

RC- 6012595

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Arthur D. Little, Inc. ACORN PARK · CAMBRIDGE, MA 02140 · (617) 864-5770 · TELEX 921436

May 20, 1977

J. Bowen Ross, Esq.  
Liggett Group, Inc.  
P. O. Box 1572  
Durham, North Carolina 27704

Dear Mr. Ross:

Enclosed for your review is a draft copy of the first of several reports on previous work which Dr. Mold has requested be prepared.

Sincerely yours,



Richard T. Murphy, Jr.  
Vice President and  
General Counsel

RTM/tjb

Enc.

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#### SUMMARY

This experiment was conducted to assess the relative tumorigenic potency of: (1) the heptane-solubles from the smoke of regular Chesterfield cigarettes; (2) a concentrate of the polycyclic aromatic hydrocarbon (PCH) fraction of smoke; and (3) a mixture of (1) and (2), such that the PCH content would be doubled over that of the heptane-solubles alone. The principal point of the comparison was to ascertain if the combination resulted in an increase in tumor incidence, which would be indicative of a deficit of tumor initiators in the heptane-soluble fraction.

The heptane-solubles were prepared at Arthur D. Little, Inc. by a simplified procedure, and the PCH fraction was provided by Liggett & Myers. Average application levels of all preparations were comparable over the duration of the experiment. Animal survival in the treated groups was closely comparable for the first year, differing thereafter previously because of removal with tumors. Health and weights of the animals were satisfactory throughout, with only slight retardation of growth in the treated groups compared to non-test controls.<sup>P</sup> Total tumor incidence (papillomas and carcinomas) was quite high in all groups: heptane-solubles, 48.9%; PCH fraction, 68.8%; and combination, 72.7%. The corresponding figures for carcinomas were 14.9%, 39.6%, and 29.6%. The incidence produced by the PCH fraction alone was so high as to diminish the likelihood of there being an additive effect from the combination.

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#### INTRODUCTION

This experiment was designed to determine whether the addition of a preparation containing the polycyclic aromatic hydrocarbon (PCH) fraction of cigarette smoke to the heptane-soluble fraction of such condensate would demonstrate an increase in tumorigenic potency. Test materials were applied three times weekly for 104 weeks. These experiments were conducted from July 1967 to July, 1969.

#### METHODOLOGY

Application of test materials, animal maintenance, and observation of tumor incidence were performed by the procedures previously reported. The preparation of test materials is described below.

##### Test Animals

For each test group, 50 Swiss albino female mice (purchased from Millerton Farms) were housed in clear plastic cages, 7-8 per cage. There were three experimental groups, plus a vehicle control (10% benzene in acetone--see below), and a non-test control group. Animals were maintained on a standard laboratory ration, and received water ad libitum. At the start of the experiment, the animals were 8 to 12 weeks of age, and the average weight in each group of 50 was 25 grams. The experimental room was maintained at a temperature of 75°F and 50% relative humidity.

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#### Test Procedure

The test procedure involved the clipping of hair from the dorsal test area prior to each test painting to remove "excess" hair using an Oster Company (Racine, Wis.) Model A-2 small animal clipper with a size 40 blade. The non-test control animals were hair-clipped only at the time of necropsy. Special care was exercised to minimize skin abrasions and other evidence of irritation. The test site is described as the dorsal area extending between the axillary and pelvic regions and between both lateral abdominal margins. For test material application, No. 5 camel's hair paint brushes were used throughout. Individual test paintings had a target weight of 100 mg (range 80-120) of the test solution per animal.

#### Condensate Preparation

Cigarettes, 70 mm Chesterfields, were stored in the laboratory, and were smoked on the L&M wheel-type smoker, with one 35 ml puff per minute to a butt-length of 30 mm. Condensate was collected at liquid air temperature in a two-trap train, and overall yield determined by increase in weight of the traps. The condensate was removed from the traps with acetone, and the acetone removed under reduced pressure at a temperature of 40-50°C on a Büchler rotary evaporator.

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#### Preparation of Heptane-Solubles

To the "dry" condensate in the evaporating flask (after weight determination), there was added 1 ml of acetone and 8 ml of n-heptane per gram of condensate. The flask was shaken for two minutes, and the contents transferred to a separatory funnel. This was shaken, then the heptane and acetone phases allowed to separate. The lower layer (acetone) was drained out and discarded, and the heptane layer decanted into a round-bottomed evaporating flask. The heptane was evaporated on the rotary evaporator at 45°C.

#### Polycyclic Hydrocarbon Concentrate

This material (designated FO-99A-143) was provided by Liggett & Myers as a benzene solution in sealed ampules. Fifteen separate preparations were submitted, containing 292 to 1072 mg of solids, representing the product of approximately 5800 to 12,000 70 mm cigarettes, and 0.049 to 0.098 mg/cigarette.

#### Preparation of Painting Solutions

The design of the experiment called for application of the following levels of each component and the mixture:

Heptane-solubles alone--40 mg per application.

Mixture--40 mg of heptane-solubles plus an "equal cigarette" amount of FO-99A-143.

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PCAH alone--an amount of FO-99A-143 equal to that used in the mixture.

Since the yield of heptane-solubles (per cigarette) was found to vary, and in turn the number of cigarette-equivalents per 40 mg application, it was desirable to adjust the amount of FO-99A-143 used in the mixture. The formula by which this was done was based on the average yield of heptane-solubles in ten smokings just completed. The factor from one group of ten was used for each mixture prepared until another ten smokings had been completed.

The usual solutions for painting were thus made up in the following ratios:

- (1) Heptane-solubles: 28 grams of a 50% solution in acetone plus 7 grams of benzene.
- (2) Mixture: 28 grams 50% heptane-solubles in acetone, plus calculated amount of benzene solution of FO-99A-143 (1.85-7.0 grams) plus benzene to total 7 grams (5.15-0 grams).
- (3) PCAH alone--28 grams acetone, plus same amounts of FO-99A-143 solution and benzene used in mixture.
- (4) Vehicle control--28 grams acetone and 7 grams benzene.

#### Observations

Animal health was continuously monitored and any seriously ill animals

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were sacrificed whenever possible. Animal weights were determined monthly throughout the experiments.

The following summarizes the survival experience and mode of removal of animals during the experiment.

	<u>Sacrificed</u>	<u>Died</u>	<u>Alive at Termination (104 Weeks)</u>
Heptane-solubles	32	14	4
FO-99A-143	40	10	0
Heptane-solubles and FO-99A-143	33	17	0
Benzene/Acetone	28	16	6
Non-test Controls	<u>19</u>	<u>24</u>	<u>7</u>
	152	81	17

Death from disease and other causes was essentially as expected from previous experience.

Observations of outgrowths and tumors in an experimental group began when any were observed during a condensate application. Formal checking was then performed at regular intervals. Gross visual observations include diagnosis of several kinds of non-tumorous outgrowths (warts, spicules, etc.), positive papillomas, and possible or probable carcinomas. At death or sacrifice, all outgrowths were examined histopathologically to confirm or extend the visual observations.

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#### RESULTS

The yields of total condensate and heptane-solubles from the collections used in this study are presented in Table 1A. (Heptane-solubles were already being prepared for other studies.) The yield of heptane-solubles as a fraction of total condensate was quite consistent, ranging from 22.0 to 26.5% with a per collection average of  $23.7 \pm 1.6$  (C.V. = 6.8%). The yield per cigarette was similarly consistent (although independent), ranging from 6.38 to 8.49 mg, averaging  $7.44 \pm 0.69$  (C.V. = 9.3%). The number of cigarettes represented in the average 40 mg applied was  $5.42 \pm 0.51$  (C.V. = 9.5%).

The PCAH preparations used varied in concentration, but the yields per cigarette ranged only from 0.049 to 0.098 mg, averaging  $0.080 \pm 0.014$  (C.V. = 17.5%), as shown in Table 1B. Also shown there are the amounts of FO-99A-143 used with successive lots of heptane-solubles, and in the corresponding control, i.e., PCAH's alone.

The distribution of amounts of test solution applied over the entire course of the study is given in Table 2. The average in each experiment is close to the desired value of 100 mg, with a good clustering of values within  $\pm 20$  mg (80-90%).

Gross observations on tumor incidence and on survival of animals are presented in Table 3. Survival up to one year was similar in all groups (82-92%). At 18 months and later, some differences are noted, largely due

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to removal of animals with tumors. Tumors appeared at a similar and higher rate in both groups receiving the PCAH concentrate (alone and in combination) than in the group receiving heptane-solubles alone. One papilloma was observed in the non-test control group, but none in the benzene-acetone solvent control.

The final tumor data, including the results of histopathological observations, are given in Table 4. The "total tumor positive animals" is the sum of the total number of tumor positives seen during the experiment, plus the number found with previously undiagnosed papillomas or carcinomas at necropsy. The effective number of animals is the number alive at the time of first occurrence of a positive papilloma in that experimental group. FO-99A-143A is significantly more tumorigenic than the heptane-solubles (on an equal cigarette basis), producing a 50% larger incidence of papillomas and a more than doubled carcinoma incidence. The mixture, however, did not result in an increase in total tumor incidence and showed a somewhat reduced incidence of carcinomas.

Since it can be used for a more refined analysis of tumor incidence, the time of death of tumor-free animals is given in Table 5. This excludes all animals with a positive tumor, whether observed during the duration of the experiment or only at necropsy.

The survival and animal weights at 6-month intervals are presented in

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Table 6. None of the experimental groups showed the final weight gain of the non-test controls, but the comparability of the groups receiving smoke products with each other and with the vehicle control indicates no strongly adverse effects.

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TABLE 1A

## Yield of Heptane-Solubles from Chesterfield Cigarettes

Group	Coll. No.	Total Cartons	Total Condensate	Total Heptane- Solubles	% H Total	Hept. Sol. per Cig.	Cig. per 40 mg. Applic.
			(gm.)	(gm.)		(mg.)	
1	86- 90	41	256.63	64.95	25.3	7.92	5.05
2	94-100	49	281.27	64.66	23.0	6.60	6.06
3	101-105, 107-110	69	401.72	98.77	24.6	7.16	5.59
4	111-120	70	391.56	89.26	22.8	6.38	6.27
5	121-130	60	409.91	96.33	23.5	8.03	4.98
6	131-140	62	341.45	83.65	24.5	6.75	5.93
7	141-150	60	318.00	79.56	25.0	6.63	6.03
8	151-160	60	409.75	89.98	22.0	7.50	5.33
9	161-170	60	396.09	101.85	25.1	8.49	4.71
10	171-180	60	433.76	96.39	22.2	8.03	4.98
11	181-190	60	412.28	90.77	22.0	7.56	5.29
12	191-200	66	436.61	91.47	21.0	6.93	5.77
13	201-210	60	400.80	94.54	23.6	7.88	5.08
14	211-223	90	561.54	148.62	26.5	8.26	4.84
Totals		867	5451.37	1290.80	23.7	7.44	5.37

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**TABLE 1B**  
**PREPARATIONS OF PCAH CONCENTRATE (FO-99A-143)**

<u>Preparation</u>	<u>Solids (mg)</u>	<u>Cigarettes</u>	<u>mg/cig.</u>	<u>mg FO-99A-143 10 gm solution</u>	<u>Heptane Pool Basis</u>
A	890	11,640	0.0764	38.7	1
B	960	10,350	0.0928	56.2	2
C	951	9,710	0.098	54.8	3
D	1,002	10,718	0.0935	58.6	4
E	828	11,490	0.072	35.9	5
F	1,034	11,890	0.087	51.6	6
G	803	8,880	0.090	54.0	7
H	963	12,410	0.078	41.6	8
I	963	12,350	0.078	36.7	9
J	821	8,780	0.0935	46.6	10
K	1,072	12,420	0.086	45.5	11
L	1,012	12,400	0.082	47.3	12
M	801	11,100	0.072	36.4	13
N	308	5,850	0.053	*	
O	292	5,950	0.049	*	
Totals	12,700	155,938	0.081		

\* Not used

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TABLE 2

Distribution of Amounts of Painting Solution Applied

Preparation	Mean mg. per Application per Animal						Total	Mean	x 80-120
	<80	80<90	90<100	100<110	110<120	>120*			
Heptane solubles	11	32	89	106	52	29	319	102.6	87.5
FO-99A-143	4	21	75	101	51	58	310	106.2	80.0
Heptane-solubles + FO-99A-143	12	32	103	99	35	17	298	100.5	90.3
Benzene/Acetone	8	25	149	81	28	28	319	100.6	88.7

\* Numbers in this column are distorted by a large number of paintings of a very few animals (1-10) for which the precision of determination of the painting weight is poor.

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TABLE 3

## Gross Tumor Incidence and Survival During Experiment

A = Cumulative number of tumor-bearing animals  
S = Surviving number of animals

Weeks on Test	Heptane Solubles		FO-99A-143		Heptane-Solubles + FO-99A-143		Benzene/Acetone	NTC
	A	S	A	S	A	S		
27		47		49	1	44	48	48
28								
29								
31			1	48				
33								46
35	1				2			
37	2		2				47	
39			3		3	43		45
41			4	47		42	45	
43			5					
45		46	7	46	4		43	
48	3		11	45	8			
50				44	12		41	
52	5				15			44
55	6	45	12	41	17			
57	7	42	15		19	40		43
59			18	38	20		40	
61		39	20	36		38	39	42
63	12	36	22	32	21	34	38	
65		34		28	23	33		41
67			24			29	36	37
69	15	32		24		27	33	35
71		28	26	20		25		31
74	17	22	28	19	24	19	28	28*
76	18	21		18	25	17	25	27
78		20		13		15		25
81	20	19	29	11	28	13		22
83		17	30	8	29	12	24	19
86		15		7	30	9		16
89		13	32	5		6	23	14
92		12	33	4	31	4	18	11
94	21					3	15	10
96		11		3		2	11	
98	22	9		2		1	8	9
102		6		1		0	7	
104		4		0			6	7

\* One confirmed papilloma observed.

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TABLE 4

Incidence of Papillomas and Carcinomas During Experiment  
and at Necropsy

	Heptane- Solubles	FO-99A-143	Heptane-Solubles + FO-99A-143
<u>During Experiment</u>			
Animals with tumor			
Total	22	33	31
Regressed	1	2	2
<u>At Necropsy</u>			
Animals with papilloma only			
Confirmation	14	12	17
New	1	0	0
Animals with carcinoma			
Confirmation	7	19	12
New	0	0	1
Total tumor positive animals	23	33	32
Effective number of animals	47	48	44
% Incidence (total)	48.9%	68.8%	72.7%
% Incidence (carcinoma)	14.9%	39.6%	29.6%
(In addition, one confirmed papilloma was found in the non-test control group.)			

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TABLE 5

## Time of Tumor-Free Deaths

Experimental Group	N	Week of Death	Survivors (104 wks)	Total
Heptane-Solubles	26	12,24,26,44,53,56,56,57,61,61,61,62,62,62,62,65,68,72,74,74,83,89,90,96,97,101	1	27
FO-99A-143	17	24,31,41,45,48,50,55,59,59,62,64,64,70,71,76,78,78	0	17
Heptane-Solubles + FO-99A-143	18	3,4,7,9,10,20,39,41,56,62,66,68,69,72,72,77,85,86	0	18
Solvent (Benzene/Acetone)	44	6,20,36,41,41,44,45,49,50,59,60,63,66,67,68,69,69,72,72,73,73,74,76,76,76,82,87,90,90,92,92,92,93,93,94,96,96,96,96,97,97,98,100,103	6	50
NTC	42	7,21,32,32,39,51,56,61,64,66,66,66,66,69,69,70,71,71,71,73,74,75,77,77,79,80,80,83,83,83,84,85,86,87,87,90,92,92,93,97,103,103	7	49

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TABLE 6

## Survival and Animal Weights

(Mean Weight in Grams: Increment from 0 Time in Parenthesis)

Group	0-Time		6 Months		12 Months		18 Months		24 Months	
	N	Wgt.	N	Wgt.	N	Wgt.	N	Wgt.	N	Wgt.
Heptane-Solubles	50	25.4	48	32.0 ( 6.6)	46	33.7 ( 8.3)	22	34.6 ( 9.1)	6	33.7 ( 8.3)
FO-99A-143	50	25.2	49	31.2 (6.0)	44	33.5 (8.3)	19	34.5 (9.3)	1	39.0 (13.8)
Heptane-Solubles + FO-99A-143	50	24.9	44	31.0 (6.1)	42	33.0 (8.1)	19	33.1 (8.2)	1	<sup>23 mos.</sup> 34.0 (9.1)
Benzene/ Acetone	50	24.9	48	33.5 (8.6)	42	36.0 (11.1)	28	34.8 (9.9)	7	35.4 (10.5)
NTC	50	24.8	48	35.8 (11.0)	45	38.6 (13.8)	27	33.7 (8.9)	7	44.2 (19.4)

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