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Metabolism of Drugs. LXXX.[1] The Metabolic Fate of Nitrofuran Derivatives. (3). Studies on Enzymes in Small Intestinal Mucosa of Rat Catalyzing Degradation of Nitrofuran Derivatives

Kiyoshi Tatsumi, Toshiro Yamaguchi, and Hidetoshi Yoshimura

Faculty of Pharmaceutical Sciences, Kyushu University[2]

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The enzymes responsible for the degradation of nitrofuran derivatives in rat small intestinal mucosa were examined *in vitro*. As a result, it was suggested that the initial reactions in the degradation of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) and 5-nitro-2-furfural semicarbazone (nitrofurazone) are mainly catalyzed by xanthine oxidase, while that of 2-amino-5-[2-(5-nitro-2-furyl)-1-(2-furyl)vinyl]-1,3,4-oxadiazole (NF-161) is catalyzed by alternative enzyme beside xanthine oxidase, too.

Furthermore, the present results suggest that a part of metabolites formed from AF-2 by hypoxanthine-dependent or NADH-dependent reaction in rat small intestinal mucosa might be tightly bound to or physically adsorbed to the macromolecules of cellular constituents.

If drugs and food additives ingested were metabolized by the stomach and intestine, their absorption from gastrointestinal tract, effectiveness and toxicity would be altered. Accordingly, although the liver has a high capacity to perform the biotransformation of exogeneous chemicals, it is important to recognize that the gastrointestinal tract also has this ability.

Until now, the metabolism of many drugs such as adrenaline,[3] isoproterenol,[4] prontosil,[5] 1-naphthyl N-methylcarbamate[6] and L-3,4-dihydroxyphenylalanine[7] by intestine or stomach has been investigated in connection with their pharmacological activity in animal body.

On the other hand, we examined previously the factors influencing the absorption of nitrofuran derivatives from gastrointestinal tract of the rat using ^{14}C-labeled compounds. As a result, it was found that a part of nitrofuran derivatives given to the rat orally were degraded by mucosa of small intestine and that the absorption rate of these compounds has close relationship with such degradation.[1]

The present investigation was undertaken in order to explore the enzymes which catalyzed the degradation of nitrofuran derivatives in small intestinal mucosa of the rat.

Experimental

Materials——Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine), NADH, NADPH and glucose-6-phosphate were obtained from Sigma Chemical Corp. Hypoxanthine was obtained from Nakarai Chemicals Co., Ltd. The ^{14}C-labeled compounds, namely ^{14}C-AF-2 [2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide(acryl-

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amide-3-^{14}C)], ^{14}C-NF-161 [2-amino-5-(2-(5-nitro-2-furyl)-1-(2-furyl)vinyl)-1,3,4-oxadiazole(vinyl-2-^{14}C)] and ^{14}C-nitrofurazone [5-nitro-2-furfural semicarbazone(formyl-^{14}C)] were kindly supplied by Ueno Pharmaceutical Co., Ltd. The radiochemical purity of these labeled compounds was examined by the thin-layer chromatography using solvent system of AcOEt–hexane–AcOH (12: 8: 1). The specific radioactivities of the above compounds were as follows: ^{14}C-AF-2 0.20 μCi/mg, ^{14}C-NF-161 0.22 μCi/mg, and ^{14}C-nitrofurazone 0.12 μCi/mg.

Radioisotope Methods of Analysis——The radioactivity of all samples was measured using Packard Scintillation Spectrometer (Model 3375) and was corrected for quenching by an external standard method. The samples were counted in a *p*-dioxane phosphor consisting of 4 g PPO, 0.2 g POPOP, 60 g naphthalene, 100 ml methanol, 20 ml ethylene glycol and *p*-dioxane to make 1 liter or in a toluene phosphor consisting of 4 g PPO, 0.1 g POPOP and toluene to make 1 liter.

Preparation of Enzyme Sources——Male Donryu rats weighing 150 to 200 g were fasted overnight before the experiments. The rats were killed by a blow on the head, exanguinated, and the small intestine was removed irrigating with ice cold 0.3M sucrose which was adjusted to pH 7.4 by adding a small amount of $KHCO_3$. The mucosa was extruded by gentle pressure with a pincette as described previously.[1] The extruded mucosa was suspended in 9 volumes of 0.3M sucrose, pH 7.4 and homogenized by six down and six upward strokes with Teflon-glass homogenizer, according to the modified method of Hübscher, *et al.*[8] The homogenate was filtered through a double layer of cloth (calico) to separate into filtered homogenate and gelatinous material. In some experiments, the filtered homogenate and gelatinous material were dialyzed against 0.1M phosphate buffer, pH 7.4 at 4° for 24 hr using the cellulose tube.

These preparations were adjusted to appropriate volume with 0.3M sucrose, pH 7.4 prior to use.

Enzyme Assay——The incubation was carried out under an atmosphere of air or N_2 at 37° for 1 hr using a metabolic incubator.

A typical incubation mixture contained 0.6 μmoles of ^{14}C-AF-2, 0.2 μmoles of ^{14}C-NF-161 or 1.3 μmoles of ^{14}C-nitrofurazone, 900 units of Penicillin G, 5 ml of tissue preparation equivalent to 0.5 g of mucosa and 4 ml of 0.05M phosphate buffer, pH 7.4.

In the experiments on the cofactor requirement, 1.2 μmoles of hypoxanthine or 1.2 μmoles of NADH were added in the above incubation mixture. Where NADPH was used, 1.2 μmoles of NADPH and 32 μmoles of glucose-6-phosphate were added. In some experiments, the xanthine oxidase inhibitor, allopurinol was added at a final concentration of 1×10^{-5}M.

The reaction was terminated by the addition of 0.6 ml of 45% perchloric acid. After centrifugation in the cold, the supernatant was adjusted to pH 5 by adding 0.8 ml of 5N potassium carbonate. The perchlorate salts were then removed by centrifugation in the cold. The supernatant was submitted to a Sephadex G-10 column chromatography in order to separate the degradation products from the unchanged compound as follows: Sephadex G-10 soaked and swollen with 0.05M NaCl solution was packed in a glass tube (0.9 cm in inside diameter). The column sizes were 5.0×0.9 cm for AF-2, 2.0×0.9 cm for NF-161 and 8.0×0.9 cm for nitrofurazone. Above supernatants (1 ml in the cases of AF-2 and nitrofurazone, and 2 ml in the case of NF-161) charged on the column were eluted with 0.05M NaCl solution, collecting into 1 ml fractions. The degradation products of three nitrofuran derivatives were eluted in the fractions 1 to 10. The unchanged compounds were eluted in the fractions 15 to 40 for AF-2, 21 to 80 for NF-161 and 15 to 30 for nitrofurazone. Quantitative determination of the unchanged compounds and the degradation products were conducted by counting the radioactivity of the above fractions. The radioactivity of each sample was recovered quantitatively during Sephadex G-10 column chromatography.

Dialysis of Degradation Products of AF-2——AF-2 was incubated with the homogenate of mucosa under aerobic or anaerobic condition. The incubation mixture was submitted to the perchloric acid-treatment and the Sephadex G-10 column chromatography successively as described above. The fraction in which the degradation products were eluted from Sephadex G-10 was concentrated to a small volume and then adjusted to 5 ml with the deionized water. The sample obtained here was dialyzed against 100 ml of the deionized water, which was renewed at the sixth and eighteenth hours, at 4° for 24 hr using the cellulose tube. After dialysis, the 0—6 hr, 6—18 hr, and 18—24 hr outer fluids were concentrated to an appropriate volume and these were counted for radioactivity. Also, the inner fluid was counted for radioactivity.

Ion Exchange Resin Column Chromatography——The degradation products of AF-2 before and after dialysis mentioned above were submitted to strong cation exchange resin column chromatography as follows: The fractions of degradation products obtained by Sephadex G-10 chromatography, and the inner and outer fluids after dialysis of these fractions were concentrated to the appropriate volume and then adjusted to 5 ml each with the deionized water. These samples were charged on Amberlite IR-120 column (20—50 mesh, free form, 0.9×8.0 cm) and eluted with successive 40 ml of the deionized water and 40 ml of 5% NH_4OH, collecting into 5 ml fractions. These fractions obtained here were counted for radioactivity.

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Result

Effect of Gas Phase on the Degradation of AF-2

Effect of gas phase on the degradation of AF-2 was investigated using the homogenate, filtered homogenate and gelatinous material of small intestinal mucosa as enzyme sources.

TABLE I. Effect of Gas Phase on Degradation of AF-2 by Tissue Preparation of Rat Small Intestinal Mucosa

	Rate of degradation (%)	
	Air	N_2
Homogenate	28.4	83.8
Boiled homogenate	5.3	8.2
Filtered homogenate(FH)	7.5	69.1
Gelatinous material(GM)	10.2	4.9
GM plus boiled FH	12.6	59.7
FH plus boiled GM	14.3	75.3

Each value represents a mean of three experiments.

As shown in Table I, nitrogen stimulated extremely the degradation of AF-2 by homogenate and filtered homogenate, but not that by gelatinous material. However, in the case of gelatinous material there is the possibility that this tissue preparation contains the enzyme only without cofactor. Therefore, the effect of boiled filtered homogenate on the degradation of the drug by gelatinous material was investigated under anaerobic condition. As a result, the degradation of the drug increased extremely by addition of boiled filtered homogenate to gelatinous material. Also, the effect of boiled gelatinous material on the degradation by filtered homogenate was examined anaerobically. However, this boiled preparation did not effect on the above degradation. On the other hand, the degradation rate of AF-2 by the homogenate boiled for 5 min in a water bath was less than 10% either in aerobic or anaerobic condition.

From these results, it was indicated that the degradation of AF-2 by small intestinal mucosa proceeded to a greater extent under anaerobic condition than under aerobic. Furthermore, it was suggested that the enzyme responsible for the degradation of AF-2 was located not only in filtered homogenate, but also in gelatinous material.

Effect of Allopurinol on the Degradation of AF-2

The xanthine oxidase inhibitor, allopurinol, was tested for its effect on the degradation of AF-2 by homogenate, filtered homogenate and gelatinous material under aerobic or anaerobic condition.

TABLE II. Effect of Allopurinol on Degradation of AF-2 by Tissue Preparation of Rat Small Intestinal Mucosa

	Rate of degradation (%)			
	Control		Addition of allopurinol	
	Air	N_2	Air	N_2
Homogenate	35.7	83.8	7.8	11.0
FH	—	69.1	—	10.6
GM Plus boiled FH	—	59.7	—	12.7

FH=filtered homogenate
GM=gelatinous material
Each value represents a mean of three experiments.

As can be seen in Table II, allopurinol at concentration of 1×10^{-5} M effectively inhibited the degradation of AF-2 in all the cases.

From these results, it was suggested that xanthine oxidase was the initial enzyme mainly responsible for the degradation of AF-2 by small intestinal mucosa.

Relationship between Aerobic and Anaerobic Reactions

It is interesting to know whether aerobic and anaerobic degradation of AF-2 by small intestinal mucosa is catalyzed by the same enzyme. In this connection, the data shown in Table II have partly intimated the similarlity of reactions under above both conditions, because allopurinol inhibited to about the same degree the both aerobic and anaerobic degradation of the drug by homogenate of mucosa.

Further investigation was carried out in order to confirm this as follows.:

(1) Dialysis of AF-2 Degradation Products——The samples of the degradation products formed from ^{14}C-AF-2 aerobically and anaerobically were dialyzed against the deionized water for 24 hr as described in Experimental.

Table III. Dialysis of Degradation Products Formed from AF-2 by Homogenate of Rat Small Intestinal Mucosa under Aerobic and Anaerobic Conditions

	% of radioactivity	
	Outer fluid	Inner fluid
Aerobic	74.0	26.0
Anaerobic	72.2	27.8

Values in Table represent a typical result obtained by one experiment.

As shown in Table III, the percent recovery of radioactivity in the outer fluid or in the inner fluid was about the same order in the aerobic and anaerobic degradation products.

(2) Amberlite IR-120 Column Chromatography of AF-2 Degradation Products——The samples of the degradation products formed from ^{14}C-AF-2 aerobically and anaerobically were submitted to Amberlite IR-120 column chromatography as described in Experimental.

Table IV. Amberlite IR-120 Column Chromatography of Degradation Products Formed from AF-2 by Homogenate of Rat Small Intestinal Mucosa under Aerobic and Anaerobic Conditions

	% of radioactivity			
	Aerobic		Anaerobic	
	H_2O	5% NH_4OH	H_2O	5% NH_4OH
Before dialysis	37.6	40.6	37.6	42.8
After dialysis				
Outer fluid	41.3	42.0	42.2	39.7
Inner fluid	34.4	39.0	31.6	33.5

Values in Table represent a typical result obtained by one experiment.

As can be seen in Table IV, the elution pattern from this cation exchanger was similar in the aerobic and anaerobic degradation products. Furthermore, the similar results were obtained in the outer and inner fluids after dialysis of the above both samples.

From these results shown in Table III and IV, it was suggested that the aerobic and anaerobic degradation products of AF-2 were formed by the same enzymatic reaction.

Effects of Hypoxanthine, NADH and NADPH on the Activity of Tissue Preparation of Mucosa to Degrade AF-2

The effects of hypoxanthine, NADH and NADPH on the anaerobic degradation of AF-2 by the filtered homogenate and gelatinous material of mucosa were compared.

TABLE V. Cofactor Requirement for Degradation of AF-2 by Tissue Preparation of Mucosa, and Percent Recovery of Radioactivity in Incubation Mixture

Additions	Dialyzed filtered homogenate		Dialyzed gelatinous material		Gelatinous material	
	D	R	D	R	D	R
			(%)			
None	3.6	93.2	3.5	94.9	10.2	92.1
Hypoxanthine	43.7	56.1	10.9	85.3	43.8	77.0
Hypoxanthine, Allopurinol	5.7	94.4	—	—	3.6	95.4
NADH	34.0	63.1	26.8	81.3	—	—
NADH, Allopurinol	4.1	100.2	0.6	103.5	—	—
NADPH	17.0	90.3	18.3	93.2	—	—
NADPH, Allopurinol	3.7	98.5	0.6	100.3	—	—

D=rate of degradation, R=percent recovery of radioactivity in incubation mixture
Each value representsa mean of three experiments.

As can be seen in Table V, hypoxanthine was more effective in the AF-2 degradation by dialyzed filtered homogenate, but was less effective in that by dialyzed gelatinous material than NADH or NADPH. However, non-dialyzed gelatinous material degraded the drug effectively with hypoxanthine. Furthermore, the effect of allopurinol on these reactions to degrade AF-2 was examined. In the case of hypoxanthine-dependent reaction by gelatinous material, allopurinol was added to the reaction mixture containing non-dialyzed preparation. As a result, above reactions involved in the AF-2 degradation were almost completely inhibited by 1×10^{-5} M allopurinol.

Thus, the facts that the hypoxanthine-dependent, NADH-dependent and NADPH-dependent degradation of AF-2 in both filtered homogenate and gelatinous material were inhibited by allopurinol, indicate that all of these reactions are presumably catalyzed by xanthine oxidase. However, that the effectiveness of hypoxanthine, NADH and NADPH as cofactors in the AF-2 degradation were different between filtered homogenate and gelatinous material suggests the multiplicity of xanthine oxidase in rat small intestinal mucosa.

Recovery of Radioactivity in Incubation Mixture

Table V also shows the percent recovery of radioactivity in the supernatant obtained from the incubation mixture after perchloric acid treatment and centrifugation.

The radioactivity from ^{14}C-AF-2 in the supernatant was apparently decreased when hypoxanthine or NADH is added to dialyzed filtered homogenate and dialyzed gelatinous material. The similar phenomenon was observed following the addition of hypoxanthine to non-dialyzed gelatinous material. Furthermore, when allopurinol was added to above tissue preparations supplemented with hypoxanthine or NADH, such decrease of radioactivity was not observed in all the cases. On the other hand, when NADPH is added to dialyzed filtered homogenate and dialyzed gelatinous material, the radioactivity recovered from the supernatant did not decreased appreciably as compared with addition of hypoxanthine or NADH.

These facts suggest that the metabolites formed from AF-2 by hypoxanthine-dependent and NADH-dependent reaction might be tightly bound to or physically adsorbed to the perchloric acid precipitates.

Effect of Allopurinol on the Degradation of Nitrofurazone and NF-161

In the presence of 1×10^{-5} M allopurinol, ^{14}C-nitrofurazone and ^{14}C-NF-161 were incubated with the homogenate of small intestinal mucosa under anaerobic condition, respectively.

TABLE VI. Effect of Allopurinol on Degradation of Nitrofurazone and NF-161 by Homogenate of Rat Small Intestinal Mucosa

	Rate of degradation (%)	
	Control	Addition of allopurinol
Nitrofurazone	59.1 ± 9.4	16.5 ± 5.6
NF-161	86.1 ± 5.4	71.5 ± 4.3

Each value represents a mean of four experiments±S.D.

As shown in Table VI, the degradation of nitrofurazone was effectively inhibited by allopurinol. To the contrary, the degradation of NF-161 was not affected so much by this inhibitor.

These results suggest that the initial reaction in the degradation of nitrofurazone, like that of AF-2, is mainly catalyzed by xanthine oxidase, while that of NF-161 can be catalyzed by alternative enzyme system beside xanthine oxidase system, too.

Discussion

It is known that xanthine oxidase is present in various rat tissues[9] and catalyzes the reduction of nitrofuran derivatives *in vitro*.[10] However, sufficient evidence has not been presented indicating that this enzyme is important in the *in vivo* metabolic fate of nitrofuran derivatives. We showed previously that when ^{14}C-nitrofuran derivatives are given orally to rat, the absorption rate of the radioactivity from the gastrointestinal tract has close relationship with the degradation rate of these compounds in the small intestinal mucosa.[1]

In the present study, it has been shown that the enzyme responsible for the initial degradation reaction of AF-2 and nitrofurazone in rat small intestinal mucosa is probably xanthine oxidase, since allopurinol remarkably inhibited the degradation of these compounds by this tissue preparation. From these facts, it is considered that xanthine oxidase plays a important role in the *in vivo* metabolic fate of AF-2 and nitrofurazone.

On the other hand, recent experiments[11] in which we examined the degradation of AF-2 by rat liver homogenate gave results apparently different from those obtained with the rat small intestinal mucosa described in the present paper. Namely, 1×10^{-5} M allopurinol inhibited only about 19% of the AF-2 degradation by rat liver homogenate under anaerobic condition, suggesting that other enzymes as well as xanthine oxidase were responsible for the degradation of the drug in this tissue preparation. Similarly, McCalla, *et al.* showed that the reduction of nitrofurazone in $9000\times g$ supernatant of homogenized rat liver was catalyzed by two enzymes, xanthine oxidase and NADPH-dependent enzyme which probably is NADPH-cytochrome C reductase.[12]

From these results, it seems that the initial reaction in the metabolism of AF-2 and nitrofurazone is exclusively catalyzed by one enzyme in rat small intestinal mucosa, while by two enzymes in rat liver. If it is true, above results very likely reflect the difference of relative

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12) D.R. McCalla, A. Reuvers, and C. Kaiser, *Biochem. Pharmacol.*, **20**, 3532 (1971).

activity of xanthine oxidase and NADPH-cytochrome C reductase between small intestinal mucosa and liver.

We showed previously that a part of NF-161 given orally to rats is split into at least two fragments in rat intestinal tract; one contains vinyl-2-carbon and the other contains oxadiazole-2-carbon. This fact suggests that NF-161 is attacked by some enzymes at the other positions beside nitro group. In accord with this view, we found that the degradation of NF-161 by the homogenate of rat small intestinal mucosa, unlike those of AF-2 and nitrofurazone, was not inhibited appreciably by allopurinol.

In addition to the fact that allopurinol inhibits the degradation reaction of AF-2, the fact that AF-2 is degraded when hypoxanthine or NADH is added to filtered homogenate or gelatinous material, provides additional support for the role of xanthine oxidase in the AF-2 degradation by small intestinal mucosa. In this case, the enzyme in dialyzed filtered homogenate requires preferentially hypoxanthine as cofactor, while the enzyme in dialyzed gelatinous material requires NADH. Moreover, the hypoxanthine-dependent enzyme activity in gelatinous material decreases remarkably by dialysis. Although little evidence is available concerning this loss of enzyme activity, it may be attributed to removal of an unidentified factor. These findings might suggest that the enzymes in both fractions are different each other. In addition, such observation rules out the possibility that the enzyme activity in gelatinous material is due to the contamination of the enzyme in filtered homogenate.

McCalla, *et al.* have reported that the enzymes such as xanthine oxidase and NADPH-dependent enzyme which are known to reduce nitrofurazone, catalyze the binding of radioactivity from ^{14}C-nitrofurazone to trichloroacetic acid-insoluble material such as protein.[12,13] Present paper also shows the possibility that some radioactivity from ^{14}C-AF-2 is bound to perchloric acid-insoluble material when hypoxanthine or NADH is added to filtered homogenate or gelatinous material. Thus, some nitrofuran derivatives are assumed to be metabolized to the intermediates which react with macromolecules of cellular constituents. However, the physiological importance or chemical nature of these intermediates has not yet been elucidated at present.

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