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Studies on the Metabolism of Oxazolam. III.[1] Absorption from Rat Intestine and Metabolites in the Liver, Brain and Blood

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The intestinal absorption of ^{14}C-oxazolam was studied by means of rat ligated loop technique and the metabolites in the intestine, liver, blood and brain were followed by thin-layer chromatography. The results indicated that oxazolam is very easily absorbed from almost whole part along the rat intestine, with the fastest rate from the upper part of the small intestine and is transferred from the lumen into the blood stream mostly in the form of unaltered oxazolam. It was also clarified that a large part, approximately 60%, of the drug absorbed is brought back into the intestinal lumen through the biliary excretion and the extent of occurring its reabsorption appears to be insignificant. In the brain, oxazolam was the main component of the radioactivity at the earliest period after the administration, while N-desmethyldiazepam showed a gradual increase with increasing the time concomitant with a decline of the oxazolam level, reaching a maximum at a later period. A possible participation of this metabolite for the duration of anticonvulsant activity of oxazolam was pointed out. It was suggested from *in vitro* studies that N-desmethyldiazepam was derived from its gradual transfer from the blood circulation into the brain tissue after being formed from oxazolam in the liver microsomal enzyme.

The previous studies[3] on the distribution and/or excretion of oxazolam (I) in mice, rats, dogs and man indicated that oxazolam appears to be well absorbed from the gastro-intestinal tract after oral administration. On the other hand, in the studies on the biotransformation of oxazolam,[1] no oxazolam was detected in the urine and the feces after oral administration in rats, while a glucuronide of 2-amino-5-chloro-3-hydroxybenzophenone (VII) was found to be excreted as the main metabolite. Therefore, it seemed of importance to study the intestinal absorption of oxazolam in order to see in what form and from what part of the gastro-intestinal tract the drug is absorbed and, furthermore, in what form the drug is distributed in the organs and tissues.

In the present paper, the absorption of ^{14}C-oxazolam from the intestine was studied by means of rat ligated loop technique[4] and the metabolites in the intestine, liver, blood and brain were followed by thin-layer chromatography. The results indicated that oxazolam is well absorbed from the intestine mostly in the form of unaltered oxazolam, which appears to be preferentially transferred into the organs and tissues.

Material and Method

Materials——^{14}C-Oxazolam labeled at the carbon-11b was prepared from ^{14}C-2-amino-5-chlorobenzophenone which was purchased from Daiichi Chemicals, Inc., Tokyo. The specific activity was 7.89 μCi/mg and the radiochemical purity was ascertained to be over 98% by thin-layer chromatography. The standard samples of oxazolam, N-desmethyldiazepam (II), oxazepam (III), 2-(2-hydroxy-*n*-propylamino)-acetylamino-5-chlorobenzophenone (PACB, V), 2-amino-5-chlorobenzophenone (ACB, VI) and 2-amino-5-chloro-3-hydroxybenzophenone (ACHB, VII) were prepared in this laboratories. Other chemicals were of reagent grade and used without further purification.

1) Part II: A. Yasumura, H. Murata, K. Hattori, and K. Matsuda, *Chem. Pharm. Bull.* (Tokyo), **19**, 1929 (1971).
2) Location: *Hiromachi 1-chome, Shinagawa-ku, Tokyo.*
3) H. Shindo, E. Nakajima, A. Yasumura, H. Murata, T. Hiraoka, and K. Sasahara, *Chem. Pharm. Bull.* (Tokyo), **19**, 60 (1971).
4) R.R. Levine and E.W. Pelikan, *J. Pharmacol. Exp. Therap.*, **131**, 319 (1961).

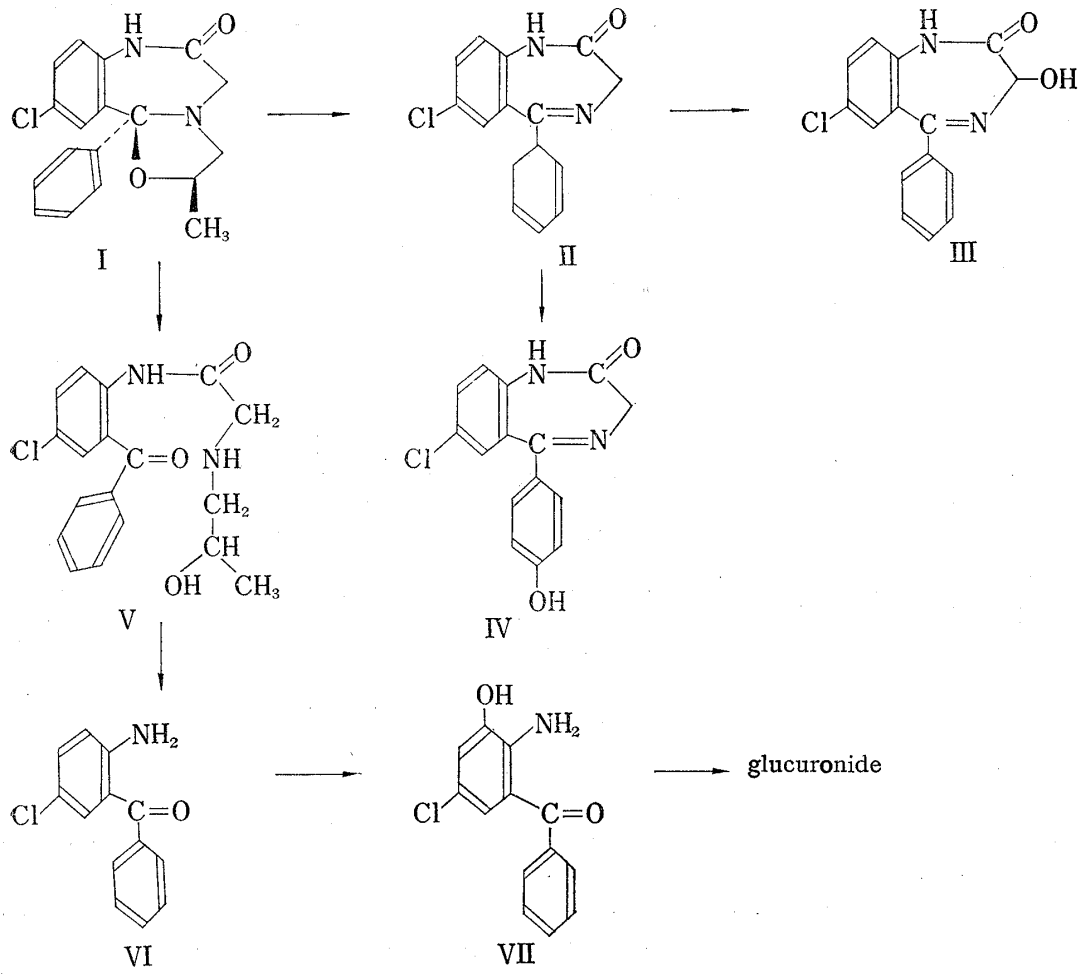


Chart 1. Possible Metabolic Pathway of Oxazolam[1)]

Experiments on Absorption from Rat Acute Loop——Male rats of Wistar-Imamichi strain weighing 180 to 240 g were used after fasting for 18 hr. After anesthetizing with ether, the intestine or stomach was exteriorized through a central mid-line incision and an acute loop of about 8 to 10 cm long was prepared from the duodenum, the upper, middle and lower part of the small intestine, rectum and whole stomach by ligatures of the both ends. The suspension[5)] of 0.5 mg ^{14}C-oxazolam in 0.5 ml of 0.5% Tragacanth solution was injected into the lumen of each loop with a syringe, the loop returned and the incision satured. After a given time, the loop was removed and the liquid contents were drained off with 10—15 ml cold saline. After cutting it open longitudinally, the mucosal surface was washed three times with each 3 ml saline using a pipett. The combined washings were centrifuged after adding 3 volumes of ethanol and the supernatant was assayed for radioactivity. The tissue was homogenized with a glass potter in 10 volumes of cold 70% ethanol and centrifuged. The residue was reextracted with 70% ethanol and the combined extracts were assayed for radioactivity. The recovery by the extraction procedure was over 97%. The amount of drug taken into the blood stream from the loop was then obtained by subtracting the residual amount in the lumen and that retained in the tissue from the dose and was referred as the amount truely absorbed.

The extracts thus obtained were evaporated to dryness at 30° under a reduced pressure with a rotary evaporator. The residue was dissolved in 0.5 ml of 70% ethanol and immediately spotted to be separated by thin-layer chromatography.[6)]

Experiments on Metabolism in Blood and Organs——Male rats of Wistar-Imamichi strain weighing 170 to 180 g were fasted for 18 hr and an acute loop of about 14 cm long was prepared from the upper part

5) In order to obtain an uniform and reproducible suspension, the crystals of ^{14}C-oxazolam was grinded in a small mortar with 0.5% Tragacanth solution for 30 min and the suspension was applied with a sonic oscillation for 10 min.

6) In order to avoid any further decomposition of oxazolam and its metabolites, the whole procedure from the extraction to the separation was achieved during less than 3 hr.

of the small intestine. One milliliter suspension containing 2 mg of ^{14}C-oxazolam was injected into the lumen and the amimals were sacrificed by bleeding from the carotid after 15, 60 and 180 min. The blood was collected in a heparinized test tube and the brain and liver were immediately removed. The blood was deproteinized and extracted with 3 volumes of cold ethanol by applying a sonic oscillation for 10 min. The brain and liver were homogenized in 70% cold ethanol with a Potter glass homogenizer. After centrifugation, the residue was reextracted with the same solvent. The combined extracts were evaporated to dryness at 30° under a reduced pressure and the residue was dissolved in 1 ml of 70% ethanol for the separation by thin-layer chromatography.[6]

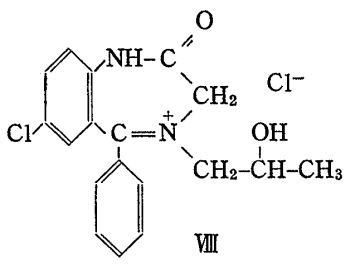
Experiments on Biliary Excretion——Male rats weighing about 200 g were used after fasting for 18 hr. Under ether anesthesia, an acute loop of about 14 cm long was prepared from the upper part of the small intestine and the common bile duct was cannulated. One milliliter suspension of 2 mg ^{14}C-oxazolam was then injected into the lumen of the loop and the bile was collected periodically every 1 to 2 hr for 24 hr period. Each 50 μl aliquot was assayed for radioactivity. In order to study the reabsorption from the intestine, the bile collected during the first 3 hr was pooled and 1 ml aliquot (about 5×10^5 dpm) was injected into the lumen of a loop prepared from the upper part of the small intestine in a normal rat. The amount of absorption in 1 hr was determined by the method described above. In order to study the metabolites, a part of the pooled bile was added with an excess of cold ethanol and after centrifugation the supernatant was separated by thin-layer chromatography.

Experiments on *in Vitro* Metabolism——Male rats weighing about 200 g were sacrificed by bleeding from the carotid and the liver and brain were removed rapidly. After rinsing in physiological saline, the tissues were homogenized in ice-cold 1.15% KCl solution (1:2 w/v) with Potter glass homogenizer. For the preparation of microsomal enzymes, the liver homogenates were centrifuged at $9000\times g$ for 20 min and then the supernatant fraction was again centrifuged at $105000\times g$ for 60 min. The precipitates were suspended in 1.15% KCl solution.

An incubation mixture, which is similar to that used by Kato, *et al.*,[7] was consisted of 2 ml of the tissue homogenate or the microsomal fraction equivalent to 1 g of the liver or brain, NADP (1.5 μmoles), glucose-6-phosphate (50 μmoles), glucose-6-phosphate dehydrogenase (0.5 units), $MgCl_2$ (25 μmoles), nicotinamide (50 μmoles), 0.5 ml of 1.15% KCl solution, 1.4 ml of 0.2M phosphate buffer of pH 7.4 and 0.5 ml suspension of 1 μmole of ^{14}C-oxazolam, the final volume being 5 ml. The mixture was incubated at 37° under air for various periods of time. The mixture was then extracted three times with 25 ml portions of chloroform. The combined extracts were concentrated under a reduced pressure and brought to the separation by thin-layer chromatography.

Separation of Oxazolam and Its Metabolites by Thin-Layer Chromatography——Oxazolam and its metabolites were separated using Aluminium oxide plate (Merck, F_{254}) which was activated by heating at 130° for 30 min prior to use. The solvent systems used are: A) chloroform: acetic acid (95: 1), B) isopropanol: conc. NH_4OH: cyclohexane (20: 1: 200) and C) *n*-heptane: chloroform: ethanol (10: 10: 1). The separated spots were detected with ultraviolet light (short wavelength) and the radioactigram was obtained with Aloka radio-chromatoscanner. The radioactive spots were quantitatively transferred into the counting vials by scratching carefully with a spatula and the radioactivity was counted after shaking in 15 ml of liquid scintillator. Average *Rf* values of oxazolam and its known metabolites[1] are shown in Table I. Pure standards were run in every separation alongside the sample for identification of the compounds.

Oxazolam gave two spots with closed *Rf* values when it was developed with solvent B, a basic solvent system. These two spots are considered to be *cis* and *trans* isomers[8] of oxazolam with respect to 11b-phenyl and 2-methyl groups which are reversible in a slow equilibration, because of the following reasons: i) The extracts from both spots gave an identical mass spectrum with a parent peak corresponding to mass number 328, ii) ultraviolet spectra of the two extracts gave an absorption maximum at 246 and 243 mμ in ethanol for the spot with a larger and a smaller *Rf* values, respectively, while the spectra of the extracts with 0.1N HCl, measured after standing for 3 hr, gave an identical spectrum with two maxima at 237 and 364 mμ, which was identical to the spectrum of the quaternary form of oxazolam (VIII), and iii) when two-dimentional chromatography was applied using the same solvent, each of the two spots separated by the first run gave a faint but apparent spot at the position corresponding to the other one of the pair, resulting in an appearence of four spots. Therefore, these two spots were regarded and treated as oxazolam.



VIII

7) R. Kato and M. Takayanagi, *Jap. J. Pharmacol.*, **16**, 380 (1966).
8) T. Miyadera, A. Terada, M. Fukunaga, Y. Kawano, T. Kamioka, C. Tamura, H. Takagi and R. Tachikawa, *J. Med. Chem.*, **14**, 520 (1971).

Table I. *Rf* Values of Oxazolam and Its Main Metabolites on Thin-Layer Chromatography

Compound	*Rf* value		
	A	B	C
Oxazolam	0.56	0.74, 0.67	0.81
N-Desmethyldiazepam	0.17	0.41	0.43
Oxazepam	0.03	0.00	0.00
PACB	0.31	0.32	0.57
ACB	0.84	0.88	0.94
ACHB	0.80	0.12	0.08

aluminium oxide plate (F_{254}, Merck)
solvent A: chloroform: acetic acid (95:1)
B: isopropanol: conc. NH_4OH: cyclohexane (20:1:200)
C: heptane: chloroform: ethanol (10:10:1)

Radioactivity Measurement——All the extracts, suspensions and bile were counted in the Beckman LS-250 liquid scintillation spectrometer using a counting medium consisted of 8 g PPO, 200 mg dimethyl-POPOP, 200 ml of toluene and 800 ml of dioxane. The counting efficiencies were determined by ^{137}Cs external standard method and the counts were converted to disintegration per minute (dpm) with Olibetti Programma 101 computor.

Identification and Analysis of ^{14}C-Oxazolam by Reverse Isotope Dilution Method——A known amount (W mg) of non-radioactive oxazolam was added to a certain amount (R dpm) of the extract from tissue with 70% ethanol and was dissolved completely by heating. After cooling the solution, the precipitated crystals were separated and recrystallized from ethanol several times. The specific activity was determined after each recrystallization. When a constant value was obtained, the content of radioactive oxazolam (W_x mg) and the percentage (X%) in the original extracts were calculated from the mean specific activity of the crystals (S dpm/mg) and the specific activity of the original ^{14}C-oxazolam (S_0 dpm/mg) according to the following equations.

$$W_x = W\left(\frac{S}{S_0 - S}\right)$$

$$X = \frac{(W+W_x)S}{R} \times 100$$

Result

Absorption of Oxazolam from Rat Intestine

Absorption of oxazolam during 1 hr after injection into the lumen of the ligated loop prepared from various sections of rat gastro-intestinal tract are shown in Table II. It was found that oxazolam is very easily absorbed from the whole part along the small intestine, in particular, from the upper part where approximately 75% of the dose (0.5 mg) was transferred into the blood stream during 1 hr. The apparent absorption of the drug from the duodenum was found to be relatively low (about 38% of the dose). However, since there was a possibility that the absorbed drug was brought back into the duodenum lumen through the biliary excretion, the same experiment was carried out in rats of which the common bile duct was ligated. The result showed a considerable increase of the absorption to about 60% of the dose in 1 hr, indicating that oxazolam is well absorbed from the duodenum to almost the same extent as that from the small intestine. The stomach showed only a slight but an appreciable absorption.

The time course of the absorption from the upper part of the small intestine is shown in Table III and Fig. 1. As early as 5 min after injection about 50% of the dose had disappeared from the lumen and after 1 and 3 hr the most of the administered drug was shown to be absorbed, indicating that the absorption of oxazolam is quite rapid and complete. As

Table II. Absorption of Oxazolam from Different Sections of Rat Gastro-intestinal Tract

Section	%-absorption in 1 hr[a)]	$n^{b)}$
Stomach	12.75±5.27	3
Duodenum	38.03	2
Duodenum[c)]	60.22±1.29	3
Small Intestine		
Upper	74.30±1.70	3
Middle	65.20±6.26	3
Lower	50.62±8.66	3
Rectum	52.23±1.76	3

a) % transferred into blood to dose ± S.E.
b) n=number of experiments
c) The common bile duct was ligated.

can be seen from the figure, the amount retained in the intestinal tissue showed a rapid increase soon after the injection and after keeping a maximum level for a period between 5 and 30 min the concentration was decreased gradually with time, while the amount of absorption into the blood stream showed a rather constant increase up to 30 min after injection. The results suggest that the transfer of oxazolam from the lumen into the blood stream is proceeded through a transient step of an accumulation of the drug in the mucosal tissue. This absorption pattern is in good accordance with that observed by Doluisio, *et al.*[9] for highly lipophilic drugs such as the phenothiazine or butyrophenone tranquilizers.

The dose-absorption relationship which characterizes the capacity for absorbing the drug is shown in Fig. 2. Up to the dose of approximately 0.3 mg/loop, the relation appears to be linear with a very high absorbability, while with increasing the dose higher than this level the slope appears to be decreased gradually.

Table III. Absorption of Oxazolam from Ligated Loop of Rat Small Intestine (Dose: 0.5 mg/loop)

	% to dose±S.E.			
	5 min (n=7)[a)]	30 min (n=6)	60 min (n=3)	180 min (n=3)
Residual amount in lumen	52.76±2.51	21.73±4.97	16.14±4.51	4.21±1.13
Retained in tissue	29.20±2.68	29.65±7.19	15.25±3.75	7.55±3.81
Absorbed into blood	18.04±3.78	48.62±6.50	68.61±3.03	88.24±1.59

a) n=number of experiments

In order to study whether oxazolam is absorbed through the mucosal tissue in the unaltered form or in some metabolized form, the extracts from the intestinal tissue and lumen 5 and 30 min after the injection were separated by thin-layer chromatography. As the results, the extracts from both the tissue and the lumen showed a dominant spot corresponding to oxazolam, concomitant with a weaker spot corresponding to PACB and a faint spot remaining at the origin. Counting of the spots revealed that, as shown in Table IV, over 80% of the total radioactivity in all the extracts was unchanged oxazolam. In a separate experiment, radioactive oxazolam was identified and analysed by reverse isotope dilution

9) J.T. Doluisio, W.G. Crouthamel, G.H. Tan, J.V. Suintosky, and L.W. Dittert, *J. Pharm. Sci.*, **59**, 72 (1970).

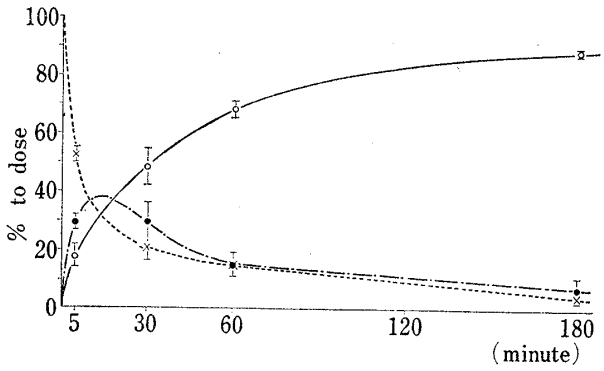


Fig. 1. Time Course of Absorption of Oxazolam from Rat Ligated Small Intestine

Each value represents the mean±S.E. from 3 to 7 experiments.
—○—: absorption into blood
·—●—·: retained in the tissue
——×——: residual amount in the lumen
dose: 0.5 mg/loop

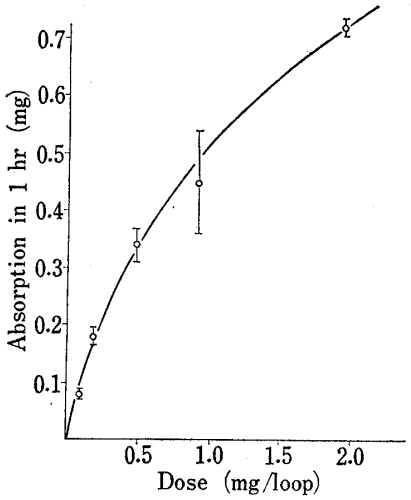


Fig. 2. Relationship between Absorption and Dose of Oxazolam in Rat Ligated Small Intestine

Each value represents the mean±S.E. from 3 experiments.

method and, as shown in Table V, over 80% of the radioactivity in the tissues was identified as oxazolam. As can be seen from Fig. 1, during the period from 5 to 30 min after injection of ^{14}C-oxazolam the radioactivity was transferred most rapidly from the lumen into the blood stream through the tissue. Therefore, the above results might be interpreted as indicating that oxazolam is absorbed through the intestine mostly in the unaltered form.

Table IV. Oxazolam and Its Metabolites in the Intestinal Tissue and Lumen after Injection of ^{14}C-Oxazolam into Ligated Loop of Rat Small Intestine (Dose: 0.5 mg/loop; average of two experiments)

Compound	% of radioactivity			
	5 min		30 min	
	Tissue	Lumen	Tissue	Lumen
Oxazolam	84.09	84.42	80.27	81.49
PACB (III)	12.44	13.46	12.88	9.55
Others	3.47	2.12	6.85	8.97

Table V. Identification and Analysis of ^{14}C-Oxazolam in the Extract from Intestinal Tissue and Lumen by Reverse Isotope Dilution Method (Dose: 0.5 mg/loop)

Min		Specific activity (dpm/mg) of the crystals			
		1st	2nd	3rd	%[a]
5	tissue	3585	3505	3539	91.09
	lumen	3939	3922	3971	79.68
30	tissue	1500	1494	1550	85.31
	lumen	1053	1045	1068	61.29

a) percentage of radioactive oxazolam to the total radioactivity of the extract

Biliary Excretion and Its Reabsorption from Intestine

The excretion of radioactivity in the bile after injection of ^{14}C-oxazolam into the ligated intestinal loop was shown in Fig. 3. A rapid excretion of radioactivity was observed soon after the injection and about 45 and 58% of the dose was recovered during the first 5 and 24 hr, respectively. In this experiment, 92% of the dose was ascertained to be transferred into the blood after 24 hr and, therefore, it can be concluded that 60 to 63% of the drug absorbed was excreted in the bile, indicating that the excretion through the biliary route is highly participating.

In order to study the reabsorption of the drug once excreted in the bile, 1 ml of the collected bile during the first 3 hr was injected into the lumen of a loop prepared from the upper part of the small intestine in a normal rat. The result revealed that only 13 to 18% of the radioactivity administered was absorbed into the blood in 1 hr, which is much lower than the absorption observed when ^{14}C-oxazolam was injected (75% of the dose). The thin-layer chromatography of the pooled bile indicated that no oxazolam is excreted, while 80 to 90% of the radioactivity was found to be remained at the origin of the plate, indicating that the most part is probably glucuronide conjugate of ACHB which was found to be the major metabolite in the feces.[1)]

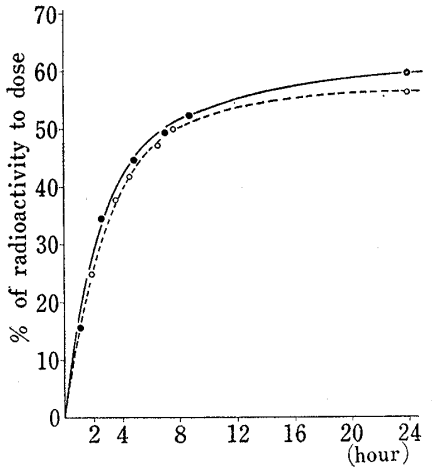


Fig. 3. Biliary Excretion of Radioactivity after Injection of ^{14}C-Oxazolam into Ligated Loop of Rat Small Intestine (Dose: 2 mg/loop, two experiments)

Metabolites in the Liver, Blood and Brain

In order to trace the fate of oxazolam in the organs after being absorbed into the blood, the radioactive materials in the blood, liver and brain were separated by thin-layer chromatography after injection of ^{14}C-oxazolam into a ligated loop of rat small intestine. The results, as shown in Table VI and Fig. 4, revealed that oxazolam was detected to a considerable amount in all the tissues investigated, particularly in the earlier period after the adminstration. Desmethyldiazepam was found to be one of the major metabolites in all the organs.

Table VI. Oxazolam and Its Metabolites in Liver, Brain and Blood after Injection of ^{14}C-Oxazolam into Ligated Loop of Rat Small Intestine (Dose: 2 mg/loop; 3 experiments)

Metabolite	% to total radioactivity ± S.E.								
	Liver			Brain			Blood		
	15 min	60 min	3 hr	15 min	60 min	3 hr	15 min	60 min	3 hr
Oxazolam	28.19 ±1.64	4.49 ±0.60	1.27 ±0.39	38.86 ±0.79	13.66 ±1.56	6.19 ±0.67	15.28 ±0.48	4.64 ±0.30	1.77 ±0.13
Desmethyldiazepam	17.03 ±4.69	16.63 ±4.25	8.98 ±1.11	22.04 ±0.09	40.38 ±4.07	31.87 ±6.12	31.48 ±5.40	24.27 ±2.78	13.69 ±2.82
PACB	18.31	11.50	5.75	13.32	13.15	10.80	20.50	15.94	9.49
ACB	3.57	1.51	1.16	2.93	2.53	2.86	3.41	1.39	1.67
Origin[a)]	23.11	43.34	53.28	18.57	16.14	31.07	16.24	34.48	55.79
Others[b)]	8.35	19.36	25.72	4.33	4.81	5.73	11.89	13.27	14.03
Concentration[c)]	40.39 ±6.88	24.08 ±1.16	12.02 ±0.70	1.93 ±0.18	2.10 ±0.19	0.78 ±0.21	1.40 ±0.05	1.74 ±0.17	1.18 ±0.25

a) aluminium oxide plate, Solvent B; mainly ACHB glucuronide in liver.
b) total radioactivity between these assigned spots
c) μg equivalents of oxazolam/g tissue or ml blood

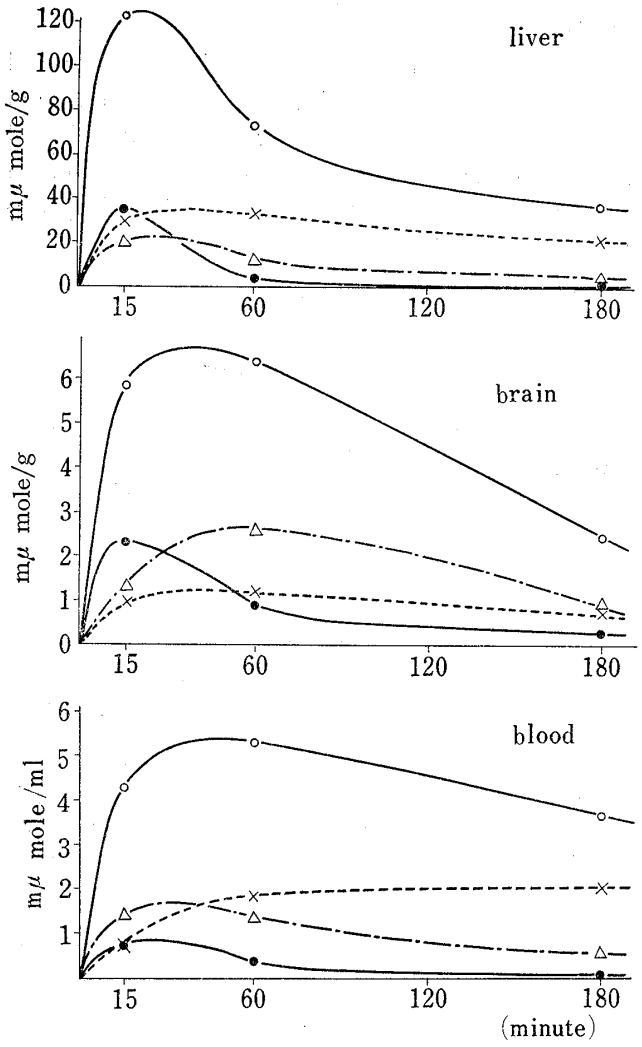


Fig. 4. Oxazolam and Its Metabolites in Liver, Brain and Blood after Injection of ^{14}C-Oxazolam into Ligated Loop of Rat Small Intestine

The radioactivity was converted to mμmole equivalents of oxazolam and the values represent the mean of 3 experiments.
—○—: total amount
—●—: oxazolam
—△—: N-desmethyldiazepam,
—×—: origin substance

After 15 min, the largest part of the tota radioactivity in both the liver and brain was detected as oxazolam (about 28 and 40%, respectively). The amount of oxazolam in the brain was decreased with increasing the time and instead that of desmethyldiazepam showed a significant increase (Fig. 4). After 60 and 180 min, the largest part of the brain radioactivity was found to be desmethyldiazepam (about 40 and 32% respectively). In the liver, an appreciable amount of desmethyldiazepam was also detected after 15 min (about 17%), but the amount was decreased gradually with time. After 60 and 180 min, the major component of the liver radioactivity was a substance which was remained at the origin of the plate after thin-layer chromatographic separation. The extracts from the plate were then separated again using two-dimentional thin-layer chromatography on silica gel plate with solvent systems B and H reported in the preceding paper[1] and the main component was found to be a glucuronide of ACHB and a small amount of oxazepam. In the circulating blood, oxazolam and desmethyldiazepam were found as the major components (about 15 and 32%, respectively) after 15 min, the former showing a rather rapid decrease with increasing the time (Fig. 4). After 180 min, the largest part was the substance remaining at the origin of the thin-layer plate, probably a glucuronide conjugate.

Metabolism after Oral Administration

The metabolites in the liver, blood and brain were again investigated after administration of ^{14}C-oxazolam not into the intestinal lumen, but into the stomach by oral administration in rats. The results, as shown in Table VII, revealed that a considerable amount of oxazolam was detected in all the tissues 1 hr after administration, confirming the above *in situ* results. It was noted that oxazolam was the main component in the brain 1 hr after oral administration when a maximum concentration of radioactivity is reached.[3] Radioactive oxazolam was further identified and analysed by reverse isotope dilution method and, as shown in Table VIII, a good coincidence was noted between the amount of oxazolam obtained from this method and that from thin-layer chromatography.

In Vtiro Metabolism

The *in vitro* biotransformation of oxazolam with rat liver and brain homogenates at 37° is shown in Table IX. As a control experiment, ^{14}C-oxazolam was incubated with the liver

Table VII. Oxazolam and Its Metabolites in Liver, Brain and Blood 1 hr after Oral Administration of ^{14}C-Oxazolam in Rats (Dose: 30 mg/kg; 3 experiments)

Metabolite	% to total radioactivity ± S.E.		
	Liver	Brain	Blood
Oxazolam	6.52±1.64	36.35±5.09	14.56±2.16
Desmethyldiazepam	10.99±0.42	17.60±4.06	14.32±2.46
PACB	9.57±1.84	8.88±3.07	14.98±1.06
ACB	2.72±0.06	2.51±0.94	1.53±0.46
Origin	40.48±3.52	12.73±6.71	32.00±5.72
Others	20.50±5.35	6.75±1.32	12.18±0.18
Concentration[a)]	59.52±7.20	7.74±1.68	4.68±0.36

a) μg equivalents of oxazolam/g tissue or ml blood

Table VIII. Identification and Analysis of Radioactive Oxazolam by Reverse Isotope Dilution Method in Rat Organs 1 hr after Oral Administration (Dose: 30 mg/kg)

	Specific activity (dpm/mg) of the crystals			% to total radioactivity	
	1st	2nd	3rd	This method	TLC method
Liver	170.0	145.6	150.4	4.87	6.22
Brain	81.9	72.3	85.9	32.40	26.29
Blood	133.6	126.4	133.7	18.96	18.69

homogenate heated at 100° for 10 min prior to use. As compared to the control, the liver homogenate showed a prominent formation of desmethyldiazepam and an increased formation of ACB, while the brain homogenate showed no appreciable difference from the control. The results thus indicated that an enzymatic degradation of oxazolam to desmethyldiazepam occurs only in the liver, but not in the brain.

With the liver microsomal enzyme, the metabolic pattern was found to be significantly different from that observed with the homogenate, as shown in Fig. 5. An incubation of ^{14}C-oxazolam in the microsomal preparation showed formation of a significant amount of desmethyldiazepam and an appreciable amount of oxazepam, which was not formed to any appreciable extent in the homogenate. The amounts of PACB and ACB formed were much lower than those with the homogenate. The ratios of the amount of PACB plus ACB to that of oxazolam after 180 min of incubation were 40.7, 43.6 and 131.5% for the control, the microsomal enzyme and the whole liver homogenate, respectively, indicating that the formation of PACB and ACB in the microsomal preparation is proceeded mainly through the chemical degradation of oxazolam at 37°, while in the homogenate some enzymatic reactions are also involved.

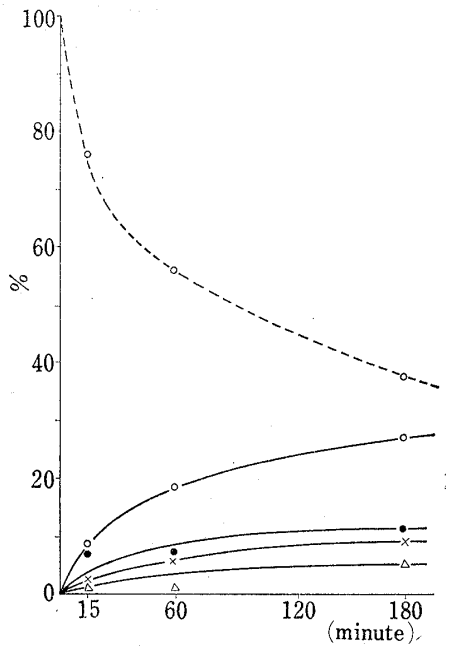


Fig. 5. *In Vitro* Metabolism of Oxazolam with Rat Liver Microsomal Enzyme (2 μmoles substrate with the Enzyme corresponding to 2 g liver, 37°, pH 7.4)

----○----: oxazolam
——○——: N-desmethyldiazepam
——×——: oxazepam,
——●——: PACB
——△——: ACB

Table IX. *In Vitro* Metabolism of Oxazolam in Rat Liver and Brain Homogenates

Metabolite	% to total radioactivity								
	Control[a]			Liver			Brain		
	15	60	180 min	15	60	180 min	15	60	180 min
Oxazolam	86.01	80.86	67.04	77.83	71.81	38.63	84.79	78.25	62.98
Desmethyldiazepam	(1.13)[b]	(1.10)	(2.43)	2.47	5.38	5.61	(0.96)	(1.22)	(1.30)
PACB	6.34	10.81	26.61	8.39	12.14	25.12	9.20	12.73	27.12
ACB	(0.61)	(0.38)	(0.69)	5.69	6.62	25.66	(0.21)	(0.54)	(0.84)

a) The liver homogenates were heated at 100° for 10 min.
b) Values in parenthesis have no significant meaning as compared to the background.

Discussion

Oxazolam (I) is an analogue of diazepam and chlordiazepoxide and has been shownto have a lower toxicity and side effects such as olcomotor ataxia than diazepam and chlordiazepoxide in animal experiments.[10] Structurally it has a characteristic of possessing an oxazolidine ring at 4 and 11b positions. The previous studies[1,3] indicated that oxazolam appears to be well absorbed from gastrointestinal tract, while that no unchanged oxazolam was detected in rat urine and feces after oral administration. It was, therefore, thought to be of importance to study in what form the drug is absorbed from intestine and is distributed in the organs, in particular in the brain—the site of the drug action.

From the present investigations, it was clarified that oxazolam is very easily absorbed from almost whole part along the rat intestine, in particular from the upper part of the small intestine in the fastest rate. It was also shown that a large part, approximately 60% of the drug absorbed is brought back into the intestinal lumen through the biliary excretion and that the extent of its reabsorption appears to be insignificant. In the bile no oxazolam was detected and the most part was considered to be a glucuronide of ACHB which is the main metabolite in the rat feces.[1] As reported previously,[3] approximately 60 and 30% of the dose was recovered in the feces and the urine, respectively, when ^{14}C-oxazolam was administered orally in rats. This can be interpreted, therefore, as that oxazolam was almost completely absorbed from the intestine and the most part of the fecal excretion was derived from the radioactivity which was excreted into the intestinal lumen through the bile.

It was indicated that oxazolam is stable enough in the intestinal lumen from the fact that over 80% of the remaining radioactivi ty in the lumen was found to be unchanged oxazolam 30 min after administration. It was also shown that approximately 10% of oxazolam is chemically transformed to PACB, the ring opening structure, during 1 hr at 37°, pH 7.4. In the intestinal tissue, also over 80% of the radioactivity was identified as unaltered oxazolam 5 and 30 min after administration of ^{14}C-oxazolam into the lumen. Since the period from 5 to 30 min after the administration was the period when the radioactivity was being transferred most rapidly from the lumen into the blood stream *via* a transient step of an accumulation in the tissue, the results might indicate that oxazolam is absorbed through the intestinal mucosa in the unaltered form. The fact that 15 min after injection of oxazolam into the intestinal loop about 30, 15 and 40% of the radioactivity in the liver, peripheral blood and brain, respectively, was found as oxazolam may further indicate that oxazolam is circulated as the unaltered form in the general circulation after passing through the liver.

10) H. Takagi, T. Kamioka, S. Kobayashi, Y. Suzuki, and R. Tachikawa, *Nippon Yakurigaku Zasshi*, **66**, 107, 134 (1970).

The uptake and the fate of oxazolam in the brain are of great importance with respect to the appearence of its pharmacological effect. The fact that a considerably higher proportion of the total radioactivity in the brain was found to be unaltered oxazolam as compared to that in the blood may indicate a high permeability of oxazolam into the brain and suggest an occurring of preferential uptake of oxazolam by the brain tissue from the circulating blood. It was shown in the previous paper,[3] that radioactivity is accumulated in a high concentration in the brain as early as 1 min after intravenous injection of ^{14}C-oxazolam into mice. In the present results, the maximum concentration of oxazolam in the brain was reached about 15 min after administration into rat intestinal lumen. Thereafter, a rather rapid fall in the level of oxazolam occurred, while the concentration of N-desmethyldiazepam gradually increased, reaching its maximum about 60 min after the administration. N-Desmethyldiazepam has been found as a major cerebral metabolite of diazepam in mice[11] and has been reported to have a high anticonvulsant activity.[12] It has been further clarified that after administration of diazepam N-desmethyldiazepam accumulates in mouse brain, while practically not in rat brain and this difference has been attributed to be responsible for a longer duration of anticonvulsant activity of diazepam in mice than in rats.[13] The present observation that an oxazolam peak was followed by an N-desmethyldiazepam peak in rat brain must, therefore, participate for a duration of anticonvulsant activity of oxazolam. Practically, however, a much stronger activity has been observed in mice than in rats[14] and this might be attributed to a higher uptake of oxazolam by mouse brain than rat brain.[3]

From the *in vitro* metabolic studies, it was shown that oxazolam was not substantially metabolized in the brain homogenate, while metabolized in the liver homogenate forming N-desmethyldiazepam. With the liver microsomal enzymes, N-desmethyldiazepam was formed as a major product with an appreciable amount of oxazepam. The results may indicate that N-desmethyldiazepam in the brain must be derived from its gradual transfer from the blood circulation into the brain after being formed in the liver microsome. In fact, a significant amount of N-desmethyldiazepam was detected in the peripheral blood, in contrast to the fact[13] that after administration of diazepam in rats almost no N-desmethyldiazepam was found in the blood. Furthermore, the concentration of N-desmethyldiazepam showed a gradual decrease in the liver and blood with increasing the time after 15 min, while in the brain the concentration showed a gradual increase to reach its maximum about 60 min after the administration.

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11) E. Van der Kleijn, *Arch. Int. Pharmacodyn.*, **178**, 193 (1969).
12) F. Marcucci, A. Guaitani, J. Kvetina, E. Mussini, and S. Garattini, *Europ. J. Pharmacol.*, **4**, 467 (1968).
13) F. Marcucci, R. Fanelli, E. Mussini, and S. Garattini, *Europ. J. Pharmacol.*, **9**, 253 (1970).
14) H. Takagi, unpublished results in this laboratories.