The Disappearance of Carcinogenic Hydrocarbons in Autoxidizing Lipids*

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Previous investigations from this laboratory have demonstrated that carcinogenic chemicals inhibit the autoxidation of aldehydes and lipoidal materials (4, 5, 15, 19). The presence of 3,4-benzpyrene in these oxidizing media resulted in the production of brown substances that were not attributable to derivatives of the lipid, and presumably originated in the oxidative degradation of the hydrocarbon. Studies have now been made on the rate of disappearance of certain carcinogenic hydrocarbons dissolved in lipids of varying degrees of autoxidizability. In addition, two colored compounds were identified in autoxidized lipid mixtures containing 3,4-benzpyrene.

METHODS

Standard lipid samples containing the carcinogens were prepared by transferring known quantities of these materials, dissolved in acetone or Skelly solve B, 1 to individual test tubes. In certain tubes 200 ~gm. of a,ß-tocopherols were added in the same manner. After all additions the volatile solvent was removed by evaporation in a vacuum desiccator. A typical test sample contained 5 mgm. of lipid and 50 ~gm. of hydrocarbon. The mixtures were then allowed to autoxidize in air at room temperature in the absence of light. At intervals duplicate samples were diluted with redistilled acetone to the proper hydrocarbon concentration, and analyzed fluorometrically for remaining hydrocarbon (12). No fluorescence was noted in the diluted lipid samples alone during the experiments, nor did the presence of lipid, fresh or oxidized, affect the fluorescence of the hydrocarbons. Hence the difference between the analyses at zero time and after a given period of autoxidation was considered to represent the amount of hydrocarbon destroyed.

The ethyl linolate was prepared by the method of Rollett (14) and further purified by distillation in vacuo, and the linoleic acid was obtained by the hydrolysis of this ester. The acid was a colorless liquid with an iodine number of 178. Cottonseed oil was largely freed from antioxidants by passing a solution of the edible commercial oil in Skelly solve B through separate columns of activated alumina and neutral-52 (17). Final purification was effected by molecular distillation. Mouse fat was extracted from minced eviscerated mouse carcasses with Skelly solve B in a Waring blender; the crude viscous oil remaining after evaporation of the solvent in vacuo was used for these experiments. A lard filtrate was obtained by the filtration of fresh commercial leaf lard through qualitative filter paper at 38° C. The semi-solid material remaining on the filter is termed the lard residue (10). The tocopherol sample was a purified mixture of a and ß tocopherols obtained from Merck and Co. The carcinogenic hydrocarbons 3,4-benzpyrene, 20-methylcholanthrene, and 1,2,5,6-dibenzanthracene were Hoffmann-LaRoche products.

RESULTS

When 3,4-benzpyrene was added to linoleic acid and the mixture allowed to autoxidize at room temperature it gradually became golden brown. Analysis of the samples at intervals throughout the period of autoxidation demonstrated a progressive destruction of the carcinogen. In a typical experiment 35 per cent of the benzpyrene was destroyed during the first week, and 74 per cent in 8 weeks (Table I). The addition of 200 ~gm. of a,ß-tocopherol to similar samples delayed the destruction of benzpyrene. Thus during the first 4 weeks only 1 per cent of the carcinogen was destroyed, as compared to 66 per cent in the sample without tocopherol. However, after this latent period a rapid disappearance of the hydrocarbon was observed, which, after 8 weeks, approached that

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1 Obtained from the Filtrol Corporation, Los Angeles, California.

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occurring in the control samples. Benzyrene was also destroyed in antioxidizing ethyl linolate, but at a slower rate than when dissolved in linoleic acid. In this mixture the addition of tocopherol also decreased the rate of destruction, but the effect was much less pronounced than in the experiment with linoleic acid. These observations are compatible with the slower autoxidative rate of ethyl linolate as compared to the free acid. When ethyl oleate was used as the solvent the disappearance of benzyrene occurred at a still slower rate. Thus only 15 per cent of the hydrocarbon was destroyed after 8 weeks of antioxidation, and the addition of tocopherol had only a slight influence on the rate of destruction.

For the separation of the colored compounds formed in oxidized mixtures of linoleic acid and benzyrene it was necessary to work with larger quantities. Mixtures of 5 ml. of pure linoleic acid and 5 mgm. of benzyrene were allowed to autoxidize in petri dishes at room temperature for 4 to 7 days. Considerable color developed during this period, without any obvious changes in the physical properties of the linoleic acid. The mixture was dissolved in benzene and adsorbed on 0.7 × 50 cm columns of activated 100 mesh aluminum oxide. The columns were standardized with a mixture of the pure 5,8- and 5,10-quinones of benzyrene obtained by chronic acid oxidation (18). The presence of linoleic acid interfered to some extent with the adsorption of the colored substances by the activated alumina; nevertheless a distinct separation of the compounds was obtained by repeating the chromatographing procedure 3 times. The third adsorption of the oxidized mixture separated the colored substances into an upper red band, which faded into a lower yellow band. Homogeneous sections of these bands were removed and eluted with acetone. The identities of these compounds were established through their adsorption characteristics and absorption spectra. Mixtures of each compound with the corresponding authentic quinone in benzene were adsorbed on columns of alumina, and found to yield single bands that gave no evidence of separation after traversing a distance of 40 cm. The absorption spectra of the acetone eluates were determined with a Cenco-Sheard spectrophotometer. An absorption maximum of approximately 460 mμ was observed with the red compound, while the yellow derivative exhibited maxima at approximately 430 and 450 mμ. The spectra of the known quinones corresponded closely to the curves ob-

### Table 1: The Disappearance of Carcinogenic Hydrocarbons in Autoxidizing Lipids

<table>
<thead>
<tr>
<th>Hydrocarbon, per cent hydrocarbon destroyed by</th>
<th>Lipid, 50 μg.</th>
<th>α,β-Tocopherols, 200 μg.m.</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4-Benzpyrene</td>
<td>Linoleic acid</td>
<td>—</td>
<td>35.0</td>
<td>74.0</td>
</tr>
<tr>
<td></td>
<td>Ethyl linolate</td>
<td>+</td>
<td>0.6</td>
<td>67.0</td>
</tr>
<tr>
<td></td>
<td>Ethyl oleate</td>
<td>+</td>
<td>7.0</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>Cottonseed oil</td>
<td>(chromatographed)</td>
<td>—</td>
<td>72.0</td>
</tr>
<tr>
<td></td>
<td>Mouse carcass fat</td>
<td>—</td>
<td>0.0</td>
<td>72.4</td>
</tr>
<tr>
<td></td>
<td>Lard filtrate</td>
<td>—</td>
<td>0.0</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>Lard residue</td>
<td>—</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Tricaprylin</td>
<td>—</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>20-Methylcholanthrene</td>
<td>Linoleic acid</td>
<td>—</td>
<td>84.0</td>
<td>94.0</td>
</tr>
<tr>
<td>1,2,5,6-Dibenzanthracene</td>
<td>Linoleic acid</td>
<td>—</td>
<td>91.8</td>
<td>93.5</td>
</tr>
<tr>
<td></td>
<td>Linoleic acid</td>
<td>—</td>
<td>92.2</td>
<td>94.0</td>
</tr>
</tbody>
</table>

When chromatographed cottonseed oil was employed as the solvent, the rate of disappearance of 3,4-benzpyrene was relatively slow, so that after 8 weeks only 22 per cent had disappeared. Similarly, only 10 per cent of the hydrocarbon was destroyed when dissolved in fat obtained from the mouse carcasses. The disappearance of benzyrene was even less rapid when tricaprylin, lard filtrate, and lard residue were employed as solvents. When these lipids were used, only 5 to 7 per cent of the carcinogen disappeared during the period of oxidation (Table 1).

20-Methylcholanthrene was found to be more rapidly destroyed than 3,4-benzpyrene in oxidizing linoleic acid while 1,2,5,6-dibenzanthracene proved to be relatively stable in this medium (Table 1). Eighty-four per cent of the methylcholanthrene disappeared in the first week of antioxidation and the originally colorless mixture became light green. The high stability of dibenzanthracene in this experiment agrees with its strong resistance to attack by chemical and physical agents (6, 11).
tain with the autoxidation derivatives. Thus it appears that in the presence of oxidizing linoleic acid benzpyrene is oxidized to the 2 known quinones. At any instant the quantity of quinones that could be isolated from such mixtures represented only a small portion of the amount of hydrocarbon found to have been destroyed. Furthermore, considerable dark unidentified material, which was derived from the hydrocarbon, was found adsorbed at the top of the chromatograph columns. Since the quinones also have been found to be rapidly destroyed in the presence of oxidizing linoleic acid (13), these substances are probably derived from the first stages in the oxidative destruction of benzpyrene.

DISCUSSION

The experiments above demonstrate that both 3,4-benzpyrene and 20-methylcholanthrene are destroyed when present in autoxidizing fats. In the case of benzpyrene, there is a coincident formation of 3,4-benzpyrene-5,8-quinone and 3,4-benzpyrene-5,10-quinone, plus unidentified substances. Apparently these oxidation products are the result of a coupled oxidation similar to that taking place when carotene (16), p-dimethylaminobenzene (7), or hemin (9) are destroyed in the presence of oxidizing fats. Part of the destruction of hydrocarbon that occurs after application to tissues may be due to a similar oxidation occurring in the vehicle employed or in the tissue lipids themselves.

Berenblum, Chalmers, and their associates (1, 2, 3) have shown that 3,4-benzpyrene is oxidized in vivo, presumably to 8-hydroxy-3,4-benzpyrene, which is readily oxidized in air to 3,4-benzpyrene-5,8-quinone. These derivatives were isolated from the excreta of rats and mice injected with the hydrocarbon. Thus the oxidations of benzpyrene in vivo and in vitro have a common product and possibly a common point of attack. These oxidation products of benzpyrene have proved to be noncarcinogenic (2), and application of the intact hydrocarbon seems necessary for the production of tumors (8). Possibly the reactions that lead to the formation of these derivatives are involved in the carcinogenic process.

SUMMARY

The rates of destruction of 3,4-benzpyrene, 20-methylcholanthrene, and 1,2,5,6-dibenzanthracene were followed fluorometrically after these hydrocarbons had been added singly to various lipids and the mixtures exposed to air at room temperature for several weeks. Benzpyrene and methylcholanthrene were rapidly destroyed in oxidizing linoleic acid, and losses of 35 and 84 per cent respectively were noted after 1 week. 1,2,5,6-dibenzanthracene, on the other hand, was relatively stable to this oxidizing agent. Benzpyrene disappeared also, though less rapidly, in ethyl linolate, ethyl oleate and chromatographed cottonseed oil. The addition of tocopherol to certain of these mixtures delayed the onset of the destruction but did not alter the final result. Benzpyrene was relatively stable when dissolved in tricaprylin, lard fractions, or mouse carcass fat.

The isolation of 2 of the colored oxidation products of benzpyrene was accomplished by chromatographic adsorption of an autoxidized benzpyrene-linoleic acid mixture on activated alumina. These compounds were identified as 3,4-benzpyrene-5,8-quinone and 3,4-benzpyrene-5,10-quinone by mixed chromatographs with the authentic quinones and by their absorption spectra.

REFERENCES


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