

# Binding and Distribution Studies in the SENCAR Mouse of Compounds Demonstrating a Route-Dependent Tumorigenic Effect

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Previous investigators have determined that benzo(a)pyrene [B(a)P] was much more effective in causing skin papillomas if applied topically than when administered orally in the initiation-promotion assay in SENCAR mouse. Conversely, urethane and acrylamide caused a higher percentage of mice to develop papillomas and induced more tumors per mouse when given orally. In an attempt to understand the reason for this discrepancy in route dependency, <sup>3</sup>H-benzo(a)pyrene, <sup>14</sup>C-urethane and <sup>14</sup>C-acrylamide were administered as single doses orally or topically to male SENCAR mice. Distribution in skin, stomach, liver, and lung was determined for time periods up to 48 hr. The binding of these compounds to DNA, RNA, and protein in these tissues was determined 6 and 48 hr after administration. For all three compounds, high concentrations were found in the skin following topical application, but very little material reached this target organ following oral administration. In contrast, the internal organs generally contained more material after oral administration. The binding of label compounds to DNA, RNA, and protein generally reflected the distribution data, thus more compound was bound in the stomach, liver, and lung after oral administration compared to topical application, whereas the opposite was true for the skin. This finding was particularly evident for B(a)P. The results suggest that differences in distribution to the skin and binding to macromolecules following oral or topical administration cannot explain the greater tumorigenicity of urethane and acrylamide after oral administration in the SENCAR mouse.

## Introduction

The initiation-promotion assay has proved to be a promising assay for determining tumorigenicity. It has been refined for use with the SENCAR mouse on the basis of the sensitivity of this strain (1). After applying the test compound to the skin followed by a promotion schedule, papilloma formation on the skin can be quantified. As more compounds have been tested using this system, differences in tumor incidence have been observed. These differences are dependent on the route of administration as well as on the strain of mouse (2).

Bull et al. (3) found that both the percentage of mice developing papillomas and the number of papillomas per mouse were greater when benzo(a)pyrene was applied topically than when administered orally. In contrast, these authors found that the oral administration of urethane (ethyl carbamate) resulted in a greater papilloma incidence and a higher proportion of animals with papillomas than when the material was applied topically. A similar finding was observed with acrylamide, which

Table 1. Tumors in SENCAR mouse skin following oral and topical administrations.<sup>a</sup>

Compound	Route	Dose, mg/kg	% Animals with tumors	No. of tumors per animal
Benzo(a)-pyrene	Oral	10	20	0.20
		30	4	0.04
		100	24	0.24
	Topical	0.01	20	0.28
		0.10	56	1.16
		1.00	92	13.24
Urethane	Oral	30	24	0.24
		100	28	0.44
		300	68	1.20
	Topical	30	16	0.16
		100	24	0.28
		300	24	0.36
Acrylamide <sup>b</sup>	Oral	75	30	0.33
		150	58	1.00
		300	75	1.30
	Topical	75	10	0.20
		150	28	0.33
		300	45	0.55

<sup>a</sup>Data are from Bull and Robinson (3,4).

<sup>b</sup>Divided into six applications over a 2-week period.

demonstrated greater activity when the oral route of administration was employed (4). These results are

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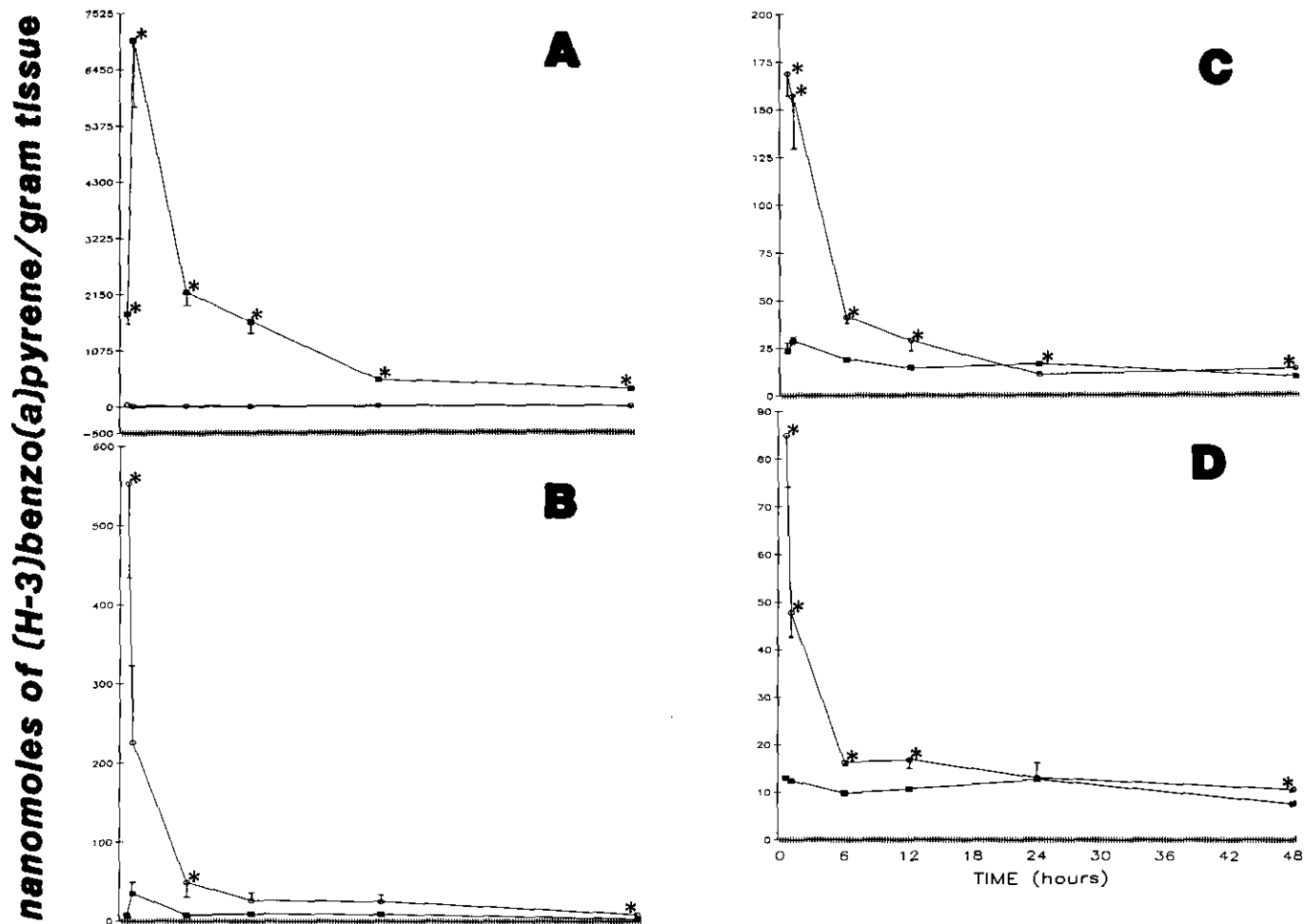


FIGURE 1. Distribution of [ $^3\text{H}$ ]-benzo(a)pyrene in tissues of SENCAR mice following oral or topical administration. [ $^3\text{H}$ ]-Benzo(a)pyrene (50 mg/kg, 630  $\mu\text{Ci}/\text{mmole}$ ) was administered (●) orally in an emulsion or (■) topically in acetone. Values are mean  $\pm$  SE for skin (A), stomach (B), liver (C), and lung (D). Asterisks denote significant differences between routes at the same time point ( $p < 0.05$ ).

summarized in Table 1, in which the cumulative papilloma formation at the end of 52 weeks is presented.

The purpose of the present studies was to determine if the route differences in tumorigenicity observed with these three compounds could be explained by differences in distribution following administration by oral and topical routes. A second hypothesis was tested to determine if the difference in sensitivity was related to the binding of the compounds to macromolecules, principally DNA, rather than related to simple distributional differences.

## Materials and Methods

### Animals

Male SENCAR mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed individually in stainless steel metabolism cages.

### Treatment of Animals

[G- $^3\text{H}$ ]Benzo(a)pyrene (Amersham Corporation, Arlington Heights, IL) was diluted with unlabeled B(a)P (Sigma Chemical Co., St. Louis, MO). It was either administered orally via intubation in an emulsion of 10% GAF Emulphor EL 620 and 90% water or was applied topically in acetone. The back of each animal was shaved 36 to 48 hr before treatment, and the compound was administered at a dose of 50 mg/kg and a specific activity of 630  $\mu\text{Ci}/\text{mmole}$  in the distribution studies and 25.2 mCi/mole in the binding studies. Groups of at least five animals were sacrificed at 0.5, 1, 6, 12, 24, and 48 hr after treatment in the distribution studies and at 6 and 48 hr in the binding studies.

Ethyl-1- $^{14}\text{C}$ -urethane (New England Nuclear, Boston, MA) was diluted with unlabeled urethane (Sigma Chemical Co.) and administered at a dose of 100 mg/kg (89  $\mu\text{Ci}/\text{mmole}$ ) for the distribution studies and 62 mg/kg (3.59 mCi/mole) for the binding studies. Water was

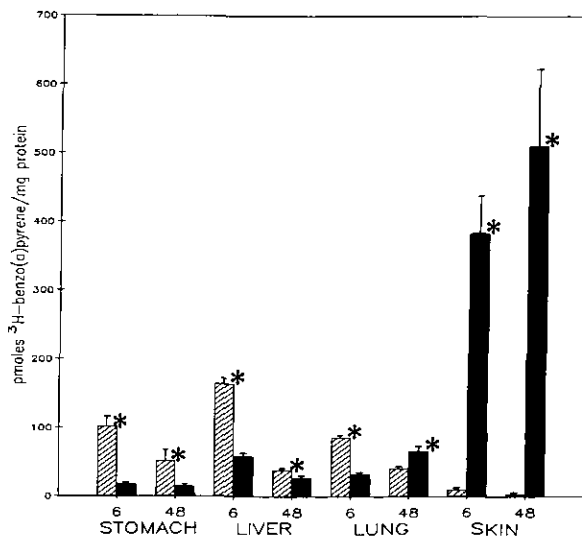
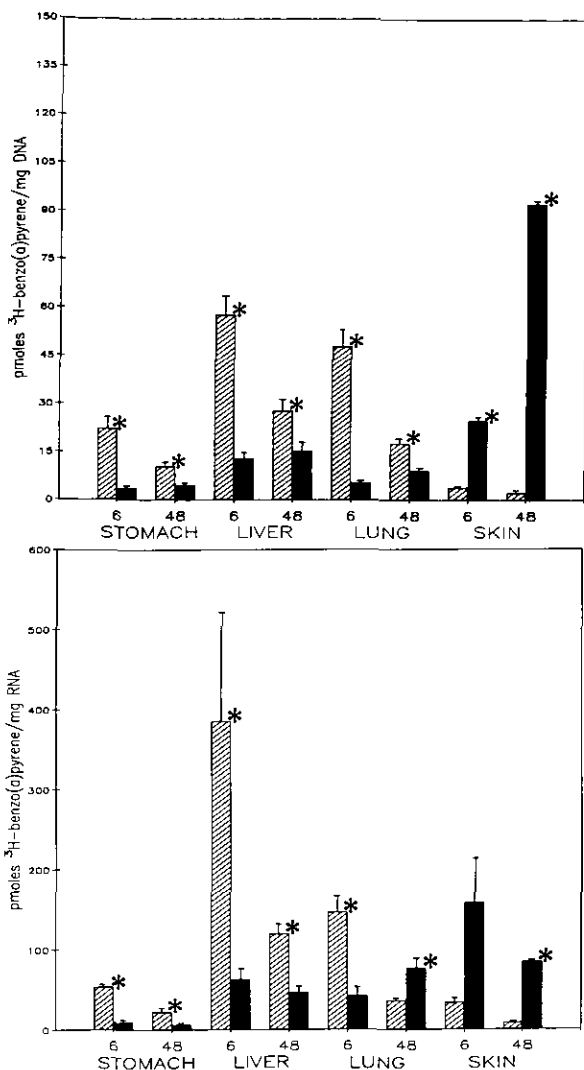


FIGURE 2. Binding of [<sup>3</sup>H]-benzo(a)pyrene to DNA, RNA, and protein in tissues of SENCAR mice 6 and 48 hr following oral or topical administration. [<sup>3</sup>H]-Benzo(a)pyrene (50 mg/kg, 25.2 mCi/m mole) was administered orally in an emulsion (hatched bars) or topically in acetone (solid bars). Values are mean ± standard error (SE). Asterisks denote significant differences between routes at the same time point for the same tissue (*p* < 0.05).

used as the vehicle for the oral studies and acetone for the topical. Observations were made at similar times starting at 1 hr. [2,3-<sup>14</sup>C]-Acrylamide (New England Nuclear, Boston, MA, for the distribution studies and Pathfinder Laboratories, St. Louis, MO, for the binding studies) was diluted with unlabeled acrylamide (Bethesda Research Laboratories, Gaithersburg, MD) and administered at a dose of 100 mg/kg with a specific activity of 56.9 μCi/m mole for the distribution studies and 1.185 mCi/m mole for the binding studies. The compound was administered in water orally or in ethanol topically. Groups of mice were sacrificed starting at 15 min after administration of the compound.

### Treatment of Tissues in Distribution Studies

Duplicate 50 to 100 mg portions of skin, liver, lung, and stomach were obtained. In the acrylamide studies, testes were also removed. The tissues were minced and digested in 1.0 mL NCS tissue solubilizer (Amersham

Corporation) for 24 hr at 50°C. After dissolution, 10 mL of Aquasol II (New England Nuclear) were added, and the samples were counted in a liquid scintillation spectrometer. [<sup>14</sup>C]-toluene and [<sup>3</sup>H]-toluene were added as internal standards and the samples recounted.

### Isolation and Analysis of Macromolecules

In the urethane studies, portions of the skin, stomach, liver, and lungs were removed and homogenized in an aqueous solution (1% NaCl, 1% triisopropyl naphthalene sulfonic acid, 6% *sec*-butyl alcohol and 6% *p*-aminosalicylate). Skin was frozen in liquid nitrogen and shattered before homogenization. Tissues including the testes were similarly treated in the acrylamide studies. In the B(a)P experiments, the epidermal layers were removed by a thermal method (5) and homogenized with a Polytron PT-10.

The DNA, RNA, and protein were isolated using a modification of the methods of Kirby and Cook (6) and Marmur (7). An equal volume of chloroform/isoamyl al-

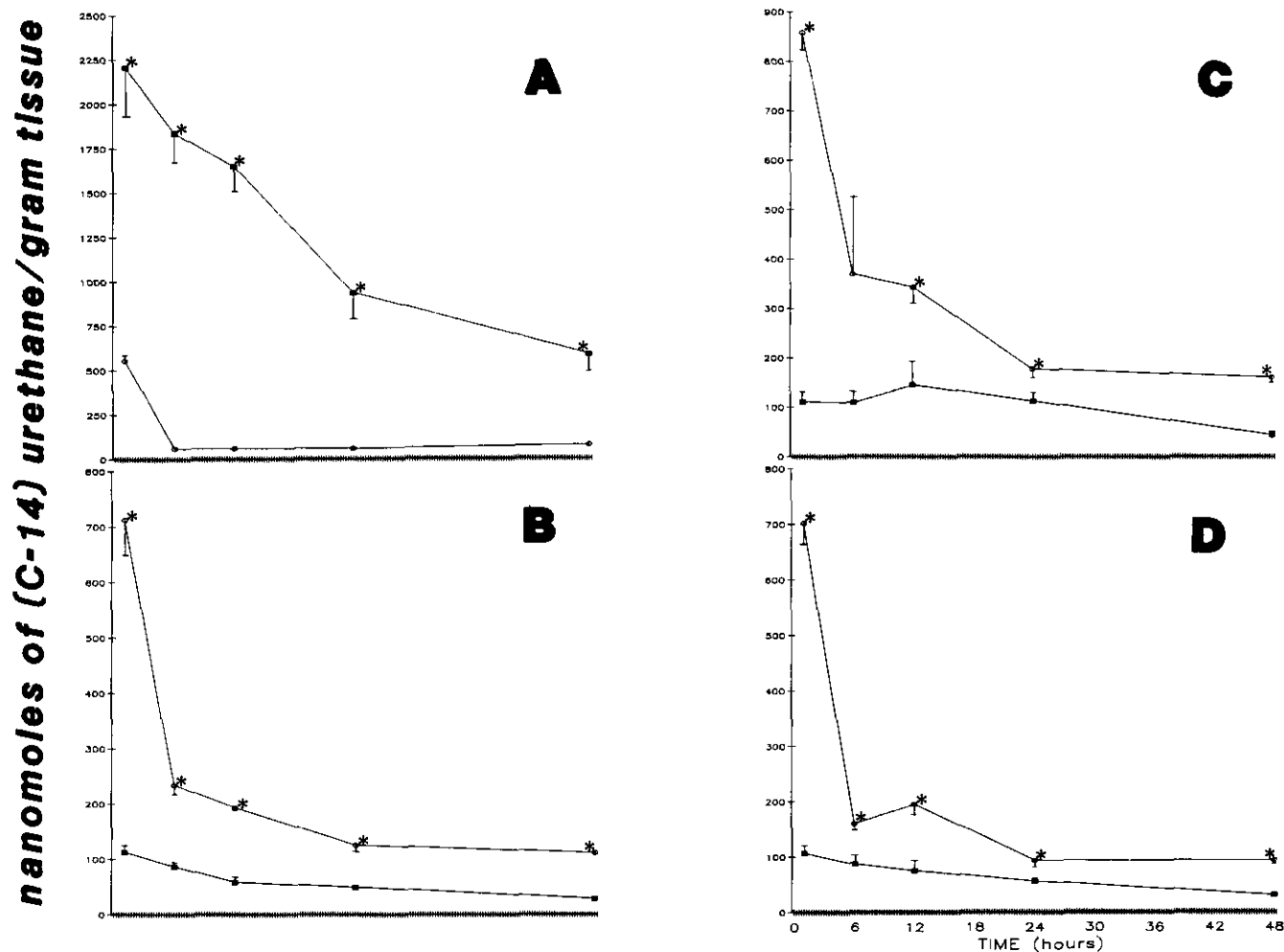


FIGURE 3. Distribution of [ $^{14}\text{C}$ ]-urethane in tissues of SENCAR mice following oral or topical administration. [ $^{14}\text{C}$ ]-Urethane (100 mg/kg, 89  $\mu\text{Ci}/\text{mmole}$ ) was administered (●) orally in water or (■) topically in acetone. Values are mean  $\pm$  SE for skin (A), stomach (B), liver (C), and lung (D). Asterisks denote significant differences between routes at the same time point ( $p < 0.05$ ).

cohol (24:1) was added to the aqueous phase. After shaking and centrifuging, the aqueous layer containing the RNA and DNA was removed, washed with the same solution, and recentrifuged. The DNA was precipitated with ethoxyethanol and the RNA with ethanol and cooling. The epidermal samples were treated with protease K and solubilized in NCS solution. Aquasol II was then added. The samples were counted as in the distribution studies. Protein from the interface was quantified using the Lowry procedure (8).

### Data Expression and Analysis

Since no attempt was made to differentiate between parent compound and metabolites or to identify the labeled material bound, data are expressed as equivalents. Differences between the two routes were analyzed using Student's  $t$ -test at the individual time points. A  $p$  value of 0.05 was selected to represent statistical significance.

### Results

When B(a)P was administered topically, the concentration in the skin peaked at 1 hr (Fig. 1). By 6 hr, the concentration had rapidly declined, and labeled material was slowly lost from the skin after that time. Very little material reached the skin when it was administered orally. In contrast, only small amounts of B(a)P were found in the stomach tissue when the compound was given topically compared to that found soon after oral administration, although little difference between the two routes was observed after 12 hr. Similar findings were observed in the liver and lung.

More B(a)P was bound to the DNA of the liver, lung, and stomach in the orally treated animals than in the topically treated animals at both 6 and 48 hr (Fig 2). However, the amount of labeled material bound to DNA after topical application was greater in the skin (epidermis) than in other tissues and was much higher than that observed after oral administration. Similar differences were exhibited in RNA and protein.

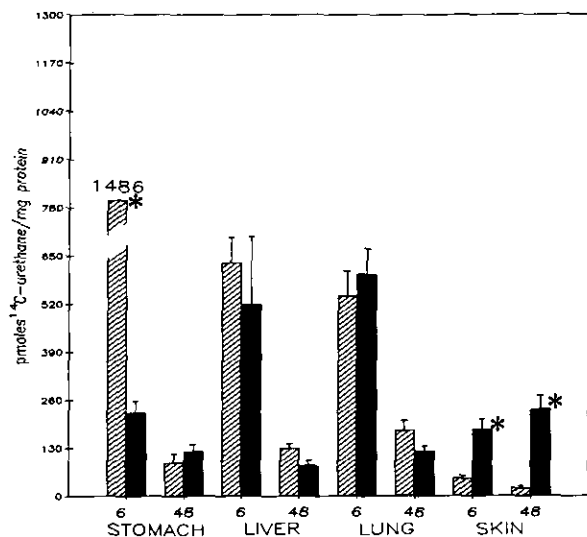
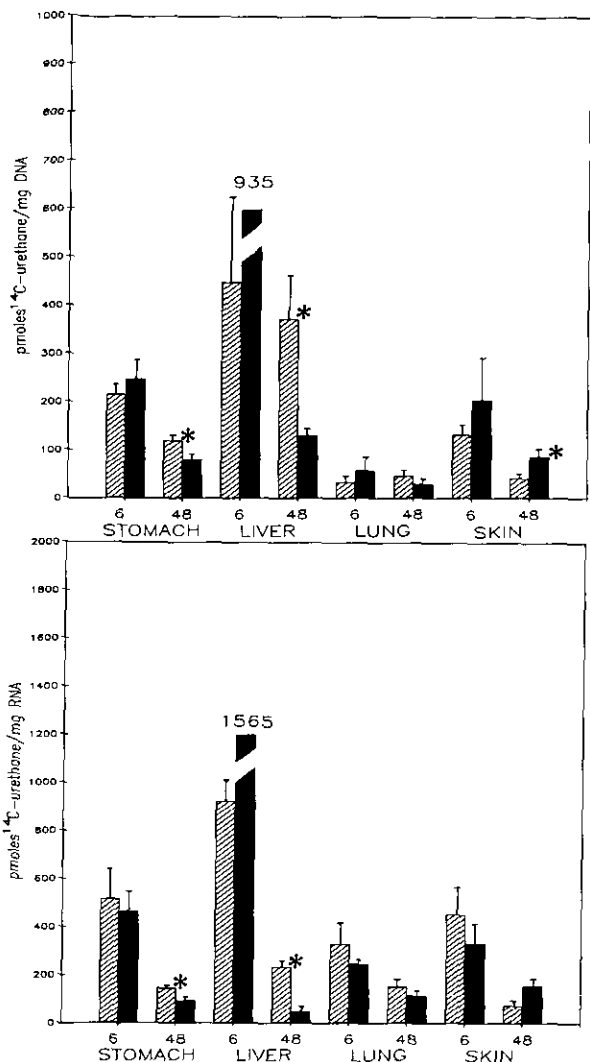


FIGURE 4. Binding of  $[^{14}\text{C}]$ -urethane to DNA, RNA and protein in tissues of SEN-CAR mice 6 and 48 hr after oral or topical administration.  $[^{14}\text{C}]$ -Urethane (62 mg/kg, 3.59 mCi/mole) was administered orally in water (hatched bars) or topically in acetone (solid bars). Values are mean  $\pm$  SE. Asterisks denote significant differences between routes at the same time point for the same tissue ( $p < 0.05$ ).

When urethane was applied to the backs of the SEN-CAR mice, the concentrations found in the skin were many times higher than those found after oral administration (Fig. 3). The decline with time was quite slow. In contrast, the concentration of labeled material found in the stomach tissue was quite low after topical application and was less than that seen after oral administration at all time points. The liver and lungs displayed a similar pattern and level, as did the stomach, suggesting a fairly even distribution of urethane among these internal organs.

When the binding of the  $[^{14}\text{C}]$  to DNA was measured, the differences between the routes of administration were not as great as those observed in the distribution studies (Fig. 4). No significant differences were observed at the 6-hr time point. However at 48 hr, more labeled material was bound to DNA in the stomach and liver of the orally treated animals and in the skin of the topically treated animals. The pattern of binding to RNA was quite similar, except that there was no difference in the pattern of binding to RNA in the skin

between the two routes. The binding of urethane to protein was greater in the stomach 6 hr after oral administration and was greater in the skin 6 and 48 hr after topical application.

Although high concentrations of  $[^{14}\text{C}]$ -acrylamide were present in the skin following topical application, very little was present in this tissue after oral administration (Fig. 5). Surprisingly high concentrations were observed in the stomach after topical application, but these decreased rapidly and from 6 to 24 hr, were lower than those measured in the orally treated animals. In liver, lung, and testes, concentrations peaked 30 to 60 min after treatment by either route and then sharply declined. In general, the oral treatment resulted in higher concentrations of acrylamide in the tissues through 24 hr, but by 48 hr, there were no differences between the groups.

The distribution data were reflected in the binding of acrylamide to macromolecules (Fig. 6). Binding to DNA, RNA, and protein in the internal organs was elevated in the orally treated mice compared to that in

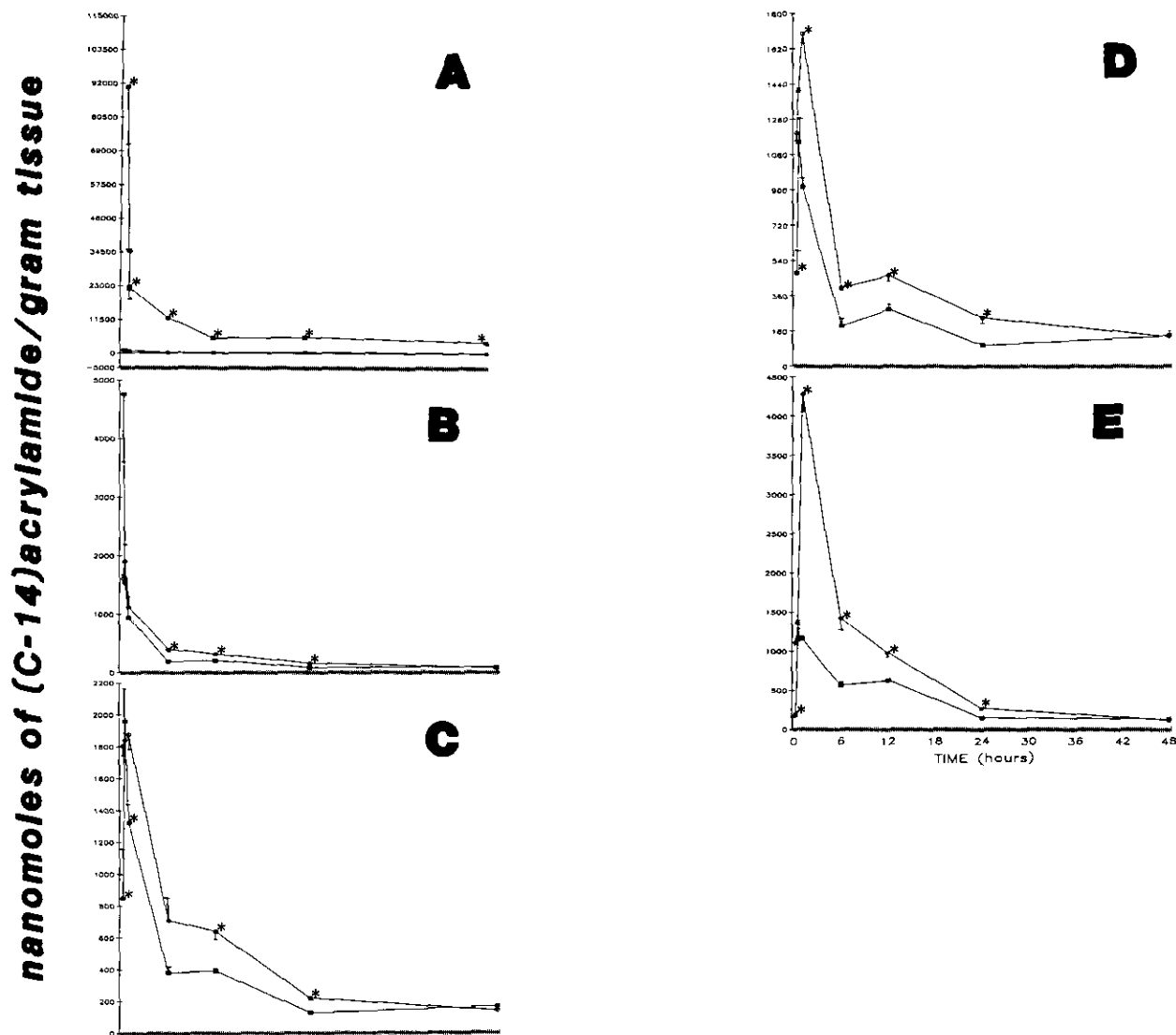


FIGURE 5. Distribution of [<sup>14</sup>C]-acrylamide in tissues of SENCAR mice following oral or topical administration. [<sup>14</sup>C]-Acrylamide (100 mg/kg, 56.9  $\mu$ Ci/mmmole) was administered (●) orally in water or (■) topically in ethanol. Values are mean  $\pm$  SE for skin (A), stomach (B), liver (C), lung (D), and testes (E). Asterisks denote significant differences between routes at the same time point ( $p < 0.05$ ).

the topically treated animals, whereas the opposite was true for the skin.

## Discussion

B(a)P is a well established carcinogen. Although much less potent, urethane has been demonstrated to cause tumors in the lung (9,10), liver (11,12), stomach (13), and skin (14,15). Acrylamide causes skin papillomas in the SENCAR mouse and lung adenomas in the A/J mouse (4) and is structurally related to vinyl carbamate hypothesized as the active metabolite of urethane (16).

The purpose of the current studies was to attempt to explain the differences in the incidence of skin papillomas in SENCAR mice. Bull et al. (3,4; personal com-

munication) found that B(a)P caused many more tumors when administered topically, whereas both urethane and acrylamide were more tumorigenic by the oral route (Table 1). One possibility was that urethane and acrylamide, which are much more hydrophilic than B(a)P, did not penetrate the skin well and that higher concentrations were achieved in this tissue after oral administration due to delivery of the material to this tissue via the blood. The data, however, did not support this hypothesis. Rather these two compounds resembled B(a)P in that higher concentrations of labeled material were found in the skin after topical application than after oral treatment.

A second possibility was examined. It was possible that the urethane and acrylamide were activated by the

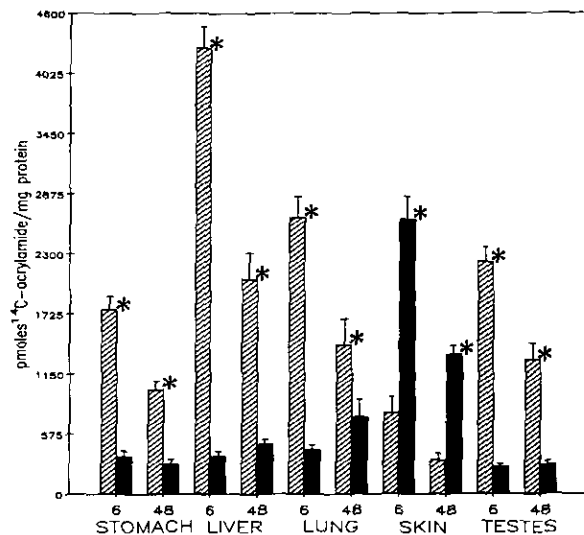
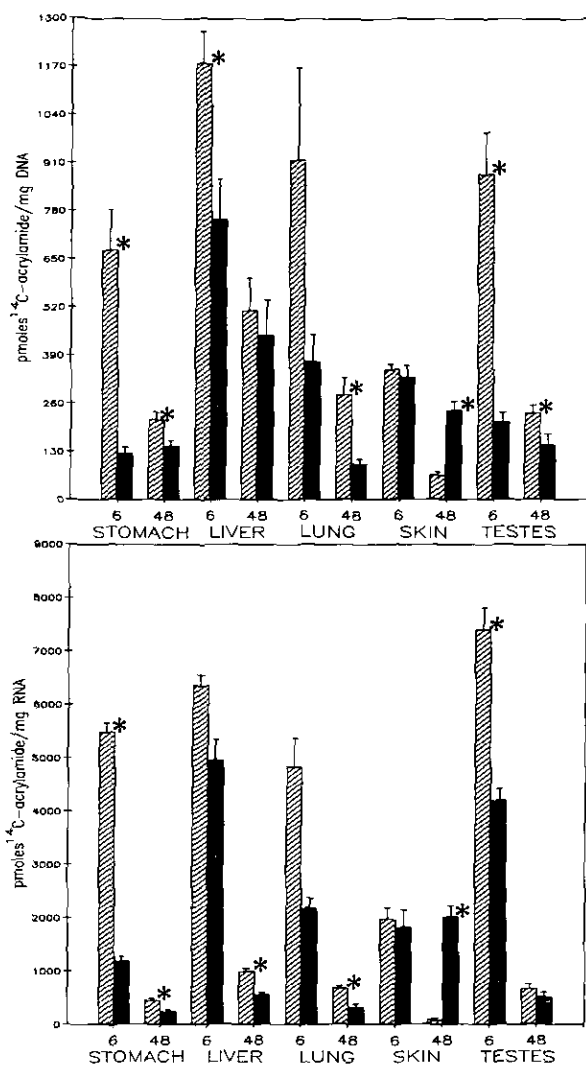


FIGURE 6. Binding of [<sup>14</sup>C]-acrylamide to DNA, RNA and protein in tissues of SENCAR mice 6 and 48 hr after oral or topical administration. [<sup>14</sup>C]-Acrylamide (100 mg/kg, 1.185 mCi/mmmole) was administered orally in water (hatched bars) or topically in ethanol (solid bars). Values are mean ± SE. Asterisks denote significant differences between routes at the same time point for the same tissue (*p* < 0.05).

liver and/or other organs to proximate carcinogens, which were subsequently transported to the target organ, the skin. This hypothesis, which was examined by determining the degree of binding to macromolecules, also does not appear to be supported by our data. Although the differences were not as striking as those found with B(a)P, in general, the binding of the urethane and acrylamide to DNA, RNA, and protein was greater in the internal organs following oral administration and was greater in the skin following topical application. Thus neither the distribution nor the binding data can adequately explain the route differences in the induction of papillomas in SENCAR mice. Further studies are necessary to evaluate a third possibility that there are differences in the DNA adducts formed following the administration of the compounds by the two routes.

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