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# Excretion and Conversion of 3-Methylcholanthrene Metabolites in the Intestinal Tract of the Mouse

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## SUMMARY

A study of the metabolism of 3-methylcholanthrene (3-MC) injected i.v. into mice has shown that the metabolites excreted in the bile could be separated into six subfractions, FIII-1, FIII-2, and FIV-1 to -4, by combined chromatography on a Sephadex LH-20 column and on a thin layer of silica gel. These metabolites were water soluble and were considered to be conjugates of mono- or dihydroxy-3-MC with glucuronic or sulfuric acid. These water-soluble conjugates were freed from the acids in the cecum and large intestine and were converted into ether-soluble substances, FI and FII, which consisted of 10 and 4 subfractions, respectively. Among the subfractions of FI, *cis*- and *trans*-1,2-dihydroxy-3-MC, 2-hydroxy-3-MC, 2-keto-3-MC, and intact 3-MC were detected. FII subfractions were considered to be metabolites of 3-MC oxidized at positions 4 to 10. 11,12-Dihydro-11,12-dihydroxy-3-MC, the so-called K-region dihydro diol, was not detected in the subfractions of FI and FII. However, a further oxidized metabolite of this dihydro diol may appear in one of the FI subfactions. Fraction FV and FV', appearing in contents of the intestine, were intermediate substances from the conjugated metabolites to FI and FII subfractions.

## INTRODUCTION

Since the first report by Chalmers and Peacock (2), the metabolism of carcinogenic hydrocarbons, especially of 3,4-benz(a)pyrene, has been studied by many workers. Although 3-MC[1] is known to be one of the most potent carcinogens, little is known of its metabolism and fate in the animal body. Dauben and Mabee (4), using 3-MC-$^{14}$C, first reported that the radioactivity of 3-MC was eliminated mainly in the feces and partly in the urine. In the experiment of Harper (8), 3-MC injected into mice appeared in the bile as conjugated metabolites with sulfuric or glucuronic acid. Recently, Sims (10) showed that 3-MC in liver homogenates of the rat and mouse was metabolized into various compounds including phenols, ketones, quinones, and glutathione conjugates. This paper describes the excretion and identification of 3-MC metabolites in the bile, in the contents of the intestinal tract, and in the feces from mice treated i.v. with this hydrocarbon, with the use of silica gel and Sephadex LH-20 for fractionation of the extracts. Radiometric and fluorometric analyses were used for determination of the 3-MC administered.

## MATERIALS AND METHODS

**Test Materials.** ICR male adult mice were used for this experiment. One mg of 3-MC with or without 0.5 μCi of 3-MC-6-$^{14}$C was suspended in 0.1 ml of bovine serum and was injected i.v. into the mice, which were sacrificed after 4 or 24 hr. The bile, feces, and contents of the small intestine, cecum, and large intestine were removed for chemical analysis. After repeated extractions of these materials with ethanol, the extracts were evaporated to dryness in a vacuum. Ethanol solutions of the dried residue of the extracts were subjected to column chromatographic analysis. As an exception, some samples of the bile were applied directly to the column without any preliminary ethanol extraction. In order to collect a large amount of the bile, we used Wistar rats, the bile ducts of which were allowed to drain by the method of Kotin *et al.* (9).

**Column Chromatography.** Sephadex LH-20 was allowed to swell in ethanol and was packed in a column (gel bed volume, 30 ml). A sample of the bile or an ethanol solution of the extracts was applied to the column and eluted with ethanol. The effluent was collected in 3-ml fractions with an automatic fraction collector. The fluorescence intensity of each fraction was measured with an excitation light of 300 nm with a Shimazu fluorospectrometer, and its radioactivity was counted with a Nuclear-Chicago liquid scintillation counter.

**TLC.** We prepared for TLC by coating a glass plate with a 20- x 20-cm width and 0.25-mm depth of Merck Silica Gel HF-254. The plates were activated at 110° for 30 min just before use. Some of the test materials dissolved in ether were applied to the plates and were developed with Solvents 1 and 2, benzene and benzene : ethanol (9:1), respectively. The other materials were diluted in water, applied to the plates, and developed with Solvent 3, 1-propanol:1-butanol:2 N $NH_4OH$ (1:2:1).

The spots on the plate were illuminated under UV light while still wet and were removed for counting radioactivity, taking fluorescence spectra, and examining UV absorption.

**Spectra.** Mass spectra were measured on the Hitachi RMU-6 mass spectrometer: ionizing voltage, 70 eV; total emission, 80 μA; ion-source temperature, 200°. UV absorption spectra were measured in ethanol on the Shimazu MPS-50 spectrometer.

**Hydrolysis by β-Glucuronidase.** About 10 μg of the test

[1] The abbreviations used are: 3-MC, 3-methylcholanthrene; TLC, thin-layer chromatography.

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materials were incubated with 500 units of β-glucuronidase from *Escherichia coli* (Sigma Chemical Co., St. Louis, Mo.) in Tris buffer of pH 7.0 at 37° for 1 hr, and the products were examined by TLC.

**Naphthoresorcinol Test.** A mixture of 10 mg of naphthoresorcinol and about 100 μg of a test material in 3 ml of sulfuric solution (15 g/dl) was boiled for 30 min and cooled at 4° for 5 min. The mixture was extracted with 2 ml of benzene, and then 1 ml of ethanol was added. If glucuronic acid is in the test material, a maximum absorption appears at 582 nm.

**Detection of Sulfuric Conjugation.** $H_2{}^{35}SO_4$ (10 μCi) and 3-MC (1 mg) were administered by intracutaneous and i.v. injections, respectively. Bile and feces were examined as above. Radioactivity on TLC was measured by a Nuclear-Chicago Actigraph III.

**3-MC and Its Standard Derivatives.** 3-MC (Chart 1, I) was obtained from Fluka (CH-9470; Buchs, Switzerland). Its melting point is 280.2° and its $R_F$ value in both Solvents 1 and 2 is 0.95. The fluorescence, excited at 300 nm, of 3-MC in ethanol was violet and had maxima at 402 and 418 nm. The UV absorption curve showed $\lambda_{max}$ at 223, 237, 262.5, 274.5, 285.5, 297, 304.5, 328, 342.5, and 359 nm. Isotope-labeled 3-MC (3-MC-6-$^{14}$C) from New England Nuclear, Boston, Mass., was commercially available.

1-Keto-3-MC (Chart I, V) was prepared by dichromate oxidation of 3-MC by the method of Fieser and Hershberg (6). In accordance with the method of Sims (10), the crude product was purified and finally crystallized from ether to yield the ketone in yellow needles: m.p. 261.7°; $\lambda_{max}$ at 217, 234, 257.5, 268.5, 295, and 307 nm. The fluorescence spectrum showed a maximum at 460 nm. Its mass spectrum showed a molecule ion peak at *m/e* 282 and no peak at *m/e* 264 = $M^+ - H_2O$. The $R_F$ values in Solvents 1 and 2 were 0.47 and 0.46, respectively.

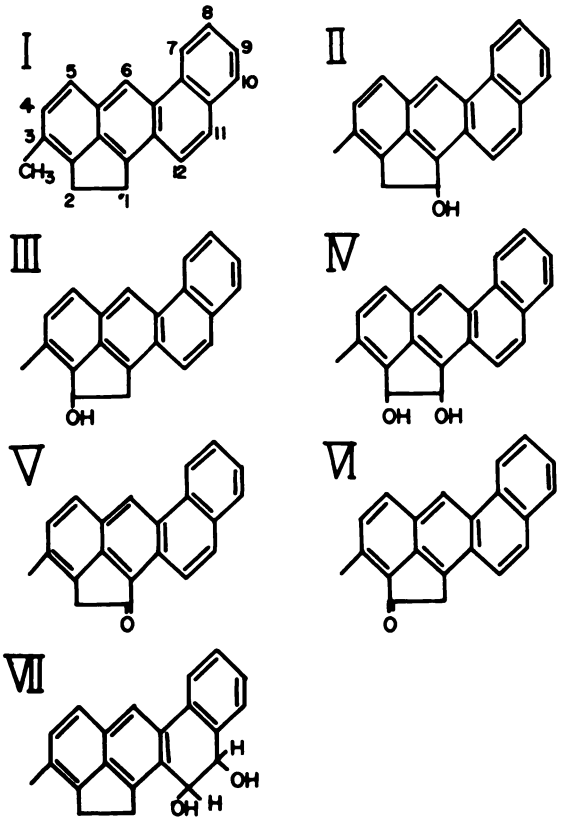


Chart 1. Chemical structures of standard hydrocarbons.

In the production of 1-hydroxy-3-MC (Chart 1, II), 1-keto-3-MC was reduced with lithium tetrahydroaluminate, according to the method of Sims (10). The crude product was purified by chromatography on silica gel in benzene and crystallized from ethanol to yield 1-hydroxy-3-MC in needles: m.p. 210.5° (decomposition); $\lambda_{max}$ at 224, 236, 256, 263, 273, 284, 295, 303, 325, 340, and 356 nm. The fluorescence spectrum showed the same curve as that of 3-MC. The mass spectrum showed a molecule ion peak at *m/e* 284 (64%) and $M^+ - H_2O$ ion at *m/e* 266 (base peak). The $R_F$ values in Solvents 1 and 2 were 0.23 and 0.34, respectively.

In the production of 2-keto-3-MC (Chart 1, VI), 1,2-dihydroxy-3MC was oxidized in acetic acid by the method of Sims (10). The product was purified by chromatography on silica gel in benzene and on alumina in benzene : ethanol (95 :5) and was crystallized from ether to yield 2-keto-3-MC in yellow needles: m.p. 201.5°; $\lambda_{max}$ at 224, 246, 277.5, 296, 301, 310, and 342.5 nm. The fluorescence spectrum showed a maximum at 495 nm. The mass spectrum showed a molecule ion peak at *m/e* 282 and no peak at *m/e* 264 = $M^+ - H_2O$. The $R_F$ values in Solvents 1 and 2 were 0.47 and 0.46, respectively.

2-Hydroxy-3-MC (Chart 1, III) was prepared by reducing 2-keto-3-MC in the same manner used in the preparation of 1-hydroxy-3-MC from 1-keto-3-MC. After chromatographic purification of the crude product, needles crystallized from ethanol were obtained. The melting point of this product was not certain because, at 180°, the product seemed to change into another compound which melted at 219.5° (decomposition). The UV absorption spectrum showed $\lambda_{max}$ at 223, 237, 256, 262, 273, 284, 295, 304, 326, 341, and 357 nm. The fluorescence spectrum showed the same curve as that of 3-MC. The mass spectrum showed a molecule ion peak at *m/e* 284 (base peak) and $M^+ - H_2O$ ion at *m/e* 266 (86.7%). The $R_F$ values in Solvents 1 and 2 were 0.15 and 0.31, respectively.

*cis*- and *trans*-1,2-Dihydroxy-3-MC (Chart 1, IV) were prepared by acetylation of 3-MC by lead tetraacetate in hot acetic acid and by subsequent hydration of the brown product by KOH, according to the method of Sims (10). The crude product was chromatographed in benzene : acetone (9 :1) on silica gel. The 2 violet fluorescent bands were evaporated and crystallized from ethanol to yield *cis*- and *trans*-1,2-dihydroxy-3-MC in microscopically visible brownish needles, with an uncertain melting point because of decomposition into 2-keto-3-MC at 190–200° and a $\lambda_{max}$ at 223, 234, 256, 262, 273, 283, 294, 302.5, 324, 338, and 354 nm. The fluorescence spectrum showed the same curve as that of 3-MC. The mass spectrum showed a molecule ion peak at *m/e* 300 (base peak) and $M^+ - H_2O$ ion at *m/e* 282 (47.7%). These data were the same for the *cis* and *trans* types of chemicals and could be differentiated by evidence of production or nonproduction of isopropylidene derivative by the method described as follows.

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A mixture of 1,2-dihydroxy-3-MC and acetone containing some 70% $HClO_4$ was allowed to stand 1 hr at room temperature. The products were examined by mass spectrometry. The faster moving band in chromatography was identified as *cis* type by the presence of a peak at *m/e* 340 (molecule ion of isopropylidene), and the second was identified as *trans* type by the absence of a peak at this position. The $R_F$ values of *cis*- and *trans*-1,2-dihydroxy-3-MC were 0 in Solvent 1 and 0.26 and 0.24, respectively, in Solvent 2.

11,12-Dihydro-11,12-dihydroxy-3-MC (Chart 1, VII) was prepared from a mixture of 3-MC and $OsO_4$ in pyridine according to the description by Cook and Schoental (3). The crude product was purified by chromatography on silica gel in benzene : actone (9 :1) and was crystallized from ethanol to yield 11,12-dihydro-11,12-dihydroxy-3-MC in a white powder which turned red at 190° and melted at 202° (decomposition), $\lambda_{max}$ at 220, 239, 255, 265, 274, 310, and 348 nm. The fluorescence spectrum showed a maximum at 370 nm. The mass spectrum showed a molecule ion peak at *m/e* 302 (79%) and $M^+ - H_2O$ ion at *m/e* 284 (base peak). The $R_F$ values in Solvents 1 and 2 were 0 and 0.25, respectively.

All samples were checked for purity by mass spectrometry.

## RESULTS

Column chromatograms of ethanol-soluble portions of bile, feces, and the contents of the small intestine, cecum, and large intestine, all from mice injected i.v. with 1 mg of 3-MC at 4 or 24 hr before sacrifice, are shown in Chart 2. One to 4 peaks of fluorescent substances, illustrated in the charts as FI, FII, FIII, FV, and FV', appeared in each chromatogram. As shown in each chromatogram, the radioactivity of these fractions after 3-MC-6-$^{14}$C application was counted in direct proportion to fluorescence intensities, indicating that the fluorescence of the fraction was due to the 3-MC injected. The bile was applied directly to the chromatograph in the same way, and 2 peaks of fluorescent substances, FIII and FIV, were separated. Although the $R_F$ value on the Sephadex of ethanol-untreated bile is much different from that of ethanol-treated bile, the faster-moving fraction was concluded to be the same material as FIII in Chart 2, based on its $R_F$ value on silica gel and on its fluorescence. The slower-moving fraction, FIV, will be discussed later. FIII was the only component furnishing both fluorescence and radioactivity in the ethanol-soluble portion of the bile and in the contents of the small intestine. In contrast, FI, FII, and FV appeared in the contents of the cecum and large intestine. Although FV appeared at the same position in the chromatograms as FIII, the 2 were identifiable by fluorospectrometry and TLC. FV' was an inflection of FV and was clearly distinct from the latter by fluorometric observations. FV' appeared only in the cecum and apparently was decomposed in the large intestine.

**TLC of FI and FII.** TLC's of FI and FII from the feces in Solvents 1 and 2 revealed 10 and 4 subfractions, respectively. Fluorescence and $R_F$ values of the FI subfractions are given in Table 1. The fluorescence specra of FI-2, -3, -4, -5, -8, and -10 were all the same and showed 2 peaks, at 402 and 418 nm, which is also characteristic of 3-MC, 1- or 2-hydroxy-3-MC, and

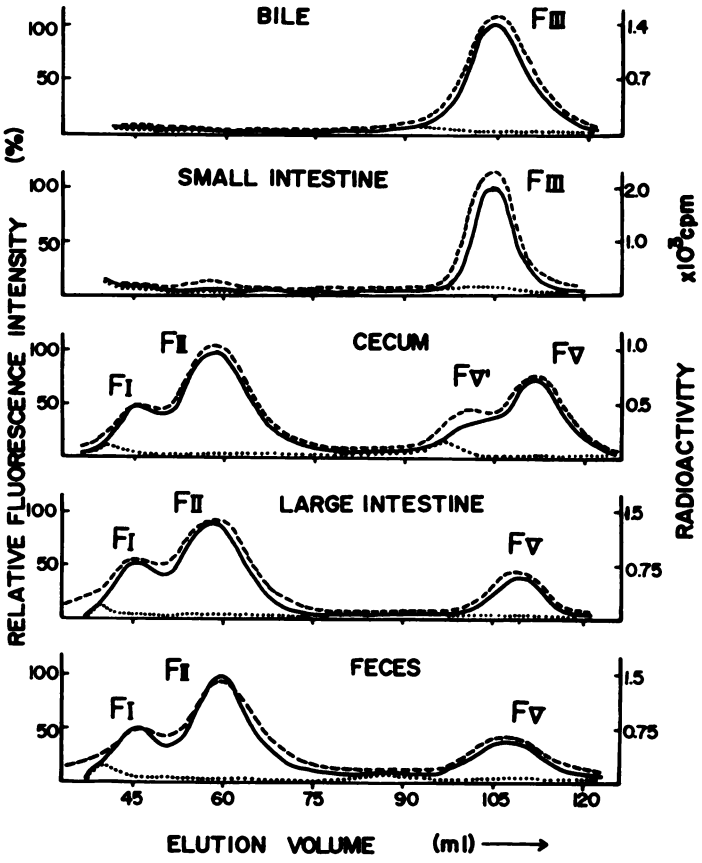


Chart 2. Column chromatograms of ethanol-soluble fractions of the bile, feces, and contents of the intestinal tract. Column: Sephadex LH-20 (gel bed volume, 30 ml); solvent: ethanol. Fluorescence intensities were measured at the excitation light of 300 nm and illumination light of 402 nm (FI), 415 nm (FII and FV'), and 425 nm (FIII and FV). — , fluorescence from mice treated with 3-MC; - - - -, radioactivity from mice treated with 3-MC-6-$^{14}$C; • • • •, fluorescence from mice treated without 3-MC.

1,2-dihydroxy-3-MC. Based on their mobilities in Solvents 1 and 2, their fluorescence spectra, and their UV absorption spectra, FI-2, -3, -5, and -10 were respectively identified as *trans*-1,2-dihydroxy-3-MC, *cis*-1,2-dihydroxy-3-MC, 2-hydroxy-3-MC, and intact 3-MC. FI-6 was also identified as 2-keto-3-MC in the same manner. FI-7 and -9 were thought to be kinds of ketones, as they showed yellow fluorescence. FI-1 was so unstable that its chemical structure has remained obscure. FI-4 and -8 are thought to be metabolites that are very close in structure to the original 3-MC. However, their $R_F$ values in Solvents 1 and 2 were not identical with any one of those of the standard derivatives prepared for identification.

The fluorescence, UV absorption spectra, and $R_F$ values of FII subfractions are shown in Table 2. None of them was identical with those of standard derivatives. Their fluorescence spectra all showed a slight shift of their peaks to long wavelengths, as compared with those of 3-MC. The chemical structures of these compounds are discussed below.

**TLC of FIII, FIV, and FV.** TLC of FII, FIV, and FV, in Solvent 3 resulted, respectively, in subfractions FIII-1 and -2, FIV-1 to -4, and FV-1 and -2. The FIII subfractions were

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Table 1
*Fluorescence and $R_F$ values on TLC of FI subfractions*

| Subfraction | Fluorescence: Color on TLC | Fluorescence: $\lambda_{max}$ (nm)[a] | $R_F$ value: Solvent 1 | $R_F$ value: Solvent 2 | Identified with |
|---|---|---|---|---|---|
| FI-1 | Violet | | 0.00 | 0.21 | |
| FI-2 | Violet | 402, 418 | 0.00 | 0.24 | *trans*-1,2-Dihydroxy-3-MC |
| FI-3 | Violet | 402, 418 | 0.00 | 0.26 | *cis*-1,2-Dihydroxy-3-MC |
| FI-4 | Violet | 402, 418 | 0.08 | 0.28 | |
| FI-5 | Violet | 402, 418 | 0.15 | 0.31 | 2-Hydroxy-3-MC |
| FI-6 | Yellow | 495 | 0.47 | 0.46 | 2-Keto-3-MC |
| FI-7 | Yellow | | 0.95 | 0.30 | |
| FI-8 | Violet | 402, 418 | 0.95 | 0.34 | |
| FI-9 | Yellow | | 0.95 | 0.38 | |
| FI-10 | Violet | 402, 418 | 0.95 | 0.95 | 3-MC |

[a] Dissolved in ethanol and excited at 300 nm.

Table 2
*Fluorescence, UV absorption, and $R_F$ value of FII subfractions*

| Sub-fraction | Fluorescence: Color on TLC | Fluorescence: $\lambda_{max}$ (nm)[a] | $R_F$ value: Solvent 1 | $R_F$ value: Solvent 2 | Absorption $\lambda_{max}$ ethanol (nm) |
|---|---|---|---|---|---|
| FII-1 | Blue-violet | 416, 432 | 0.00 | 0.28 | 283, 293, 305 |
| FII-2 | Blue-violet | 410, 430 | 0.00 | 0.34 | 250, 260, 283, 293, 303, 318, 343 |
| FII-3 | Blue-violet | 405, 425 | 0.21 | 0.41 | 232, 268, 283, 293, 306, 348, 365 |
| FII-4 | Blue-violet | 406, 425 | 0.31 | 0.42 | 282.5, 293, 305, 318, 347, 363 |

[a] Dissolved in ethanol and excited at 300 nm.

Table 3
*Physical and chemical properties of FIII, FIV and FV subfractions*

| Sub-fraction | Fluorescence: Color on TLC | Fluorescence: $\lambda_{max}$ (nm)[a] | $R_F$ value Solvent 3 | Hydrolysis by β-glucuronidase | Naphtho-resorcinol test |
|---|---|---|---|---|---|
| FIII-1 | Violet | 402, 418 | 0.86 | – | – |
| FIII-2 | Blue-violet | 425 | 0.53 | + | + |
| FIV-1 | Light blue | 420, 440 | 0.67 | – | |
| FIV-2 | Blue-yellow | 460 | 0.42 | – | |
| FIV-3 | Blue-violet | 445 | 0.35 | – | |
| FIV-4 | Blue-violet | 430 | 0.29 | – | |
| FV-1 | Blue-violet | 415 | 0.85 | – | |
| FV-2 | Light blue | 425 | 0.65 | – | |

[a] FIII and FV were dissolved in ethanol and FIV was dissolved in water and excited at 300 nm.

soluble in water, much less soluble in ethanol, and almost insoluble in ether and benzene. The FIV subfractions were soluble in water and almost insoluble in ethanol, ether, and benzene. The FV subfractions were soluble in water and slightly soluble in ethanol, ether, and benzene. Fluorescence spectra and $R_F$ values of these substances in Solvent 3 on silica gel are shown in Table 3. Susceptibility to β-glucuronidase and results of the naphthoresorcinol test are also shown in Table 3. FIII-2 was hydrolyzed by β-glucuronidase into FII-2 and was positive in the naphthoresorcinol test. Radioactivity due to $^{35}S$-labeled sulfuric acid appeared in FIII and FV. After development of these fractions on TLC, activity was found only in FIII-2 and FV-1, not in FIII-1 and FV-2. These data indicate that FIII-2 is a mixture of conjugates of 3-MC metabolites with sulfuric and glucuronic acid and that FV-1 is a conjugate with sulfuric acid.

***In Vivo* Conversion of FIII-1, FIII-2, and FIV.** For a test of the *in vivo* conversion of FIII and FIV fraction, rat bile was injected into the small intestine of mice, and their feces were collected. Data are shown in Table 4. It became clear from this experiment that FIII-1 is broken down into FII subfractions, and FV′ is considered to be an intermediate from FIII-1 to FII. In contrast, *in vivo* breakdown products of FIII-2 and FIV are FI and FII subfractions, and FV appeared as an intermediate from FIII-2 to its final products.

**Relative Amounts of 3-MC Subfractions.** Table 5 shows



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Table 4
*Breakdown products in the feces of FIII-1[a], FIII-2[a], and FIV[a] injected into the small intestine*

| Injected | Breakdown product | | |
|---|---|---|---|
| FIII-1 | ND[b] | FII-1, -2 | FV' |
| FIII-2 | FI-1, -2, -3, -4 | FII-2, -3 | FV |
| FIV | FI-1, -2, -3, -5 | FII-1, -2 | |

[a] These fractions from the rat bile were used, as they were confirmed to be similar to those from the mouse.
[b] ND, not detected.

Table 5
*Relative intensities of radioactivity in breakdown products of 3-MC-6-$^{14}$C in the feces*

| Methods | Radioactivity (%) |
|---|---|
| Ethanol fractionation | |
| Ethanol-soluble fraction | 65 |
| Ethanol-insoluble (water-soluble) fraction | 35 |
| Sephadex fractionation | |
| FI | 13 |
| FII | 31 |
| FV[a] | 12 |
| Others | 8.9 |
| TLC (subfractions) | |
| FI-1 | 0.8 |
| FI-2 | 2.1 |
| FI-3 | 0.9 |
| FI-4 | 0.9 |
| FI-5 | 4.6 |
| FI-6 | 0.3 |
| FI-7 | 0.4 |
| FI-8 | 0.4 |
| FI-9 | 0.1 |
| FI-10 | 3.0 |
| FII-1 | 3.2 |
| FII-2 | 13.2 |
| FII-3 | 12.5 |
| FII-4 | 2.5 |

[a] Variable according to dosage of 3-MC. These data in this table indicate the relative contents of fractions when 0.5 μCi of 3-MC-6-$^{14}$C was injected with 1 mg of nonlabeled 3-MC.

relative amounts of 3-MC metabolites obtainable from mice with 3-MC administration. FII-2 and -3 are the main products, and FI-2, -5, and -10 and FII-1 and -4 are the minor ones. 3-MC metabolites in ethanol-insoluble and water-soluble portions form 35% of the radioactivity in the feces; these were omitted from the present considerations.

## DISCUSSION

Since the comprehensive work of Boyland and his co-workers, it has been known that polycyclic compounds are metabolized and excreted into the urine and bile as conjugates with cysteine, glutathione, sulfuric and glucuronic acid, and others. Regarding the carcinogenic hydrocarbons, Berenblum and Schoental (1) and Weigert and Mottram (11) pointed out unknown conjugated metabolites of benzpyrene in the bile and tissues. Harper (7) and Falk *et al.* (5) elucidated that these compounds are conjugates with sulfuric and glucuronic acid. Harper (8) also suggested that 3-MC is metabolized in the same manner as is benzpyrene. Sims (10) also cited only a glutathione conjugate of this hydrocarbon in rat liver homogenates. In the present experiment, it was shown that the bile of the mouse previously dosed with 3-MC contained metabolites conjugated with sulfuric and glucuronic acids. They appear in FIII-2 and FV-1. The other subfractions, FIII-1, FIV, and FV-2, are also considered to be conjugated metabolites, although their modes of conjugation have not been confirmed as yet. This consideration is based on the similarities to FIII-2 and FV-1 of their hydrosolubility and of their conversion into nonconjugated metabolites, FI and FII subfractions, in the cecum and large intestine. The conversion has been confirmed by the chromatographic analysis of feces of the mouse after the injection of rat-conjugated metabolites into the small intestine. The *in vivo* conversion experiment shows that FIII-1 was converted only into FII subfractions. FV and FV' were intermediates from FIII to nonconjugated metabolites and therefore did not appear when the dosage with 3-MC was minimal. Conversion of the conjugated metabolites of 3-MC into simple phenolic substances in the cecum and large intestine was suggested but not examined by Harper (8). In the present experiment, subfractions of FI and FII in the cecum, large intestine, and feces were confirmed to be from decomposition of the conjugated metabolites in the bile. 2-Hydroxy-3-MC, *cis*- and *trans*-1,2-dihydroxy-3-MC, 2-keto-3-MC, and intact 3-MC were identified in the FI fraction. Two other ketones, FI-7 and FI-9, were undetectable and tentatively are considered metabolites oxidized at their aromatic nuclei. It should be noted that $R_F$ values in Solvents 1 and 2 of these subfractions were different from those of 1- or 2-keto-3-MC. Two other FI subfractions, FI-4 and -8, were thought to be metabolites intact at least at their aromatic nuclei, based on the similarity of their fluorescence spectra to that of original 3-MC. FI-1 was unstable; however, it appeared in the feces when 11,12-dihydro-11,12-dihydroxy-3-MC was injected i.v. into the mouse. Therefore, this fraction is possibly a further oxidized metabolite of the dihydro diol. Among these FI subfractions, FI-6, -7, -8, -9, and -10 were undetectable in the *in vivo* breakdown products in the mouse. Although further studies are required, this may be due at least in part to different pathways of 3-MC metabolism between heterogeneous animal strains.

Exact chemical structures of FII subfractions are unknown as yet. However, FII-3 and FII-4 are possibly monohydroxy compounds, and FII-1 and FII-2 may be dihydroxy compounds, as their $R_F$ values in Solvents 1 and 2 were identical with those of standard mono- and dihydroxy derivatives of 3-MC, respectively. They are seemingly oxidized at positions 4 to 10 of 3-MC because of the shift to long wavelength of the fluorescence spectra of these compounds.

In an *in vitro* experiment, Sims (10) indicated 1- and 2-hydroxy-3-MC, 1,2-dihydroxy-3-MC, 1- and 2-keto-3-MC, 1,2-quinone-3-MC, 11,12-dihydro-11,12-dihydroxy-3-MC, intact 3-MC, and a glutathione conjugate of 3-MC at 11,12 positions in rat liver homogenates incubated with 3-MC. Some of them were identical with those in the present experiment,

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and others were not. The discrepancy may be due to the methods used.

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