3-Methyl-2-(2-pentynyl)-2-cyclopentenone (Dehydrojasmone) (5)——A mixture of **3** (0.88 g) and NaCl (0.4 g) in DMSO (40 ml) and H_2O (0.7 g) was placed in a sealed tube. After heating at 180° for 7.5 hr, the reaction mixture was poured into a saturated NaCl solution (100 ml) and this solution was extracted with hexane. The hexane extract gave an oily substance (0.3 g), which was purified by silica gel (10 g) column chromatography, eluting with benzene, to give the product **5** as a colorless oil, 0.16 g (33%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2200, 1705, 1655. NMR (CCl_4) δ: 1.10 (3H, t, J=7 Hz, CH_3), 2.18 (3H, s, CH_3), 1.90—2.79 (6H, m), 2.98 (2H, s, 2-$CH_2C\equiv$). 2,4-Dinitrophenylhydrazone: mp 163—164° (EtOH) (lit.[7] mp 166°).

3-Methyl-2-(2-*cis*-pentenyl)-2-cyclopentenone (*cis*-Jasmone) (6)——A mixture of **5** (90 mg) and 5% Pd–$CaCO_3$[8] (50 mg) in ethanol (5 ml) was shaken in H_2 until absorption ceased (12 ml). The catalyst was filtered off, and the filtrate was concentrated. The residue (80 mg) was subjected to silica gel (2 g) column chromatography. Elution with $CHCl_3$ gave the product **6** as a pale yellow oil, 60 mg (65%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1695, 1645, 760 (*cis* CH=CH). NMR (CCl_4) δ: 0.96 (3H, t, J=7 Hz, CH_3), 2.03 (3H, s, CH_3), 2.0—2.7 (6H, m), 2.85 (2H, d, J=6 Hz, CH_2), 5.2—5.55 (2H, m, olefinic protons). 2,4-Dinitrophenylhydrazone: mp 114—115° (EtOH) (lit.[9] mp 117.5°).

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7) K. Sisido, Y. Kawashima, and T. Ishida, *Perfum. Essent. Oil Rec.*, **57**, 364 (1966).
8) J.N. Ashley and R.D. MacDonald, *J. Chem. Soc.*, **1957**, 1668.
9) L. Crombie and S.H. Harper, *J. Chem. Soc.*, **1952**, 869.

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Studies on the Metabolic Relationship between Alkyl Carbamates and Alkyl N-Hydroxycarbamates in Rats

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When methyl, ethyl or *n*-propyl N-hydroxycarbamate was administered to rats intraperitoneally, the corresponding alkyl carbamate was detected as a urinary metabolite by thin-layer chromatography. These alkyl N-hydroxycarbamates were also converted to the corresponding alkyl carbamates by rat liver slices, but not by rat liver 9000 ***g*** supernatant. This suggests that the reduction of alkyl N-hydroxycarbamates may be catalyzed by liver enzymes which are different in nature from aromatic hydroxylamine-reducing enzymes. Similarly, the metabolism of ethyl carbamate in rats was investigated *in vivo* and *in vitro*. However, no ethyl N-hydroxycarbamate could be detected in these experiments. Therefore, it appears unlikely that alkyl N-hydroxycarbamates are metabolites of the corresponding alkyl carbamates in rats.

Keywords——alkyl carbamates; alkyl N-hydroxycarbamates; metabolism; rats; thin-layer chromatography

The carbamate compounds are potentially dangerous from an environmental point of view. They have been widely used medicinally as sedatives, industrially as chemical raw materials, and agriculturally as herbicides, insecticides and fungicides. The simplest compounds among them, ethyl carbamate and ethyl N-hydroxycarbamate have almost equal

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carcinogenic activities.[2,3] On this basis and by analogy with the N-hydroxylation theory of aromatic amine carcinogenesis,[3] the possibility was considered that ethyl carbamate acts as a carcinogen by prior conversion to ethyl N-hydroxycarbamate.[2,3] This view was supported by Boyland and Nery,[4] who detected ethyl N-hydroxycarbamate and its acetyl derivative in the urine of ethyl carbamate-treated rats and other animals. The theory seemed especially attractive as ethyl N-hydroxycarbamate is chemically more reactive than ethyl carbamate.[4] On the other hand, Mirvish[5,6] showed that about 70% of the injected ethyl N-hydroxycarbamate was converted into ethyl carbamate in mice, but failed to demonstrate the reverse conversion. Thus, and because ethyl N-hydroxycarbamate is not more carcinogenic than ethyl carbamate, it was suggested that the carcinogenicity of ethyl N-hydroxycarbamate is due to its conversion to ethyl carbamate, rather than *vice versa*.

Such conflicting views led us to investigate the metabolic relationship between alkyl carbamates and the corresponding N-hydroxy compounds in detail.

Materials and Methods

Chemicals——Methyl carbamate (mp 54°) and ethyl carbamate (mp 50°) of guaranteed reagent grade were purchased from Wako Pure Chemical Industries, Ltd., and Nakarai Chemicals, respectively. *n*-Propyl carbamate (mp 61°) was prepared by the method of Thiele and Dent.[7] Methyl N-hydroxycarbamate (mp 51°), ethyl N-hydroxycarbamate (bp 86—88° (0.6 mmHg)), *n*-propyl N-hydroxycarbamate (bp 90—92° (0.6 mmHg)) and ethyl N-acetoxycarbamate (bp 73° (3 mmHg)) were prepared by the method of Boyland and Nery.[8] Prior to use, the purity of these compounds was cheked by thin-layer chromatography (TLC). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide were obtained from Sigma Chemical Co.

Animals——Female Wistar and Donryu strain rats weighing 150—160 g were used in this study. They were given a commercial pellet diet (Oriental Kobo Co., Ltd.) and tap water *ad libitum*.

TLC——TLC was conducted on Silica gel plates (Wako gel B-5FM, 0.25 mm thick) using two solvent systems: (A) $CHCl_3$–acetone (9: 1) and (B) $CHCl_3$–acetone–petroleum ether (2: 1: 7). Spots were visualized by spraying the plates with *p*-dimethylaminocinnamaldehyde reagent (2 g in 100 ml of 6 N HCl and 100 ml of EtOH).[4] Alkyl carbamates and alkyl N-hydroxycarbamates examined gave red spots with this reagent. The *Rf* values of these compounds in solvent systems A and B (in that order) are given in parentheses: methyl carbamate (0.30, 0.11), methyl N-hydroxycarbamate (0.15, 0.02), ethyl carbamate (0.37, 0.17), ethyl N-hydroxycarbamate (0.20, 0.04), ethyl N-acetoxycarbamate (0.48, 0.29), *n*-propyl carbamate (0.42, 0.20) and *n*-propyl N-hydroxycarbamate (0.27, 0.06).

***In Vivo* Experiments**——Rats were injected intraperitoneally with methyl N-hydroxycarbamate, ethyl N-hydroxycarbamate, *n*-propyl N-hydroxycarbamate or ethyl carbamate as a 20% solution in water. The animals were then placed in metabolic cages and the urine was collected under toluene in receivers immersed in solid CO_2–acetone. The urine was adjusted to pH 7.0 and extracted three times with an equal volume of ether by shaking. The ether extract was dried over anhydrous Na_2SO_4, evaporated to dryness *in vacuo* and the residue was subjected to TLC. In these experiments, the normal urine collected before dosing was used as a control.

In Vitro* Experiments**——Rats were killed by cervical fracture and exsanguinated. Livers were removed and sliced to 0.5 mm thickness with a sharp blade. Liver 9000 ***g supernatant was prepared as follows: Livers were homogenized with a Teflon-glass homogenizer in three volumes of 1.15% KCl and the homogenate was centrifuged for 20 min at 9000 ***g*** in a Kubota KR–6L–D centrifuge. The incubation mixture consisted of 100 μmol of an alkyl carbamate or 5 μmol of an alkyl N-hydroxycarbamate, 1 g of slices and 10 ml of 0.05 M phosphate buffer (pH 7.4). In some experiments, the incubation mixture consisted of 100 μmol of an alkyl carbamate or 5 μmol of an alkyl N-hydroxycarbamate, 4.2 ml of 9000 ***g*** supernatant (equivalent to 1 g of liver), 1000 μmol of nicotinamide, a NADPH-generating system (10 μmol of NADPH, 200

2) I. Berenblum, D. Ben-Ishai, N. Haran-Ghera, A. Lapidot, E. Simon, and N. Trainin, *Biochem. Pharmacol.*, **2**, 168 (1959).
3) J.A. Miller, J.W. Cramer, and E.C. Miller, *Cancer Res.*, **20**, 950 (1960).
4) E. Boyland and R. Nery, *Biochem. J.*, **94**, 198 (1965).
5) S.S. Mirvish, *Biochim. Biophys. Acta*, **93**, 673 (1964).
6) S.S. Mirvish, *Biochim. Biophys. Acta*, **117**, 1 (1966).
7) J. Thiele and F. Dent, *Ann.*, **302**, 245 (1898).
8) E. Boyland and R. Nery, *Analyst*, **89**, 520 (1964).

μmol of glucose-6-phosphate, 20 units of glucose-6-phosphate dehydrogenase) or 100 μmol of NADH, and 5.8 ml of 0.05 M phosphate buffer (pH 7.4). The incubation was carried out for 1 hr at 37° under an atmosphere of air or nitrogen. After incubation, the mixture was immediately extracted three times with an equal volume of ether. The combined extract, after removal of the solvent, was subjected to TLC as described for the *in vivo* experiments.

Results and Discussion

Three groups, each of four rats, were injected with methyl, ethyl or *n*-propyl N-hydroxycarbamate at a single dose of 1 g/kg, respectively. In all cases, thin-layer chromatograms showed that the corresponding alkyl carbamate together with the unchanged compounds were excreted in the 48 hr urine, in agreement with a similar earlier study using the colorimetric determination method, which was reported by Boyland and Nery.[4]

It is known that aromatic nitro compounds and aromatic hydroxylamines are reduced to the corresponding aromatic amines by nitroreductases which are localized mainly in the microsomal and the supernatant fractions of mammalian livers.[9] However, the reduction of alkyl N-hydroxycarbamates to the corresponding alkyl carbamates has not previously been demonstrated *in vitro*. In the present study, therefore, we investigated the *in vitro* metabolism of alkyl N-hydroxycarbamates using slices or the 9000 ***g*** supernatant from rat livers. Methyl, ethyl or *n*-propyl N-hydroxycarbamate was incubated with liver slices under aerobic conditions. Figure 1 shows typical thin-layer chromatograms of the ether extracts from the incubation mixtures, which were developed in solvent system A.

In all cases, the upper and the lower spots were identified as alkyl carbamates and the unchanged substrates, respectively, on the basis of the *Rf* values. Anaerobic conditions, that is the replacement of air with nitrogen, did not affect the thin-layer chromatograms shown in Fig. 1. In control experiments using boiled liver slices, the unchanged substrates were observed, but not the corresponding alkyl carbamates, indicating that the conversion of alkyl N-hydroxycarbamates to alkyl carbamates is due to an enzymic reaction. This is the first evidence of enzymic N-dehydroxylation of aliphatic compounds *in vitro*. Boyland and Nery[10] suggested that the oxidation of alkyl N-hydroxycarbamates with iodine in aqueous ammonium carbonate gives the corresponding alkyl carbamates, so the metabolic reduction of these N-hydroxy compounds may be mediated by an oxidation mechanism. However, since the *in vitro* reduction of alkyl N-hydroxycarbamates proceeds even under anaerobic conditions, as described above, N-dehydroxylation of alkyl N-hydroxycarbamates in animal bodies may occur by a reduction mechanism, rather than an oxidation mechanism.

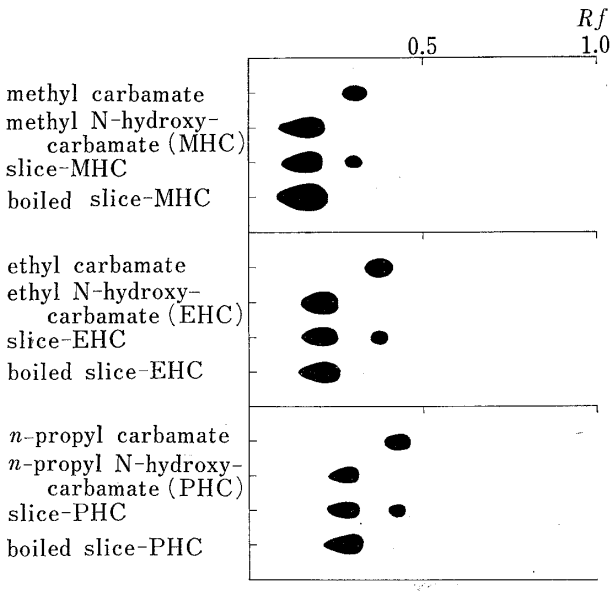

Fig. 1. Thin-Layer Chromatograms of Ether Extracts from Reaction Mixtures after Incubation of Alkyl N-hydroxycarbamates with Rat Liver Slices

Solvent system: chloroform-acetone (9:1).

9) R. Kato, T. Oshima, and A. Takanaka, *Mol, Pharmacol.*, **5**, 487 (1969).
10) E. Boyland and R. Nery, *J. Chem. Soc.* (*C*), **1966**, 354.

Next, in order to elucidate the nature of the enzyme (s) involved in the reduction of alkyl N-hydroxycarbamate, ethyl N-hydroxycarbamate was incubated aerobically or anaerobically with 9000 ***g*** supernatant in the presence of NADPH or NADH. In these cases, no spot corresponding to ethyl carbamate could be detected, in contrast to the slice experiments, indicating the importance of cellular organization in the reduction of ethyl N-hydroxycarbamate by rat livers. It is known that the 9000 ***g*** supernatant from rat livers has activity for the NADPH- or NADH-linked reduction of *p*-hydroxylaminobenzoic acid to *p*-aminobenzoic acid.[9] In fact, our experiments showed that the 9000 ***g*** supernatant used in this study could reduce methyl *p*-hydroxylaminobenzoate and *p*-hydroxylaminoacetophenone to the corresponding arylamines in the presence of NADPH or NADH, respectively (data not shown). These findings suggest that the reduction of alkyl N-hydroxycarbamates and aromatic hydroxylamines may be catalyzed by liver enzymes which are different in nature.

Previously, Boyland and Nery[4] showed that when ethyl carbamate was administered to rats, rabbits and man, ethyl N-hydroxycarbamate and its acetyl derivative were excreted in the urine. However, Mirvish[5] reported that when ^{14}C-ethyl carbamate was injected into mice, only the radioactive peak of ethyl carbamate administered was detected in the blood. In connection with studies on the mode of action of ethyl carbamate, it is clearly important to determine whether ethyl carbamate is indeed converted to ethyl N-hydroxycarbamate in animal bodies. In the present study, four rats were given ethyl carbamate at a single dose of 1 g/kg and the 48 hr urine was examined by TLC as described in "Materials and Methods." However, ethyl N-hydroxycarbamate or its acetyl derivative, ethyl N-acetoxycarbamate, could not be found, though amounts of these compounds equivalent to 0.1% of the ethyl carbamate administered could be detected in the 48 hr urine of rats under the present experimental conditions. Nery[11] reported that N-hydroxylation of ethyl carbamate *in vivo* was stimulated by pretreatment of the animals with 3-methylcholanthrene and by repeated injection of ethyl carbamate. Therefore, additional experiments were carried out as follows: two groups, each of four rats, were given 3-methylcholanthrene subcutaneously at a single dose of 25 mg/kg or phenobarbital sodium intraperitoneally at a dose of 70 mg/kg/day for three days in order to stimulate the drug-metabolizing enzyme activity. After pretreatment, these groups were injected with ethyl carbamate in a single dose, and the 48 hr urine was subjected to TLC examination as described above. The third group, consisting of five rats, was given ethyl carbamate at a dose of 1 g/kg/day for five days successively. The urine was collected for six days after dosing, extracted continuously with ether for 16 hr and the ether extract was examined by TLC. However, TLC failed to reveal any ethyl N-hydroxycarbamate in these ether extracts.

Furthermore, ethyl carbamate was incubated with liver slices or the liver 9000 ***g*** supernatant from normal rats or from pretreated rats which had received phenobarbital sodium or 3-methylcholanthrene as described above, under aerobic conditions. Portions (2 ml) of each incubation mixture were treated with 0.5 ml of 36% trichloroacetic acid and the ethyl N-hydroxycarbamate content was determined by the iodine oxidation method.[12] If ethyl N-hydroxycarbamate were formed from ethyl carbamate, this colorimetric method could detect 0.01 μmol (1.05 μg) of the N-hydroxy compound per ml of the incubation mixture. The remaining portions (~8 ml) of the incubation mixture were extracted with ether and the extract was examined by TLC. However, no alkyl N-hydroxycarbamate could be detected in these *in vitro* experiments.

In all the experiments described in this paper, no strain differences between Wistar and Donryu rats were observed. The present study shows that alkyl N-hydroxycarbamates are easily converted to the corresponding alkyl carbamates in rats, but the conversion of

11) R. Nery, *Biochem. J.*, **106**, 1 (1968).
12) R. Nery, *Analyst*, **91**, 388 (1966).

ethyl carbamate to ethyl N-hydroxycarbamate could not be demonstrated, supporting the previous work of Mirvish.[5,6] In conclusion, it appears unlikely that alkyl N-hydroxycarbamates are metabolites of the corresponding alkyl carbamates in rats. From a chemical point of view, however, it seems that ethyl carbamate, which has little chemical reactivity,[13] should require some metabolic activation before producing its biological effects. As regards possible carcinogenic metabolites of this compound, the recent work of Dahl *et al.*[14] has suggested that candidates include vinyl carbamate, which might be epoxidized to yield the ultimate carcinogen. Considering from these facts, therefore, the activation mechanism of ethyl carbamate must be investigated extensively without adhering to the N-hydroxylation of this compound.

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13) R. Nery, *J. Chem. Soc.* (*C*), **1969**, 1860.
14) G.A. Dahl, J.A. Miller, and E.C. Miller, *Cancer Res.*, **38**, 3793 (1978).