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TECHNICAL REPORT

TUMORS INDUCED IN C_3H/HeJ MICE BY COAL TAR NEUTRAL SUBFRACTIONS

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Center for Disease Control
National Institute for Occupational Safety and Health

TUMORS INDUCED IN C_3H/HeJ MICE

BY COAL TAR NEUTRAL SUBFRACTIONS

Donald E. Richards

William P. Tolos

Jag B. Lal

Division of Biomedical and Behavioral Science

Charles V. Cooper

Division of Physical Sciences and Engineering

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ABSTRACT

The biologic activity of seven coal tar neutral subfractions was investigated by repeated applications on mouse skin. Four of the subfractions induced skin tumors--identified histopathologically as squamous cell papillomas or squamous cell carcinomas--in one or more mice in their respective test groups. Pulmonary metastases from a squamous cell carcinoma of the skin were seen in one mouse. The two subfractions that induced squamous cell carcinomas were shown by gas chromatographic-mass spectrometric analyses to contain many 4- and 5-ring polynuclear aromatic hydrocarbons, some of which are known to be carcinogenic in mouse skin.

CONTENTS

INTRODUCTION

In FY 1972, the National Institute for Occupational Safety and Health undertook a series of studies designed to develop toxicity data that could provide a basis for the assessment of hazards associated with human exposure to organic emissions from the coking process. As a test material for these studies, a composite coal tar, which was prepared by blending condensed, high-boiling coke-oven effluents from several different coking ovens in the greater Pittsburgh area, was obtained from a commercial source. The coking ovens from which the coal tar was obtained were of different designs and used coal from different sources as their starting materials. The composite coal tar thus served as a model material, typical of complex organic mixtures found in coke oven emissions.

This composite coal tar sample was tested for biologic activity using the mouse-skin bioassay (1,2). Results of these studies showed that the coal tar stimulated neoplastic proliferation in the skin of mice of the C_3H/HeJ strain and that a dose-response relationship existed. In a subsequent investigation (3), a portion of the composite coal tar was separated by chemical means into coal tar acids, coal tar bases, and a neutral fraction. The neutral fraction was tested in NIOSH laboratories and was found to be as biologically active in mouse skin as the composite coal tar. A 2-kg quantity of the neutral fraction was then separated into 16 subfractions by

chromatographic techniques (4). These subfractions were characterized by spectroscopic analysis and gas chromatography.

The purpose of the investigation described in this report was to screen all 16 coal tar neutral subfractions for biologic activity by the mouse-skin bioassay. However, it was possible to test only seven of the subfractions prior to the expiration of the project. The results of the screening tests on the seven subfractions are described in this report.

MATERIALS AND METHODS

TEST MATERIALS

The following subfractions, as characterized by spectroscopic analysis and gas chromatography (5), were evaluated for biologic effect:

Subfraction 2 - Approximately 50% naphthalene.

Subfraction 3 - Probably a mixture of naphthalene, substituted naphthalenes, and anthracene.

Subfraction 6 - Possibly substituted 3-ring systems with some 4-ring compounds.

Subfraction 7 - Possibly pyrene, phenanthrene, and substituted 3-ring compounds.

Subfraction 9 - Mostly condensed 4-ring systems, with possibly benz(a)anthracene and/or chrysene as major components.

Subfraction 10 - Probably 5-ring systems and higher. Possibly benzo(a)pyrene.

Subfraction 13 - Possibly polar polynuclear compounds.

These seven test materials were also analyzed qualitatively in NIOSH laboratories by combined gas chromatographic-mass spectrometric techniques. The gas chromatograph-mass spectrometer system consisted of a Perkin-Elmer

Model 900 gas chromatograph interfaced through an all-glass, single-stage jet separator to a differentially pumped DuPont Model 21-492B double-focusing mass spectrometer. The gas chromatograph was equipped with either a 3-meter x 2.1-mm i.d. stainless steel column packed with 5% Dexsil 400 on 100/120-mesh Supelcoport, or a 33-meter x 0.5-mm i.d. SCOT glass capillary column coated with SE-30. Helium was used as the carrier gas at flow rates of 30 cc/min and 5 cc/min, respectively, through the packed column and the capillary column. Make-up helium was introduced after the capillary column at a flow rate of 28 cc/min. The temperature of the gas chromatograph oven was programmed at 6°C/min or 8°C/min over a range which varied with the sample, as shown in Tables 1 through 7. Electron impact mass spectra were obtained for each sample component as it eluted from the gas chromatograph and entered the ion source of the mass spectrometer. The ion source pressure was maintained at 1×10^{-5} torr. The mass spectrometer, operating with an accelerating voltage of 3.5 kV and an electron energy of 70 eV, was scanned from 15 to 1000 amu at 2 sec/decade. The resolution of the mass spectrometer, $M/\Delta M$ with a 10% valley, was 2000 at the operating source pressure. A DuPont Model 21-094B data system was used to acquire and process all gas chromatographic-mass spectrometric data.

Compound identifications were made by matching mass spectra of the chromatographed components of the subfractions with spectra of authentic compounds in the DuPont mass spectral library and in the Registry of Mass Spectral Data (6). However, differentiation between certain isomers is not possible by such comparisons alone, and uncertainty exists in some identifications. The compounds identified in each of the seven subfractions are given in Tables 1 through 7.

ANIMALS AND TREATMENTS

Young adult male mice of the C_3H/HeJ strain, weighing 18 to 22 g, were obtained from the Jackson Laboratory, Bar Harbor, Maine. After a 2-week quarantine and acclimation period, these animals were placed under test. Eight groups of 15 mice each, closely matched by weight, were established, and the animals were housed individually in suspended wire-mesh cages in air-conditioned quarters. Water and a diet of Purina Laboratory Chow were given ad libitum. The skin of the interscapular region of each mouse was clipped free of hair with electric clippers. Because of the possibility of mechanical irritation, the hair of the mice was not clipped again after the test was started.

A solution of each subfraction was prepared in toluene ("Distilled in Glass," Burdick and Jackson Laboratories, Inc.) at a concentration of 50 mg/ml. A 100-µl volume of each solution, containing 5 mg of the test material, was applied twice weekly, at least 3 days apart, to the clipped interscapular area of each mouse of the respective test group for a maximum of 26 weeks--a time period in which 100% (70/70) of the C_3H/HeJ mice treated with an equal concentration of the original unfractionated coal tar in a previous study (2) developed skin tumors. Each mouse of the vehicle control group was treated with 100 µl of toluene on the same time schedule.

Each mouse was weighed immediately prior to undergoing test and prior to the first weekly application throughout the test period. Observations of the mice were made daily, 5 days a week, for evidence of systemic toxicity, mortality, and for the appearance of tumors. A growth was recorded as a tumor when it reached 1 mm x 1 mm in size.

All surviving animals were sacrificed and autopsied 2 weeks after completion of the scheduled treatment. Gross and histopathologic examinations were made of skin from the test site, lungs, liver, kidneys, urinary bladder, spleen, adrenal glands, and axillary lymph nodes.

RESULTS

GENERAL OBSERVATIONS

The interscapular skin of mice treated with subfractions 6, 7, 9, 10, and 13 became dry and scaly after only four applications of the test materials. Horny growths appeared on the skin of two mice after treatment with subfraction 7 for 17 weeks. Treatment with subfraction 9 resulted in the development of growths on the skin of 11 of 15 mice after 6 to 12 weeks. Some of these growths became ulcerated upon continued treatment. All animals in this group became moribund after 15 weeks and were sacrificed for pathology. All mice treated with subfraction 10 developed growths after 9 to 14 weeks. These growths subsequently became ulcerated. In the twenty-third week of the test, 8 of 15 mice in the group treated with subfraction 10 died suddenly. The carcasses were not submitted to pathologic examination because of autolysis. Repeated skin applications of subfraction 13 stimulated the formation of a growth on one mouse after 25 weeks.

The average total body-weight gain of 6 g by the mice in each test group paralleled that of the control mice over the 26-week test period.

PATHOLOGY

Neoplasms developed in the interscapular skin area of one or more mice in the groups treated with four of the seven materials tested. Squamous cell papillomas were present in mice treated with subfractions 7, 9, and 13, and squamous cell carcinomas were observed in animals receiving subfractions 9 and 10. Metastases to the lungs occurred in one animal treated with subfraction 10.

The pertinent pathology for animals in each group is presented below:

Controls - Histopathology showed the lungs of three mice to exhibit mild peribronchial lymphocytic infiltrates.

Subfraction 2 - Histopathology revealed moderate mast cell infiltration, primarily near the blood vessels in the dermis of the interscapular skin of all mice. The urinary bladders of two animals showed mild focal areas of lymphocytic infiltrates in the muscular coat.

Subfraction 3 - Histopathology showed the skin of all mice to exhibit moderate mast cell infiltration which was confined to areas near the blood vessels in the dermis. The lungs of one animal showed a focal abscess. The urinary bladder of one mouse showed a mild focal area of lymphocytic infiltrates in the muscular coat.

Subfraction 6 - Histopathology of the skin revealed a few mast cells around the blood vessels in the dermis. The lungs of three mice showed mild peribronchial lymphocytic infiltrates.

Subfraction 7 - Gross pathology of the skin of four mice showed discrete to confluent horny growths of varying size at the test sites. Histopathology showed a squamous cell papilloma characterized by marked hyperkeratosis in two of these mice. A mild to moderate inflammatory infiltrate, mostly lymphocytes, and some polymorphs were present. Mitotic figures were common. The other two animals having horny growths showed mild hyperkeratosis.

Subfraction 9 - Gross pathology showed the skin of all animals except one to exhibit numerous confluent cauliflower-like growths having rough surfaces. Some of the growths were ulcerated. The skin of one animal had a crusty appearance. Histopathology of the skin of six mice showed squamous cell papillomas characterized by marked hyperkeratosis. A mild to moderate inflammatory infiltrate, mostly lymphocytes, and some polymorphs were present in the dermis. Mitotic figures were common. The skin of five other mice showed squamous cell carcinomas. The criteria for malignancy included invasion of the dermis and striated muscle, many anaplastic tumor

cells with nuclei of varying sizes and shapes, several mitotic figures (many of which were abnormal), and lack of organization of cells in patterns typical for benign tumors. A mild to moderate inflammatory infiltrate was usually present.

Subfraction 10 - Gross pathology of the skin of all mice showed ulcerated horny growths with rough surfaces and narrow stalks. Histopathologically, these growths were malignant. The lungs of one mouse showed a metastatic lesion composed of anaplastic tumor cells arising from the skin. The kidneys of another mouse showed mild focal interstitial nephritis.

Subfraction 13 - Gross pathology showed a horny confluent growth attached to the interscapular skin of one animal. Histopathologically, this growth was identified as a squamous cell papilloma. The skin of six other animals showed mild inflammatory changes and hyperkeratosis.

The tumorigenic response and mortality of this screening test of seven coal tar neutral subfractions are summarized in Table 8.

DISCUSSION

As previously noted, only 7 of the 16 coal tar neutral subfractions were evaluated prior to expiration of the project. The potential biologic activity of the remaining nine subfractions remains to be studied. Such a study, however, is not being pursued by NIOSH at this time.

Not all mice that were placed under test survived until the end of the 26-week experimental period. However, with the possible exception of mice comprising the groups that underwent test with subfraction 9 and 10, morbidity and mortality of the mice were considered unrelated to the treatment. The deaths in the control group and in the groups treated with subfractions 2, 3, 6, 7, and 13 occurred after a weekend when environmental conditions in the animal facility were subnormal. Autopsies revealed that the deaths were due to respiratory complications. No pathologic evidence was found for the moribund state of the 13 animals sacrificed after 15 weeks of treatment with subfraction 9. The cause of death of the eight mice that died suddenly in the twenty-third week of treatment with subfraction 10 could not be established because of severe autolysis.

As shown in Table 8, subfractions 7, 9, and 13 induced squamous cell papillomas of the skin in one or more mice, and subfractions 9 and 10 induced squamous cell carcinomas. Histopathologic examination of the skin lesions resulting from treatment with subfraction 9 showed various stages of

progression of squamous cell papillomas to squamous cell carcinomas, contributing to the belief that squamous cell carcinomas arise originally from squamous cell papillomas (7). In addition, histopathologic examination revealed pulmonary metastases from the squamous cell carcinomas of the skin of one mouse treated with subfraction 10. Negative results were obtained with subfractions 2, 3, and 6.

Although the purpose of this project was not specifically to correlate biologic activity of the subfractions with their chemical composition, the results do show that the two subfractions possessing the greatest biologic activity (subfractions 9 and 10) are composed principally of 4- and 5-ring polynuclear aromatic hydrocarbons, some of which are known to possess oncogenic activity.

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TABLE 1

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 2

Molecular weight	Compound
116	indene
118	indan
120	trimethylbenzene isomer
128	naphthalene
130	1-methyl-1H-indene
134	tetramethylbenzene isomer
134	tetramethylbenzene isomer
142	methylnaphthalene isomer
142	methylnaphthalene isomer
152	acenaphthylene
154	acenaphthene
156	1-ethylnaphthalene
156	dimethylnaphthalene isomer
156	dimethylnaphthalene isomer
156	2-ethylnaphthalene
168	methylbiphenyl isomer
170	isopropylnaphthalene isomer
170	trimethylnaphthalene isomer
170	trimethylnaphthalene isomer
170	trimethylnaphthalene isomer
170	trimethylnaphthalene isomer

* Components were separated on a 3-meter x 2.1-mm i.d. stainless steel column of 5% Dexsil 400 on 100/120-mesh Supelcoport, temperature programmed from 130°C to 300°C at a rate of 6°C/min.

TABLE 2

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 3

Molecular weight	Compound
116	indene
118	indan
128	naphthalene
128	C_9H_{20} hydrocarbon isomer
128	C_9H_{20} hydrocarbon isomer
130	1-methyl-1H-indene
132	methylbenzofuran isomer
134	tetramethylbenzene isomer
134	unidentified
142	methylnaphthalene isomer
144	dimethylindene isomer
148	methylbenzothiophene isomer
152	acenaphthylene
154	acenaphthene
154	biphenyl
156	dimethylnaphthalene isomer
156	dimethylnaphthalene isomer
166	methylacenaphthylene isomer
166	fluorene
168	dibenzofuran
168	methylbiphenyl isomer
168	methylbiphenyl isomer
170	n-dodecane
170	trimethylnaphthalene isomer
170	trimethylnaphthalene isomer
170	trimethylnaphthalene isomer
178	anthracene or phenanthrene
182	dimethylbiphenyl isomer
182	hydroxyfluorene isomer
184	methoxybiphenyl isomer
184	methoxybiphenyl isomer
184	methoxybiphenyl isomer
184	dibenzothiophene
190	methylenephenanthrene isomer
192	methylanthracene isomer
192	methylanthracene isomer
196	methoxyfluorene isomer
198	methyldibenzothiophene isomer
198	methyldibenzothiophene isomer
202	fluoranthene
202	pyrene
206	dimethylanthracene isomer
206	dimethylanthracene isomer
208	anthraquinone
208	unidentified
210	unidentified
216	methylpyrene isomer
216	methylpyrene isomer

* Components were separated on a 33-meter x 0.5-mm i.d. SE-30 SCOT glass capillary column, maintained at 90°C for 4 min and then programmed to 270°C at a rate of 6°C/min.

TABLE 3

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 6

Molecular weight	Compound	Molecular weight	Compound
120	propylbenzene	192	methylanthracene isomer
120	trimethylbenzene isomer	194	hydroxyphenanthrene isomer
128	naphthalene	196	methoxyfluorene isomer
132	4-methylindan	202	fluoranthene
142	methylnaphthalene isomer	202	pyrene
142	methylnaphthalene isomer	204	2-phenylnaphthalene
152	acenaphthylene	206	dimethylanthracene isomer
154	biphenyl	206	dimethylanthracene isomer
166	fluorene	206	dimethylphenanthrene isomer
168	methylbiphenyl isomer	216	benzo(a)fluorene
168	dibenzofuran	216	benzo(e)fluorene
178	anthracene or phenanthrene	216	methylfluoranthene isomer
180	methylfluorene isomer	216	methylpyrene isomer
182	dimethylbiphenyl isomer	230	dihydrochrysene isomer
182	2-ethylbiphenyl	230	dihydrobenzo(c)phenanthrene isomer
184	dibenzothiophene	234	dihydroxypyrene isomer
184	methylisopropylnaphthalene isomer		

* Components were separated on a 3-meter x 2.1-mm i.d. stainless steel column of 5% Dexsil 400 on 100/120-mesh Supelcoport, temperature programmed from 130°C to 290°C at a rate of 6°C/min.

TABLE 4

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 7

Molecular weight	Compound	Molecular weight	Compound
120	propylbenzene	202	pyrene
120	trimethylbenzene isomer	206	dimethylphenanthrene isomer
128	naphthalene	206	ethylphenanthrene isomer
134	tetramethylbenzene isomer	206	ethylanthracene isomer
166	fluorene		
168	2-methylbiphenyl	216	benzo(a)fluorene
168	dibenzofuran	216	benzo(c)fluorene
168	methylbiphenyl isomer	216	methylfluoranthene isomer
174	trimethyl-1-indanone isomer	216	methylpyrene isomer
178	anthracene or phenanthrene	218	dihydrobenzo(c)fluorene isomer
180	1,1-diphenylethene	218	dihydrobenzo(c)fluorene isomer
182	2-ethylbiphenyl		
184	methylisopropylnaphthalene isomer	220	trimethylphenanthrene isomer
192	methylanthracene isomer	226	benzo(ghi)fluoranthene
192	methylphenanthrene isomer	228	triphenylene
		228	chrysene
196	methoxyfluorene isomer	234	hexahydrochrysene isomer
202	fluoranthene		

* Components were separated on a 3-meter x 2.1-mm i.d. stainless steel column of 5% Dexsil 400 on 100/120-mesh Supelcoport, temperature programmed from 130°C to 290°C at a rate of 6°C/min.

TABLE 5

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 9

Molecular weight	Compound	Molecular weight	Compound
128	naphthalene	192	methylanthracene isomer or methylphenanthrene isomer
134	diethylbenzene isomer	196	methoxyfluorene isomer
134	dimethyl-4-ethylbenzene isomer	196	unidentified
134	tetramethylbenzene isomer	198	n-tetradecane
142	methylnaphthalene isomer	202	fluoranthene
142	methylnaphthalene isomer	202	pyrene
154	biphenyl	204	1-phenylnaphthalene
156	n-undecane	204	2-phenylnaphthalene
156	dimethylnaphthalene isomer	210	unidentified
156	dimethylnaphthalene isomer	212	$C_{15}H_{32}$ hydrocarbon isomer
166	fluorene	212	$C_{15}H_{32}$ hydrocarbon isomer
168	methylbiphenyl isomer	216	benzofluorene isomer
168	dibenzofuran	216	benzofluorene isomer
170	n-dodecane	218	dihydrobnezofluorene isomer
178	anthracene or phenanthrene	218	dibydrobenzofluorene isomer
178	diphenylacetylene	218	$C_{16}H_{10}O$ isomer
180	methylfluorene isomer	218	$C_{16}H_{10}O$ isomer
182	hydroxyfluorene isomer	218	$C_{16}H_{10}O$ isomer
182	xanthene	226	n-hexadecane
182	2-ethylbiphenyl	228	naphthacene
184	n-tridecane	230	diphenylbenzene isomer
191	9H-fluorene-2-carbo-nitrile	230	diphenylbenzene isomer
192	methylanthracene isomer or methylphenanthrene isomer	234	naphtho(1,2-b)thia-naphthene
192	methylanthracene isomer or methylphenanthrene isomer	242	methylchrysene isomer
192	methylanthracene isomer or methylphenanthrene isomer	242	methylchrysene isomer
		242	methylchrysene isomer
		244	unidentified
		252	benzofluoranthene isomer
		252	benzo(a)pyrene or benzo(e)pyrene

* Components were separated on a 33-meter x 0.5-mm i.d. SE-30 SCOT glass capillary column, maintained at 90°C for 4 min and then programmed to 270°C at a rate of 6°C/min.

TABLE 6

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 10

Molecular weight	Compound	Molecular weight	Compound
130	2-ethylhexanol-1	252	benzo(a)pyrene or benzo(e)pyrene
178	anthracene or phenanthrene	252	perylene
191	unidentified	254	binaphthyl isomer
192	methylanthracene isomer	254	binaphthyl isomer
202	fluoranthene	254	binaphthyl isomer
206	2-ethylanthracene	264	unidentified
216	benzofluorene isomer	264	unidentified
218	dihydrobenzo(c)fluorene isomer	266	methylbenzo(a)fluoranthene isomer
228	chrysene	266	methylbenzo(a)pyrene isomer
229	benz(a)acridine	268	3-methylcholanthrene
230	dihydrochrysene isomer	268	methylcholanthrene isomer
240	$C_{14}H_8S_2$ (possibility)	276	anthanthrene
		241	unidentified
242	2-methylbenz(a)anthracene or methylchrysene isomer	276	benzo(ghi)perylene
		276	indeno(1,2,3-cd)pyrene
246	2,4,-diphenylphenol	278	dibenzanthracene isomer
252	benzo(k)fluoranthene	278	picene
		278	benzo(b)chrysene

* Components were separated on a 3-meter x 2.1-mm i.d. stainless steel column of 5% Dexsil 400 on 100/120-mesh Supelcoport, temperature programmed from 250°C to 325°C at a rate of 6°C/min.

TABLE 7

COMBINED GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC QUALITATIVE ANALYSIS* OF COAL TAR NEUTRAL SUBFRACTION 13

Molecular weight	Compound	Molecular weight	Compound
117	indole	202	fluoranthene
133	hydroxyindole isomer	202	pyrene
152	acenaphthylene	212	n-pentadecane
153	1-naphthylisocyanide	217	11H-benzo(a)carbazole
167	carbazole	226	benzo(ghi)fluoranthene
170	methylundecane isomer	226	dimethyltetradecane isomer
170	methylundecane isomer		
170	n-dodecane	226	dimethyltetradecane isomer
178	anthracene or phenanthrene	228	benz(a)anthracene
181	2-methylcarbazole	228	chrysene
184	n-tridecane	229	benz(a)acridine
192	methylanthracene	252	benzo(a)pyrene
195	2-aminophenazine	252	benzo(e)pyrene
		252	benzo(k)fluoranthene

* Components were separated on a 3-meter x 2.1-mm i.d. stainless steel column of 5% Dexsil 400 on 100/120-mesh Supelcoport, temperature programmed from 130°C to 325°C at a rate of 8°C/min.

TABLE 8

SKIN TUMORIGENESIS IN C_3H/HeJ MICE TREATED WITH COAL TAR NEUTRAL SUBFRACTIONS

Subfraction	Weeks of treatment	No. of mice undergoing test	No. of mice autopsied	Number of mice bearing skin tumors: Squamous cell papillomas	Squamous cell carcinomas	Total	Average time of appearance of first tumor (weeks)
2	26	15	12	0	0	0	--
3	26	15	12	0	0	0	--
6	26	15	11	0	0	0	--
7	26	15	13	2	0	2	17
9	15	15	13	6	5	11	9
10	26	15	6	0	6	6	11.5
13	26	15	12	1	0	1	25
Controls	26	15	11	0	0	0	--

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