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In Vivo Metabolism and Anti-inflammatory Activity of Benzydamine Hydrochloride in Rats treated with Carrageenin

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The present study was made to evaluate the anti-inflammatory action of benzydamine hydrochloride (BZY-HCl) in relation to its metabolism. In rats given BZY·HCl orally, unconjugated metabolites (consisting mainly of benzydamine N-oxide) were predominantly excreted in urine, but conjugated metabolites (mainly consisting of glucuronides) were predominant in the bile. Moreover, it appeared that enterohepatic circulation occurred during the metabolism of BZY·HCl, then the levels of conjugated metabolites decreased greatly with time. In rat blood, unconjugated BZY and benzydamine N-oxide (BZY-NO) were predominant, whereas in the blood of rabbits, a conjugated metabolite (G-1) was predominant. However, in rats treated with carrageenin as an inflammation model, the levels of BZY-NO apparently increased in the blood and inflamed paw.

The anti-edematous and analgesic potencies of BZY-NO hydrogen maleate (BZY-NO maleate) were determined simultaneously with the anti-inflammatory action. In the case of intravenous injection, BZY-NO maleate did not show anti-edematous or analgesic action, whereas in the case of oral administration, it showed anti-edematous action amounting to two-thirds of that of the parent drug BZY·HCl, and analgesic action similar to that of BZY·HCl.

In addition, it was confirmed that BZY-NO maleate was well absorbed in the body by oral administration, and was reduced to the parent drug BZY. It is suggested that the effect of oral administration of BZY-NO maleate was due to BZY formed by the reduction of BZY-NO in the rat body.

Keywords——benzydamine; benzydamine N-oxide; disposition; enterohepatic circulation; species difference; carrageenin-induced edema; anti-edematous activity; analgesic activity

Benzydamine hydrochloride (BZY·HCl, 1-benzyl-3-(3-dimethylaminopropoxy)-1H-indazole hydrochloride, Fig. 1) has been used clinically as a nonsteroidal analgesic anti-inflammatory agent.

The metabolism of BZY·HCl in the rabbit has already been investigated by the present authors.[2] The metabolism of the drug in rats[3] has also been studied. The major urinary metabolites in rabbits given BZY·HCl were benzydamine N-oxide (BZY–NO), 1-(*p*-hydroxybenzyl)-3-(3-dimethylaminopropoxy)-1H-indazole (HO·BZY) and three unknown glucuronides (G-1, G-2 and G-3), together with several minor metabolites as shown in Fig. 1.

However, no information is available on the behavior of BZY and its metabolites in the rat body, except for reports that dosed BZY was distributed to various organ tissues without undergoing metabolism in the body,[4] and was concentrated unchanged in carrageenin-induced rat paw edema.[5]

Therefore, in the present paper, the authors sought to clarify the relationship between the metabolism and anti-inflammatory actions of BZY·HCl by using rats treated with the

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3) B. Catanese, R. Lisciani, and B. Silvestrini, *Arzneim.-Forsch.* (Drug Res.), **22**, 882 (1972).
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5) R. Lisciani, B.P. Scorza, and B. Silvestrini, *Eur. J. Pharmacol.*, **3**, 157 (1968).

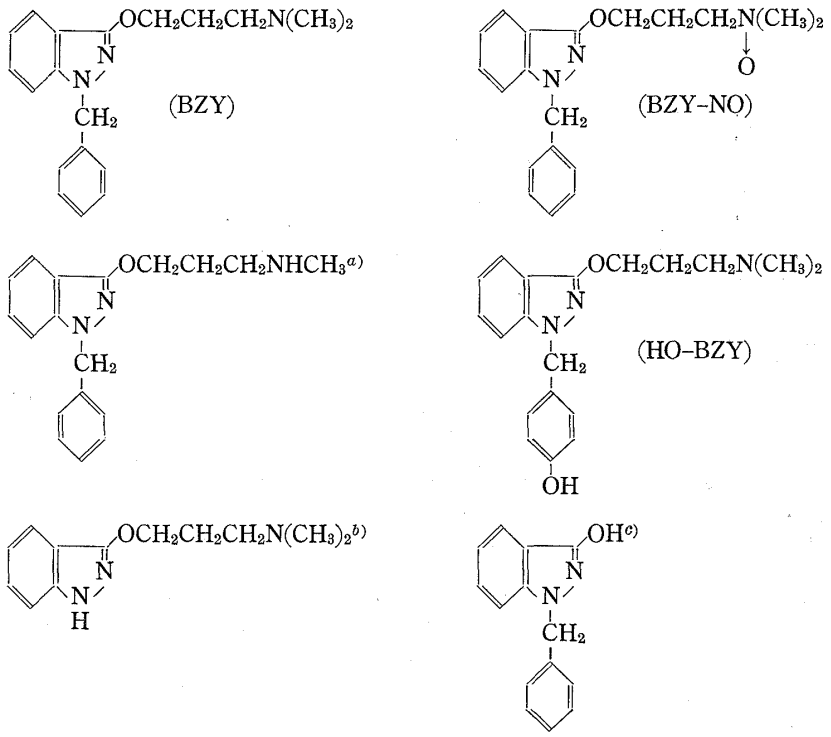

Fig. 1. Structures and Abbreviation of Benzydamine and Its Metabolites

a) 1-Benzyl-3-(3-methylaminopropoxy)-1H-indazole; nor-BZY.
b) 3-(3-Dimethylaminopropoxy)-1H-indazole; deB-BZY.
c) 1-Benzyl-3-indazolone; BID.
Nor-BZY, deB-BZY and BID were identified as minor metabolites in the previous papers.[2]

phlogogenic agent carrageenin as an inflammation model. BZY metabolites in the urine, blood, bile and inflamed paw of rats given BZY·HCl were first determined in intact and carrageenin-treated rats. It was found that BZY–NO levels increased in the blood and urine, and conjugated metabolites were largely excreted in the bile rather than in the urine.

Finally, the anti-inflammatory action of the major metabolite BZY–NO was examined. The anti-inflammatory action of BZY·HCl is discussed in relation to species differences in its *in vivo* metabolism.

Materials and Methods

Chemicals——BZY·HCl was a gift from Yoshitomi Pharmaceutical Co., Ltd. and Daiichi Pharmaceutical Co., Ltd. 1-Benzyl-3-(3-dimethylaminopropoxy)-1H-indazole N-oxide hydrogen maleate (BZY-NO maleate) was synthesized as described in the previous paper.[2] Other chemicals were of analytical grade and were obtained commercially.

***In Vivo* Metabolism**

1) **Surgical Operation for Determining Urinary and Biliary Metabolites of Rats**——Rats (wt. 200—250 g) were anesthetized with sodium pentobarbital (40 mg/kg body wt., *i.p.*) and cannulated to determine the urinary and or biliary excretion of BZY metabolites in rats. Four kinds of operations were carried out as follows: A) a polyethylene catheter was inserted into the bladder, and the ureter was ligated, B) a catheter was inserted into the bladder and the common bile duct, and then the ureter was ligated, C) a catheter was inserted into the bladder, and the common bile duct and the ureter were ligated, D) in two rats submitted to operation B, the bile duct catheter of one rat was inserted into the duodenum of the other rat. In the present paper, treatments A, B, C and D refer to the above operations A, B, C and D, respectively.

BZY·HCl was administered orally to the rats at a dosage of 100 mg/kg body wt. 2 ml water at 30 min after surgical operations. The rats were subjected to bile duct and/or bladder cannulation, immobilized in restraining cages, and maintained on a diet of commercial rat cubes and water *ad libitum*. After dosing

BZY·HCl, urine and bile were collected for 24 hours, and used for the determination of BZY and its metabolites.

2) **BZY and Its Metabolite Levels in Blood, and Normal and Inflamed Paws of Rats treated with Carrageenin**——Rats (150—200 g) used in these experiments were starved overnight. Next, 0.1 ml of 2% (w/v) carrageenin suspended in saline, and the same volume of saline were injected subcutaneously under the plantar surface of the left hind paw and the right hind paw, respectively, of the same rat.

To determine the levels of BZY and its metabolites in the blood, and in normal and inflamed paws at selected times, the subject rats were treated as follows: 1) BZY·HCl (100 mg/kg body wt.) was administered to rats treated 1 hour earlier with carrageenin, 2) BZY·HCl and carrageenin were administered simultaneously, 3) BZY·HCl was administered to the rats 1 hour after treatment with carrageenin, 4) BZY·HCl was administered to the rats 2 hours after treatment with carrageenin. In the present paper, treatments 1, 2, 3 and 4 refer to the above four kinds of treatments, 1, 2, 3 and 4, respectively. The rats were sacrificed hourly during 4 or 5 hours after the administration of BZY·HCl, then blood samples were taken from the carotid, and collected in test tubes coated with 1000 units/ml of heparin. BZY-NO maleate (100 mg/kg body wt.) was also given simultaneously with carrageenin to rats in the same way as in treatment 2 described above, and the rats were sacrificed after 3 hours. The normal and inflamed hind legs were removed at the knee joint and weighed. The paws, both normal and inflamed, were each chopped, crushed mechanically in a mortar with a pestle, and then extracted four times with 50 ml of 0.1 N HCl. The blood was centrifuged at 3000 rpm for 10 min. The resulting plasma and combined HCl extracts were used to determine BZY and its metabolites.

To examine the anti-inflammatory effects of BZY·HCl on carrageenin-induced edema, the rats were sacrificed 3 hours after the treatment with carrageenin. The swelling (percent) was calculated from the normal and inflamed paw weights according to the following formula:

$$S\,(\%) = \frac{W_t - W_n}{W_n} \times 100$$

where S is the swelling (percent); W_n is the mean weight of the right hind paws; W_t is the mean weight of the left hind paws. The inhibitory effect (%) of BZY·HCl on inflamed paw edema was calculated as follows:

$$I\,(\%) = \frac{S_n - S_t}{S_n} \times 100$$

where I is the inhibition (percent); and S_n and S_t are the swelling (percent) in control rats and BZY·HCl-dosed rats, respectively.

3) **Experiments in Intact Rats and Rabbits**——After starvation overnight, BZY·HCl was given orally at a dose of 100 mg/kg body wt./2 ml of water to rats (150—200 g) and at a dose of 100 mg/kg body wt./4 ml of water to rabbits (2.5—3.0 kg). Blood was taken 0.5, 1, 2, 3 and 4 hours later.

4) **Determination of BZY Metabolites in Urine, Bile, Plasma and Paw**——(a) Extraction of Metabolites: In order to separate metabolites roughly, the urine, bile, plasma and HCl extract from paw were diluted with 10—20 volumes of water. These samples were worked up according to the reported procedure[2] as shown in Fig. 2.

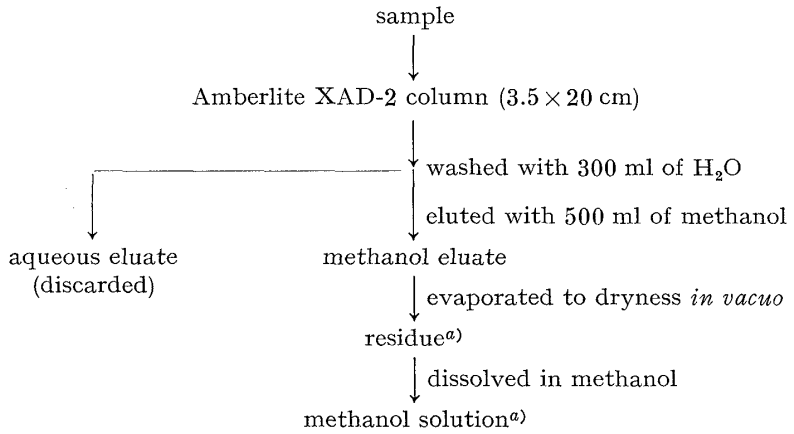

Fig. 2. Procedure employed for the Separation of BZY Metabolites from Urine, Bile, Plasma and Extracts from Paws

a) The residue was dissolved in 0.5 ml of methanol for samples from plasma and extracts from paws, or in 5.0 ml of methanol for those from urine and bile. These methanol solutions were subjected to TLC for identification and determination of metabolites.

More than 97.5% of the applied material was recovered from the Amberlite XAD-2 in the methanol eluate.

(b) Thin-Layer Chromatography: Thin-layer chromatography (TLC) was carried out on plates precoated with Kieselgel 60 F_{254} (E. Merck, Art. 5554) 0.25 mm thick, or Aluminumoxid 60 F_{254} neutral (Typ E) (E. Merck, Art. 5550) 0.20 mm thick. The solvent systems used were A: benzene–$CHCl_3$–MeOH–EtOH–NH_4OH (Sp. gr. 0.9) (15: 15: 10: 5: 0.5, v/v); B: BuOH–MeOH–H_2O–AcOH (35: 20: 10: 4, v/v); C: toluene–EtOH–H_2O–AcOH (20: 30: 10: 1, v/v, upper layer); and D: benzene–EtOH–H_2O–AcOH (20: 20: 10: 1, v/v, upper layer). A and B were used with silica gel, and C and D with aluminum oxide. Solvents A, C and D were used for the identification of free metabolites, and B was used for the identification of glucuronides.

Metabolites on TLC plates were detected under ultraviolet light (254 nm) or by spraying with Dragendorff, Gibbs, Folin-Ciocalteu, or naphthoresorcinol reagents. The *Rf* values and the color reactions of BZY and its metabolites have already been reported by the authors.[2] The spots of each metabolite were located by comparison with reference compounds. The adsorbent at positions corresponding to each reference compound was well shaken with 4 ml of methanol to remove BZY metabolites.

(c) Quantitative Determination by Fluorometry: The fluorescence of the methanol eluate was measured at 355 nm with excitation at 305 nm for all metabolites except deB-BZY (excitation at 300 nm and emission at 343 nm), using a Hitachi spectrofluorometer (model 512), and the amount of each metabolite was calculated from the fluorescence intensity relative to that of the reference compound. HO-BZY, G-1, G-2 and G-3 were calculated as BZY·HCl equivalents, since authentic samples were not available. This TLC procedure gave recoveries of fluorescent reference compounds exceeding 75%.

After diluting the sample 1000 times with 0.1 M acetate buffer, pH 5, the total fluorescent metabolites excreted into urine or bile was directly determined with excitation at 305 nm and emission at 360 nm.

Anti-Inflammatory Actions of BZY-NO Maleate and BZY·HCl

1) **Anti-Edematous Activity**——At least six rats (about 120 g) were used so as to measure the activity at each dose level. BZY-NO maleate and BZY·HCl were dissolved in water for oral administration or in 0.9% saline for injection *via* the tail vein. The phlogistic agent carrageenin was injected into rats as described for *in vivo* metabolism 2) for the preparation of an experimental inflammation model. Rats were simultaneously treated with carrageenin and a test drug. The volume of the foot treated with carrageenin was measured by water displacement to the knee joint immediately (at 0 time) and then at the indicated times. The inflammation model induced by injecting carrageenin was also used to measure simultaneously the analgesic potencies of BZYHCl and BZY-NO maleate as described later.

The swelling of inflamed paw and the inhibitory actions of BZY-NO maleate and BZY·HCl on inflamed paw edema were calculated as described for the *in vivo* metabolism 2), except that volume was used instead of weight.

2) **Analgesic Activity**——Using the same inflammation model as for the measurement of anti-edematous activity, the analgesic activities of BZY-NO maleate and BZY·HCl were measured according to Randall and Selitto.[6] After giving BZY-NO maleate or BZY·HCl orally or intravenously, the pain threshold was measured with an Analgesy-Meter (Ugo Basile, Milano, Italy) at hourly intervals over a period of 4 hours. Pressure was applied to the normal and inflamed paws at a rate of 60 g/min. The end-point, or pain threshold, was defined as the value (g) necessary to cause a rat to struggle and/or vocalize. The analgesic potency of both drugs was evaluated on the basis of analgesic indexes calculated by dividing the sum of individual pain thresholds at hourly intervals over a period of 4 hours after a subject drug had been given by the sum of those obtained after giving the vehicle alone.

Results

In Vivo Metabolism

1) **Urinary and Biliary Excretion of Metabolites**——The urinary and biliary excretions of BZY metabolites were studied by administering BZY·HCl orally to rats which had been subjected to bile duct cannulation and/or bladder fistulation. The results are given in Table I. The total metabolites excreted in the urine amounted to about 25% of the dose in 24 hours (Table I, A). This is in good agreement with the values reported by Catanese *et al.*[7] and Kataoka *et al.*[2]

In the experiments in rats (treatment B) subjected to bile duct canulation and bladder fistulation, the total amounts of BZY metabolites in the urine and bile were larger than the

6) L.O. Randall and J.J. Selitto, *Arch. Int. Pharmacodyn. Ther.*, **111**, 409 (1957).
7) B. Catanese, A. Grasso, and B. Silvestrini, *Arzneim.-Forsch.* (Drug Res.), **16**, 1354 (1966).

Table I. Amounts of BZY Metabolites excreted in the Urine and Bile by Rats[a]

Metabolites	A Urine	B Urine	B Bile	C Urine
	Percentage of total dose			
	25.0±1.2	17.7±3.5	24.9±2.9	25.5±1.1
	Percentage of each metabolite			
G-1	7.4	9.0	47.2	33.0
Sum of G-2 and G-3	5.8	9.5	46.5	23.0
BZY–NO	70.5	54.3	3.5	33.5
nor-BZY	7.9	6.9	t[b]	5.0
deB–BZY	1.1	t[b]	t[b]	t[b]
HO–BZY	6.3	t[b]	t[b]	t[b]
Unchanged BZY	1.1	20.7	2.8	5.5

a) A, B and C correspond to surgical treatments A—C described in "Materials and Methods," respectively. Percentages of dose are expressed as the means±S.E. of six rats.
b) "t" indicates a trace amount.

amounts present in the urine of rats (treatment A) subjected to bladder fistulation alone. In addition, the ratio of total unconjugated metabolites to total conjugated metabolites in the urine was almost equal in treatment A and B rats. However, compared with treatment A rats, the excretion of the major metabolite BZY–NO in the urine was decreased in treatment B rats, and unchanged BZY in the urine of treatment B rats largely accounted for the decrease of BZY–NO. Biliary metabolites in treatment B rats were mainly conjugated.

On the other hand, in the rats (treatment C) with ligated bile duct, 25% of the dose was excreted in the urine (Table I, C). This value is the same as that obtained by collecting only urine (Table I, A), but compared with the urinary metabolites obtained in the two experiments mentioned above, more conjugated metabolites were present in this urine.

These experiments suggest that biliary excretion is more prominent in BZY metabolism than urinary excretion. Therefore, the treatment D rats described in "Materials and Methods" (*in vivo* metabolism 1) were used to examine the reabsorption of BZY metabolites in the intestine. When the biliary excreta from the first rat dosed with BZY·HCl were passed into the duodenum of a second rat by cannulation, the amount of the urinary metabolites in the first rat as a percentage of the dose of BZY·HCl was almost equal to the value presented in Table I, B. In contrast, 3.2% appeared in the urine and 7.3% in the bile (expressed as a percentage of dose given to the first rat) of the second rat. No metabolites were detectable in the feces (not shown).

Table II. Reabsorption of BZY Metabolites from the Intestine[a]

Metabolites	Percentage of each metabolite		
	Urine	Bile	Sum of urine and bile
G-1	38.4	60.8	53.6
Sum of G-2 and G-3	30.8	39.3	36.6
BZY–NO	15.4	t[b]	4.9
Unchanged BZY	15.4	t[b]	4.9

a) Urine and bile were collected from the second rat over a period of 24 hours after bile excreta from the first rat receiving BZY·HCl had been passed into the duodenum of the second rat. (Two rats in total.)
Nor-BZY, deB-BZY and HO-BZY were omitted since they were present only in trace amounts.
b) "t" indicates a trace amount.

2) **Plasma and Paw Levels of BZY Metabolites**——a) Plasma Level of BZY Metabolites in Intact Rats: After giving BZY·HCl orally, the plasma levels of each metabolite reached a peak 1 hour later, and then declined over the next 3 or 4 hours (Fig. 3). The data in intact rats (Fig. 3) clearly showed that the bulk of the metabolites was present in plasma in unconjugated form (BZY–NO and BZY), while the remaining metabolites (deB–BZY, nor-BZY and HO·BZY) were each present at a concentration of about 0.1 μg/ml. However, the sum of G-2 and G-3 was almost equal to the concentration of G-1. The minor metabolites are omitted in Fig. 3.

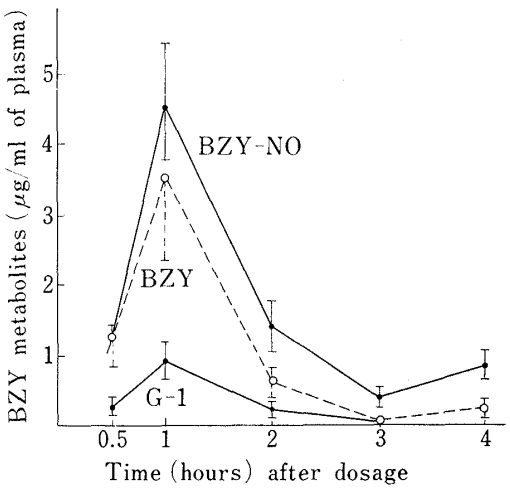

Fig. 3. Plasma Levels of Metabolites after Oral Administration of BZY·HCl to Intact Rats[a]

a) Intact rats were given 100 mg/kg of BZY·HCl orally. Data are means±S.E. of five rats. The combined concentration of G-2 and G-3 was almost identical to that of G-1.

b) Plasma and Paw Levels of BZY Metabolites in Rats subjected to Treatment 1: After treating the rats with BZY·HCl and then with carrageenin, the plasma levels of BZY metabolites decreased in the same way as in Fig. 3 by 2 hours after the administration of BZY·HCl, but the plasma level of BZY–NO reached a peak 3 hours later (Fig. 4, A). BZY metabolites in the normal and inflamed paws differed from those in plasma quantitatively and qualitatively (Fig. 4, B and C). In particular, unchanged BZY was present at a very low concentration in the plasma, but was present at high levels in the normal and inflamed paws.

c) Plasma and Paw Levels of BZY Metabolites in Rats subjected to Treatment 2: As shown in Fig. 5, BZY–NO was predominantly present in the blood over a period of 3 hours after simultaneous treatment with carrageenin and BZY·HCl. Metabolites in the normal and inflamed paws showed similar patterns, and also resembled those in Fig. 4, B and C.

d) Plasma and Paw Levels of BZY Metabolites in Rats subjected to Treatment 3: When BZY·HCl was administered orally to rats 1 hour after carrageenin treatment, BZY–NO appeared mainly in the plasma over a period of 3 hours (Fig. 6, A). In the normal and inflamed paws, the increases of BZY and BZY–NO took place rapidly after 3 hours (Fig. 6, B and C). This is in contrast to Fig. 4, B and C, and Fig. 5, B and C.

e) Plasma and Paw Levels of BZY Metabolites in Rats subjected to Treatment 4: In rats subjected to treatment 4, the BZY–NO level in the plasma reached a peak after 1 hour and then fell to nearly half over a period of 3 hours (Fig. 7, A). In both normal and inflamed paws, the level of BZY–NO was much larger than that of unchanged BZY after 3 hours (Fig. 7, B and C).

3) **Plasma and Paw Levels of BZY Metabolites, and Inhibitory Effect of BZY·HCl on Carrageenin-induced Edema in Rats**——On the basis of the results of the experiments described above (Fig. 4—7), plasma and inflamed paw levels of BZY metabolites are listed in Table III together with the anti-edematous activities of BZY·HCl at 3 hours after carrageenin injection. As indicated in Table III, the inhibitory effect on edema was highest in treatment 1 rats and decreased in the order of treatments 2, 3 and 4. In addition, the degree of the inhibition of edema apparently does not correspond to BZY and BZY–NO levels in plasma, though there may be some relation in the rats subjected to treatments 1 and 2. The inhibition of edema in the treatment 2 rats was in approximate agreement with the result (20%) of Silvestrini *et al.*,[8] even though the dose of BZY·HCl (60 mg/kg *p.o.*) and

8) B. Silvestrini, A. Garau, C. Pozzatti, and V. Cioli, *Arzneim.-Forsch.* (Drug Res.), **16**, 59 (1966).

the time of measurement of edema volume (4 hours after injecting carrageenin) were different in their study from those in the present study.

4) **Plasma and Paw Levels of BZY-NO Metabolites in Intact and Carrageenin-Treated Rats**——Metabolites in plasma were mainly BZY–NO, BZY and glucuronides 3 hours after BZY–NO maleate had been orally administered to intact or carrageenin-treated rats. The data in Table IV show that, in paws also, the level of BZY–NO was highest, followed by BZY.

5) **Plasma Levels of BZY Metabolites in Intact Rabbits**——Fig. 8 shows plasma levels of BZY metabolites after oral administration of BZY·HCl to intact rabbits. G-1 was much larger than the sum of G-2 and G-3, and unconjugated metabolites, in contrast to the case in intact rats (Fig. 3). This is in accord with the findings reported in our previous paper[2] that unconjugated metabolites are largely excreted in rat urine, but conjugated metabolites are largely excreted in rabbit urine.

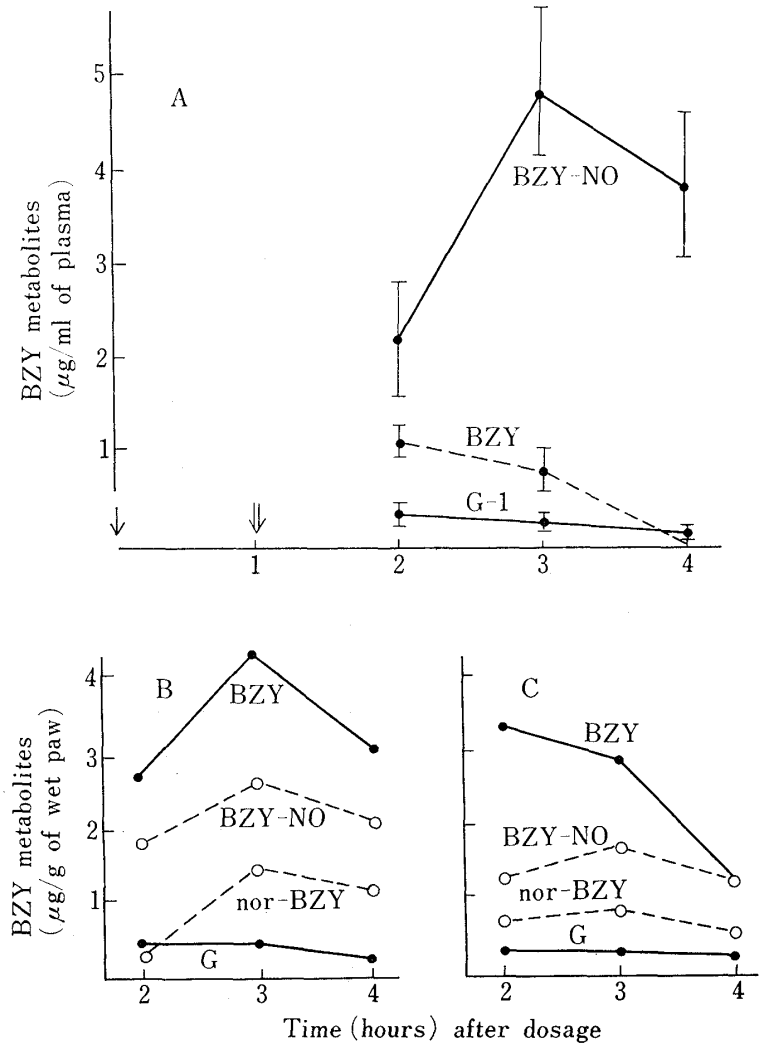

Fig. 4. Plasma and Paw Levels of BZY Metabolites in Treatment 1 Rats[a]

a) Data in panel A are means±S.E. of four rats, and data in panels B and C are means of two groups (one group consisted of two rats). Panels A, B and C represent plasma, normal and inflamed paw levels of metabolites, respectively. Downward arrows ↓ and ⇓ indicate the administration of BZY·HCl and the injection of carrageenin, respectively. G-2 and G-3 are omitted because the combined concentration of G-2 and G-3 was almost equal to that of G-1. "G" indicates the combined concentration of G-1, G-2 and G-3. Other free metabolites (deB-BZY, HO-BZY) are not shown because their concentrations were less than 0.2 μg/ml.

Anti-Inflammatory Actions of BZY-NO Maleate and BZY·HCl

1) **Anti-Edematous Activity**——Fig. 9 shows the swelling calculated from the volumes of rat hind paw edema at hourly intervals for 4 hours after simultaneous treatment with carrageenin and the drug. Panel A shows the progression of swelling after oral administration of BZY·HCl and BZY–NO maleate, and panel B shows that after injection of BZY–NO maleate through the tail vein (Fig. 9). BZY–NO maleate possessed less anti-edematous potency than BZY·HCl on oral administration. However, it is known that BZY–NO reduced to the parent drug BZY in rats (Table IV). Accordingly, it is not clear whether the anti-edematous action of BZY–NO maleate is due to BZY–NO itself or to BZY formed in the body. Therefore, BZY–NO maleate was injected through the rat tail vein to avoid secondary metabolism of the drug in the body. As shown in panel B, in this case BZY–NO maleate

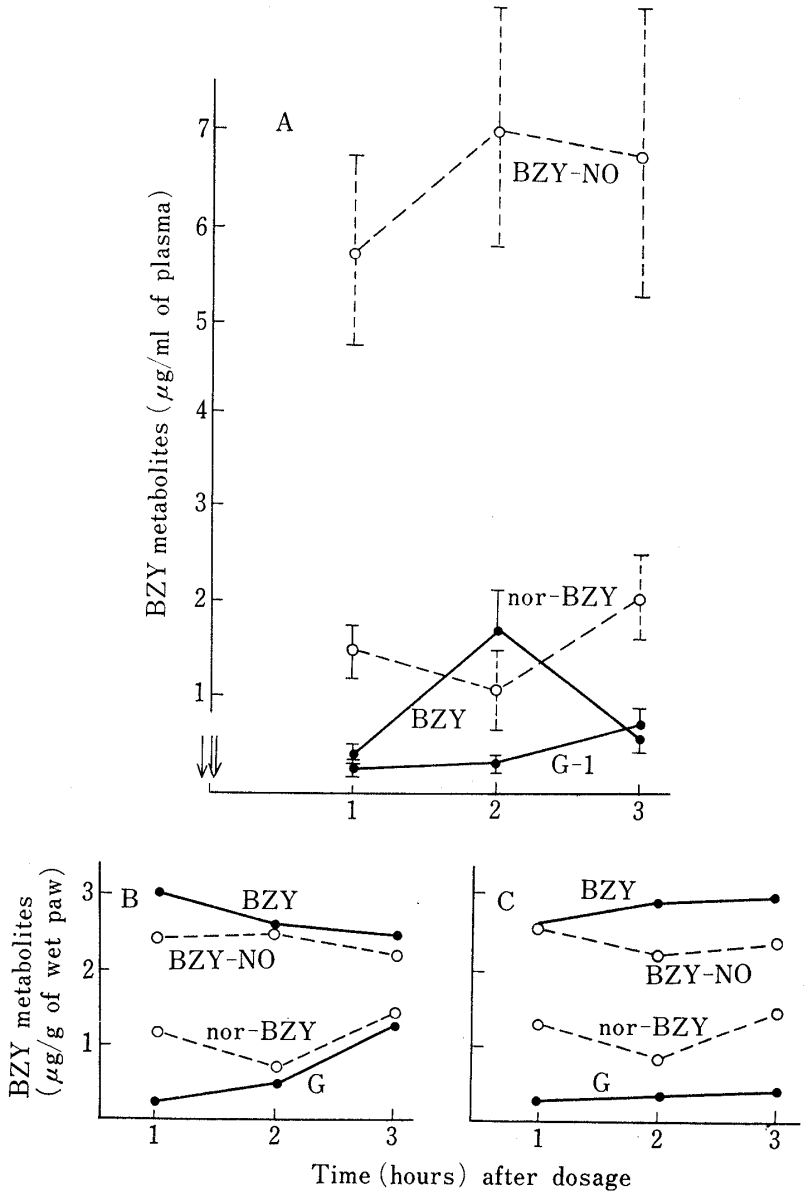

Fig. 5. Plasma and Paw Levels of BZY Metabolites in Treatment 2 Rats[a]

a) Details are as in Fig. 4.

did not exert any inhibitory effect on carrageenin-induced edema even at the high dosage of 64 mg/kg of body wt.

These results suggest that BZY·NO itself does not have anti-edematous activity, and that BZY resulting from the reduction of BZY–NO in the body caused the pharmacological action. Thus, on the basis of the inhibition of swelling 3 hours after simultaneous treatment of rats with carrageenin and either BZY–NO maleate or BZY·HCl, inhibitory dose-response curves of BZY–NO maleate and BZY·HCl were prepared to compare their anti-edematous potencies after oral administration (Fig. 10).

As indicated in Fig. 10, the inhibitory effect of BZY–NO maleate or BZY·HCl on swelling increased with the dose of the drug, that is, dose-dependent anti-edematous action was seen with both drugs. The doses necessary to cause 50% inhibition were found to be 68.7 mg/kg for BZY·HCl, and 135.2 mg/kg for BZY–NO maleate.

2) **Analgesic Activity**——The same carrageenin-treated rats were used to evaluate the analgesic potency of BZY–NO maleate and BZY·HCl by the Randall-Selitto method. An

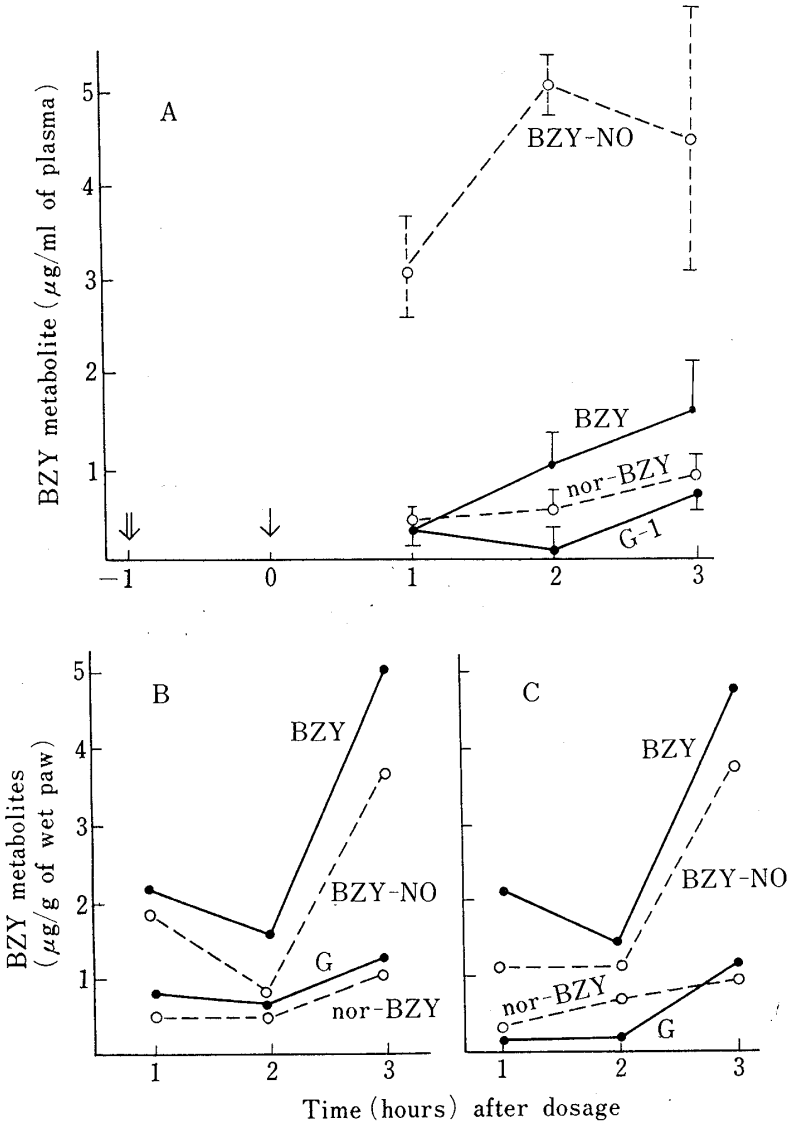

Fig. 6. Plasma and Paw Levels of BZY Metabolites in Treatment 3 Rats[a]

a) Details are as in Fig. 4.

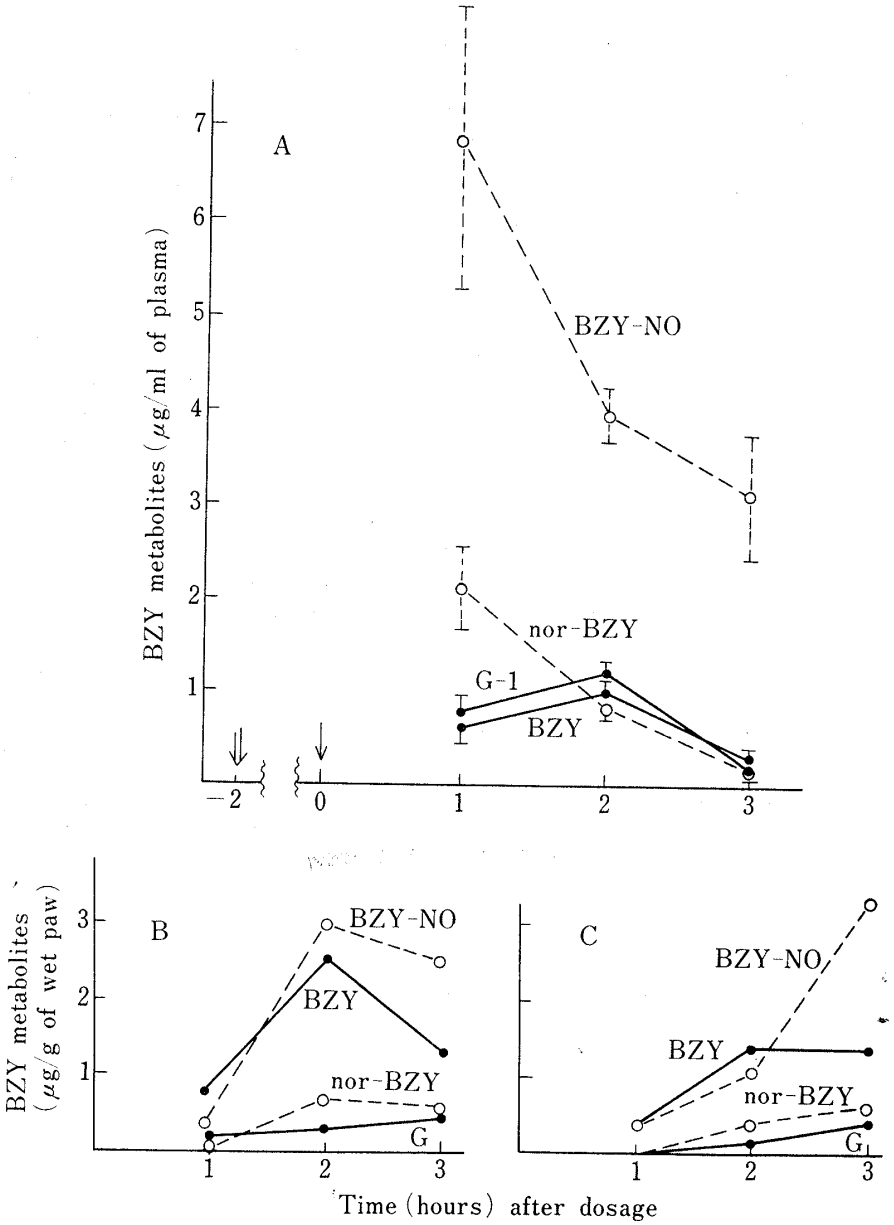

Fig. 7. Plasma and Paw Levels of BZY Metabolites in Treatment 4 Rats[a]

a) Details are as in Fig. 4.

TABLE III. Levels of BZY Metabolites in Plasma and Inflamed Paw, and the Inhibitory Effects of BZY·HCl on Carrageenin-Induced Edema in Rats treated under Various Conditions[a]

Treatment	Inhibition (%) of edema	Metabolites in Plasma (μg/ml of plasma)		Metabolites in Edema (μg/g of wet tissue)	
		BZY-NO	BZY	BZY-NO	BZY
1	77.5	4.91±0.78	0.77±0.25	1.70	2.87
2	27.4	6.80±1.55	0.65±0.15	2.35	2.94
3	10.7	5.07±0.31	1.01±0.33	1.10	1.40
4	−7.1	6.80±1.55	0.63±0.15	0.40	0.04

a) Treatemnts correspond to those indicated in "Materials and Methods." Data were obtained from rats sacrificed 3 hours after injecting carrageenin, and levels of metabolites in the plasma and inflamed paw are taken from Fig. 4—7.

analgesic index was calculated as a parameter of analgesic effectiveness of both drugs. A typical example is shown in Table V. The data confirm the earlier report of Silvestrini *et al.*[8] that BZY·HCl displayed a noncentral analgesic action.

The dose-analgesic index plots of both drugs gave very similar dose-dependent straight lines (Fig. 11). On the other hand, when BZY–NO maleate was given at high doses of 32 and 64 mg/kg *via* the rat tail vein, dose-dependence was not well maintained, strongly suggesting that the analgesic effectiveness of BZY–NO maleate is caused not by BZY–NO itself, but by BZY formed from it in the body.

TABLE IV. Plasma and Paw Levels of BZY–NO Metabolites in Rats[a]

Metabolites	Intact rat Plasma (μg/ml)	Carrageenin-treated rat		
		Plasma (μg/ml)	Normal paw (μg/g of wet tissue)	Inflamed paw (μg/g of wet tissue)
BZY–NO	2.09±0.19	2.31±0.52	2.51	2.99
deB–BZY	0.14±0.03	0.18±0.03	0.04	0.01
nor-BZY	0.12±0.01	0.11±0.03	0.47	0.37
BZY	0.61±0.03	1.10±0.21	1.47	1.01
HO–BZY	0.15±0.01	0.16±0.03	0.10	0.01
G-1	0.28±0.08	0.29±0.14	0.17	0.15
Sum of G-2 and G-3	0.75±0.06	0.81±0.15	0.44	0.39

a) After oral administration of BZY-NO maleate (100 mg/kg body wt.) to intact and carrageenin-treated rats, the rats were sacrificed 3 hours later. Plasma levels are expressed as the means±S.E. of four rats, and the values of paw levels are the means of 2 groups (1 group consisted of two rats).

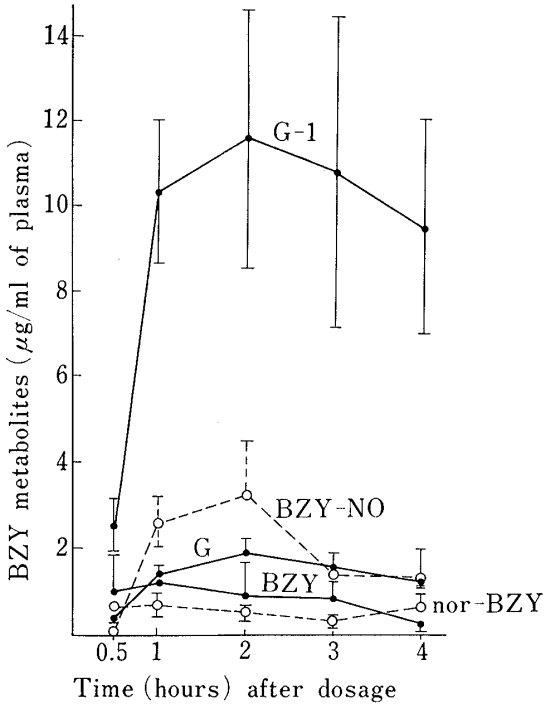

Fig. 8. Plasma Levels of Metabolites after Oral Administration of BZY·HCl to Intact Rabbits[a]

a) Intact rabbits were treated with BZY·HCl (100 mg/kg of body weight). Data are means±S.E. of five rabbits. "G" indicates the combined concentration of G-2 and G-3.

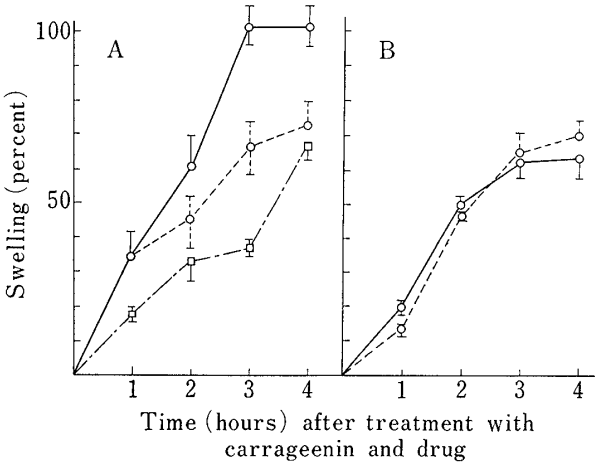

Fig. 9. Typical Example of the Inhibitory Effects of BZY·HCl and BZY–NO Maleate on the Swelling of Rat Hind Paw induced by Carrageenin[a]

a) Panels A and B show curves for oral and intravenous dose routes, respectively. Bars show standard errors of the means. Each group consisted of 6 male rats.
—○—○—: control (A; H_2O, B; saline).
----○----: BZY-NO maleate (A; 128 mg/kg/2 ml of H_2O, *p.o.*, B; 64 mg/kg/2 ml of saline, *i.v.*).
—□—: BZY·HCl (100 mg/kg/2 ml of H_2O, *p.o.*).

Table V. A Typical Example of Pain Threshold and Analgesic Index according to the Randall-Selitto Method

	Noninflamed paw (hr)					Inflamed paw (hr)				
	1	2	3	4	Index	1	2	3	4	Index
			(×10 g)					(×10 g)		
Control	10.6	9.7	10.2	9.5	1.00	9.1	6.5	5.9	3.0	1.00
BZY·HCl (100 mg/kg *p.o.*)	10.3	11.0	8.0	8.5	0.97	10.9	6.9	6.5	6.5	1.26
BZY–NO maleate (128 mg/kg *p.o.*)	10.1	11.4	9.4	7.7	0.97	8.9	9.5	7.1	6.0	1.29[a]

a) $I=\frac{8.9+9.5+7.1+6.0}{9.1+6.5+5.9+3.0}=1.29$

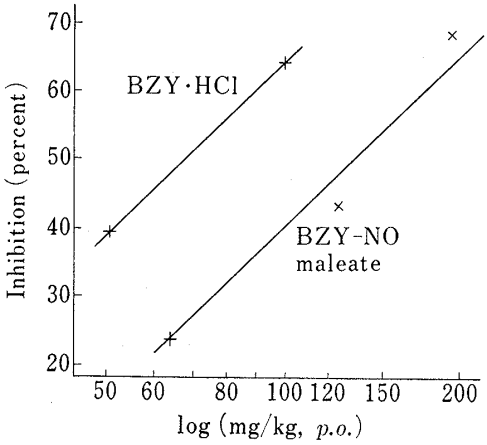

Fig. 10. Inhibitory Dose-Response Curve of BZY·HCl and BZY–NO Maleate on Carrageenin-Induced Edema in Rats

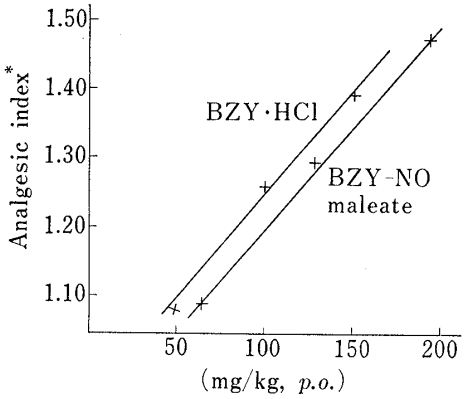

Fig. 11. Comparison of the Analgesic Effects of BZY·HCl and BZY–NO Maleate according to the Randall-Selitto Method[a]

a) An analgesic index was calculated as shown in footnote of Table V.

Discussion

The present study was designed to investigate BZY metabolism in rats in order to understand its anti-inflammatory action. The behavior of BZY and its metabolites in the body (Table I and II), the effect of carrageenin on the *in vivo* metabolism of BZY·HCl (Fig. 3—5 and 8, and Tables III and IV), and the anti-inflammatory action of the major metabolite BZY–NO (Fig. 9 and 10, and Table V) were examined under various conditions.

For drugs where the biliary or urinary route is an important route of excretion, it is evident that quantitative alterations in these pathways could have marked effects on the duration of action or even the intensity of action. In general, it is well known that the extent of biliary excretion of a compound is influenced by a number of factors.[9] In particular, it was proposed on the basis of studies on the biliary or urinary excretion of many aromatic compounds in rats that the minimum threshold molecular weight for biliary excretion was about 325±50,[10] while compounds with molecular weights less than about 300 were mainly eliminated by the kidney.[11] The present results that unconjugated BZY (molecular weight; 309) and its major metabolite BZY–NO (molecular weight; 325) were mainly excreted in urine, and that conjugated metabolites (G-1, G-2 and G-3; molecular

9) R.L. Smith, "The Excretory Function of Bile," Chapman and Hall, London, 1973, p. 16.
10) P. Millburn, R.L. Smith, and R.T. Williams, *Biochem. J.*, **105**, 1275 (1967).
11) M.M. Abou-El-Makarem, R.L. Smith, and R.T. Williams, *Biochem. J.*, **105**, 1269 (1967).

weights of perhaps more than about 400) were excreted in the bile, are in good accord with these reports.[10,11] In addition, it was found that the conjugated metabolites were circulated between liver and intestine, being further decomposed during the enterohepatic circulation. This will reduce the anti-inflammatory effectiveness of BZY·HCl, and should be reflected in the metabolic characteristics in the blood, inflamed paw and normal paw. In fact, in rats treated with carrageenin, which is frequently used for the preparation of an experimental inflammation model, the BZY–NO level in plasma apparently increased, compared with that in the plasma of intact rats. Moreover, there was a tendency for BZY–NO to increase not only in the plasma but also in the paws, as time passed after carrageenin treatment (Fig. 7). The inhibitory effect of BZY·HCl on edema seems to depend on the presence of BZY–NO and/or BZY itself in the inflamed paw, but not in plasma (Table III), although it remains unclear whether the apparent increase of BZY–NO level due to carrageenin treatment is caused by the stimulation of BZY N-oxygenation, by the inhibition of BZY–NO N-deoxygenation in the blood and/or liver, or by the inhibition of BZY–NO excretion. However, a more detailed discussion of this question is given in the accompanying paper.[12]

On the other hand, it is known that there are marked differences in anti-inflammatory action in different animal species: BZY·HCl only partially suppressed the inflammatory response of rabbit skin induced by X-rays,[13] and displayed no activity on the erythema induced by ultraviolet irradiation of guinea-pigs,[8] whereas reddening of inflamed area disappeared more rapidly and the functional recovery improved in rats injected with silver nitrate into the ankle articulation.[8] Unconjugated BZY and BZY–NO were mainly present in rat blood, but the level of a conjugated metabolite (G-1) was lower (Fig. 3). On the other hand, in rabbit blood, a conjugated metabolite (G-1) was predominant (Fig. 8). Such results are in accord with reports[8,13] of species differences in the anti-inflammatory action, and further with the results of the previous paper[2] showing that conjugated metabolites were predominant in rabbit urine, in contrast to the situation in rat urine.

As shown in Fig. 9,—11, it was confirmed that BZY–NO possessed anti-inflammatory action only when administered orally, and did not exhibit any action when given intravenously. Silvestrini *et al.*[8] have already reported that the minimal effect of BZY·HCl was produced with an intravenous dosage of about 5 mg/kg on the basis of the dose-analgesic activity curve in rats using the Randall and Selitto method, and the maximal effect was seen within 30—90 minutes. In view of the activity of BZY·HCl at this low level, and the fact that BZY–NO maleate did not exhibit any action when given intravenously, it seems very likely that the anti-inflammatory action of BZY·HCl may be caused by BZY itself, and not by BZY–NO. BZY–NO appears to be reduced only when given orally, and hardly at all when given intravenously. However, the reason for this is not clear.

Although the major metabolite BZY–NO did not possess anti-inflammatory action itself, its apparent anti-inflammatory action suggests that BZY and its metabolite BZY–NO coexist in metabolic equilibrium in the body, and cause anti-inflammatory action directly by transfer from the blood stream to the inflamed tissue.

It is known that the N-deoxygenation of aliphatic tertiary amine N-oxides can occur anaerobically in a system of cytochrome P-450[14] and/or xanthine oxidase (XOD).[15] Accordingly, the possible participation of the XOD system in the N-deoxygenation of aromatic

12) S. Kataoka, K. Nishimura, T. Naito, and K. Taira, *Chem. Pharm. Bull.* (Tokyo), **27**, 2904 (1979).
13) R.G. Harrison and P.J. O'Donnell, *Toxicol. Appl. Pharmacol.*, **17**, 355 (1970).
14) M. Sugiura, K. Iwasaki, H. Noguchi, and R. Kato, *Life Sci.*, **15**, 1433 (1974); R. Kato, K. Iwasaki, and H. Noguchi, *Hoppe-Sayler's Z. Physiol. Chem.*, **357**, 1033 (1976); R. Kato, K. Iwasaki, and H. Noguchi, R. Kato, Y. Imai, and R. Sato, *Biochem. Biophys. Res. Comm.*, **77**, 1143 (1977).
15) S. Kataoka, T. Naito, T. Ariyoshi, and E Takabatake, the Ninth Symposium on Drug Metabolism and Action, p77, Kumamoto, Japan, November, 1977; S. Kataoka and T. Naito, *Chem. Pharm. Bull.* (Tokyo), **27**, 2913 (1979).

and aliphatic tertiary amine N-oxides requires examination.

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