribution of Benzydamine N-Oxide Reducing Enzyme(s)[a]

Subfraction	BZY formed (nmol/g liver/min) Mercaptoethanol[b]	
	Without	Added
Whole homogenate	836.7±11.9(100)	885.3± 9.5(100)
600×*g* pallet	571.8± 2.2(68.5)	760.3± 9.8(86.2)
9000×*g* pellet	200.3±15.3(24.0)	379.3± 7.9(42.9)
9000×*g* sup.	854.0±15.0(102.1)	1011.2± 7.0(114.6)
Microsomes	517.7±12.0(62.1)	875.7±15.9(99.0)
Soluble fraction	300.7± 6.0(35.9)	563.5± 9.5(63.8)

a) Preparation from the pooled livers of three rats. Reaction mixtures with an NADPH-generating system, 125 mg of liver and 0.5 mM of FMN were incubated under N_2 for 15 min at 37°. The acivity is presented as the mean±S.E. of triplicate values. Numbers in parentheses indicate % of whole homogenate.
b) 1.1×10^{-1} M of 2-mercaptoethanol (final concentration) was added as indicated.

reduction of various N-oxides, including BZY–NO, is not only due to the action of NADH- and/or NADPH-dependent XOD, but also to the action of NADPH-dependent cytochrome P-450 located in the microsomal fraction of rat liver, as proposed by Sugiura *et al.*[6]

Effect of *n*-Octylamine or Allopurinol on the NADPH-, or Xanthine-Dependent Reduction of BZY-NO

In order to clarify to what extents cytochrome P-450 and XOD are involved in the reduction of BZY–NO, the effects of *n*-octylamine (NOA)[7] and allopurinol (ALP),[15] potent inhibitors of cytochrome P-450 and XOD, respectively, were examined on the reduction of the N-oxide in rat liver subfractions (Table IV).

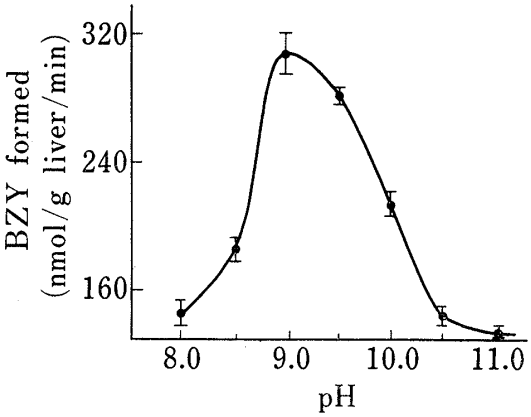

Fig. 3. pH Profile of the Xanthine-Dependent Reduction of Benzydamine N-Oxide under N_2[a]

a) Reaction mixtures consisting of liver 9000×*g* sup. corresponding to 125 mg of liver, 0.5 mM FMN, 0.5 mM xanthine, 1.1×10^{-1} M 2-mercaptoethanol, 1.0 mM EDTA and Tris-phosphate-carbonate buffer were incubated under N_2 for 15 min at 37°. The final concentration of buffer components was 133 mM. The vertical lines indicate mean±S.D. of triplicate values.

The xanthine-dependent reduction of BZY–NO was greater than the NADPH-dependent reduction in the soluble fraction. NOA at a concentration of 1 mM effectively inhibited the NADPH-dependent reduction of BZY–NO in all the subfractions, including the soluble fraction, in which cytochrome P-450 was absent. This may mean that NOA inhibits not only the cytochrome P-450 dependent reduction, but also NADPH-dependent XOD. On the other hand, the xanthine-dependent reduction in the 9000×*g* supernatant fraction was little affected by ALP (1 mM) and that in the microsomal or soluble fraction was rather stimulated by ALP. In general, XOD preparations have been dialyzed so as to remove endogenous purines prior to assay.[16] Therefore, when the soluble fraction including XOD was dialyzed for 20 hr at 0° against 200 volumes of water, the specific activity in the xanthine-dependent reduction of BZY–NO increased and was inhibited by ALP. On the basis of the inhibitory effect of ALP on the xanthine-dependent reduction of BZY–NO by the dialyzed soluble

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Table IV. Effects of *n*-Octylamine and Allopurinol on NADPH- and Xanthine-Dependent Benzydamine N-Oxide Reduction in Rat Liver 9000×*g* Sup., Microsomal and Soluble Fractions[a]

Cell fraction	Inhibitor NOA (1 mM)	NADPH-dependent reduction: BZY formed (nmol/mg prot./15 min)	NADPH-dependent reduction: Inhibition (%)	Inhibitor ALP (1 mM)	Xanthine-dependent reduction: BZY formed (nmol/mg prot./15 min)	Xanthine-dependent reduction: Inhibition (%)
9000×*g* sup.	−	90.3±4.2		−	97.2±1.4	
	+	45.6±2.6	48.4	+	92.0±1.6	5.4
Microsomes	−	118.5±0.6		−	141.2±4.6	
	+	52.1±2.4	56.2	+	212.6±5.4	−50.8[b]
Soluble fractions (Undialyzed)	−	36.6±1.7		−	71.1±2.3	
	+	10.4±0.5	71.5	+	110.1±3.0	−55.0[b]
(Dialyzed prior to assay)[c]				−	212.3±6.5	
				+	151.8±2.2	28.5

a) Preparation from the pooled livers of three rats. Reaction mixtures for NADPH- and xanthine-dependent reduction were prepared as described in the legends to Table III and Fig, 3, but the former included 2-mercaptoethanol, while the latter was adjusted to pH 9.0 with Tris-phosphate-carbonate buffer. The activity is presented as the mean±S.E. of triplitate values.
b) Stimulated compared with the reaction in the absence of ALP.
c) Dialyzed against H_2O at 0° for 20 hr.

fraction, it is suggested that XOD may play an important role in the reduction of BZY–NO, even though the activities attributable to cytochrome P-450 and XOD are not distinguishable. However, at present we cannot explain why the xanthine-dependent reduction of BZY–NO in the undialyzed preparations was stimulated by ALP.

Effects of *n*-Octylamine and Allopurinol on the Reduction of BZY-NO by Milk Xanthine Oxidase Preparation

The effects of NOA and ALP on the reduction of BZY–NO by milk XOD preparation were examined in the NADPH- and xanthine-dependent reactions (Table V).

Table V. Effects of *n*-Octylamine and Allopurinol on NADPH- and Xanthine-Dependent Benzydamine N-Oxide Reduction by Milk Xanthine Oxidase[a]

Inhibitor	BZY formed (nmol/mg prot./min)	Inhibition (%)
	NADPH-dependent	
—	29.7±0.7	
NOA	11.6±0.1	61.0
ALP	31.9±1.1	−7.4
	Xanthine-dependent	
—	69.0±1.3	
NOA	47.7±1.8	30.9
ALP	18.6±1.8	73.0

a) The incubations were carried out with 0.189 unit of milk xanthine oxidase (0.3 mg prot.) under N_2 at 37° for 30 min. Reaction mixtures for NADPH- and xanthine-dependent reductions are the same as those described in the legend to Table IV, but water was used instead of liver preparations. The activity is presented as the mean±S.E. of triplicate values.

As shown in Table V, NADPH- and xanthine-dependent reduction of BZY–NO evidently took place with the milk XOD, and were inhibited in the same way as in rat liver fractions by NOA and ALP, except that ALP had no inhibitory effect on NADPH-dependent reduction. These findings suggested strongly that the enzymatic reduction of BZY–NO in rat

liver is dependent on XOD. In addition, it is interesting that NOA also showed an inhibitory effect on XOD.

Purification and Activity of Rat Liver XOD

The above results suggest that XOD might also participate to some degree in the reduction of BZY–NO. Accordingly, XOD from rat liver was purified to compare the activities of XOD and the xanthine-dependent BZY–NO reductase system at each purification step (Table VI). The specific activities of both XOD and xanthine-dependent BZY–NO reductase increased in parallel, although it appears that the yield of xanthine-dependent BZY–NO reductase is larger in the hydroxylapatite eluate than in the ammonium sulfate fraction. In particular, the specific activities of XOD and xanthine-dependent BZY–NO reductase in the DEAE-cellulose eluates (the combined fractions 36 to 45 in Fig. 4) showed 186- and 655-fold increases in XOD and xanthine-dependent BZY–NO reductase, respectively, compared with the starting crude extract (purification step 1). Further, it was confirmed that each fraction from purification steps 1 to 4 contained various amounts of cytochrome P-450 and/or P-420 (Table VII). Therefore, it seems clear that BZY–NO is reduced in a xan-

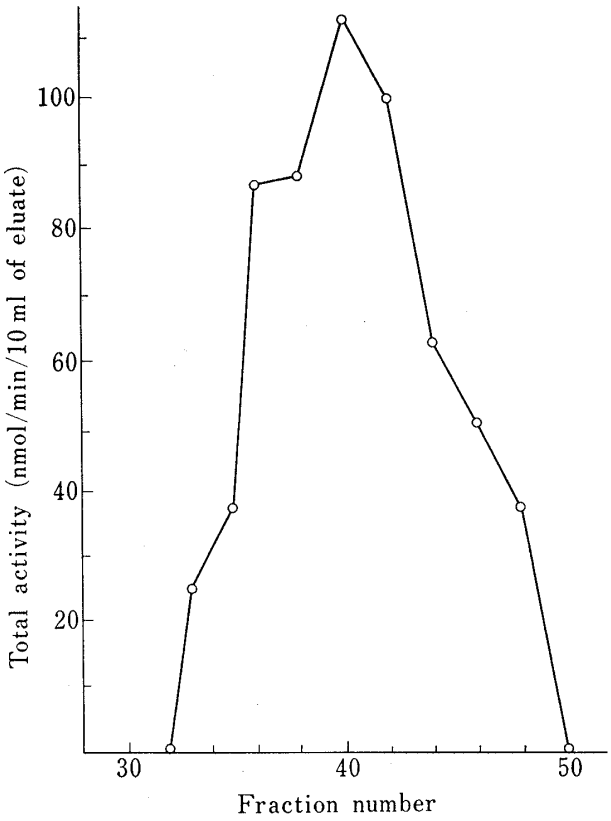

Fig. 4. Elution Pattern of Rat Liver XOD from DEAE-Cellulose[a]

a) 43% acetone precipitate was dissolved in 5 mM phosphate buffer, pH 7.4 (containing 1 mM EDTA), and dialyzed against this buffer for 24 hr. The dialyzed solution was then applied to a 12×2.5 cm DEAE-cellulose (Brown) column. The column was eluted with a linear granient of 5 mM to 50 mM phosphate buffer, pH 7.4 (containing 10 μM EDTA), at a flow rate of about 10 ml/30 min. The volume of each buffer was 300 ml, and 10 ml fractions were collected. Total activities for the early and late fractions were not calculated since the XOD activity was very low.

Table VI. Activities of Xanthine Oxidase and Xanthine-Dependent Benzydamine N-Oxide Reductase

Purification steps[a]	Volume (ml)	Protein (mg/ml)	Enzyme activity		Total enzyme activity		Specific enzyme activity		Yields		Relative activity	
			XOD (nmol/ml/min)	NOR[b] (nmol/ml/min)	XOD (μmol/min)	NOR[b] (μmol/min)	XOD (nmol/min /mg prot.)	NOR[b] (nmol/min /mg prot.)	XOD (%)	NOR[b] (%)	XOD	NOR[b]
1. 27000×*g* supernatant	105	12.4	71.9	627.3	7.56	65.8	5.8	50.5	100	100	1	1
2. $(NH_4)_2SO_4$ precipitate	25	5.24	297.2	628.7	7.42	15.7	56.8	120.0	98.2	23.8	9.8	2.4
3. Hydroxyl-apatite eluate	73.7	0.457	51.9	500.7	3.82	36.9	113.2	1090.0	50.6	56.0	19.5	21.6
4. 43% acetone precipitate	35.4	0.182	17.5	379.3	0.62	13.4	96.2	2080.0	8.2	20.4	16.6	41.1
5. DEAE-cellulose eluate[c]	4.25	0.013	14.4	440.0	0.06	1.9	1080.0	33100	0.8	2.9	186.0	655.0

a) All fractions except for the DEAE-cellulose eluate were dialyzed for 24 hr against 200 volumes of 1 mM phosphate buffer, pH 7.4, containing 1 mM EDTA.
b) NOR; xanthine-dependent benzydamine N-oxide reductase. Reaction mixture and conditions for xanthine-dependent reduction were as in Table IV.
c) Fractions 36 to 46 shown in Fig. 4 were pooled, and concentrated with a Millipore XX42-025 ultrafiltration cell fitted with a Pellicon PSED membrane (Millipore Co., Bedford, Massachusetts).

Table VII. Contents of Cytochrome P-450 and P-420 in Each Fraction

Purification steps	Absorbance/3 ml of assay mixture[a]		nmol/3 ml of assay mixture[b]		Total amounts nmol	
	P-450	P-420	P-450	P-420	P-450	P-420
1. 27000×*g* supernatant	negative absorbance	0.340	—	9.28	—	975.0(100)[c]
2. $(NH_4)_2SO_4$ precipitate	0.013	0.055	0.440	1.50	11.0	37.5(3.9)[c]
3. Hydroxylapatite eluate	0.007	0.008	0.231	0.218	17.0	16.1(1.7)[c]
4. 43% acetone precipitate	0	0.006	—	0.164	—	5.8(0.6)[c]
5. DEAE-cellulose eluate[d]	No measurable		—	—	—	—

a) Three ml of assay mixture contained 1.0 ml of enzyme preparation in 50 mM phosphate buffer, pH 7.6.
b) Contents of cytochrome P-450 and P-420 were computed from the absorbance as nmoles of P-450 and P-420 using extiction coeffcients of 91 cm^{-1} mM^{-1} at 450—490 nm for the former and of 110 cm^{-1} mM^{-1} at 420—490 nm for the latter.
c) Values in parentheses are expressed as percentages of the 27000×*g* supernatant fraction.
d) Fractions 36 to 46 in Fig. 4 were pooled, and concentrated by ultrafiltration.

thine-dependent reaction system by the purified preparation (DEAE-cellulose eluate) of rat liver XOD as well as milk XOD.

Although it is well known that NADH is generally more effective than NADPH with XOD,[17] in view of the findings that NADPH exhibited the same effect as NADH (Table II) and that the crude enzyme preparations contained cytochrome P-540 and/or P-420 (Table VII), we can not rule out the possibility that NADPH-dependent reduction of BZY–NO is catalyzed not only by XOD but also by cytochrome P-450, at least in the microsomal fraction of rat liver.

Further studies on N-oxide reduction in purified rat liver XOD are now in progress with various types of tertiary amine N-oxides, including heteroaromatic amines, and will be reported elsewhere.

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17) R.C. Bray, "The Enzymes," Vol. 12 (Part B), ed. by P.D. Boyer, Academic Press, 1975, p. 299.