Copy From D. V. Stroop For Information of Members of Medical Advisory Committee and Subcommittee on Carcinogenicity May 6,1954

ESSO LABORATORIES
STANDARD OIL DEVELOPMENT COMPANY
P. O. BOX 51, LINDEN, N. J.

May 4, 1954

TO: Members of the Subcommittee on Carcinogenicity

Gentlemen:

I am attaching a copy of a manuscript forwarded to me by Dr. Horton entitled "Carcinogenesis of the Skin: I. Basic Methods Employed in Testing Complex Hydrocarbon Type Tars and Oils, and the Development of a Quantitative Scale to Express Their Relative Potencies to Mice". This paper has been written by Dr. Horton and his associate, Dorothy T. Denman. It is his plan, I believe, to submit this paper to Cancer Research for publication, although he has not yet indicated to me just to which journal he plans to submit it for publication.

Dr. Horton has indicated that any date in May except the period May 11-20 would be all right for a meeting of the Subcommittee. After having reviewed the manuscript, I believe a meeting of the Subcommittee with Dr. Horton would be advisable, and would suggest that this meeting be held after each of you has had ample time to review the manuscript both personally and possibly with some of your technical people, so that at the time of the meeting with Dr. Horton we will be in a position to express all suggestions to him. With this in mind I tentatively propose the dates of Friday and Saturday, May 21 and 22, or Thursday and Friday, May 27 and 28 for a meeting with the Kettering group to discuss this manuscript. I would appreciate it if each of you would let me know as soon as possible which of these two dates would be satisfactory so that final arrangements can be made with the Kettering group. As soon as your wishes are in, I shall send you definite notice of the meeting dates.

Sincerely yours,

/s/ R. E. Eckardt

R. E. ECKARDT, M. D.

REE:ilk

cc: Members of the Medical Advisory Committee
Dr. A. Wesley Horton

API 05770

FOR INFORMATION ONLY NOT FOR PUBLICATION

API RESEARCH PROJECT MC-1

At Kettering Laboratory - University of Cincinnati (1946-1954)

Manuscript of paper submitted for review prior to publication.

Title: "Carcinogenesis of the Skin, I"

Proposed for publication in "Cancer Research"

Status of Manuscript

May 4, 1954 - Received from Kettering Laboratory.

May 6, 1954 - Copies mailed to Subcommittee on Carcinogenicity (and to MAC).

API 05771

EB 005516

CARCINOGENESIS OF THE SKIN, I. Basic Methods Employed in Testing Complex Hydrocarbon-type Tars and Oils, and the Development of a Quantitative Scale to Express Their Relative Potencies to Mice.

by A. Wesley Horton and Dorothy T. Denman

The investigation of skin cancer in this Laboratory has been concerned with the estimation of the extent of the potential hazard of this disease in certain industries. As in any other aspect of the general field of toxicology, it must be recognized that a specific hazard is the product of several variables, two of which assume dominant importance. These may be termed conveniently the physiological activity of the material or mixture in question, and the conditions of exposure. The latter aspect involves the practices of personal and industrial hygiene. This paper will be devoted primarily to the development of reproducible methods of determining the first variable, the level of activity, or relative carcinogenic potency, of hydrocarbon-type oils and tars.

Mice were chosen as the primary test species since neoplasms of the skin, comparable in certain respects to those of man in the same tissue, can be reproduced in these animals under suitable experimental conditions. Certain specific materials, which have induced an abnormally high incidence of cancer of the skin of men as a result of repeated exposure in their occupation, have been found capable of producing epitheliomas in the skin of mice. Conversely, in our experience certain inbred mice, such as the C3H strain, have not developed "spontaneous" skin tumors, nor have

they reacted by skin tumor formation to repeated experimental application of a number of materials, including water, benzene, white mineral oil, etc., which are presumed to be non-carcinogenic to the skin of man on the basis of extensive practical experience.

The specification, "suitable experimental conditions", in the previous paragraph was used advisedly. It is evident from perusal of the literature, that too often the problem of excessive mortality due to the toxicity of the material under examination, as well as that due to the occurrence of incidental disease among the experimental animals, has been met only by increasing the number of animals on test. Such an approach ignores the probability that the survivors of these conditions are not at all representative of the population involved with respect to their susceptibility to carcinogenesis. Hence, stress will be laid upon the necessity for standards of selection and care of the experimental animals which will maintain their health at as high a level as possible.

EXPERIMENTAL METHODS

Most of our work has been carried out with male mice of the C3H strain, purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. A number of experiments have also been carried out with male mice of the CFW strain obtained from Carworth Farms, but the results were not as reproducible as those derived from the C3H strain. Although the difference in genetic homogeneity is undoubtedly a factor, the fact that the C3H mice are more docile probably contributes to the reproducibility of experiments in which they are employed. The frequent occurrence of bites and scratches

of the skin of the CFW mice, inflicted by their companions, probably results in occasional direct intraepithelial introduction of carcinogens. Added to this complication is the possibility that wound-healing may play an accelerating role in carcinogenesis (1). The same trouble has been experienced in recent experiments with Swiss males, although the females seem to be more congenial.

The mice were received at six to eight weeks of age and placed under observation for approximately four weeks before use in any experiment. To control infestation of the animals with mites or lice (which when uncontrolled affects adversely their health), the mice were dusted routinely with Aramite[1] by the supplier prior to shipment and then twice again with the same miticide during the period of observation in this laboratory. Any group in which there was evidence of poor health during this period was withheld from use in tests in which quantitative results were needed.

In all experiments described herein, the mice were fed on Purina Laboratory Chow and water without restriction. At six months of age, C3H mice fed on this diet and subjected to no adverse experimental procedure reach an average weight of about 29-31 g., while corresponding CFW mice weigh about 33-35 g. Since growth of both the hosts and their tumors might vary with the temperature of their surroundings, an effort has been made to maintain a relatively constant temperature of 75±2° in the animal rooms.

For a given experiment, a group of 20 to 30 mice was divided into three to five cages. The animals were assigned individual numbers for identification and marked by clipping their toes.

[1] Aramite - 15W, U. S. Rubber Company, Naugatuck, Conn.

The exposure of the mice to any given material involved repeated applications upon the interscapular region. In preparation for the application of dosages of 100 mg. of a material, the fur was left intact, but when the dosage was to be less than 100 mg., the fur was removed by means of electric clippers. A small camel's hair brush of a selected size to deliver the desired dosage was used to apply the material, which was then simply allowed to spread according to its individual physical characteristics. Applications were repeated in accordance with a predetermined schedule (one, two, or three times each week) until the animals died. Care was taken to keep the dosage and the location of each application as constant as possible throughout the experiment.

Normal growth of the mice could not be maintained in testing certain of the materials, particularly those of high potency, when the experiment involved three 100 mg. applications each week. Hence, when there was some physicochemical or biological evidence that a given sample was likely to have a relatively high carcinogenic potency to the mice (equivalent to that of a solution of 0.15 percent, or greater, of methylcholanthrene in benzene), the material was applied at a lower frequency, usually once each week. In the case of materials of lower potency, more frequent applications were required to obtain sufficient tumors. Hence, when toxicity of such samples proved a serious problem at the 100 mg. level, the dose was reduced, in some cases to as low as 5 mg. per application.

Experience has shown that, even when the mortality among an experimental group was low, the time of appearance of tumors was sometimes delayed seriously by conditions of variable or uncertain origin which also interrupted the normal growth of the animals. As a measure of such conditions, therefore, the mice were weighed at frequent intervals throughout the experiments. Since early observations gave evidence that interruptions of growth were usually the result of intercurrent infections of low severity affecting most of the animals in a given cage, the weighing of individual animals was discontinued in favor of determining the average weight of the survivors in each group. Various precautions, such as regular cleaning and sterilization of cages, feeders and water bottles, decontamination (with a bacteriostatic agent) of gloves used to handle animals from one cage before proceeding to another, and isolation of sick mice, were taken to minimize infections and to prevent their spread from one group to another.

Throughout each experiment, the animals were examined at least once each week by one of us (D.T.D.), and the presence and time of appearance of any abnormal gross changes in the skin were recorded. Epilation and crusting of small areas were frequently noted and, in the early stages of tests on solutions of greater than 0.2 percent of methylcholanthrene or benzopyrene in benzene, large swellings, of the type previously described by Cramer (2), and small ulcerated areas, were occasionally observed. In response to the application of solutions of these synthetic carcinogens, papillomas eventually developed on the skin of practically all of

the surviving animals except in the case of the lowest level of concentration tested (0.01 percent). In general, the induced neoplasms progressed through the familiar pattern from papilloma to squamous cell carcinoma, with gross evidence of invasion. In the first test of a given material, sections of any epidermal tumors produced were prepared for microscopic examination.

The date of which each typical papilloma reached the arbitrary size of 1 mm. in diameter and elevation was recorded as the "time of appearance" of the papilloma. In addition, the date on which it was grossly apparent that the tumor was invading the subcutaneous tissues was noted (rolled border, generally accompanied by cratering necrosis of the center). When a mouse developed such an apparently malignant tumor, it was killed. Usually, the others were maintained on test until death from "natural causes" occurred. The data, thus obtained, were recorded on graphs such as those shown in Figures 1 through 4.

As experience was gained in associating the physical and chemical characteristics of various types of materials with their relative toxicity and with their ability to induce tumors of the skin in C3H mice, certain criteria were developed for selecting optimum conditions for any given biological test. As a general practice, a schedule of application was chosen which would lead to induction of papillomas in an average time of not less than 12 weeks. The restriction was based on repeated observations that this average time was unduly variable in experiments in which the severity of exposure resulted in more rapid induction of tumors.

On the other hand, experimental conditions which involved very long periods of exposure before tumors were induced proved to be even more unsatisfactory than those associated with very short latent periods, because of the increased possibility of intercurrent infections or other unidentified interferences with the normal growth of the mice. Even when these interruptions resulted in little or no loss of animals, the irregular effects on the growth of the induced tumors made it very difficult to interpret the results of the experiment. Therefore, as a general rule the frequency and severity of the applications were scheduled to produce an average latent period as close to 15 weeks as possible. When the materials were low in potency, this obviously meant the use of as severe conditions as were compatible with the normal growth and survival of the mice.

INTERPRETATION OF DATA OBTAINED

In general, under these experimental conditions one of two results has been obtained in response to the applications of any one of a series of materials, i.e., most of the animals which lived long enough developed tumors of the skin at the site of the application, or none of them did so. The outcome of the experiments, therefore, has been essentially unequivocal, and the relative potencies of the different active materials could be expressed in terms of the relative rates of induction of tumors.

The calculation of the "mean time of appearance of tumors" involved the determination, by the method of least squares, of the linear function which best fitted the plot of the cumulative frequency of tumor response (in probits) versus the time (in weeks) after the first application (shown on the right side of Figures 1 through 4). The latter coordinate is equivalent to cumulative dosage. It is to be noted that, for these experiments involving repeated applications of carcinogenic materials to the skin, the response varied linearly with the dosage itself, and not with the logarithm of dosage. It is assumed that the exponentially decreasing rate of appearance of tumors usually observed following single or limited numbers of exposures to carcinogens (3) was not seen in these experiments because the more resistant animals in any given group were given a larger effective dosage than were the more susceptible.

Calculation of Cumulative Frequencies of Response

The convention is adopted (after Bliss, 4) that, at the midpoint of the period between two given observations, the number of mice bearing tumors is equal to the average of the numbers at the times of the observations. At the midpoint of each period during which one or more mice develop their first papilloma, a calculation is made of the cumulative percentage of the "effective group of mice" which bear tumors on that date. The "effective group" at a given time is defined as the sum of the number of survivors plus the number of

mice which died previously after developing tumors of the skin, except that after the appearance of a tumor in the "average" mouse this sum is held constant. The "average" mouse is designated by the median in a simple arithmetic array of the times of appearance of the first tumor in each animal.

As an example, the calculations used to obtain the cumulative frequencies of tumor response from the data of Figure 3 are shown in the following table:

TABLE I

TIME* OF OBSERVATION OF NEW POSITIVES	MIDPOINT* OF PERIOD BETWEEN OBSERVATIONS, x	NUMBER OF NEW POSITIVES, R'	TOTAL NUMBER OF MICE BEARING TUMORS,** R' + ΣR	CUMULATIVE RESPONSE, $\Sigma R + \frac{(\Sigma R + R')}{2}$	EFFECTIVE NUMBER OF MICE	FREQUENCY OF RESPONSE, r (probits)
	19.5			0.5	18	3.08
20		1	1			
	23.5			1.5	18	3.62
24		1	2			
	24.5			2.5	18	3.91
25		1	3			
	33.5			4.0	16	4.33
34		2	5			
	35.5			6.5	16	4.76
36		3	8			
	37.5			9.0	16	5.16
38		2	10			
	38.5			11.0	16	5.49
39		2	12			
	41.5			12.5	16	5.78
42		1	13			
	46.5			14.0	16	6.15
47		2	15			

* Number of weeks after first application. **ΣR is the total number of mice with tumors at the time of previous observation.

Calculation of Mean Time of Appearance of Tumors, $\bar{X}$:

1. Assume that the tumor response in probits, r, is a linear function of the time, x,

   or $r = mx + b$

2. Determine constants, m and b, by method of least squares (5). Normal equations:

   $$\Sigma rx = m\Sigma x^2 + b\Sigma x$$

   $$\Sigma r = m\Sigma x + 9b$$

   (Values of x in weeks and r in probits are underlined in preceeding table)

   $$1485.67 = 10{,}688.25m + 300.5b$$

   $$42.28 = 300.5m + 9b$$

   $$b = 0.960$$

   $m = 0.112$ = slope of dose-response relationship

   $\therefore r = .112x + .960$

3. $\bar{x}$ = time of 5-probit response = 36.1 weeks

   s, standard deviation of mean,

   = $\bar{x}$ minus time of 4-probit response

   = 8.9 weeks.

CLASSIFICATION OF EXPERIMENTAL RESULTS ON THE BASIS OF THE HEALTH OF THE ANIMALS

In the appraisal of data derived from these experiments, consideration has been given to the following apparent relationships between the average growth and survival of the mice and the mean "time of appearance" of their epidermal tumors.

1. If, in the course of an experiment, an intercurrent infection overtakes a group of mice at about the time when papillomas would be expected to appear (on the basis of other comparable experiments), there is usually a delay in the mean time of appearance of tumors. The infection need not be lethal to any of the mice, a change in sign of the slope of the average growth curve frequently being an indication of such a condition (see Figure 1). It is believed that, in many such instances, the mice would probably be unaffected were their resistance not diminished somewhat by systemic toxic effects induced by the hydrocarbons being applied upon their skin. Thus, untreated controls may not show any symptoms of the infection.

Not infrequently, as the animals recover from such disturbances, tumors grow to measurable size in a large proportion of the animals in a relatively short time. The slope of the plot of the frequency of response versus time is then exceedingly steep. In other words, the standard deviation of the mean time of appearance of tumors in the group is much smaller than normal (see Figure 2). Thus, it could be assumed mistakenly, if it were not for the evidence from the weight curves of the presence of uncontrolled variables, that such an experiment had defined the statistical limits of the mean better than another in which such disturbances had not occurred.

2. In some experiments the cumulative toxic effects of the material applied, or chronic infections, or a combination of both, result in prolonged inhibition of the growth of the mice. A number of examples have been observed in which the average weights have increased for a few weeks, but became stationary at a point 10 percent or more below that of adult controls, and then, in many instances, gradually declined (see Figure 3). Thus, whether or not these conditions influence the timing of the initial changes resulting in the neoplasms they may retard the growth of the induced tumors to visibly discernible size and, accordingly, lengthen the apparent average period of exposure required for the induction of tumors by the material under test. In such experiments, the standard deviation of this mean time is frequently much greater than normal.

This somewhat extended discussion of the apparent relationships between the irregularities in growth due to the toxicity of applied materials and to intercurrent infections, and the rate of induction of tumors, does not relate to the originality of this observation but rather from our recognition of its importance in experiments in which various materials are being compared. Occasional mention of the phenomenon has appeared in the literature on experimental carcinogenesis (i.e., 6,7,8) and, in the field of chemotherapy of cancer, recognition has been given to the necessity of considering the systemic toxicity of a material in evaluating the specificity of its apparent "anticarcinogenic" effects (i.e., 9).

further, the relationships between general health and susceptibility to "spontaneous" tumors in man are currently receiving attention. Hence, it is believed that, in the experimental assay of the relative potencies of various materials or of the susceptibility of various species or strains of one species to a given material, information on the health and survival of the groups of animals being compared is essential to any quantitative interpretation of the data.

The results of all experiments described herein have been examined, therefore, in the light of these relationships. Each one has been classified according to the estimated significance of such effects in the following manner.

Class A. Those characterized by normal growth and survival of the mice at least until the time when most of the animals have developed papillomas (see Fig.4).

Class B. Those in which all or most of the mice lived long enough to develop tumors, but in which there was evidence (from average weights) of intercurrent infection at times prior to or during the period when the new growths were appearing. So long as recovery from the infection was prompt, and no obvious skewing of the distribution of the times of appearance of tumors occurred, it was felt that reliable estimates of the mean time of appearance could reasonably be made (see Figure 1). However, when no tumors had appeared prior to the onset of an infection, and when with recovery tumors developed

somewhat precipitously, the reliability of the apparent mean time of appearance was not determinable, and therefore such tests were classified X (see Figure 2).

Class X. Those characterized by poor growth (as discussed in Section 2 above) or high rate of mortality. The confidence limits of the apparent mean time for the induction of tumors could not be calculated by standard statistical methods, because of inability to assign proper weight to the effects of the uncontrolled variables.

Several types of moderately toxic materials failed to yield experiments of the Class A category. On the other hand, experimental results of the frankly unsatisfactory type (Class X) were usually found to be avoidable through the exercise of appropriate care in the initial choice and the subsequent handling of the mice.

REFERENCE STANDARDS

To provide a background of reference which might permit a more nearly quantitative interpretation of the mean times of appearance of tumors induced in C3H mice by certain complex oils and tars under various conditions of exposure, a number of experiments have been carried out over the past five years employing solutions of the synthetic carcinogens, 3-methylcholanthrene, benzo[a]pyrene, and 7,12-dimethylbenz[a]anthracene, in various solvents. It has been found that one of the essential requirements

for obtaining reproducible and logically consistent results is the maintenance of the animals in a good state of health as measured by growth curves. Thus, in Table II, the results of Experiments 205 show the precision of the determination of the mean time, $\bar{x}$, when Class B experiments are involved. Similarly, it has been found that even in the case of a complex material, so long as the conditions of the experiments permitted reasonably normal growth of the animals, the repeatability of the determination of the mean time, $\bar{x}$, has been quite satisfactory (within ± 10%).

To determine the effect of variations in the level of concentration and in the frequency of exposure, comparable experiments were conducted with other solutions of methylcholanthrene in benzene, and the results are shown in Table II.

The purity of the polycyclic hydrocarbon is obviously an important consideration in obtaining consistent results. The usual chemical criteria are necessary, but not always sufficient, requirements. In one case in particular, the methylcholanthrene, as received, had a sharp melting point, 176-7°C., and no indication was apparent by its ultraviolet absorption spectrum of any contamination. Yet, on repeated bioassay, it proved to be excessively irritating and much more rapidly carcinogenic than the normal material. Unfortunately the quantity available did not permit determination of a satisfactory procedure for purification. Since no further sample with such unusual potency has been obtained, bioassay remains the only way of acertaining that the methylcholanthrene is free of the material responsible for this activity.

TABLE II

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CONCENTRATION OF METHYLCHOLANTHRENE IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF METHYLCHOLANTHRENE PER UNIT AREA[1] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, $\bar{X}$ (weeks)	CLASSIFICATION
201	0.012	100	3	0.036	72.6	X
202	0.044	100	3	0.132	30.4±3.8[2]	B
203	0.086	100	3	0.258	29.9±2.2	B
203	0.086	100	3	0.258	26.3±3.4	B
204	0.172	100	3	0.516	15.7±1.6	B
204	0.172	20	3	0.516	16.4±1.1	B
204	0.172	20	3	0.516	16.6±1.2	B
204	0.172	20	3	0.516	15.5±1.1	B
205	0.345	100	3	1.035	11.0±1.9	B
205	0.345	100	2	0.69	15.1±1.5	B
205	0.345	100	1	0.345	22.4±2.7	B
205	0.345	100	3	1.035	14.7	X
206	0.69	100	1	0.69	17.8	X
206	0.69	100	3	1.98	6.7±4.1	B

[1] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.

[2] 5% fiducial limits.

TABLE II. (page 2)

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CONCENTRATION OF METHYLCHOLANTHRENE IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF METHYLCHOLANTHRENE PER UNIT AREA[1] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, $\bar{X}$ (weeks)	CLASSIFICATION
207	1.15	100	3	3.45	8.9	X
209	0.517	20	1	0.517	18.3±1.9[2]	B
209	0.517	100	1	0.517	16.2±2.7	B
209	0.517	100	3	1.551	10.4	X
219	0.115	100	3	0.345	24.0	X
220	1.034	100	1	1.034	9.7±1.6	B
238	0.23	20	2	0.46	18.7±2.3	B
238	0.23	100	2	0.46	16.4±2.0	B
243	0.15	100	3	0.45	17.9±2.0	A
243	0.15	20	3	0.45	18.2±2.1	B
268	0.287	20	2	0.574	17.6	X

[1] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.
[2] 5% fiducial limits.

In Table II it is apparent that variation in the dosage of solution per application from 20 to 100 mg., other factors remaining constant, had no effect on the rate of induction of tumors by methylcholanthrene in benzene. Similar results have been observed when solutions of benzopyrene in benzene were used as the carcinogenic stimulus. This constancy of potency with variation in the quantity of material applied (or area of skin exposed) also holds for some complex oils and tars, but for others there is significant change of potency with changes in the quantity used for each application. The chemical basis for these differences will be discussed in a later paper.

The values in column 5, Table II, were calculated from the concentration of methylcholanthrene in the solutions and the number of applications per week. It has been determined experimentally that variations in the concentration of the carcinogen did not affect the area covered by a given weight of the solutions. It was further assumed that the dosage per unit area was not significantly different when 20 or 100 mg. of the same solution were applied. Comparison of the data in columns 5 and 6 shows a consistent inverse relationship between the dosage of carcinogen per unit area per week and the mean time of appearance of tumors. The data (omitting Class X experiments) are plotted in Figure 5 together with the curve of the corresponding hyperbolic function (calculated by the method of least squares),

$$(\bar{x} - 3.7)(d + 0.10) = 8.2$$

or,

$$\bar{x} = \frac{8.2}{(d + 0.10)} + 3.7 \quad .$$

It is interesting to note the similarity between this equation and that calculated from theoretical considerations of the mechanism of carcinogenesis by Iversen and Arley (10),

$$\overline{t_{ap}} = \frac{1}{kc_o} + T, \text{ where k and T are constants.}$$

The constant, 0.10, in the experimental equation might be interpreted as a correction required by the definite, though very small, probability of the "spontaneous" excitation of a cell of the dorsal skin of a C3H mouse to the rate of proliferation necessary for the development of a new growth.

Similar experiments have been carried out with solutions of two other commonly used carcinogenic hydrocarbons, benzopyrene and 7,12-dimethylbenzanthracene. The results are shown in Table III, together with some data on solutions of methylcholanthrene in solvents other than benzene. As is evident from the data, it has been difficult to obtain satisfactory experiments with benzopyrene on C3H mice. The growth of the animals has tended to be extremely erratic. Even in tests in which the average weight eventually reached 30 g., the curve was, more often than not, interrupted at critical times by sharp downward breaks. In such experiments, recovery from these periods of poor health was sometimes accompanied by the appearance of tumors in a large percentage of the animals in a relatively short time (relationship exemplified in Figure 2). It will also be noted that, even at high levels of concentration, the mean time of appearance of tumors was in no case less than 20 weeks.

TABLE III

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF SYNTHETIC CARCINOGENS IN VARIOUS SOLVENTS UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CARCINOGEN[1]	SOLVENT	CONCENTRATION OF CARCINOGEN IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF CARCINOGEN PER UNIT AREA[2] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, X (weeks)	CLASSIFICATION	RELATIVE CARCINOGENIC POTENCY, P_{MC}
215	DMBA	Benzene	0.115	100	3	0.345	15.6±3.3[3]	B	0.19
214	DMBA	Benzene	0.345	100	3	1.035	7.4±1.1	B	0.62
244	BP	Benzene	0.172	100	3	0.516	25.1±2.5	B	0.10
244	BP	Benzene	0.172	20	3	0.516	~27.3	X	(0.09)
252	BP	Benzene	0.322	20	1	0.322	~35.0	X	(0.18)
252	BP	Benzene	0.322	20	3	0.966	~23.0	X	(0.11)
252	BP	Benzene	0.322	100	3	0.966	~20.1	X	(0.13)
282	BP	Benzene	0.092	20	3	0.276		X	
264	BP	White oil	0.175	20	3	0.525	~25.4	X	(0.10)
270	BP	White oil	0.35	20	3	1.05	~21.5	X	(0.12)
285	BP	Cotton-seed oil	0.50	20	3	1.50		X	
286	BP	Cotton-seed oil	1.00	20	3	3.00	~20.4	B(X)	(0.13)
281	MC	Cottonseed oil	0.042	100	3	0.126	~41.0	B	0.047

[1] Carcinogens are 7,12-dimethylbenz[a]anthracene, DMBA; benzo[a]pyrene, BP; 3-methylcholanthrene, MC.
[2] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.
[3] 5% fiducial limits.

USE OF REFERENCE STANDARDS AS A SCALE OF RELATIVE POTENCY OF COMPLEX TARS AND OILS

Various methods of expressing the relative potencies of the pure polycyclic carcinogens and complex materials have been reviewed by Badger (11). Many of these are simply rough classifications based upon a particular investigator's personal experience. The Iball index (12) and a recent adaptation (13) attempted a more quantitative approach, involving the use of the reciprocal of the mean time of appearance of tumors. Such methods are useful in comparing results obtained in a given laboratory using a standardized and inflexible schedule of application of various materials. However, experimental techniques differ significantly from one research organization to the next, thus rendering the comparison of data on this basis rather difficult. Further, as was pointed out previously in this article, it was sometimes necessary in the current investigation to change either or both the frequency and severity of exposure to obtain satisfactory conditions for the measurement of the potency of a sample while minimizing its toxic side effects. Hence, a scale of relative potencies which would permit such flexibility in experimental design was needed.

The use of the data on relative rates of induction of tumors by the pure carcinogens, methylcholanthrene and benzopyrene, as external standards of reference, provided a practical solution to this problem. The latter is one of the important contributors to the potency of cracked residua (14); the former has not been identified in complex tars and oils but might be regarded as a

representative of the 4-ringed carcinogens supposed to be present (15,16). Thus, the mean time of appearance of tumors induced by some complex tar or oil may be translated into a value of Relative Carcinogenic Potency, P_{MC} (potency as compared to methylcholanthrene), by use of Figure 5. The dosage of methylcholanthrene corresponding to the given mean time is either read off the graph or calculated from the equation,

$$d = \frac{8.2}{\bar{x} - 3.7} - 0.10 .$$

Then, the value of the relative potency, P_{MC}, for the complex material in question is

$P_{MC} = d$, for an experiment involving one application each week,

$= \frac{d}{2}$, for an experiment involving two applications each week,

$= \frac{d}{3}$, for an experiment involving three applications each week.

Thus, by comparing the results of tests on complex materials with those involving methylcholanthrene as an external standard of reference, one may obtain the relative carcinogenic potencies of the different materials, even though the experiments may have involved differing frequencies of applications. It should be noted, however, that the description of the potency of a complex oil cannot be considered complete until it has been tested at two appreciably different dosages, i.e., 20 mg. and 100 mg. per application, unless negative results were obtained at the higher level of dosage in a test in which the mice survived and grew at a normal rate.

Thus, a given oil may be much more potent than another under severe conditions of exposure, but the former may actually have a lower potency than the latter if the comparison is carried out under mild conditions. Chemical research on such oils has led to an understanding of this apparent paradox, and the matter will be dealt with in a later publication.

The technique of relating results on complex materials to those on standard synthetic carcinogens should facilitate comparison of results from different laboratories. The synthetic compound chosen should, of course, be comparable to the type of carcinogens supposed to be present in the complex mixtures. Under appropriate conditions of dosage and exposure, it must be capable of inducing tumors at least as rapidly as the most potent of the samples to be tested. Thus, benzopyrene has not proved to be a suitable standard for work with C3H mice, since it will not induce tumors sufficiently rapidly even at high levels of concentration. 7,12-Dimethylbenz(a)anthracene on the other hand, is very satisfactory from this standpoint, but has the disadvantage of requiring special care in handling to prevent its oxidation to the non-carcinogenic 7,12-photooxide (7).

It should be reemphasized that this consideration of a quantitative scale of potencies applies only to the results of experiments which may be classified as satisfactory (A and B by our criteria). Those in our Class X have only a limited value. If positive results are obtained, the material may be classified qualitatively as carcinogenic; if survival is good and most of the mice eventually develop tumors (X classification based on certain irregularities in growth) the estimation of the apparent potency may be useful as a minimum value.

RELATIVE POTENCIES OF POLYCYCLIC AROMATIC COMPOUNDS

The data available to the writers on experiments involving repeated applications of solutions of various polycyclic carcinogens in solvents such as benzene, acetone, and white mineral oil of high viscosity, upon the skin of mice, indicate that the average rate of induction of papillomas is independent of the quantity of solution used in each application so long as it exceeds 10 mg. The controlling variables are the frequency of application and the dosage of carcinogen per unit area of the skin. The latter will depend upon the concentration of carcinogen in the solution applied and the relative spreading coefficient of the solution.

Solutions of polycyclic hydrocarbons in different solvents have different capacities for spreading over the skin of a mouse because of variations in such physical properties as volatility, interfacial tension, and viscosity. Since much of the previous experimental testing of polycyclic compounds for their carcinogenicity to the skin has been carried out with solutions in benzene, this solvent was used for the reference standards described herein. An intensive investigation of the effects of variation of the composition of the solvent has also been carried out and will be described in a later paper.

By comparing data on the rate of induction of tumors by solutions of known concentration of various aromatic hydrocarbons in benzene under suitable experimental conditions, with such data as those shown in Table II and Figure 5, it should be possible to arrive at logical estimates of the relative carcinogenic potencies

of the different compounds. By inference, the relative potency of methylcholanthrene on the P_{MC} scale would be 100. Corresponding numerical values for benzopyrene and 7,12-dimethylbenzanthracene may then be derived from the data of Table III. Thus, since 0.345 percent 7,12-dimethylbenzanthracene produced tumors at the same rate as 0.62 percent methylcholanthrene under comparable conditions, the relative potency of this dimethyl- derivative is

$$P_{MC}^{\circ} = \frac{100}{0.345} \times 0.62 = 180$$

From Experiment 215, the relative potency, P_{MC}°, for 7,12-dimethylbenzanthracene is 165. The higher value is preferred at the present time, since, if autoxidation of the hydrocarbon caused any reduction in activity, it might reasonably be assumed that the effect would have been greater in Experiment 215.

Similarly, the relative potency of benzopyrene on this scale is 57 - 65 (Experiments 244, 252, and 282). The data from Experiment 252 involving three applications each week cannot be used since apparently the maximum effective dosage of this carcinogen had been reached at a lower level. Thus, as a general rule the determination of the relative potency of any given compound should be based upon the results of experiments at at least two different concentrations, which yielded significantly different mean times of appearance of tumors under otherwise comparable conditions. At least one and preferably both of these should meet our classification A or B from the standpoint of growth and survival.

LITERATURE CITED

(1) Pullinger, B. D., J. Path. Bact., 55, 301 (1943).

(2) Cramer, W. and Stowell, R. E., J. Nat. Cancer Inst., 2, 369 (1942).

(3) Bryan, W.R. and Shimkin, M.B., J.Nat.Cancer Inst., 1, 807 (1941).

(4) Bliss

(5) Whittaker, E. and Robinson, G., The Calculus of Observation, London, Blackie and Sons, Ltd., 1924, p. 209.

(6) Watson, A. F. and Mellanby, E., Brit. J. Exp. Path., 11,267 (1930).

(7) Bradbury, J. T., Bachmann, W.E., and Lewisohn, M.G., Cancer Research, 1, 685 (1941).

(8) Cook, J.W. and Kennaway, E.L.

(9)

(10) Iversen, S. and Arley, N., Acta Pathol. Microbiol. Scand., 27, 1 (1950).

(11) Badger, G.M., Brit. J. Cancer, 2, 309 (1948).

(12) Iball, J., Am. J. Cancer, 35, 188 (1939).

(13) Blanding, F.H., King, W.H., Priestley, W., and Rehner, J., Arch. Ind. Hyg. Occ. Med., 4, 335 (1951).

(14) Tye, R., Graf, M.J., and Horton, A.W., (Anal. Chem., 1954).

(15) Berenblum, I. and Schoental, R., Brit. J. Cancer, 1, 157 (1947).

(16) Fischer, H.G.M., Priestley, W., Eby, L.T., Wanless, G.G., and Rehner, J., Arch. Ind. Hyg. Occ. Med., 4, 315 (1951).

EXPERIMENTS INVOLVING THE APPLICATION OF MATERIALS UPON THE SKIN OF C3H MICE

LEGEND

Symbols used in connection with average weight curves:

- $\overline{\nabla}_i$ Time of death (from disease) of i th tumor-free mouse.
- i Time of death (from disease) of i th tumor-bearing mouse
- $\overline{\nabla}_i$ or $\overline{i}$ Time mouse killed.
- * Painting discontinued for 1 week at each indicated time.

Gross and microscopic pathology:

- $\boxed{i}$ Time of appearance of first papilloma in i th mouse. When this symbol is first used for a given mouse after its death, the presence of non-invading carcinoma (intra-epithelial) or small areas of benign neoplasm was determinable only by histopathology.
- $\textcircled{i}$ Time of appearance of gross changes indicative of malignancy in tumor of i th mouse. Diagnosis confirmed except in cases where no tissue section available. When this symbol is first used for a given mouse after its death, the invasive malignancy of the tumor was determinable only by histopathology.
- $\overset{\#\$}{\boxed{i}}$ or $\overset{\#\$}{\textcircled{i}}$ No tissue section available for histopathology because of extensive post-mortem decomposition or cannibalism.

API 05799

EB 005544

FIGURE 1

EXPERIMENT 220, SOLUTION OF METHYLCHOLANTHRENE, 1.034 PERCENT IN BENZENE

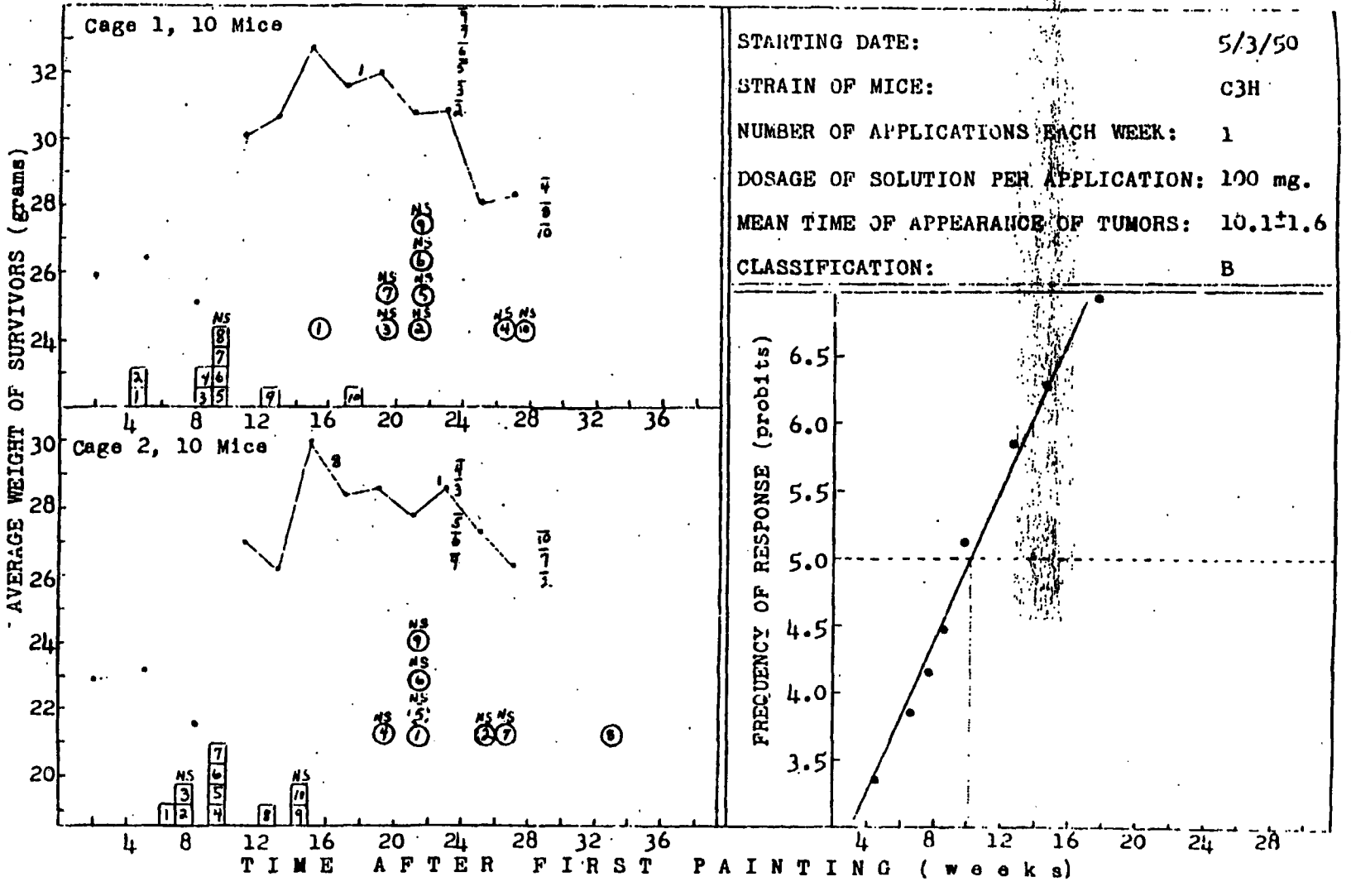


FIGURE 2

EXPERIMENT 268, SOLUTION OF METHYLCHOLANTHRENE, 0.287 PERCENT IN BENZENE

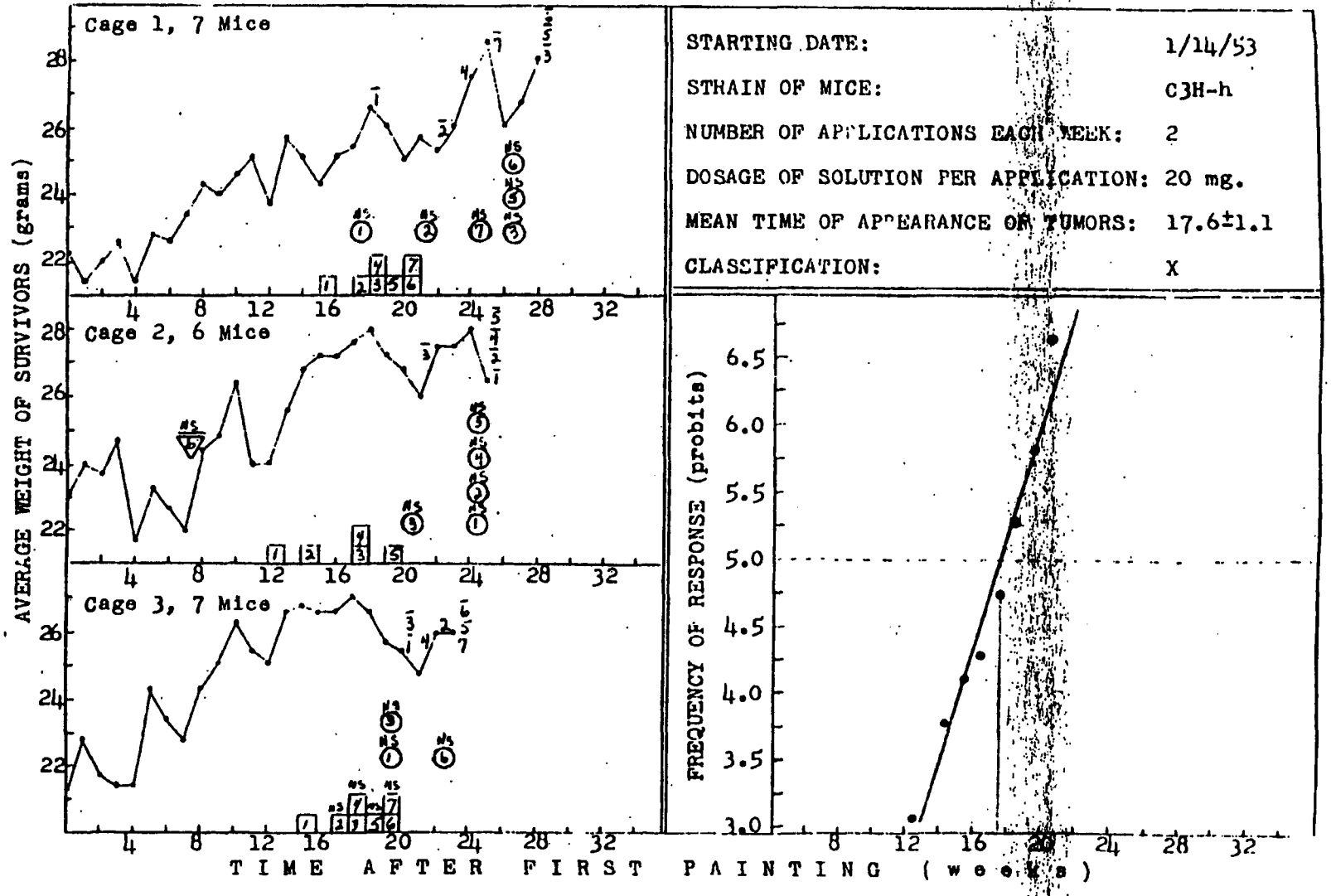
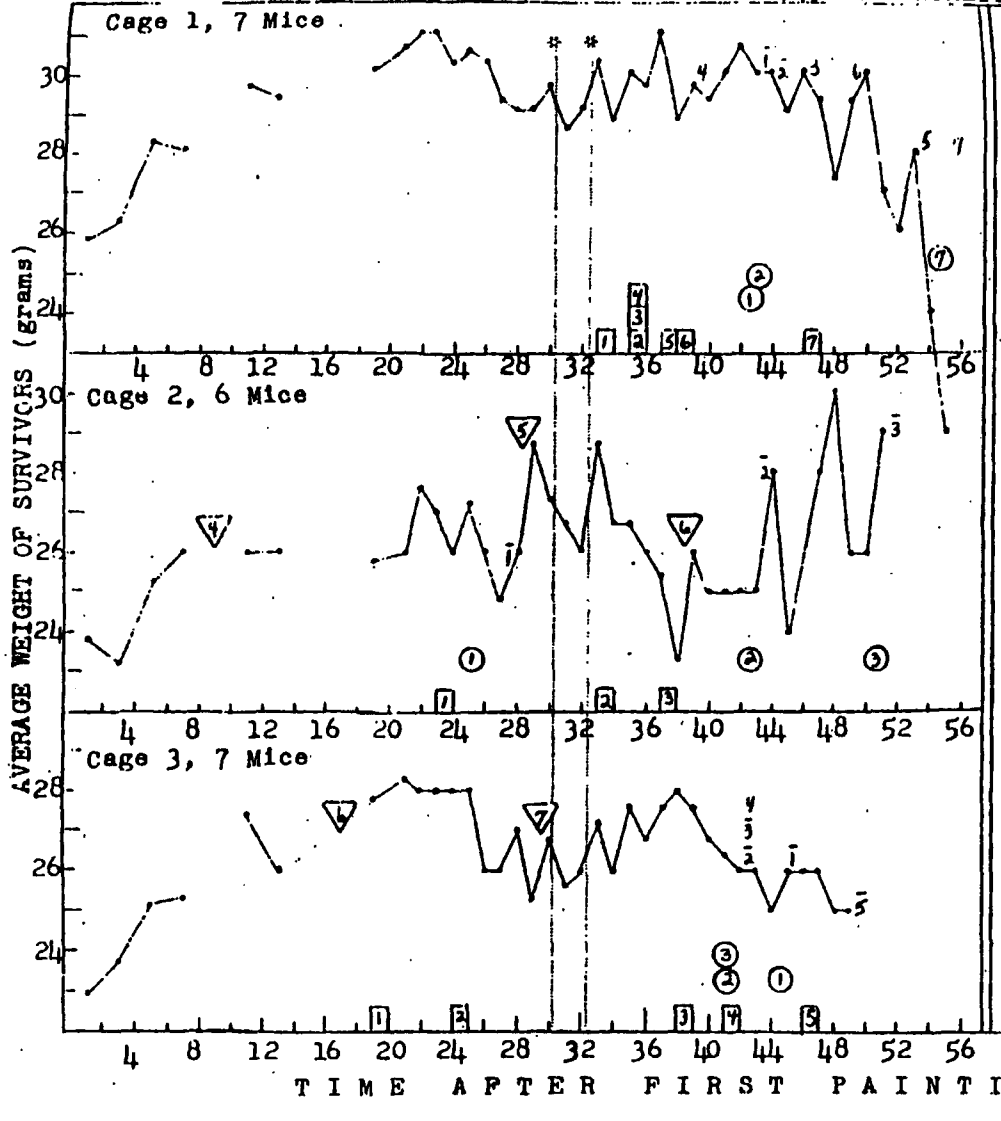


FIGURE 3 - EXPERIMENT 74. NON-CATALYTICALLY CRACKED OIL



STARTING DATE: 8/7/51

STRAIN OF MICE: C3H

NUMBER OF APPLICATIONS EACH WEEK: 2

DOSAGE OF SOLUTION PER APPLICATION: 100 mg.

RELATIVE CARCINOGENIC POTENCY, P_{MC}: (0.08)

CLASSIFICATION: X

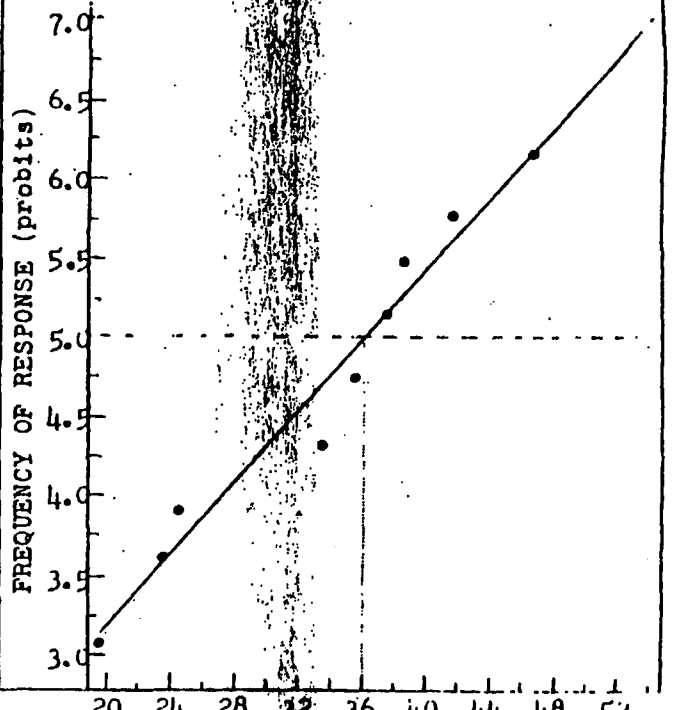
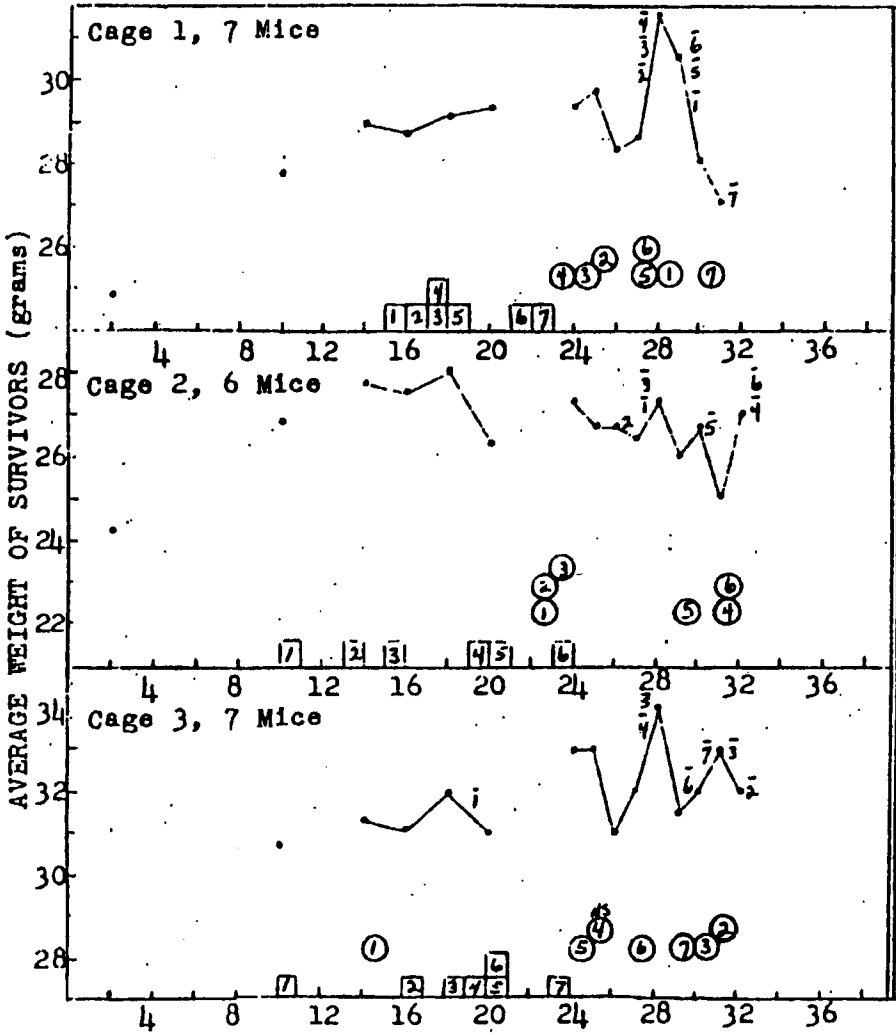


FIGURE 4

EXPERIMENT 243, SOLUTION OF METHYLCHOLANTHRENE, 0.15 PERCENT IN BENZENE



STARTING DATE:	7/28/51
STRAIN OF MICE:	C3H
NUMBER OF APPLICATIONS EACH WEEK:	1
DOSAGE OF SOLUTION PER APPLICATION:	100 mg.
MEAN TIME OF APPEARANCE OF TUMORS:	18.1±1.8
CLASSIFICATION:	A

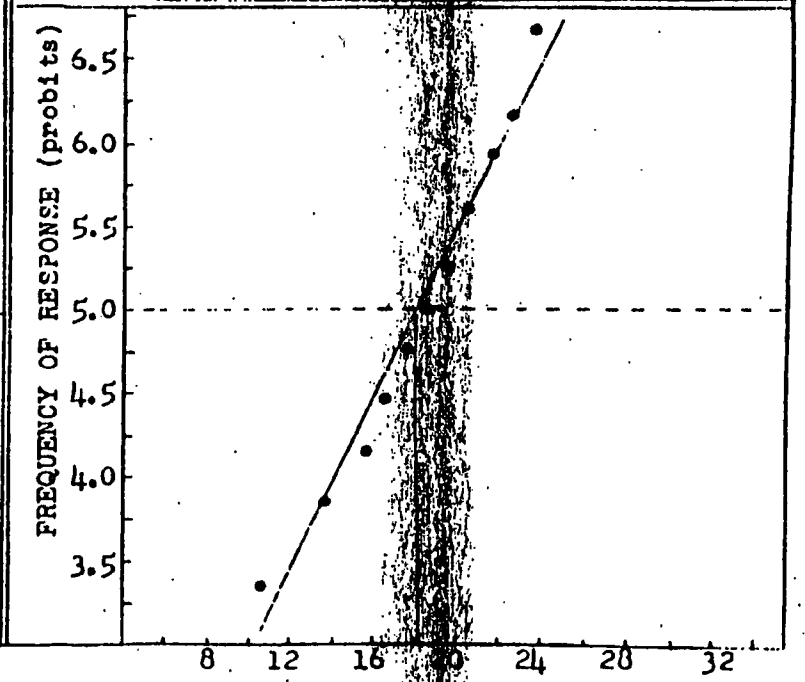
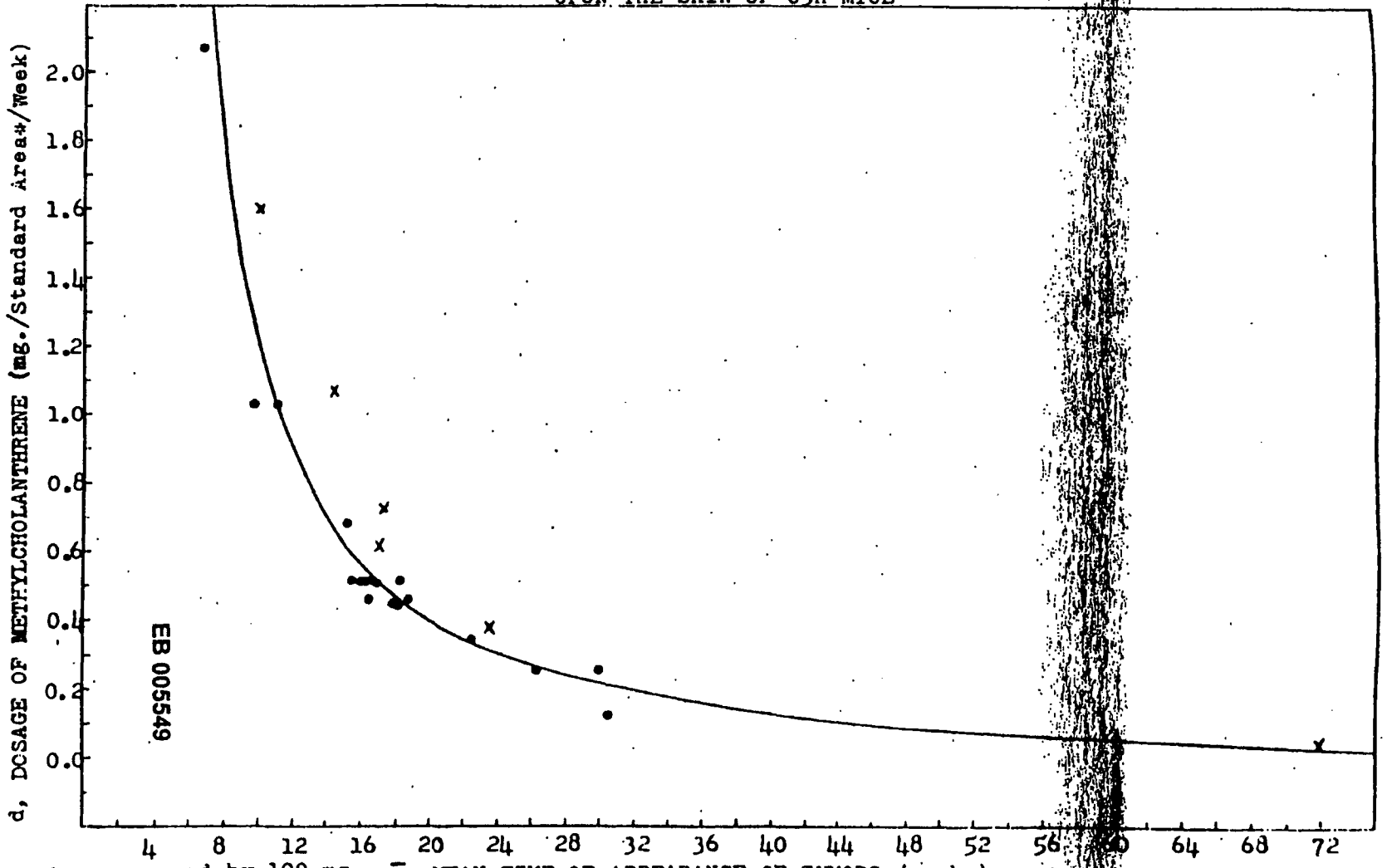


FIGURE 5

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE



* Area covered by 100 mg. of the solution.

$\bar{x}$, MEAN TIME OF APPEARANCE OF TUMORS (weeks)

FOR INFORMATION ONLY

NOT FOR PUBLICATION

API RESEARCH PROJECT MC-1

At Kettering Laboratory - University of Cincinnati (1946-1954)

Manuscript of paper submitted for review prior to publication.

Title: "Carcinogenesis of the Skin, I"

Proposed for publication in "Cancer Research"

Status of Manuscript

May 4, 1954 - Received from Kettering Laboratory.

May 6, 1954 - Copies mailed to Subcommittee on Carcinogenicity (and to MAC).

EB 005550

API 05805

CARCINOGENESIS OF THE SKIN, I. Basic Methods Employed in Testing Complex Hydrocarbon-type Tars and Oils, and the Development of a Quantitative Scale to Express Their Relative Potencies to Mice.

by A. Wesley Horton and Dorothy T. Denman

The investigation of skin cancer in this Laboratory has been concerned with the estimation of the extent of the potential hazard of this disease in certain industries. As in any other aspect of the general field of toxicology, it must be recognized that a specific hazard is the product of several variables, two of which assume dominant importance. These may be termed conveniently the physiological activity of the material or mixture in question, and the conditions of exposure. The latter aspect involves the practices of personal and industrial hygiene. This paper will be devoted primarily to the development of reproducible methods of determining the first variable, the level of activity, or relative carcinogenic potency, of hydrocarbon-type oils and tars.

Mice were chosen as the primary test species since neoplasms of the skin, comparable in certain respects to those of man in the same tissue, can be reproduced in these animals under suitable experimental conditions. Certain specific materials, which have induced an abnormally high incidence of cancer of the skin of men as a result of repeated exposure in their occupation, have been found capable of producing epitheliomas in the skin of mice. Conversely, in our experience certain inbred mice, such as the C3H strain, have not developed "spontaneous" skin tumors, nor have

they reacted by skin tumor formation to repeated experimental application of a number of materials, including water, benzene, white mineral oil, etc., which are presumed to be non-carcinogenic to the skin of man on the basis of extensive practical experience.

The specification, "suitable experimental conditions", in the previous paragraph was used advisedly. It is evident from perusal of the literature, that too often the problem of excessive mortality due to the toxicity of the material under examination, as well as that due to the occurrence of incidental disease among the experimental animals, has been met only by increasing the number of animals on test. Such an approach ignores the probability that the survivors of these conditions are not at all representative of the population involved with respect to their susceptibility to carcinogenesis. Hence, stress will be laid upon the necessity for standards of selection and care of the experimental animals which will maintain their health at as high a level as possible.

EXPERIMENTAL METHODS

Most of our work has been carried out with male mice of the C3H strain, purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. A number of experiments have also been carried out with male mice of the CFW strain obtained from Carworth Farms, but the results were not as reproducible as those derived from the C3H strain. Although the difference in genetic homogeneity is undoubtedly a factor, the fact that the C3H mice are more docile probably contributes to the reproducibility of experiments in which they are employed. The frequent occurrence of bites and scratches

of the skin of the CFW mice, inflicted by their companions, probably results in occasional direct intraepithelial introduction of carcinogens. Added to this complication is the possibility that wound-healing may play an accelerating role in carcinogenesis (1). The same trouble has been experienced in recent experiments with Swiss males, although the females seem to be more congenial.

The mice were received at six to eight weeks of age and placed under observation for approximately four weeks before use in any experiment. To control infestation of the animals with mites or lice (which when uncontrolled affects adversely their health), the mice were dusted routinely with Aramite[1] by the supplier prior to shipment and then twice again with the same miticide during the period of observation in this laboratory. Any group in which there was evidence of poor health during this period was withheld from use in tests in which quantitative results were needed.

In all experiments described herein, the mice were fed on Purina Laboratory Chow and water without restriction. At six months of age, C3H mice fed on this diet and subjected to no adverse experimental procedure reach an average weight of about 29-31 g., while corresponding CFW mice weigh about 33-35 g. Since growth of both the hosts and their tumors might vary with the temperature of their surroundings, an effort has been made to maintain a relatively constant temperature of 75±2° in the animal rooms.

For a given experiment, a group of 20 to 30 mice was divided into three to five cages. The animals were assigned individual numbers for identification and marked by clipping their toes.

[1] Aramite - 15W, U. S. Rubber Company, Naugatuck, Conn.

The exposure of the mice to any given material involved repeated applications upon the interscapular region. In preparation for the application of dosages of 100 mg. of a material, the fur was left intact, but when the dosage was to be less than 100 mg., the fur was removed by means of electric clippers. A small camel's hair brush of a selected size to deliver the desired dosage was used to apply the material, which was then simply allowed to spread according to its individual physical characteristics. Applications were repeated in accordance with a predetermined schedule (one, two, or three times each week) until the animals died. Care was taken to keep the dosage and the location of each application as constant as possible throughout the experiment.

Normal growth of the mice could not be maintained in testing certain of the materials, particularly those of high potency, when the experiment involved three 100 mg. applications each week. Hence, when there was some physicochemical or biological evidence that a given sample was likely to have a relatively high carcinogenic potency to the mice (equivalent to that of a solution of 0.15 percent, or greater, of methylcholanthrene in benzene), the material was applied at a lower frequency, usually once each week. In the case of materials of lower potency, more frequent applications were required to obtain sufficient tumors. Hence, when toxicity of such samples proved a serious problem at the 100 mg. level, the dose was reduced, in some cases to as low as 5 mg. per application.

Experience has shown that, even when the mortality among an experimental group was low, the time of appearance of tumors was sometimes delayed seriously by conditions of variable or uncertain origin which also interrupted the normal growth of the animals. As a measure of such conditions, therefore, the mice were weighed at frequent intervals throughout the experiments. Since early observations gave evidence that interruptions of growth were usually the result of intercurrent infections of low severity affecting most of the animals in a given cage, the weighing of individual animals was discontinued in favor of determining the average weight of the survivors in each group. Various precautions, such as regular cleaning and sterilization of cages, feeders and water bottles, decontamination (with a bacteriostatic agent) of gloves used to handle animals from one cage before proceeding to another, and isolation of sick mice, were taken to minimize infections and to prevent their spread from one group to another.

Throughout each experiment, the animals were examined at least once each week by one of us (D.T.D.), and the presence and time of appearance of any abnormal gross changes in the skin were recorded. Epilation and crusting of small areas were frequently noted and, in the early stages of tests on solutions of greater than 0.2 percent of methylcholanthrene or benzopyrene in benzene, large swellings, of the type previously described by Cramer (2), and small ulcerated areas, were occasionally observed. In response to the application of solutions of these synthetic carcinogens, papillomas eventually developed on the skin of practically all of

the surviving animals except in the case of the lowest level of concentration tested (0.01 percent). In general, the induced neoplasms progressed through the familiar pattern from papilloma to squamous cell carcinoma, with gross evidence of invasion. In the first test of a given material, sections of any epidermal tumors produced were prepared for microscopic examination.

The date of which each typical papilloma reached the arbitrary size of 1 mm. in diameter and elevation was recorded as the "time of appearance" of the papilloma. In addition, the date on which it was grossly apparent that the tumor was invading the subcutaneous tissues was noted (rolled border, generally accompanied by cratering necrosis of the center). When a mouse developed such an apparently malignant tumor, it was killed. Usually, the others were maintained on test until death from "natural causes" occurred. The data, thus obtained, were recorded on graphs such as those shown in Figures 1 through 4.

As experience was gained in associating the physical and chemical characteristics of various types of materials with their relative toxicity and with their ability to induce tumors of the skin in C3H mice, certain criteria were developed for selecting optimum conditions for any given biological test. As a general practice, a schedule of application was chosen which would lead to induction of papillomas in an average time of not less than 12 weeks. The restriction was based on repeated observations that this average time was unduly variable in experiments in which the severity of exposure resulted in more rapid induction of tumors.

on the other hand, experimental conditions which involved very long periods of exposure before tumors were induced proved to be even more unsatisfactory than those associated with very short latent periods, because of the increased possibility of intercurrent infections or other unidentified interferences with the normal growth of the mice. Even when these interruptions resulted in little or no loss of animals, the irregular effects on the growth of the induced tumors made it very difficult to interpret the results of the experiment. Therefore, as a general rule the frequency and severity of the applications were scheduled to produce an average latent period as close to 15 weeks as possible. When the materials were low in potency, this obviously meant the use of as severe conditions as were compatible with the normal growth and survival of the mice.

INTERPRETATION OF DATA OBTAINED

In general, under these experimental conditions one of two results has been obtained in response to the applications of any one of a series of materials, i.e., most of the animals which lived long enough developed tumors of the skin at the site of the application, or none of them did so. The outcome of the experiments, therefore, has been essentially unequivocal, and the relative potencies of the different active materials could be expressed in terms of the relative rates of induction of tumors.

The calculation of the "mean time of appearance of tumors" involved the determination, by the method of least squares, of the linear function which best fitted the plot of the cumulative frequency of tumor response (in probits) versus the time (in weeks) after the first application (shown on the right side of Figures 1 through 4). The latter coordinate is equivalent to cumulative dosage. It is to be noted that, for these experiments involving repeated applications of carcinogenic materials to the skin, the response varied linearly with the dosage itself, and not with the logarithm of dosage. It is assumed that the exponentially decreasing rate of appearance of tumors usually observed following single or limited numbers of exposures to carcinogens (3) was not seen in these experiments because the more resistant animals in any given group were given a larger effective dosage than were the more susceptible.

Calculation of Cumulative Frequencies of Response

The convention is adopted (after Bliss, 4) that, at the midpoint of the period between two given observations, the number of mice bearing tumors is equal to the average of the numbers at the times of the observations. At the midpoint of each period during which one or more mice develop their first papilloma, a calculation is made of the cumulative percentage of the "effective group of mice" which bear tumors on that date. The "effective group" at a given time is defined as the sum of the number of survivors plus the number of

mice which died previously after developing tumors of the skin, except that after the appearance of a tumor in the "average" mouse this sum is held constant. The "average" mouse is designated by the median in a simple arithmetic array of the times of appearance of the first tumor in each animal.

As an example, the calculations used to obtain the cumulative frequencies of tumor response from the data of Figure 3 are shown in the following table:

TABLE 1

TIME* OF OBSERVATION OF NEW POSITIVES	MIDPOINT* OF PERIOD BETWEEN OBSERVATIONS, x	NUMBER OF NEW POSITIVES, R'	TOTAL NUMBER OF MICE BEARING TUMORS, R' + ΣR**	CUMULATIVE RESPONSE, $\Sigma R + \frac{(\Sigma R + R')}{2}$	EFFECTIVE NUMBER OF MICE	FREQUENCY OF RESPONSE, r. (probits)
	19.5			0.5	18	3.08
20		1	1			
	23.5			1.5	18	3.62
24		1	2			
	24.5			2.5	18	3.91
25		1	3			
	33.5			4.0	16	4.33
34		2	5			
	35.5			6.5	16	4.76
36		3	8			
	37.5			9.0	16	5.16
38		2	10			
	38.5			11.0	16	5.49
39		2	12			
	41.5			12.5	16	5.78
42		1	13			
	46.5			14.0	16	6.15
47		2	15			

* Number of weeks after first application. ** ΣR is the total number of mice with tumors at the time of previous observation.

Calculation of Mean Time of Appearance of Tumors, $\bar{x}$:

1. Assume that the tumor response in probits, r, is a linear function of the time, x,
   or $r = mx + b$
2. Determine constants, m and b, by method of least squares (5). Normal equations:

$$\Sigma rx = m\Sigma x^2 + b\Sigma x$$

$$\Sigma r = m\Sigma x + 9b$$

(Values of x in weeks and r in probits are underlined in preceeding table)

$$1485.67 = 10{,}688.25m + 300.5b$$

$$42.28 = 300.5m + 9b$$

$$b = 0.960$$

$$m = 0.112 = \text{slope of dose-response relationship}$$

$$\therefore r = .112x + .960$$

3. $\bar{x}$ = time of 5-probit response = 36.1 weeks
   s, standard deviation of mean,
   = $\bar{x}$ minus time of 4-probit response
   = 8.9 weeks.

CLASSIFICATION OF EXPERIMENTAL RESULTS ON THE BASIS OF THE HEALTH OF THE ANIMALS

In the appraisal of data derived from these experiments, consideration has been given to the following apparent relationships between the average growth and survival of the mice and the mean "time of appearance" of their epidermal tumors.

1. If, in the course of an experiment, an intercurrent infection overtakes a group of mice at about the time when papillomas would be expected to appear (on the basis of other comparable experiments), there is usually a delay in the mean time of appearance of tumors. The infection need not be lethal to any of the mice, a change in sign of the slope of the average growth curve frequently being an indication of such a condition (see Figure 1). It is believed that, in many such instances, the mice would probably be unaffected were their resistance not diminished somewhat by systemic toxic effects induced by the hydrocarbons being applied upon their skin. Thus, untreated controls may not show any symptoms of the infection.

Not infrequently, as the animals recover from such disturbances, tumors grow to measurable size in a large proportion of the animals in a relatively short time. The slope of the plot of the frequency of response versus time is then exceedingly steep. In other words, the standard deviation of the mean time of appearance of tumors in the group is much smaller than normal (see Figure 2). Thus, it could be assumed mistakenly, if it were not for the evidence from the weight curves of the presence of uncontrolled variables, that such an experiment had defined the statistical limits of the mean better than another in which such disturbances had not occurred.

2. In some experiments the cumulative toxic effects of the material applied, or chronic infections, or a combination of both, result in prolonged inhibition of the growth of the mice. A number of examples have been observed in which the average weights have increased for a few weeks, but became stationary at a point 10 percent or more below that of adult controls, and then, in many instances, gradually declined (see Figure 3). Thus, whether or not these conditions influence the timing of the initial changes resulting in the neoplasms they may retard the growth of the induced tumors to visibly discernible size and, accordingly, lengthen the apparent average period of exposure required for the induction of tumors by the material under test. In such experiments, the standard deviation of this mean time is frequently much greater than normal.

This somewhat extended discussion of the apparent relationships between the irregularities in growth due to the toxicity of applied materials and to intercurrent infections, and the rate of induction of tumors, does not relate to the originality of this observation but rather from our recognition of its importance in experiments in which various materials are being compared. Occasional mention of the phenomenon has appeared in the literature on experimental carcinogenesis (i.e., 6,7,8) and, in the field of chemotherapy of cancer, recognition has been given to the necessity of considering the systemic toxicity of a material in evaluating the specificity of its apparent "anticarcinogenic" effects (i.e., 9).

further, the relationships between general health and susceptibility to "spontaneous" tumors in man are currently receiving attention. Hence, it is believed that, in the experimental assay of the relative potencies of various materials or of the susceptibility of various species or strains of one species to a given material, information on the health and survival of the groups of animals being compared is essential to any quantitative interpretation of the data.

The results of all experiments described herein have been examined, therefore, in the light of these relationships. Each one has been classified according to the estimated significance of such effects in the following manner.

Class A. Those characterized by normal growth and survival of the mice at least until the time when most of the animals have developed papillomas (see Fig.4).

Class B. Those in which all or most of the mice lived long enough to develop tumors, but in which there was evidence (from average weights) of intercurrent infection at times prior to or during the period when the new growths were appearing. So long as recovery from the infection was prompt, and no obvious skewing of the distribution of the times of appearance of tumors occurred, it was felt that reliable estimates of the mean time of appearance could reasonably be made (see Figure 1). However, when no tumors had appeared prior to the onset of an infection, and when with recovery tumors developed

somewhat precipitously, the reliability of the apparent mean time of appearance was not determinable, and therefore such tests were classified X (see Figure 2).

Class X. Those characterized by poor growth (as discussed in Section 2 above) or high rate of mortality. The confidence limits of the apparent mean time for the induction of tumors could not be calculated by standard statistical methods, because of inability to assign proper weight to the effects of the uncontrolled variables.

Several types of moderately toxic materials failed to yield experiments of the Class A category. On the other hand, experimental results of the frankly unsatisfactory type (Class X) were usually found to be avoidable through the exercise of appropriate care in the initial choice and the subsequent handling of the mice.

REFERENCE STANDARDS

To provide a background of reference which might permit a more nearly quantitative interpretation of the mean times of appearance of tumors induced in C3H mice by certain complex oils and tars under various conditions of exposure, a number of experiments have been carried out over the past five years employing solutions of the synthetic carcinogens, 3-methylcholanthrene, benzo[a]pyrene, and 7,12-dimethylbenz[a]anthracene, in various solvents. It has been found that one of the essential requirements

for obtaining reproducible and logically consistent results is the maintenance of the animals in a good state of health as measured by growth curves. Thus, in Table II, the results of Experiments 205 show the precision of the determination of the mean time, $\bar{x}$, when Class B experiments are involved. Similarly, it has been found that even in the case of a complex material, so long as the conditions of the experiments permitted reasonably normal growth of the animals, the repeatability of the determination of the mean time, $\bar{x}$, has been quite satisfactory (within $\pm$ 10%).

To determine the effect of variations in the level of concentration and in the frequency of exposure, comparable experiments were conducted with other solutions of methylcholanthrene in benzene, and the results are shown in Table II.

The purity of the polycyclic hydrocarbon is obviously an important consideration in obtaining consistent results. The usual chemical criteria are necessary, but not always sufficient, requirements. In one case in particular, the methylcholanthrene, as received, had a sharp melting point, 176-7°C., and no indication was apparent by its ultraviolet absorption spectrum of any contamination. Yet, on repeated bioassay, it proved to be excessively irritating and much more rapidly carcinogenic than the normal material. Unfortunately the quantity available did not permit determination of a satisfactory procedure for purification. Since no further sample with such unusual potency has been obtained, bioassay remains the only way of acertaining that the methylcholanthrene is free of the material responsible for this activity.

TABLE II

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CONCENTRATION OF METHYLCHOLANTHRENE IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF METHYLCHOLANTHRENE PER UNIT AREA[1] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, $\bar{x}$ (weeks)	CLASSIFICATION
201	0.012	100	3	0.036	72.6	X
202	0.044	100	3	0.132	30.4±3.8[2]	B
203	0.086	100	3	0.258	29.9±2.2	B
203	0.086	100	3	0.258	26.3±3.4	B
204	0.172	100	3	0.516	15.7±1.6	B
204	0.172	20	3	0.516	16.4±1.1	B
204	0.172	20	3	0.516	16.6±1.2	B
204	0.172	20	3	0.516	15.5±1.1	B
205	0.345	100	3	1.035	11.0±1.9	B
205	0.345	100	2	0.69	15.1±1.5	B
205	0.345	100	1	0.345	22.4±2.7	B
205	0.345	100	3	1.035	14.7	X
206	0.69	100	1	0.69	17.8	X
206	0.69	100	3	1.98	6.7±4.1	B

[1] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.

[2] 5% fiducial limits.

TABLE II (page 2)

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CONCENTRATION OF METHYLCHOLANTHRENE IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF METHYLCHOLANTHRENE PER UNIT AREA[1] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, $\bar{X}$ (weeks)	CLASSIFICATION
207	1.15	100	3	3.45	8.9	X
209	0.517	20	1	0.517	18.3±1.9[2]	B
209	0.517	100	1	0.517	16.2±2.7	B
209	0.517	100	3	1.551	10.4	X
219	0.115	100	3	0.345	24.0	X
220	1.034	100	1	1.034	9.7±1.6	B
238	0.23	20	2	0.46	18.7±2.3	B
238	0.23	100	2	0.46	16.4±2.0	B
243	0.15	100	3	0.45	17.9±2.0	A
243	0.15	20	3	0.45	18.2±2.1	B
268	0.287	20	2	0.574	17.6	X

[1] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.
[2] 5% fiducial limits.

In Table II it is apparent that variation in the dosage of solution per application from 20 to 100 mg., other factors remaining constant, had no effect on the rate of induction of tumors by methylcholanthrene in benzene. Similar results have been observed when solutions of benzopyrene in benzene were used as the carcinogenic stimulus. This constancy of potency with variation in the quantity of material applied (or area of skin exposed) also holds for some complex oils and tars, but for others there is significant change of potency with changes in the quantity used for each application. The chemical basis for these differences will be discussed in a later paper.

The values in column 5, Table II, were calculated from the concentration of methylcholanthrene in the solutions and the number of applications per week. It has been determined experimentally that variations in the concentration of the carcinogen did not affect the area covered by a given weight of the solutions. It was further assumed that the dosage per unit area was not significantly different when 20 or 100 mg. of the same solution were applied. Comparison of the data in columns 5 and 6 shows a consistent inverse relationship between the dosage of carcinogen per unit area per week and the mean time of appearance of tumors. The data (omitting Class X experiments) are plotted in Figure 5 together with the curve of the corresponding hyperbolic function (calculated by the method of least squares),

$$(\bar{x} - 3.7)(d + 0.10) = 8.2$$

or,

$$\bar{x} = \frac{8.2}{(d + 0.10)} + 3.7 \quad .$$

It is interesting to note the similarity between this equation and that calculated from theoretical considerations of the mechanism of carcinogenesis by Iversen and Arley (10),

$$\overline{t_{ap}} = \frac{1}{kc_o} + T, \text{ where k and T are constants.}$$

The constant, 0.10, in the experimental equation might be interpreted as a correction required by the definite, though very small, probability of the "spontaneous" excitation of a cell of the dorsal skin of a C3H mouse to the rate of proliferation necessary for the development of a new growth.

Similar experiments have been carried out with solutions of two other commonly used carcinogenic hydrocarbons, benzopyrene and 7,12-dimethylbenzanthracene. The results are shown in Table III, together with some data on solutions of methylcholanthrene in solvents other than benzene. As is evident from the data, it has been difficult to obtain satisfactory experiments with benzopyrene on C3H mice. The growth of the animals has tended to be extremely erratic. Even in tests in which the average weight eventually reached 30 g., the curve was, more often than not, interrupted at critical times by sharp downward breaks. In such experiments, recovery from these periods of poor health was sometimes accompanied by the appearance of tumors in a large percentage of the animals in a relatively short time (relationship exemplified in Figure 2). It will also be noted that, even at high levels of concentration, the mean time of appearance of tumors was in no case less than 20 weeks.

TABLE III

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF SYNTHETIC CARCINOGENS IN VARIOUS SOLVENTS UPON THE SKIN OF C3H MICE

EXPERIMENT NUMBER	CARCINOGEN[1]	SOLVENT	CONCENTRATION OF CARCINOGEN IN SOLUTION (% by weight)	DOSAGE OF SOLUTION PER APPLICATION (mg.)	NUMBER OF APPLICATIONS PER WEEK	DOSAGE OF CARCINOGEN PER UNIT AREA[2] PER WEEK (mg.)	MEAN TIME OF APPEARANCE OF TUMORS, $\bar{X}$ (weeks)	CLASSIFICATION	RELATIVE CARCINOGENIC POTENCY, P_{MC}
215	DMBA	Benzene	0.115	100	3	0.345	15.6 ± 3.3[3]	B	0.19
214	DMBA	Benzene	0.345	100	3	1.035	7.4 ± 1.1	B	0.62
244	BP	Benzene	0.172	100	3	0.516	25.1 ± 2.5	B	0.10
244	BP	Benzene	0.172	20	3	0.516	~ 27.3	X	(0.09)
252	BP	Benzene	0.322	20	1	0.322	~ 35.0	X	(0.18)
252	BP	Benzene	0.322	20	3	0.966	~ 23.0	X	(0.11)
252	BP	Benzene	0.322	100	3	0.966	~ 20.1	X	(0.13)
282	BP	Benzene	0.092	20	3	0.276		X	
264	BP	White oil	0.175	20	3	0.525	~ 25.4	X	(0.10)
270	BP	White oil	0.35	20	3	1.05	~ 21.5	X	(0.12)
285	BP	Cotton-seed oil	0.50	20	3	1.50		X	
286	BP	Cotton-seed oil	1.00	20	3	3.00	~ 20.4	B(X)	(0.13)
281	MC	Cottonseed oil	0.042	100	3	0.126	~ 41.0	B	0.047

[1] Carcinogens are 7,12-dimethylbenz[a]anthracene, DMBA; benzo[a]pyrene, BP; 3-methylcholanthrene, MC.

[2] Area of skin covered by 100 mg. of solution of methylcholanthrene in benzene.

[3] 5% fiducial limits.

USE OF REFERENCE STANDARDS AS A SCALE OF RELATIVE POTENCY OF COMPLEX TARS AND OILS

Various methods of expressing the relative potencies of the pure polycyclic carcinogens and complex materials have been reviewed by Badger (11). Many of these are simply rough classifications based upon a particular investigator's personal experience. The Iball index (12) and a recent adaptation (13) attempted a more quantitative approach, involving the use of the reciprocal of the mean time of appearance of tumors. Such methods are useful in comparing results obtained in a given laboratory using a standardized and inflexible schedule of application of various materials. However, experimental techniques differ significantly from one research organization to the next, thus rendering the comparison of data on this basis rather difficult. Further, as was pointed out previously in this article, it was sometimes necessary in the current investigation to change either or both the frequency and severity of exposure to obtain satisfactory conditions for the measurement of the potency of a sample while minimizing its toxic side effects. Hence, a scale of relative potencies which would permit such flexibility in experimental design was needed.

The use of the data on relative rates of induction of tumors by the pure carcinogens, methylcholanthrene and benzopyrene, as external standards of reference, provided a practical solution to this problem. The latter is one of the important contributors to the potency of cracked residua (14); the former has not been identified in complex tars and oils but might be regarded as a

representative of the 4-ringed carcinogens supposed to be present (15,16). Thus, the mean time of appearance of tumors induced by some complex tar or oil may be translated into a value of Relative Carcinogenic Potency, P_{MC} (potency as compared to methylcholanthrene), by use of Figure 5. The dosage of methylcholanthrene corresponding to the given mean time is either read off the graph or calculated from the equation,

$$d = \frac{8.2}{\bar{x} - 3.7} - 0.10 .$$

Then, the value of the relative potency, P_{MC}, for the complex material in question is

$P_{MC} = d$, for an experiment involving one application each week,

$= \frac{d}{2}$, for an experiment involving two applications each week,

$= \frac{d}{3}$, for an experiment involving three applications each week.

Thus, by comparing the results of tests on complex materials with those involving methylcholanthrene as an external standard of reference, one may obtain the relative carcinogenic potencies of the different materials, even though the experiments may have involved differing frequencies of applications. It should be noted, however, that the description of the potency of a complex oil cannot be considered complete until it has been tested at two appreciably different dosages, i.e., 20 mg. and 100 mg. per application, unless negative results were obtained at the higher level of dosage in a test in which the mice survived and grew at a normal rate.

Thus, a given oil may be much more potent than another under severe conditions of exposure, but the former may actually have a lower potency than the latter if the comparison is carried out under mild conditions. Chemical research on such oils has led to an understanding of this apparent paradox, and the matter will be dealt with in a later publication.

The technique of relating results on complex materials to those on standard synthetic carcinogens should facilitate comparison of results from different laboratories. The synthetic compound chosen should, of course, be comparable to the type of carcinogens supposed to be present in the complex mixtures. Under appropriate conditions of dosage and exposure, it must be capable of inducing tumors at least as rapidly as the most potent of the samples to be tested. Thus, benzopyrene has not proved to be a suitable standard for work with C3H mice, since it will not induce tumors sufficiently rapidly even at high levels of concentration. 7,12-Dimethylbenz[a]anthracene on the other hand, is very satisfactory from this standpoint, but has the disadvantage of requiring special care in handling to prevent its oxidation to the non-carcinogenic 7,12-photooxide (7).

It should be reemphasized that this consideration of a quantitative scale of potencies applies only to the results of experiments which may be classified as satisfactory (A and B by our criteria). Those in our Class X have only a limited value. If positive results are obtained, the material may be classified qualitatively as carcinogenic; if survival is good and most of the mice eventually develop tumors (X classification based on certain irregularities in growth) the estimation of the apparent potency may be useful as a minimum value.

RELATIVE POTENCIES OF POLYCYCLIC AROMATIC COMPOUNDS

The data available to the writers on experiments involving repeated applications of solutions of various polycyclic carcinogens in solvents such as benzene, acetone, and white mineral oil of high viscosity, upon the skin of mice, indicate that the average rate of induction of papillomas is independent of the quantity of solution used in each application so long as it exceeds 10 mg. The controlling variables are the frequency of application and the dosage of carcinogen per unit area of the skin. The latter will depend upon the concentration of carcinogen in the solution applied and the relative spreading coefficient of the solution.

Solutions of polycyclic hydrocarbons in different solvents have different capacities for spreading over the skin of a mouse because of variations in such physical properties as volatility, interfacial tension, and viscosity. Since much of the previous experimental testing of polycyclic compounds for their carcinogenicity to the skin has been carried out with solutions in benzene, this solvent was used for the reference standards described herein. An intensive investigation of the effects of variation of the composition of the solvent has also been carried out and will be described in a later paper.

By comparing data on the rate of induction of tumors by solutions of known concentration of various aromatic hydrocarbons in benzene under suitable experimental conditions, with such data as those shown in Table II and Figure 5, it should be possible to arrive at logical estimates of the relative carcinogenic potencies

of the different compounds. By inference, the relative potency of methylcholanthrene on the P_{MC} scale would be 100. Corresponding numerical values for benzopyrene and 7,12-dimethylbenzanthracene may then be derived from the data of Table III. Thus, since 0.345 percent 7,12-dimethylbenzanthracene produced tumors at the same rate as 0.62 percent methylcholanthrene under comparable conditions, the relative potency of this dimethyl- derivative is

$$P_{MC}^{\circ} = \frac{100}{0.345} \times 0.62 = 180$$

From Experiment 215, the relative potency, P_{MC}°, for 7,12-dimethylbenzanthracene is 165. The higher value is preferred at the present time, since, if autoxidation of the hydrocarbon caused any reduction in activity, it might reasonably be assumed that the effect would have been greater in Experiment 215.

Similarly, the relative potency of benzopyrene on this scale is 57 - 65 (Experiments 244, 252, and 282). The data from Experiment 252 involving three applications each week cannot be used since apparently the maximum effective dosage of this carcinogen had been reached at a lower level. Thus, as a general rule the determination of the relative potency of any given compound should be based upon the results of experiments at at least two different concentrations, which yielded significantly different mean times of appearance of tumors under otherwise comparable conditions. At least one and preferably both of these should meet our classification A or B from the standpoint of growth and survival.

LITERATURE CITED

(1) Pullinger, B. D., J. Path. Bact., 55, 301 (1943).

(2) Cramer, W. and Stowell, R. E., J. Nat. Cancer Inst., 2, 369 (1942).

(3) Bryan, W.R. and Shimkin, M.B., J.Nat.Cancer Inst., 1, 807 (1941).

(4) Bliss

(5) Whittaker, E. and Robinson, G., The Calculus of Observation, London, Blackie and Sons, Ltd., 1924, p. 209.

(6) Watson, A. F. and Mellanby, E., Brit. J. Exp. Path., 11,267 (1930).

(7) Bradbury, J. T., Bachmann, W.E., and Lewisohn, M.G., Cancer Research, 1, 685 (1941).

(8) Cook, J.W. and Kennaway, E.L.

(9)

(10) Iversen, S. and Arley, N., Acta Pathol. Microbiol. Scand., 27, 1 (1950).

(11) Badger, G.M., Brit. J. Cancer, 2, 309 (1948).

(12) Iball, J., Am. J. Cancer, 35, 188 (1939).

(13) Blanding, F.H., King, W.H., Priestley, W., and Rehner, J., Arch. Ind. Hyg. Occ. Med., 4, 335 (1951).

(14) Tye, R., Graf, M.J., and Horton, A.W., (Anal. Chem., 1954).

(15) Berenblum, I. and Schoental, R., Brit. J. Cancer, 1, 157 (1947).

(16) Fischer, H.G.M., Priestley, W., Eby, L.T., Wanless, G.G., and Rehner, J., Arch. Ind. Hyg. Occ. Med., 4, 315 (1951).

EXPERIMENTS INVOLVING THE APPLICATION OF MATERIALS UPON THE SKIN OF C3H MICE

LEGEND

Symbols used in connection with average weight curves:

$\overset{i}{\triangledown}$ Time of death (from disease) of i th tumor-free mouse.

i Time of death (from disease) of i th tumor-bearing mouse

$\overline{\overset{i}{\triangledown}}$ or $\overline{i}$ Time mouse killed.

* Painting discontinued for 1 week at each indicated time.

Gross and microscopic pathology:

$\boxed{i}$ Time of appearance of first papilloma in i th mouse. When this symbol is first used for a given mouse after its death, the presence of non-invading carcinoma (intra-epithelial) or small areas of benign neoplasm was determinable only by histopathology.

$\textcircled{i}$ Time of appearance of gross changes indicative of malignancy in tumor of i th mouse. Diagnosis confirmed except in cases where no tissue section available. When this symbol is first used for a given mouse after its death, the invasive malignancy of the tumor was determinable only by histopathology.

$\overset{\#3}{\boxed{i}}$ or $\overset{\#3}{\textcircled{i}}$ No tissue section available for histopathology because of extensive post-mortem decomposition or cannibalism.

API 05833

EB 005578

FIGURE 1

EXPERIMENT 220, SOLUTION OF METHYLCHOLANTHRENE, 1.034 PERCENT IN BENZENE

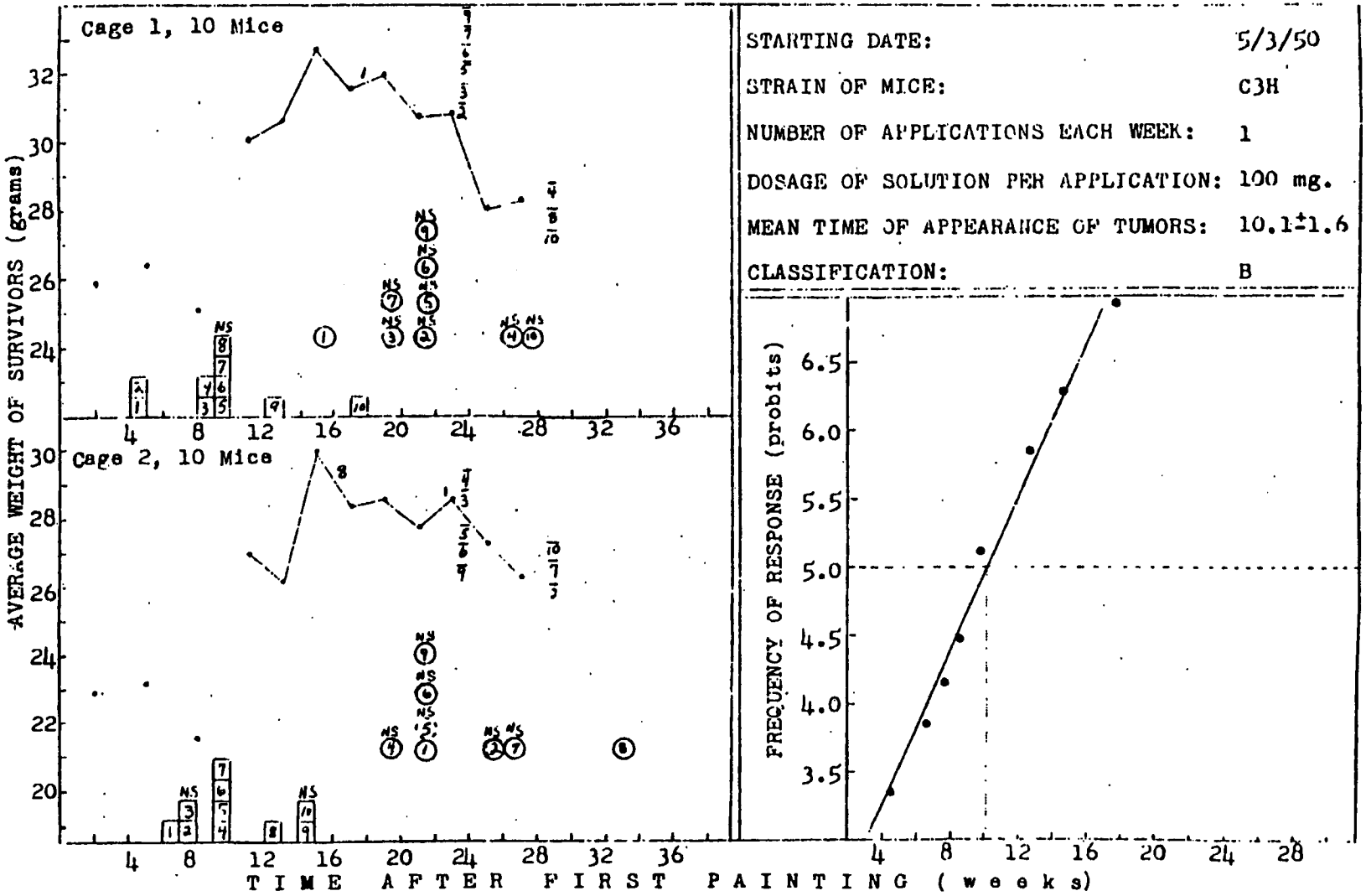


FIGURE 2

EXPERIMENT 268, SOLUTION OF METHYLCHOLANTHRENE, 0.287 PERCENT IN BENZENE

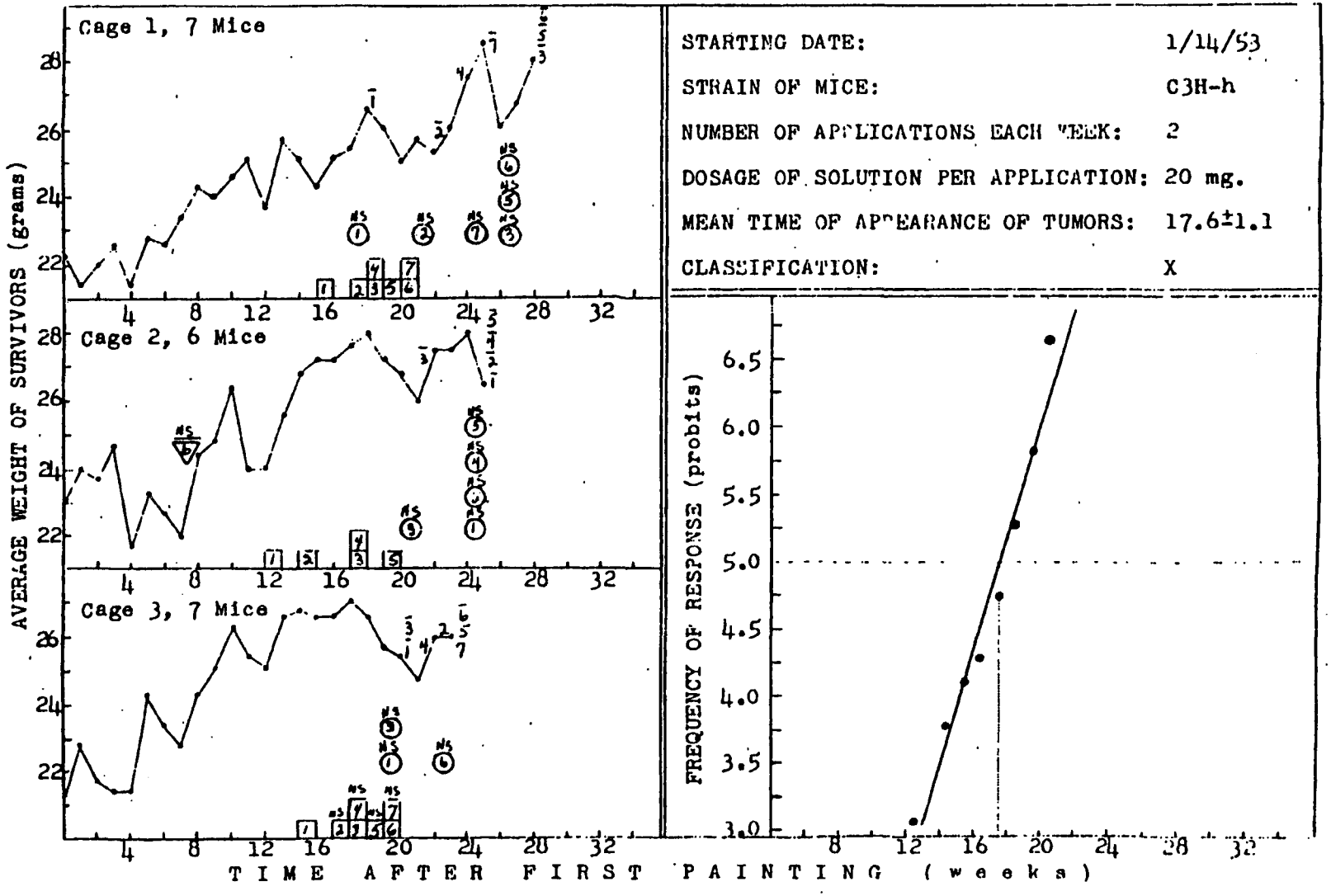
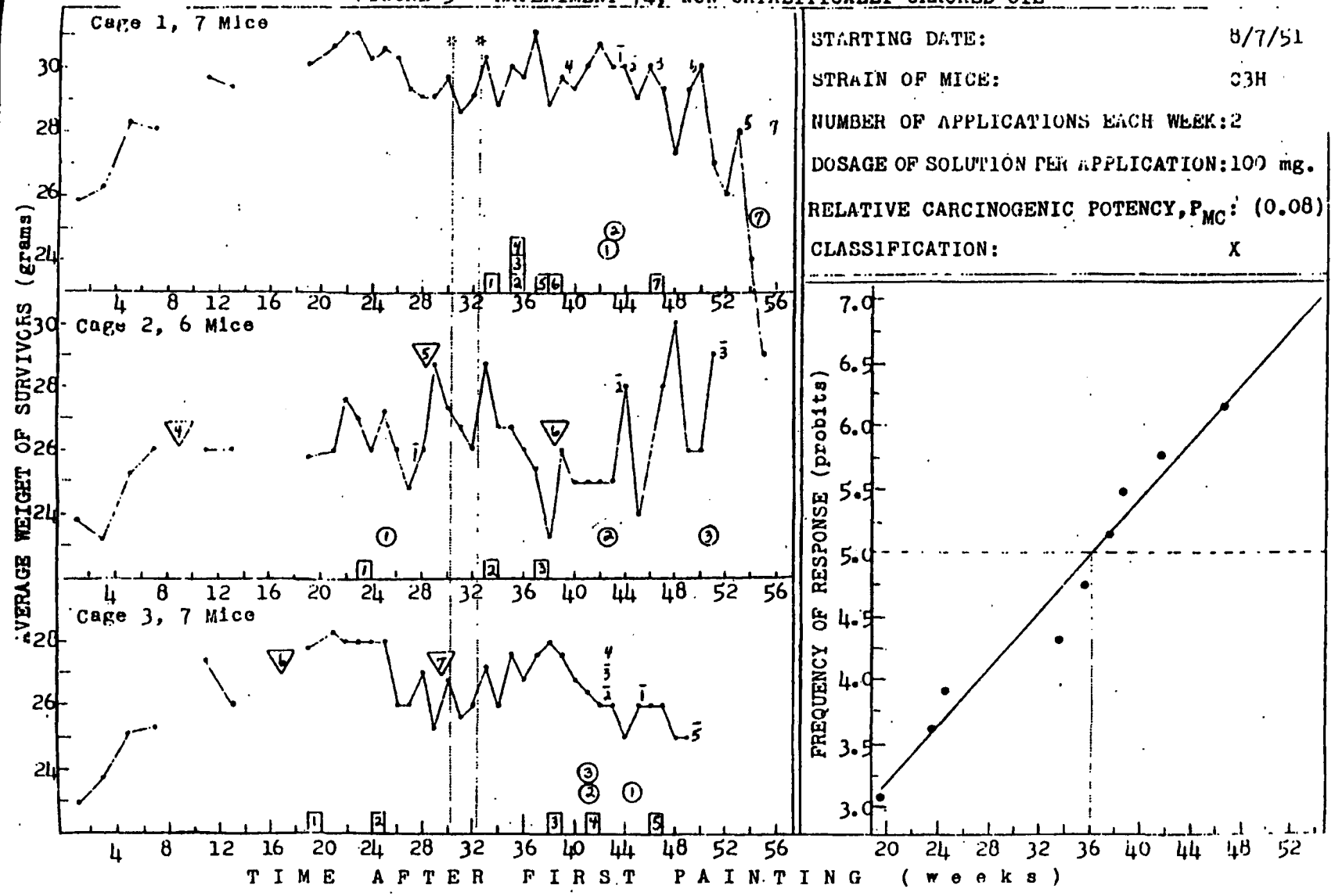


FIGURE 3 - EXPERIMENT 74, NON-CATALYTICALLY CRACKED OIL



STARTING DATE: 8/7/51

STRAIN OF MICE: C3H

NUMBER OF APPLICATIONS EACH WEEK: 2

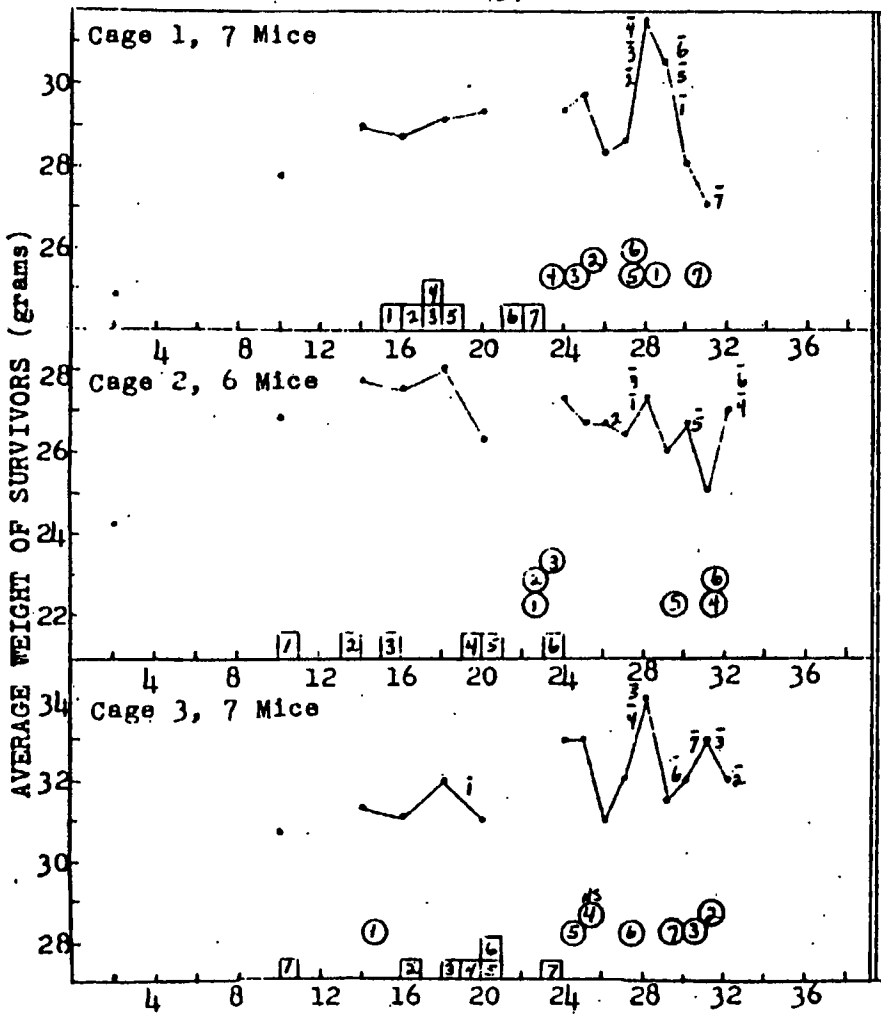
DOSAGE OF SOLUTION PER APPLICATION: 100 mg.

RELATIVE CARCINOGENIC POTENCY, P_{MC}: (0.08)

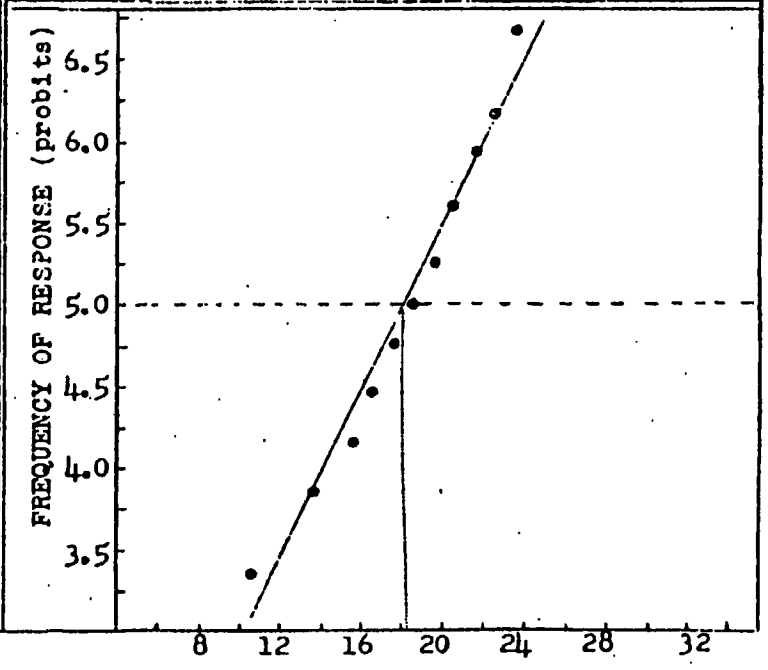
CLASSIFICATION: X

FIGURE 4

EXPERIMENT 243, SOLUTION OF METHYLCHOLANTHRENE, 0.15 PERCENT IN BENZENE



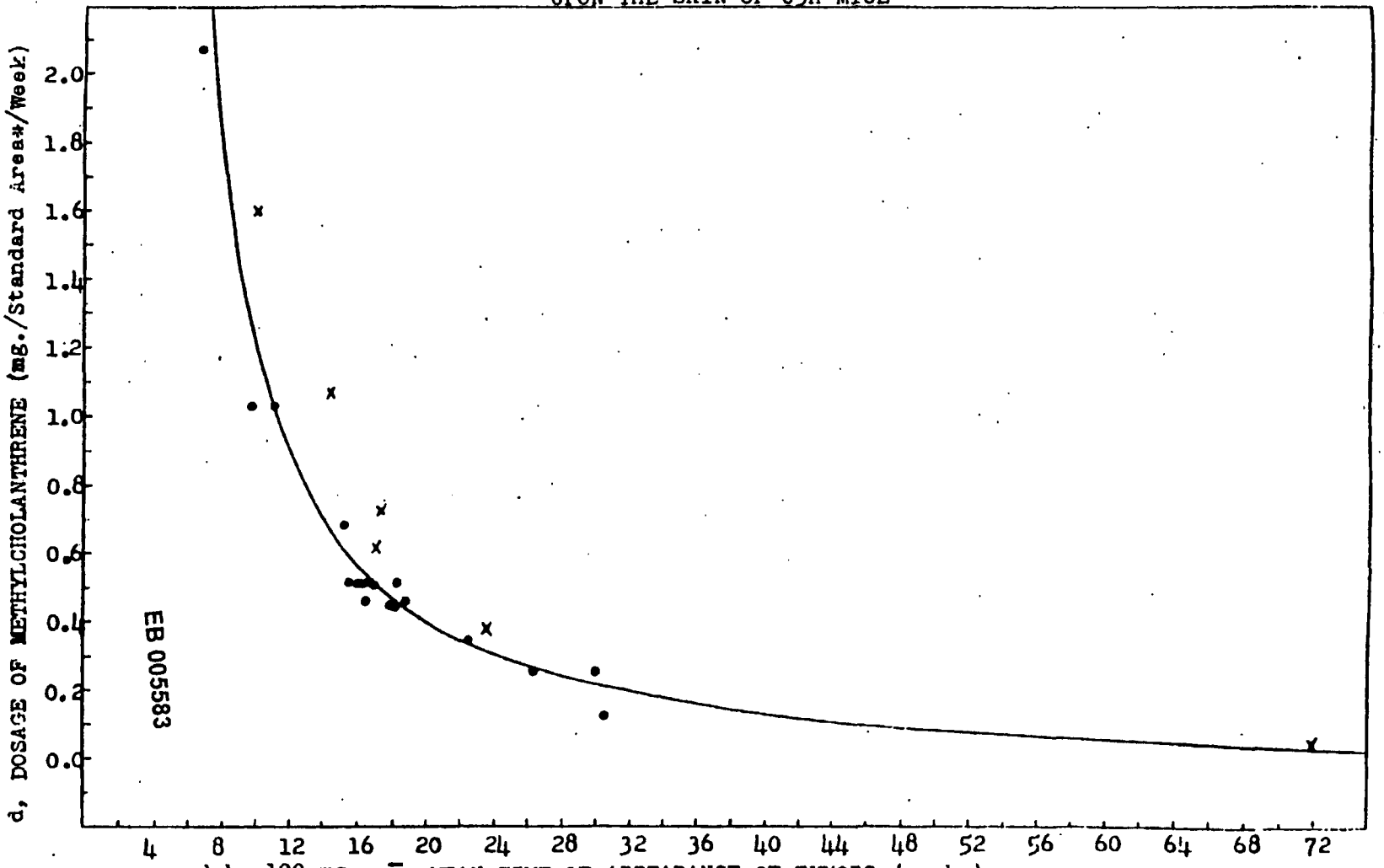
STARTING DATE:	7/28/51
STRAIN OF MICE:	C3H
NUMBER OF APPLICATIONS EACH WEEK:	1
DOSAGE OF SOLUTION PER APPLICATION:	100 mg.
MEAN TIME OF APPEARANCE OF TUMORS:	18.1±1.8
CLASSIFICATION:	A



TIME AFTER FIRST PAINTING (weeks)

FIGURE 5

EXPERIMENTS INVOLVING THE APPLICATION OF SOLUTIONS OF METHYLCHOLANTHRENE IN BENZENE UPON THE SKIN OF C3H MICE



* Area covered by 100 mg. of the solution.

just an acknowledgment

Copy From D. V. Stroop to Recipients of the Kettering Manuscript With Copies of Summary for Attachment to Manuscript Which Had Been Fully Prepared for Shipment Prior to Receipt of Summary May 10, 1954

UNIVERSITY OF CINCINNATI
The Kettering Laboratory
College of Medicine - Eden Avenue
Cincinnati 19, Ohio

May 8, 1954

Mr. D. V. Stroop, Director
Technical Service Division
American Petroleum Institute
50 West 50th Street
New York 17, New York

Dear Mr. Stroop:

Attached are the stencils of the summary of the paper on "Carcinogenesis of the Skin". These pages should be inserted in the manuscript just before the bibliography.

Thanks again for your cooperation in these matters.

Sincerely yours,

/s/ Wes

A. Wesley Horton

AWH:mjg
cc: Dr. R. E. Eckardt
Enc.

API 05839

EB 005584

SUMMARY

Repeated applications of solutions of carcinogenic hydrocarbons upon the dorsal skin of male mice of the C3H strain under suitable experimental conditions result in the induction of epitheliomas in essentially all of the exposed animals. The average period of exposure ($\bar{x}$) to solutions of 3-methylcholanthrene in benzene required to induce papillomas of the skin of these mice varies inversely with the dosage (d) of the carcinogen per unit area per week, according to the following relationship:

$$\bar{x} = \frac{8.2}{d + 0.10} + 3.7$$

For experiments involving combinations of synthetic carcinogens with non-accelerating solvents, the mean time of induction of tumors is independent of the total area of skin exposed, so long as a certain minimum is exceeded (about 10 sq. mm.). The good health of the mice, as measured by their growth, has proved to be an essential factor in obtaining reproducible results.

A quantitative scale for expressing the relative carcinogenic potencies of various synthetic carcinogens and complex tars and oils has been derived, using the experiments on solutions of methylcholanthrene in benzene as reference standards. Utilizing the relationship between the dosage of methylcholanthrene and the rate of induction of tumors shown above, a value for the relative potency, P_{MC}, is obtained. The numerical value of the P_{MC} of a tar or oil is equal to the level of concentration, in percent by weight, of the solution of methylcholanthrene in benzene which would induce papillomas at the same rate as the material in question under

comparable experimental conditions. The potencies, P_{MC}°, of pure compounds, relative to that of methylcholanthrene, taken as 100, may be derived in a similar manner. From the data in this paper, the relative potencies of 7,12-dimethylbenz[a]-anthracene and benzo[a]pyrene are 180 and 60, respectively.

SUMMARY

Repeated applications of solutions of carcinogenic hydrocarbons upon the dorsal skin of male mice of the C3H strain under suitable experimental conditions result in the induction of epitheliomas in essentially all of the exposed animals. The average period of exposure ($\bar{x}$) to solutions of 3-methylcholanthrene in benzene required to induce papillomas of the skin of these mice varies inversely with the dosage (d) of the carcinogen per unit area per week, according to the following relationship:

$$\bar{x} = \frac{8.2}{d + 0.10} + 3.7$$

For experiments involving combinations of synthetic carcinogens with non-accelerating solvents, the mean time of induction of tumors is independent of the total area of skin exposed, so long as a certain minimum is exceeded (about 10 sq. mm.). The good health of the mice, as measured by their growth, has proved to be an essential factor in obtaining reproducible results.

A quantitative scale for expressing the relative carcinogenic potencies of various synthetic carcinogens and complex tars and oils has been derived, using the experiments on solutions of methylcholanthrene in benzene as reference standards. Utilizing the relationship between the dosage of methylcholanthrene and the rate of induction of tumors shown above, a value for the relative potency, P_{MC}, is obtained. The numerical value of the P_{MC} of a tar or oil is equal to the level of concentration, in percent by weight, of the solution of methylcholanthrene in benzene which would induce papillomas at the same rate as the material in question under

comparable experimental conditions. The potencies, P_{MC}^{o}, of pure compounds, relative to that of methylcholanthrene, taken as 100, may be derived in a similar manner. From the data in this paper, the relative potencies of 7,12-dimethylbenz[a]-anthracene and benzo[a]pyrene are 180 and 60, respectively.