

ORIGINAL REPORT

FLUOROMETRIC MEASUREMENT OF 3-METHYLCHOLANTHRENE AND ITS METABOLITES IN TISSUE. I. MEASUREMENT OF 3-METHYLCHOLANTHRENE IN ORGANIC SOLVENTS

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INTRODUCTION

In our previous experiment¹⁾, 3-methylcholanthrene (3-MC) in Freund's incomplete adjuvant was introduced into the bronchial trees of rats to produce lung tumors. Epidermoid or other tumors developed at the site of instillation of the carcinogen. However, in considering the mechanism of tumor production many questions arose: how long and how much of the carcinogen is retained in the lung; to what other organs is it transported from the lung; what keeps the tumor limited to the lung. Some of these problems have been clarified, at least in part, by histological examinations of carcinogenesis²⁾, especially by the illumination of tissue sections under ultraviolet (UV) light, which indicated the presence in or the elimination from tissues of the carcinogen whose fluorescent characteristics are well known. The experiments described in the present paper were carried out to investigate the method of fluorospectrophotometry of the hydrocarbon in various organic solvents.

MATERIALS AND METHODS

Chemicals: Yellowish crystalline 3-MC was prepared by E. Merck A. G. (Darmstadt, Ger.). The solvents, benzene and ethyl alcohol, were extra-pure and produced by Nakarai Chem. Co. (Kyoto) for use in fluorometric analysis. Liquid paraffin was produced by the same company. Anhydrous lanolin was purchased from Maruishi Chem. Co. (Osaka). Sesame and corn oils were obtained commercially.

Fluorometry: Shimadzu Fluorospectrophotometer Model GF-16 was used to measure fluorescence. A test solution was put in a cubic cell 1 cm in diameter and illuminated by UV light. At the best emission, an excitation curve was obtained by changing the excitation light from 200 to 650 nm wavelength. The fluorescence curve was obtained at the best excitation. The sensitivity of this apparatus for fluorometry was near 1 ng 3-MC per ml at the smallest concentration.

RESULTS

Fluorometry of 3-MC in benzene

Profiles of excitation and fluorescence curves of 3-MC are dependent on its concentration in benzene as illustrated in Figs. 1 and 2. The maximum of excitation is at 300, 303, 367 and 393 nm wavelength of emission light, for solutions of 0.1, 1.0, 10 and 100 μg 3-MC per ml concentrations respectively. The highest peaks of fluorescence are 400 nm for 0.1, 1.0 and 10 μg per ml solutions and 418 nm for 100 μg . The peak observed at 300 nm of fluorescence is due to the leakage of excitation light. As shown in Fig. 3, the luminescence intensity of fluorescence is a function of the concentration of 3-MC. These two are linearly proportional in concentrations less than 1 μg per ml.

Fig. 4 indicates decrease of the luminescence under continuous emission by UV light of 3-MC solutions. The diminution of fluorescence depends on the intensity of light and concentration and depth of solutions.

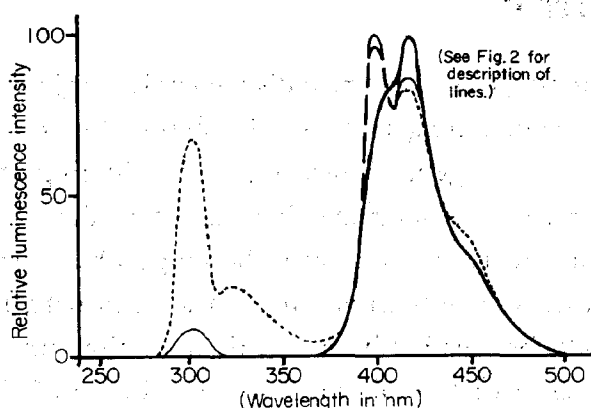


Fig. 1 Fluorescence curve of MC in benzene

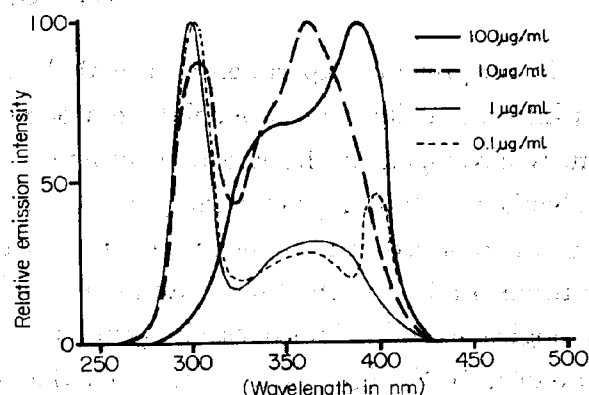


Fig. 2 Excitation curve of MC in benzene

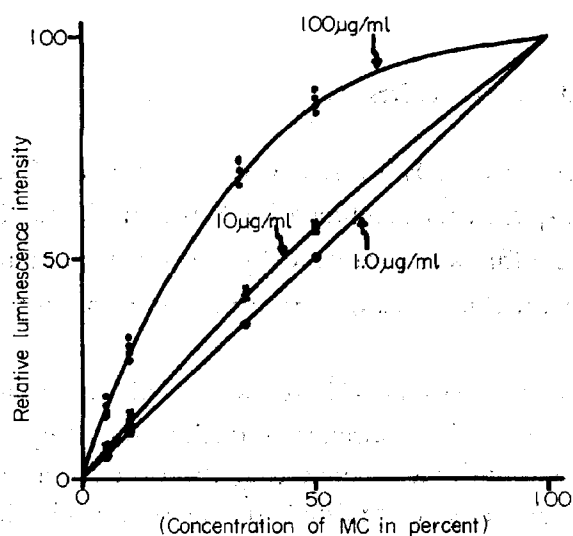


Fig. 3 Fluorescence intensity of graded concentrations of MC in benzene

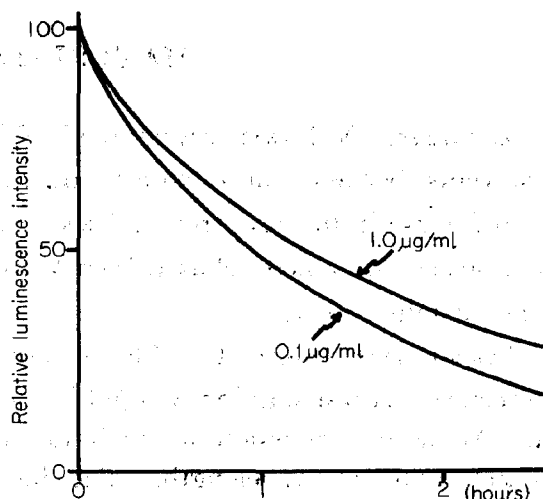


Fig. 4 Decreasing intensity of MC fluorescence under UV light

Fig. 5 illustrates the excitation and fluorescence curves of lanolin, a solvent of 3-MC at animal application, in benzene.

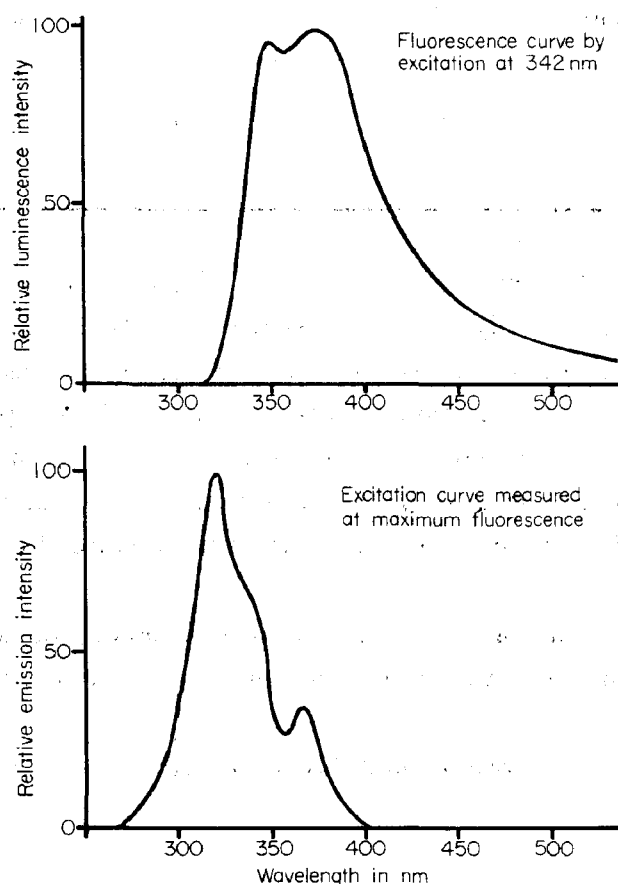


Fig. 5 Fluorescence of lanolin in benzene

Fluorescence of 3-MC in Freund's incomplete adjuvant (FIA) or some other solvents

Table 1 shows the suppression by lanolin of 3-MC fluorescence. Liquid paraffin does not interfere with fluorescence but does accelerate it slightly. A 3.3 percent solution of lanolin, with or without 3.3 percent liquid paraffin, decreases the fluorescence intensity by about a half. The table also indicates that lanolin has its own fluorescence. The quenching by lanolin of 3-MC fluorescence is shown also in Table 2 whose data indicate that the quenching activity is propor-

Table. 1 Quenching of MC fluorescence by lanolin

MC (0.1 μ g/ml)	Composition of solution		Benzene	Relative fluorescence intensity
	Liquid paraffin (10%)	Lanolin (100 mg/ml)		
3	—	—	—	100
1	1	—	1	35—38
—	1	—	2	0
1	—	1	1	15—17
1	1	1	—	15—17
—	—	1	2	11—15

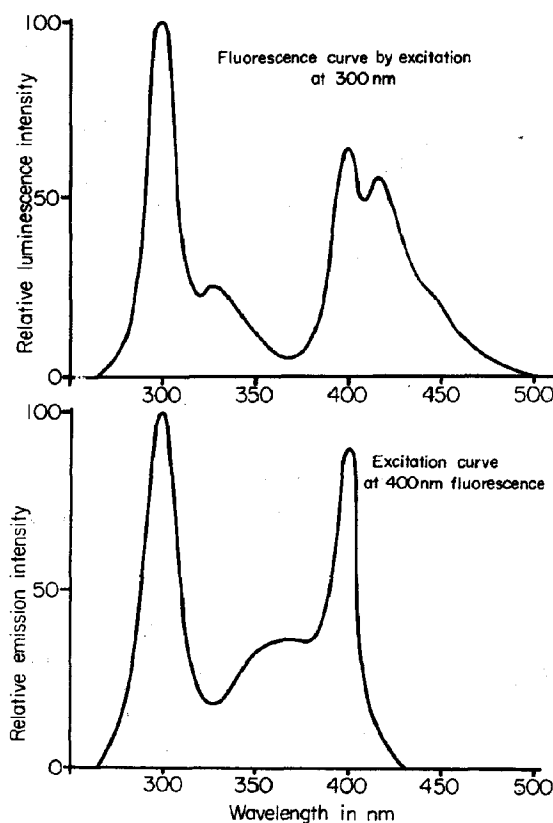
Table. 2 Quenching activity of different concentrations of MC

Composition of solution			Fluorescence intensity		
MC (1 $\mu\text{g/ml}$)	Lanolin (100 mg/ml)	Benzene	1 fold	Dilution 10 folds	100 folds
2	—	—	100	100	100
1	—	1	55	53	50
1	1	—	2	51	85

tional to the concentration of lanolin. The fluorescence of 1 μg of 3-MC is reduced by 5 percent lanolin to only 2 percent of the original luminescence intensity. However, the reduction rate of fluorescence by lanolin is markedly decreased in low concentrations of the oil, that is, 0.05 percent lanolin in solution reduced the luminescence of 0.01 μg of 3-MC per ml only to 85 percent. The reduction may be due to competition of excitation energy acquisition between 3-MC and lanolin whose excitation maximum is at 320 nm and fluorescence peak is at 370 nm. In a very dilute concentration, therefore, fluorescence of 3-MC is not disturbed even in lanolin-containing FIA, as shown in Fig. 6.

Fig. 7 shows the quenching of 3-MC fluorescence by sesame oil which has its own slight fluorescence. Its interference with the fluorescence of 3-MC is similar to that of lanolin, as can be seen in the figure.

Fig. 8 shows the influence of corn oil on 3-MC fluorescence. The oil in benzene has its own

**Fig. 6** Fluorescence of MC (0.1 $\mu\text{g/ml}$) in benzene with diluted FIA

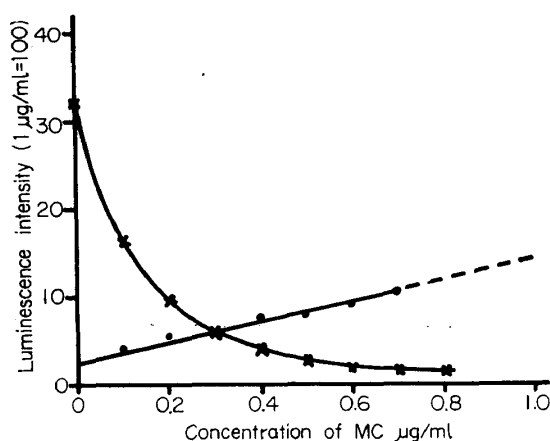


Fig. 7 Quenching of MC fluorescence by sesame oil

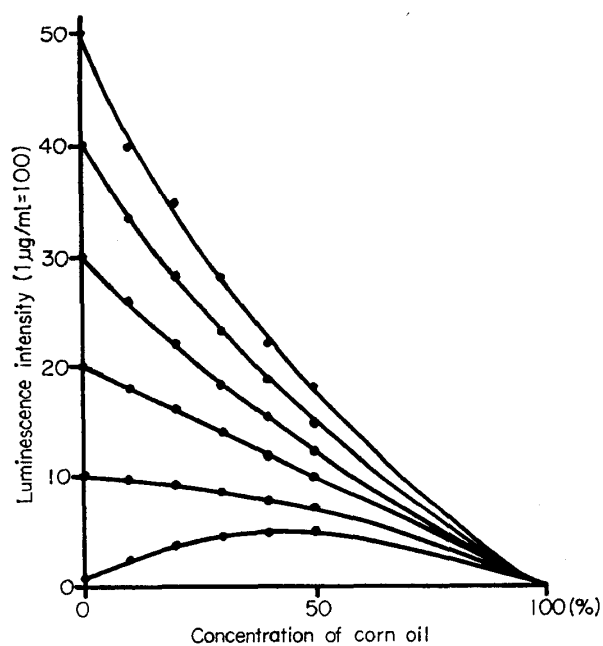


Fig. 8 Fluorescence of MC in benzene-corn oil

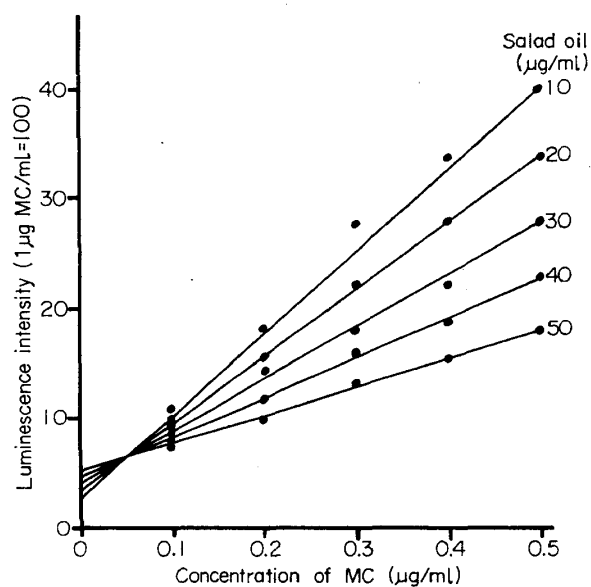


Fig. 9 Suppression of MC fluorescence by corn oil

fluorescence which affects the 3-MC fluorescence by increasing its intensity at concentrations lower than $0.2 \mu\text{g}$. When the concentration of 3-MC is changed in a solvent containing a certain amount of corn oil, the intensity of fluorescence is linearly proportional to the concentration of 3-MC although the fluorescence appears to be suppressed by the oil. These data are shown in Fig. 9.

Stability of 3-MC in FIA

Ten mg of 3-MC per ml was prepared in FIA (liquid paraffin and lanolin 2 : 1 mixture). The solution stood at room temperature, and the concentration of 3-MC in it was measured periodically. No reduction of the concentration was detected for 6 weeks. A diluted concentration ($1 \mu\text{g}$ per ml) of 3-MC in benzene with or without lanolin (10 mg per ml) was also found to be stable.

DISCUSSION

The fluorescence curve of 3-MC in benzene has two main peaks at 400 and 418 nm wavelength. The fluorescence is excited most by UV light at 300 nm for solutions with concentrations less than 1 microgram per ml. The luminescence intensity of 3-MC fluorescence is linearly proportional to its concentration when less than 1 microgram per ml. Therefore, fluorometric measurement of 3-MC should be carried out at concentrations less than 1 microgram per ml and by excitation at 300 nm and fluorometry at 400 nm. The decrease of fluorescence under UV light is rather significant, so the measurement should be performed as quickly as possible. The solvent, benzene, has its own fluorescence, but this does not affect the measurement of 3-MC when the fluorescence is measured under the conditions described above.

In our biological experiments, 3-MC has been used as a concentrated solution in FIA. Therefore, the possibility of destruction of 3-MC and interference with its fluorescence by FIA were examined. It was found that the hydrocarbon is stable in the solvent for at least 6 weeks after preparation, although lanolin in FIA interferes with the fluorescence significantly. The fluorescence of 3-MC is restored at low concentrations of lanolin.

Sesame oil has been used as a vehicle for 3-MC and other carcinogenic hydrocarbons in experimental carcinogenesis. However, it was found that the oil has as much quenching activity as the lanolin in FIA. Corn oil also showed quenching activity, but slightly less than that of sesame oil. Therefore, there is no advantage in substituting these oils for FIA in the fluorometry of 3-MC.

It has been pointed out by Simpson et al. (1954)³, Rusch et al. (1945)⁴ and Grabtree (1945)⁵ that hydrocarbons, when they are applied to animals in a form of suspension in lanolin, reduces their carcinogenic activity markedly. This is supported in part by data reported by Mueller et al. (1945)⁶ that 3-MC and 3,4-benzopyrene but not 1 : 2, 5 : 6-dibenzanthracene are destroyed fast in a solution containing lauroleic acid. In our experiment 3-MC appeared to be stable in FIA or lanolin-benzene solution. The apparent suppression of 3-MC fluorescence by concentrated lanolin could be removed by simple dilution of the solution with benzene. Weil-Malherbe and Weiss (1942)⁷ pointed out that the quenching of fluorescence of polycyclic hydrocarbons was due to oxygen in the solvent, and, therefore, complete recovery of fluorescence was seen when the measurement was done in vacuo. Findings in this report and in our experiments confirm the opinion that 3-MC is not destroyed in lanolin. The fast disappearance of 3-MC at the site of application in rats, as described in the following paper⁸, is, therefore, due to the metabolizing activity of the tissue and the transferring of the hydrocarbon from the lung to the other organs.

SUMMARY

1. Fluorometry of 3-MC was carried out with a Shimadzu Fluorospectrophotometer Model GF-16 with a sensitivity for this hydrocarbon of about 1 ng.

2. Fluorometry of the hydrocarbon in benzene should be done at concentrations less than 1 μ g per ml by excitation at 300 nm wavelength and fluorescence measurement at 400 nm.

3. To avoid degradation by UV light illumination, the measurement should be performed as quickly as possible.

4. Lanolin, as well as sesame and corn oils, has its own fluorescence and this results in a significant decrease of 3-MC fluorescence. However, the quenching can be avoided by simple dilution with benzene of the solution containing these substances.

5. 3-MC is stable in Freund's incomplete adjuvant for at least 6 weeks.

ACKNOWLEDGEMENT

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