Studies on the Formation of Protein-bound Derivatives of 3,4-Benzpyrene in the Epidermal Fraction of Mouse Skin

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INTRODUCTION

The general metabolism of the carcinogenic hydrocarbons has been studied intensively by a number of workers (2, 4, 5). However, the reactions occurring in the tissues susceptible to their carcinogenic activity have received relatively little attention, although such studies should be of particular value in determining the mechanism by which the tumors are initiated. The late F. Weigert and his associates (15, 17—19) have shown the presence of certain derivatives of 3,4-benzpyrene in sites where it was injected subcutaneously or applied to the skin of mice. Most of the metabolites in the skin were present in a form which could not be extracted prior to alkaline hydrolysis of the tissue. Recently, we have observed protein-bound derivatives of 3,4-benzpyrene in the epidermis of mice following topical application of the hydrocarbon (7). Studies on the occurrence and properties of these derivatives form the subject of this paper. The search for these derivatives was undertaken, since the carcinogenic aminoazo dyes (or azo metabolites thereof) have been shown to combine with certain proteins of the liver (the susceptible tissue) in the rat. This reaction appears to have causal significance in liver carcinogenesis (8, 9).

METHODS

Purification of 3,4-benzpyrene.—All the 3,4-benzpyrene (Hoffman LaRoche, Inc.) was purified in a dark room by chromatography on activated alumina (10) in 20- to 45-mg. lots. Either pure petroleum ether (Skelly Solve B, b.p. 66°—68° C.) or petroleum ether containing up to 5 per cent of benzene (depending on the activity of the alumina) was used as solvent and eluant. After elution the solvent was removed in vacuo (water bath temperature less than 45° C.), and the 3,4-benzpyrene was dissolved in ethanol and precipitated by the addition of water. This procedure removed two fluorescent bands, one moving at a faster and one at a slower rate than the main band of 3,4-benzpyrene; the resulting product appeared homogeneous when rechromatographed on alumina. While it was necessary to follow the band by its fluorescence under ultraviolet light, extreme care was used to prevent photo-oxidation of the hydrocarbon. In particular, the chromatographic column was covered and was viewed with ultraviolet light only momentarily through a vertical slit a few mm. wide. Furthermore, throughout the study all solutions containing the carcinogen or its derivatives were always shielded from light by black cloth or paper covers.

Animal experiments.—Female albino mice1 about 2—4 months of age were used throughout. The mice were kept in screen-bottom cages in groups of three to seven and were fed a stock diet2 and water ad libitum. The hair was removed from a 12—15 sq. cm. area on the backs of the mice with a surgical clipper prior to each experiment, and individual mice were reclipped when necessary during the experiments. In general, 4—6 drops of either benzene or a 0.2 per cent solution of 3,4-benzpyrene in benzene were applied as uniformly as possible to the clipped area of each mouse. This corresponded to 50—75 mg. of hydrocarbon per application, and the average dose was recorded for each experiment. Where large quantities of epidermal protein were needed for chemical studies, 50—75 mice were treated daily for 5 days with either benzene or a 0.2 per cent solution of 3,4-benzpyrene, and the mice were killed on the sixth day. For studies on the rate of accumulation of the derivatives under different conditions, groups of

1 Purchased from Arthur Sutter, Springfield, Mo.
2 Friskie Dog Biscuits, purchased from the Carnation Co., Milwaukee, Wis.
mice were treated with benzene or the hydrocarbon solution, and groups of three to six mice were killed at the intervals indicated for each experiment.

In all cases the mice were killed with ether, and the treated area of the skin was excised. The skins were immersed in n/3 ammonia solution (1) for 20—30 minutes, and the epidermal area was then scraped away from the dermal portion with a scalpel. Both portions were collected in ice-cold beakers protected from the light. The dermal fraction was ground through the thin blades of a household meat grinder, and then both fractions were ground separately in a Waring Blendor in an 85 per cent ethanol solution containing 10 per cent of trichloroacetic acid. The suspension was centrifuged, and the precipitate was washed 3 times with ethanol. For the preparation of protein from the internal organs, the tissues were homogenized in water in the Waring Blendor, and aqueous trichloroacetic acid was added to give a final concentration of 9 per cent. The proteins were sedimented by centrifugation and then washed successively once with a pH 5, 1 m acetate buffer and twice with ethanol. Finally, all protein fractions were wrapped individually in filter paper, extracted at 60° C. for 48 hours with ethanol in a Soxhlet apparatus, and dried in vacuo over sulfuric acid. The resultant crude preparations thus consisted chiefly of a mixture of protein and nucleoprotein.

All the solvents used for purifying and applying the hydrocarbon, washing and extracting the protein preparations, and carrying out the analytical work were redistilled from an all-glass apparatus and were essentially free of fluorescent materials. All the glassware for these experiments was cleaned with sulfuric acid—sodium dichromate solution maintained at 50°—60° C. Contact with corks, rubber stoppers, and stopcock grease was scrupulously avoided.

**Analytical procedures.**—For analysis, 25 mg. of the crude protein preparation and 2 ml. of ethanol, 5 ml. of 4 N potassium hydroxide, 5 ml. of toluene, and about 1.6 gm. of zinc dust were heated in a 1 × 8-inch test tube at reflux on a sand bath for 2 hours. After cooling, the hydrolysate was extracted 3 times with 15-ml. aliquots of benzene. The zinc dust was then packed by light centrifugation, the aqueous layer was decanted into a clean test tube, and the zinc was washed with 2 ml. of 3 N potassium hydroxide in 30 per cent ethanol. The combined aqueous layer and washings were acidified by the addition of 5 ml. of 7 N HCl, and the solution was extracted 3 times with 15-ml. aliquots of benzene. A motor-driven assembly fitted with ten spiral glass stirrers was used for the extractions, while a suction device consisting of a capillary tube equipped with a standard taper glass joint was used to transfer the benzene extracts from the test tubes to 100-ml. graduated cylinders fitted with complementary joints. The benzene extracts from the alkaline and from the acid solutions, i.e., the neutral and acidic fractions, respectively (see below), were each diluted to 70 ml. and dried with sodium sulfate, and their fluorescent intensities were determined in a Coleman fluorophotometer equipped with the B-B filters. All the samples were read against a standard quinine sulfate solution, and, since the identities of the derivatives are not known, all the results are expressed in terms of "fluorescence units." These units are identical to the galvanometer readings with this instrument. Duplicate analyses for each fraction usually agreed within 10 per cent.

Hydrolysis of the protein with 4 N KOH under these conditions liberated essentially all the fluorescent derivatives within 90 minutes, and no destruction was found within hydrolysis periods up to 4 hours. The 2-hour hydrolysis was used routinely, since troublesome emulsions formed upon extraction of samples hydrolyzed for shorter periods. Rehydrolysis of the neutral derivative under these conditions gave quantitative recoveries, while only 70—90 per cent of the acidic derivative was recovered. Neither derivative gave rise to appreciable levels of the other on rehydrolysis. When 20- to 50-mg. samples of the protein were hydrolyzed and extracted under the conditions listed above for 25-mg. samples, the amounts of each fluorescent derivative were approximately in proportion to the amount of protein used.

The reductive hydrolysis was suggested by Martin's modification (6) of the Clemmensen reduction, in which anthracene was obtained in 98 and 80 per cent yields by heating anthrone and anthraquinone, respectively, in the presence of alkali, toluene, and zinc dust activated with copper sulfate. The importance of the zinc dust in the estimation of the fluorescent derivatives of 3,4-benzpyrene is shown in Table 1. When the zinc dust was omitted, very little or none of the neutral derivative could be extracted, while the fluorescence of the acidic extracts was usually unchanged but occasionally increased up to 25 per cent. The extracts from the alkaline hydrolysates had a maximum fluorescence when 0.8 or 1.6 gm. of zinc dust were added per tube. Similar results were also obtained with as little as 0.1 gm. of zinc dust which had been activated by suspension for 15 minutes in 15 ml. of a 0.2 per cent solution of copper sulfate;
the copper sulfate was then decanted, and the moist zinc was used immediately. Small amounts of the copper sulfate solution in the absence of zinc did not give rise to the fluorescent neutral derivative. Although the addition of toluene to the hydrolysis mixture was not essential, the analyses for the neutral component appeared to be more reproducible when it was used. The nature of the reactions occurring under these reducing conditions has not been investigated. However, alkaline hydrolysis in the presence of sodium hydrosulfite did not yield a neutral fluorescent derivative and reduced the yield of the acidic fluorescent derivative.

### RESULTS

**Properties of the fluorescent derivatives.**—For studies on the properties of the fluorescent derivatives, 1- to 2-gm. batches of epidermal protein were prepared, as described above, from groups of 50–75 mice which had been treated topically with either benzene or a benzene solution containing 0.2 per cent of 3,4-benzpyrene on 5 successive days. The preparations from the hydrocarbon-treated mice contained a high level of both fluorescent derivatives, while those from the benzene-treated mice had very little fluorescence and were used as control material. The solutions of the hydrocarbon derivatives were obtained by the procedures described under “Methods.”

The hydrocarbon derivatives, obtained when the hydrolysis of the protein was carried out in the presence of zinc dust, had similar fluorescent properties but different solubilities. The neutral derivative could be extracted from alkaline, neutral, or acidic solutions with benzene, but for convenience was routinely extracted from the alkaline hydrolyzate. The compound appeared to have some polar character, since, on shaking a benzene solution of the derivative with an aqueous solution, the compound distributed itself between the two phases. The acidic derivative could not be extracted with benzene from alkaline solutions, but was extracted gradually from neutral solutions and readily from acidic solutions. This derivative was quantitatively removed from a benzene solution by extraction with a small amount of alkali.

Like 3,4-benzpyrene, both the neutral and acidic derivatives had a blue fluorescence upon irradiation with the 360 m\(\mu\) band from a mercury lamp; the fluorescent light was transmitted by a filter (Coleman B2) with maximum transmission at 440 m\(\mu\) (range, 420–460 m\(\mu\)). However, the fluorescence of the derivatives was much less readily quenched by oxygen than that of 3,4-benzpyrene (18). Thus, when a benzene solution of 3,4-benzpyrene was saturated with nitrogen by bubbling the gas through a fine capillary, the fluorescence of the solution was 9 times greater than when the same solution was saturated with oxygen. However, when benzene solutions of the neutral and acidic derivatives were similarly gassed, their fluorescent intensities were only 2.5 and 2.5 times greater when the solution was saturated with nitrogen instead of oxygen. When 3,4-benzpyrene was added to either of these solutions, its fluorescence was still 9 times greater when the solution was saturated with nitrogen rather than oxygen.

**Evidence that the derivatives are combined chemically with the protein.**—Most of the chemical studies reported in this paper were carried out to determine whether the fluorescent derivatives of 3,4-benzpyrene were combined chemically with the protein, adsorbed on the protein, or combined with a contaminant in the crude protein preparation. For these studies 1- to 2-gm. batches of hot-ethanol-extracted crude epidermal protein were prepared, by the methods described above, from mice treated either with benzene or with a benzene solution containing 0.2 per cent of 3,4-benzpyrene on 5 successive days. After the treatments described, in each case the preparations were analyzed by the routine analytical procedure.

In one experiment 60-mg. samples of epidermal protein were extracted with three 90-ml. changes of boiling solvents over a 10-hr. period. If the derivatives had been only adsorbed to the protein, significant losses would have been expected, but the content of the 3,4-benzpyrene derivatives was not altered when the boiling solvent was ethanol, ethyl ether (peroxide-free), or benzene (Table 2).
Similar treatment with boiling pyridine caused a 20 per cent loss of the fluorescent derivatives, but this decrease may have been caused by the basic properties of the pyridine rather than by a simple extraction.

Studies on the rate of liberation of the fluorescent derivatives also indicated that the neutral derivative, at least, was held by a bond stronger than that of the usual adsorption complex. Thus, when the hydrolysis was carried out with ethanolic 4 N KOH at boiling, the cleavage of both fluorescent derivatives was complete within 20 minutes. However, when the hydrolysis was carried out in a 70°C oil bath, only 40-50 per cent of the neutral derivative was liberated within 20 minutes, although the maximum fluorescent intensity of the extract was the same as that extracted after a 3- to 5-hour hydrolysis. However, solvent over a 10-hr. period.

Of KOH were used. Thus, with 1 N KOH and in a 60°C bath only 50 and 60 per cent as much of the neutral derivative appeared to be relatively unaffected by the concentration of alkali and the time of hydrolysis.

For a more rigorous test of chemical combination, 100- to 200-mg. samples of epidermal protein from either benzene- or 3,4-benzpyrene-treated mice were suspended in 14 ml. of a 1:2.5 mixture of ethanol and 0.1 N KOH/100 mg of sample and finely dispersed with a loose Potter-Elvehjem homogenizer. Each preparation was then transferred to a 125-ml. flask and incubated as described below. At the end of the incubation period the suspension was cooled quickly, and the residual protein was sedimented by centrifugation. The clear supernatant solution was decanted carefully, the soluble protein was precipitated by the addition of trichloroacetic acid to a final concentration of 10 per cent, and the precipitated protein was separated by centrifugation. Both the residual protein and the protein precipitated with trichloroacetic acid were washed once with a pH 5, 1 M acetate buffer and twice with ethanol, extracted for 48 hours with ethanol at 60°C. in a Soxhlet apparatus, dried in vacuo, and analyzed. All transfers were made quantitatively so that the recovery of protein could be determined.

Essentially the same results were obtained whether the protein was incubated in a 55°C bath for 18 minutes or in a 45°C bath for 80 minutes (Table 3). In the latter case, the reprecipitated proteins had the same concentration of the acidic derivative as the original protein but only 26 per cent as high a concentration of the neutral derivative. The concentrations of the neutral and acidic derivatives in the residual protein were 41 and 56 per cent, respectively, of those in the original protein. The over-all recoveries of the hydrocarbon derivatives in the two fractions were 81 per cent for the neutral derivative and 63 per cent for the acidic derivative. These recoveries were increased to 60 and 90 per cent, respectively, by analysis of the combined aqueous and ethanolic solutions obtained in the preparation and washing of the protein fractions. The solubilized derivatives were about equally distributed between the aqueous and ethanolic solutions, and about half of those in the aqueous solutions could not be extracted prior to alkaline hydrolysis. Although some of the derivatives of 3,4-benzpyrene were solubilized by these treatments, the retention of very significant quantities by the reprecipitated proteins is strong evidence in favor of a chemical linkage between derivatives of the carcinogen and the epidermal protein. It should be noted that these conditions were drastic enough to degrade 20-25 per cent of the protein to fragments no longer precipitable with trichloroacetic acid, and there is suggestive evidence that the fluorescent derivatives were attached to some of these fragments.

With more rigorous conditions the yield of fluorescent derivatives remaining on the protein decreased, but even after 60 minutes in an 80°C. bath the concentration of the acidic derivatives on

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>Neutral derivative</th>
<th>Acidic derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-Benzpyrene-treate</td>
<td>None</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Benzene</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Ethyl ether</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Pyridine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Bensene-treated</td>
<td>None</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Benzene</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ethyl ether</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Pyridine</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

* Sixty mg. of protein extracted with three 50-ml. changes of boiling solvent over a 10-hr. period.

† Peroxide-free.
the reprecipitated proteins was 50 per cent of that in the original protein (Table 3). However, the neutral derivative could just be detected in the reprecipitated protein, and neither fraction was found in the residual protein. In this case 55 per cent of the protein was degraded to a size which was no longer precipitated with 10 per cent trichloroacetic acid. When an 80° C. bath was used for 30 minutes, the concentration of the acidic derivative on the reprecipitated protein was 85 per cent of that on the original sample. Although the levels were low, the neutral derivative could be detected in the reprecipitated protein, and both types could be detected in the residual protein. Thirty-four per cent of the protein was not recovered in this experiment.

In all these experiments the total protein recovery was essentially identical for the epidermal proteins from mice treated with either benzene or 3,4-benzpyrene in benzene solution. However, the yield of reprecipitated protein was consistently greater and the amount of residual protein consistently less when the proteins from 3,4-benzpyrene-treated mice were used (Table 8).

Preliminary experiments with Schneider's hot trichloroacetic acid extraction of nucleic acids (16) indicated that the fluorescent derivatives were probably not attached to the nucleic acid fraction of the crude protein mixture. Although the extraction procedure solubilized about 30 per cent of the original crude protein so that considerable amounts of protein as well as nucleic acid were apparently degraded, the remaining extracted protein still contained about one-third and one-half as great concentrations of the neutral and acidic fluorescent compounds as the original protein.

Content of protein-bound derivatives of 3,4-benzpyrene in various tissues.—To determine the distribution of the protein-bound derivatives of 3,4-benzpyrene in various tissues, a group of ten mice was given daily applications of 220 mg. of 3,4-benzpyrene in benzene solution on the skin of the back for 5 days, and a control group received similar applications of benzene alone. The mice were killed 20 hours after the last application, and protein preparations were made from various tissues. On analysis the epidermal protein from the treated area contained 35 and 30 units, respectively, of the neutral and acidic derivatives per 25 mg., while neither derivative could be detected in the dermal protein from the treated skin or the proteins from untreated skin obtained from the same animals (Table 4). Furthermore, no protein-bound derivatives of the hydrocarbon were found in the proteins from the kidneys, hearts, lungs, intestines, or livers from these mice.

Rate of accumulation of protein-bound fluorescent derivatives in epidermis.—A single application of a 0.2 per cent solution of 3,4-benzpyrene in benzene (approximately 300 µg.) was sufficient to give rise to appreciable levels of the protein-bound derivatives (Chart 1). Within 3 hours after the application of the carcinogen, detectable levels had been formed, and maximum levels of 20 and 10 units, respectively, of the neutral and acidic derivatives per 25 milligrams of protein were found in epidermal samples taken at 24 hours. Thereafter, the amounts of both derivatives decreased slowly. Samples from mice killed at 4 and 7 days still contained approximately one-half and one-fourth, respectively, of the maximum levels. At 2 weeks, the derivatives could just be detected; no protein-

**Table 3**

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Neutral</th>
<th>Acidic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight (mg.)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>30 min. at 45° C.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original, BP-treated</td>
<td>200</td>
<td>39</td>
</tr>
<tr>
<td>Residue, BP-treated</td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td>Reprecipitated, BP-treated</td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td><strong>18 min. at 55° C.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original, BP-treated</td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td>Residue, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td>Reprecipitated, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td><strong>50 min. at 65° C.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original, BP-treated</td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td>Residue, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td>Reprecipitated, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td><strong>60 min. at 65° C.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original, BP-treated</td>
<td>150</td>
<td>26</td>
</tr>
<tr>
<td>Residue, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
<tr>
<td>Reprecipitated, BP-treated</td>
<td>123</td>
<td>16</td>
</tr>
</tbody>
</table>

* Fourteen ml. of a 1:4.5 mixture of ethanol and 0.1 M KOH were used for each 100 mg. of protein. Samples were incubated in 150-ml. Erlenmeyer flasks, and temperatures reported are for the water bath.

† BP = 3,4-benzpyrene.
Table 4

The Distribution of Protein-bound Derivatives of 3,4-Benzpyrene in the Mouse Following Application of the Carcinogen to the Skin Once Daily for 5 Days*

(Expressed as fluorescence units per 25 milligrams of protein)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Neutral Fraction from Mice Treated with 3,4-Benzpyrene</th>
<th>Acidic Fraction from Mice Treated with 3,4-Benzpyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin from back (treated area):</td>
<td>Epidermis: 3 units, 35 units</td>
<td>Dermis: 3 units, 3 units</td>
</tr>
<tr>
<td>Skin from abdomen (untreated area):</td>
<td>Epidermis: 5 units, 4 units</td>
<td>Dermis: 5 units, 3 units</td>
</tr>
<tr>
<td>Kidney</td>
<td>3 units, 3 units</td>
<td>3 units, 3 units</td>
</tr>
<tr>
<td>Heart</td>
<td>3 units, 3 units</td>
<td>3 units, 3 units</td>
</tr>
<tr>
<td>Lung</td>
<td>3 units, 3 units</td>
<td>3 units, 3 units</td>
</tr>
<tr>
<td>Small intestine</td>
<td>3 units, 3 units</td>
<td>3 units, 3 units</td>
</tr>
<tr>
<td>Large intestine</td>
<td>3 units, 3 units</td>
<td>3 units, 3 units</td>
</tr>
<tr>
<td>Liver</td>
<td>4 units, 3 units</td>
<td>4 units, 3 units</td>
</tr>
</tbody>
</table>

*0.2 per cent solution in benzene; total dose approximately 1.1 mg. per mouse.

Miller—Protein-bound Derivatives of 3,4-Benzpyrene

bound fluorescence was found in samples collected 3 or 6 weeks after the single application of the hydrocarbon.

Considerably higher levels of the protein-bound derivatives of 3,4-benzpyrene were obtained when the carcinogen was applied on several successive applications of 50 µg. of 3,4-benzpyrene on the skin of the back 2 hours before the end of the irradiation period, while five controls were treated only with benzene. Groups of five mice treated with the carcinogen were killed 24 hours after the first, third, and fifth applications; the control mice were killed after the fifth application. The levels of the neutral derivative in the epidermal protein from each of these groups are plotted in Chart 3. A similar plot for the acidic derivative is not given, since the shapes of the curves were very similar to those for the neutral derivative. After five applications 25 mg. of epidermal protein from the mice kept in the dark contained 42 and 39 units, respectively, of the neutral and acidic derivatives. At the same time 25 mg. of epidermal protein from the mice irradiated by incandescent lamps contained only 18 and 19 units, respectively, of these derivatives, while only 5 units of each derivative were found in 5 mg. of epidermal protein from the mice maintained in the direct sunlight. In other experiments it was found that mice maintained in the diffuse light of the animal room had essentially the same levels of the fluorescent derivatives as those kept in darkness.

Attempts to produce the protein-bound derivatives in vitro.—A few preliminary studies were carried out to determine whether the protein-bound derivatives of 3,4-benzpyrene could be formed in vitro.

With daily applications of 200 µg. of 3,4-benzpyrene, the levels rose rapidly following the first five applications and maintained a plateau at approximately 40 units of each derivative per 25 milligrams of protein from the fifth to the eighth application (Chart 2). When 300 µg. were applied daily, the curve rose more steeply and plateaued at approximately the same level after the third or fourth application. Experiments of longer duration were impractical, since exfoliation of some of the treated areas began to occur following the ninth or tenth application.

The levels of both the protein-bound fluorescent derivatives were greatly decreased when the mice were kept in bright light. In this study three groups of mice were treated similarly, except that the mice of one group were kept in darkness, those of another group were directly in front of an east window (in December), and those of the third were placed 15 inches below 40-watt incandescent lamps for 7 hrs/day. In the latter case, each cage of mice was centered below a different lamp. The animals were kept in cages made entirely of ½-inch wire mesh, and only five mice were kept in each cage. Fifteen mice in each group received daily applications of 250 µg. of 3,4-benzpyrene on the skin of the back 2 hours before the end of the irradiation period, while five controls were treated only with benzene. Groups of five mice treated with the carcinogen were killed 24 hours after the first, third, and fifth applications; the control mice were killed after the fifth application. The levels of the neutral derivative in the epidermal protein from each of these groups are plotted in Chart 3. A similar plot for the acidic derivative is not given, since the shapes of the curves were very similar to those for the neutral derivative. After the irradiations, 25 mg. of epidermal protein from the mice kept in the dark contained 42 and 39 units, respectively, of the neutral and acidic derivatives. At the same time 25 mg. of epidermal protein from the mice maintained in the direct sunlight. In other experiments it was found that mice maintained in the diffuse light of the animal room had essentially the same levels of the fluorescent derivatives as those kept in darkness.

Attempts to produce the protein-bound derivatives in vitro.—A few preliminary studies were carried out to determine whether the protein-bound derivatives of 3,4-benzpyrene could be formed in vitro.

In one experiment the mice were killed, their hair clipped, and the skin from the back excised. The skins were pinned on a filter paper-covered board and kept at room temperature in a tightly covered glass dish. The filter paper was moistened with a small amount of 0.9 per cent saline solution con-
taining 0.5 per cent phenol. Twenty hours after a
single application of 300 µg. of 3,4-benzpyrene in
benzene solution, the epidermis was separated
from the dermis of each skin, and the proteins
were prepared from each fraction in the usual man-
ner. No protein-bound fluorescent derivatives
could be detected in the epidermis or dermis from
the mice treated either with 3,4-benzpyrene or
with benzene alone. Eviscerated dead mice were
used in the second series. To reduce bacterial con-

amination, the inside of the body cavity was cov-
ered with a penicillin ointment, the cavity was
filled with glass wool, and the opening was sutured
and covered with the ointment. The mice were
kept in tightly covered glass dishes as before, and
given two applications of 300 µg. each of 3,4-benz-
pyrene. Twenty-four hours elapsed between treat-
ments, and the mice were killed 20 hours after the
second application. Groups of six such mice were
kept in the dark or in bright sunlight, and no pro-
tein-bound fluorescent derivatives could be de-
tected in the epidermis from either the hydrocarbon-
treated or control mice. Both experiments
were complicated by obvious contamination with
microorganisms, but this appeared to be consider-
ably less in the second series.

Analyses for protein-bound fluorescent derivatives
following the application of croton oil or 20-methyl-
cholanthrene.—As evidence that the fluorescent
compounds were derived from 3,4-benzpyrene rather
than being a nonspecific hyperplastic re-
sponse, several mice were treated daily for 5 days
with benzene solutions containing 0.5 per cent of
croton oil, 0.2 per cent of 20-methylcholanthrene,
or 0.2 per cent of 3,4-benzpyrene. Croton oil was
used since it is noncarcinogenic but causes an in-
tense hyperplastic response, while 20-methylcho-
lanthrene is a more potent skin carcinogen and
hyperplastic agent than 3,4-benzpyrene but emits
only one-third to one-fourth as much fluorescent
light as the latter carcinogen (11). On analysis
both the neutral and acidic extracts from 25 mg. of
the epidermal proteins from control and croton oil-
treated mice contained about 2 fluorescence units,
while the extracts from 3,4-benzpyrene-treated
epidermis contained about 35 units each. The neu-
tral and acidic extracts from 25 mg. of epidermal
protein from the mice treated with 20-methylcho-
lanthrene each contained 4 fluorescence units; a
weak fluorescence might be expected in this case if
protein-bound derivatives of 20-methylcholan-
threne were formed in the treated epidermis.

DISCUSSION

Although final proof will rest with the character-
ization of the fluorescent compounds from mouse
epidermis, the evidence presented here suggests

strongly that the compounds are derivatives of
3,4-benzpyrene which were combined chemically
in vivo with the epidermal protein. Their relation-
ship to 3,4-benzpyrene is indicated by the rapid
appearance of the derivatives after only one appli-
cation of the carcinogen and by the lack of this
response following the application of 20-methyl-
cholanthrene or croton oil, which produce a similar
or greater hyperplastic response but are much less
fluorescent. The derivatives can be differentiated
from 3,4-benzpyrene by their different solubility properties, and, furthermore, the fluorescence of 3,4-benzpyrene solutions is quenched to a much greater extent by dissolved oxygen than the fluorescence of solutions of the derivatives. The evidence that the fluorescent derivatives are combined chemically with the epidermal protein rests in part on the isolation of a hot ethanol-extracted crude protein fraction from the epidermis prior to analysis. Further evidence was obtained by the lack of extraction of the derivatives from the protein by boiling solvents, and even stronger evidence was furnished by the finding of a high concentration of the acidic derivative and significant quantities of the neutral derivatives on protein which had been dissolved in weak alkali and reprecipitated with trichloroacetic acid. Further studies will be necessary to determine the relationship of these derivatives of 3,4-benzpyrene to the metabolites found by the British workers (15, 17–19) following the application of the carcinogen to mouse skin in vivo or in vitro. While they were unable to extract these metabolites from skin unless the tissue was first hydrolyzed with alkali, the nature of the tissue components with which the hydrocarbon was combined has not been reported.

Although the role of the 3,4-benzpyrene derivatives in the carcinogenic process cannot be properly assessed at this time, several observations suggest that there might be a causal relationship between the formation of these derivatives and the eventual appearance of tumors. First, following topical application of the carcinogen the protein-bound derivatives were found only in the epidermis of the treated area; this is the site at which tumors eventually arise. The derivatives were not found in the dermal portion of the treated skin, in the skin from untreated areas, or in any other tissue that was analyzed. Secondly, a single application of approximately 20 μg of 3,4-benzpyrene per square centimeter of skin gave rise to very significant levels of the protein-bound derivatives; and Boutwell, Brush, and Rusch have shown that a single application of about this size induces papillomas in approximately 30 per cent of the mice of this stock if the hydrocarbon treatment is followed by repeated applications of the noncarcinogenic irritant, croton oil, for 200 days. Following 45 semiweekly applications of the carcinogen, about 67 per cent of these mice develop carcinomas at the site of treatment within 235 days. Thirdly, the decreased levels of the protein-bound derivatives of 3,4-benzpyrene in the epidermis from mice exposed either to sunlight or to incandescent lights are paralleled by the lower incidence of skin tumors in mice kept under similar conditions. Thus, Doniach and Mottram (3) found a marked inhibition of the formation of papillomas and carcinomas in mice treated topically with 3,4-benzpyrene when the mice were exposed to sunlight rather than being maintained in the dark, and Morton, Luce-Clausen, and Mahoney (18) obtained a similar decrease in tumor incidence when the mice were exposed to fluorescent lamps. Both the decreased levels of the 3,4-benzpyrene derivatives and the lower tumor incidence may be the result of photo-oxidation of a part of the carcinogen, so that the effective dose for the mice exposed to light was lower than for those maintained in the dark. If so, the binding of the hydrocarbon to the epidermal protein and the carcinogenic reaction are probably not mediated through surface oxidation of the hydrocarbon. Rather, it would appear that the hydrocarbon is taken into the cells and then metabolized to a derivative which unites with the proteins within the cells, and, possibly, is involved in the initiation of the carcinogenic process.

The means by which a reaction between derivatives of a carcinogen and cell protein might initiate a carcinogenic process can be visualized in the following manner. If the various proteins of the cell were attacked in a more or less random manner, some cells would undoubtedly lose so many or such vital proteins that the cells would die. However, among those cells which survived, a few might retain the capacity for growth but lose the capacity to respond to the growth controls of the body; these cells would be classed as tumor cells. Whether the proteins so affected were in nuclear genes, cytoplasmic genes (plasmagenes), or enzymes would seem to be of little importance so long as they were autosynthetic; in this way, the capacity for unlimited growth would be transmitted upon cell division. Furthermore, precancerous cells might arise by deletion or alteration of only a part of these critical proteins. If, in subsequent cell divisions, the rate of duplication of the remaining critical proteins did not keep pace with the rate of cell division, a few cells might eventually arise which completely lacked these proteins and might hence become the initial tumor cells. Such concepts as these can apply to any carcinogen and have already been discussed in more detail in connection with our studies on the protein-bound derivatives of the hepatic carcinogen 4-dimethylaminoazobenzene (8, 9, 14).

SUMMARY

1. Crude epidermal protein preparations from the skin of mice treated with benzene solutions of
3,4-benzpyrene contain fluorescent substances which appear to be derived from the carcinogen and combined through chemical bonds with the protein. The bound fluorescent derivatives were not removed from the epidermal protein preparations by exhaustive extraction with boiling solvents, and a high concentration of an acidic derivative and significant quantities of a neutral derivative were found on proteins which had been dissolved in dilute alkali and reprecipitated with trichloroacetic acid.

2. Both the neutral and acidic fractions could be extracted with benzene following alkaline hydrolysis in the presence of zinc dust, while only the acidic derivative was obtained when the zinc dust was omitted. Both derivatives were more polar than 3,4-benzpyrene, and the fluorescence of their benzene solutions was less sensitive to quenching by dissolved oxygen than the fluorescence of solutions of the carcinogen.

3. The protein-bound derivatives were found only in the epidermal fraction of 3,4-benzpyrene-treated skin. No protein-bound derivatives were found in the dermal portion of 3,4-benzpyrene-treated skin or in untreated skin or in kidney, heart, lung, liver, or intestine from benzene- or 3,4-benzpyrene-treated mice.

4. A single application of 300 µg. of the carcinogen gave rise to detectable levels of the bound derivatives within 8 hours and a maximum of 10 to 20 units of each derivative at 24 hours. Thereafter, the level dropped slowly; one-fourth of the maximum level was still present after 1 week, and none could be detected at 3 weeks. When 200 µg. of 3,4-benzpyrene was applied daily, a maximum level of about 40 units of each derivative was reached after four or five applications. If the mice were kept in direct sunlight or directly under incandescent lamps, the level of each derivative was reduced to one-tenth to one-third of that found in the skin of mice maintained in the dark or in diffuse light.

5. The protein-bound derivatives were not found following one or two applications of 3,4-benzpyrene to the skin of freshly killed mice.

6. Although preliminary, the data are consistent with the idea that the binding of 3,4-benzpyrene (or a derivative) with the epidermal protein might be involved in the genesis of skin tumors.

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Studies on the Formation of Protein-bound Derivatives of 3,4-Benzpyrene in the Epidermal Fraction of Mouse Skin

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