STUDIES IN CARCINOGENESIS *

XI. Development of Skin Tumors in Mice Painted with 3:4-Benzpyrene and Creosote Oil Fractions

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Industrial experience with coal tar products for a period of more than 30 years has enabled one of us (S. C.) to make observations on the effect of coal tar products in producing dermatitis. It appeared that while the higher boiling fractions and residuals, which contained anthracene and related substances, were strikingly irritating to the skin of workmen exposed to these fractions alone, no appreciable irritation was noted when the lower boiling members of this series were also present. In one workman, exposure to material containing only the lower boiling fractions was followed by regression of a previously existing skin wart. The impression was gained that coal tar pitch or high boiling anthracene oil frequently produced irritation of the skin in workmen handling these materials, but that no appreciable irritation occurred when these materials also contained 10 per cent, or more, of the lower boiling fractions. These latter fractions contain most of the benzene and naphthalene derivatives, including their phenols.

The experiments described in this paper were designed to ascertain whether such low-boiling fractions would exert an inhibiting effect on the production of skin tumors in mice painted with 3:4-benzpyrene.

Materials and Methods

The various fractions were prepared from a creosote oil (designated as Fraction I) obtained from a vertical retort coal tar; 90 per cent of the creosote oil distilled between 160° and 300° C. The basic constituents (Fraction II) were removed with aqueous

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hydrochloric acid; the phenols (Fraction III) were removed with aqueous sodium hydroxide. The remaining neutral material (Fraction IV) was steam-distilled. About 70 per cent of this material was obtained in the distillate (Fraction V); the residue was designated as Fraction VI. These fractions, dissolved in benzene, were painted on the skins of mice to obtain information on their toxicity; the concentrations employed had approximately the same relative proportions which they had in the original creosote oil. Fractions V and VI were also tested without dilution with benzene.

After the preliminary tests for toxicity, solutions in benzene were prepared which contained 0.2 per cent of 3:4-benzpyrene in addition to the selected concentrations of the coal tar fractions. A duplicate set of solutions was prepared in which the benzpyrene concentration was 0.05 per cent. Female albino market mice, in groups of 20 animals each, were painted 3 times weekly with these solutions. The control groups were painted with solutions containing 0.2 and 0.05 per cent, respectively, of benzpyrene.

Before starting the painting the hair on the back was clipped short. The solutions were applied to the skin of the clipped areas by means of swabs, which were made by attaching a small amount of absorbent cotton to the end of a narrow, cylindrical wooden applicator. For each mouse the swab was dipped into the solution and the excess drained by pressing the swab lightly against the wall of the container; as a rule, five strokes, without redipping, were made with the swab on each mouse.

Painting with the solutions containing the creosote oil fractions and benzpyrene was begun 4/14/36, and was carried out 3 times weekly until 9/1/36 (20 weeks); from this time on, the solutions containing 0.2 per cent benzpyrene were applied only twice weekly until 10/14/36 (6 weeks), when all painting with this concentration of benzpyrene was discontinued. Thus, over a period of 26 weeks, the mice had been painted 72 times. With the solutions containing the lower concentration of benzpyrene, painting was continued until 1/6/37, for a total of 114 times over a period of 38 weeks. When a papilloma appeared, painting was continued until the skin growth attained an "average diameter" (Shear [1]) of about 4 mm.

The mice were examined weekly as a routine, and oftener on occasion. Each mouse was identified and individual records were

TABLE I

Tumor Production in Mice Painted with 0.2 Per Cent Benzpyrene and Creosote Oil Fractions

Group*	Creosote oil		Number of mice which developed tumors (+), and number of negative survivors (−), after the intervals shown (in weeks)														Tumor-bearing mice (total number)	Regressions‡
	Fraction	Concentration	12	13	14	15	16	17	18	19	20	22	24	26	28	32	36	
		%																
I b	Unfractionated	33.3	..	..	1+	..	.	2+	..	..	1+	..	3+	2+	1+	1+	2+	13	2
			..	..	18−	..	.	16−	..	..	14−	..	8−	6−	5−	4−	2−		
II b	Basic	1.0	3+	..	2+	3+	..	1+	..	..	3+	1+	3+	3+	..	1+	..	20	0
			17−	..	15−	12−	..	11−	..	..	8−	7−	4−	1−	..	0−	..		
III b	Phenolic	6.6	..	..	..	2+	..	1+	1+	2+	..	4+	5+	..	..	3+	..	18	0
			..	..	..	18−	..	17−	16−	14−	..	9−	4−	..	..	0−	..		
IV b	Neutral	25.0	1+	1+	..	2+	..	1+	..	..	1+	2+	6+	2+	..	..	..	16	0
			19−	17−	..	15−	..	12−	..	..	11−	9−	3−	0−	..	..	..		
V b	Neutral distillate	25.0	1+	..	1+	1+	1+	1+	..	2+	..	7+	2+	1+	2+	..	1+	20	0
			19−	..	18−	17−	16−	15−	..	13−	..	6−	4−	3−	1−	..	0−		
V d	Neutral distillate	99.8	..	..	..	..	..	..	..	..	1+	..	..	..	..	1+	3+	7†	4
			..	..	..	..	..	..	..	..	16−	..	..	..	..	9−	5−		
VI b	Neutral residue	99.8	..	1+	..	..	..	..	..	2+	1+	1+	3+	1+	1+	3+	1+	14	1
			..	19−	..	..	..	..	..	17−	15−	14−	11−	9−	8−	5−	4−		
VII b	**	..	..	..	..	1+	2+	3+	3+	1+	3+	..	..	2+	1+	..	..	19	0
			..	..	18−	16−	13−	10−	7−	6−	3−	..	..	1−	0−	..	..		

* 20 mice in each group.
** Control (0.2% benzpyrene).
† Tumors appeared in 2 mice after 37 weeks.
‡ Number of mice in which papillomas regressed without subsequent development of progressively growing tumors; these mice were not included, in the preceding columns, as tumor-bearing mice.

TABLE II

Tumor Production in Mice Painted with 0.05 Per Cent Benzpyrene and Creosote Oil Fractions

Group*	Creosote oil		Number of mice which developed tumors (+), and number of negative survivors (−), after the intervals shown (in weeks)														Tumor-bearing mice (total number)	Regressions‡
	Fraction	Concentration	21	22	23	24	25	26	27	28	30	32	36	40	44	48	52	
		%																
I c	Unfractionated	33.3	..	..	..	..	..	..	..	..	..	..	2+	2+	1+	..	3+	8	3
			..	..	..	..	..	..	..	..	..	..	16−	14−	12−	..	7−		
II c	Basic	1.0	..	1+	1+	1+	2+	..	1+	..	3+	5+	4+	1+	..	..	..	19	0
			..	19−	18−	17−	15−	..	13−	..	10−	5−	1−	0−	..	..	..		
III c	Phenolic	6.6	..	..	..	..	..	..	..	..	1+	..	4+	4+	2+	..	1+	12	2
			..	..	..	..	..	..	..	..	19−	..	15−	11−	8−	..	6−		
IV c	Neutral	25.0	1+	1+	1+	1+	..	3+	2+	2+	2+	2+	..	2+	1−	..	..	18	0
			18−	17−	16−	14−	..	11−	9−	7−	5−	3−	..	1−	0−	..	..		
V c	Neutral distillate	25.0	1+	..	..	..	..	..	..	3+	2+	3+	6+	1+	1+	..	..	17	0
			19−	..	..	..	..	..	..	15−	13−	10−	3−	2−	1−	..	..		
V e	Neutral distillate	99.95	..	..	..	..	..	..	..	..	..	1+	1+	..	..	..	0+	5†	3
			..	..	..	..	..	..	..	..	..	17−	15−	..	..	..	14−		
VI c	Neutral residue	99.95	..	..	..	..	..	..	..	..	2+	..	4+	1+	3+	..	1+	11	6
			..	..	..	..	..	..	..	..	15−	..	10−	8−	4−	..	3−		
VII c	**	..	..	..	1+	..	..	..	..	2+	1+	2+	4+	5+	1+	..	..	16	1
			..	..	17−	..	..	..	..	15−	14−	12−	7−	1−	0−	..	..		

*20 mice in each group.
** Control (0.05% benzpyrene).
† Tumors developed in 3 mice during the 13th month.
‡ Number of mice in which papillomas regressed without subsequent development of progressively growing tumors; these mice were not included, in the preceding columns, as tumor-bearing mice.

kept for each. The histological material obtained at autopsy will be described in a later communication.

Not all of the papillomas which appeared on the painted areas developed into large tumors. In a significant number of cases the papillomas were sloughed off; other papillomas developed later in many instances. In this report the designation "tumor" is restricted to those neoplasms which grew progressively and did not regress. In comparing the rates of production of skin tumors in the different groups, the time of first appearance of the papillomas which continued to grow progressively until death occurred is given in the tables and charts. The mice in which papillomas regressed without subsequent development of progressively growing tumors were not considered as tumor-bearing mice in these comparisons.

RESULTS

Toxicity Tests

In the preliminary tests for toxicity, groups of 5 mice each were painted 3 times weekly with the solutions having the composition described in the tables, except that they contained no benzpyrene. When evidence of lethal or severe toxic effects was not noted after 7 weeks, the experiments with the benzpyrene-containing solutions were begun.

The painting with the benzpyrene-free solutions was continued, however, for the duration of the investigation (38 weeks). No tumors were produced by any of these solutions except for solution VI, which produced papillomas in 1 mouse after 9 months; these papillomas grew very slowly and were not malignant when the mouse was killed 36 weeks after their first appearance. This solution consisted entirely of Fraction VI, with no added benzene or benzpyrene; it was the residue of the neutral fraction remaining after distillation of Fraction V, and is the fraction that would be likely to contain whatever hydrocarbon carcinogen might be present in the creosote oil.

Benzpyrene Control Solutions

For the control solution it was considered desirable to use a concentration of benzpyrene which would be high enough to produce tumors in a large proportion of the mice, and yet not so high

that any retarding effect of the test materials would be masked. A concentration of 0.05 per cent benzpyrene was selected for this purpose. However, since it appeared possible, at the time these experiments were begun, that this concentration might be too low, a concentration of 0.2 per cent benzpyrene was also employed.

The results are summarized in the tables and charts, in which the 0.2 and 0.05 per cent benzpyrene control experiments are designated VII b and VII c, respectively. With the higher concentration of benzpyrene, tumors were obtained in 19 of the 20 mice; 16 of these tumors developed in 22 weeks, at which time no tumors had yet been obtained with the lower concentration (compare Curves VII b and VII c in Charts 1 and 2). With 0.05 per cent benzpyrene, tumors were obtained in 16 mice; a papilloma that developed after 25 weeks in a 17th mouse later regressed.

Retarding Solutions

In spite of the pronounced difference in potency of the two concentrations of benzpyrene, the solutions that exhibited a retarding

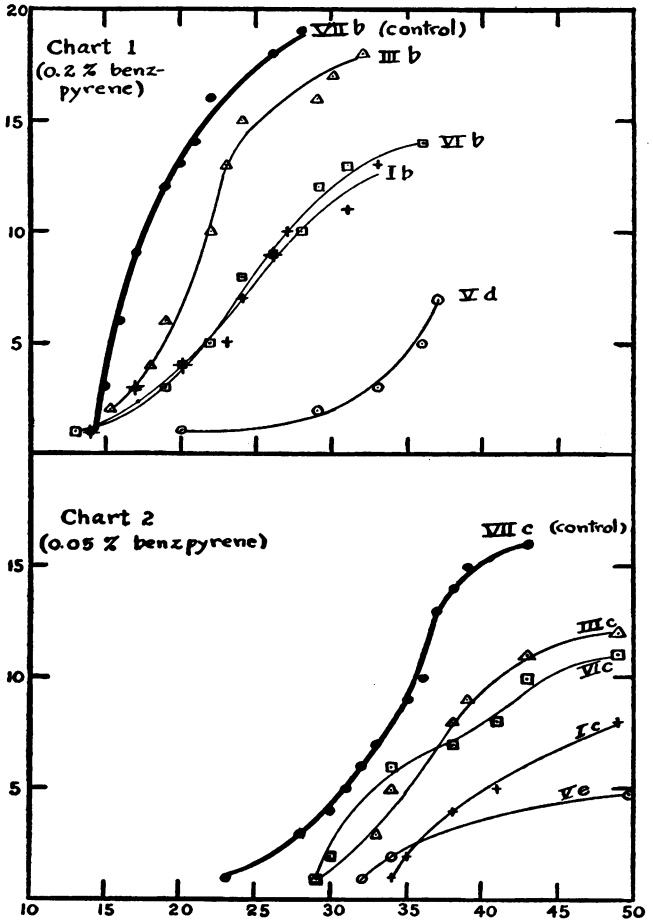
TABLE III

Creosote Oil Fractions which Retarded Tumor Production

Solution	Composition			Solvent
	Benzpyrene	Creosote oil fraction		
	%		%	
III b	0.20	Phenolic	6.6	Benzene
III c	0.05	Phenolic	6.6	Benzene
VI b	0.20	Neutral residue	99.8	....
VI c	0.05	Neutral residue	99.95	....
I b	0.20	Unfractionated	33.3	Benzene
I c	0.05	Unfractionated	33.3	Benzene
V d	0.20	Neutral distillate	99.8	....
V e	0.05	Neutral distillate	99.95	....

effect on tumor production by 0.05 per cent benzpyrene gave entirely analogous results in the experiments with 0.2 per cent benzpyrene. Four of the test solutions delayed tumor production; the results of these experiments are summarized in Charts 1 and 2, in which the heavy, full lines (Curves VII b and VII c) represent the benzpyrene control experiments.

In the order of increasing retardation the solutions and their composition are shown in Table III.



RETARDING EFFECT

Rate of production of skin tumors in mice painted with solutions containing fractions of creosote oil.

CHART 1. *All solutions contained 0.20 per cent benzpyrene.*

III b =	6.6% phenol fraction	plus	0.20%	benzpyrene
VI b =	99.8% neutral residue	"	"	"
I b =	33.3% unfractionated oil	"	"	"
V d =	99.8% neutral distillate	"	"	"
VII b =	(control)		"	"

CHART 2. *All solutions contained 0.05 per cent benzpyrene.*

III c =	6.6% phenol fraction	plus	0.05%	benzpyrene
VI c =	99.95% neutral residue	"	"	"
I c =	33.3% unfractionated oil	"	"	"
V e =	99.95% neutral distillate	"	"	"
VII c =	(control)		"	"

Damage to the skin, consisting of denudation of hair, reddening, induration and some ulceration, occurred in many of the mice. The extent of this damage was greatest in the mice in groups I b, V d and VI b. Very much less damage to the skin was produced in groups I c, V e and VI c, although these received the same concentrations of creosote oil constituents as I b, V d and VI b.

The retardation produced by the phenolic fraction (III b and III c) did not, however, appear to be attributable to obvious skin damage. In fact, remarkably little visible skin damage was produced by III c; no more evidence of damage was noted in this group than in II c, which exhibited a promoting effect on tumor production.

Promoting Solutions

With the higher concentration of benzpyrene, 3 of the solutions gave results which paralleled closely those given by the control

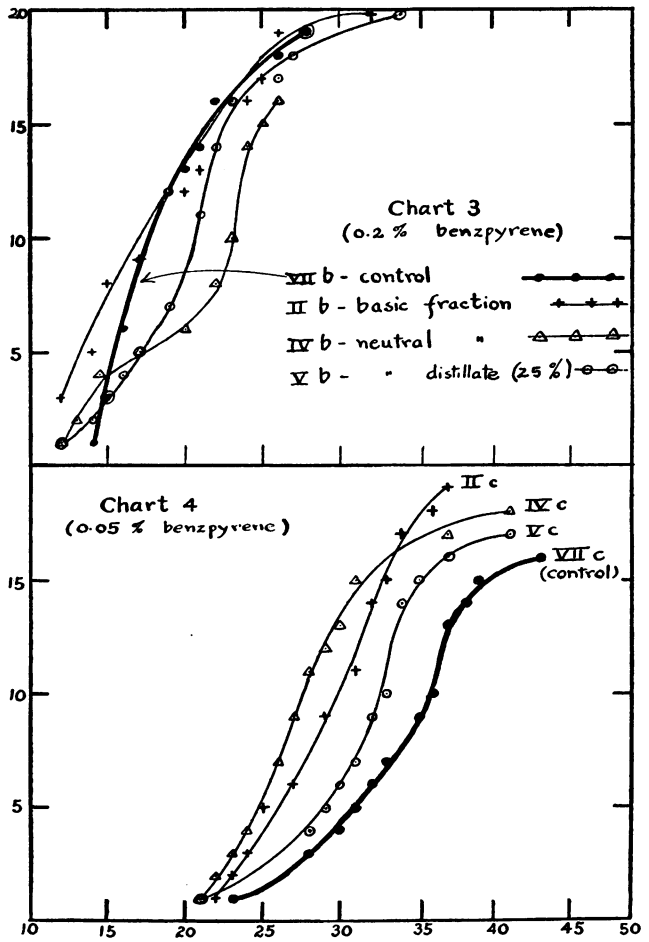
TABLE IV

Creosote Oil Fractions which Accelerated Tumor Production

Solution	Composition			Solvent
	Benzpyrene	Creosote oil fraction		
	%		%	
II c	0.05	Basic	1.0	Benzene
II b	0.20	Basic	1.0	Benzene
IV c	0.05	Neutral	25.0	Benzene
IV b	0.20	Neutral	25.0	Benzene
V c	0.05	Neutral distillate	25.0	Benzene
V b	0.20	Neutral distillate	25.0	Benzene

solution (see Chart 3). With the lower concentration of benzpyrene, however, these 3 solutions produced tumors more rapidly than did the control solution (see Chart 4). The composition of these solutions is shown in Table IV.

The amount of benzpyrene at the higher concentration was apparently sufficiently great to mask the promoting effect which was more evident at the lower benzpyrene concentration. Examination of the curves in Chart 3 reveals, however, that all 3 of these solutions began to produce tumors earlier than the control. This was most pronounced in the case of the basic fraction (see



PROMOTING EFFECT

Rate of production of skin tumors in mice painted with solutions containing fractions of creosote oil.

CHART 3. *All solutions contained 0.20 per cent benzpyrene.*

II b =	1% basic fraction	plus 0.20%	benzpyrene
IV b =	25% neutral fraction	" "	"
V b =	25% neutral distillate	" "	"
VII c =	(control)	"	"

CHART 4. *All solutions contained 0.05 per cent benzpyrene.*

II c =	1% basic fraction	plus 0.05%	benzpyrene
IV c =	25% neutral fraction	" "	"
V c =	25% neutral distillate	" "	"
VII c =	(control)	"	"

Curve II b) which, in 15 weeks, produced tumors in 8 mice; in the control group (VII b) only 3 mice bore tumors at this time.

Discussion

The retarding effect produced by solutions V d and V e appears to be attributable to the skin damage produced, rather than to a specific inhibitory effect, for when the concentration of the neutral distillate was reduced from 100 to 25 per cent (solutions V b and V c) the damage to the skin was less and no retardation was noted. Whether the slightly more rapid tumor production by V c, as compared with the control solution (VII c), represents a significant difference cannot be deduced from these data.

The entire creosote oil (I b and I c) and the neutral residue (VI b and VI c) also retarded tumor production but here, too, damage to the skin was greater than in the corresponding control mice. Moreover, the concentrations of the fractions employed were high (33.3 and 100 per cent, respectively).

The retarding effect obtained with the phenolic fraction (III b and III c) appears to be of greater interest for two reasons. In the first place, little skin damage was noted and, in the second place, the comparatively low concentration of 6.6 per cent was used. At the end of 43 weeks tumors had been produced in only 11 mice of group III c as compared with tumors in 16 of the control mice (see Chart 2). This was not due to higher mortality in this test group, since 8 mice in III c were still alive and negative at 43 weeks, whereas there were no negative survivors in the control group at this time (see Table II).

In further investigations of the retarding action of such material it would appear to be preferable to use concentrations of benzpyrene in the vicinity of 0.05 per cent rather than 0.2 per cent, for the benzpyrene itself contributes to the damage to the skin which is greater the higher the benzpyrene concentration. It would, moreover, be of interest to employ lower concentrations of the other fractions, particularly of the phenolic fraction. Apart from the possible value of such information for the prevention of dermatitis and skin cancer in workmen in the coal tar and related industries, it would be of interest for a better understanding of the mechanism of the production of skin tumors to ascertain

whether any coal tar constituents act as "anti-carcinogens" (Berenblum [2]).

The basic fraction exhibited a promoting action at both levels of benzpyrene. This is the only one of these creosote oil fractions that it has so far been feasible to investigate further. In subsequent painting experiments,* in which strain A mice and solutions containing 0.05 and 0.02 per cent benzpyrene were employed, skin tumors developed in mice painted with solutions containing 2 per cent of the basic fraction and benzpyrene more rapidly than in the control mice painted with benzpyrene alone. Other control mice, painted with a solution containing only 2 per cent of the basic fraction, developed no skin tumors.

Experiments have also been carried out to ascertain whether an analogous promoting effect would be obtained in the production of subcutaneous tumors by benzpyrene. In mice injected with 0.5 mg. of benzpyrene in lard containing 2 per cent of the basic fraction, tumors did not develop any more rapidly than in mice that received the benzpyrene alone, but even the control mice developed tumors quite rapidly. When the dose was reduced to 0.1 mg. of benzpyrene, however, subcutaneous tumors developed, slowly, in 45 per cent of the control mice, as contrasted with rapid development of tumors in 95 per cent of the mice which had received, in addition, the basic fraction.

This line of inquiry has been extended to other materials and individual compounds in tests of possible "co-carcinogenic" activity (Shear [3, 4]) and to other carcinogens. The results obtained so far have been complicated by what appears to be a contributory, and variable, action of different batches of the lard, used as solvent, upon the rate of tumor production. Other experiments are in progress [5, 6] in which tricaprylin is being employed as the solvent.

Summary

1. Albino market mice were painted with benzene solutions containing various fractions of creosote oil together with 0.2 and 0.05 per cent, respectively, of 3:4-benzpyrene.

2. The 0.05 per cent benzpyrene control solution began to produce skin tumors after most of the mice painted with the 0.2 per cent benzpyrene control solution already bore tumors.

* In collaboration with Mr. Robert D. Sall of the Harvard Medical School.

3. Four of the test solutions exhibited a retarding effect on tumor production. In 3 of these the retardation may have been due to damage to the skin.

4. The phenolic fraction, in a concentration of 6.6 per cent, exhibited a retarding effect which was not due to obvious skin injury.

5. Three of the test solutions exhibited a promoting effect. The basic fraction, in a concentration of 1 per cent, exhibited an accelerating effect at both benzpyrene levels.

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