

## 4,4'-Methylene-bis-ortho-chloro-aniline (MBOCA): Absorption and Excretion after Skin Application and Gavage

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4,4'-methylene-bis-ortho-chloro-aniline (MBOCA) is an aromatic amine and industrial chemical that has been shown to cause cancer of several different organs in rats and mice and bladder cancer in dogs. The purpose of this study was to determine the efficacy of using urinary concentrations of MBOCA as a means for evaluating extent of exposure. Male Sprague-Dawley rats were given MBOCA and [ $^{14}\text{C}$ ]MBOCA by either gavage or skin application. Concentrations and amounts of  $^{14}\text{C}$  were measured in urine, feces, skin and total carcasses, and parent MBOCA in urine at several intervals after application. The percentages of administered doses excreted and retained in the animals were calculated and comparisons made. Within 72 hr after gavage 16.5% of the administered compound was excreted in urine as  $^{14}\text{C}$  but only 0.25% as parent MBOCA. In the same interval after skin application a maximum of 2.54% of administered MBOCA was excreted as  $^{14}\text{C}$  but only 0.008% as parent MBOCA. Seventy-two hours after gavage 13.7% of the administered dose was retained in the tissues, and after skin absorption 5-13% was retained. With gavage the rate of excretion of  $^{14}\text{C}$  in urine and feces was very high in the first 24 hr (68.3%) but fell off rapidly (2.07%) by the third day. After skin absorption the rates of excretion of  $^{14}\text{C}$  were fairly constant over a 3-day period. Less MBOCA was absorbed from the skin if the skin was washed within 8 hr after application, as compared to waiting 24 hr or not washing at all. The amount of parent MBOCA detected in urine is a very small amount of that applied or absorbed. The percentage detected and the rates of excretion depend upon the route of administration, and the interval between exposure and sampling. For these reasons urinary analysis for MBOCA can be used only as very imprecise indicators of extent of recent exposure.

### INTRODUCTION

4,4'-methylene-bis-ortho-chloro-aniline (MBOCA) is an aromatic amine which causes cancer of the lungs, mammary glands, livers, and other organs in rats and/or mice (Kommineni *et al.*, 1979; Steinhoff and Grundmann, 1969; Stula *et al.*, 1975; Russfield *et al.*, 1975) and bladder cancer in dogs (Stula *et al.*, 1977). It is commercially important as a curing agent for diisocyanate-based polymers (polyurethanes) and epoxy resin systems used in the manufacture of several thousand different products, e.g., semirigid foam crash padding in vehicles, industrial tires, gear blanks, rollers, and conveyor belts. It has been widely used since the early 1960s. In 1972 the production figures approximated 8 million pounds (NIOSH, 1977; NIOSH, 1978). Although it is no longer produced in the United States, it is reported that more than 1000 U.S. companies import 4 million pounds of MBOCA yearly (Business Week, 1980). NIOSH estimated through a national survey that in the early 1970s approximately 55,000 U.S. workers were potentially

exposed to MBOCA (NIOSH, 1977). Although MBOCA was one of the 14 substances for which the Occupational Safety and Health Administration promulgated an emergency temporary standard on May 3, 1973 the standard was remanded for procedural reasons by the U.S. Court of Appeals on December 17, 1974, and at the present time there is no Federal standard (NIOSH, 1978).

In 1971 Linch *et al.* published a health hazard report on employees engaged in the manufacture of MBOCA (Linch *et al.*, 1971). They concluded that skin absorption from direct contact with MBOCA was the major method of exposure to the employees, and recommended biological monitoring for MBOCA by urinalysis. The experiments reported herein were initiated to determine the relationship between exposures by gavage and skin application and the concentration of MBOCA in urine.

## MATERIALS AND METHODS

### *General*

Two millicuries (58 mCi/mmol) of [<sup>14</sup>C]methylene-bridged MBOCA (Amersham Co.) were received as a solid compound from the State of Michigan Toxic Substance Commission. It was dissolved in acetone to provide a stock solution with a concentration of 2.1  $\mu$ Ci/0.1 ml. This was stored in a refrigerator and used for each experiment. The stock solution was subsequently analyzed for radiochemical purity by high performance liquid chromatography with a Waters Model ALC/GPC-201 liquid chromatograph using a 30-cm-long  $\times$  3.9-mm-i.d. stainless-steel column packed with 10- $\mu$ m particle size micro Bondapak (Waters Associates, Milford, Mass.). Chromatograms were developed using a 1-ml/min water:methanol solvent system gradient programmed from 20–100% methanol at a linear rate during a 45-min period. Radioactive peaks were detected using a Tri-Carb RAM 7500 radioactivity monitor (Packard Instrument Co., Downers Grove, Ill.) equipped with a 100- $\mu$ l quartz flow cell packed with a solid scintillator and digital peak plotter. Analysis of the stock solution revealed that 96.31% ( $\pm$ 0.51) of the <sup>14</sup>C activity appeared in one peak and 0.17 to 1.03% in each of seven other minor peaks.

The unlabeled MBOCA was received from the Anderson Development Company, Adrian, Michigan. It was a pale-yellow, pelleted (5-mm) powder, used commercially in the manufacturing of polyurethane products. The unlabeled MBOCA was analyzed for purity using a uv detector (Schoeffel Model SF770 Spectroflow, Kratos Analytical Instruments, Ramsey, N.J.) set at 254 nm. The methanol:water gradient was the same as that used for the purity analysis on [<sup>14</sup>C]MBOCA. With these conditions only two uv absorbing peaks were found. One peak accounted for 92.36% and the other for 7.56% of the total uv absorbance.

Male Sprague–Dawley rats weighing 151–175 g were received from the Charles River Breeding Laboratories in two separate shipments. They were housed three/cage in stainless-steel wire-meshed cages and fed Purina rat chow and tap water *ad lib*. Two weeks after receiving they were ear tagged with individual numbers (1–460). The rats for each experiment were selected by a formal randomization procedure using those numbers. During each experiment the treated rats were

housed individually in stainless-steel metabolism cages. Urine was collected in 60-ml, screw-cap, polyethylene bottles containing 2 ml of 30% citric acid (unless otherwise indicated). At each 24-hr interval the urine and feces from each animal were measured and weighed and saved for analysis, and the animals placed in clean metabolism cages.

All animals were sacrificed with overdoses of phenobarbital.

### *Gavage Experiments*

For the gavage experiments 62.5 mg of MBOCA was dissolved in 10 ml of propylene glycol in a 25-ml volumetric flask by swirling and warming to 50°C for 10 min. It was stored overnight at room temperature and protected from light. On the following day (treatment day) 2.5 ml of stock [<sup>14</sup>C]MBOCA in acetone was added and dissolved. Additional propylene glycol was then added to the 25-ml mark on the flask and the contents were mixed. A new solution was made prior to each experiment.

Rats were fasted for 18 hr prior to gavage. They were weighed and the volume of solution was calculated to provide a 1-ml vol of solution (containing 2.5 mg of MBOCA and 2.1 μCi of <sup>14</sup>C) per 200 g of body wt. This solution was aspirated into an 18-gauge, 4-inch-long gavage needle connected to a 3-cc polystyrene syringe. The needle was wiped clean and the needle and syringe with their contents were weighed to the nearest 0.01 g. After administering the solution, the needle and syringe was reweighed and the dose delivered was calculated. In addition an aliquot of the treatment solution was taken in the same manner of <sup>14</sup>C analysis. The animals were then individually housed in metabolism cages and the urine and feces collected at 24-hr intervals.

Experiments 1 and 2 were pilot studies in which glass cylinders were glued to the skin to protect the skin application sites. After 24 hr these devices fell off and the application sites were no longer protected. Results from those studies will not be presented in this paper.

In Experiment 3 eight rats weighing 191–243 g were gavaged with 11 μg of MBOCA (containing 1932 DPM <sup>14</sup>C/μg unlabeled MBOCA) per 1 g of body wt. Each rat was then individually housed in a stainless-steel metabolism cage and the urine collected from each rat in a plastic bottle containing 2 ml of 30% citric acid. The urine was then collected and volumes were measured 24, 48, 72, 96, and 120 hr after administering the MBOCA, and analyzed for the parent compound and <sup>14</sup>C activity.

In Experiment 4 eight rats weighing 273–327 g were gavaged with 12 μg of MBOCA (containing 1951 DPM [<sup>14</sup>C]MBOCA/μg unlabeled MBOCA) per 1 g of body wt. Each rat was individually housed as in Experiment 3, but the urine was collected in plastic bottles (no citric acid added) surrounded by dry ice packed in an insulated container. Both urine and feces were collected 24, 48, 72, and 96 hr after MBOCA administration. During the collection of the urine in these bottles, the tubes from the metabolism cages to the bottles became obstructed by frozen urine. As a result, the urine which was backed up in the tubes was probably not cooled. Immediately after collecting the urine it was thawed, the volumes were measured, and adjusted to pH 3.0 with 30% citric acid solution. Aliquots

were then adjusted to pH 1.0 with N HCl and hydrolyzed at 90°C for 1 hr, and then analyzed for MBOCA. Other aliquots of urine were taken for <sup>14</sup>C analysis. The feces were weighed and aliquots analyzed for <sup>14</sup>C activity.

In Experiment 5 six rats weighing 196–210 g were given 11.7 µg of MBOCA per 1 g of body wt by gavage. The urine was collected as in Experiment 4 except modifications were made to prevent the urine from freezing in the collection tube. Urine was collected 24 and 48 hr after administration and stored in a freezer. Within 2 weeks after collection samples were removed from the freezer (about six samples at a time), thawed overnight in a refrigerator or gently warmed in warm water. Two milliliters of 30% citric acid was then added to each sample. Seven 2-ml aliquots of each urine sample were then diluted to 10 ml with water and processed according to the following scheme. One aliquot was analyzed for MBOCA within 2 hr after dilution. Five aliquots were titrated to pH 1.0 with 10 N HCl, incubated at 38, 50, 60, 70, or 80°C for 30 min immediately after dilution and then analyzed. The seventh aliquot was stored in a refrigerator (5°C) for 72 hr and then analyzed.

#### *Skin Application*

Fifty milligrams of MBOCA was put in a 3-ml glass vial, 1 ml of the stock [<sup>14</sup>C]MBOCA solution was added, and the mixture agitated until all the MBOCA dissolved. A new solution was made 1 hr prior to each experiment. Acetone was used as the vehicle in the skin absorption experiments for two reasons: MBOCA is insoluble in water and therefore unsuitable for quantitative experimentation; acetone is used in industry to clean out the reaction vessels in which MBOCA is synthesized.

Eighteen hours prior to skin application a 6 × 6-cm area on the back of each rat was clipped with an electric clipper with a No. 40 blade. Care was taken to avoid nicking the skin.

Experiment 6 consisted of five groups with six rats in each group. Fifty microliters of the treatment solution containing 2.5 mg of MBOCA and [<sup>14</sup>C]MBOCA ( $2.2 \times 10^6$  dpm) were dropped on the shaved skin of each rat with the aid of a micro pipette. A 50-µl aliquot was also pipetted into a 25-ml flask for <sup>14</sup>C analysis. After allowing 2 min for the solution to dry a 1-inch-square gauze pad with adhesive margins was placed over the site. This was then secured in place with a 3-inch-wide elastic adhesive tape which was wrapped two times around the abdomen and back. They were then placed in metabolism cages. The groups were sacrificed 1, 2, 4, 8, and 24 hr postapplication. The patches were removed and the clipped skin was excised, put on a piece of filter paper, and washed with soap and water and dried. The patch, skin, filter paper, and gauzes used to wash and dry the skin and carcass of each rat were collected for <sup>14</sup>C analyses. The urine in each bladder was aspirated with a needle and syringe and combined with any urine collected in the metabolism cage collection bottle.

Experiment 7 consisted of six groups with six rats in each group. The procedure was the same as in Experiment 6 with the following modifications. At the time of application of MBOCA a 2-cm-diameter circle was made near the center of the shaved skin by pressing the rim of a plastic vial previously inked with black

ink from an ink pad. Fifty microliters of acetone containing 2.5 mg of MBOCA and [ $^{14}\text{C}$ ]MBOCA ( $2.2 \times 10^6$  dpm) was dropped onto the skin in the center of the circle on each rat. Two minutes later a 2-inch-square gauze pad was placed over the surface. This was kept in place by wrapping a 3-inch-wide elastic adhesive over the patch, back, and abdomen of the rat two times. The rats were then put in metabolism cages and the urine and feces collected at 24, 48, and 72 hours. The elastic bandages and gauzes were removed from the rats in the groups at either 1, 2, 4, 8, or 24 hr postadministration. The application area was washed with soap and warm water on a 4-inch-square gauze and wiped dry with another gauze. All the gauzes were saved for  $^{14}\text{C}$  analyses. The skin was then dried with a hair drier held 18–24 inches from the skin for 90 sec. A 2-inch-square gauze was then applied to the area and an elastic adhesive wrapped around as before. The rats were then put back in metabolism cages. In one group the skin was not washed or the bandages removed.

At 72 hr postadministration all rats were sacrificed. The gauze patch was removed and the skin application site with a wide margin was excised from each rat for  $^{14}\text{C}$  analyses. The whole rat was saved for digestion and  $^{14}\text{C}$  analysis. To the skin of each of five control rats 50  $\mu\text{l}$  of acetone only was applied in the same manner as described above. Only the urine from these rats was saved for parent MBOCA analyses 24 hr postadministration. No parent MBOCA was found in any of these control urine samples.

#### SAMPLE PREPARATION FOR $^{14}\text{C}$ ANALYSES

The excised skin was weighed and solubilized in two volumes by weight of a 1 M NaOH methanol–water solution (30:70) at 60°C for 16 hr. Duplicate 0.5-ml aliquots of this were combusted and the  $\text{CO}_2$  was trapped and counted. Duplicate 0.5-ml aliquots of urine were combusted and the trapped  $\text{CO}_2$  was counted.

Feces were ground with approximately two parts by weight of microcrystalline cellulose powder (EM reagents) in a microanalytical mill (Teckmar Industries). Triplicate 0.5-g samples were weighed and combusted.

The gauze patch (that covered the skin application site) was placed in a glass vial and 10 ml of methanol was added. The patch was submerged and allowed to soak overnight or longer. After the solution was thoroughly mixed, duplicate 1-ml samples were counted.

The gauzes that were used to wash and dry the application site on each animal were combined and extracted with 50 ml of methanol overnight or longer. Duplicate 1-ml samples were counted.

Each carcass was digested for 72 hr at 60°C in approximately 3 vol of 1 M NaOH methanol–water (30:70) in a 2-liter glass jar. Triplicate 0.5-ml aliquots were prepared for counting by the method of Weigel *et al.* (1978).

Samples were combusted in a Packard Tri-Carb sample oxidizer, Model B306. The  $\text{CO}_2$  was trapped in Carbo-Sorb and counted in Permafluor V. Combustion times were adjusted to ensure complete combustion. Recovery of  $^{14}\text{C}$  was greater than 99% based upon analyses of standard samples. One standard was analyzed with each batch of 25–50 samples.

All samples were counted in a Beckman Model LS8100 liquid scintillation counter. Samples were corrected for quenching using the external standard method employing the H-number concept. Random samples were internally standardized with  $^{14}\text{C}$  toluene to check the quench correction curve (Horrocks, 1977).

The results were calculated using the following equations.

(a) Counting efficiency:

$$\%CE = 96 + X_1H + X_2H^2 + X_3H^3,$$

where

$\%CE$  = percentage counting efficiency,

96 = nominal counting efficiency of an unquenched standard,

$X_1, X_2, X_3$  = factors obtained by the solution of simultaneous equations from data obtained from a series of quench standards.

(b) dpm/sample aliquot:

$$\text{counted} = 100 \frac{(\text{cpm}_S - \text{cpm}_B)}{\%CE}$$

where

dpm = disintegrations/minute,

$\text{cpm}_S$  = counts per minute of the sample,

$\text{cpm}_B$  = background counts/minute.

$$(c) \text{dpm}_{TS} = \frac{\text{dpms}}{\text{Alq}} \times \text{TSMF}$$

where

$\text{dpm}_{TS}$  = disintegrations/minute of total sample,

dpms = disintegrations/minute of sample counted,

Alq = weight or volume counted,

TSMF = total sample in weight or volume.

$$(d) \% \text{Dose} = \frac{\text{dpm}_{TS} \times 100}{\text{dpm}_D}$$

$\% \text{Dose}$  = percentage of dose given the animal,

$\text{dpm}_{TS}$  = disintegrations/minute in total sample,

$\text{dpm}_D$  = disintegrations/minute in dose given the animal.

#### URINE ANALYSES FOR PARENT MBOCA

Urine samples were analyzed by a method utilizing gas chromatography (NIOSH, 1981). Briefly, 2 or 5 ml of urine from each rat was diluted 1:1 with distilled water. This was then extracted with diethyl ether:hexane (1:1) solution and concentrated to 1 ml. Derivatization was carried out with pentafluoropropionic anhydride. The derivative was extracted with hexane and duplicate 5- $\mu\text{l}$  vol were injected into a gas chromatograph. In most cases the concentration of MBOCA was too low to be detected and required further separation by Florisil. The gas-liquid chromatography was done on a Perkin-Elmer Model 3920 equipped with a nickel-63 electron capture detector and was separated in a glass column packed with 3% OV-1 on Gas Chrom Q. A Perkin-Elmer PEP-II data

TABLE I  
PERCENTAGE OF ADMINISTERED MBOCA IN URINE (MEANS  $\pm$  SD) EXPERIMENT 3

Collection interval (hr)	Parent MBOCA (% $\pm$ SD)	<sup>14</sup> C (% $\pm$ SD)	Parent MBOCA $\div$ <sup>14</sup> C $\times$ 100 $\pm$ SD
0-24	0.225 $\pm$ 0.058	14.30 $\pm$ 2.33	1.59 $\pm$ 0.39
24-48	0.021 $\pm$ 0.013	1.95 $\pm$ 1.22	1.68 $\pm$ 1.61
48-72	0.0007 $\pm$ 0.00017	0.205 $\pm$ 0.128	0.96 $\pm$ 1.51
72-96	ND <sup>a</sup>	0.086 $\pm$ 0.014	—
92-120	ND <sup>a</sup>	0.053 $\pm$ 0.006	—
0-24	0.225 $\pm$ 0.058	14.30 $\pm$ 2.33	—
0-48	0.246 $\pm$ 0.047	16.26 $\pm$ 1.94	—
0-72	0.246 $\pm$ 0.047	16.47 $\pm$ 1.90	1.51 $\pm$ 0.32
0-96	—	16.56 $\pm$ 1.91	—
0-120	—	16.61 $\pm$ 1.91	—

<sup>a</sup> ND = not detectable.

system was used to collect all data. Our detection limit by this method of analysis was 1 ng MBOCA/ml of urine.

## RESULTS

### Gavage Experiments

*Experiment 3.* The micrograms of parent MBOCA found in the urine (expressed in means and ranges) were 5.23  $\mu$ g (2.45–6.89), 0.488  $\mu$ g (0.045–1.05), and 0.018  $\mu$ g (<0.016–0.024) in the 0–24, 24–48, and 48–72 hr intervals, respectively. No parent compound was detected in the urine in any of the rats in the 72–96 and 96–120 hr intervals. The mean values and standard deviations of the percentage of administered compound excreted in the urine as parent MBOCA and as <sup>14</sup>C activity are presented in Table 1. In addition the percentage of labeled compound in the urine excreted as parent MBOCA is also given. Although 14.3% of the administered <sup>14</sup>C label was excreted in the urine in the first 24 hr, only 1.6% of the urinary <sup>14</sup>C label was present as parent MBOCA.

*Experiment 4.* The micrograms of parent MBOCA excreted in the urines in the 0–24, 24–48, 48–72 hr intervals were (means and ranges) 4.45  $\mu$ g (2.56–8.01), 0.345  $\mu$ g (0.05–0.593), and 0.048  $\mu$ g (0.015–0.164). No MBOCA was detectable in any of the urines in the 72 to 96-hr interval. The mean values and standard deviations of the percentage of administered compound excreted in the urine as parent MBOCA and as <sup>14</sup>C activity are presented in Table 2. In addition the percentage of labeled compound in the urine excreted as parent MBOCA is also given. Although 14.8% of the administered <sup>14</sup>C label was excreted in the urine in the first 24 hr, only 0.86% of the urinary <sup>14</sup>C label was present as parent MBOCA.

Data on the fecal excretion are also presented in Table 2. By far the largest percentage of <sup>14</sup>C label was excreted in the feces (53.5% in the first 24 hr). By 96 hr it was apparent that the rate of excretion in both the urine and feces had been reduced to a very low level. In the urine this was 0.098%/day and in the feces 0.55%/day. The total amount excreted (urine and feces) in the 96-hr period was

TABLE 2  
PERCENTAGE ADMINISTERED MBOCA EXCRETED (MEANS  $\pm$  SD) EXPERIMENT 4

Collection interval (hr)	Urine			Feces $^{14}\text{C}$ (% $\pm$ SD)	Urine + feces $^{14}\text{C}$ (% $\pm$ SD)
	Parent MBOCA (% $\pm$ SD)	$^{14}\text{C}$ (% $\pm$ SD)	Parent MBOCA $\div$ $^{14}\text{C} \times 100 \pm$ SD		
0-24	0.128 $\pm$ 0.052	14.80 $\pm$ 1.50	0.86 $\pm$ 0.30	53.5 $\pm$ 4.5	68.3 $\pm$ 4.2
24-48	0.010 $\pm$ 0.007	1.41 $\pm$ 0.31	0.68 $\pm$ 0.40	14.4 $\pm$ 9.3	15.8 $\pm$ 9.5
48-72	0.0014 $\pm$ 0.0016	0.30 $\pm$ 0.10	0.43 $\pm$ 0.36	1.76 $\pm$ 0.34	2.07 $\pm$ 0.38
72-96	ND <sup>a</sup>	0.098 $\pm$ 0.014	—	0.55 $\pm$ 0.08	0.65 $\pm$ 0.08
0-24	0.128 $\pm$ 0.052	14.80 $\pm$ 1.50		53.5 $\pm$ 4.5	68.3 $\pm$ 4.2
0-48	0.138 $\pm$ 0.055	16.21 $\pm$ 1.64		67.9 $\pm$ 6.7	84.1 $\pm$ 7.8
0-72	0.139 $\pm$ 0.055	16.52 $\pm$ 1.68	0.84 $\pm$ 0.28	69.7 $\pm$ 6.7	86.3 $\pm$ 7.9
0-96	—	16.61 $\pm$ 1.69		70.2 $\pm$ 6.7	86.9 $\pm$ 7.9

<sup>a</sup> ND = not detectable.

86.9%. By subtracting from 100%, one can then estimate that 13% was retained and would be excreted very slowly (rate of less than 0.5%/day).

*Experiment 5.* The results of the urine analyses for parent MBOCA appear in Table 3. Because there was insufficient urine, the 60°C incubation was performed on only three samples. These data are not included. Those samples labeled "immediate" are those analyzed within 2 hr of dilution with no incubation. The largest amount of parent MBOCA was recovered in the urine stored for 72 hr in the refrigerator. A comparable amount was found in samples incubated at 80°C for one-half hour. Immediate analysis or incubation at lower temperatures for one-half hour at pH 1.0 resulted in lower values (18-34% lower in the 0- to 24-hr samples). The percentage of administered MBOCA excreted in the urine (in the samples stored in the refrigerator for 72 hr) in the 0-24 and 24-48 hr intervals were 0.184% ( $\pm$  0.037) and 0.026% ( $\pm$  0.015), respectively. The levels at the same intervals in Experiment 3 were 0.225 and 0.021% and in Experiment 4, 0.128 and 0.010%. It would appear that the collection of urine in 2 ml of 30% citric acid at room temperature is the best method for enhancing the detection of parent MBOCA. The amount of time the sample should be left at room temperature, however, was not tested, nor is it known.

The actual urinary concentrations of the parent compound in all three gavage experiments appear in Table 4.

TABLE 3  
MICROGRAMS OF ADMINISTERED MBOCA IN URINE (MEANS  $\pm$  SD) EXPERIMENT 5

Analysis group	0-24 hr ( $\mu\text{g}$ )	24-48 hr ( $\mu\text{g}$ )	0-48 hr ( $\mu\text{g}$ )
Immediate	3.013 $\pm$ 0.645	0.313 $\pm$ 0.302	3.326 $\pm$ 0.495
72 hr	4.318 $\pm$ 0.928	0.612 $\pm$ 0.357	4.931 $\pm$ 1.072
38°C	2.860 $\pm$ 0.544	0.293 $\pm$ 0.184	3.153 $\pm$ 0.593
50°C	3.540 $\pm$ 0.946	0.468 $\pm$ 0.266	4.008 $\pm$ 0.954
70°C	3.332 $\pm$ 0.692	0.609 $\pm$ 0.324	3.941 $\pm$ 0.648
80°C	4.143 $\pm$ 0.903	0.659 $\pm$ 0.330	4.803 $\pm$ 0.977

TABLE 4  
CONCENTRATION OF PARENT MBOCA IN URINE (MEANS  $\pm$  SD)

Collection interval (hr)	Experiment 3 <sup>a</sup>		Experiment 4 <sup>b</sup>		Experiment 5 <sup>c</sup>	
	Urine vol (ml)	MBOCA Conc. ( $\mu$ g/ml)	Urine vol (ml)	MBOCA Conc. ( $\mu$ g/ml)	Urine vol (ml)	MBOCA Conc. ( $\mu$ g/ml)
0-24	14.5 $\pm$ 3.8	0.388 $\pm$ 0.152	19.6 $\pm$ 2.7	0.227 $\pm$ 0.085	17.4 $\pm$ 3.4	0.346 $\pm$ 0.125
24-48	19.5 $\pm$ 6.5	0.027 $\pm$ 0.018	23.9 $\pm$ 8.4	0.017 $\pm$ 0.016	23.8 $\pm$ 4.5	0.030 $\pm$ 0.019
48-72	16.8 $\pm$ 3.9	0.001 $\pm$ 0.002	26.6 $\pm$ 8.8	0.002 $\pm$ 0.003	—	—

Note. See text for discussion of statistical differences.

<sup>a</sup> Collected in 2 ml of 30% citric acid.

<sup>b</sup> Collected without 30% citric acid, then acid hydrolyzed (pH 1.0 for 60 min at 90°C).

<sup>c</sup> Collected frozen, citric acid added, stored at 5°C for 72 hr, and then analyzed.

To determine whether or not there were significant differences between experiments in the percentage of administered parent MBOCA and the percentage of administered <sup>14</sup>C in the urine, the data were analyzed utilizing the repeated-measures multivariate analysis approach (MANOVA) (Winer, 1971). There were no differences between Experiments 3 and 4 in the percentage of applied <sup>14</sup>C found in the urine at 0-24, 24-48, 48-72, or 72-96 hr. However, the MANOVA result for the percentage of administered MBOCA detected in the urine as *parent* MBOCA was significant ( $P < 0.01$ ). The significant difference appears to be due to a difference between Experiments 3 and 4 in the 0- to 24-hr interval and a difference between Experiments 4 and 5 in the 24- to 48-hr interval.

#### Skin Absorption Experiments

*Experiment 6.* In Table 5 are presented the means and standard deviations of the percentage of applied <sup>14</sup>C activity in the urine, feces, skin application site, gauze patch, gauzes used to wash and dry the application site (and filter paper), and the carcass for each group of rats. In addition the amounts found in the urine, feces, and carcass for each rat were totalled and the means derived for each group. This represents the percentage of administered dose that was absorbed. The results were compared between groups utilizing ANOVA with Duncan multiple range tests for significant results. These results are presented in Table 5. Two observations should be made. <sup>14</sup>C was detected in the urine within 1 hr after skin application, and much greater amounts of MBOCA were found in the urine, feces, and carcass at the 24-hr interval than at the other intervals.

The higher amounts of <sup>14</sup>C found in the carcasses in the 1- and 2-hr groups than in the 4-hr group might be a function of technique. The spot for the skin application of MBOCA was not marked on the skin and it is possible that not all the MBOCA-containing skin was excised in some cases. This deficiency was corrected in Experiment 7.

*Experiment 7.* The data from four animals are not included because part of the bandages were chewed off or samples were lost.

Since the entire animal as well as the excretory products and gauzes were analyzed for <sup>14</sup>C activity, it was believed that no or negligible radioactivity was lost, and that any deviation from 100% recovery was probably related to initial

TABLE 5  
 PERCENTAGE OF APPLIED  $^{14}\text{C}$  ACTIVITY (MEANS  $\pm$  SD) EXPERIMENT 6

Group (sacrifice interval) (hr)	Urine	Feces	Carcass	Skin	Filter paper + wash gauzes	Patch gauze	Urine + feces + carcass	Total recovery (% of calc. dose)
1	0.016 $\pm$ 0.005	No samples	4.80 $\pm$ 1.04	7.62 $\pm$ 5.27	47.8	22.7 $\pm$ 6.75	4.81 $\pm$ 1.10	83.0
2	0.073 $\pm$ 0.090	No samples	8.43 $\pm$ 1.41 <sup>b</sup>	10.1 $\pm$ 10.6	46.4	15.6 $\pm$ 6.66	8.50 $\pm$ 1.42 <sup>b</sup>	80.7
4	0.28 $\pm$ 0.12 <sup>a</sup>	No samples	2.90 $\pm$ 0.96	27.9 $\pm$ 13.9 <sup>b</sup>	40.0	10.9 $\pm$ 5.76	3.18 $\pm$ 1.06	81.9
8	0.28 $\pm$ 0.10 <sup>a</sup>	0.41 $\pm$ 0.24	9.03 $\pm$ 1.87 <sup>b</sup>	32.8 $\pm$ 10.3 <sup>b</sup>	42.5	7.90 $\pm$ 3.69	9.71 $\pm$ 1.96 <sup>b</sup>	92.9
24	2.12 $\pm$ 0.37 <sup>c</sup>	1.70 $\pm$ 0.32 <sup>a</sup>	13.5 $\pm$ 4.31 <sup>c</sup>	23.5 $\pm$ 10.2 <sup>b</sup>	31.2	17.7 $\pm$ 5.97	17.33 $\pm$ 4.40 <sup>c</sup>	89.8

<sup>a</sup> Significantly higher than the lowest value in the column.

<sup>b</sup> Significantly higher than the two lowest values in the column.

<sup>c</sup> Significantly higher than the four lowest values in the column.

TABLE 6  
<sup>14</sup>C ANALYSES (PERCENTAGE OF ADMINISTERED DOSE) (MEANS ± SD) EXPERIMENT 7

Wash interval and No. rats/Group	Gauze A	Wash gauze	Gauze B	Skin	Carcass	Urine <sup>f</sup> (0-72 hr)	Feces <sup>f</sup> (0-72 hr)	Urine <sup>f</sup> + feces (0-72 hr)	Urine + feces + carcass (0-72 hr)
1 hr (4 rats)	21.2 ± 8.4	60.1 ± 6.5 <sup>b</sup>	5.05 ± 3.20	2.24 ± 1.35	5.01 ± 0.75	1.72 ± 0.50	4.81 ± 0.92	6.53 ± 1.21	11.5 ± 1.9
2 hr (6 rats)	26.8 ± 12.4	45.3 ± 7.6 <sup>a</sup>	7.51 ± 4.19 <sup>b</sup>	6.73 ± 3.94	5.88 ± 1.05	1.63 ± 0.39	6.25 ± 1.33	7.88 ± 1.68	13.8 ± 1.9
4 hr (4 rats)	29.5 ± 5.7	54.9 <sup>a</sup> ± 6.0	2.74 ± 0.95	1.26 ± 0.74	5.88 ± 0.43	1.20 ± 0.13	4.54 ± 0.71	5.74 ± 0.77	11.6 ± 1.2
8 hr (6 rats)	30.0 ± 14.5	49.4 ± 16.8 <sup>a</sup>	4.33 ± 3.19	4.48 ± 3.28	5.04 ± 0.76	1.66 ± 0.35	5.10 ± 1.19	6.76 ± 1.53	11.8 ± 2.0
24 hr (6 rats)	49.4 <sup>d</sup> ± 6.4	27.3 ± 6.0	2.10 ± 0.87	5.09 ± 6.92	7.91 <sup>d</sup> ± 1.09	2.15 ± 0.83	6.12 ± 1.31	8.27 ± 2.02	16.2 <sup>c</sup> ± 2.8
No wash (6 rats)	47.1 <sup>d</sup> ± 14.9	—	—	31.1 <sup>e</sup> ± 15.0	13.0 <sup>e</sup> ± 3.3	2.54 ± 1.32	6.35 ± 2.44	8.90 ± 3.69	21.9 <sup>e</sup> ± 6.7

<sup>a</sup> Significantly higher than the lowest value in the column.

<sup>b</sup> Significantly higher than the two lowest values in the column.

<sup>c</sup> Significantly higher than the three lowest values in the column.

<sup>d</sup> Significantly higher than the four lowest values in the column.

<sup>e</sup> Significantly higher than the five lowest values in the column.

<sup>f</sup> No significant differences in this column.

TABLE 7  
<sup>14</sup>C ANALYSES, PERCENTAGE ADMINISTERED DOSE EXCRETED (MEANS ± SD) EXPERIMENT 7

Wash interval and No. rats/Group	Urine* (hr)			Feces* (hr)			Urine + feces* (hr)		
	0-24	24-48	48-72	0-24	24-48	48-72	0-24	24-48	48-72
1 hr (4 rats)	0.94 ± 0.32	0.45 ± 0.11	0.34 ± 0.18	1.81 ± 0.13	1.89 ± 0.36	1.11 ± 0.51	2.75 ± 0.36	2.34 ± 0.47	1.45 ± 0.62
2 hr (6 rats)	0.76 ± 0.13	0.49 ± 0.13	0.37 ± 0.17	1.90 ± 0.39	2.60 ± 0.72	1.75 ± 0.44	2.66 ± 0.48	3.09 ± 0.84	2.13 ± 0.58
4 hr (4 rats)	0.68 ± 0.05	0.32 ± 0.16	0.20 ± 0.07	1.56 ± 0.43	2.05 ± 0.64	0.94 ± 0.29	2.23 ± 0.47	2.37 ± 0.66	1.14 ± 0.35
8 hr (6 rats)	0.64 ± 0.13	0.66 ± 0.13	0.37 ± 0.12	1.27 ± 0.46	2.40 ± 0.45	1.43 ± 0.40	1.90 ± 0.52	3.07 ± 0.57	1.80 ± 0.50
24 hr (6 rats)	0.80 ± 0.42	0.86** ± 0.33	0.49 ± 0.24	1.71 ± 0.57	2.29 ± 0.71	2.12** ± 0.73	2.51 ± 0.87	3.15 ± 0.99	2.61** ± 0.92
No wash (6 rats)	0.94 ± 0.53	0.84** ± 0.45	0.77** ± 0.37	1.80 ± 0.99	2.36 ± 0.79	2.18** ± 0.85	2.74 ± 1.42	3.20 ± 1.23	2.95** ± 1.19

\* MANOVA results significant at  $P \leq 0.05$ .

\*\* Based upon univariate analyses. these appear to be the sources of difference found by MANOVA.

pipetting errors in applying the [<sup>14</sup>C]MBOCA. The percentages of <sup>14</sup>C recoveries ranged from 79.9 to 114.3 in individual rats and from 89 to 108.9 in group means. In order to be able to compare groups all individual data were first normalized (to 100% recovery) by multiplying each <sup>14</sup>C percentage of administered dose result by  $100 \div \% \text{ recovery}$  for each rat.

Group means for the samples analyzed for <sup>14</sup>C activity expressed in percentage of administered dose are shown in Tables 6 and 7. For the data in Table 6 a one-way analysis of variance (ANOVA) was used to detect group differences and was followed by a Duncan multiple range test to assess pairwise differences if the ANOVA results were significant. The percentage of administered dose in the carcass was significantly higher in the no-wash and the 24-hr-wash groups compared to the other groups ( $P \leq 0.05$ ). When the percentages of administered dose found in the urine + feces + carcass were combined in each group, the no-wash group was found to be significantly higher than all other groups and the 24-hr-wash group was significantly higher than the 1-, 4-, and 8-hr-wash groups ( $P \leq 0.05$ ).

As expected, the skin of the rats in the no-wash group contained significantly higher amounts of <sup>14</sup>C than the skin of rats in the other groups, but there were no significant differences between any of the other groups. Gauze A contained more than <sup>14</sup>C in the 24-hr and no-wash groups than in the other groups ( $P \leq 0.05$ ).

Table 7 shows results of MANOVA analysis using repeated measures, and subsequent univariate analyses of <sup>14</sup>C analyses of percentage administered dose excreted.

The amount of parent MBOCA detected in urine and the percentage of the applied dose it represents are presented in Table 8. There were no significant differences between groups at any interval. Although there appeared to be a trend toward a decreasing urinary excretion with time, this was not very dramatic and at the 48- to 72-hr interval the group urines contained between 53 and 100% of

TABLE 8  
URINE ANALYSIS FOR PARENT MBOCA AT SPECIFIED INTERVALS (MEANS  $\pm$  SD) EXPERIMENT 7

Wash interval and No. rats/Group	0-24 hr		24-48 hr		48-72 hr		0-72 hr	
	ng	% Dose $\times$ 1000	ng	% Dose $\times$ 1000	ng	% Dose $\times$ 1000	ng	% Dose $\times$ 1000
1 hr (4 rats)	60.8 $\pm$ 14.9	2.43 $\pm$ 0.60	48.0 $\pm$ 43.5	1.92 $\pm$ 1.74	40.3 $\pm$ 30.7	1.61 $\pm$ 1.23	149 $\pm$ 63	5.96 $\pm$ 2.93
2 hr (6 rats)	74.4 $\pm$ 24.0	2.98 $\pm$ 0.96	39.8 $\pm$ 14.8	1.59 $\pm$ 0.59	57.6 $\pm$ 35.7	2.30 $\pm$ 1.43	179 $\pm$ 34	6.87 $\pm$ 1.69
4 hr (4 rats)	65.2 $\pm$ 47.7	2.61 $\pm$ 1.91	49.9 $\pm$ 34.9	2.00 $\pm$ 1.40	64.9 $\pm$ 61.6	2.60 $\pm$ 2.46	180 $\pm$ 66	7.20 $\pm$ 3.05
8 hr (6 rats)	101 $\pm$ 60	4.05 $\pm$ 2.39	69.7 $\pm$ 49.1	2.79 $\pm$ 1.96	60.5 $\pm$ 38.6	2.42 $\pm$ 1.55	231 $\pm$ 94	9.25 $\pm$ 3.77
24 hr (6 rats)	100 $\pm$ 77	4.02 $\pm$ 3.11	55.6 $\pm$ 23.6	2.23 $\pm$ 0.95	52.7 $\pm$ 30.2	2.11 $\pm$ 1.21	202 $\pm$ 63	8.35 $\pm$ 2.66
No wash (6 rats)	92.0 $\pm$ 36.7	3.68 $\pm$ 1.47	61.1 $\pm$ 27.2	2.45 $\pm$ 1.09	66.4 <sup>a</sup> $\pm$ 43.1	2.65 $\pm$ 1.72	209 $\pm$ 62	8.35 $\pm$ 2.78

<sup>a</sup> Only five samples at this interval.

the amount they contained at the 0- to 24-hr interval. It should be noted that the urines collected over any 24-hr period contained only 0.0016 to 0.0045% (group means) of the administered compound, i.e., the rats were exposed to 25,000 to 63,000 times more MBOCA than could be detected in the urine in any 24-hr period.

To further obtain an idea of how indicative the amount of parent MBOCA in the urine is for the extent of exposure, calculations were made to determine the percentage of the *absorbed dose* that appeared in the urine as parent MBOCA and as <sup>14</sup>C compounds over the 72-hr period. The absorbed dose can be reliably determined from the amount of <sup>14</sup>C activity found in the carcass, urine, and feces from 0- to 72-hr (see Table 6). The results are presented in Table 9. Even though 10.3 to 15.0% (group means) of the absorbed MBOCA was excreted in the urine, only 0.038 to 0.078% (group means) of the absorbed dose was excreted as parent

TABLE 9  
PERCENTAGE ABSORBED DOSE EXCRETED (URINE + FECES) AND RETAINED (CARCASS)  
(0-72 hr; MEANS) EXPERIMENT 7

Wash interval (hr)	Urine		Feces <sup>14</sup> C (%)	Urine + feces <sup>14</sup> C (%)	Carcass (% Retained) (72 hr)
	<sup>14</sup> C (%)	Parent MBOCA (%)			
1	15.0	0.052	41.8	56.8	43.6
2	11.8	0.050	45.3	57.1	42.6
4	10.3	0.062	39.1	49.5	50.7
8	14.1	0.078	43.2	57.3	42.7
24	13.3	0.052	37.8	51.0	48.8
No wash	11.6	0.038	29.0	40.6	59.4

MBOCA. It should also be noted that 42.6 to 59.4% (group means) of the absorbed dose was still present in the carcass 72 hr after administration.

## DISCUSSION

In 1983 Farmer *et al.* reported results of experiments in which he gave MBOCA by gavage to two rats. They found that 83.25% of the dose was excreted within 48 hr after administration (23.75% in urine, 59.5% in feces). This compares favorably with our results in Experiment 4 with eight rats, where 84.1% of the administered dose was excreted in the first 48 hr (16.2% in the urine and 67.9% in the feces). However, in the next 2 days the rate of excretion decreased rapidly so that on the fourth day only 0.65% of the dose was excreted. We estimated that at the end of the fourth day 13.1% of the dose was retained and that the rate of excretion after that would be  $\leq 0.65\%/day$ . We conclude that when MBOCA is given by gavage there is evidence for long-term retention of [ $^{14}C$ ]MBOCA and its metabolites in rats.

Manis *et al.* (1982) determined the rate of excretion of [ $^{14}C$ ]MBOCA after skin application in dogs. They found that 1.3% of the administered dose was excreted in the urine in the first 24 hr. This compares favorably with the results we obtained in Experiment 7 where 0.80 and 0.94% of the dose (24-hr-wash and no-wash groups, respectively) were excreted in the urine within 24 hr after skin application. In Experiment 6 where we aspirated urine from the bladders of the rats and combined it with the urine from the collection bottles we obtained a higher percentage (2.12% in the 24-hr group). Manis *et al.* (1982) also measured the rate of excretion of the parent compound. They found that 0.0052% of the administered dose was excreted within 24 hr as the parent compound. We found that 0.0040 and 0.0037% (24-hr-wash and no-wash groups, respectively, in Experiment 7) of the administered dose was excreted as the parent compound.

As with the gavage experiments, we also found evidence for long-term retention of MBOCA and its metabolites after skin application. Whereas, 11.5 to 21.9% of the applied dose had been absorbed (Experiment 7, all groups) within 72 hr after application, only 5.74 to 8.9% had been excreted, and 42.6 to 59.4% of the *absorbed* dose was still present in the animal 72 hr after application (Table 9).

There were markedly different patterns for rates of excretion (urine + feces) between the gavage and skin application experiments. The rate in the gavage groups was very high initially and by the third day the rate (2.07%/day) had fallen below that of some of the skin absorption groups (e.g., 2.13, 2.61, and 2.95%). Also, the rates of excretion in the skin absorption groups were fairly constant over a 3-day period, being 1.90–2.75% on Day 1 and 1.14–2.95% on Day 3. The higher rates of excretion in the no-wash and 24-hr-wash groups on the third day were probably because more of the MBOCA was absorbed than in those groups that were washed at earlier intervals.

It is also of some interest to note that within 1 hr after administration 4.8% of the dose had been absorbed and 0.016% of the dose was present in the urine. Similarly, Manis *et al.* (1982) found in dogs that within 2 hr 0.02% of the dose had been excreted in the urine.

TABLE 10  
 PERCENTAGE OF  $^{14}\text{C}$  IN URINE PRESENT AS PARENT MBOCA (MEANS  $\pm$  SD)

Collection interval (hr)	Gavage (Expt. 3)	Skin absorption					
		1-hr Wash	2-hr Wash	4-hr Wash	8-hr Wash	24-hr Wash	No wash
0-24	1.59 $\pm$ 0.39	0.29 $\pm$ 0.13	0.40 $\pm$ 0.17	0.38 $\pm$ 0.27	0.74 $\pm$ 0.70	0.56 $\pm$ 0.55	0.49 $\pm$ 0.30
24-48	1.68 $\pm$ 1.61	0.38 $\pm$ 0.30	0.33 $\pm$ 0.09	0.70 $\pm$ 0.35	0.43 $\pm$ 0.28	0.31 $\pm$ 0.21	0.31 $\pm$ 0.10
48-72	0.96 $\pm$ 1.51	0.56 $\pm$ 0.41	0.59 $\pm$ 0.21	1.20 $\pm$ 0.80	0.75 $\pm$ 0.74	0.55 $\pm$ 0.53	0.40 $\pm$ 0.22

We found that washing MBOCA off the skin between 1 and 8 hr postadministration did reduce the amount of MBOCA absorbed in those rats compared to those whose skin was not washed or was washed 24 hr after application. However, washing had no significant effect on the amount excreted in the 0-24 and 24-48 hr periods.

It is apparent from these studies that a significant amount of MBOCA dissolved in acetone is absorbed from the skin in a relatively short period of time, and that the rate of absorption in the first hour is much higher than the rates in succeeding hours. This effect can probably be explained on the basis that acetone permits rapid penetration of the MBOCA into the vascularized tissue, and that after the acetone on the surface evaporates, MBOCA is absorbed at a much slower but still significant rate. It would be informative to study the rate of absorption of the dry compound.

Can the analysis for parent MBOCA in urine be used as an accurate indicator of extent of exposure? In our experiments the amount of MBOCA given by gavage and the amount placed on the skin were about the same (2.5 mg). The percentage of administered dose present as parent MBOCA in the urine in the first 24 hr was 0.225% by gavage and 0.004% by skin absorption. It can be estimated that 50 mg applied to the skin would result in the same urinary concentration of parent MBOCA as 1 mg taken orally. Therefore, the analysis for parent MBOCA in urine cannot be used as an accurate indicator of extent of exposure, unless the route of exposure is known.

If the urinary concentration of MBOCA and its metabolites is the only consideration, we are in a slightly better position to estimate the risk. In the skin absorption experiments the percentage of  $^{14}\text{C}$  in urine present as parent MBOCA (Table 10) varied between 0.29 and 0.74 (all groups) in the first 24-hr period. In the gavage Experiment 6 this was 1.59%. To determine the amount of MBOCA and metabolites in the urine after skin application one must multiply the amount of parent MBOCA detected by a number between 158 and 387. To determine the same for the gavage study the number is 64.

Does the urinary concentration of parent MBOCA indicate the amount in the tissues? Comparable amounts of administered MBOCA were retained in the tissues 72 hr after gavage (13.7%) and skin application (5.01-13.0%). The amount

of parent compound excreted in the gavage experiment in the 0- to 72-hr period was 0.139%, whereas in the skin absorption experiments it was 0.006–0.009%. Also, 96 hr after gavage, when 13.1% of the dose was retained, *no* parent compound could be detected in the urine. Therefore, without knowing the route of exposure and the exact time of exposure, the urinary concentration of MBOCA cannot be used to estimate tissue concentration.

The concentration of parent compound found in the acid-hydrolyzed, 24-hr urines of rats fed 125 ppm MBOCA in the diet for 75 weeks was 0.63  $\mu\text{g}$  MBOCA/ml (Kommineni *et al.*, 1979). This is approximately 8.38  $\mu\text{g}$  of MBOCA excreted in 24 hr (mean urine volume  $\times$  mean MBOCA urine concentration) and represents an estimated 0.342% of the amount of MBOCA consumed in the 24-hr period prior to urine collection. In Experiment 3 reported here the amount of parent compound excreted in urine in the same interval was 0.225% of the dose. The amount of MBOCA excreted in the urine in the 24-hr period after ceasing administration in these two different experiments are remarkably similar. This is rather remarkable considering the animals in the feeding study had been ingesting MBOCA daily for 75 weeks. Apparently MBOCA is rapidly metabolized even after 75 weeks of exposure.

For these reasons we conclude that the urinary analysis for MBOCA can be used only as a very imprecise indicator of extent of *recent* exposure, and cannot be used at all as an indicator of exposures that occurred days or weeks prior to sampling.

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