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

January 26, 1954

TO: The Members of the Medical Advisory Committee

I am attaching a copy of a manuscript of a paper which Dr. A. Wesley Horton wishes to submit to the Journal of Analytical Chemistry in the near future. This paper was presented to the RPA Committee at its meeting in Cincinnati on January 21, 1954. Certain minor modifications were made at that time, and these modifications have been incorporated into the attached manuscript, the revised copy having been received in my office on January 25, 1954. It was the unanimous agreement of those of the RPA Committee present in Cincinnati (Dr. L. C. Beard and Dr. C. H. Hine were absent) that this manuscript was acceptable for publication.

I would greatly appreciate it if each of you would review this manuscript in detail and transmit any criticisms or suggestions which you may have concerning it to Mr. Stroop within the next 20 days*. At the end of that time, if no major objections to the publication of this manuscript have been received from any of you, it is the plan to transmit it to the Health Committee of the Board for their approval. As you know, the total time for clearance of this manuscript is 60 days, at the end of which time Kettering Laboratory can submit this paper for publication, so that anything you can do to expedite comments would be greatly appreciated.

Very truly yours,

/s/ R. E. Eckardt

R. E. ECKARDT, M. D.

REE:ilk
Attachment

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* Note by D. V. Stroop
As we have been delayed in transmitting this letter until February 3, we shall defer reference to Medical and Health Committee of the Board until March 1. Three copies of manuscript are enclosed for each committee member. None is being sent to any technical advisor.

DVS

AN ANALYTICAL METHOD FOR DETERMINATION OF BENZO[a]PYRENE IN COMPLEX MIXTURES[1]

by Russell Tye, Mary Jane Graf, and A. Wesley Horton

Kettering Laboratory, Department of Preventive Medicine and Industrial Health, College of Medicine, University of Cincinnati, Cincinnati, Ohio

PREFATORY SUMMARY

This paper describes the isolation and identification of the polycyclic hydrocarbon, benzo[a]pyrene, in an oil produced by catalytic cracking of petroleum, together with the analytical method developed for the estimation of the concentration of this compound in products of refining operations. A selected fraction of a given sample is obtained by the use of a standardized chromatography. Two equal portions of the fraction are taken, and one of them is subjected to a catalytic iodination on a column of activated alumina. Spectrophotometric measurement of the difference in absorbance between the iodinated portion and its mate is then used to determine the concentration of benzo[a]pyrene present.

INTRODUCTION

A prolonged search for the carcinogenic element in coal tar culminated in the isolation of a pure hydrocarbon carcinogen from coal tar pitch by Hieger (1). Cook and Hewett (2) synthesized benzo[a]pyrene and proved its identity with the hydrocarbon from coal tar. In the course of an investigation of the possible carcinogenic properties of high boiling products from petroleum refining operations, it was observed that a distillate fraction of a catalytically cracked residuum had an ultraviolet absorption

[1] This work was carried out largely as part of the American Petroleum Institute Research Project MC-1.

spectrum which strongly suggested the presence of benzo[a]pyrene in significant concentration. This hydrocarbon was isolated by the following procedure in a sufficient state of purity for positive identification.

Fourteen hundred grams of the oil were subjected to a simple vacuum distillation. A fraction, boiling 205 to 275°C. at 0.5 mm. pressure, and weighing 175 g., was chromatographed on alumina, and benzo[a]pyrene-enriched fractions selected spectrophotometrically. The chromatographic procedure was repeated five times, resulting in 6.5 g. of a red semi-solid, the ultraviolet absorption spectrum of which indicated a content of benzo[a]pyrene between 0.8 and 2.5 g. Fractional adduction by iodine (3) was used to remove perylene and other unidentified compounds, reducing the weight of the concentrate to 5 g. This was dissolved in benzene and extracted by cold concentrated H_2SO_4. The acid was diluted with ice, and the resulting precipitate was dissolved in benzene. An additional series of chromatographies and fractional crystallizations produced 0.116 g. of crude benzo[a]pyrene, having substantially the same ultraviolet absorption as that of an authentic sample.

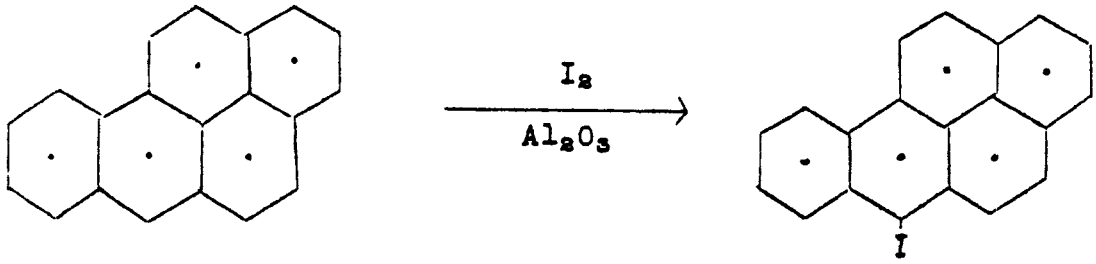
Further recrystallization produced 12 mg. of crystals, m.p. 173-174°C. (uncor.). The melting point of a mixture with synthetic benzo[a]pyrene was 174.5-175.5°C.(uncor.), as compared with that of tne pure material, 177.5-178.0°C.

The method of Goulden and Tipler (4), as adapted by Waller (5) for the determination of benzo[a]pyrene in soot and city dusts, utilizes chromatography and fluorescence spectroscopy, the final estimation of concentration being made by visual comparison of the spectrum of the unknown with that of a known sample compositied of benzo[a]pyrene and suitable impurities. Falk (6) and Wedgwood (7) have used chromatography and ultraviolet spectrophotometry to separate and identify polynuclear aromatic hydrocarbons from complex mixtures.

The method described in this paper attempts to utilize the chromatographic technique not only to concentrate the benzo[a]-pyrene but to aid in identifying it by its rate of movement along a carefully standardized column. Secondly, a selection is made by catalytic iodination, and, finally, identification and measurement are accomplished on the basis of the change in the ultraviolet spectrum caused by the iodination. The method is relatively fast, requiring about three hours per analysis, or less if multiple analyses are made simultaneously.

CATALYTIC IODINATION

Activated alumina catalyses the substitution of iodine for hydrogen of the benzo[a]pyrene molecule. A convenient method of accomplishing the reaction is to pass a solution of benzo[a]-pyrene and iodine in suitable concentration in benzene through a short column of activated alumina in the manner of elution chromatography. Under the conditions of the procedure used in this analysis, 6-iodobenzo[a]pyrene is produced in about 65% yield.



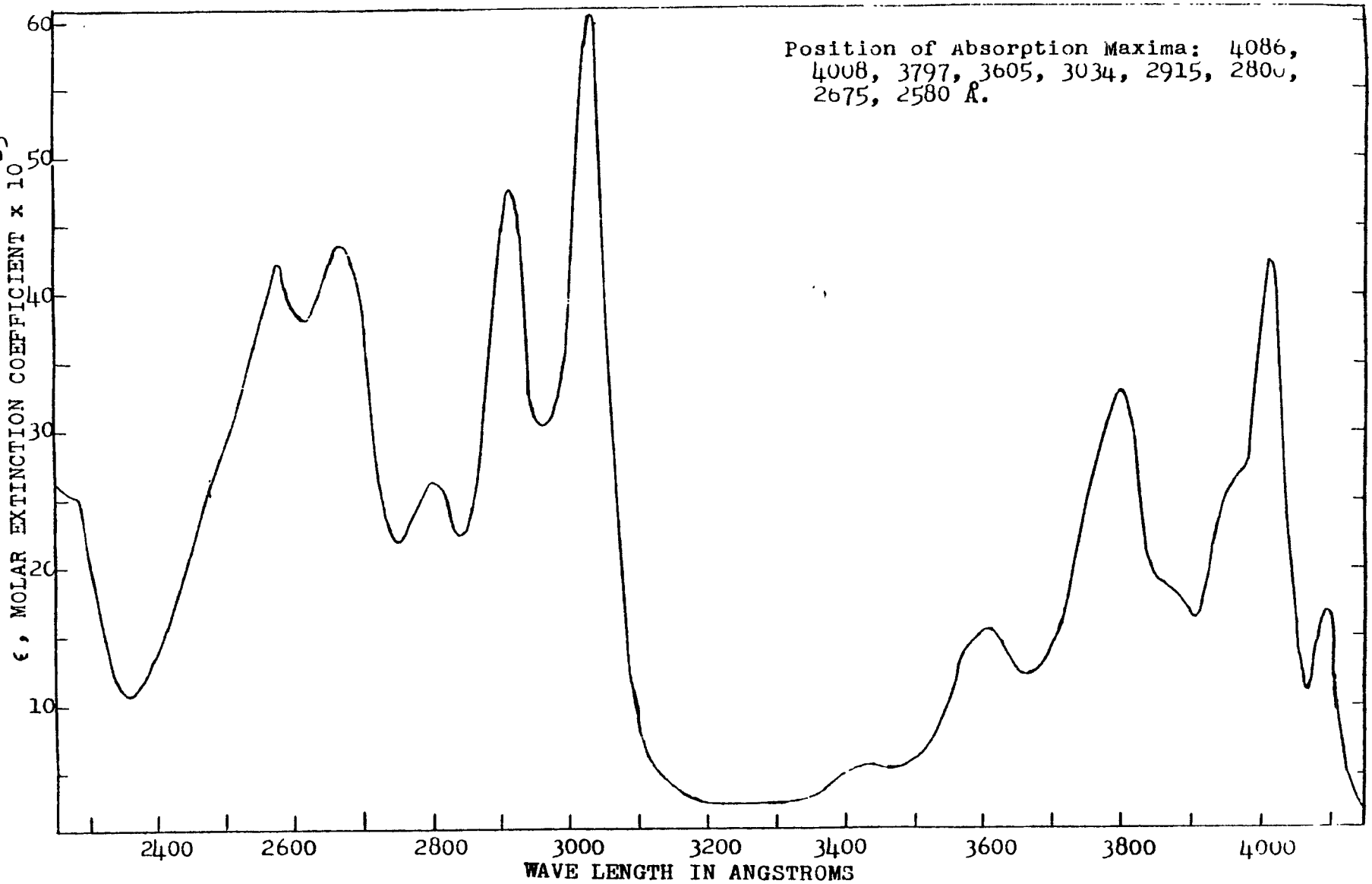
Benzo[a]pyrene — 6-Iodobenzo[a]pyrene

The iodo- compound has been purified by chromatography and recrystallization. Its melting point was 211-212°C. (uncor.); its iodine content (calculated as 33.6% for $C_{20}H_{11}I$) was 33.1%. The ultraviolet absorption spectrum is shown in Fig. I; it is noteworthy that the compound does not have any visibly perceptible fluorescence.

The position of substitution was established by conversion of the iodo derivative to the nitrile by heating with a small excess of $Cu_2(CN)_2$ in a sealed glass tube at 306°C. for one and one-half hours. A yield of 97% of crude product was obtained. This product, when carefully purified, melted at 234-235°C. (uncor.). 6-Cyanobenzo[a]-pyrene, prepared by the method of Windaus and Raichle (8), melted at 231-232°C. (uncor.), and in a mixture with the first cyano compound, melted at 233-234°C.(uncor.). The ultraviolet spectra of the two samples were in agreement and corresponded with the spectrum obtained by Jones (9).

FIGURE I

ULTRAVIOLET ABSORPTION SPECTRUM OF 6-IODOBENZO[a]PYRENE (IN ISOOCTANE)



Other activated adsorbents also catalyze the iodination of benzo[a]pyrene. The pH of the adsorbent appears to be important. Under conditions similar to those of the analysis, attapulgus fuller's earth of low activity and of a lower pH than Grade F-20 alumina (as measured by pH meter on aqueous extracts) produces roughly equal yields of 6-iodo-benzo[a]pyrene and an additional probable iodobenzo[a]pyrene. Activated magnesia, having a pH much higher than Grade F-20 alumina, and activated silica gel, having a pH much lower than the attapulgus fuller's earth, promote only faint reactions. The addition of $NaHCO_3$ to the silica gel enhances its catalytic activity markedly, although $NaHCO_3$ shows no activity alone. A large increase in the quantity of iodine causes the alumina-catalyzed reaction to produce a mixture similar to that from attapulgus fuller's earth.

The ability of aromatic hydrocarbons to form molecular complexes with Lewis acids has been discussed by several authors (10,11,12,13,14) and related by Mulliken (13) and Brown (14) to the ease with which these compounds may be halogenated. Brown found a simple linear relationship between the logarithms of the relative rates of halogenation of several methyl benzenes and the logarithms of the relative stabilities of their deeply colored onium-ion complexes with $HF-BF_3$.

The solubility of various polycyclic aromatics in cold, concentrated sulfuric acid (3) is doubtless a function of the basic properties of the hydrocarbons. These solutions have vivid colors ranging from yellow to wine-red, suggesting the formation of onium-ion complexes (13,14). Perylene, 3-methylcholanthrene,

benzo[a]pyrene, pyrene, and anthracene can be extracted from benzene with varying degrees of completeness by an equal volume of concentrated sulfuric acid at 10°C. Under the conditions of the analytical procedure, the same compounds react with iodine to similar relative extents. Benz[a]anthracene can be extracted by sulfuric acid but reacts negligibly with iodine. Chrysene, benzo[c]phenanthrene, dibenz[a,h]anthracene and phenanthrene cannot be extracted by cold concentrated sulfuric acid, and are not iodinated by the analytical procedure.

A more generalized check on the apparent rule that polycyclic aromatic hydrocarbons not soluble in cold, concentrated sulfuric acid are not substituted by iodine under the conditions of this analytical method, has been made. A typical residuum, resulting from catalytic cracking of petroleum, believed to contain a wide variety of polycyclic aromatics including alkylated and hydrogenated derivatives, was exhaustively extracted by cold concentrated sulfuric acid. Two equal portions of the non-extract were taken, one portion iodinated, and the difference spectrum measured according to the standard analytical procedure. No apparent reaction had occurred.

These data suggest that the iodine substitution occurs through formation of an acid-base reaction intermediate, probably an onium-ion complex.

APPARATUS

The chromatographic column which is used to prepare a suitable fraction for the analysis is composed essentially of a reservoir and two separate sections of adsorbent, as shown in Fig. II. The spectrophotometer employed in the development of the method was the Beckman Model DU equipped with 1 cm. cells and a tungsten lamp. It is assumed that any comparable instrument could be used equally well.

MATERIALS

ISOOCTANE, Phillips Petroleum Company, pure grade, re-distilled.

BENZENE, C.P. thiophene-free, re-distilled.

SOLUTION OF BENZO[a]PYRENE, Edcan Laboratories, C.P., 5.0 mg. in 100.0 ml. of benzene.

DEVELOPING SOLVENT, Mixture of 200 ml. of isooctane with 200 ml. of benzene.

STANDARD SOLUTION OF IODINE (reagent grade), 1 g. in 10 ml. of benzene.

ACTIVATED ALUMINA, Alcoa, Grade F-20, 80-200 mesh.

SOLUTION OF SODIUM THIOSULFATE, C.P., approximately 5 g. in 200 ml. of distilled water.

SAND, non-porous, white, silica.

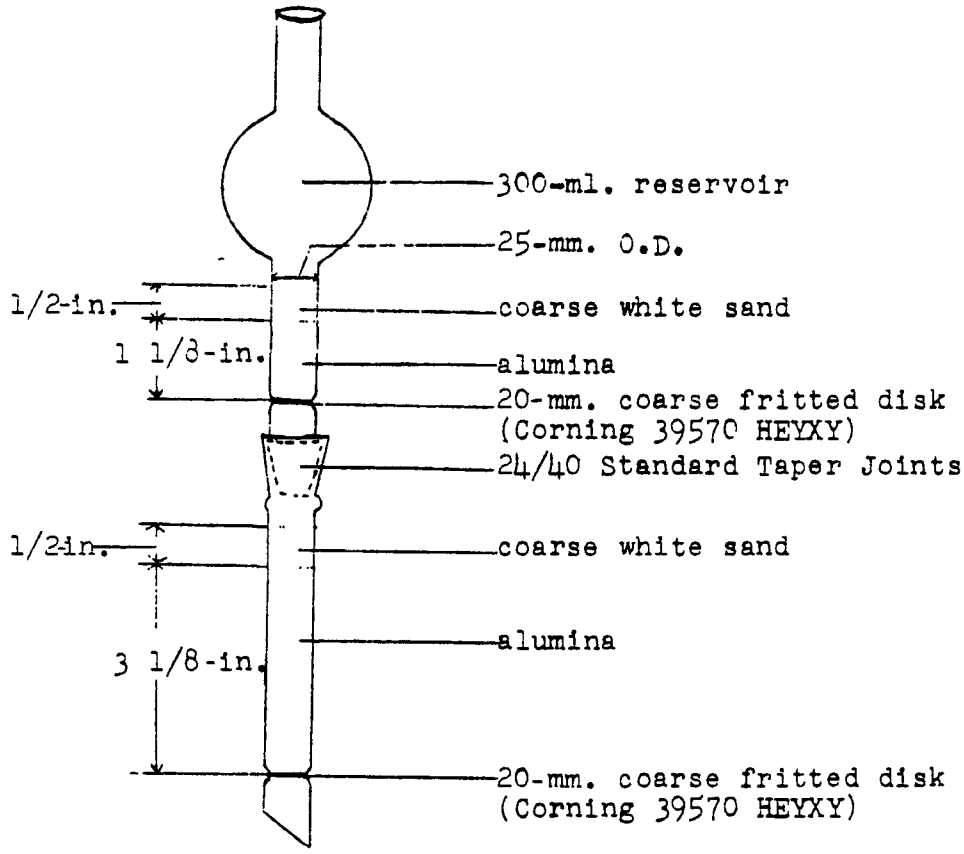

300-ml. reservoir
25-mm. O.D.
1/2-in.
coarse white sand
1 1/8-in.
alumina
20-mm. coarse fritted disk
(Corning 39570 HEYXY)
24/40 Standard Taper Joints
1/2-in.
coarse white sand
alumina
3 1/8-in.
20-mm. coarse fritted disk
(Corning 39570 HEYXY)

FIGURE II

PROCEDURE

A. Determination of activity of alumina and corresponding volume of developing solvent required for satisfactory chromatographic fractionation.

A chromatographic column of the alumina is prepared as shown in Fig. II. Ten milliliters of the developing solvent are poured on to the column followed by 10±.05 ml. of the solution of benzo[a]pyrene and then by an additional 100 ml. of the solvent. Each portion of liquid should be allowed to enter the top layer of sand before the next is added. Additional solvent is then added, 10 ml. at a time, until the top of the benzo[a]pyrene band, as indicated by blue-violet fluorescence under ultraviolet light, is approximately 1/4 in. below the top of the lower section of alumina. At this point, the fluorescent compound will usually be spread over about one-third of the lower section of alumina. With adsorbent of normal activity, this development will require a total volume of about 120 to 150 ml. of the solvent (not including the two 10 ml. portions used to prewet the column and as solvent for the benzo[a]pyrene). The total volume of solvent thus determined is used as the "standard volume" for the separatory chromatography (Step B below). The use of alumina requiring a "standard volume" of solvent less than 100 ml. is to be avoided if possible, as the effectiveness of the separation of benzo[a]pyrene from interfering components is thereby reduced. The only known disadvantage in the use of alumina requiring a "standard volume" greater than 170 ml. is the additional cost in time and material. Alumina of unusually

high or low activity could probably be used satisfactorily by modification of the composition of the developing solvent, but should in that case be checked further with pure benzo[a]pyrene to determine whether it has a satisfactory catalytic action in the iodination reaction described in Step C.

B. Preparation of fraction for iodination.

One hundred milligrams of the material to be analysed are dissolved in 5 ml. of benzene. After the material is completely in solution, 5 ml. of isooctane are added. (If part of the material is insoluble in benzene or if precipitation occurs on addition of isooctane the sample should receive special procedure - see Modification # 1.) The material is then fractionated on a freshly packed chromatographic column exactly as described in Step A above, substituting this solution of the unknown for the solution of benzo-[a]pyrene used to calibrate the alumina.

After the "standard volume" of the developing solvent has run through the column, the sections are separated and the material adsorbed on the lower section is eluted by 50 ml. of a solution of 20% ethanol, 80% benzene, by volume. The solvent is removed from the eluate by careful evaporation under a stream of nitrogen, and the residue, representing a concentrate of any benzo[a]pyrene present in the sample, is then ready for Step C.

C. Iodination.

The residue from Step B is dissolved in 15±.05 ml. of benzene, and two 5 ml. portions, labeled I and II, of the resulting solution, are transferred to two clean 25-ml. flasks, using the

same pipette for each. Five milliliters of the standard solution of iodine are added to I, and 5 ml. of benzene added to II. Two parallel columns of alumina are prepared, each one inch deep and one inch in diameter, covered with 1/2 in. of sand. Each column of adsorbent is wet by 15 ml. of benzene and allowed to drain until the frequency of drops is less than one in ten seconds. Clean, tared, 125-ml. flasks are then placed under the columns. Five milliliters of the standard solution of iodine, diluted with 5 ml. of benzene, are poured on to the first column, 10 ml. of benzene alone poured on to the second. Solutions I and II are then added to the respective columns, and the flasks which had contained them rinsed by 10 ml. of benzene, and the rinsings added to the proper columns. Each is then eluted by 70 ml. of benzene and allowed to drain as before.

The eluate from column I, containing the iodine, is then shaken with 200 ml. of the aqueous sodium thiosulfate in a 500-ml. separatory funnel, after which it is washed with two 100 ml. portions of distilled water. The eluate obtained from column II is used directly in Step D without further handling.

The processed fractions, I and II, should be subjected to the spectrophotometric analysis of Step D without delay, since the iodinated sample occasionally proves to be unstable on standing overnight.

D. Determination and analysis of difference spectrum.

The algebraic difference between the ultraviolet absorption spectra of the final fractions, I and II, is now measured directly by use of a Beckman DU spectrophotometer. Sample I is placed in

the 1-cm. cell normally used for solutions, and sample II in the 1-cm. cell used for a blank. Obviously, the cells used must be well matched as to length. The instrument is operated in the normal fashion except that when II has the higher absorbance, the machine is balanced on I, and the absorbance is recorded as a negative value (see Fig. III). The slit width must be kept moderately low, below 0.1 mm. being desirable. The sensitivity knob should therefore be turned to a point near its extreme counterclockwise position, and the tungsten lamp should be used. The reduced slit widths are necessary because the iodinated solution I usually has a markedly lower level of fluorescence than the solution II. This difference assumes critical importance when compared with the difference in absorption rather than with the usual total absorption. Hence, if the slit width required at 380 mμ exceeds 0.15 mm., 10.00 ml. of each solution should be diluted with 50.00 ml. of benzene prior to spectral analysis.

Satisfactory analyses may be obtained from difference spectra in which the measured absorbance at 389 or 405 mμ is 0.02, or greater, so long as each point is determined carefully and the absorbance is plotted on a suitable scale. However, if the absorbance is less than 0.02, the weights of the solutions should be determined; then they are concentrated sufficiently to meet this requirement, and their reduced weights adjusted to equal percentages of their respective original weights with benzene (this percentage is used as the value of the factor, V, in the equation below).

The difference in absorbance is determined at 380, 389, and 405 mμ and at every 2 mμ in the region 410 to 370 mμ. The

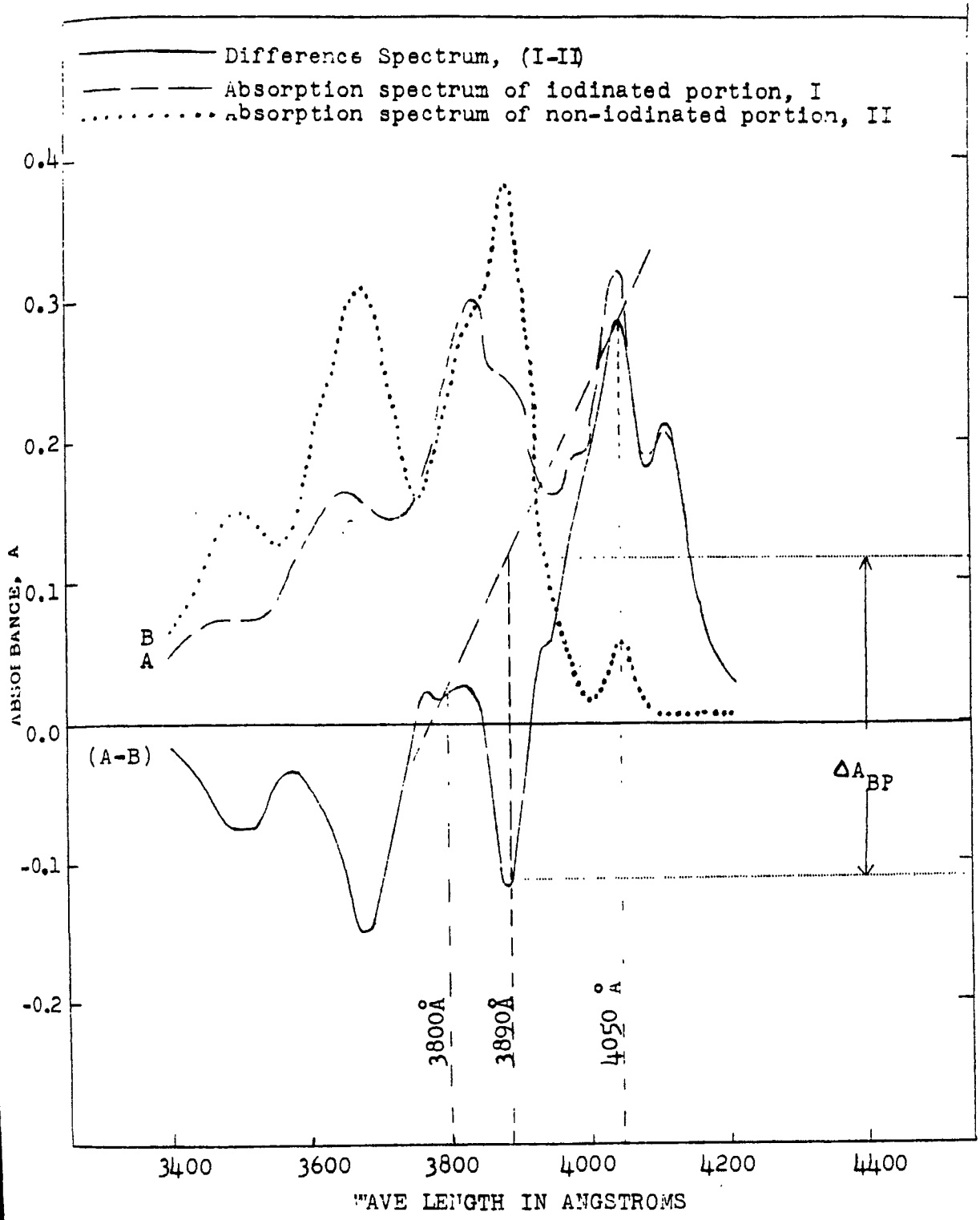


FIGURE III - STANDARD DIFFERENCE SPECTRUM OF BENZO[a]PYRENE AND IODINATED BENZO[a]PYRENE

NOTE: ΔA_{BP} = 0.23 in difference spectrum obtained from standard analytical procedure on a sample containing 1 mg. of benzo[a]pyrene.

following base-line technique (15,16) is applied to the difference spectrum obtained. A line is drawn intersecting the curve at the points corresponding to 405 and 380 mμ. The distance (in absorbance units) along the vertical coordinate at 389 mμ from the curve to this line is designated as ΔA_{BP} (see Fig. III). The percentage of benzo[a]pyrene in the original sample is calculated from the equation:

$$\text{Percent Benzo[a]pyrene} = \frac{\text{V, ml.}}{\text{Sample Weight, mg.}} \times \frac{\Delta A_{BP}}{0.23}$$

A value of 100 ml. is used for the factor, V, unless dilution or concentration of solutions I and II was required to correct for excessive or insufficient absorbance in Step D. A value of 600 ml. is used in the former case. If it was necessary to reduce the volume, the value of V to be used in the calculation is equal to the percentage determined from the process of concentration.

Thus, when 100 mg. of the original material was used in the analysis and such corrections of volume were unnecessary,

$$\text{Percent Benzo[a]pyrene} = 4.35\ \Delta A_{BP}$$

Modification # 1 - The suspension of 100 mg. of the sample in either 5 ml. of benzene or in 10 ml. of the 50:50 mixture of benzene and isooctane, as the case may be, is poured on to a column of alumina one inch in diameter and one inch deep (comparable to the upper section of the column in Fig. II). The flask in which the suspension was prepared is washed thoroughly with 10 ml. portions of benzene, and the washings added to the column. After this material has passed into the adsorbent the column is eluted with 100 ml. of benzene.

Most of the solvent is removed from the eluate by evaporation on a steam bath, the remainder by blowing under a stream of nitrogen. The residue obtained is dissolved in 5 ml. of benzene, 5 ml. of isooctane are added, and the analysis resumed at the point of interruption (Step A).

REPEATABILITY AND INTERFERENCES

Quantities of pure benzo[a]pyrene ranging from 0.0025 to 0.5% were dissolved in several different oils of widely varying type, to which this analytical procedure had been applied previously. In each case in which the amount of added benzo[a]pyrene represented a significant increase in the total present, the standard analysis yielded a reasonably precise measure of the quantity added. The examples, shown in the following table, indicate that other components of typical petroleum products did not create a serious interference with the analysis.

OIL NUMBER	BENZO[a] PYRENE FOUND IN ORIGINAL SAMPLE	CONCENTRATION OF BENZO[a] PYRENE ADDED	TOTAL DETERMINED BY ANALYSIS
1	0.02%	0.10%	
2	0.38%	0.50%	(to
3	0.003%	0.05%	be
4	0.0005%	0.01%	determined)
5	0.10%	0.20%	

Data from fifty analyses involving either duplication or known amounts of added benzo[a]pyrene, indicate a reproducibility within the limits of about ± 20% for samples with benzo[a]pyrene concentrations around 0.01%, ± 10% for concentrations of 0.10%, and ± 5% for concentrations of 0.50%.

Many compounds other than benzo[a]pyrene could react with iodine under the conditions of Step C of the analysis. These include anthracene, pyrene, methylcholanthrene, perylene, etc. The majority of such compounds are separated from benzo[a]pyrene by the chromatographic fractionation of Step B. Of the compounds which accompany benzo[a]pyrene through this separation and the subsequent iodination, the alkyl derivatives of benzo[a]pyrene are perhaps the most important potential interferers, if present in any significant concentration. It is not known how their presence would effect the final analysis. The following experiments were carried out in an effort to obtain some estimate of the extent of such interference, if any.

Analysis of a 20% residuum from distillation of the oil from which benzo[a]pyrene was isolated indicated a content of 1.8% of this hydrocarbon. A second sample of this residuum was fractionated by the procedure of Step B. One-fifth (termed Fraction A in the following table) of the chromatographic fraction containing the benzo[a]pyrene was then subdivided by a more refined chromatography into three fractions containing approximately equal portions of benzo[a]pyrene, and these analysed by iodination and difference spectra. A measured quantity of synthetic benzo[a]pyrene was then added to a similar one-fifth portion of the standard chromatographic

fraction from the oil. This sample was then subdivided by a careful reproduction of the previous chromatography, and the three resulting fractions analysed. Duplications were then made for both experiments. Finally, pure benzo[a]pyrene was processed chromatographically in duplicate experiments similar to the preceding ones, and the resulting fractions analyzed. The results of these experiments are shown in the following table.

	PERCENTAGE OF TOTAL BENZO[a]PYRENE PRESENT IN:			
SAMPLE	Fraction 1	Fraction 2	Fraction 3	Fraction 1 + 2 + 3
1. Fraction A of Cracked Residuum	29	39	31	99
	31	42	27	100
2. Fraction A + added benzo[a]pyrene	31	42	25	98
	34	41	22	97
3. Pure benzo-[a]pyrene	31	47	20	98
	28	46	26	100

In the cases of samples 1. and 2., percentages are based on the total benzo[a]pyrene indicated oy summation of the weight of synthetic benzo[a]pyrene added and that determined by independent ordinary analysis of Fraction A. The table shows that the apparent benzo[a]pyrene from the oil does not differ chromatographically from synthetic benzo[a]pyrene. There is some reason, therefore, to believe that alkylbenzo[a]pyrenes are creating no serious interference in the analysis of the oils.

In the analysis of certain types of materials, such as crude residua, difference spectra are occasionally obtained which lack sharp maxima and minima or well defined inflections and do not furnish satisfactory identification of this hydrocarbon. Hence, the significance of results obtained by application of the base-line technique to such spectra is somewhat uncertain. Various methods of eliminating the obscuring interference are being examined.

A technique has been devised which, applied to such materials, provides evidence that the actual concentration of benzo[a]-pyrene is, at most, no higher than that indicated by the standard procedure. Since the values in question have usually been less than 0.003%, and therefore possibly below the range of carcinogenic significance, such evidence may prove very useful. It has been found that a significant proportion of the compounds responsible for the obscuration of the difference spectra may be modified in composition (possibly polymerized) by subjection of a weighed quantity (approximately 100 mg.) of the sample to what amounts to a flash distillation at atmospheric pressure without reflux. Application of the standard method of analysis to the distillate then yields a much improved difference spectrum. In our experience, the value of the concentration of benzo[a]pyrene, thus determined, has not proved to be higher than that obtained by the routine method. Indeed, agreement between the values obtained by the two procedures has been rather striking.

SENSITIVITY AND APPLICABILITY

Consideration of various factors inherent in the procedure indicates that the limit of sensitivity of the method under ideal conditions is about 0.0001 mg. of benzo[a]pyrene. In actual analyses, the presence of other compounds, which modify the shape of the final difference spectra, limits the practical sensitivity to about 0.002 mg.

The method was developed primarily for application to various petroleum products resulting from refining operations. It has been applied to more than thirty such materials which have been subjected to biological testing for carcinogenic potency. All analytical data thus far obtained have been plausible in the light of the observed potencies and the known physical properties of the materials. The use of the method has been extended, in a limited manner, to substances derived from other sources, including coal tars and fractions thereof. No apparent difficulties were encountered with these materials. It seems likely that the analysis may be applied to mixtures extracted by benzene from a variety of additional materials such as soot, carbon black, condensates from atmospheric samples, and other materials which are derived from fossil fuels or the incomplete combustion of organic mixtures. It should be recognized that the results of such analyses would not necessarily indicate the total amount of benzo[a]pyrene in the original sample, since the complete extraction of benzo[a]pyrene from such materials is frequently very difficult to achieve.

LITERATURE CITED

(1) Hieger, I., J. Chem. Soc., 1933, 395.

(2) Cook, J. and Hewett, C., J. Chem. Soc., 1933, 398.

(3) Clar,E., "Aromatische Kohlenwasserstoffe", Berlin, Springer-Verlag, 1941.

(4) Goulden, F. and Tipler, M., Brit. J. Cancer, 3, 157 (1949).

(5) Waller, R. E., Brit. J. Cancer, 6, 8 (1952).

(6) Falk, H.L., Steiner, P.E., Goldfein, S., Breslow, A., and Hykes, R., Cancer Research, 11, 318 (1951).

(7) Wedgwood, P. and Cooper, R., Analyst, 78, 170 (1953).

(8) Windaus, A. and Raichle, K., Ann., 537, 157 (1939).

(9) Jones, R. N., J. Am. Chem. Soc., 67, 2127 (1945).

(10) Benesi, H.A. and Hildebrand, J.H., J. Am. Chem. Soc., 70, 2832 (1948).

(11) Ibid., 71, 2703 (1949).

(12) Mulliken, R.S., J. Am. Chem. Soc., 72, 600 (1950).

(13) Ibid., 74, 811 (1952).

(14) Brown, H.C. and Brady, J.D., J. Am. Chem. Soc., 74, 3570 (1952).

(15) Wright, N., Ind. Eng. Chem., Anal. Ed., 13, 1 (1941).

(16) Seyfried, W. D. and Hastings, S. H., Ind. Eng. Chem., Anal. Ed., 19 , 298 (1947).

UNIVERSITY OF CINCINNATI
DEPARTMENT OF PREVENTIVE MEDICINE AND INDUSTRIAL HEALTH

...KETTERING LABORATORY
...EGE OF MEDICINE—EDEN AVENUE
...NNATI 19 OHIO

CABLE ADDRESS KETLAB CINCINNATI
TELEPHONE CAPITOL 1414

March 3, 1954

Mr. D. V. Stroop, Director
Department of Technical Services
American Petroleum Institute
50 West 50th Street
New York City 20

Dear Mr. Stroop:

I am sending you herewith, for your information, a statement of expenditures made on behalf of the American Petroleum Institute for the fourth quarter of 1953. For your information, this does not include the payment of $38,283.00 received on December 28, 1953. I believe that otherwise the statement will be self-explanatory, but if you have any questions or comments, Doctor Kehoe would appreciate your bringing them to his attention.

Very truly yours,

E. R. Fortlage, Secretary to
Dr. Kehoe

ef

Enc.

API 05767

UNIVERSITY OF CINCINNATI
KETTERING LABORATORY

ACCOUNT OF AMERICAN PETROLEUM INSTITUTE

FOR 4th Quarter 1953

SALARIES (Based on Proportion of Time Actually Spent on Project)

Direct Salaries	6,392.42	
Indirect Salaries		
Histopathological Preparation	212.00	
Other Services	7,068.08	13,672.50

MISCELLANEOUS EXPENSE

Purchase of Animals	246.80	
Special Laboratory Supplies	375.41	
Travel	1,259.19	
Overhead (Proportion of Heat, Gas, Electricity, Steam, Telephone, General Laboratory Supplies, Postage, Annuities, Pensions, Maintenance, etc.	4,095.60	5,977.00
TOTAL		19,649.50

Balance Available for Further Work at End of 3rd Quarter 1953		12,213.57
Balance Due Kettering Laboratory at End of		
Receipts		
Expenditures 4th Quarter 1953		19,649.50
Balance Available for Further Work at End of		
Balance Due Kettering Laboratory at End of 4th Quarter 1953		7,435.93

FORM 1230 KET. LAB. 3M—11-53

API 05768

April 29, 1954

Dr. Robert Eckardt
Standard Oil Development Company
Esso Laboratories
P.O. Box 51
Linden, New Jersey

Dear Doctor Eckardt:

There was no opportunity to discuss the manuscript on carcinogenesis in any detail in Chicago with the subcommittee members. Dave Stroop suggested that I send him the stencils so that he could distribute copies to members of the subcommittee and perhaps also to the general committee. At any rate, I expect that you will have had a chance to judge from the copy sent to you whether a meeting will be needed to discuss the paper or if you can depend upon an adequate response by correspondence.

I believe any date in May except the period from May 11 to 20 would be OK at this end if you want to call a meeting.

Sincerely yours,

A. Wesley Horton

AWH:mjg
CC: Mr. David V. Stroop

API 05769

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

FOR INFORMATION ONLY NOT FOR PUBLICATION

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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 favcr 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, $\frac{\Sigma R + (\Sigma R + R')}{2}$	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 ± 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),

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

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

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

From Experiment 215, the relative potency, P_{MC}^{o}, 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:

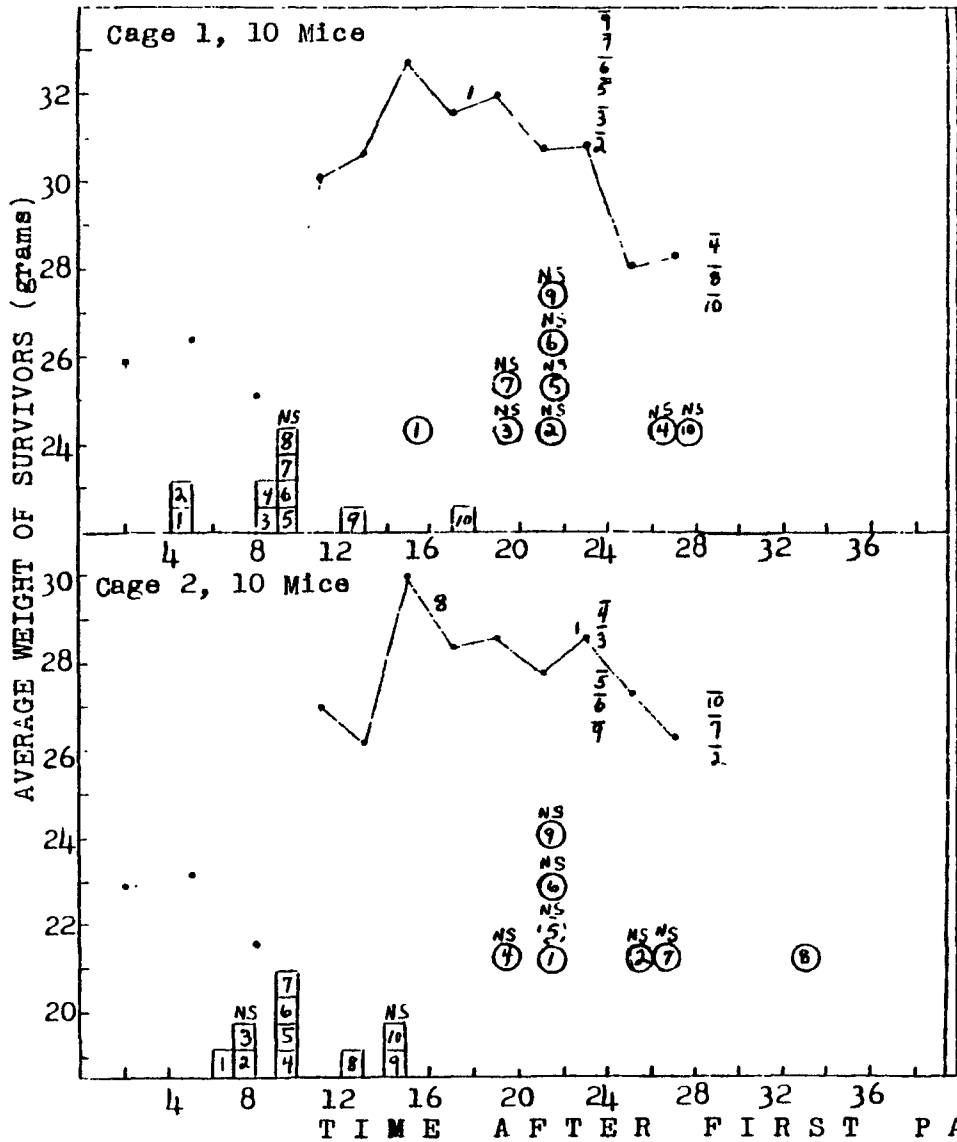
$\boxed{i}$ Time of appearance of first papilloma in i th mouse. When this symbol is first used for a given mouse after its death, the presence of non-invading carcinoma (intra-epithelial) or small areas of benign neoplasm was determinable only by histopathology.

$\textcircled{i}$ 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.

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

FIGURE 1

EXPERIMENT 220, SOLUTION OF METHYLCHOLANTHRENE, 1.034 PERCENT IN BENZENE



STARTING DATE:	5/3/50
STRAIN OF MICE:	C3H
NUMBER OF APPLICATIONS EACH WEEK:	1
DOSAGE OF SOLUTION PER APPLICATION:	100 mg.
MEAN TIME OF APPEARANCE OF TUMORS:	10.1±1.6
CLASSIFICATION:	B

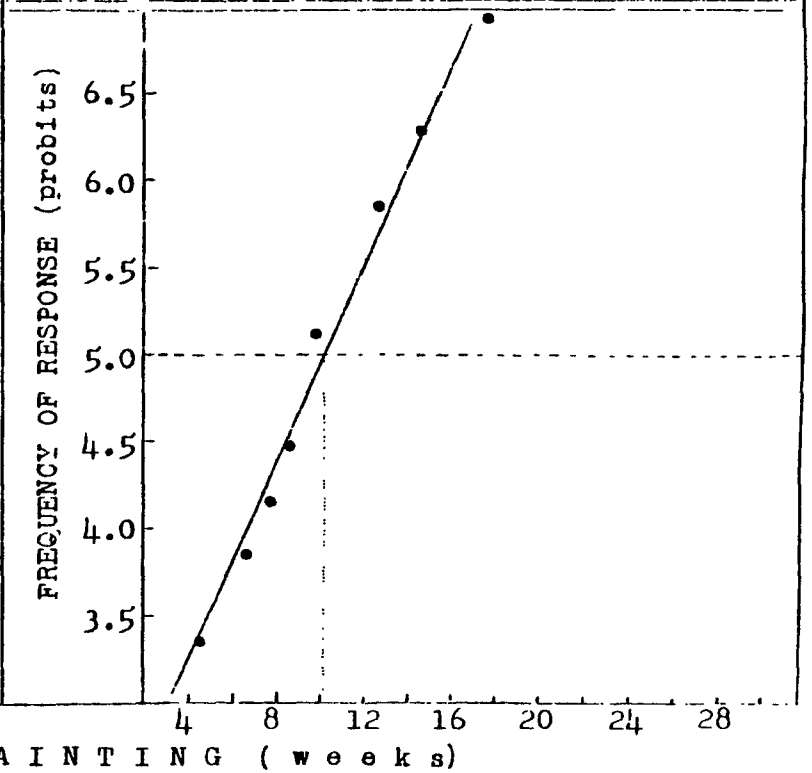


FIGURE 2

EXPERIMENT 268, SOLUTION OF METHYLCHOLANTHRENE, 0.287 PERCENT IN BENZENE

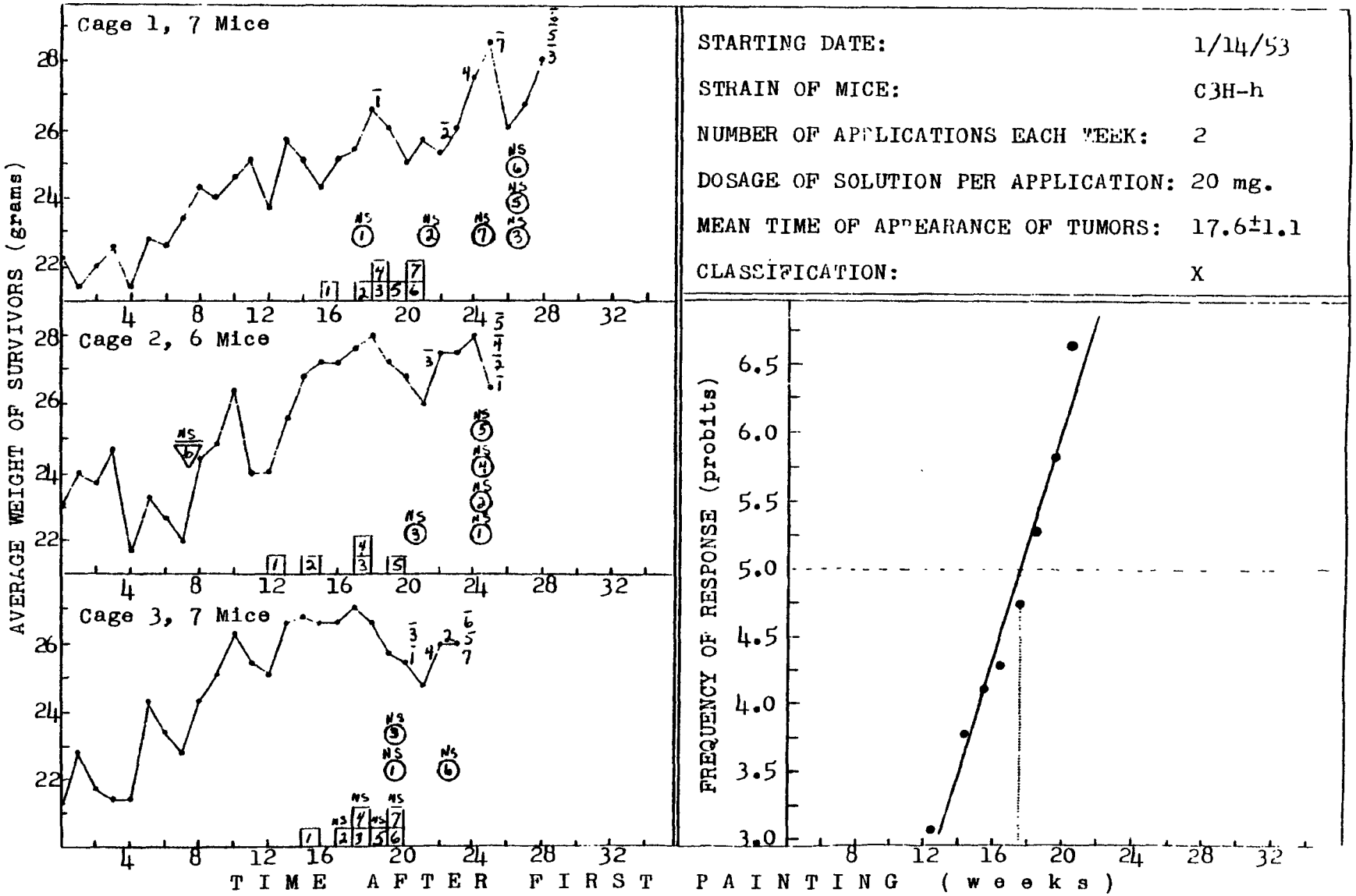
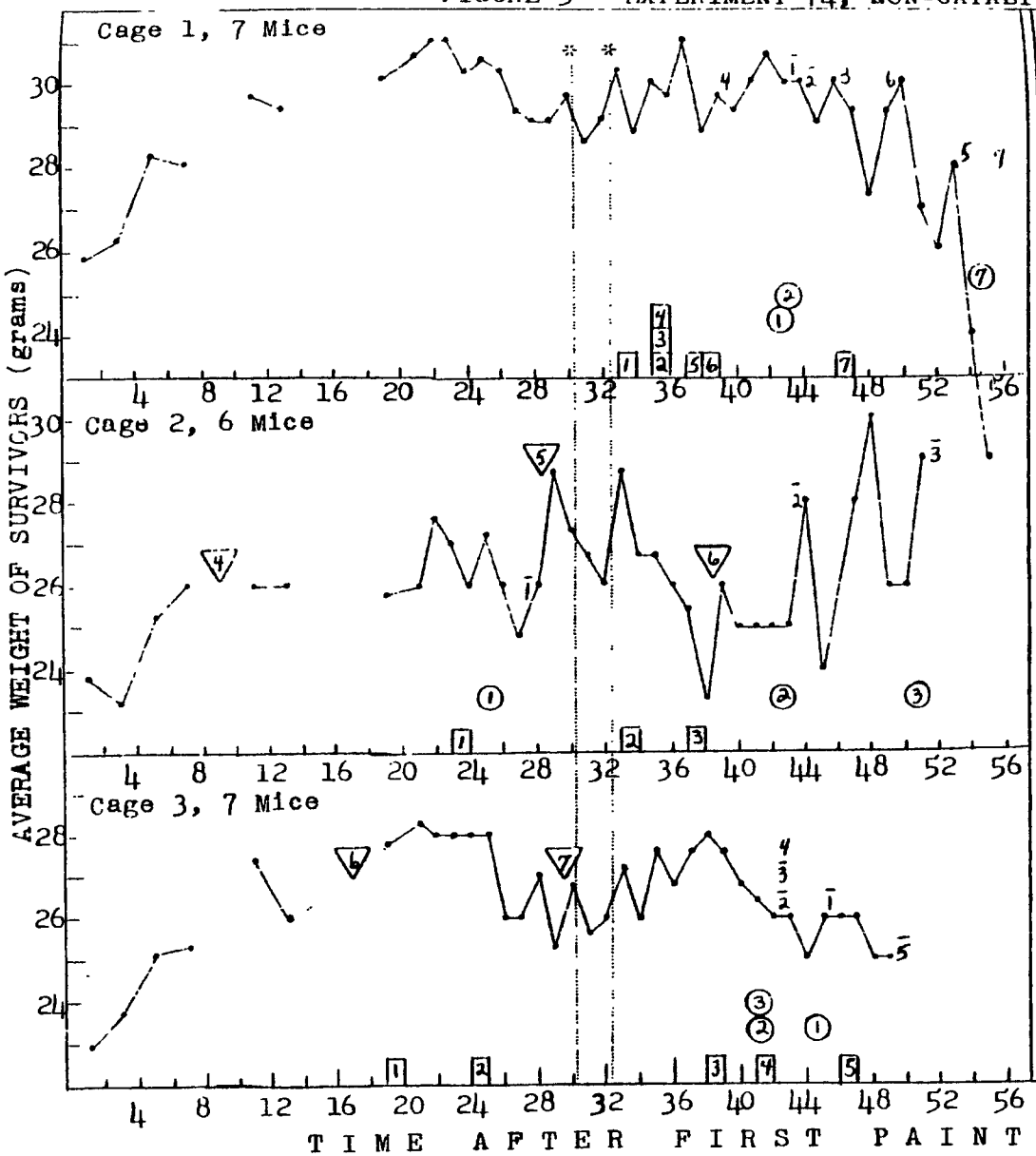


FIGURE 3 - EXPERIMENT 74, NON-CATALYTICALLY CRACKED OIL



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

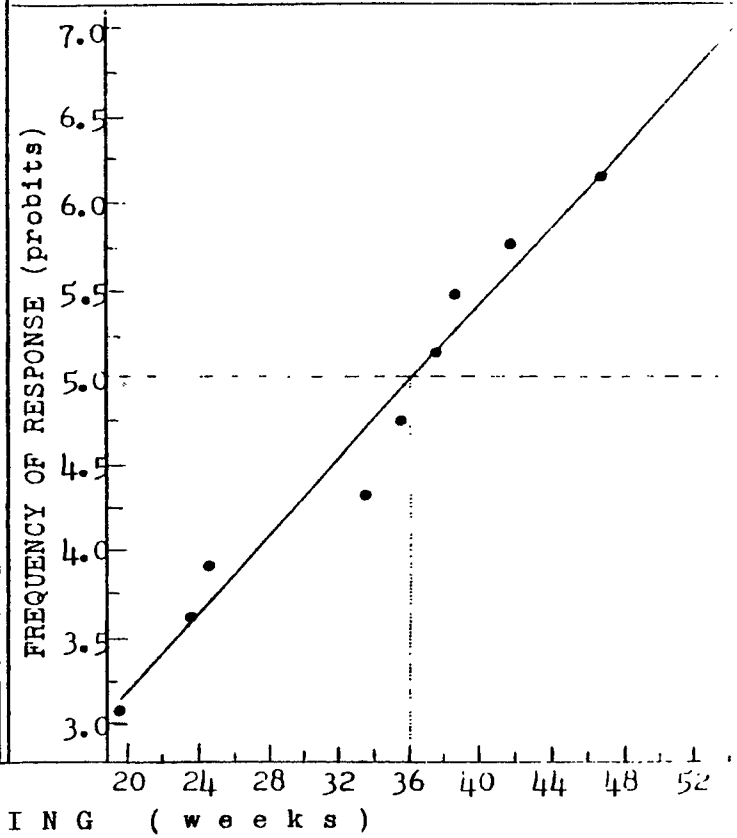
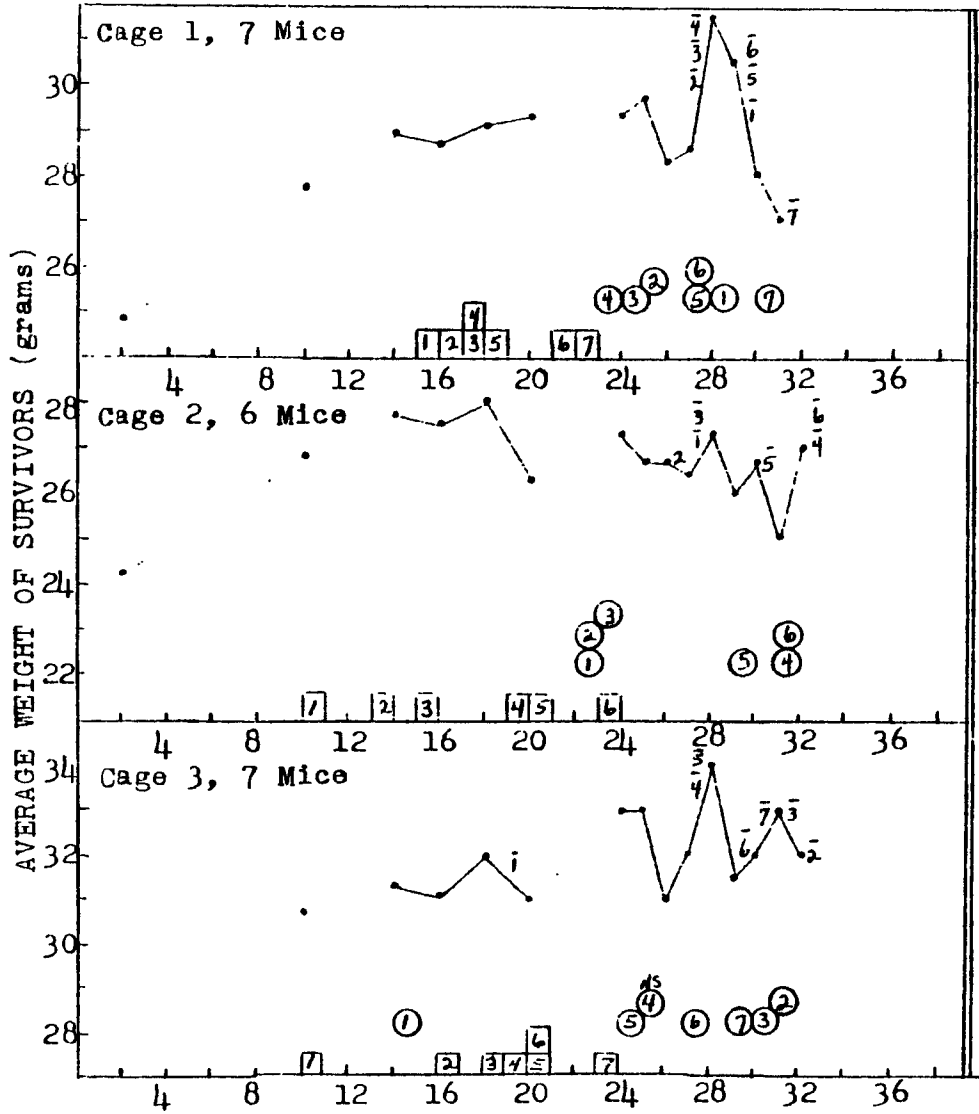


FIGURE 4

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

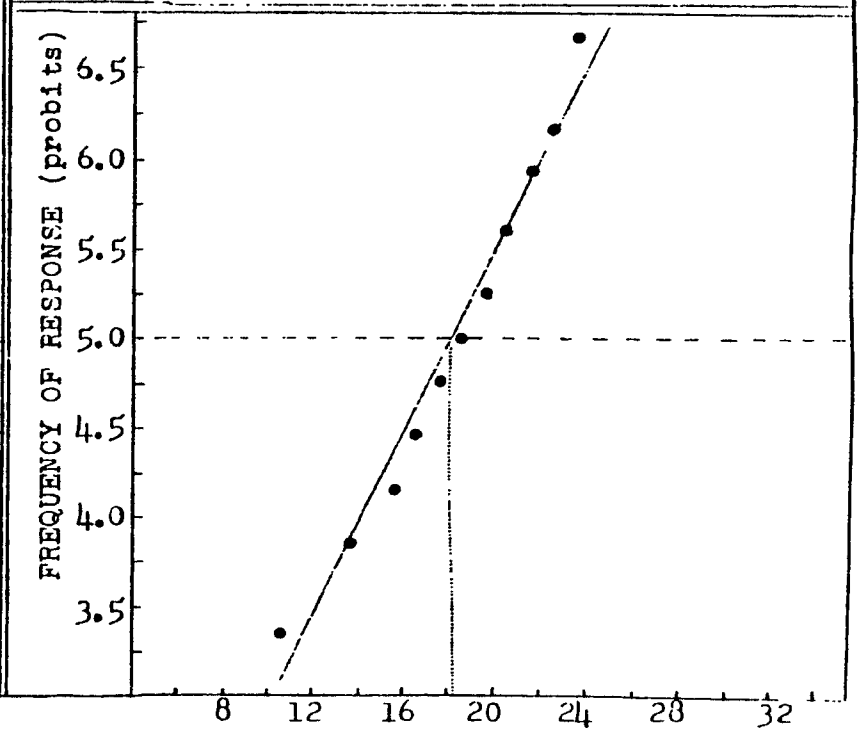


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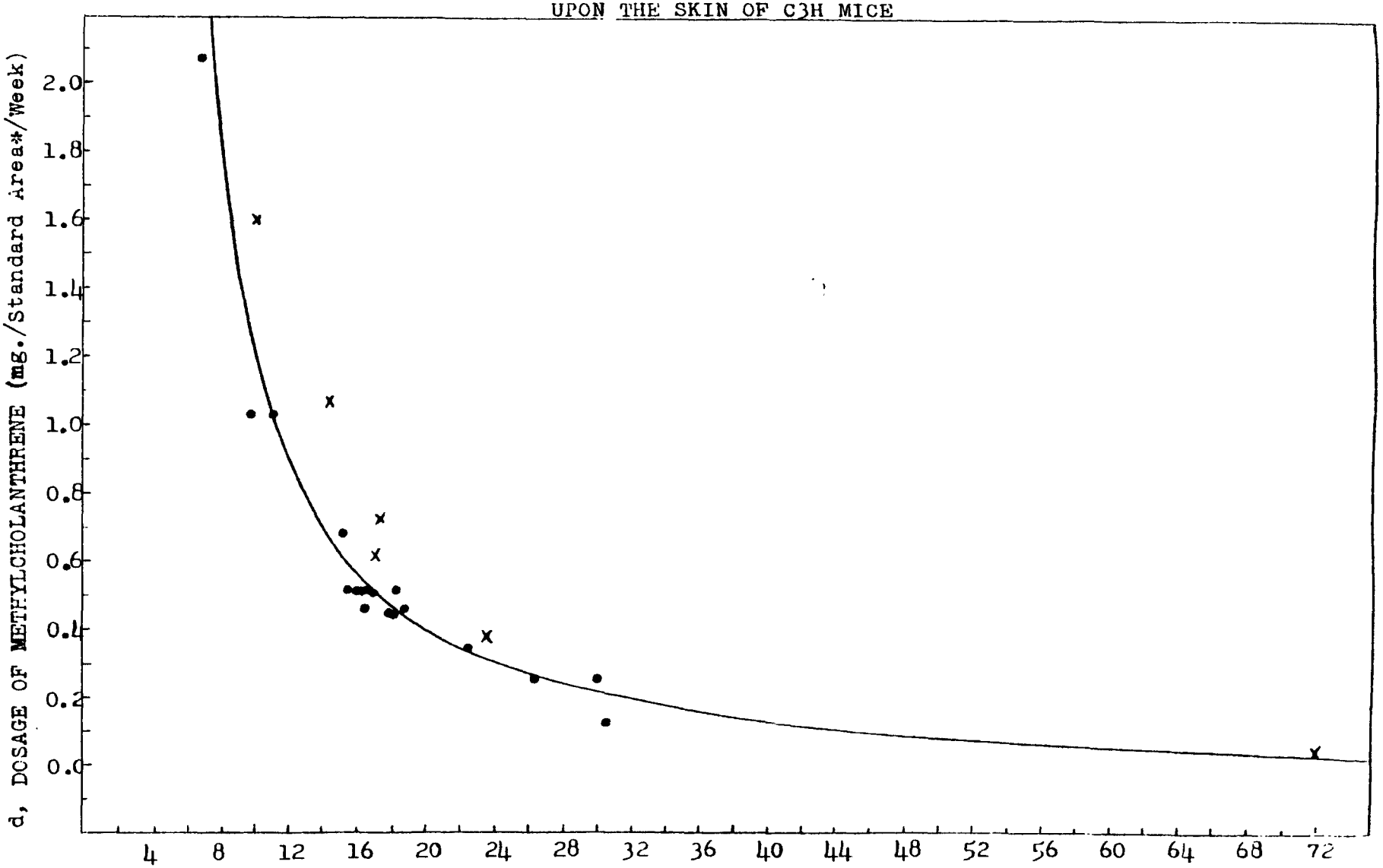
FIGURE 5

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



* Area covered by 100 mg. of the solution.

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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, $\frac{\Sigma R + (\Sigma R + R')}{2}$	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.

4. 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, $\bar{x}$ (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:

$\overline{\nabla}_i$ 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:

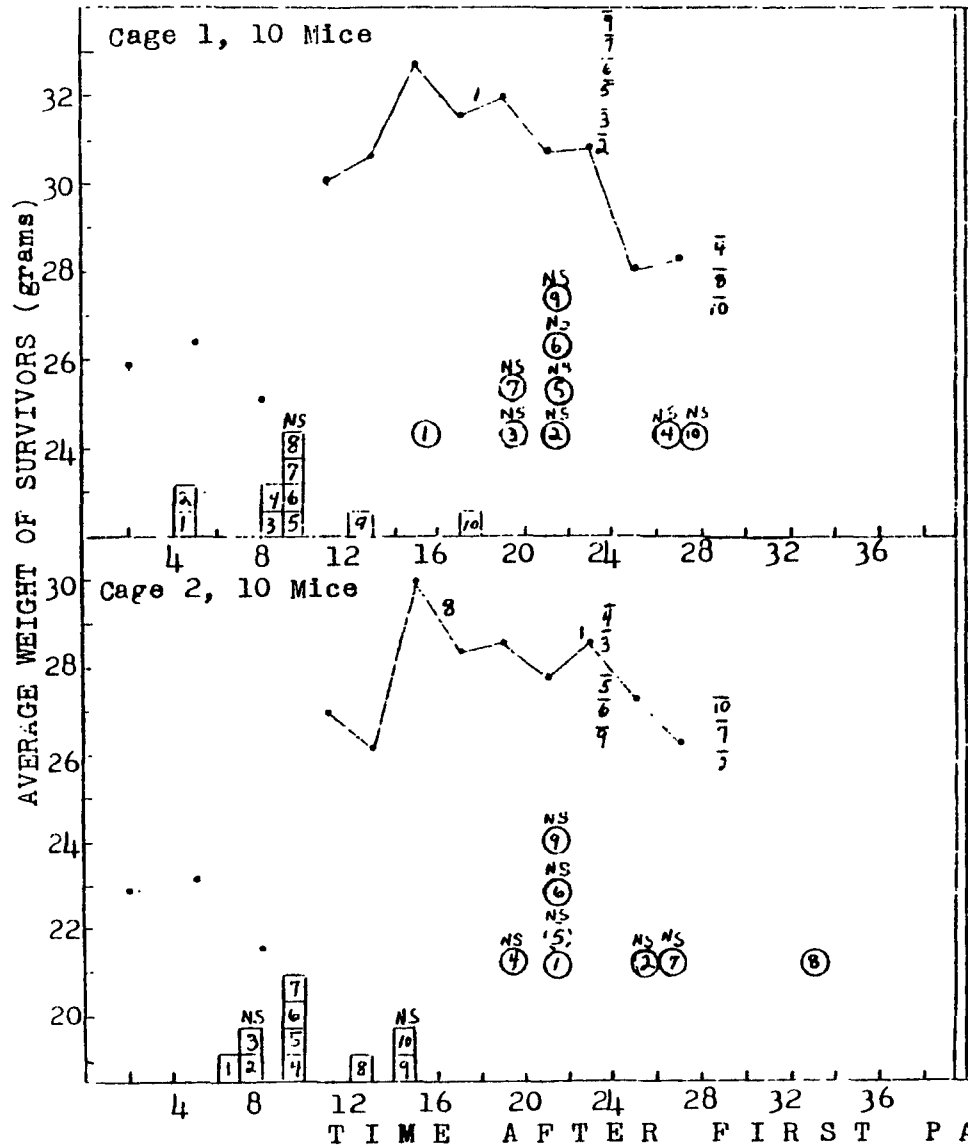
$\boxed{i}$ Time of appearance of first papilloma in i th mouse. When this symbol is first used for a given mouse after its death, the presence of non-invading carcinoma (intra-epithelial) or small areas of benign neoplasm was determinable only by histopathology.

$\textcircled{i}$ 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.

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

FIGURE 1

EXPERIMENT 220, SOLUTION OF METHYLCHOLANTHRENE, 1.034 PERCENT IN BENZENE



STARTING DATE:	5/3/50
STRAIN OF MICE:	C3H
NUMBER OF APPLICATIONS EACH WEEK:	1
DOSAGE OF SOLUTION PER APPLICATION:	100 mg.
MEAN TIME OF APPEARANCE OF TUMORS:	10.1±1.6
CLASSIFICATION:	B

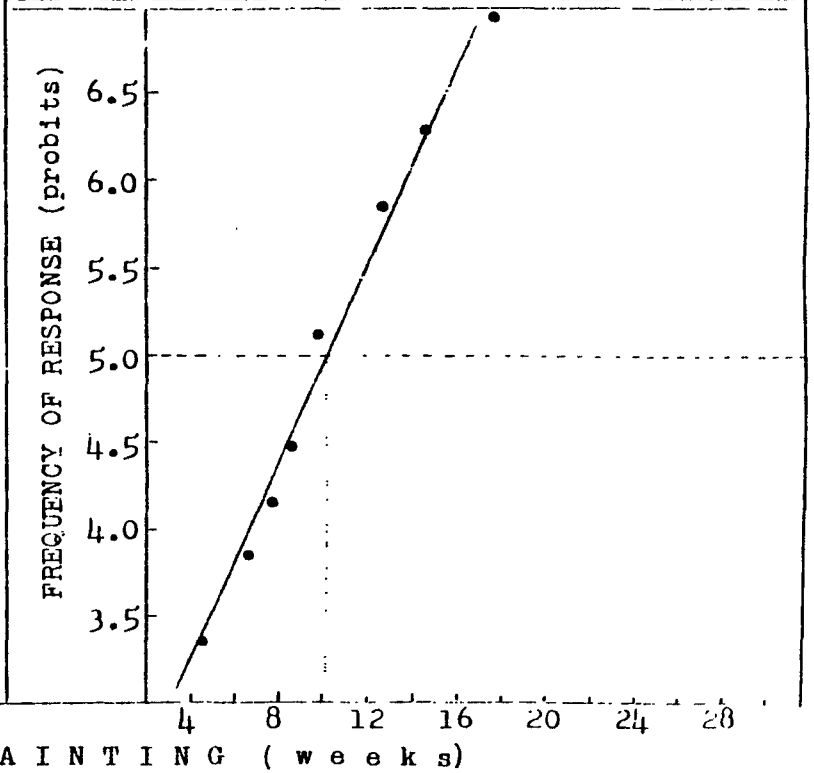
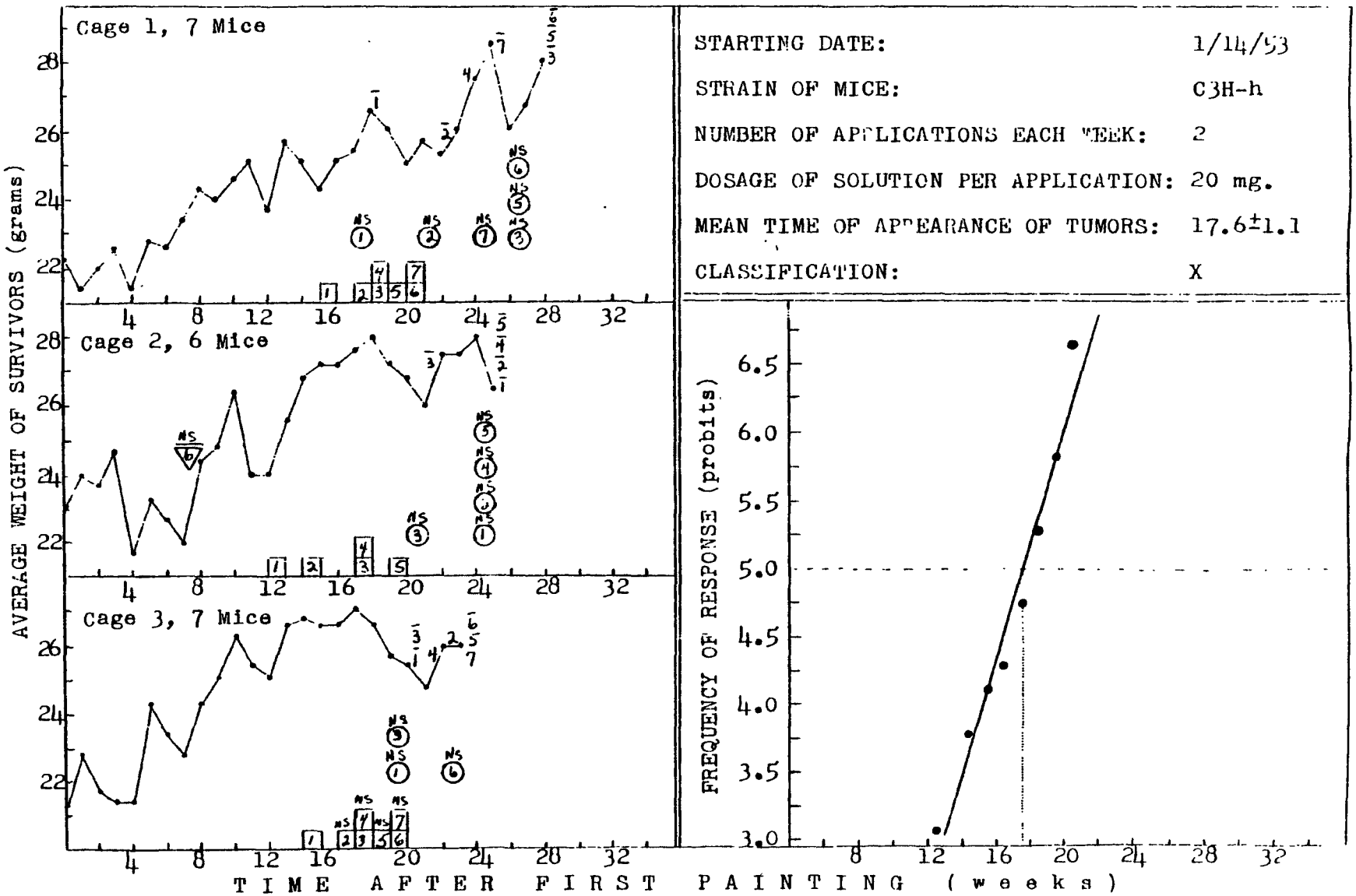


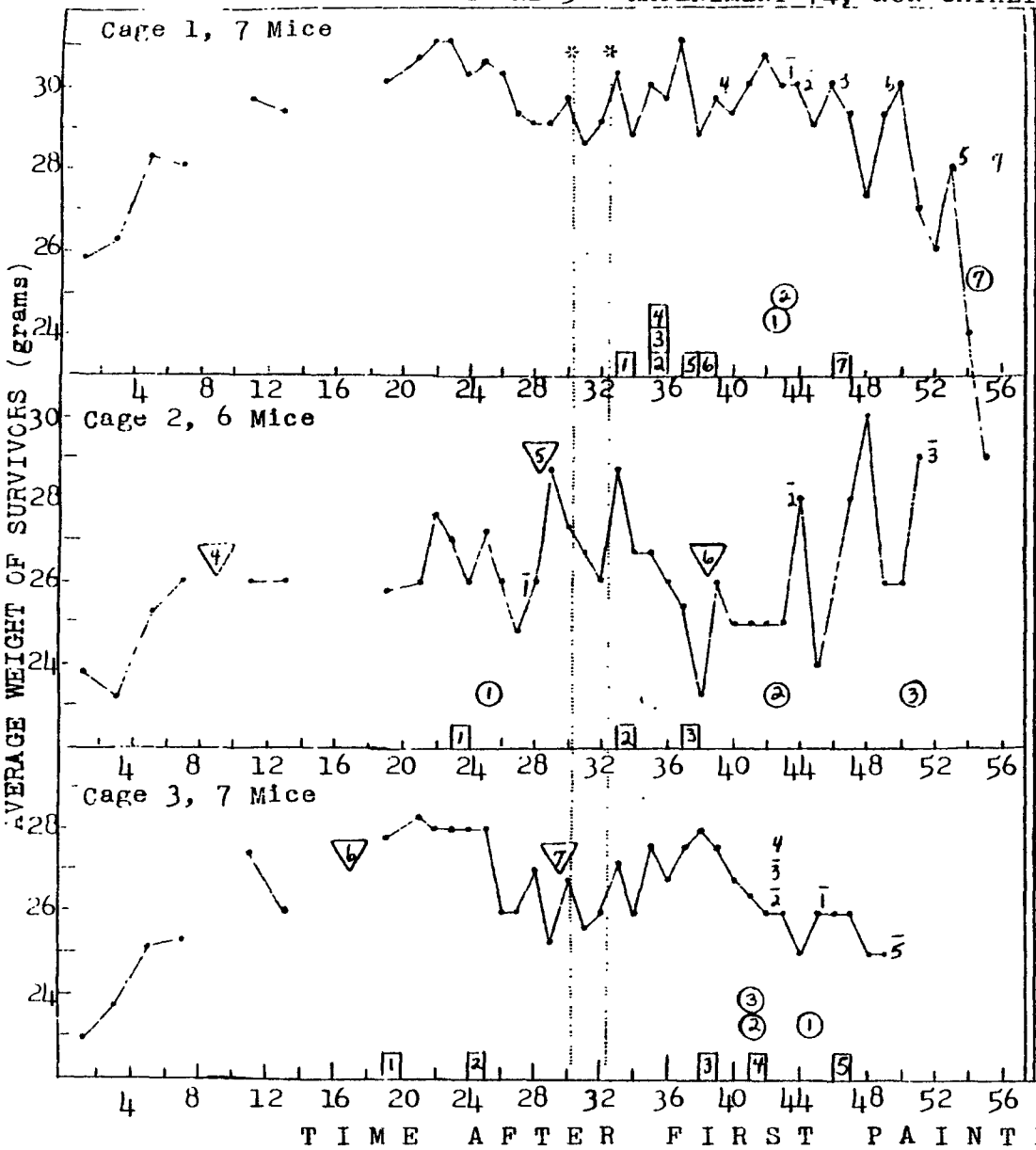
FIGURE 2

EXPERIMENT 268, SOLUTION OF METHYLCHOLANTHRENE, 0.287 PERCENT IN BENZENE



STARTING DATE:	1/14/53
STRAIN OF MICE:	C3H-h
NUMBER OF APPLICATIONS EACH WEEK:	2
DOSAGE OF SOLUTION PER APPLICATION:	20 mg.
MEAN TIME OF APPEARANCE OF TUMORS:	17.6±1.1
CLASSIFICATION:	X

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

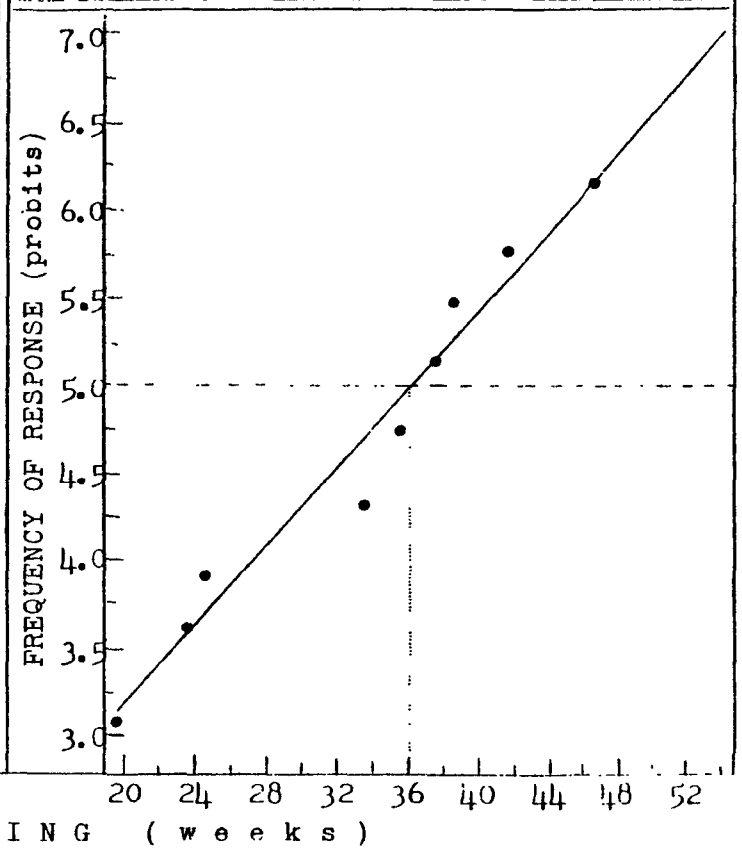
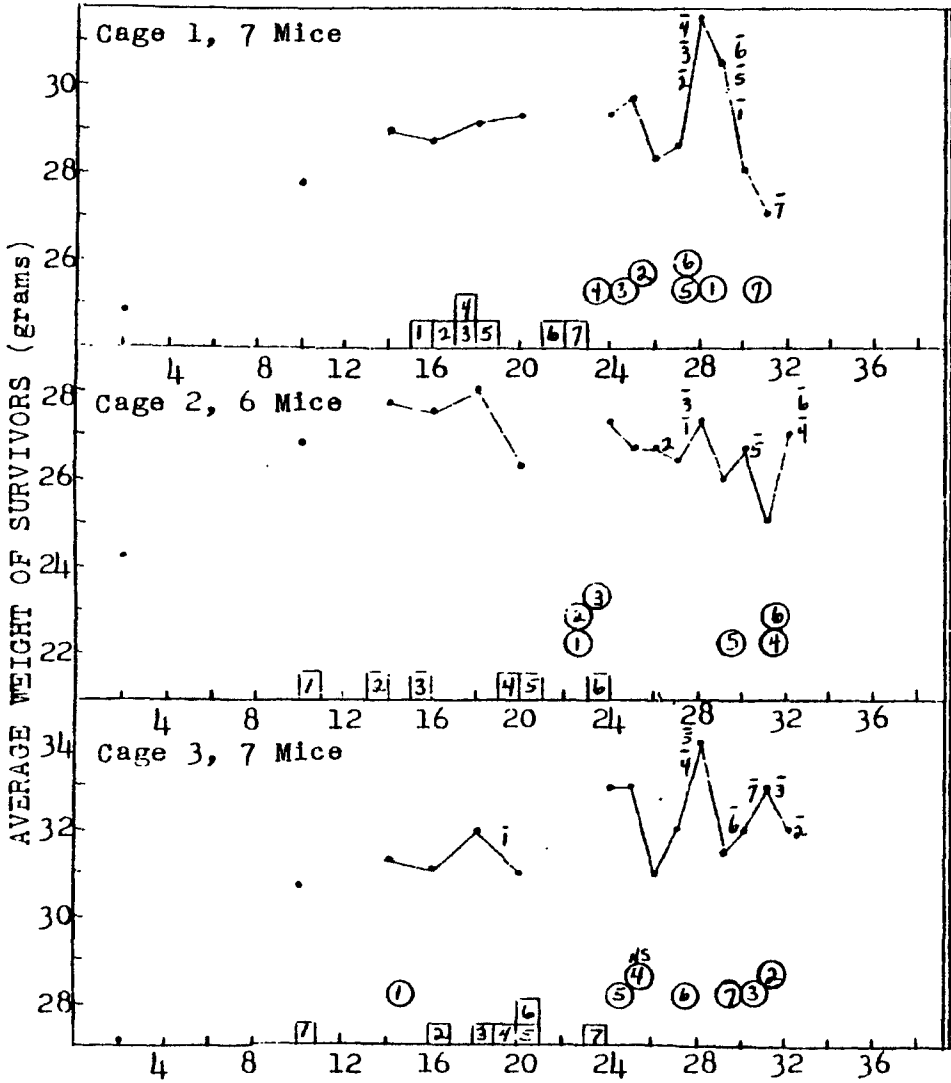


FIGURE 4

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

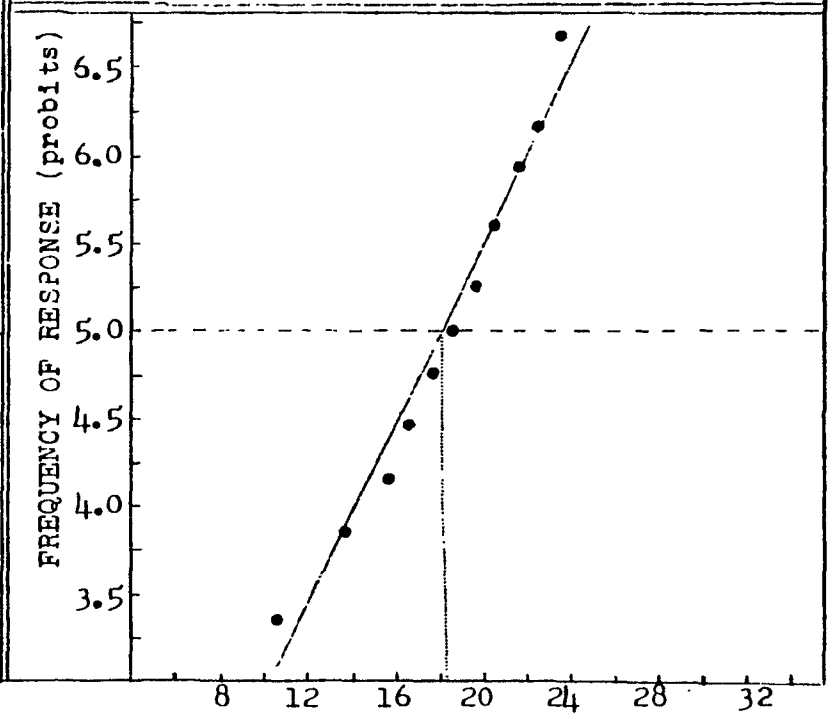
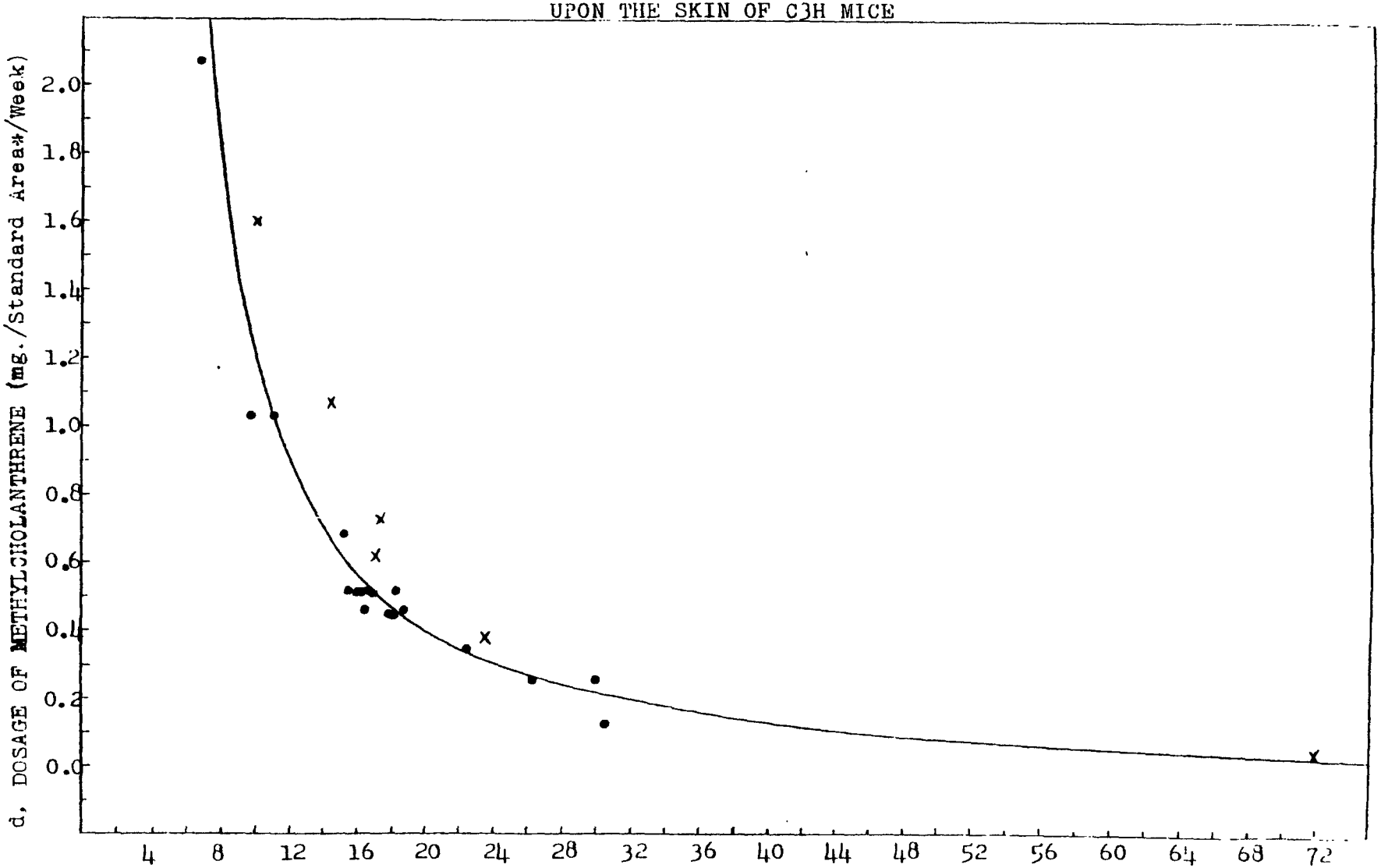


FIGURE 5

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



* Area covered by 100 mg. of the solution.

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.

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.

file: University of Cincinnati Expenditures

May 24, 1954

Dr. Robert A. Kehoe
University of Cincinnati
The Kettering Laboratory
Cincinnati 19, Ohio

Dear Dr. Kehoe:

This will acknowledge the receipt of the invoice covering the American Petroleum Institute's share of expense for investigation of fluorine and fluorine compounds for the year ending June 30, 1955.

We plan to issue a check in payment of the invoice on or about June 24.

Very truly yours,

DVS:ja
cc: Mr. Lacey Walker

API 05844

UNIVERSITY OF CINCINNATI
DEPARTMENT OF PREVENTIVE MEDICINE AND INDUSTRIAL HEALTH

THE KETTERING LABORATORY
COLLEGE OF MEDICINE—EDEN AVENUE
CINCINNATI 19, OHIO

May 17, 1954

CABLE ADDRESS: KETLAB, CINCINNATI
TELEPHONE: CAnyon 1414

Mr. D. V. Stroop, Director
Department of Technical Services
American Petroleum Institute
50 West 50th Street
New York City 20

Dear Mr. Stroop:

I am enclosing herewith an invoice covering American Petroleum Institute's share of expenses for the investigation of fluorine and fluorine compounds for the year 1954, (July 1, 1954 to June 30, 1955).

Very truly yours,

E. R. Fortlage

E. R. Fortlage, Secretary to
Dr. Kehoe

ef

Enc.

API 05845

UNIVERSITY OF CINCINNATI
DEPARTMENT OF PREVENTIVE MEDICINE AND INDUSTRIAL HEALTH

THE KETTERING LABORATORY
COLLEGE OF MEDICINE—EDEN AVENUE
CINCINNATI 19, OHIO

CABLE ADDRESS: KETLAB, CINCINNATI
TELEPHONE: CAPITOL 1414

May 25, 1954

Mr. D. V. Stroop, Director
Department of Technical Services
American Petroleum Institute
50 West 50th Street
New York City 20

Dear Mr. Stroop:

I am sending you herewith, for your information, a statement of expenditures made on behalf of the American Petroleum Institute for the first quarter of 1954. I believe that this statement will be self-explanatory, but if you have any questions or comments, Doctor Kehoe would appreciate your bringing them to his attention.

Very truly yours,

E. R. Fortlage

E. R. Fortlage, Secretary to
Dr. Kehoe

ef

Enc.

API 05848

UNIVERSITY OF CINCINNATI
KETTERING LABORATORY

ACCOUNT OF AMERICAN PETROLEUM INSTITUTE

FOR 1st Quarter 1954

SALARIES (Based on Proportion of Time Actually Spent on Project)

Direct Salaries	4990.83	
Indirect Salaries		
Histopathological Preparation	242.00	
Other Services	6537.99	11,770.82

MISCELLANEOUS EXPENSE

Purchase of Animals	193.80	
Special Laboratory Supplies	298.90	
Travel	784.49	
Overhead (Proportion of Heat, Gas, Electricity, Steam, Telephone, General Laboratory Supplies, Postage, Annuities, Pensions, Maintenance, etc.	2046.24	3,323.43

TOTAL 15,094.25

Balance Available for Further Work at End of _____

Balance Due Kettering Laboratory at End of 1953 — 7,435.93

Receipts January 1954 (December 1953) — 38,283.00

Sum Available 30,847.07

Expenditures 1st Quarter 1954 . . . . . 15,094.25

Balance Available for Further Work at End of 1st Quarter 1954 . . . . — 15,752.82

Balance Due Kettering Laboratory at End of _____

FORM 1230 KET. LAB. 3M—11-53

API 05849

Mimeographed Copies From D. V. Stroop To Those Indicated In Letter. June 9, 1954

ESSO LABORATORIES
Standard Oil Development Company
P. O. Box 51, Linden, N. J.

Medical Research Division
Robert E. Eckardt, PH.D., M.D.
Director

May 28, 1954

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

Dear Mr. Stroop:

I am attaching the minutes and attachments to the minutes of the April 29th meeting of the Committee on the Carcinogenic Action of Mineral Oils of the Medical Research Council of Great Britain. This was forwarded to me by Dr. Newquist who received it from Colonel Auld. It would be my suggestion that this material be duplicated for circulation to the members of the Subcommittee on Carcinogenicity and to the members of the Medical Advisory Committee. In addition, I would appreciate it if a copy of this material is forwarded to Dr. Horton at the Kettering Laboratory.

Sincerely yours,

/s/ R. E. Eckardt

R. E. ECKARDT, M. D.

REE:ilk
Encls.

MEDICAL RESEARCH COUNCIL

For Specified (Normal Committee) Circulation

MRC.54/277
CAMO.Ag.15

Committee on the Carcinogenic Action of Mineral Oils

A G E N D A

for the Fifteenth Meeting to be held at 38 Old Queen Street, Westminster, S.W.1 on Thursday, 29th April, 1954 at 4.00 p.m.

1. Minutes of the last meeting. (MRC.53/621 - already circulated)

2. Report by Professor J. W. Cook. (CAMO.54/1 - Enclosure A)

3. Report by Professor F. Morton. (To be circulated)

4. Other reports. (CAMO.54/2 - Enclosure B) (CAMO.54/3 - Enclosure C)

5. Future plans.

6. Any other business.

7. Date of next meeting.

API 05851

MEDICAL RESEARCH COUNCIL

For Specified (Normal Committee) Circulation

MRC.54/275
CAMO.54/1

Committee on the Carcinogenic Action of Mineral Oils

PROGRESS REPORT NO. X

from Dr. W. Carruthers and Professor J. W. Cook,
Glasgow University

We have received from Professor Morton two of the fractions prepared at Birmingham by distillation of the Kuwait No. 1 side stream. These are the fractions designated K_4S_2b (b.p. 357½-360°) and K_4S_6b (b.p. 377½-380°) of which we had 3.75 ℓ. and 6.32 ℓ. respectively. Fraction K_4S_2b was a mobile orange-yellow oil; K_4S_6b contained some waxy material which was filtered off before proceeding further.

'Aromatic extracts' of these fractions were prepared by shaking them with 5% aqueous acetone as previously arranged. This yielded 940 c.c. of a dark coloured extract from K_4S_2b (25%), and 1430 c.c. from K_4S_6b (23%). These were washed with dilute acid and alkali (which removed only a trace of material) and the absorption spectra of distilled samples examined. The spectra showed the characteristics noted in previous Kuwait fractions. Further work has been entirely confined to these extracts. The almost colourless rafinnates have been stored.

It is, perhaps, of interest to note that the separation with acetone into 'aromatic' and 'non-aromatic' fractions is by no means clear cut. Chromatography of the 'aromatic' part of K_4S_2b on silica gel showed that it contained about 25% of material of refractive index below 1.5000 and thus to be regarded as largely non-aromatic in character. Similarly the 'non-aromatic' part was shown to contain 20% of material with refractive index above 1.5000.

The extracts were heated with maleic anhydride at 100° for several hours. This treatment was designed to remove anthracene type compounds from those of other structure present in the oil through their alkali soluble maleic anhydride adducts. 'Anthracene fractions' were then recovered from the separated adducts by distillation over sodium hydroxide.

Thus, from the aromatic extract of K_4S_2b 3.8 g. of an 'anthracene fraction' was obtained, b.p. 175-180°/0.4 mm. This deposited a small amount of crystalline material which, after purification, formed pale yellow plates, m.p. 235°. The ultra-violet absorption spectrum showed it was a derivative of anthracene and elementary analysis suggested the formula $C_{16}H_{14}$. The quinone melted at 163-165°. It was identified as 2:7-dimethylanthracene by mixed melting point determination (literature m.p. 241° cor.; quinone, m.p. 170° cor.). The remaining oily part of the 'anthracene fraction' was chromatographed, but no further crystals were obtained. The fractions showed well defined anthracene-type spectra.

The adduct from K_4S_6b similarly yielded 11 g. of a dark oil b.p. 190-210°/2 mm. From this three crystalline hydrocarbons were isolated by a combination of low temperature crystallisation and chromatography. The first formed pale yellow plates, m.p. 244-245°. Its absorption spectrum confirmed that it was a derivative of anthracene, and analysis indicated a molecular formula $C_{16}H_{14}$, $C_{17}H_{16}$, $C_{18}H_{16}$ or $C_{19}H_{18}$. Oxidation with chromic acid in acetic acid yielded a quinone, m.p. 236-237°. Comparison of the compound with the known di- and tri-methylanthracenes suggests that it may be 2:3:6-trimethylanthracene (literature m.p. 255° cor.; quinone, m.p. 240° cor.). We hope to confirm this by mixed melting point.

Another compound formed colourless blades, m.p. 169-170°. The absorption spectrum again indicated an anthracene structure. The quinone, obtained as above, had m.p. 169-170°. Analyses of the two compounds suggested a trimethylanthracene, or its equivalent, as the most likely structure. The third hydrocarbon from the 'anthracene fraction' of K_4S_6b crystallised as pale yellow blades, m.p. 224-225°. Elementary analysis is in agreement with the formulae $C_{17}H_{16}$, $C_{18}H_{18}$ or $C_{19}H_{18}$, and the absorption spectrum confirmed the anthracene structure. Oxidation yielded a quinone m.p. 225-226°. Neither of these hydrocarbons corresponds to any known anthracene derivative. Further efforts are being made to identify them.

The great bulk of the aromatic extract in each case remained unattacked by the maleic anhydride and was recovered. It was shown by its absorption spectrum to be free of anthracene compounds. It was treated with picric acid in ethanol, and thereby separated into a picrate-forming part and a (greater) part which did not form a crystalline picrate under these conditions. By this means about 7% of K_4S_2b (30% of the aromatic extract) and 5% of K_4S_6b (25% of the extract) have been concentrated through their crystalline picrates. Present work is centred on the further subdivision of these picrate forming fractions by chromatography, fractional distillation and other means. The non-picrate-forming parts of the aromatic extracts have been set aside for the time being.

Mention was made in a previous report of experiments being done with the residue K5 from distillation of the original Kuwait crude. These experiments were pursued at some length, but gave very unpromising results. Some idea of the complexity of this material can be gathered from the fact that small fractions prepared by distillation, extraction and chromatography, and representing 1 part in 10^5 of the original material gave absorption spectra which were completely devoid of any characteristic features. In view of this, and since this material was found in any case to be non-carcinogenic, further chemical investigation of it has been discontinued.

MEDICAL RESEARCH COUNCIL

For Specified (Normal Committee) Circulation

MRC.54/284
CAMO.54/2

Committee on the Carcinogenic Action of Mineral Oils

STATISTICAL ANALYSIS OF 1952-3 EXPERIMENTS AT BIRMINGHAM

by Dr. J. O. Irwin

Experiments with 21 treatments were carried out with both mice and rabbits. The treatments consisted of applications with the 15 sub-fractions separated under the supervision of Professor Morton and shown in Table 1 of the first report of the Committee (CAMO.53/1) and the 6 further substances:-

9:10-dimethyl-1:2-benzanthracene, 0.2% and 0.05% in liquid paraffin (D_2 and $D_{\frac{1}{2}}$)

Methylcolanthrene 0.2% and 0.05% in liquid paraffin (M_2 and $M_{\frac{1}{2}}$)

Kuwait fraction 4, neat and diluted 4 times with liquid paraffin (K_4)

The 15 were sub-fractions of Kuwait 4, Lagunillas 4 and Oklahoma 4 and are designated

$K_{4.1}$, $K_{4.2}\cdots K_{4.5}$; $L_{4.1}$, $L_{4.2}\cdots L_{4.5}$; $O_{4.1}$, $O_{4.2}\cdots O_{4.5}$

Experiments with Mice

The 21 specimens were each tested on 50 mice; the results are shown in Table 1. They show that the mice responded only very slightly to the oil fractions in comparison with their response to the carcinogenic hydrocarbons. However, the higher Lagunillas sub-fractions show a significant increase in carcinogenicity over the lower. In general the fractions show a very slightly lower average carcinogenicity than that obtained in the previous year, when the average values were:-

	Expectation of Tumourless Life (limited to 52 weeks)	Date when 10% of survivors had tumours	Total number of Tumours
K_4	50.2 ± 1.3	not reached	2
L_4	49.0 ± 1.7	31.8 ± 1.2	3
O_4	49.9 ± 1.4	46.5 ± 4.3	2

but the difference might well be due to the mice. As in 1951-2 the order of carcinogenicity is L>K>O; however, L is more distinctly differentiated from K and O in the 1952-3 than in the 1951-2 results

for mice. While $K_{4.3}$ appears to be the most carcinogenic Kuwait sub-fraction, L and O show a slight increase of concentration in the higher boiling fractions.

From the data of Table 1, estimates may be made of the relative carcinogenic potencies of D and M. These are as follows:

		Estimated Potency (D/M)
(1)	From E.T.L.	10.5
(2)	From 10% dates	6.5
(3)	From total number of tumours	4.8

The errors of these estimates cannot be very precisely calculated, but the fiducial limits (P = 0.95) are about 50-200% for (1) and (2) and about 25-400% for (3). Thus we can conclude that, judged by the mice used, D is about 7 times as potent as M.

Experiment with Rabbits

The design of this experiment is shown in Table 2. It is of the same type as that employed in 1951-2, a Youden square. There are 21 groups of 5 rabbits. In each group, each rabbit was painted with the same 5 fractions, one on each of the sites I, II, III, IV, and VII (see diagram in Appendix I to the First Report of the Committee*). Each of the 21 sub-fractions or standards was painted on each site in one and only one group. Each fraction was used in exactly 5 groups and every pair of fractions was used in just 1 group.

The percentage of each group of 5 animals which got tumours on each site was calculated. The method of analysis was to transform percentages to degrees by means of the angular transformation**(which approximately equalises variance at different levels) and then to perform an analysis of variance of the results.

Table 3 shows the mean values of the transformed variables and the probabilities of getting a tumour, estimated for each fraction and standard. The general order of carcinogenicity is again L>K>O. The 5 sub-fractions O show no significant differences among themselves; the K sub-fractions show a maximum at $K_{4.2}$; the L sub-fractions increase in carcinogenicity up to $L_{4.4}$. The residue is in each case less carcinogenic than the original K_4, the value for which is in agreement with that obtained in 1951-2. The probability value obtained for L_4 in 1951-2 was 0.473 and none of the sub-fractions

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* Sites I and II, Ears, outer surface near base.
Sites III and IV, Lateral Thoracic.
Site VII Interscapular.

** The actual transformation used was $y=\sin^{-1}\sqrt{\frac{r+3/8}{n+3/4}}$ where r is the number of positive responses out of n. In this case n=5 and r is the number of animals out 5 which got tumours. This is an improvement on $y=\sin^{-1}\sqrt{\frac{r}{n}}$ (used for the 1951-2 data) when n is small, because it removes bias in the mean, and more nearly equalises variance at different levels.

in 1952-3 are significantly more carcinogenic than this. The O sub-fractions on the other hand suggest a slight increase of carcinogenicity compared with O_4 in 1951-2.

From the data of Table 3, the standard D can be estimated to be 9 times as potent as M with fiducial limits (P = 0.95) of 25-400%. This is in agreement with the value found from mice.

The analysis of variance is shown in Table 4. There are significant differences in response between the different sites of application, and between the different groups of rabbits. Table 5 shows the number of tumours out of 105 possible on the 5 different sites. The results agree with those of 1951-2 in showing that the greatest number of tumours is on site VII (middle of the back nearest the ears) and that sites I and II (ears) come next in order.

Comparison of Results with Mice and Rabbits

The relative potencies of the standards D and M do not differ significantly in mice and in rabbits. The sub-fractions on the other hand are much less potent in mice than in rabbits, relative to the two standards. For example, $L_{4.3}$ appears to be very nearly equal in potency to $M\frac{1}{2}$, or say $D_{1/14}$, as judged by the mice experiments, but in the rabbit experiments it is about equal in potency to D_1.

Nevertheless the relation between the sub-fractions themselves seems much the same in mice and rabbits. This is exemplified by $L_{4.1}$, $L_{4.2}$, $L_{4.3}$, $L_{4.4}$, though $L_{4.5}$ appears a little more potent than these in the mice and definitely less potent in the rabbit experiments.

As judged by the limits of error of the potency determinations of M and D made above, 20 rabbits painted on 5 sites give about the same amount of information as 50 mice. This might suggest that it was more profitable to use mice, but the response of the mice to the sub-fractions is too slight to make differences of carcinogenic effect easily ascertainable. For example, the difference between $L_{4.2}$ and $L_{4.4}$ in rabbits is about 2.4 times its standard error, in mice there is no significant differentiation at all.

Table 1 - Experiments with mice at Birmingham 1952-3

Two pure carcinogens and one crude (at two dilutions each) and fifteen derived fractions

Standard, crude or fraction	Expectation of Tumourless Life (limited to 52 weeks)	Date when 10% of survivors had tumours and standard error	Total number of tumours
M_2	33.7 ± 1.8	20.9 ± 0.9	23
$M_{\frac{1}{2}}$	48.3 ± 1.4	37.1 ± 0.7	6
D_2	13.9 ± 0.4	11.4 ± 0.8	38
$D_{\frac{1}{2}}$	24.8 ± 1.6	17.1 ± 0.6	25
K_4	52	not reached	0
$K_{4.\frac{1}{4}}$	52	not reached	0
$K_{4.1}$	52	not reached	0
$K_{4.2}$	51.4 ± 0.6	not reached	1
$K_{4.3}$	49.4 ± 1.3	44.9 ± 1.3	4
$K_{4.4}$	52	not reached	0
$K_{4.5}$	52	not reached	0
$L_{4.1}$	52	not reached	0
$L_{4.2}$	50.0 ± 1.2	40.5 ± 8.9	3
$L_{4.3}$	47.3 ± 2.2	37.4 ± 4.3	3
$L_{4.4}$	48.0 ± 2.2	34.1 ± 0.8	3
$L_{4.5}$	44.6 ± 2.5	23.9 ± 6.4	7
$O_{4.1}$	52	not reached	0
$O_{4.2}$	52	not reached	0
$O_{4.3}$	51.8 ± 0.3	not reached	1
$O_{4.4}$	51.4 ± 0.6	not reached	1
$O_{4.5}$	51.3 ± 0.7	not reached	1

Table 2 - Design of experiment on 21 fractions

(1952-3 tests)

Rabbits	I	II	III	IV	VII
1 - 5	d	p	n	u	a
6 - 10	e	q	o	a	b
11 - 15	f	r	p	b	c
16 - 20	g	s	q	c	d
21 - 25	h	t	r	d	e
26 - 30	i	u	s	e	f
31 - 35	j	a	t	f	g
36 - 40	k	b	u	g	h
41 - 45	l	c	a	h	i
46 - 50	m	d	b	i	j
51 - 55	n	e	c	j	k
56 - 60	o	f	d	k	l
61 - 65	p	g	e	l	m
66 - 70	q	h	f	m	n
71 - 75	r	i	g	n	o
76 - 80	s	j	h	o	p
81 - 85	t	k	i	p	q
86 - 90	u	l	j	q	r
91 - 96	a	m	k	r	s
97 - 100	b	n	l	s	t
101 - 105	c	o	m	t	u

Key to fractions a - u

$K_{4.1}$ = k
$K_{4.2}$ = b
$K_{4.3}$ = u
$K_{4.4}$ = m
$K_{4.5}$ = g

$O_{4.1}$ = o
$O_{4.2}$ = t
$O_{4.3}$ = r
$O_{4.4}$ = j
$O_{4.5}$ = e

$L_{4.1}$ = f
$L_{4.2}$ = s
$L_{4.3}$ = a
$L_{4.4}$ = l
$L_{4.5}$ = d

M_2 = h
$M_{\frac{1}{2}}$ = p
D_2 = c
$D_{\frac{1}{2}}$ = q
K_4 = n
$K_{4.\frac{1}{4}}$ = i

a = $L_{4.3}$
b = $K_{4.2}$
c = D_2
d = $L_{4.5}$
e = $O_{4.5}$

f = $L_{4.1}$
g = $K_{4.5}$
h = M_2
i = $K_{4.\frac{1}{4}}$
j = $O_{4.4}$

k = $K_{4.1}$
l = $L_{4.4}$
m = $K_{4.4}$
n = K_4
o = $O_{4.1}$

p = $M_{\frac{1}{2}}$
q = $D_{\frac{1}{2}}$
r = $O_{4.3}$
s = $L_{4.2}$
t = $O_{4.2}$
u = $K_{4.3}$

Table 3 - Experiments with rabbits at Birmingham 1952-3

Two pure carcinogens and one crude (at two dilutions each) and fifteen derived fractions

Mean Values corrected for group differences

Fraction or Standard	Transformed Variable (°)	Probability of tumour	Total number of Tumours (out of 25 possible)
$K_{4.1}$	36.52	.332	8
$K_{4.2}$	44.79	.496	13
$K_{4.3}$	30.19	.216	7
$K_{4.4}$	30.41	.220	7
$K_{4.5}$	21.77	.158	2
$L_{4.1}$	32.48	.257	7
$L_{4.2}$	37.57	.353	9
$L_{4.3}$	49.79	.521	14
$L_{4.4}$	54.43	.686	17
$L_{4.5}$	29.60	.206	5
$O_{4.1}$	23.55	.109	2
$O_{4.2}$	20.27	.063	3
$O_{4.3}$	26.61	.156	3
$O_{4.4}$	25.21	.134	3
$O_{4.5}$	21.13	.074	2
M_2	23.55	.109	3
$M_{\frac{1}{2}}$	11.92	0	0
D_2	60.62	.798	20
$D_{\frac{1}{2}}$	34.63	.296	7
K_4	37.00	.342	9
$K_{4.\frac{1}{4}}$	19.76	.056	2
S.E. one mean	4.96		
Difference between two means	7.01		

Table 4 - Experiment with rabbits on fifteen sub-fractions and six "standards" (1952-3)

Analysis of variance on angular transformation of proportion of tumours out of 5

	Sums of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Sites	4020.09	4	1005.02	9.03 ($P < 0.001$)
Groups of rabbits	4917.58	20	245.88	2.21 ($.05 < P < .01$)
Fractions	15358.24	20	767.91	6.90 ($P < 0.001$)
Error	6678.57	60	111.31	
Total	30974.47	104		

Table 5 - Experiment with rabbits on fifteen sub-fractions and six "standards" (1952-3)

Number of tumours out of 105 on the five different sites

Site	Number of tumours
I	25
II	24
III	23
IV	22
VII	51

MEDICAL RESEARCH COUNCIL

For Specified (Normal Committee) Circulation

MRC.54/286
CAMO.54/3.

Committee on the Carcinogenic Action of Mineral Oils

PROGRESS REPORT NO. X. (APRIL 1954)

by

D. L. Woodhouse and J. R. Squire

TESTS CARRIED OUT BY D. L. WOODHOUSE 1953-54

The tests have been carried out satisfactorily for 32 weeks, and the observations of tumours, etc., are recorded in the Tables for the period of 30 complete weeks of applications. The survival of the mice has been somewhat better than in previous years and the weekly weighing has demonstrated that their health has been maintained throughout without any period of epidemic infections. Two rabbits were lost through accidental injury.

The fractions have been warmed to 40°C for each application to liquify the waxy constituents. This mild treatment does not appear to have affected the properties or appearance.

Results.

(a) Mice. The mice as usual reacted with many tumours to the 9:10-dimethyl-1:2-benzanthracene (DBA) at both concentration levels. Few tumours are being obtained with the other fractions, K_4S_8A being the most potent at present.

(b) Rabbits. All sites have reacted somewhat earlier than in last year's tests of.

	1953-54	1952-53
D(2) at 30 weeks	sites affected 8/25	sites affected 1/25
K_44 at 30 weeks	5/25	1/25

However, many of the fractions prepared by Professor Morton show even greater potencies, and the results have therefore been examined in some detail in that they may help to guide planning for 1954-55 tests. None of the conclusions have as yet been checked for statistical significance by Dr. Irwin.

As these 30 weeks results are preliminary, one may consider the K_4S_1-8 -K_4S_1-8A series together. These show that the main tumour-producing compound (s) is concentrated in fractions 4-8 inclusive, the bulk (about 45%) being in fraction 6 and 7 (which make up together about one third of the total volume distilled).

Solvent extraction with acetone has resulted in a much greater concentration in the aromatic fractions in fractions 4 - 8 (K_4S_1A - 3A have only similar potencies to K_4S_{1-3}). The estimated amount present agrees closely with the proportion of aromatics obtained normally, the exceptions being K_4S_6A (about double the potency expected) and K_4S_7A (about half the potency expected). Otherwise there is no need to postulate very marked accelerator or inhibitor action interfering with the biological assay. The potency of the pooled paraffinic residue is low, but unfortunately not zero. In view of the large volume of the fractions made up by this residue (about 80%), it may be that appreciable <u>amounts</u> of carcinogen have been left in this residue.

The concentration of carcinogen in K_4S6A and K_4S8A leads to a potency of $2\frac{1}{2}$ x $1\frac{1}{2}$ x the potency of 0.2% 9:10-dimethyl-1:2-benzanthracene (D(2)) respectively. The potencies of K_4S_4A, K_4S_5A, and K_4S_7A are simllar to that of this standard. Plotting % sites bearing tumours as probits against log (time in weeks) the K_4S_6A graph runs parallel to the D(2) standard. This is interesting since most of the Kuwait fractions so far tested have shown a divergence of slope in this kind of plotting, making precise potency grading difficult.

Table 1

Fraction $K_4$4 — Mouse Experiment 1953-54 — (after 30 weeks)

Weeks	Survivors	Survivors with tumours	% Survivors with tumours	New Tumours	Total dead with tumours
20	38				
21	32				
22	31	1	3.2	1	
23	30	1	3.3		
24	28	2	7.1	1	
25	28	2	7.1		
26	27	2	7.4		
27	27	1	3.7		1
28	26	1	3.8		1
29	24	1	4.2		1
30	24	0	-		2

Fraction K_4S_1A

20	34				
21	32				
22	31	1	3.2	1	
23	30	1	3.3		
24	28	2	7.1	1	
25	28	2	7.1		
26	27	2	7.4		
27	27	1	3.7		1
28	26	1	3.8		1
29	24	1	4.2		1
30	24	0	-		2

Fraction K_4S_4A

5	45				
6	43				
7	43				
8	43				
9	43	1	2.3	1	
10	43	1	2.3		
11	43	1	2.3		
12	43	1	2.3		
13	43	1	2.3		
14	43	1	2.3		
15	43	2	4.6	1	
16	42	2	4.7		
17	42	2	4.7		
18	40	2	5.0		
19	40	2	5.0		
20	40	2	5.0		
21	40	2	5.0		
22	40	2	5.0		

C

Mouse experiment (Contd.)

Weeks	Survivors	Survivors with tumours	% Survivors with tumours	New Tumours	Total dead with tumours
Fraction K4S4A (Contd.)					
23	40	2	5.0		
24	40	2	5.0		
25	40	2	5.0		
26	40	2	5.0		
27	40	2	5.0		
28	40	2	5.0		
29	39	2	5.1		
30	38	3	7.9	1	
Fraction K4S5A					
20	42				
21	42				
22	42				
23	41				
24	39	1	2.5	1	
25	39	1	2.5		
26	38	1	2.6		
27	38	1	2.6		
28	38	1	2.6		
29	38	1	2.6		
30	38	1	2.6		
Fraction K4S6A					
18	42				
19	41				
20	41	1	2.4	1	
21	41	1	2.4		
22	40	1	2.5		
23	40	1	2.5		
24	39	1	2.56		
25	39	1	2.56		
26	38	1	2.63		
27	37	1	2.7		
28	26	1	2.77		
29	36	1	2.77		
30	36	1	2.77		
Fraction K4S7A					
20	40				
21	40				
22	40				
23	40	1	2.5	1	
24	40	1	2.5		

Mouse experiments (Contd.)

Weeks	Survivors	Survivors with tumours	% Survivors with tumours	New Tumours	Total dead with tumours
Fraction K_4S_7A (Contd.)					
25	40	1	2.5		
26	40	1	2.5		
27	38	1	2.63		
28	34	1	2.9		
29	30	0	-		1
30	27	0	-		1
Fraction K_4S8A					
20	37				
21	37				
22	37	1	2.7	1	
23	36	2	5.5	1	
24	35	3	8.6	1	
25	35	3	8.6		
26	33	3	9.9		
27	33	3	9.9		
28	31	5	16.1	2	
29	30	5	16.6		
30	30	8	26.6	3	
Standard D(2)					
5	48				
6	43				
7	41				
8	41	1	2.4	1	
9	41	4	9.76	3	
10	40	13	30.8	9	
11	40	17	42.5	4	
12	40	23	57.5	6	
13	40	26	65.0	3	
14	40	26	65.0		
15	40	26	65.0		
16	40	26	65.0		
17	40	26	65.0		
18	40	39	97.5	13	
19	38	37	97.4		2
20	28	28	100.0	1	12
21	27	27	100.0		13
22	0	0	-		40
Standard D($\frac{1}{2}$)					
6	48				
7	48				
8	48				
9	48	1	2.1	1	

Mouse experiment (Contd.)

Weeks	Survivors	Survivors with tumours	% Survivors with tumours	New tumours	Total dead with tumours
Standard D(½) (Contd.)					
10	48	4	8.3	3	
11	48	7	14.6	3	
12	48	11	22.9	4	
13	48	19	39.6	8	
14	48	19	39.6		
15	48	20	40.7	1	
16	48	20	40.7		
17	48	23	47.9	3	
18	47	31	65.9	8	
19	47	31	65.9		
20	40	28	70.0	3	6
21	40	28	70.0	1	7
22	39	33	84.6	5	7
23	39	33	84.6		7
24	36	30	83.3		10
25	24	18	75.0		22
26	24	18	75.0		22
27	24	22	91.7	4	22
28	24	22	91.7		22
29	24	22	91.7		22
30	23	22	95.6		22
31	5	3	60.0		43

Table 2 — Rabbit Experiment 1953-54 — (after 30 weeks)

Site and Time of Appearance of Tumours

K4S1A		K4S2A		K4S3A		K4S4A		K4S5A		K4S6A		K4S7A		K4S8A	
Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site
20	II	18	III	27	VII	13	III	13	II	11	VII	10	VII	11	III
20	IV	25	VII	1 tumour		13	IV	13	III	12	IV	11	VII	11	IV
22	I	27	I			17	II	13	VII	13	III	17	III	11	IV
30	III	30	III			20	VII	18	I	17	IV	17	IV	11	VII
4 tumours		4 tumours				20	VII	18	II	17	IV	17	IV	11	VII
						22	I	20	I	18	II	18	III	13	III
						25	VII	20	III	18	II	20	I	17	II
						28	I	22	III	20	I	25	IV	18	III
						8 tumours		22	IV	20	I	27	I	20	III
								27	I	20	III	30	III	22	VII
								29	I	20	VII	10 tumours		27	VII
								11 tumours		22	II			28	I
										22	IV			28	I
										22	VII			13 tumours	
										27	II				
										15 tumours					

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Table 2 (Contd.)

Rabbit Experiment 1953-54 (after 30 weeks)

Site and Time of Appearance of Tumours

K_4S1		K_4S2		K_4S3		K_4S4		K_4S5		K_4S6		K_4S7		K_4S8	
Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site
25	1	18	1	18	II	11	I	27	III	18	III	14	II	10	IV
1 tumour		20	VII	25	III	13	VII	30	I	20	VII	17	III	11	II
		22	I	2 tumours		25	IV	2 tumours		22	II	18	VII	30	VII
		22	II			3 tumours				25	VII	18	VII	3 tumours	
		4 tumours								4 tumours		4 tumours			

D(2)		$D(\frac{1}{2})$		K44		$K44(\frac{1}{4})$		K_4SP	
Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site	Weeks	Site
17	I	22	VII	10	II	-	-	20	VII
17	II	27	VII	13	VII	0 tumours		25	II
18	II	30	II	18	I			2 tumours	
18	VII	3 tumours		27	III				
22	I			27	III				
22	II			5 tumours					
25	IV								
27	VII								
8 tumours									

Table 3 Approximate concentrations and amounts of carcinogen in oil fractions (expressed as 9:10-dimethyl-1:2-benzanthracene equivalents) deduced from Dr. Woodhouse's 30 week results of rabbit tests 1953-54.

		STILL FRACTIONS			SOLVENT EXTRACTS			
I Fraction	II Boiling range °C	III Carcinogen concn. g/l.	IV Volume l.	V Carcinogen amount. g.	VI Fraction	VII Carcinogen concn. g/l.	VIII Volume l.	IX Carcinogen amount. g.
K_4S_1	350-355	0.3	8.7	2.6	K_4S_1A	0.6	2.8	1.7
K_4S_2	355-360	0.6	12.2	7.3	K_4S_2A	0.6	2.3	1.4
K_4S_3	360-365	0.3	16.4	4.9	K_4S_3A	0.3	3.1	0.9
K_4S_4	365-370	0.5	15.3	7.6	K_4S_4A	2.0	3.3	6.3
K_4S_5	370-375	0.3	23.0	6.9	K_4S_5A	2.0	4.6	9.2
K_4S_6	375-380	0.6	18.1	10.9	K_4S_6A	5.0	3.4	17.0
K_4S_7	380-385	0.6	27.6	16.6	K_4S_7A	2.0	4.4	8.8
K_4S_8	385-390	0.5	19.4	9.7	K_4S_8A	3.0	3.3	9.9
Total			140.7	66.3			27.2	55.2

Notes (a) If assay were perfect and all the carcinogen were transferred to solvent extracts, columns V and IX should be identical. In fact, the correlation coefficient between them r= 0.61, and since n-7, the probable significance of this is 0.10-0.05.

(b) Recovery by totalling solvent extracts (55.2 g.) is 82%. Slight carcinogenicity was found in pooled paraffin residue (about 0.3 g./l. in 113.5l = 34 g.). If this is added to the calculation recovery is well over 100%. In general, the results in fractions with low potencies should be treated with reserve until the tests are complete.

file: Medical Advisory Committee - UNIV. OF CINCINNATI Expenditures

June 23, 1954

Dr. Robert A. Kehoe
University of Cincinnati
The Kettering Laboratory
College of Medicine
Cincinnati 19, Ohio

Dear Doctor Kehoe:

Pursuant to understanding reached by our letters of October 28 and November 5, 1947, we are enclosing the Institute's check in the amount of $2,500 payable to the University of Cincinnati as our contribution toward the development of information on fluorine and fluorine compounds for the twelve-month period ending June 30, 1955.

Very truly yours,

DVJ:js
Enclosure
cc: George M. Saunders, M. D.
Lacey Walker

MEMO TO MR. WALKER:

This expenditure is to be charged against the Medical Research Fund.

API 05870

File: Medical Advisory Committee - UNIV. OF CINCINNATI Expenditures

June 23, 1954

Dr. Robert A. Kehoe
University of Cincinnati
The Kettering Laboratory
College of Medicine
Cincinnati 19, Ohio

Dear Doctor Kehoe:

Pursuant to the action taken at the Medical Advisory Committee meeting on September 30, 1953, we are enclosing the Institute's check in the amount of $5,000 payable to the order of the University of Cincinnati as our contribution toward the skin physiology project for the twelve-month period ending June 30, 1955.

Very truly yours,

DVS:js
Enclosure
cc: George M. Saunders, M. D.
Lacey Walker

MEMO TO MR. WALKER:

This expenditure is to be charged against the Medical Research Fund.

API 05871

UNIVERSITY OF CINCINNATI
DEPARTMENT OF PREVENTIVE MEDICINE AND INDUSTRIAL HEALTH

THE KETTERING LABORATORY
COLLEGE OF MEDICINE—EDEN AVENUE
CINCINNATI 19, OHIO

CABLE ADDRESS: KETLAB, CINCINNATI
TELEPHONE: CAPITOL 1414

June 26, 1954

Mr. D. V. Stroop, Director
Department of Technical Services
American Petroleum Institute
50 West 50th Street
New York City 20

Dear Mr. Stroop:

I herewith acknowledge with thanks receipt of the following checks from American Petroleum Institute:

$5,000.00 - in support of the investigation of the physiology of the skin for the year ending June 30, 1955;

$2,500.00 - in support of the investigation of fluorides for the year ending June 30, 1955.

Very truly yours,

E. R. Fortlage

E. R. Fortlage, Secretary to
Dr. Kehoe

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API 05872