Preparation of 3,6-Diisobutyl-1-methoxy-2-oxo-1,2-dihydropyrazine (XVIII)——Colorless crystals, prepared from 480 mg (2 mmol) of XXII by treatment with CH_2N_2, were warmed at 40° for 30 min with 1 ml of PCl_3 in 80 ml of anhydr. AcOEt. The reaction mixture was poured into ice-water, made alkaline with K_2CO_3, and the AcOEt layer was separated. The usual work-up gave a yellowish oil (259 mg), which indicated two peaks (intensity ratio, 4: 1) in GLC (1.5% SE-30 on Shimalite; 1.5 m×3 mm; column temp., 75°; N_2 flow rate, 40 ml/min) and was chromatographed over silica gel (Wakogel C-200, 5 g), eluted with a mixture of benzene and acetone to give 178 mg (42%) of XVIII as a colorless semisolid and 45 mg (11%) of XIV. XVIII thus obtained was used for the demethylation reaction without further purification.

Acknowledgement The authors are grateful to Mrs. Yoshiko Baba, Mrs. Keiko Isobe and Mr. Shigeru Suzuki for elemental analyses, and Mr. Yasuo Shida for mass spectral measurements.

[Chem. Pharm. Bull. 25(4) 821—825 (1977)]

UDC 547.269.3.09 : 615.263.076.9

Percutaneous Absorption of α-Olefin Sulfonate (AOS) in Rats[1]

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(Received July 8, 1976)

Percutaneous absorption of α-olefin sulfonate (AOS) was investigated in rats by using ^{14}C-labeled compound. The solution of ^{14}C–AOS was applied to the dorsal skin under various conditions: (a) the intact skin dried naturally after application, (b) the intact skin wiped off 0.5 hr after application, (c) the intact skin wiped off 1.5 hr after application, (d) the intact skin with a plastic cup containing ^{14}C–AOS solution and (e) the damaged skin without the *stratum corneum* dried naturally after application.

When rats were applied with 0.5 ml of a 0.2% solution of ^{14}C–AOS under the condition of (a), the recoveries of radioactivity were 0.33% in the urine, 0.08% in the bile and 0.21% in the main organs at 24 hr after application. It was thus estimated that the total amount absorbed through the skin was about 0.6% of the applied dose. Comparing the results obtained under the conditions of (a), (b) and (c), the percutaneous absorption of ^{14}C–AOS applied on the skin was almost finished by 1.5 hr after application. The excretion of radioactivity into the urine and bile was approached to the highest rate around 3—6 hr, then gradually decreased, and continued even 70—90 hr after application. When a 0.02% solution of ^{14}C–AOS was always in contact with the skin under the condition of (d), a small amount of the surfactant was continuously absorbed from the skin.

On the other hand, when the skin was damaged and ^{14}C–AOS was applied on it under the condition of (e), a greater amount of radioactivity was excreted into the urine and bile, and the recoveries were 36.26% in the urine, 1.83% in the bile and 12.28% in the main organs 30 hr after application, being about 50% in total.

Keywords——surfactant; percutaneous absorption; α-olefin sulfonate; dermal application; damaged skin; biliary excretion; uriary excretion

The main surfactant used previously as domestic detergent was tetrapropylene benzene sulfonate (ABS). Since it was pointed out that ABS was resistant to biodegradation and caused the pollution in environment, more biodegradable surfactants such as linear alkyl benzene sulfonate (LAS), higher alcohol sulfate (AS), alkyl ethoxy sulfonate (AES), and α-olefin sulfonate (AOS) have been widely used.

In order to assess the safety of these surfactants, many toxicological studies have been performed. As for AOS which was introduced as a new surfactant with the claims of

1) A part of this work was presented at the 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1976.

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mildness to the skin[3] and rapid biodegradability,[4] studies on acute toxicity,[5] teratogenicity,[6] and long-term toxicity[7] were reported by some investigators.

It is important to estimate the extent of absorption of surfactant through the skin when the percutaneous toxicity is discussed. Therefore, this report is to determine the extent of percutaneous absorption of ASO under various conditions, by using rats applied ^{14}C-labeled compound.

Materials and Methods

Radioactive AOS——Radioactive C_{14}-AOS-(1-^{14}C) was prepared by Daiichi Pure Chemicals Co., Ltd., Tokyo. It was composed of a mixture of about 55% 3-hydroxytetradecane-1-^{14}C sulfonate ($C_{11}H_{23}CH(OH)$-$CH_2{}^{14}CH_2SO_3Na$) and about 45% β-tetradecene-1-^{14}C sulfonate ($C_{11}H_{23}CH=CH{}^{14}CH_2SO_3Na$) which were not separable by thin-layer chromatography. The ^{14}C-AOS preparation consisting of the above two components gave one peak by thin-layer chromatography on Silica gel plate developed with the following solvent systems; *n*-buthanol–acetic acid–water (4: 1: 2), *n*-propanol–conc. ammonia water (3: 2) and ethanol–conc. ammonia water (7: 3). The specific activity was 6.55 μCi/mg.

Animals and Treatments——Groups of 3 male Wistar rats weighing between 360 and 380 g were used unless otherwise stated. The dorsal hair of each animal was closely clipped to expose the skin and 0.5 ml of a 0.2% (w/v) ^{14}C-AOS solution (6.00 μCi or 5.88 μCi/0.5 ml) was applied to a marked area (4 cm × 3 cm) of the clipped skin with a stomach tube, except the experiment of the condition of (d) described below (4 cm × 4 cm).

The following five conditions were employed for the application of ^{14}C-AOS: (a) the intact skin dried naturally after application, (b) the intact skin wiped off 0.5 hr after application, (c) the intact skin wiped off 1.5 hr after application, (d) the intact skin with a plastic cup containing ^{14}C–AOS solution (cup method) and (e) the damaged skin without the *stratum corneum* dried naturally after application.

In the conditions of (b) and (c), the applied area was cleanly wiped off ten times with an absorbent cotton ball wet with water (diam. 1 cm). Sixty to seventy % of the total radioactivity applied was recovered by wiping off the surfactant with wet absorbent cotton balls. The residual radioactivity was not removed from the skin. The condition of (d) was employed to bring the surfactant solution into contact with the skin at all the time. A plastic ice-making cup (4 cm × 4 cm × 3 cm) was reversely attached to the dorsal clipped area of rats with Aron Alpha, a quick set adhesive (Toa Gosei Chemical Industries Co., Ltd.). A little hole was made on the bottom of the cubic cup and 10 ml of 0.02% (w/v) ^{14}C-AOS (11.7 μCi/10 ml) was poured through the hole which was subsequently shielded with a cellophane tape. The damaged skin was made by twenty-time treatments of the dorsal skin with a cellophane adhesive tape (width: 50 mm, length: 70 mm, Nichiban Co., Ltd.) to remove the *stratum corneum*, according to the method of Washitake *et al.*[8]

Biliary and Urinary Excretion—— In experiments using the rats with bile-duct and bladder cannulae, animals were anaesthetized with pentobarbital in a dose of 40 mg/kg intraperitoneally. The bile-duct was surgically exposed by a mid-line incision and cannulated with a fine polyethylene tubing (Imamura Rubber Co., Ltd. the inside diam. 0.8 mm). The bladder was also directly cannulated and the urethral was ligated tighly. Then, the abdomen was stitched and binded with Aron Alpha for protecting against the effusion of body fluid. Animals were fixed on the abdomen and maintained by giving occasionally a 5% (w/v) glucose solution *per os* through the experimental time. Bile and urine were collected at regular intervals after application.

Determination of Radioactivity in Urine and Bile——An Aloka LSC-751 scintillation spectrometer was used for the measurement of radioactivity. Radioactivities of urine and bile samples were measured using a dioxane scintillator containing 0.7% 2,5-di-phenyloxazole (PPO), 0.03% 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) and 10% naphthalene, and were corrected for quenching by an external standard source.

Determination of Radioactivity in Organs——The animals were sacrificed 24 hr after application unless otherwise stated. Organs were removed and weighed. Each organ sample (0.1—0.5 g) was dissolved completely in 3 ml of Soluene 100 (Packard Instrument Co., INC.) by heating at 50—60° for 6 hr. Each portion (0.1 ml) of the organ solution was transfered to a vial and a toluene scintillator containing 1% PPO and 0.05% POPOP was added. The radioactivities were measured as described above.

3) M. Iimori, E. Iwata, and Y. Sato, *J. Japan Oil Chem. Soc.*, 20, 584 (1971); K. Oba, A. Mori, and S. Tomiyama, *J. Japan Oil Chem. Soc.*, 17, 628 (1968).
4) K. Oba, A. Mori, and S. Tomiyama, *J. Japan Oil Chem. Soc.*, 17, 517 (1968).
5) K. Oba and J. Tamura, *Agr. Biol. Chem.*, 31, 1509 (1967).
6) A.K. Palmer, M.A. Readshaw, and A.M. Neuff, *Toxicology*, 3, 107 (1975).
7) B. Hunter and H.G. Benson, *Toxicology*, 5, 359 (1976).
8) M. Washitake, T. Yajima, T. Anmo, T. Arita, and R. Hori, *Chem. Pharm. Bull.* (Tokyo), 21, 2444 (1973).

Results and Discussion

Excretion and Distribution of ^{14}C-AOS Applied on Intact Skin

Table I shows the biliary and urinary excretion of radioactivity after application of ^{14}C-AOS under the condition of (a).

Table I. Biliary and Urinary Excretion of Radioactivity in Rats Applied ^{14}C-AOS

Time after application (hr)	Intact skin[a] % of applied dose		Damaged skin[b] % of applied dose	
	Bile	Urine	Bile	Urine
0— 1	trace	trace	0.203+0.138	3.457±3.165
1— 3	0.006±0.003	0.022±0.017	0.480±0.277	11.305±3.942
3— 6	0.013±0.005	0.048±0.017	0.361±0.249	7.359±3.486
6— 9	0.010±0.003	0.054±0.014	0.196±0.110	5.003±2.322
9—12	0.009±0.005	0.047±0.011	0.127±0.032	3.907±1.782
12—24	0.019±0.007	0.127±0.024	0.329±0.063	4.169±3.868
24—30	—	—	0.130±0.032	1.059±0.559

a) Five animals were used. Mean values are given ±S.E.
b) Three animals were used.

The radioactivity appeared in the bile and urine within 1 hr after application, but it was very low. Total recoveries of the radioactivity were 0.298% in the urine and 0.057% in the bile 24 hr after application.

The distribution of radioactivity was examined in the brain, lung, liver, kidney and spleen 24 hr after applicaton. As shown in Table II, the radioactivity did not show significant affinity for those organs under this experimental condition.

Table II. Distribution and Total Recovery of Radioactivity in Rats Applied ^{14}C-AOS

Organ	Intact skin (24 hr)[a]		Damaged skin (30 hr)[a]	
	dpm/g	% of applied dose	dpm/g	% of applied dose
Brain	856± 100	0.010±0.001	3066± 800	0.042± 0.010
Lung	998± 173	0.012±0.002	93051±62822	1.461± 0.861
Liver	1419± 547	0.123±0.037	113536±17262	9.876± 2.013
Kidney	2975±1561	0.059±0.032	36383±10871	0.871± 0.316
Spleen	2136± 535	0.004±0.001	12336± 676	0.027± 0.004
Urine	—	0.327±0.077	—	36.259±18.203
Bile	—	0.083±0.032	—	1.826± 0.823
Total	—	0.618	—	50.362

a) Three animals were used. Mean values are given ± S.E.

Since ^{14}C-AOS absorbed from the intact skin was very low, it might be rapidly metabolized in the liver to be excreted into urine through the kidney. Total recovery of radioactivity from the urine, bile and main organs was about 0.6% dose 24 hr after application. In the preliminary experiment, no unchanged AOS was detected in the urine and bile by thin-layer chromatography, suggesting the rapid biodegradation of the surfactant.

Comparison of Biliary and Urinary Excretion under Various Conditions

Table III shows the biliary and urinary excretion of radio-activity 24 hr after application under various conditions. The recoveries of radioactivity in the bile and urine under the condition of (b) were considerably lower than the conditions of (a) and (c), though there

was no significant difference between the latter two. This fact showed that the percutaneous absorption of AOS was almost finished at least by 1.5 hr after application, because the surfactant applied on the skin was dried naturally between 0.5 and 1 hr.

Table III. Comparison of Biliary and Urinary Excretion of Radioactivity in Rats Applied ^{14}C-AOS under Various Conditions

	Condition	Animal number	% of applied dose 24 hr after application	
			Bile	Urine
(a)	Applied alone	(8)	0.051±0.015	0.329±0.044
(b)	Applied and wiped off 0.5 hr after application	(3)	0.023±0.011	0.100±0.008
(c)	Applied and wiped off 1.5 hr after application	(3)	0.043±0.007	0.367±0.069
(d)	Cup method	(3)	0.025±0.012	0.092±0.032

Mean values are given ± S.E.

When 10 ml of a 0.02% ^{14}C-AOS solution (11.76 μCi) was continuously contacted with the skin under the condition of (d), the recoveries of radioactivity were rather low compared with the condition of (a) where the initial concentration of AOS was 0.2%.

Time-Coures of Biliary and Urinary Excretion of ^{14}C-AOS under Various Conditions

The excretory patterns of radioactivity into the bile and urine of rats after application of ^{14}C-AOS under the conditions of (a), (b) and (c) were similar each other. Fig. 1 shows a typical excretion pattern in the condition of (a). The excretions were approached to the highest rates arount 3—9 hr, then gradually decreased, and continued even 70—90 hr after application.

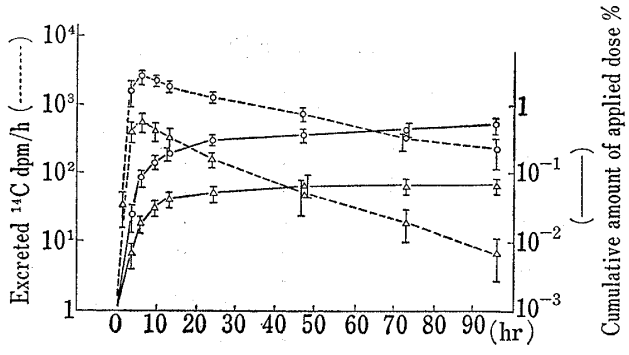

Fig. 1. Time-Course of Biliary and Urinary Excretion of ^{14}C-AOS under the Intact Skin Dried naturally after Application

△: bile ○: urine

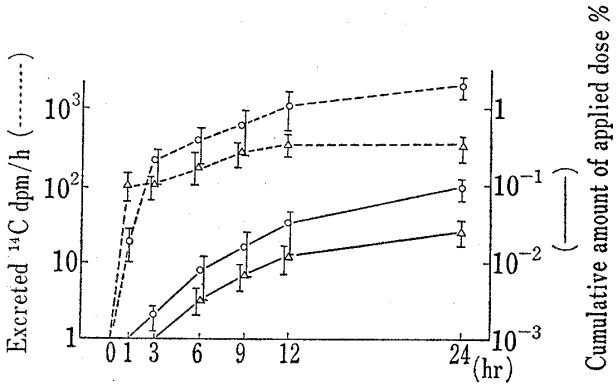

Fig. 2. Time-Course of Biliary and Urinary Excretion of ^{14}C-AOS under the Intact Skin with a Plastic Cup Containing ^{14}C-AOS Solution (Cup Method)

△: bile ○: urine

On the other hand, when a solution of ^{14}C-AOS was always in contact with the the skin under the condition of (e), it seemed to be continuously absorbed from the skin (Fig. 2). The excretion was gradually increased by 24 hr after application without a maximum peak, though the rate was not so high.

Percutaneous Absorption of ^{14}C-AOS from Damaged Skin

Percutaneous absorption of ^{14}C-AOS from the damaged skin wasstudied under the condition of (e). Table I shows the excretion patterns of radioactivity into the urine and bile, and Table II presents the distribution of radioactivity in the main organs. These data,

however, show the considerable differences between the individual animals, based on the difficulty in obtainning the reproducible results by treatment with the cellophane tape.

As shown in Table II, the total amont of absorption from the damaged skin was approximately 80 times that from the intact skin. The recoveries of radioactivity were 36.26% in the urine and 1.83% in the bile 30 hr after application. The distribution of radioactivity in the main organs was much larger than that of the intact skin.

Since a large amount of ^{14}C–AOS was absorbed from the damaged skin, the distribution of radioactivity in the liver was in excess compared with other organs. The significance of the high accumulation of radioactivity in the lung was obscure, because the values varied remarkably in each animal. The total recovery of radioactivity in the urine, bile and main organs of damaged skin rat was about 50% dose. Such a high absorption from the damaged skin shows that the *stratum corneum* functions as a kind of barrier controlling the percutaneous absorption.

Acknowledgement The authors are grateful to Dr. Oba of Lion Fat and Oil Co., Ltd. and Dr. M. Washitake of Taisho Pharmaceutical Co., Ltd. for their kind advice of this work. Thanks are also to Mr. F. Takamura for technical assistance.

[Chem. Pharm. Bull. 25(4) 825—830 (1977)]

UDC 547.546.04 : 542.98.04

NADH-dependent O-Demethylation of *p*-Nitroanisole with Rabbit Liver Microsomes

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(Received July 9, 1976)

Following the previous study on deethylation of *p*-nitrophenetole with rabbit liver microsomes, demethylation of *p*-nitroanisole was analogously investigated, using reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and NADH plus NADPH as the cofactor. It was found that this reaction proceeded well with NADH as well as NADPH, and this NADH-dependent demethylation was different from those of other two systems. For example, optimum pH was 6.0 in the NADH system, but was 7.4 in the NADPH or NADH plus NADPH system, and the reaction was inhibited by CO in the NADPH or NADH plus NADPH system, but not in the NADH system. Furthermore, KCN did not inhibit the demethylation in any one of three systems at 10^{-4}M. These results were just the same as obtained in the deethylation of *p*-nitrophenetole and suggested also a possible involvement of a new type of NADH-dependent oxygenase which was different from cytochrome P-450 and cyanide-sensitive factor, in the demethylation of *p*-nitroanisole with rabbit liver microsomes. The NADPH- and NADH plus NADPH-dependent demethylations, on the other hand, were assumed to be catalyzed by the enzyme system involving cytochrome P-450.

Keywords——*p*-nitroanisole; microsomal O-demethylation; rabbit liver; NADH-dependent oxygenase; NADPH-dependent oxygenase; cytochrome P-450

Most of oxidative metabolisms of foreign compounds are known to be catalyzed by the mixed-function oxidase system in the liver microsomes. In these reactions, the cytochrome P–450 generally participates as a terminal oxidase and reduced nicotinamide adenine di-

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