7,12-Dimethylbenz(a)anthracene Retention in the Rat Submandibular Gland following Intraglandular Injection

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SUMMARY

The relative concentration of radioactive carbon retained in the rat submandibular gland and its cell fractions following the intraglandular injection of 7,12-dimethylbenz(a)-anthracene-12-14C was determined at periods ranging from 6 hr to 6 weeks by liquid scintillation spectroscopy. Ethyl acetate extracts of gland homogenates and cell fraction suspensions were similarly analyzed to determine the degree of binding of the carcinogen in this tissue. Less than 30% of the total hydrocarbon injected was found in the glands of animals sacrificed during the 1st week. The carcinogen content of the gland declined to 13, 7, and 5%, respectively at 2, 4, and 6 weeks after treatment. A minimum of 65% of the radioactivity in this tissue was extractable from the homogenates of all the groups examined. The nuclear fraction contained higher values of 14C than other fractions during the 1st day, whereas, at subsequent time periods, more isotope was found in the cytosol fraction.

INTRODUCTION

The tumorigenicity of DMBA3 in the rat SMG is well known (5, 6, 8, 18–20). Following a single intraglandular injection of a solution of DMBA or the implantation of crystalline DMBA, tumors are induced in a minimum of about 8 weeks. The majority of the neoplasms in this species are squamous cell carcinomas, although, in a few instances, sarcomas are produced.

The histological sequence of events in the induction of the tumors conforms to a consistent pattern (5, 6, 8). Direct injection of the carcinogen into the surgically exposed SMG initially invokes a marked inflammatory reaction and necrosis of gland tissue which is most evident between 3 and 7 days. This is followed by a reparative process which involves the removal of the necrotic debris and its replacement by granulation tissue and eventual fibrosis. Small viable ducts persist and proliferate at the periphery of the lobules. Some of these ducts become dilated and show comitant epithelial hyperplasia and squamous metaplasia with the formation of keratin cysts. Neoplastic changes of the squamous lining follow and lead to the eventual development of invasive squamous cell carcinoma.

While the morphological changes are well outlined there have been no biochemical studies directed at SMG carcinogenesis in the rat. The present work is, therefore, designed to study some of the biochemical parameters related to the induction of malignant epithelial tumors in this experimental model. Its purpose is to determine the amount of DMBA in the gland and the various cell fractions of this tissue at certain time intervals after injection of the hydrocarbon. In addition, the study is intended to provide some preliminary information on the degree of binding of the carcinogen or its metabolites to constituents of the cell fractions. This information will hopefully provide a base for further study of the mechanism of SMG carcinogenesis by this agent.

Significantly, DMBA induces fibrosarcomas in the SMG of Syrian hamsters under the same conditions of administration (4, 7). The initial histopathological changes parallel those seen in the rat gland. There are similar inflammatory changes with necrosis and early fibrosis. The proliferation of small ducts showing some evidence of squamous metaplasia is also apparent. Eventually, however, the mesenchymal reaction assumes the aggressive role in the hamster to become the malignant tissue component. Future studies are planned to compare the biochemical responses of the SMG to DMBA in the 2 species.

MATERIALS AND METHODS

Forty male Sprague-Dawley rats, 2 to 3 months of age, were singly caged in a room maintained at 72 ± 3°F and provided with a 12-hr dark-light cycle. The animals were fed a standard laboratory diet and water ad libitum. Under ether anesthesia, the submandibular glands were exposed through a ventral midline incision. Radioactive carbon-labeled DMBA [1.0 μCi of DMBA-12-14C (specific activity, 6.53 mCi/mmol); Amersham-Searle Corp., Des Plaines, Ill.] in a carrier of 50 μl of 2.0% unlabeled DMBA (1.0 mg) (Fisher Scientific Co., Pittsburgh, Pa.) in paraffin oil was injected into the body of the gland. The animals were divided into 7 groups. The 1st 3 groups, consisting of 8 animals each, were sacrificed by decapitation at 6, 24, and 72 hr after treatment. The remaining 4 groups each...
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contained 4 animals and were killed at 1, 2, 4, and 6 weeks posttreatment. The glands were removed and frozen at -20° until they were prepared for assay.

Pairs of glands from a common group were combined and minced in 0.25 M sucrose-0.015 M Tris buffer adjusted to pH 7.4, and a 5% homogenate (w/v) was prepared. Radioactivity determinations were done on homogenate and cell fraction digests as well as on EtAc extracts of these tissues. Cell fraction samples were prepared by differential centrifugation as outlined in Chart 1. Three portions of the homogenate were separately evaluated. A 1.0-ml homogenate digest aliquot was prepared and counted. A 10% volume of the homogenate was extracted with purified EtAc (Fisher Scientific Co.) and counted. The remaining volume was separated into subcellular fractions by differential centrifugation.

All of the fractions except the soluble protein were washed and resuspended in buffer. A tissue digest sample was then prepared from a 1.0-ml aliquot of each of the fractions and the remainder of the suspensions was extracted.

Digest samples were solubilized overnight at 37° in 3.0 ml of NCS solubilizer (Amersham-Searle Corp.). Scintillation fluid (10 ml) containing BioSolv solubilizer, Formula BBS-3 (Beckman Instruments Co., Fullerton, Calif.), 25 ml/liter of scintillation grade toluene (Matheson, Coleman, and Bell Co., Norwood, Ohio), 4 g PPO per liter (Amersham-Searle Corp.), and 0.04 g POPOP per liter (Amersham-Searle Corp.), of scintillation grade toluene were added and the mixture was counted.

The 10% homogenate aliquot and the cell fraction suspensions were extracted with two 10-ml volumes of EtAc and washed with 0.1 N NaOH and distilled water. The organic phase was evaporated under vacuum and the extract dissolved in 2.5 ml of benzene/methanol (1/1, v/v). This solution was then counted in scintillation fluid (10 ml) containing PPO and POPOP in the concentration previously mentioned.

All samples were counted for a minimum of 10 min at 5° in a Packard Tri-Carb Model 3003 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples were corrected for quenching by the channels ratio method as described by Bush (3). Disintegrations per min for all samples were calculated on the basis of the total homogenate or suspension volume.

The group mean percentage of injected DMBA-14C recovered from the digests of the gland homogenates at the selected time periods and the percentage of extractable DMBA in the homogenate are shown in Chart 2. Only 28.5% of the carcinogen could be accounted for in the 6-hr glands. This value remained relatively constant throughout the 1st week except for the 24-hr group in which 21.3% of the carcinogen was recovered. At 2, 4, and 6 weeks, the DMBA recovery was 12.1, 6.7, and 4.7%, respectively.

The percentage of DMBA in the homogenates that was the efficiency of the analyses. The tritiated hydrocarbon was pipeted into the homogenizers in a solution of benzene/ethanol (1/1, v/v) and the solvent was evaporated under a stream of nitrogen. The SMG tissue mince was then added and a 5% homogenate prepared as previously outlined. The mean 4H recovery from the digest samples of all groups was 78.8% with a range of 72.1 to 89.4%. A separate in vitro series was done in which 4 pairs of SMG's were separately minced and added to individual homogenizers containing recrystallized DMBA-3H. The tissue was then homogenized and 1.0-ml solubilized aliquots were counted. The mean 4H recovery was 77.7% with a range of 67.2 to 84.4%.

RESULTS

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The percentage of DMBA in the homogenates that was

![Chart 1. Scheme of sample preparation and cell fractionation.](chart1.png)

![Chart 2. Mean DMBA-14C content in SMG homogenates. The lower plot represents the percentage of the injected dose of DMBA-14C recovered from SMG homogenate digests at the time intervals indicated on the abscissa. The percentage of 14C recovered from the homogenates is shown in the upper plot. Range = standard error of the mean.](chart2.png)

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extractable with EtAc is also shown in Chart 2. During the 1st 24 hr, more than 95% of the carcinogen was extracted but only 69% was available in the solvent at 72 hr. An increase in extractable isotope to 86% was seen in the 1-week series. The values ranged from 67 to 71% at 2, 4, and 6 weeks, respectively.

A comparison of the percentage of isotope concentration in the digest sample of the cell fractions is shown in Table 1. There was a higher radioactivity associated with the nuclear fraction between 6 and 24 hr; this was equivalent to 23 and 30%, respectively, of the initial dose of the carcinogen. At 72 hr the nuclear digests represented 20.5% of the challenge dose and then declined to 10% at 6 weeks.

The mitochondrial and microsomal digest fractions contained relatively similar amounts of radioactivity throughout the series. There was an apparent increase in the radioactive carbon in both fractions at 72 hr. The mitochondrial fraction remained relatively stable thereafter. The microsomal fraction, however, gradually decreased from the highest value of 16.3% at 72 hr to 10.6% at 6 weeks.

There was an increase with time in the amount of radioactivity in the cytosol fraction. A distinct rise was first evident at 72 hr. The figures for the 1st- and 2nd-week samples were calculated values rather than counted values and, therefore, their significance cannot be evaluated.

The percentage of the EtAc-extractable carcinogen in the various fractions is shown in Table 2. All of the hydrocarbon was extracted from the nucleus and mitochondria fractions at 6 and 24 hr. Thereafter, the nuclear extracts showed a gradual decline of extractable isotope. There was a similar downward trend with mitochondrial extracts notwithstanding a marked fluctuation ranging between 31% at 72 hr and 67.5% at 1 week.

Extracts of the microsomal fractions showed the highest activity at 1 and 2 weeks but appeared comparatively stable at other periods.

The cytosol extracts remained relatively consistent and showed the highest value of 24.0% in the 24-hr specimens.

**DISCUSSION**

The maximum quantitative recovery of the carcinogen from the submandibular gland was less than 30%. The amount of 14C recovered during the 1st week following injection ranged from 21 to 28% of the 1.0-mg dose of DMBA. At 2, 4, and 6 weeks, the values were 12.7, 6.7, and 4.7%, respectively. Weist and Heidelberger (25) were able to recover 35% of a tricaprylin solution of 1,2,5,6-dibenzanthracene-9,10-14C injected in the SMG of mice 2 hr after injection. Their highest recovery rate of 44% was achieved from specimens examined 1 week postinjection. At 5 weeks, 12% of the carcinogen remained in the mouse gland.

Our low recovery rates appeared to be a function of the fluidity of the solution injected, the inflammatory process it invoked, and the physiological elimination of the compound from the gland. Some amount of carcinogen was undoubtedly lost postoperatively through reflux leakage from the needle puncture wound into the surrounding tissue. The marked inflammatory reaction induced by the surgical procedure and the carcinogen itself caused a dis-

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclei</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>22.8 ± 3.14\a</td>
<td>7.3 ± 1.52</td>
<td>8.4 ± 1.79</td>
<td>16.9 ± 1.22</td>
</tr>
<tr>
<td>24 hr</td>
<td>30.2 ± 3.20</td>
<td>6.8 ± 0.46</td>
<td>9.0 ± 0.59</td>
<td>12.7 ± 1.08</td>
</tr>
<tr>
<td>72 hr</td>
<td>20.5 ± 1.23</td>
<td>13.8 ± 1.65</td>
<td>16.3 ± 1.66</td>
<td>29.9 ± 1.56</td>
</tr>
<tr>
<td>1 wk</td>
<td>13.1 ± 1.07</td>
<td>12.2 ± 1.36</td>
<td>13.2 ± 3.71</td>
<td>46.6 ± 2.40\b</td>
</tr>
<tr>
<td>2 wk</td>
<td>14.4 ± 1.32</td>
<td>12.3 ± 0.93</td>
<td>13.5 ± 1.64</td>
<td>42.2 ± 6.47\c</td>
</tr>
<tr>
<td>4 wk</td>
<td>8.0 ± 0.60</td>
<td>12.6 ± 1.30</td>
<td>11.9 ± 0.97</td>
<td>38.0 ± 4.80</td>
</tr>
<tr>
<td>6 wk</td>
<td>10.0 ± 1.09</td>
<td>14.0 ± 0.08</td>
<td>10.6 ± 1.73</td>
<td>34.9 ± 7.01</td>
</tr>
</tbody>
</table>

\a Percentage isotope concentration = fraction dpm/homogenate dpm × 100.
\b Mean ± S.E.
\c Because of the loss of samples the 1- and 2-week cytosol fractions were calculated using the values obtained from extracts of these fractions and assuming a ratio of extract/digest of 0.388 which was the mean value in this fraction at other time periods.

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Nuclei</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>100.0 ± 15.85\a</td>
<td>100.0 ± 13.18</td>
<td>25.5 ± 4.94</td>
<td>19.2 ± 2.47</td>
</tr>
<tr>
<td>24 hr</td>
<td>100.0 ± 3.30</td>
<td>100.0 ± 17.23</td>
<td>34.7 ± 1.11</td>
<td>24.3 ± 2.78</td>
</tr>
<tr>
<td>72 hr</td>
<td>71.0 ± 4.96</td>
<td>30.6 ± 4.55</td>
<td>24.2 ± 3.10</td>
<td>10.8 ± 2.09</td>
</tr>
<tr>
<td>1 wk</td>
<td>68.2 ± 6.63</td>
<td>67.4 ± 7.79</td>
<td>38.7 ± 3.03</td>
<td>13.0 ± 1.76</td>
</tr>
<tr>
<td>2 wk</td>
<td>58.4 ± 14.84</td>
<td>50.7 ± 6.42</td>
<td>35.2 ± 4.42</td>
<td>16.7 ± 1.91</td>
</tr>
<tr>
<td>4 wk</td>
<td>45.5 ± 14.46</td>
<td>35.0 ± 3.48</td>
<td>28.6 ± 4.34</td>
<td>18.3 ± 0.11</td>
</tr>
<tr>
<td>6 wk</td>
<td>53.3 ± 3.37</td>
<td>40.9 ± 1.57</td>
<td>25.3 ± 1.12</td>
<td>18.6 ± 0.01</td>
</tr>
</tbody>
</table>

\a Percentage of extractable isotope = dpm fraction extract/dpm fraction digest × 100.
\b Mean ± S.E.
Distinct swelling of the glands which was most prominent during the 1st week. Consequently, the glands of animals sacrificed at these earlier time periods were difficult to handle and some of the hydrocarbon could have been lost by simple manipulation. In addition, it appears possible that the increased volume of fluid associated with the inflammatory exudate enhanced the physiological elimination of the carcinogen.

It is interesting to reflect on the amount of carcinogen present in the gland during the initial time periods. If it were assumed that approximately 30% of the 1.0-mg injected dose of DMBA was available to the gland during the 1st week, as little as 300 μg or about 1.2 μmoles of this hydrocarbon may be the effective dose required for the induction of tumors.

Several investigators have shown that DMBA binds with DNA, RNA, and protein of target cells (10, 13, 15). It is also likely that similar interactions between carcinogens or their derivatives take place with other macromolecules (10) and with components of lower molecular weight. The significance of carcinogen binding to DNA versus RNA and protein is not known. It has been postulated (17) that polycyclic hydrocarbons could be expected to interfere with DNA function by virtue of its size and thereby cause point mutations or other permanent alterations of the template. Conversely, others (13, 16) propose that cellular protein–carcinogen interaction may be the significant event in the mechanism of tumor induction by chemical carcinogens. The present study was not designed to investigate specific intracellular binding of DMBA with the various cellular proteins or nucleoproteins. It was felt, however, that by the simple extraction of the tissues with EtAc, some knowledge of the reactivity of the carcinogen with tissue components might be achieved.

Essentially all of the DMBA-14C contained in the homogenate was extracted with EtAc during the 1st day of the experiment. Therefore, there seems to be no significant covalent binding of the carcinogen with cellular constituents. Less carcinogen was extracted from the homogenate at 72 hr (approximately 65%). About 70% of the injected dose of DMBA was EtAc-soluble in the glands studied at 2, 4, and 6 weeks. It has been previously noted that the inflammatory response reached a peak at about 72 hr and that during this period the histological appearance of the tissue is characterized by a marked acute inflammatory exudate and the presence of abundant necrotic debris. The amount of DMBA that was incorporated in this debris by chemical or physical means cannot be determined. It might be possible that the inflammatory cells, as well as fibrin, could have been responsible, in part, for the higher apparent binding of the carcinogen at 72 hr.

No more than 30% of the radioactivity accounted for in the homogenate was recovered in the nuclear fraction and this was seen at 24 hr. All of the material, however, was extractable. At the later time periods less radioactivity was associated with the nuclei and less of it was soluble in EtAc. Thirteen, 14, and 10% of the homogenate dpm were counted in the nuclear fraction in the 1-3, 2-, and 6-week samples, respectively. This is in agreement with Weist and Heidelberger (25), who reported 14% of the homogenate 14C in the SMG cell nuclei 1 week following intraglandular injection of 1,2,5,6-dibenzanthracene-9,10-14C with a slight decline during the subsequent 5 weeks. A uniform homogenate, with the preservation of the nuclear fraction, was difficult to obtain in the 1st 4 groups because of the intense inflammatory response. The nuclei were undoubtedly contaminated by a considerable amount of cellular debris and, consequently, an appreciable amount of radioactivity may have been entrapped. A true evaluation of the data relative to the earlier groups is, therefore, tenuous at best.

The combined radioactivity associated with the mitochondrial and microsomal fractions was less than that contained in either the nuclei or cytosol fractions. An approximate 2-fold increase in the total radioactivity in the mitochondrial and microsomal fractions was noted from 6 to 72 hr. The smaller and more constant values of extractable DMBA presented in the microsomal fractions were perhaps of greater significance. It is known that the microsomes are responsible for the synthesis of a wide variety of mixed function oxidases (12) which are functional in the metabolism of numerous drugs. The work of Conney et al. (9) has shown a 3-fold increase in the activity of 1 such system, aryl hydroxylase, in rat liver homogenates following treatment with 1.0% DMBA. These systems are known to exist in a variety of species and tissues (24). It might, therefore, be speculated that DMBA association with the microsomes is instrumental in the oxidation or hydroxylation of the hydrocarbon.

Several metabolites of DMBA, the most prominent of which are hydroxylated derivatives, have been isolated from rat liver homogenates (1, 2). Opinions differ as to whether DMBA or a metabolite of DMBA is the proximate carcinogen. There is evidence to support the concept that hydroxylation of the parent hydrocarbon is inhibitory to carcinogenesis (21–23). Flesher and Sydnor (11), on the other hand, believe that the proximate carcinogen may be 7-hydroxymethyl-12-methylbenz(a)anthracene. Efforts in this laboratory to isolate hydroxylated metabolites of DMBA from EtAc extracts of SMG tissue have been unrewarding; however, the use of more sophisticated extraction techniques may be more revealing. In addition, it is known that DMBA induces fibrosarcomatous changes in the SMG of hamsters (4, 7) as opposed to the carcinomas found in the rat gland. It is possible that a metabolite of DMBA might be responsible for this dicotomy.

Increasing proportional amounts of DMBA-14C were noted in the cytosol digest fractions. Cytosol radioactivity represented 17% of the homogenate dpm in the 6-hr glands and approximately 13% at 24 hr. The percentage increased to 30% at 3 days and was about 35% at 6 weeks. Despite the fluctuation of the extract values during the period of maximum inflammation, the amount of EtAc-resistant carcinogen was rather constant.

A comparison between the ratios of cytosol digest dpm to nuclear digest dpm of the groups is shown in Table 3. During the 1st day there was higher radioactivity in the nuclear fraction. A permanent reversal occurred between 1 and 3 days at which time more hydrocarbon was found in
the soluble fraction. An increase in $^{14}$C concentration in the cytosol fraction would infer that some metabolic pathway is active in the formation of a more polar metabolite or conjugated form of DMBA to account for its increased solubility. Carcinogen activity associated with the cytosol fraction is compatible with the mechanism of depression of genetic expression as theorized by Heidelberger (13) and Pitot and Heidelberger (16) through alteration of the basic Jacob-Monod (14) model of enzyme induction.

### REFERENCES

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