The Interaction of Carcinogenic Hydrocarbons with Tissues
VI. Studies on Zero-Time Binding to Proteins*
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(McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.)

SUMMARY
A comparison has been made of the methods used for homogenizing and processing tissues in hydrocarbon-skin protein binding studies. The procedure of choice is to use a glass homogenizer cooled in ice and to precipitate the proteins with acetone. The zero-time binding of a number of hydrocarbons has been studied and has been found to be negligible. The zero-time binding of several derivatives of 1,2,5,6-dibenzanthracene has been studied, and in general they bind much more than the parent hydrocarbon, especially 2-phenylphenanthrene-3,3'-dialdehyde, which is not carcinogenic. An attempt was made to find out whether this compound is a metabolite of 1,2,5,6-dibenzanthracene in mouse skin in vivo. Only negligible amounts could be detected. The significance of these results for the hypothesis that the formation of a tissue-carcinogen complex is an early step in carcinogenesis is discussed.

Previous contributions from this laboratory (4-8) have been concerned with the way in which polycyclic hydrocarbons and their derivatives become bound to mouse skin proteins and the correlation between this binding and the carcinogenic potency of the hydrocarbons. In general the binding of the carcinogenic hydrocarbons starts at a low value and rises to a maximum 2 days after application, indicating the production of some metabolite which forms covalent bonds with the skin proteins (5). The noncarcinogenic hydrocarbons, with the notable exception of 1,2,3,4-dibenzanthracene, are bound only to a very small extent at all time intervals after application (5).

Hadler, Darchun, and Lee (2, 3) have reported that the noncarcinogenic hydrocarbons, anthracene and 9-methylanthracene, are bound extensively to skin proteins when mixed with the denatured protein in a homogenizer. The implications of their work are twofold: first, that under appropriate conditions homogenization alone is sufficient to induce binding between hydrocarbons and protein; and, second, that although the initial or zero-time binding of a hydrocarbon may be low it is still possible that a metabolite produced from it might have a high zero-time binding. Under these circumstances the binding as measured by our technic might therefore be an artifact of the experimental procedure, rather than the result of an in vivo process.

In the present work we have examined the zero-time binding of some hydrocarbons and their derivatives under our own experimental conditions in order to re-assess the validity of our previous results (4-8).

MATERIALS AND METHODS
The following compounds were prepared by the methods of Oliverio and Heidelberger (6), and their specific activities are given in parentheses: 1,2,5,6-dibenzanthracene-9,10-C₁⁴ (1,2,5,6-DBA) (4.95 × 10⁶ counts/min/mg, diluted 1:10 before use), 9,10-dimethyl-1,2,5,6-dibenzanthracene-9,10-C₁⁴ (4.88 × 10⁶ counts/min/mg), 3-hydroxy-1,2,5,6-dibenzanthracene-9,10-C₁⁴ (6.76 × 10⁶ counts/min/mg), 1,2,5,6-dibenzanthracene-3,4-dihydrodiol-9,10-C₁⁴ (5.83 × 10⁶ counts/min/mg), 1,2,5,6-dibenz-3,4-anthraquinone-9,10-C₁⁴...

1,2,5,6-Dibenzanthracene-3,4-dihydrodiol was purified by conversion to the diazoxy derivative, chromatography of this on alumina, elution with chloroform in benzene, and reduction of the diazoxy compound to the dihydrodiol with LiAlH₄. A similar degree of purification could also be achieved by chromatography of the dihydrodiol on Florisil previously washed with 6 N HCl and dried at 100°C, and elution with 5 per cent ethyl acetate in benzene.

1,2,3,4-Dibenzanthracene-9,10-C₁⁴ (4.12 X 10⁷ counts/min/mg) was prepared by the method of Heidelberger and Moldenhauer (5).

Anthracene-9,10-C₁⁴ (2.08 X 10⁷ counts/min/mg) and 9-methylanthracene-9,10-C₁⁴ (1.92 X 10⁷ counts/min/mg) were generously supplied by Dr. H. I. Hadler and were purified by chromatography on Florisil and alumina, respectively.

2-Phenylphenanthrene-3,2'-dialdehyde-9,10-C₁⁴ (PDA dialdehyde) (1.04 X 10⁷ counts/min/mg) was prepared, in collaboration with Dr. J. A. LaBudde, from 1,2,5,6-dibenzanthracene-3,4-dihydrodiol-9,10-C₁⁴ (20 mg., purified as above) by oxidation with NaIO₄ (4.5 mg.) at room temperature overnight in a mixture of acetic acid (4.5 ml.), water (2.5 ml.), and dioxan (2 ml.). The dialdehyde was precipitated with water and crystallized from ethanol; yield, 12.8 mg., m.p., 156°–59°C. Chromatography on Florisil (previously washed with 6 N HCl), elution of the aldehyde with 15 per cent chloroform in petroleum ether (Skellysolve B, b.p., 65°–66°C.), and final recrystallization from benzene-petroleum ether gave pale yellow needles, m.p., 157°–60°C. Larger quantities of nonradioactive material were made for carcinogenic testing by the same method, and could be oxidized to 2-phenylphenanthrene-3,2'-dicarboxylic acid (PDA) (1) with CrO₃ in acetone at 0°C.

Measurement of protein binding.—The methods used by Wiest and Heidelberger (7) for processing the skin have been modified and are therefore described again below:

The backs of female albino mice¹ were shaved, and 0.1 mg. of the labeled compounds (1 mg/ml) in benzene (dioxan for the 3,4-quinone) was applied to an area of approximately 5 sq. cm. The mice were killed after the appropriate time interval, and the excess compound was wiped off with cotton soaked in benzene unless otherwise stated. In the case of the zero-time experiments, the animals were killed as rapidly as possible after application of the compound, and the skins were not wiped. The skins were removed with the aid of a stencil, 2 X 2.5 cm., frozen in liquid air, and the fat and connective tissue were scraped off as before (7). Each pair of skins was minced with scissors and homogenized in 2 ml. of 0.154 M KCl with an all-glass homogenizer cooled in ice water unless otherwise specified. One hundred strokes of the pestle were made in 2–3 min. In the case of liquid air homogenization the tissue was pulverized under liquid air and then suspended in isotonic KCl. After centrifugation, the particulate fraction was washed with 2 ml. of isotonic KCl, and the supernatant fractions were combined. The supernatant and particulate fractions were then processed by one of the following methods:

TCA precipitation.—Trichloroacetic acid (TCA) was added to the supernatant fractions to a final concentration of 5 per cent, and the precipitate of “soluble” proteins was centrifuged. Both soluble and particulate fractions were washed twice with 5 per cent TCA, with 5 per cent NaCl until the washings were neutral, and then with water followed by 70 per cent ethanol. Up to this point the processing was carried out in a cold room at ca. 5°C. Then the following washes were carried out at room temperature: once with 5 ml. of 70 per cent ethanol, twice with 5 ml. of 90 per cent ethanol, and twice with 5 ml. of absolute ethanol. These washes were followed by 5 ml. of ethanol-ether (3:1) at the boiling point, 5 ml. of dioxan at 50°C. for 5 minutes, 5 ml. of benzene at 50°C. for 5 minutes, and finally 5 ml. of ether at room temperature.

Acetone method.—The supernatant fraction (5 ml.) was diluted to 40 ml. with cold acetone, and the precipitate of “soluble” protein was centrifuged. The particulate fraction was washed with 5 ml. of acetone, and then both fractions were washed with 5 ml. of acetone and 5 ml. of 70 per cent ethanol (to remove salt). These steps were carried out in the cold room at 5°C. The rest of the washes were as follows: once with 5 ml. of absolute ethanol at room temperature, once with 5 ml. of ethanol-ether (3:1) at the boiling point, once with 5 ml. of dioxan at 80°C. for 5 minutes, twice with 5 ml. of benzene at the boiling point, and once with 5 ml. of ether at the boiling point.


(3.92 X 10⁶ counts/min/mg), 1,2,5,6-dibenz-9,10-anthraquinone-9,10-C₁⁴ (5.44 X 10⁶ counts/min/mg).

...
The proteins were then air-dried and suspended in 70 per cent ethanol for plating on aluminum discs, and their radioactivity was measured with a standard deviation of less than 10 per cent. Correction was made for self-absorption, specific activity, and molecular weight of the compound used, and the binding is expressed as $10^{-6}$ mole compound equivalent/mg dry weight of protein.

In the experiment with denatured skin, the skins were excised and defatted as above and then heated with benzene for 2 hours at 70°C. They were then soaked in isotonic KCl to soften them, and, after blotting, the PDA dialdehyde in benzene was applied to the dorsal side. Processing was by the acetone precipitation method.

In the experiments with polylysine, “Polytide 20” hydrochloride (Kremers-Urban Co., Milwau-
kee) was used. The PDA dialdehyde in benzene was added to 5–10 mg. of polylysine in an all-glass homogenizer, under the conditions stated in Table 4, and homogenized for 100 strokes with the homogenizer cooled in ice water. The solid was washed with hot acetone or alcohol, benzene, and ether, then dissolved in 70 per cent ethanol and plated.

For the experiment in which aqueous alkali was used as the condensing agent (Table 5), 24.6 mg. of polylysine hydrochloride was dissolved in a few drops of water, and an equal volume of 6 N NaOH was added. The dialdehyde in 0.2 ml. benzene (1.98 X $10^6$ counts/min) was added and stirred with a glass rod for a few minutes, after which the polylysine was reprecipitated by addition of acetone and alcohol. The solid was isolated by centrifugation, washed as above, and plated. For the subsequent acid treatment it was dissolved of the DBA. The backs of the mice were protected by adhesive tape to prevent licking. Three days later the mice were killed, and the defatted skins from half of each group were extracted whole with a mixture of benzene and ethanol containing 10 mg. of carrier (nonradioactive) dialdehyde per group of four skins. The other skins were homogenized with isotonic KCl containing 10 mg. of carrier dialdehyde per group, and the homogenates were extracted with benzene. All these extracts were dried and re-extracted with benzene. Five mg. of carrier 1,2,5,6-DBA was then added to each extract, and the dialdehyde was separated by chromatography on acid-washed Florisil as above. Further purification was carried out by crystallization from benzene-petroleum ether and benzene-ethanol, and by conversion to the phenylhydrazone, which could be chromatographed on alumina, by eluting with pe-
troleum ether-benzene, 1:1, and then with benzene. The crystallizations were complicated by the presence of fats extracted from the skins.

RESULTS
Table 1 shows the binding of 1,2,5,6-DBA under various conditions of homogenization, and it can be seen that at zero time the use of the stainless steel homogenizer resulted in much higher binding than occurred with the other methods, although at 2 days after application the difference was not in consistently higher specific activities, which indicate that the acetone method is to be preferred. In addition, the acetone method gave higher yields of soluble proteins.

The zero-time binding of 1,2,5,6-DBA, 1,2,3,4-DBA, and 9,10-dimethyl-1,2,5,6-DBA shown in Table 2 was clearly negligible in comparison with the in vivo binding at longer time intervals (see Chart 2, also [6]). The binding of anthracene and 9-methylnaphthalene, while somewhat higher, was still of a low order, and their binding at other

<table>
<thead>
<tr>
<th>CONDITIONS</th>
<th>Dose (COUNTS/HR/MOUSE)</th>
<th>AMOUNT BOUND (10^-6 µMOL/MG)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stainless steel homogenizer</td>
</tr>
<tr>
<td>Zero time:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenized at ice temp.</td>
<td>0.93 × 10^6</td>
<td>19.8</td>
</tr>
<tr>
<td>Homogenized at room temp. (27.5 °C.)</td>
<td>0.88 × 10^6</td>
<td>31</td>
</tr>
<tr>
<td>2 Days (homogenized at ice temp.):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skins not washed</td>
<td>0.85 × 10^6</td>
<td>25.2</td>
</tr>
<tr>
<td>Skins washed and boiled with benzene/acetone (1:1)</td>
<td>1.2 × 10^6</td>
<td>32.8</td>
</tr>
<tr>
<td>Skins washed with benzene</td>
<td>1.2 × 10^6</td>
<td>55</td>
</tr>
<tr>
<td>Skins washed with benzene and the mince extracted with boiling benzene/acetone (1:1) prior to homogenization</td>
<td>1.2 × 10^6</td>
<td>69</td>
</tr>
</tbody>
</table>

significant. Pulverization of the skins under liquid air was found to give consistently lower yields of soluble proteins than by the other methods, and so the use of the all-glass homogenizer cooled in ice water was adopted as the procedure of choice. The effect of washing the excess hydrocarbon from the surface of the skin was compared with that of boiling the minced skins with solvents, and there was little difference.

In Table 2, zero-time binding figures are given for a number of hydrocarbons and oxidation products of 1,2,5,6-DBA, together with a comparison of the acetone and TCA methods for precipitating the soluble proteins. The TCA method resulted in strong contrast to the results obtained by Hadler et al. (3) at 30 minutes, which are included in Table 3 for comparison. Their data have been recalculated to correspond to the same units as ours. 2-Phenylphenanthrene-3,2′-dialdehyde (PDA dialdehyde) was found to have a zero-time binding nearly 100 times that of any hydrocarbon tested and, in fact, several times higher than the maximum binding obtained with 1,2,5,6-DBA in vivo. A similar order of binding was observed with the 1,2,5,6-DBA-3,4-dihydrodiol if it was not purified by the method described above. The dialdehyde was also bound to denatured skin and to synthetic
polylysine (Table 4). In the latter case the binding was increased in the presence of alkali, but much of this radioactivity could be extracted after suspending the polylysine in cold dilute HCl, as would be expected of a Schiff's base. By contrast, no radioactivity was extractable from dialdehyde-bound protein under the same conditions (Table 5).

The zero-time binding found after the application of varying amounts of PDA dialdehyde in the same volume of solvent is shown in Table 6, and in Chart 1 the zero-time and in vivo binding of dialdehyde is compared with that of 1,2,5,6-DBA. In this experiment the backs of the mice were protected by adhesive tape to prevent licking.

Since it seemed possible that PDA dialdehyde might be a metabolite produced from 1,2,5,6-DBA in vivo, a carrier experiment was performed to test this hypothesis. The final specific activities of the carriers and the corresponding percentages of the applied dose are shown in Table 7. The radioactivity of the skin proteins from this experiment was also measured and found to correspond
to 0.2 per cent of the applied dose, so the amounts retained by the carriers were clearly negligible in comparison to the amount bound to the protein, except in the case of extract CHO 1. This extract was difficult to purify because of the presence of large amounts of fat, and no significance is attached to the high value obtained in this case.

PDA dialdehyde was also tested for carcinogenic activity, both by subcutaneous injection and by skin painting under various conditions designed to test initiating and promoting ability. The results, given in Table 8, show no evidence of carcinogenic activity except for one malignant lymphoma with extensive leukocytic infiltration produced at the site of injection of 1 mg. of aldehyde. The number of papillomas produced in the treated animals was similar to that produced in the controls given croton oil alone, and hence is of no significance.

**DISCUSSION**

In the zero-time experiments described above, the excess compound was not removed by washing the mouse skins before homogenization. Consequently, the amount of radioactive material present in the homogenizer is much larger than in the normal in vivo experiments in which the skins are washed and in which much is also removed by licking. Nevertheless, the zero-time binding under these conditions was negligible when compared with the maximum values obtained in vivo, although the use of steel homogenizers is undesirable and cooling of the homogenizer is necessary. Also,

**TABLE 4**

Binding of PDA Dialdehyde to the Soluble and Particulate Proteins from Denatured Mouse Skin, and to Synthetic Polylysine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total activity applied</th>
<th>Binding (10^-4 μmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin denatured by heating in benzene (70°C / 2 hr.)</td>
<td>4.16×10^6</td>
<td>76 (soluble) 119 (particulate)</td>
</tr>
<tr>
<td>Polylysine HCl in acetone</td>
<td>1.04×10^6</td>
<td>57</td>
</tr>
<tr>
<td>Polylysine HCl in acetone + alcoholic KOH</td>
<td>1.04×10^6</td>
<td>163</td>
</tr>
<tr>
<td>Polylysine HCl in acetone + potassium acetate</td>
<td>1.04×10^6</td>
<td>143</td>
</tr>
</tbody>
</table>

**TABLE 5**

The Effect of Acid Treatment on Polylysine and Protein Bound to PDA Dialdehyde

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total counts/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylysine HCl</td>
<td>&gt;50,000*</td>
</tr>
<tr>
<td>Benzene extract of polylysine before acidification</td>
<td>158</td>
</tr>
<tr>
<td>Benzene extract of polylysine after acidification with 2 N HCl</td>
<td>8,290</td>
</tr>
<tr>
<td>Particulate protein bound to dialdehyde</td>
<td>8,500</td>
</tr>
<tr>
<td>Benzene extract of protein</td>
<td>88</td>
</tr>
<tr>
<td>Benzene extract of protein after cold air treatment</td>
<td>171</td>
</tr>
<tr>
<td>Benzene extract of protein after hot acid treatment</td>
<td>2,300</td>
</tr>
</tbody>
</table>

* Samples were too hot for radioactivity to be measured accurately.

**TABLE 6**

Binding of PDA Dialdehyde to Mouse Skin at Zero Time after Application of Varying Amounts in the Same Volume of Solvent

<table>
<thead>
<tr>
<th>Amount applied/skin (μg)</th>
<th>Binding Soluble/particulate (10^-4 μmole/mg)</th>
<th>Amount bound as per cent of amount applied*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18/10</td>
<td>6.6</td>
</tr>
<tr>
<td>10</td>
<td>191/97</td>
<td>6.7</td>
</tr>
<tr>
<td>100</td>
<td>536/920</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* Assuming a total of 10 mg. soluble and 70 mg. particulate proteins in the four mice used.

**TABLE 7**

Carrier Experiment To Measure the Production of PDA Dialdehyde in Mouse Skin 3 Days after Application of Labeled 1,2,5,6-DBA

<p>| (1.48×10^7 counts/min/group of four mice; 12 mg. carrier dialdehyde added to each group) |
|---------------------------------------------|---------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Extract</th>
<th>Total counts/min</th>
<th>Counts/min/mg dialdehyde after each purification step</th>
<th>Per cent of amount applied corresponding to final carrier activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA 1</td>
<td>2.41×10^6</td>
<td>66</td>
<td>0.005</td>
</tr>
<tr>
<td>DBA 2</td>
<td>5.45×10^6</td>
<td>63, 27, 46</td>
<td>0.004</td>
</tr>
<tr>
<td>CHO 1</td>
<td>5.25×10^6</td>
<td>2950, 16100, 5950, 6270</td>
<td>0.5</td>
</tr>
<tr>
<td>CHO 2</td>
<td>2.60×10^6</td>
<td>506, 471, 80</td>
<td>0.006</td>
</tr>
</tbody>
</table>
under our conditions anthracene and 9-methylan-
thracene did not give the high zero-time binding
values found by Hadler et al. (2 3), and so far
we have been unable to explain this difference.
There is, therefore, nothing in the present experi-
ments to support the idea that the results previously
reported from this laboratory (4-8) were invalid
because of high zero-time binding of hydrocarbons.
However, the work with the hydrocarbon deriva-
tives, especially PDA dialdehyde, tends to suggest
the possibility that a metabolite might become
bound in this way. If such were the case, the bind-
ing values would reflect the production of the
binding is likely to be more extensive than that
following external application. This might explain
the failure to detect significant amounts of free
dialdehyde in the carrier experiments and might
also explain the lack of carcinogenic activity of
the dialdehyde, since the externally applied com-
 pound could become bound to extracellular sub-
stances without ever reaching the active center
where it might be produced as a metabolite in
vitro.

It seems to us, therefore, that the most useful
working hypothesis is still that a metabolite-
protein complex is involved in the carcinogenic
process, although there is no conclusive evidence
at present as to the stage of hydrocarbon metabo-
lism at which this complex is formed.

TABLE 8
Carcinogenic Testing of PDA Dialdehyde

<table>
<thead>
<tr>
<th>Groups</th>
<th>Duration of test (weeks)</th>
<th>No. animals with tumors</th>
<th>No. alive at end/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls: 0.2 ml. tricaprylin injected s.c.</td>
<td>58</td>
<td>0</td>
<td>18/30</td>
</tr>
<tr>
<td>0.1 mg. of PDA dialdehyde in tricaprylin injected s.c.</td>
<td>58</td>
<td>0</td>
<td>18/30</td>
</tr>
<tr>
<td>1 mg. of PDA dialdehyde in tricaprylin injected s.c.</td>
<td>53</td>
<td>1*</td>
<td>19/30</td>
</tr>
<tr>
<td>PDA dialdehyde, twice weekly on skin (1 drop 0.5% in benzene, 40 drops/ml)</td>
<td>57</td>
<td>0</td>
<td>20/30</td>
</tr>
<tr>
<td>PDA dialdehyde, 2 applications (1 drop, 0.5% in benzene), followed by croton oil, twice weekly on skin (1 drop, 0.5% in benzene)</td>
<td>58</td>
<td>9 (paps.)</td>
<td>19/31</td>
</tr>
<tr>
<td>Controls: croton oil twice weekly (1 drop, 0.5% in benzene)</td>
<td>58</td>
<td>10 (paps.)</td>
<td>19/30</td>
</tr>
<tr>
<td>9,10-Dimethyl-1,2-benzanthracene (0.125 mg.) once, then PDA dialdehyde twice weekly on skin (1 drop, 0.5% in benzene)</td>
<td>57</td>
<td>1 (pap.)</td>
<td>16/30</td>
</tr>
</tbody>
</table>

* Malignant lymphoma (slides read by Dr. Henry Plot and Dr. Paul Kotin).

metabolite and not necessarily the participation
of a metabolite-protein complex in the carcinogen-
process. After application to the skin, only
about 1/15 of the dialdehyde was bound at zero
time, and if aldehyde produced metabolically were
to be bound in the same way we might expect
the same relation to hold. In the case of the carrier experiment described in Chart 1, 14 times
the amount of bound radioactivity would have
been available for extraction. From the results
in Table 7 this was clearly not the case, and if
one takes this experiment at its face value it would
mean that the dialdehyde is not a metabolite
of DBA in skin. We feel, however, that any
compound as reactive as PDA dialdehyde may be
bound as soon as it is formed in vivo, probably
to the enzyme which forms it, and that this

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