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Effect of Carrageenin Treatment on the Metabolism of Benzydamine and Its N-Oxide in Rat Organ Preparations

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The present study was performed to clarify the *in vitro* metabolism of benzydamine hydrochloride (BZY·HCl) and benzydamine N-oxide hydrogen maleate (BZY-NO maleate) in rat tissues and the livers of several animals, and to examine the effects of carrageenin on the N-oxygenation and N-demethylation of BZY·HCl, as well as on the N-deoxygenation of BZY-NO maleate in rat blood and liver. The results were as follows. 1) BZY·HCl was mainly metabolized by liver microsomal fraction in the presence of an reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system and in air. 2) The main reactions were N-oxygenation and N-demethylation, and the former proceeded more rapidly than the latter in all animal species except the guinea pig. 3) No effect of carrageenin on the N-oxygenation and N-demethylation of BZY·HCl was found on comparing liver preparations of control and carrageenin-treated rats. 4) The N-deoxygenation of BZY-NO maleate occurred to the extent of only a few percent in blood, but was significant in the hemolysate (hemoglobin) of erythrocytes, however, the production of methemoglobin was very slight. 5) On the other hand, carrageenin treatment inhibited the N-deoxygenation of BZY-NO maleate in liver preparations, but not in the hemolysate of erythrocytes. The present results account for our previous observation that the N-oxygenation of BZY·HCl in rats is apparently enhanced in the body by carrageenin treatment. Namely, it is suggested that the phenomenon was ascribable not to stimulation of the N-oxygenation of BZY, but to inhibition of the N-deoxygenation of BZY-NO in the liver.

These results are discussed in connection with the anti-inflammatory actions of BZY·HCl.

Keywords——benzydamine; benzydamine N-oxide; fluorimetric determination; N-oxygenation; N-deoxygenation; carrageenin-treatment

In order to understand the mechanism of anti-inflammatory action of the nonsteroidal analgesic and anti-inflammatory agent, benzydamine hydrochloride (BZY·HCl, 1-benzyl-3-(3-dimethylaminopropoxy)-1*H*-indazole hydrochloride), the authors have investigated the *in vivo* metabolism of the drug using several animals.[2,3]

In these previous studies, it has been found that benzydamine N-oxide (BZY–NO, 1-benzyl-3-(3-dimethylaminopropoxy)-1*H*-indazole N-oxide) and 1-(*p*-hydroxybenzyl)-3-(3-dimethylaminopropoxy)-1*H*-indazole (HO–BZY) were excreted as major unconjugated metabolites together with three unknown glucuronides in the urine of rabbits given BZY·HCl.[2] Further, it was recognized that the ratio of conjugated metabolites to unconjugated ones in the urine varied from species to species: more unconjugated metabolites were present than conjugated ones in cats, dogs and rats, whereas the opposite was the case in guinea pigs, mice and rabbits.[2] Similar features of benzydamine (BZY) metabolites were noted in the blood of rat and rabbit; that is, in rats, the unconjugated metabolites BZY and BZY–NO

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2) S. Kataoka, K. Taira, and E. Takabatake, *Chem. Pharm. Bull.* (Tokyo), **19**, 1511 (1971); S. Kataoka, K. Taira, T. Ariyoshi, and E. Takabatake, *Chem. Pharm. Bull.* (Tokyo), **21**, 358 (1973).
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were predominant, but in rabbits, glucuronide (G-1) was the major metabolite.[3] Moreover, it was reported that BZY–NO was present in the blood and the inflamed paw of rats treated with the phlorogogenic compound carrageenin to prepare an inflammation model.[3] These results provide a basis for evaluating the anti-inflammatory action of BZY·HCl in the light of its metabolism.

However, the *in vitro* metabolism of BZY·HCl has not been investigated so far. Therefore, an *in vitro* study was first carried out to identify the enzyme system involved, using isolated tissues of rat and liver preparations of various animal species. Secondly, the effects of carrageenin on the N-oxygenation and N-demethylation of BZY·HCl and on the N-deoxygenation of BZY–NO hydrogen maleate were examined, using blood and liver preparations of control and carragennin-treated rats.

These results are discussed in connection with the anti-inflammatory action of BZY·HCl.

Materials and Methods

Chemicals——BZY·HCl was a gift from Yoshitomi Pharmaceutical Co. Ltd. and Daiichi Pharmaceutical Co. Ltd. 1-Benzyl-3-(3-methylaminopropoxy)-1*H*-indazole hydrochloride (Nor-BZY·HCl) was kindly provided by Dr. B. Silbestrini, A.C.R. Angelini, Rome, Italy. 1-Benzyl-3-(3-dimethylaminopropoxy)-1*H*-indazole N-oxide hydrogen maleate (BZY-NO maleate) was synthesized according to the previous paper.[2] Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate (G6P) and glucose 6-phosphate dehydrogenase (G6PDH) were obtained from Boehringer Mannheim Co. Ltd., and other chemicals were of analytical grade, obtained commercially.

Animals——The animals used were ddN mice (30—35 g), Wistar rats (120—150 g), guinea pigs (350—450 g) and white rabbits (2.5—3.0 kg). All were male, and were maintained on commercial chow and water *ad libitum*.

Enzyme Preparation——The enzyme preparations used in the present paper were prepared from the livers of the mouse, guinea pig and rabbit, and from various organs including blood of the rat. The animals were sacrificed by decapitation or by means of a blow on the head. Various organs of rats were homogenized in a Potter–Elvehjem homogenizer with aliquots of ice-cold 1.15% KCl, pH 7.4, while 25% liver homogenates were prepared with 0.25 M sucrose (containing 1 mM Na ethylenediaminetetraacetic acid (EDTA)), pH 7.4 for subcellular fractionation. The fractionation was carried out as follows: the 25% liver homogenate was differentially centrifuged at $600 \times g$ and $9000 \times g$ for 10 min. The resulting supernatant fraction was then centrifuged at $105000 \times g$ for 60 min. The microsomal pellet was suspended in 1.15% KCl/25 mM Tris buffer, pH 7.4, and diluted to the original volume. In some experiments, Ca^{2+}-sedimentable microsomes were prepared according to the Ca^{2+} precipitation method of Cinti *et al.*[4] as follows: livers were homogenized in 3 volumes of 0.25 M sucrose solution (omitting Na EDTA), and the $9000 \times g$ supernatant fraction was prepared as described above, diluted with 5 volumes of 1.25 mM sucrose solution containing 9.6×10^{-3} M $CaCl_2$, well shaken, and centrifuged at $3000 \times g$ for 10 min. The microsomal pellet obtained was suspended in 1.15% KCl/25 mM Tris buffer, pH 7.4, diluted to a final concentration of 0.4 g liver/ml, and used as the enzyme source, in addition to each fraction described above.

BZY·HCl Metabolism by Various Tissue Preparations——a) Assay of N-Demethylation by Liver $9000 \times g$ Supernatant and Microsomal Fraction: The total volume of the incubation mixture was 3 ml, and the constituents were 50 mM Tris buffer, pH 7.4, 10 mM $MgCl_2$, 2 mM G6P, 0.1 mM NADP, 0.5 mM BZY·HCl as a substrate and $9000 \times g$ supernatant fraction corresponding to 200 mg of liver, or alternatively 50 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, 2.4 mM G6P, 0.33 mM NADP, 1.4 units G6PDH, 0.5 mM BZY·HCl and microsomal fraction corresponding to 0.5 or 1.0 g of liver. With the $9000 \times g$ supernatant fraction, incubation was performed at 37° for a selected time in air. With the microsomal fraction, incubation was carried out at 37° for 5 min prior to the addition of the substrate, then continued after addition of the substrate for a selected time, and was terminated by the addition of 1.5 ml each of saturated $Ba(OH)_2$ and 20% $ZnSO_4$, or by the addition of 1.5 ml of 20% trichloroacetic acid solution. The mixture was centrifuged at 10000 rpm for 10 min. The supernatant fraction was used to determine the formaldehyde liberated into the reaction mixture according to the method of Nash.[5] Control incubations were run omitting BZY·HCl. The standard flasks consisted of known amounts of formaldehyde and the incubation mixture except for the substrate, and were incubated in the same manner. Protein was determined by the Lowry method[6] using bovine serum albumin as a standard.

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b) Assay of BZY·HCl Metabolism by the Fluorimetric Method: The constituents of the incubation mixture were the same as in method a), but the total volume was 0.6 ml and the amount of whole homogenate or $9000\times g$ supernatant fraction used was equivalent to 2.5—40.0 mg of tissues. The incubation was started by the addition of 2.0 μg of BZY·HCl, and was stopped by immersing the incubation flask in boiling water for 5 min. The activity of BZY·HCl metabolism was calculated by measuring the rate of BZY·HCl disappearance from the incubation mixture. The unchanged BZY·HCl was extracted according to the procedure in Chart 1, and was determined fluorimetrically by the method reported previously.[2]

c) Determination of BZY Metabolites: After adding 0.2 ml of 1 N NaOH to the incubation mixture, BZY metabolites (BZY, BZY-NO and Nor-BZY) were extracted twice with two volumes of ethyl acetate. The combined extracts were dried over sodium sulfate, and evaporated down under reduced pressure. The separation and determination of BZY metabolites from the residue (methanol solution) were performed as described in the previous papers.[2,3]

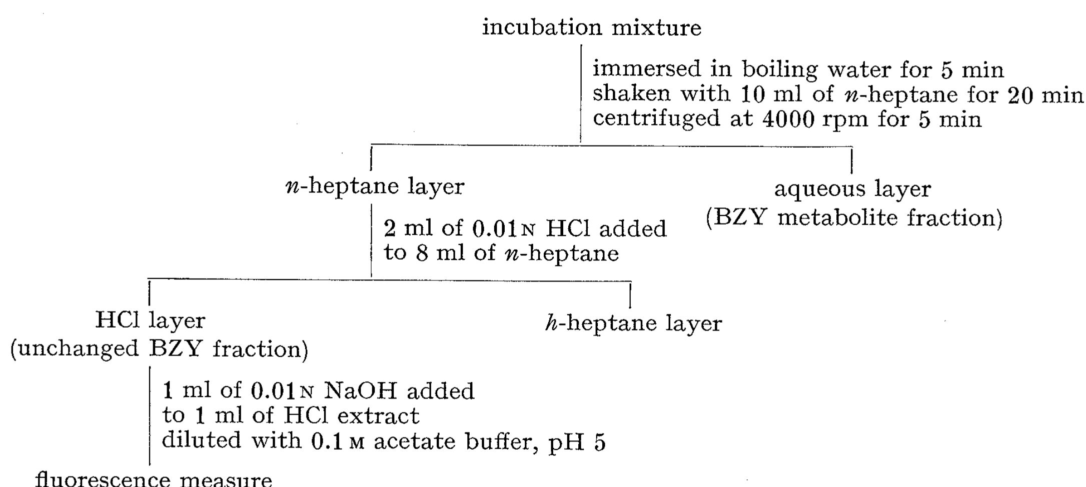

Chart 1. Extraction of Unchanged BZY HCl from Incubation Mixtures

N-Deoxygenation of BZY-NO Maleate by Whole Blood, Plasma, Erythrocytes or Hemoglobin——The N-deoxygenation of BZY-NO maleate by whole blood, plasma or erythrocytes was examined. Further, the ability of hemoglobin to deoxygenate BZY-NO maleate was measured together with the amount of hemoglobins released from the erythrocytes in a hypotonic solution. The hemoglobin was prepared by the procedure of Seeman and Weinstein[7] with minor modifications. In brief, plasma and erythrocytes were separated by centrifuging heparinized blood at 3000 rpm for 10 min, and the erythrocytes were washed three times with saline. A stock suspension of rat erythrocytes was prepared as a 50% suspension in saline. The isotonic salt solution consisted of 154 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4. The hypotonic solution contained 20—120 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4. The experiments on the hemolysis of erythrocytes were carried out as follows: 0.2 ml of the erythrocyte suspension (5% erythrocytes in saline) was mixed with 3.8 ml of hypotonic solution, then the mixture was incubated in air at 37° for 10 min, and centrifuged for 10 min at 3500 rpm. The percentage of hemolysis was calculated after measuring the extinction of the mixture at 543 nm. The experiments on the N-deoxygenation of BZY-NO maleate were carried out as follows: 0.25 ml of the erythrocyte suspension (50% erythrocytes in saline) and 2.5 μg of BZY-NO maleate were mixed with 1.75 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 20, 50, 80, 120 or 154 mM NaCl. After incubation for 10 min at 37°, BZY formed was extracted (Chart 1), and determined by means of the fluorimetric assay system.[2,3]

The contents of hemoglobin and methemoglobin were determined by the cyanomethemoglobin method[8] and Fujisawa method,[9] respectively.

N-Deoxygenation of BZY-NO Maleate by Hemoglobin and Liver Preparations of Control or Carrageenin-Treated Rats——The carrageenin treatment, the methods for preparing rat liver whole homogenates and hemoglobin, and the analytical procedure for BZY formed were all the same as presented above. The components and conditions for the incubation are given in the legends to tables or figures.

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Results

BZY·HCl Metabolizing Activities in Various Tissues of the Rat

BZY·HCl metabolism in whole homogenates of various tissues corresponding to 5—40 mg of rat tissue was determined at 37° for 10 min in air. The BZY·HCl metabolizing activity was calculated as the loss of BZY·HCl determined from the decrease of fluorescence intensity of BZY·HCl during incubation. As shown in Table I, it was found that liver and kidney were able to metabolize BZY·HCl, but no activity was present in the brain, heart or blood (1 ml of whole blood). The activity in the liver was 6—7 times higher than that in the kidney.

Table I. Metabolism of Benzydamine Hydrochloride in Various Tissue Whole Homogenates from Male Rat[a]

Tiseue	Wet weight of various tissues (mg)	Amount of BZY·HCl metabolized (μg/mg of tissue/10 min)
Liver	10	0.143
	5	0.188
Kidneys	40	0.023
	20	0.026
Brain	40	0
Heart	40	0
Blood (whole)	1 (ml)	0

a) Each incubation mixture (0.6 ml) consisted of whole homogenate (equivalent to 5—40 mg of tissue), 2.0 μg of BZY·HCl as a substrate (about 1×10^{-6} M), 10 mM $MgCl_2$, 2 mM G6P, 0.1 mM NADP and 50 mM Tris buffer (pH 7.4). The metabolizing activities were calculated as loss of BZY·HCl from the decrease of fluorescence in *n*-heptane extracts during the incubation.

BZY·HCl Metabolizing Enzyme System

As described above, the liver was found to be the major tissue responsible for the metabolism of BZY·HCl. The enzymatic properties of BZY·HCl metabolism were examined using rat liver preparations. In whole homogenate or 9000×***g*** supernatant fraction, the BZY·HCl metabolizing activity changed by an amount corresponding to that of liver preparation added. However, no activity was found in heatdenaturated preparations. In the microsomal fraction, the activity was about six times higher in the presence of NADPH than NADH, but no activity was found in the absence of both cofactors (Table II).

Table II. Subcellular Localization and Cofactor Requirements of Benzydamine Hydrochloride Metabolizing Enzyme Systems in Rat Liver[a]

Liver preparation	Liver weight (mg)	Treatment	Amount of BZY·HCl metabolized (μg)	
			10 min	20 min
Whole homogenate	10.0	Heat	0	0
	10.0	Intact	1.52	1.78
	2.5	Intact	0.58	1.09
9000×***g*** supernatant	10.0	Heat	0	0
	10.0	Intact	1.54	1.86
	2.5	Intact	0.74	1.00
Microsomes	10.0	No addition	0	
	10.0	NADPH	1.60	
	10.0	NADH	0.26	

a) The constituents and conditions for the incubation were as in Table I. Calculations: 2.0—total amount of BZY·HCl in *n*-heptane extracts.

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Species Difference in BZY·HCl Metabolism

Some attempts were made to determine species difference in BZY·HCl metabolism using the liver microsomes of rat, mouse, guinea pig and rabbit. The results are summarized in Table III. The metabolism of BZY·HCl consisted mainly of N-oxygenation and N-demethylation. In addition, the enzyme activity was greater for N-oxygenation than N-demethylation in all animal species except the guinea pig, and varied from species to species in both reactions.

Table III. Liver Microsomal Metabolizing Activities of Benzydamine Hydrochloride in Several Animals[a]

Animals	Number of animals	Metabolites (formed) (nmol/mg prot./15 min)		
		BZY–NO	Nor-BZY	HCHO
Mouse	4 groups (1 group; 8 animals)	115.9±3.4	41.4±3.1	44.8±1.3
Guinea pig	4 groups (1 group; 2 animals)	89.4±8.8	73.1±5.2	89.6±3.6
Rat	4 groups (1 group; 2 animals)	58.0±4.5	17.4±1.3	39.4±0.7
Rabbit	1 animal	55.3	36.5	32.5

a) Each incubation mixture (final volume, 3.0 ml) contained microsomal fraction (equivalent to 0.2 g of liver, prepared according to the Ca^{2+} precipitation method), 1.0 mM BZY·HCl as a substrate, 10 mM $MgCl_2$, 50 mM Tris buffer (pH 7.4), 2 mM G6P, 0.1 mM NADP and 1.4 units of G6PDH. The incubation was carried out at 37° in air. The extraction and separation of metabolites (BZY-NO and Nor-BZY) were achieved with ethyl acetate and by TLC, respectively. Each metabolite was determined fluorimetrically. Each value is the mean±S.E.

N-Oxygenation of BZY·HCl by Rat Liver

Table III shows that the N-oxygenation of BZY·HCl in rats is predominant in the presence of an NADPH generating system at pH 7.4. This is consistent with the finding in the previous papers that BZY–NO was the major metabolite in the *in vivo* metabolism of BZY·HCl.[2,3] However, Ziegler *et al.*[10] have reported that the N-oxygenation was more

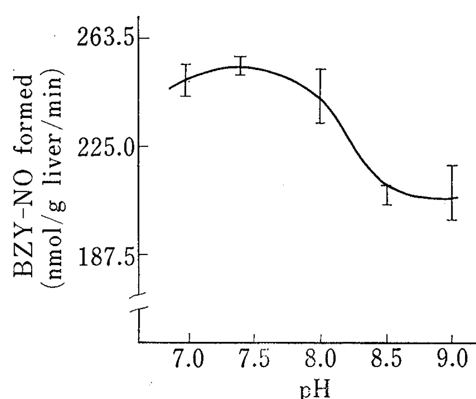

Fig. 1. Effect of pH on N-Oxygenation of BZY·HCl[a]

a) The components of the incubation mixture and the determination of BZY–NO were as in Table III. The incubation was carried out at 37° for 20 min. The vertical lines indicate the means ± S.D. of triplicate values.

Table IV. Effect of NADPH on the N-Oxygenation of BZY·HCl[a]

NADPH	BZY–NO formed (nmol/g liver/min)	
	pH 7.4	pH 8.5
–	144.3±2.3	158.0±5.0
+	215.0±2.6	216.0±2.0

a) The components of the incubation mixture, the conditions for incubation and the determination of BZY–NO were as in Table III and Fig. 1. The activity is presented as the mean±S.E. of triplicate values.

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efficient at pH 8.4 in the presence of an NADPH generating system. Therefore, the effects of pH and NADPH on the N-oxygenation of BZY·HCl were examined using rat liver microsomes. As shown in Fig. 1, the optimal pH for the N-oxygenation of BZY·HCl was in a broad range from 7.0 to 9.0. Therefore, the effect of NADPH on the N-oxygenation of BZY·HCl was examined at pH 7.4 and 8.5, and the results were compared. The activity was almost the same at both pHs, and increased further with the addition of NADPH (TableIV).

As described in the previous paper,[3] BZY–NO levels were greatly increased in the urine and blood of rats given BZY·HCl with carrageenin treatment. The effects of pretreatment with carrageenin on the N-oxygenation and the N-demethylation of BZY·HCl were determined using rat liver microsomes at pH 7.4 in the presence of an NADPH generating system. However, the data in Table V show that the pretreatment with carrageenin had no effect on the N-oxygenation and N-demethylation of BZY·HCl.

TABLE V. Effect of Carrageenin Treatment on N-Oxygenation and N-Demethylation of BZY HCl in Liver Microsomal Preparations[a]

Carrageenin treatment	BZY–NO (nmol/mg prot./20 min)	HCHO (nmol/mg prot./20 min)
−	39.9±3.3	23.8±0.8
+	42.3±3.0	21.1±0.8

a) The components and conditions for incubation were as in Table III. Each activity was determined in five rats, and is expressed as the mean±S.E.

N-Deoxygenation of BZY-NO Maleate in Whole Blood, Plasma or Erythrocytes

The N-deoxygenation of BZY–NO maleate was attempted in air using whole blood, plasma or erythrocytes of rats. In blood, it was clearly found that N-deoxygenation of BZY–NO maleate occurred only to the extent of a few percent. Next, we tried to clarify the relationship between the N-deoxygenation of BZY–NO maleate and the hemolysis of erythrocytes. Fig. 2A shows that the concentration of phosphate-buffered NaCl solution required to cause 50% hemolysis was about 65 mM. In addition, the N-deoxygenation of BZY–NO maleate increased in proportion to an extent of hemolysis of erythrocytes, as shown in Fig. 2B.

TABLE VI. N-Deoxygenation of BZY–NO by Whole Blood, Plasma and Erythrocytes[a]

Blood and its components	BZY formed (%)
Whole blood	1.2±0.3
Plasma	0.7±0.1
Erythrocytes	2.6±0.9

a) Whole blood, plasma and 50% erythrocyte suspension were diluted with 4 volumes of saline. To the diluted sample solutions (total volume, 2.5 ml), BZY–NO maleate was added at a concentration of 8 or 5 μg per 1 ml of parent whole blood in whole blood, or in plasma and erythrocyte suspension, respectively. Incubation was carried out at 37° for 20 min under air. Data are the means±S.D. of triplicate determinations.

Relationship between N-Deoxygenation of BZY-NO Maleate and Production of Methemoglobin from Hemoglobin

The N-deoxygenation of BZY–NO maleate was followed for a selected period using the hemolysate (hemoglobin) produced by complete hemolysis of the erythrocytes. It was observed that BZY–NO maleate added was N-deoxygenated to the extent of about 50% in

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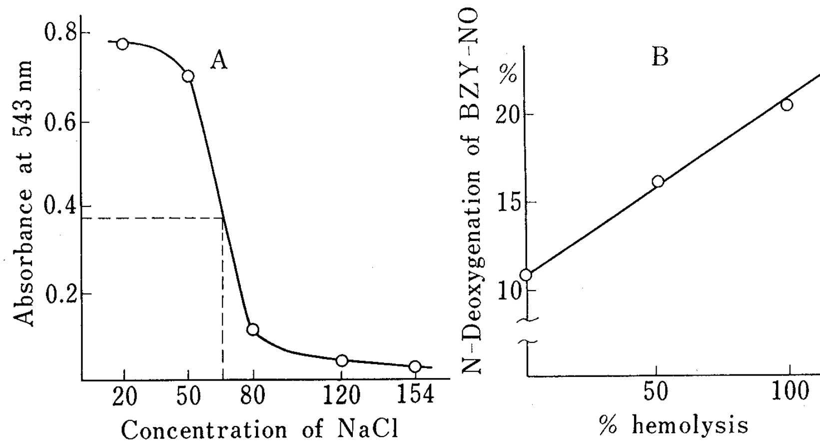

Fig. 2. Relationship between N-Deoxygenation of BZY–NO and Hemolysis of Erythrocytes[a]

a) Panel A: hemolysis of erythrotytes, B: N-deoxygenation of BZY–NO.

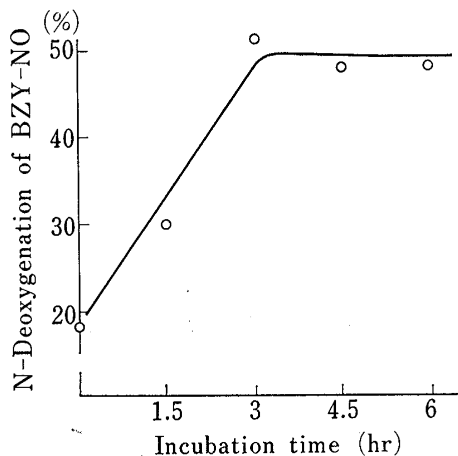

Fig. 3. N-Deoxygenation of BZY–NO in Hemolysate of Erythrocytes[a]

a) 0.25 ml of 50% erythrocyte suspension and 1.75 ml of 20 mM NaCl in 10 mM phosphate buffer, pH 7.4, were mixed with 2.5 μg of BZY–NO maleate (equivalent to about 10 μg of BZY–NO maleate per 1 ml of whole blood). The mixtures were incubated at 37° for selected time in air. The content of hemoglobin was 12.1 g per dl of whole blood at the starting time, but that of methemoglobin was about 0.08 g per dl of whole blood during the whole period. Each point represents the mean of triplicate determinations.

Table VII. N-Deoxygenation of BZY–NO Hemoglobin from Erythrocytes of Control and Carrageenin-Treated Rats[a]

Control (%)	Carrageenin treatment (%)
54.1±1.3	54.0±0.9

a) The components and the conditions for incubation were as in Fig. 3 except that the incubation time was 4 hr. Percentages are presented as the means±S.E. of 5 rats per group. About 1.75 μg of BZY formed equals 100% N-deoxygenation of BZY–NO maleate (2.5 μg). Carrageenin (0.1 ml of 2% suspension in saline) was injected subcutaneously under the plantar surface of the rat hind paw, and then 4 hr later, the rat was sacrificed. Blood was collected immediately.

3 hr (Fig. 3). Nevertheless, the transformation from hemoglobin into methemoglobin was less than 0.7% even at incubation times longer than 7 hr (Fig. 3).

Lastly, the effect of carrageenin treatment on the N-deoxygenation of BZY–NO maleate was examined using the hemolysate produced from the erythrocytes of control or carrageenin-treated rats. No effect of carrageenin treatment on the N-deoxygenation of BZY–NO maleate was recognized (Table VII).

N-Deoxygenation of BZY-NO Maleate in Liver Preparations of Control or Carrageenin-Treated Rats

The above results show that the N-deoxygenation of BZY–NO maleate in the blood was not affected by carrageenin treatment. Accordingly, the effect of carrageenin treatment on the N-deoxygenation of BZY–NO maleate was examined using liver preparations of control

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or carrageenin-treated rats. As shown in Table VIII, BZY–NO reductase (s) activity was inhibited significantly in the rat group sacrificed 2 hr after carrageenin treatment. On the other hand, in the rat groups sacrificed 1 and 3 hr after carrageenin treatment, no significant difference was found between the control and treatment groups, but carrageenin treatment tended to inhibit the action of BZY–NO reductase (s).

Table VIII. Effect of Carrageenin Treatment on the N-Deoxygenation of BZY–NO in Rat Liver Whole Homogenate[a]

Time after treatment with carrageenin (hr)	N-Deoxygenation of BZY–NO (% of 3 μmol)	
	Control	Carrageenin treatment
1	71.5±4.2	68.6±1.8
2	93.8±2.1	82.7±2.5[b]
3	45.0±3.8	38.8±6.0

a) Total incubation volume was 3 ml and the components were 50 mM Tris buffer, pH 7.4, rat liver whole homogenate (equivalent to 125 mg of liver), 10 mM $MgCl_2$, 2 mM G6P, 0.5 mM NADP, 0.5 mM FMN and 1 mM BZY–NO maleate. The incubation was carried out at 37° for 15 min in an N_2 atmosphere. The results are the means S.E. of 5—6 rats per group.
b) Significantly different from the control ($p < 0.01$).

Discussion

BZY·HCl has been widely used in the clinical field as an analgesic and anti-inflammatory agent, and various data on its action have been reported: a) The anti-inflammatory activity rather than the analgesic activity of BZY·HCl is associated with tight BZY binding to protein in the blood,[11] b) BZY·HCl may act on the vascular component of an inflammatory process, and also concentrates preferentially in experimentally inflamed tissues,[12,13] c) Large quantities of unchanged BZY are found in animals treated with BZY·HCl, together with small quantities of urinary metabolite which reacted with the Folin–Ciocalteu reagent.[14] The present authors have reported in previous papers[2,3] that BZY·HCl is significantly metabolized in several animals, and the major metabolite, BZY–NO, apparently increases in the urine and blood of rats treated with the phlogogenic compound carrageenin. However, BZY metabolites in carrageenin-induced paw edema were almost the same as those in normal paw, qualitatively and quantitatively. On the other hand, even though the inflammation models used in the present and previous work were different, the pharmacological action of BZY·HCl has been found to differ among animal species: BZY·HCl more rapidly removed the reddening of the inflamed area and improved the functional recovery in rats injected with silver nitrate into the ankle articulation, but displayed no evidence of activity on UV-induced erythema in guinea pigs,[15] while the drug only partially suppressed the inflammatory response of rabbit skin induced by X-rays.[16] Such differences in the pharmacological action of BZY·HCl correspond to differences in the *in vivo* metabolism of BZY·HCl as follows: conjugated metabolites were predominant in the urine[2] or the blood[3] of rabbit and guinea pig, whereas unconjugated ones were predominant in the rat.

In the present paper, we have confirmed that BZY·HCl is mainly metabolized by N-oxygenation and N-demethylation in rat liver microsomes, and that the former is predominant

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in all animal species except the guinea pig (Tables I, II, and III). Moreover, there was no causal connection between the species difference in the *in vitro* metabolism and the pharmacological action of BZY·HCl.

However, the effect of carrageenin on the N-oxygenation of BZY·HCl or on the N-deoxygenation of BZY–NO maleate must be examined in the light of the previous information[3] that carrageenin treatment caused an increase of BZY–NO in experiments on the anti-inflammatory action of BZY·HCl. Based on the results in the present paper, it appears that an increase of BZY·NO due to carrageenin treatment is not induced by stimulation of the N-oxygenation of BZY·HCl in the blood and the liver of rats. N-Deoxygenation of BZY–NO maleate occurred slightly in whole blood, plasma and erythrocytes in air (Table VI), but was moderate in the hemolysate (hemoglobin) of erythrocytes (Fig. 2 and 3). Uehleke *et al.*[17] reported that N,N-dimethylaminoazobenzene N-oxide was reduced to tertiary amine by erythrocytes or hemoglobin, with the production of methemoglobin. However, the data in the present paper show that amount of methemoglobin was very small, even at incubation times longer than 7 hr. Even if the N-deoxygenating enzyme system of BZY–NO in hemolysate is different from that of N,N-dimethylaminoazobenzene N-oxide, carrageenin treatment still does not exert any effect on the deoxygenation of BZY–NO maleate (Table VII).

In regard to the BZY–NO reductase(s) system, it has been reported that xanthine oxidase (XOD)[18] and cytochrome P-450[19] anaerobically catalyzed the N-deoxygenations of BZY–NO maleate and some aliphatic tertiary amine N-oxides in rat liver preparations. The data in Table VIII indicate that the N-deoxygenation of BZY–NO maleate by NADPH-dependent BZY–NO reductase(s) is inhibited in liver preparations of rats treated with carrageenin. Even though it is not clear whether the effect of carrageenin treatment on the N-deoxygenation of BZY–NO maleate is dependent on cytochrome P-450 or XOD, it seems very likely that the phenomenon reported in previous paper,[3] that BZY–NO levels in the blood and the urine of carrageenin-treated rats apparently increased, was ascribable not to the stimulation of BZY N-oxygenation, but to the inhibition of BZY–NO N-deoxygenation in the liver. Therefore, these results suggest that carrageenin may be a specific inhibitor of BZY–NO N-deoxygenation, at least in rat liver. Further, the results of the present study on the *in vitro* metabolism of BZY–HCl and BZY–NO maleate support the previous suggestions[3] that BZY and BZY–NO are biotransformed into each other in the body, and that carrageenin treatment may result in a weakening of the anti-inflammatory action of BZY–HCl.

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