Environmental Health Perspectives
Vol. 45, pp. 103-109, 1982

Assessment of the Mutagenicity of Phthalate Esters

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The Ames assay was used to investigate the mutagenicity of several phthalate esters as an approximation of their carcinogenic potential. The *ortho* diesters, dimethyl phthalate (DMP) and diethyl phthalate (DEP) produced a positive dose-related mutagenic response with Salmonella TA100, but only in the absence of S-9 liver enzymes. Dibutyl, di(2-ethylhexyl), mono(2-ethylhexyl), and butyl benzyl phthalate as well as the dimethyl isophthalate and terephthalates and the trimethyl ester, trimellitate, were not mutagenic with TA100 or TA98 in the presence or absence of S-9. In a host-mediated assay, extracts of 24-hr urines of rats injected IP with DMP (2 g/kg) were not mutagenic to TA100 at levels up to 8 equivalent-ml of urine/plate (representing 30% of their daily urinary output). *In vitro* studies revealed that S-9 associated esterase hydrolyzed DMP to the monoester and methanol and eliminated its mutagenicity. Whole rat skin was shown to have about 1.5% of the DMP-esterase activity of liver, when compared on a wet weight basis. An *in vitro* binding study indicated that epidermal macromolecules bound DMP at a severalfold greater rate than hepatic macromolecules. Thus, both the mutagenicity and binding of DMP are inversely related to the metabolism of this compound. These results suggest that skin could be at high risk for a mutagenic/carcinogenic insult.

Introduction

Since there are scant data on the mutagenic and carcinogenic potential of a variety of phthalate esters, the Ames assay was used to investigate the mutagenicity of some of these compounds as an approximation of their carcinogenic potential. This paper presents evidence for the mutagenesis of dimethyl phthalate (DMP) and diethyl phthalate (DEP), along with additional studies on the metabolism of DMP and its binding to macromolecules.

DMP is a widely used insect repellent which is available to the general public as an active ingredient in commercial products. In its pure form it is used by agricultural workers in doses up to 100 mg/kg applied directly to the skin (*1*) and by the U.S. military in tropical regions of the world. Considering the many people exposed to DMP as well as the potentially large doses to which they may be exposed, further research on the mutagenic effects of this chemical is well justified.

A literature search in the area of mutagenicity of DMP revealed relatively few studies. One study by Pilinskaya et. al. (*2*) reported no increase in chromosomal aberrations in bone marrow cells of white mice administered DMP intraperitoneally (dose not cited). Another study by Tsuchiya and Hattori (*3*) also reported no increase in chromosomal damage in human leukocytes treated in culture with 20 μg/ml of DMP. On the other hand, Yurchenko (*4*) did report a significant increase in chromosomal aberrations in hepatocytes from rats treated topically with 1250 mg/kg DMP five times per week for one month. Rats administered only a single topical dose displayed no significant increase in chromosomal aberrations. Thus, DMP's topical use as an insect repellent and the observation of hepatic chromosomal damage would suggest skin and liver, respectively, as potential sites for mutagenic/carcinogenic effects.

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Methods

Mutagenesis

The standard Ames bacterial mutagenicity assay (*5*) was used with several modifications as described by Batzinger et al. (*6*). The major modifications included shifting the histidine and biotin (at concentrations of 50 and 15 g/plate, respectively) from the top to the bottom agar, and reducing the glucose to 67.5 mg/plate in the bottom agar. Each phthalate ester was tested with *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of S-9 liver microsomal fraction from male Sprague-Dawley rats induced with Aroclor 1254.

Host-Mediated Assay

Male Sprague-Dawley rats (approximately 350 g) were injected IP with 2 g/kg of DMP and 24-hr urine was collected. The urine from 12 animals was pooled. Five such pools were collected. The urine (not further acidified) was extracted once with *n*-butanol which was then evaporated. The residue was dissolved in DMSO and tested in the Ames assay. Aliquots representing various equivalent volumes of urine from each pool were tested.

Chemical Analysis of Urine

The 24-hr pooled urine from a group of 12 rats (approximately 350 g) was acidified to pH 1-2 with HCl and then exhaustively extracted with *n*-butanol until no UV-absorbing phthalate moieties could be detected in the urine by direct thin-layer chromatographic (TLC) analysis. The butanol extracts were combined and examined by TLC. The UV absorbant spots corresponding to the R_f values of monomethyl phthalate (MMP) and DMP standards were scraped from the plates and extracted with methanol. The spectrophotometric absorbance of the methanol extracts at 275 nm was used to determine the amount of phthalate present. No other UV-absorbing spots were seen on the TLC plates.

Preparation, Incubation and Treatment of Skin Slices

The backs of male Sprague-Dawley rats between 300 and 400 g were shaved 24 hr prior to sacrifice by cervical dislocation. Dorsal skin was removed immediately after sacrifice and the fat and loose connective tissue adhering to the hypodermis was scraped away with a scalpel. The skin, now consisting of dermis and epidermis, was sliced, weighed and placed into phosphate buffered saline (PBS), pH 7.4, at a ratio of 1 to 5 (w/v). DMP was added to a final concentration of 1.5 mg/ml. The incubation mixture was shaken at 37°C for various time periods up to 2 hr before the reaction was terminated with the addition of sufficient 1.0*N* HCl to reduce the pH to approximately 1.5. The acidified medium was decanted and the skin slices were washed several times with PBS while maintaining the pH at 1-2. The washes were pooled with the original medium and the total volume was extracted with chloroform until no phthalate moieties could be detected by direct TLC analysis of the aqueous pooled fraction. In addition, the skin slices were also rinsed several times with chloroform. The chloroform extracts of both the skin slices and aqueous medium were pooled and centrifuged at 1000*g* for 10 min. The supernatant was then fractionated by TLC and the content of MMP and DMP determined as above.

Preparation, Incubation and Treatment of Liver S-9

A 0.15 ml aliquot of DMP (4000 μg/ml water) was added to 2 ml of E medium plus 0.5 ml of S-9 mix (*5*). The mixture was shaken at 37°C for various time periods up to 1 hr. The reaction was terminated by the addition of 0.4 ml of 1.0 *N* HCl. After centrifugation at 1000 *g* for 10 min, 2 ml of the acidified supernatant (pH 1.5) was extracted twice with chloroform.

Chemical Analysis of *In Vitro* Skin and Liver Extracts

The rate of *in vitro* metabolism of DMP to MMP and methanol was measured by the formation of MMP. The chloroform extracts from the liver S-9 and the skin incubations were analyzed for MMP by the TLC-spectrophotometric method described in the above section on chemical analysis of urine. The rate of metabolism was determined over the linear range of metabolism for each tissue.

In addition, the *in vitro* metabolism of DMP to methanol by skin slices was investigated using the basic method of Wood and Siddiqui (*7*). A slight modification of the method allowed for simultaneous determination of formaldehyde formation. This was accomplished by measuring the amount of formaldehyde formed in the presence and absence of $KMnO_4$, which is used to convert methanol to formaldehyde. In the presence of $KMnO_4$, the total of methanol plus formaldehyde is measured. In the absence of $KMnO_4$, only the presence of formaldehyde is detected. The difference between the two procedures yields the amount of methanol formed.

Preparation of Epidermal and Hepatic Homogenates

The homogenized epidermis was prepared by modifications of previously described techniques (*8*). After the removal of the entire dorsal skin as described above, it was immersed in distilled water at 50°C for 30 sec and then immediately transferred to iced water for another half minute. The epidermal layer was then scraped off with a scalpel, suspended in 2-3 ml of ice cold PBS (pH 7.4) and homogenized. The homogenate was then strained through a fine nylon mesh.

A rat was decapitated and following exsanguination the *in situ* liver was perfused with 30-40 ml of PBS (pH 7.4) via the hepatoportal vein. The liver was then removed and homogenized in PBS at a ratio of 1 to 3 (w/v). The homogenate was strained through fine nylon mesh.

A 1-ml aliquot of each of epidermal and liver homogenates was mixed with 1 ml of ^{14}C-carbonyl-labeled DMP solution (3.0 mg/ml of DMP in PBS), representing from 1.4×10^5 to 2.0×10^6 dpm per vial. The incubation mixtures were shaken at 37°C for 2 hr and the reaction terminated and the macromolecules precipitated with the addition of 4.0 ml of 0.9*M* trichloroacetic acid (TCA).

Determination of Macromolecular Binding

To determine macromolecular binding, the method of Jollow et al. (*9*) was employed. Successive washings with TCA, 80% ethanol and acetone were continued until the 1 ml aliquots of the wash solution (dissolved in ACS Aquasol scintillation fluid) produced counts equivalent to background on a Packard Tricarb liquid scintillation spectrometer.

The final acetone washed TCA precipitate was taken to dryness and weighed to determine the mg of dry weight. Finally, the macromolecular residue was solubilized in NCS tissue solubilizer (Amersham) for counting.

Results

The results from the modified Ames mutagenic assay of phthalic acid (PA), DMP and DEP in TA100 in the absence of S-9 are shown in Table 1. Hycanthone was used as the positive control; it consistently produced a response of greater than 600 net revertants at a concentration of 30 μg/plate. PA, the de-esterified product of phthalate esters, proved negative in this assay. However, both DMP and DEP registered a positive and dose-related response. At 1000 μg the mutagenic response rep-

Table 1. Mutagenesis assay of PA, DMP and DEP in *Salmonella typhimurium* TA100.

Test compound	μg/plate	Revertants per treated plate / Revertants per control plate ±SEM[a]
Hycanthone	30	3.49 ± 0.14
PA	100	1.11 ± 0.07
	300	0.95 ± 0.02
	1000	0.90 ± 0.07
DMP	100	0.82 ± 0.03
	500	1.45 ± 0.10[b]
	1000	1.84 ± 0.12[b]
DEP	100	1.06 ± 0.05
	500	1.26 ± 0.09[b]
	1000	1.90 ± 0.09[b]

[a]The control or background reversion frequency was 291 ± 20 (n = 14). Each value represents the mean from 3-5 replicate experiments. In each experiment duplicate plates were run for each condition.

[b]$p < 0.05$ by Student's t-test when tested for difference from 1.00.

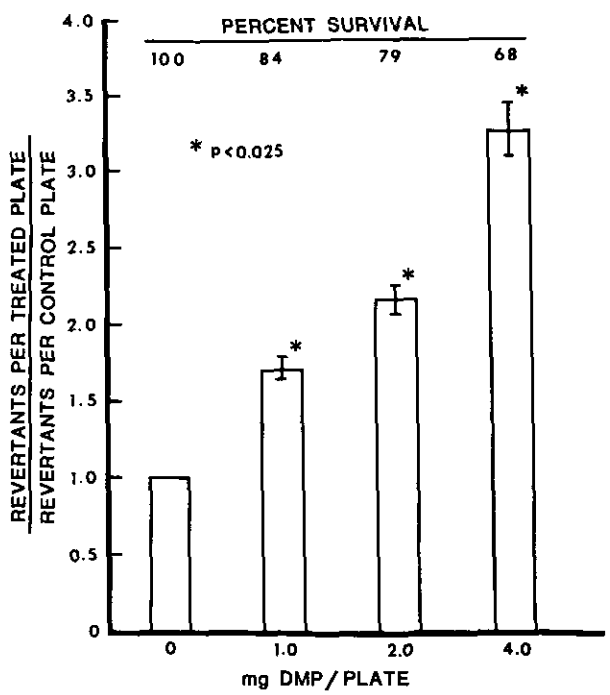


FIGURE 1. Mutagenic dose response for DMP. Each mutagenic value represents a mean from two or more replicate experiments in which duplicate plates (histidine-deficient) were used at each concentration. Percent survival was determined in the following way: known dilutions of TA100 were plated on the nutrient agar (histidine-containing) either in the absence of DMP (control) or in the presence of known amounts of DMP (treated). After 48 hr of incubation, numbers of surviving mutant colonies on the treated plates were compared to control. The asterisk denotes $p < 0.025$ by Student's t-test when tested for differences from 1.0.

Table 2. Effect of S-9 supplementation on the Ames assay of DMP and DEP in TA100.

		Revertants per treated plate / Revertant per control plate ± SEM[a]			
			With S9		
Test compound	μg/plate	No S-9	+NADP	+NAD	No cofactors
Lucanthone	30	1.04 ± 0.04	2.34 ± 0.13	–	–
DMP	1000	1.72 ± 0.14[b]	1.00 ± 0.04	1.03 ± 0.04	1.11 ± 0.04
DEP	1000	1.58 ± 0.21[b]	0.76 ± 0.07	0.69 ± 0.08	0.77 ± 0.08

[a]The control or background reversion frequency was 253 ± 16 (N = 11) in the absence of S-9 and 209 ± 10 in the presence of S-9.
[b]p 0.05 by Student's t-test when tested for difference from 1.00.

resented essentially a doubling of the basal level of reversion. These three compounds were not mutagenic when tested with the TA98 strain of Salmonella (data not shown).

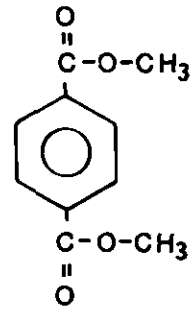
The effect of S-9 liver extract on DMP- and DEP-induced mutations is presented in Table 2. Lucanthone (Luc) was used as the positive control to demonstrate the effectiveness of the S-9 fraction in activating a nondirect-acting mutagen. In the absence of S-9, DMP and DEP produced a positive mutagenic response, confirming the data presented in Table 1. With S-9 and NAD or NADP added to the system, the positive response was reversed. It is important to note that S-9 alone (without the cofactors) also eliminated the mutagenic response.

A number of other phthalate esters were tested in our modified Ames assay. Each phthalate was assayed with bacterial strains TA100 and TA98, both in the presence and absence of S-9 liver extract. All of these phthalate esters failed to produce a positive response at concentrations up to 1000 μg/plate. The compounds tested were: di-n-butyl phthalate, di(2-ethylhexyl) phthalate, mono(2-ethylhexyl) phthalate and butyl benzyl phthalate (data not shown).

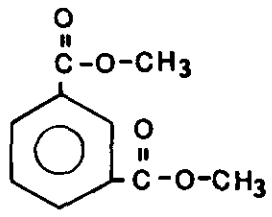
Figure 1 shows the mutagenic dose response for DMP extended over a broader range of concentrations, i.e., from 1 to 4 mg/plate. A positive dose-related mutagenic response was associated with a dose-related decrease in survival of the cells of the TA100 tester strain. If the absolute number of revertants per plate were corrected for percent survival, an even greater mutagenic response would have been seen than the 3- to 3.5-fold response at 4 mg/plate.

The structure–activity relationship (SAR) of DMP was investigated by testing three other methyl phthalates in the Ames assay with TA100 and TA98, either in the absence or presence of S-9 activation. Dimethyl terephthalate (I), dimethyl isophthalate (II) and trimethyl trimellitate (III) were all negative in the mutagenesis assay.

The results of a host-mediated mutagenic assay of DMP in rats are presented in Figure 2. There were no statistically significant differences in the number of revertants per plate for control or treated



I



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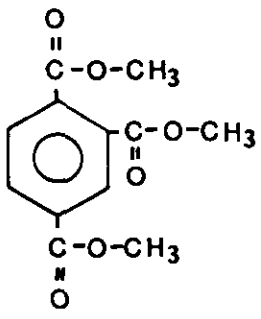

III

urines (over a range of 1 to 8 ml urine) when compared to blank plates. A volume of 8 ml of urine represents approximately 50% of the daily output of control rats and about 30% of the daily output of treated rats. This difference in the fraction of the total daily urinary output represented by a given volume of the two urines was due to a marked diuresis in the DMP-treated rats.

The lack of a mutagenic response to extracts equivalent to up to 8 ml of treated urine (approximately 30% the daily output) led us to assay the extracts for DMP and other phthalic acid-containing metabolites. These extracts were found to have 0.06 mg/ml of DMP and 1.90 mg/ml of the mono-de-esterified product, monomethyl phthalate (MMP). No other phthalic acid-containing derivatives were found. Thus the mutagenesis plates containing the equivalent of 8 ml of treated urine would have contained 480 μg of DMP and 15.2 mg of MMP. Previous experiments (data not shown) had shown that MMP was not mutagenic in TA100 at levels up to 24 mg per plate.

Since the urinary extracts used in the host-mediated assay were obtained by a single *n*-butanol extraction of urine that was not further acidified beyond its native pH (see methods), further experiments were conducted to determine the phthalic acid-containing components in urine of treated rats using more exhaustive extractions from samples that were acidified to pH 1-2 with HCl. The data are shown in Table 3. DMP and MMP were found at levels of 0.14 and 15.70 mg/ml, representing 0.6 and 67.0% of the injected dose, respectively. Once again, no other phthalic acid-containing derivatives were detected. Thus, DMP represented less than 1% of all phthalic acid-containing molecules excreted in 24 hr.

Although these results suggest systemic inactivation of the mutagenic activity of DMP, it should be recalled that the major route of human exposure is topical. Therefore, the ability of rat skin to metabolize DMP to MMP and thus inactivate the diester was investigated. The results from three separate experiments performed to study the *in vitro* metabolism of DMP by rat skin are presented in Table 4. In the first experiment, only MMP formation was assayed; it was found to be 3.47 nmole/hr/mg tissue. Since the de-esterification of DMP should also result in the formation of methanol, the rate for this product was measured in a second experiment. It was found to be 2.58 nmole/hr/mg tissue. In a third experiment the stoichio-

Table 3. DMP and MMP in the 24-hr urine of rats injected IP with DMP at a dose of 2 g/kg.

Compound	Ester in urine, mg/ml	% of injected dose
MMP	15.70	67.0
DMP	0.14	0.6

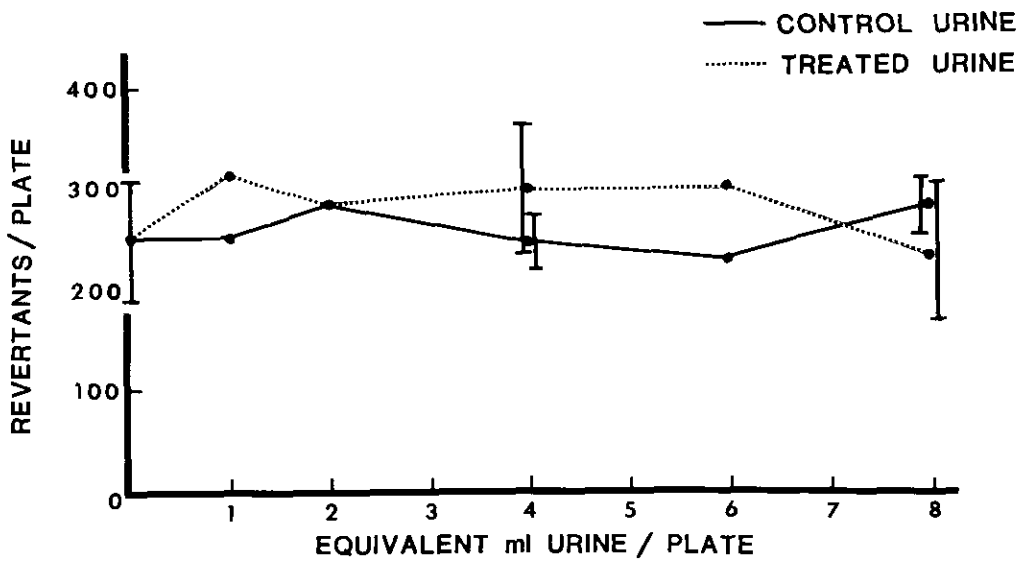

FIGURE 2. Host-mediated mutagenic assay of DMP in rats. Blank plates contained no urine and the data for these are shown on the *y*-axis. No toxicity (as measured by percent survival on nutrient agar) was seen in TA100 with extracts of either control or treated urine at levels up to 8.0 ml. Data points without standard error bars are the means of values from two independent experiments. Those with standard error bars represent an *n* of 3 or more. Student's *t*-test for these values showed no statistical significance when compared to the data from the blank plates.

Table 4. *In vitro* metabolism of DMP by whole slices of rat skin.[a]

Dimethyl Phthalate → Monomethyl Phthalate + Methanol
(DMP) (MMP)

Exp. no.	*MMP formed,* nmole/hr/mg tissue (wet)	*MeOH formed,* nmole/hr/mg tissue (wet)	*HCHO formed,* nmole/hr/mg tissue (wet)
1	3.47	—	—
2	—	2.58	—
3	2.16	2.18	0

[a]*In vitro* rate of metabolism of DMP by rat liver S-9: 165 *nmole MMP formed/hr/mg liver (wet).*

Table 5. *In vitro* binding of DMP.[a]

	DMP bound, nmole/mg tissue (TCA precipitable dry weight) $\bar{X} \pm$ SEM
Liver	0.17 ± 0.01
Epidermis	*0.98 ± 0.19*[b]

[a]Each value represents the mean of four separate assays.
[b]*p* 0.025 by Student *t*-test when tested for difference from 0.17 (mean binding value of CMP for liver homogenate).

metric relationship between MMP and methanol was examined and found to be 1:1. The rate of formaldehyde formation was also investigated in this latter experiment, but none of this product could be detected. It should be noted that the rate of de-esterification of DMP by whole skin is approximately 1.5% of the rate seen in the liver, when compared on a mg wet weight basis.

Given the markedly lower rate of metabolism of DMP to a nonmutagenic metabolite in skin relative to liver, the *in vitro* binding of DMP to tissue macromolecules in these two tissues was investigated. The results are shown in Table 5. ^{14}C-Carbo nyl-labeled DMP was found to bind to hepatic and epidermal macromolecules at levels of 0.17 and 0.98 nmole/mg tissue (dry weight-TCA precipitable), respectively.

Discussion and Conclusion

The positive dose-related mutagenic response of TA100 to DMP and DEP (Table 1) in the Ames assay was shown to be affected or modified by two factors. One of these is the molecular structure of DMP, as revealed by the SAR study on the effect of position and number of methyl ester moieties on the benzene ring. The failure of the *meta* and *para* dimethyl esters to elicit a positive response in the Ames assay emphasizes the importance of the *ortho* configuration. The addition of a third methyl ester to the 4-position of DMP and resultant elimination of the mutagenic response indicates the necessity for the *ortho* diester arrangement. However, testing of the 1,2,3-trimethyl phthalate is necessary before any conclusive statements can be made in reference to the mutagenic potential of trimethyl esters. Another SAR-related effect is important to note. From the series of phthalate esters tested only the shorter chained and more hydrophilic of the aliphatic phthalate diesters, DMP and DEP, are mutagenic in the bacterial assay.

The elimination of the positive mutagenic response by S-9 liver microsomes (Table 2) implicates metabolism as a second important modifying factor. Lack of involvement of the cofactors NAD and NADP indicates that the classical mixed-function oxidase (MFO) system is not involved in the apparent metabolic elimination of the mutagenic response. Liver S-9 (Table 4) readily metabolizes DMP to MMP, which has been shown to be a nonmutagenic compound in the Ames assay. These results suggest that a S-9 associated esterase activity is responsible for the elimination of the mutagenic effect. Results from the host-mediated mutagenic assay (Fig. 2) support this idea. In this assay approximately 97% of the phthalate ester content of the urine extract is MMP. Thus, the lack of a positive mutagenic response along with the preponderance of monoester indicate the importance of DMP-monoesterase activity in eliminating the mutagenic effect and possibly in affording protection to tissue exposed to DMP.

It should be noted that 8 equivalent ml of treated urine are negative in the mutagenic assay in spite of containing approximately 480 μg of DMP, an amount which results in a statistically significant increase, albeit small, in the mutation rate in the direct assay. It is possible that an interaction with other components of the urine extract might have suppressed the mutagenic response to DMP in the Ames assay. For example, in the extract of 8 ml of urine there was a 33-fold greater level of MMP relative to DMP. This preponderance of monoester could have affected the tester organism, interfering with or eliminating its mutagenic response to DMP.

The *in vitro* ability of skin and liver to metabolize DMP was investigated because of the demonstrated importance of metabolism in determining the mutagenic effect of DMP and the recognized fact that these two tissues represent potential mutagenic/carcinogenic sites for this compound. When the rates of metabolism of DMP were compared on a wet weight basis, liver had a 60-fold greater rate than whole skin. This marked metabolic difference highlights liver's enhanced capacity to metabolize DMP to a nonmutagenic phthalate form and thus to be protected from a potential mutagenic effect. Conversely, skin, perhaps the only organ exposed

to the entire body dose of DMP, may have little inherent metabolic protection against the mutagenic effects of DMP.

In considering the above along with the known ability of many mutagens to bind macromolecules, an investigation of *in vitro* macromolecular binding of ^{14}C-labeled DMP was compared in liver and skin. The results from the binding experiments indicate that DMP binds to epidermal macromolecules at a severalfold greater rate than to liver macromolecules. Thus, DMP's lower rate of de-esterification in the skin relative to liver was related inversely to the higher rate of DMP's binding in the epidermis.

In spite of the substantial hepatic metabolism of DMP by rats, the only positive mammalian mutagenic response to this compound was observed by Yurchenko (*4*) in rat liver. In light of the inverse correlation of binding as well as of mutagenicity to metabolism of DMP, one would have expected the liver to be relatively refractory to a mutagenic event. Thus, based on Yurchenko's observation, either the hepatic metabolism of rats is insufficient to protect the liver from chronic administration of DMP or neither the Ames assay nor the TLC analytical methodology used in these studies is adequate to detect a mutagenic metabolite formed from the products of the mono-esterase activity. If the former explanation prevails, then skin with its considerably lower level of mono-esterase activity might well be at even greater risk than liver to incur a mutagenic/carcinogenic event. On the other hand, if the latter possibility is correct, then the greater rate of hepatic monoesterase activity would suggest liver, rather than skin, as the primary target site.

In conclusion, based on the results presented, we would recommend an *in vitro* bioassay for the carcinogenesis of DMP, particularly in view of the wide spread exposure of the human population to this compound. These results would argue for a bioassay protocol that would include the topical application of DMP.

This work was supported by a training grant (ES07067) and a research grant (ES00454) from the National Institute of Environmental Health Sciences.

REFERENCES

1. Casida, J. personal communication.
2. Pilinskaya, M. A., Kurinngi, A. I., and Kondratenko, T. I. Results from a study of the cytogenetic activity of certain pesticides (Russian). In: Molekulyarnye Mekhanizmy Geneticheskikh Protsessov Trudy, 1976, pp. 295-299.
3. Tsuchiya, K., and Hattori, K. A chromosomal study of cultured human leukocytes treated with phthalic acid esters. Hokkaido Eisei Kenkgusho Ho. 26: 114 (1976).
4. Yurchenko, V. V. A cytogenetic study of the Mutagenic properties of the repellents dimethylphthalate and phenoxyacetic acid *N*, *N*-diethylamide. Farmikol. Toksikol. 40 (4): 454-457 (1977).
5. Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31: 347-364 (1975).
6. Batzinger, R. P., Suh-Yun, L. Ou., and Bueding, E. Antimutagenic effects of 2 (3)-*tert*-butyl-4-hydroxyanisole and of antimicrobial agents. Can. Res. 38: 4478-4485 (1978).
7. Wood, P. J., and Siddiqui, I. R. Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. Anal. Biochem. 39: 418-428 (1971).
8. Thompson, S., and Slaga, T. J. Mouse epidermal aryl hydrocarbon hydroxylase. J. Invest. Dermatol. 66: 108-111 (1976).
9. Jollow, D. J., Mitchell, J. R., Potter, W. Z., Davis, D. C., Gillette, J. R., and Brodie, B. B. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. J. Pharm. Exptl. Therap. 187: 195-202 (1973).