Fractionation of Skin Tumor-initiating Activity in Coal Liquids

D. Dennis Mahlum, Cherylyn W. Wright, Edward K. Chess, and Bary W. Wilson

ABSTRACT

Narrow temperature range distillates from biologically active solvent refined coal-I and -II heavy-end coal liquids were fractionated according to chemical class and assayed for initiation of skin carcinogenesis in CD-1 mice. In addition, instrumental chemical analyses were performed on the distillates and their chemical fractions. Results showed that initiation activity in these complex fuel mixtures could be segregated both by boiling point and chemical class. Neutral polycyclic aromatic hydrocarbon fractions were the most active of the chemical classes, with some initiating activity being shown by nitrogen-containing polycyclic aromatic hydrocarbon. Aliphatic and hydroxylated polycyclic aromatic hydrocarbon fractions showed little or no initiating activity. For the two solvent refined coal-II distillates studied, initiating activity was substantially higher in the material boiling above 850°F than in that boiling 800–850°F, although both contained essentially the same concentrations of benzo[a]pyrene. These data indicate that the overall initiating activity of these complex mixtures is highly dependent on interactions of the many chemical carcinogens and that relative concentrations of known carcinogenic polycyclic aromatic hydrocarbons, such as benzo[a]pyrene and dimethylbenz[a]anthracene, are not the sole determinants of initiating activity.

INTRODUCTION

Studies have shown that the biological activity of liquids derived from direct coal liquefaction processes increases as the b.p. increases (4, 13, 14, 17, 21). For example, Pelroy et al. (17, 21), studying the mutagenic activity of liquids derived from both SRC-I and -II processes, found essentially no activity in distillates boiling below 700°F. However, substantial activity was found in distillates with b.p. above 700°F, with peak activity occurring around 800°F for SRC-I and at >850°F for SRC-II materials. Cell transformation studies with SHE cells (4) and forward mutation studies conducted with CHO cells (15), using many of the same materials studied by Pelroy et al. (17, 21), found low levels of activity below 600°F, with significantly higher activity in distillation distillates boiling above 700°F. The highest activity in the mammalian cell assays was observed in material boiling above 850°F. Mahlum (14) also studied several SRC-I and -II distillates for skin tumor-initiating activity and found that, in general, initiating activity also increased with increasing distillation temperature.

Microbial mutagenesis studies have resulted in convincing evidence implicating APAH as the determinant mutagens in coal-derived liquids. For example, Pelroy and Stewart (16) have shown that most of the mutagenic activity in high-boiling SRC coal liquids can be eliminated by treatment with nitrous acid, a procedure which converts the primary aromatic amino functionality into an aromatic hydroxy group. Chemical fractionation studies using the method of Later et al. (9, 22) have also shown that microbial mutagenic activity can be segregated into fractions enriched in APAH. Similar studies using SHE and CHO cells (11) show a somewhat different pattern of activity from that found with the Ames system. Although the fractions enriched in nitrogen-containing compounds, including APAH, contain significant activity in both the SHE and CHO cell systems, substantially higher activity was found in PAH fractions.

Both the Ames assay and mammalian cell culture systems are now being evaluated for their ability to predict the potential carcinogenicity of complex synfuel mixtures. We have, therefore, tested fractions prepared from high-boiling distillation distillates of SRC-I and -II liquids for skin tumor initiating activity to determine correlations between in vitro and in vivo studies, as well as to evaluate the relative initiating activity of the major chemical classes present in the coal liquids.

MATERIALS AND METHODS

Distillates with 50°F b.p. ranges were prepared from wide-boiling-range (300–850°F) liquids obtained from the SRC-I and -II processes. Preparation of these distillates has been described previously (21). The 800–850°F distillates from SRC-I process solvent and the 800–850°F and >850°F distillates from the SRC-II materials were separated on an alumina column according to the method of Later et al. (9) to provide 4 fractions. The first fraction (A1) contained primarily aliphatics and olefinic compounds; the second (A2), neutral PAHs; the third (A3), nitrogen-containing polycyclic aromatic compounds; and the fourth (A4), hydroxy-PAH. The weight percentage yields for each fraction from each distillation distillates are shown in Table 1.

Crude distillates and their alumina-separated fractions were analyzed by high-resolution gas chromatography and probe-inlet MS. Quantitative determinations of the PAH compounds listed in Table 2 were made by high-resolution gas chromatography using an external standard calibration program with response factors calculated from standards at 8 concentrations ranging over 2 orders of magnitude. Specific conditions for gas chromatographic analyses have been published elsewhere (10, 17).

Probe-inlet MS was used to characterize the average molecular weights of the 2 series of b.p. distillates, since many of the compounds in these materials, particularly the 850°F+ materials, cannot be determined by gas chromatography because of their low volatility. The ZAB-1F mass spectrometer (Vacuum Generators Analytical, Ltd., United Kingdom) was operated in the electron impact mode (70 eV) with an accelerating voltage of 5000 V, a magnet scan rate of 3 sec/mass decade, a source temperature of 250°C, and at a dynamic resolving power (as determined by the VG-2035 data system) of 1:2000. Each sample (10 to 20 μg) was loaded into a glass capillary tube, which was inserted into...
the source affixed to the end of a direct insertion probe. The probe was heated in a linear fashion from ambient to 250–280°C, slowly desorbing the sample from the capillary tube in what amounted to fractional distillation. The MS was scanned repetitively throughout this desorption period, and data were collected across the entire desorption profile. By using the data system to sum the intensities of each mass across the entire profile, an average spectrum representing the entire sample was generated. These averaged spectra were then used to estimate the mean molecular weight of each sample and were compared to gas chromatography data to confirm the rank order of average molecular weights assigned to the various samples.

Charles River CD-1 female mice (groups of 30) were used to determine initiating activity. They were housed 5/cage and given food and water ad libitum. At the beginning of each experiment, they were individually ear-tagged and their backs shaved. Test materials were applied to the sample from the capillary tube in what amounted to fractional distillation. The MS was scanned repetitively throughout this desorption period, and data were collected across the entire desorption profile. By using the data system to sum the intensities of each mass across the entire profile, an average spectrum representing the entire sample was generated. These averaged spectra were then used to estimate the mean molecular weight of each sample and were compared to gas chromatography data to confirm the rank order of average molecular weights assigned to the various samples.

RESULTS

Results from instrumental analyses and chemical fractionation carried out to characterize these materials are shown in Tables 1 and 2. Mass spectral data of the SRC-II materials showed that the 850°F+ distillate consisted of substantially higher molecular weight materials than did the 800–850°F distillate, with the average molecular weight of the former approximately 30 atomic mass units higher than that of the latter by these analyses. With the exception of several pericondensed PAH, such as BaP and BeP, which were distributed fairly evenly between the 2 cuts, the distillations appear to have effectuated good separation of the PAH according to molecular weight. The lower boiling distillate contained primarily compounds having 5 or fewer aromatic rings, while the 800°F+ material was composed mainly of compounds having 5 or more rings, together with some contribution from higher alkyl homologues of several 4-ringed species. Chemical class fractionation showed that the relative PAH content of the 2 distillates were essentially the same, while the nitrogen polycyclic aromatic compound content of the 850°F+ distillates was somewhat higher.

The tumorigenic responses of the positive and negative control groups are shown in Table 3. The tumor incidence for BaP rose very quickly beginning at about 50 days and reached 90% by 90 days, while the incidence in the negative control was 13% by the end of the experiment. The BaP and negative-control groups had 164 and 5 tumors (normalized to n = 30), respectively.

Table 3 and Chart 3 show the response of the skin initiated with the SRC-I 800–850°F+ distillate and its fractions. When incidence is considered (Chart 3), the animals initiated with the 800–850°F+ material began to show tumors at about 75 days, with the incidence increasing steadily thereafter. Tumor incidence among animals initiated with the PAH (A2) fraction of the 800–850°F distillate increased rapidly at about 90 days to a level above that for the crude 800–850°F+ liquid, and remained at a higher level for the duration of the experiment. A significant number of the mice initiated with the nitrogen-rich (A3) fraction developed tumors, although at a lower rate than for the A2 or the crude 800–850°F+ group. The incidences for groups initiated with aliphatic (A1) or PAH (A4) fractions were not above background. When total tumors within the group are considered...
Chart 1. Averaged probe-inlet mass spectrum of the PAH fraction from SRC-II 850°F+ distillate from coal liquid. Ions in the lower envelope of both spectra are mainly doubly charged species typical of PAH spectra. Starred isomers have been identified specifically by high-resolution gas chromatography.

Chart 2. Averaged probe-inlet mass spectrum of the PAH fraction from SRC-II 800-850°F distillate. Ions in the lower envelope of both spectra are mainly doubly charged species typical of PAH spectra. Starred isomers have been identified specifically by high-resolution gas chromatography.

(Table 3), the order of increasing response is the same as for incidence; the A2 > 800–850°F crude > A3 with the A1 and A4 groups at background. However, the difference in response between the A2 and A3 groups is emphasized when total tumor yield is considered, with about 3-fold as many tumors in the A2 as in the A3 group by the end of the experiment.

The tumor incidences for the SRC-II 800–850°F distillate and its chemical fractions are shown in Chart 4. The A2 fraction was by far the most active of the materials tested, including the 800–850°F liquid from which it was prepared. Substantial activity was again found with the A3 fraction, although the incidence was lower than with the crude 800–850°F distillate and its A2 fraction. The A1 and A4 groups had an incidence of about 25%. This was not significantly above the negative controls, but suggests the...
possibility of a low level of initiating activity. Examination of the tumor yield (Table 3) shows the same relative order of activity as determined by the tumor incidence numbers; i.e., A2 > crude 800–850°F distillate > A3 > A1 and A4.

The pattern of response for the SRC-II-850°F+ distillate and its fractions is shown in Chart 5. The incidences are very similar for the crude 850°F+ and its A2 fraction; the incidence rose rapidly about 55 days after initiation to above 90% for both groups by 90 days. The incidence in the A3 group reached about 80% by the conclusion of the experiment, although the rate at which mice showed tumors was slower than for the crude and A2 groups. In contrast to the SRC-I materials, there was also a significant incidence of mice with tumors in the A1 and A4 groups. When total tumor yield is examined (Table 3), differences between groups are even more readily apparent. The A2 fraction had the highest level, followed closely by the 850°F+ crude. The A3 fraction was significantly lower in total tumor production than the A2 fraction or the 850°F+ crude (approximately 60 tumors versus 180 to 210 for the 850°F+ distillate and A2 groups). Only a few tumors were found in the A1 and A4 groups.

**DISCUSSION**

Three high-boiling distillates, one (800–850°F+) from the SRC-I process and 2 (800–850° and 850°F+) from the SRC-II process were tested for their skin-tumor initiating activity. Of these, the SRC-II 850°F+ was the most active, as judged either by the rate at which mice first showed tumors or by the total tumor yield.

Although the other 2 distillates (SRC-I 800–850°F+ and SRC-II 800–850°F) were not matched exactly in temperature range represented, their initiating activities (tumor incidence and yield) were similar.

Chemical fractionation of the 3 distillates on alumina yielded chemically well-defined fractions, which could be evaluated independently for initiating activity. Cross-contamination among the fractions was minimal. Gas chromatographic analysis of the A2 fractions using a nitrogen-specific detector showed that essentially the only contamination was from carbazoles, which were present in total concentrations of less than 200 ppm. There

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**Table 3**

<table>
<thead>
<tr>
<th>Initiator</th>
<th>Dose (mg)</th>
<th>% of mice with tumors</th>
<th>Tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>14</td>
<td>0.17 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>BaP</td>
<td>0.05</td>
<td>97</td>
<td>5.23 ± 0.57</td>
</tr>
<tr>
<td>800–850°F+, SRC-I</td>
<td>17.0</td>
<td>76</td>
<td>2.24 ± 0.39</td>
</tr>
<tr>
<td>A1</td>
<td>0.82</td>
<td>20</td>
<td>0.30 ± 0.13</td>
</tr>
<tr>
<td>A2</td>
<td>3.23</td>
<td>88</td>
<td>3.04 ± 0.32</td>
</tr>
<tr>
<td>A3</td>
<td>6.12</td>
<td>60</td>
<td>0.76 ± 0.16</td>
</tr>
<tr>
<td>A4</td>
<td>6.80</td>
<td>12</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>800–850°F+, SRC-II</td>
<td>17.0</td>
<td>72</td>
<td>1.43 ± 0.26</td>
</tr>
<tr>
<td>A1</td>
<td>0.88</td>
<td>27</td>
<td>0.27 ± 0.08</td>
</tr>
<tr>
<td>A2</td>
<td>7.82</td>
<td>92</td>
<td>1.93 ± 0.23</td>
</tr>
<tr>
<td>A3</td>
<td>4.76</td>
<td>61</td>
<td>0.97 ± 0.20</td>
</tr>
<tr>
<td>A4</td>
<td>3.06</td>
<td>25</td>
<td>0.27 ± 0.10</td>
</tr>
<tr>
<td>850°F+, SRC-II</td>
<td>17.0</td>
<td>79</td>
<td>4.58 ± 0.62</td>
</tr>
<tr>
<td>A1</td>
<td>0.17</td>
<td>43</td>
<td>0.93 ± 0.34</td>
</tr>
<tr>
<td>A2</td>
<td>7.82</td>
<td>100</td>
<td>6.88 ± 0.59</td>
</tr>
<tr>
<td>A3</td>
<td>6.12</td>
<td>83</td>
<td>2.07 ± 0.30</td>
</tr>
<tr>
<td>A4</td>
<td>4.25</td>
<td>41</td>
<td>0.40 ± 0.12</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*
were no detectable hydroxylated compounds in the A2 fractions. The A2 fractions did, however, contain low concentrations of neutral sulfur- and oxygen-containing heteroatomic polynuclear compounds.

When the alumina-derived fractions were tested for initiating activity, the neutral-PAH fraction consistently showed the highest activity. Moreover, the activity was as great, or greater, than that of the crude distillates from which they were derived. The A3 fractions, containing nitrogen polycyclic aromatic compounds such as azaarenes, aromatic amines, and carbazoles, also showed initiating activity, but the activity was always substantially below that of the corresponding A2 fraction. The A3 fractions of higher-molecular-weight coal liquids boiling above 800°F contained trace amounts of BaP, which had b.p.s within the temperature range of the cut. These results strongly support the concept that the neutral PAHs are the major determinants of skin-tumor-initiating activity in these distillates.

The 850°F+ distillate and its A2 fraction are significantly more active than the 800–850°F distillate and its A2 fraction, but the identities of the components responsible for this difference are unknown at this time. Although BaP has been suggested as a dominant carcinogen in high-boiling coal liquids, examination of Table 2 shows that the BaP contents of the A2 fractions from the 800–850°F and 850°F+ distillates are essentially identical. This indicates that BaP is not the determinant factor in the initiating activity of these A2 fractions and that other factors must be considered. This observation is consistent with our earlier finding that the initiating activity of solvent-separated fractions from SRC-II heavy distillate did not correlate with their BaP content (6). In the case of these solvent-extracted fractions, the tar fractions, which contained between 10 and 50 ppm of BaP, were far more active than the PAH fraction, which contained about 1000 ppm of BaP. These data suggest that the coal-liquid matrix in which a compound such as BaP is administered may have an important influence on the expression of its carcinogenicity.

The data in Table 2 indicate that the overall levels of other known carcinogenic PAH such as DMBA and methylycrysene are as high or higher in the 800–850°F as in the 850°F+ A2 fraction, although the 800–850°F A2 fraction is substantially less active as an initiator of skin tumorigenesis. This observation suggests that components that have little or no carcinogenic activity are influencing the expression of the activity of strong carcinogens such as BaP and DMBA and that these components may be as important in determining the response to the complex mixtures as the carcinogenic constituents themselves. It must be recognized that these quantitative chemical analyses are limited to a relatively few PAH and that an extremely active but unidentified carcinogen could be present in the 850°F+ A2. It seems more likely, however, that there are certain components in the 800–850°F A2 fraction, which prevent complete expression of its carcinogenic constituents. Some credence is lent to this suggestion by the results of a number of studies (3, 5–8, 19) that have shown that the simultaneous administration of 2 or more PAH may result in the inhibition of carcinogenesis. Thus, Falk et al. (3, 19) found that benzantracene and benzofluoranthene inhibited the carcinogenic effects of BaP and dibenz[a]anthracene. Similarly, Hill et al. (5, 6) found that a number of PAH including 6,8-DMBA, benzanthracene, BaP, dibenzanthracene, and methylcholanthrene decreased the tumorigenic activity of DMBA. It has also been reported that tumor initiation by DMBA is inhibited by phenanthrene (8) and dibenzanthracene (18). Investigations by Lijinsky et al. (12) showed that several hydrogenated derivatives of dibenz[a,h]anthracene decreased the tumor response to the parent compound. Additional discussion of the interactions of PAH in the carcinogenic process may be found in reviews by DiGiovanni and Slaga (1, 2). The 800–850°F A2 fraction in our study contained about 3,000 ppm of benz[a]anthracene and 13,500 ppm of benzo(a)pyrene, compared to less than detectable amounts of benz[a]anthracene and 2,700 ppm of benzo(a)pyrenes in the 850°F+ A2 fraction. If these materials in the complex milieu used in this study inhibit tumor initiation by BaP, DMBA, methylchlysene, and other carcinogens, it might be expected that their effects would be greater in the 800–850°F-derived material, since the concentrations are greater in it than in the 850°F+ A2 fraction.

While certain components of these complex mixtures may be suspected of inhibiting the carcinogenic expression of other components, there may also be compounds which increase the activity of carcinogens. Benzo[ghi]perylenes and BeP, for example, have been reported by Van Duuren and Goldschmidt (20) and others (2) to enhance the carcinogenic activity of BaP. Although the BeP levels are similar for the 800–850°F and 850°F+ A2 fractions, there is far more benzo[ghi]perylenes in the 850°F+ A2 fraction which could enhance the activity of this fraction.

We can only speculate at this time about the biological interactions of the many components of these coal-derived liquids. However, the alternatives raised here lend themselves to experimental resolution. It should be possible using mixing experiments to determine whether certain components of the 800–850°F A2 fraction such as benzanthracene and benzofluoranthrene indeed inhibit the expression of its carcinogenic constituents or whether the benzo[ghi]perylenes contained in the 850°F+ A2 enhance the activity of BaP and other carcinogens.

There was a small amount of initiating activity in the A1 (aliphatics and olefins) and A4 (hydroxy-PAH) fractions from the SRC-II 850°F+ distillate. These fractions did not show significant activity when they were derived from the lower-boiling distillates. Examination of the chemical constituents in the A1 and A4 fractions from the SRC-II 850°F+ distillates does not readily reveal the components responsible for the initiating activity.

Another purpose of this study was to provide skin tumor data on a number of complex materials which could be used for comparison with Ames and mammalian cell culture data. Data obtained with SHE and CHO cells (4, 15) with these distillates and their chemical fractions correlated very well with initiation results. The SRC-II 850°F+ distillate was the most active in both mammalian cell systems and in all cases the A2 fraction was more active than the A3. As with the initiation/promotion system, the mammalian cells exhibited detectable but lower activity for the A1 and A4 fractions from the 850°F+ distillates. A low level of mammalian cell activity was also found in the A1 fraction from SRC-I 800–850°F+ distillates, although no initiating activity was found.

Results from the Ames system correlate less well, both qualitatively and quantitatively with initiation/promotion results, than do those from the mammalian cell culture studies. In the case of the SRC-I distillation distillates, for example, the microbial mutagenesis activity appears to peak in the 750–800°F boiling range (21). In the Ames test, the A3 fractions from these distillates were extremely active compared to the A2 fractions. The
Ames system detected only slight activity in the A2 fractions, and it appears to have relatively little sensitivity in general to neutral PAH in complex mixtures. A modification of the Ames assay, called the fluctuation test, is more sensitive to PAH, but the amount of fluctuation-test data available is presently limited. Examination of microbial-mutagenesis data obtained with a number of synfuel materials suggests that the Ames system agrees qualitatively with skin tumorigenesis results when crude materials are tested (15). However, there is less agreement between microbial mutagenesis and whole-animal results when chemically separated fractions are tested. This observation appears to relate to the high sensitivity of the Salmonella tester strains to APAH and its relative insensitivity to complex mixtures of neutral PAH.

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