FILE NAME: Oil Industry and American Petroleum Institute (API)

DATE: 1957 Apr 10

DOC#: API133

DOCUMENT DESCRIPTION: Preparation of the Interim Report for the API Research Project MC-1

PREPARATION OF THE INTERIM REPORT FOR THE API RESEARCH PROJECT MC-1

APRIL 10,1957

Preliminary Comment

This status report is being prepared so as to include details reserved hitherto for the annual report presented in September after the close of the fiscal year. In order to assemble the large mass of experimental data in a useful document, the form normally followed in scientific journals is being used.

An introductory statement of the background and broad objectives of the investigation is followed by a description of the pursuit of an experimental program toward the specific goal of the analytical tool required for a rapid estimation of the hazard of skin cancer associated with the handling of any type of hydrocarbon oil. The introduction is concluded with a very brief summary of the main contributions made thus far to the achievement of the objectives as set forth, and to the portrayal of the general picture of the relationship between composition and carcinogenic potency as it is understood at this time. The experimental methods employed in the current investigation and the results of their application to a variety of petroleum oils are discussed in detail in a series of appendices.

The presentation of the proposed program for 1957-58 is divided into a statement of the general program for the further development of correlations between the composition and potency

of these oils and an outline of the specific areas to be attacked and the experimental techniques to be used.

It is hoped that this method of formulating the report will serve the dual needs of the American Petroleum Institute for a reasonably concise statement of the progress towards the objectives of the investigation, on the one hand, and a detailed technical discussion of the experimental methods and results, on the other.

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API RESEARCH PROJECT MC-1

Introduction

Repeated contact with certain petroleum oils is known to have produced cancer of the skin of man under certain occupational conditions. Investigators in various countries have gathered evidence of the existence in these oils of certain types of aromatic hydrocarbons of complex structure, which are capable of inducing cancer of the skin of mice when applied thereon under suitable experimental conditions. Some years ago, further experimental evidence from a number of sources indicated, or at least suggested, that certain products of modern refining processes (e.g. catalytic cracking) in the petroleum industry were capable of inducing experimental cancer of the skin of mice with considerable speed and uniformity. This experimental fact, while not alarming in itself, and while lacking any confirmation of its practical significance in the terms of experience in the petroleum industry, came to be regarded, among responsible people in the industry, as the justification for a fairly comprehensive investigation of a wide range of products and processes, from the aspect of the potential hazard of such cancer inducing substances as might be found therein. Such an investigation was initiated in 1946 in The Kettering Laboratory, in the Department of Preventive Medicine and Industrial Health of the University of Cincinnati, under the financial sponsorship of the American Petroleum Institute.

The objectives of this investigation, as they were established initially, were three in number:

1. The first of these was that of determining, on the basis of the experimental response of the skin of mice to applications of specific and well identified petroleum products, those which possessed carcinogenic capacity. It was hoped, of course, that such capacity could be estimated in quantitative or, at least, comparative terms.

2. The second objective was that of developing an analytical method or tool, or possibly a series of such tools, whereby the carcinogenic capacity of various oils could be detected, and whereby ultimately, if possible, carcinogenic substances could be classified, identified, and assessed quantitatively.

3. A third and crucial objective was that of ascertaining the extent of the correlation which might exist between the experimental induction of cancer in the skin of mice and the capacity of petroleum oils to induce human cancer of the skin under occupational or other environmental conditions which might be found to have arisen historically or currently in industry or elsewhere.

The first two of these objectives were to be pursued by the application, adaptation and refinement of biological and technological methods, while the third required the gathering of the available information from the literature and from contemporary investigators, as well as from the past and current experience of the petroleum industry. The approach to the experience of the industry contemplated an epidemiological investigation of extensive scope which will be the subject of a later report.

In the background of the specific objectives which determined the pattern of the experimental work, were two general purposes, (1) that of pin-pointing the foci and severity of the hazard of skin cancer, if such hazard existed in fact, rather than as a potentiality, within the industry, on the one hand; and (2) that of revealing general or specific techniques for controlling such hazard as might be found, whether by the methods of technology or by those of industrial hygiene and preventive medicine. As the work in the Laboratory progressed, these purposes underwent some further definition. Thus, out of the biological side of the work came indubitable evidence of the existence and the nature of factors which influenced significantly the ability of specific

petroleum oils to induce cancer in the skin of mice. Moreover out of the technological work came correlations between the carcinogenic potencies of oils (for the induction of cancers of the skin of mice) and certain physical and chemical characteristics of these oils that led to the identification of certain of their components as being actually carcinogenic or capable of promoting or inhibiting carcinogenic activity. It is evident that such experimental results, which will be described in suitable detail later, may provide strategic means for the control or perhaps the elimination of certain occupational hazards, to the extent that the latter are comparable in type or degree to the conditions imposed experimentally upon the mouse. Thereby some basis for the selection of process or product may be afforded to the petroleum technologist, in accordance with his needs, purposes and judgments or, alternatively, for the application of safeguards by the industrial hygienist.

It is of some significance to recognize that a considerable body of information, gathered by experience and experiment over the years, has revealed strong correlations between human experience with the cancer-inducing properties of coal tar, shale oil, and crude paraffinic distillates, and the experimental results of the application of these materials upon the skin of mice. It is necessary to maintain a carefully guarded critique against too literal an acceptance of the view that the human lesions of this type that have occurred can be reproduced in the mouse under precisely parallel conditions, or vice versa. Nevertheless, the use of these animals in following the objectives of this and other comparable investigations has been justified fully, and it is fair to say that the correlations between human experience and animal experiments in this field have been strengthened by improvements in the design and technique of experiments. Thus one tends to assume, with some reservation of judgment, that the more rapidly an oil will produce cancer of the skin of mice, the more active it will be upon the skin of men.

On the foregoing background, the status of the investigative program may be sketched briefly in the following manner:

The rate at which tumors of the skin of the mouse can be induced by a given oil depends basically upon the concentration of certain active carcinogens which are polycyclic aromatic hydrocarbons. The basic rate may be increased as much as five-fold by the influence of the other components of the oil even though the latter are completely non-carcinogenic when freed of the polycyclic aromatic fraction. This catalytic activity of the medium is contributed in part by alkyl derivatives of the lower aromatics (naphthalenes and benzenes) as well as by saturated compounds, such as the normal paraffins. On the other hand, the effect of these "accelerators" may be counteracted almost completely by other saturated hydrocarbons (the polycycloparaffins) which in some manner inhibit the catalysis and limit the potency of the oil to a level little higher than that due to the carcinogens.

A few years ago research in the area of saturated hydrocarbons of high molecular weight was extremely difficult. However, the development of high mass spectrometry during the past four years has made it possible to obtain useful information on the composition of saturated fractions of refinery streams. This technique of analysis has also played an important role, together with absorption spectrophotometry, in the concurrent investigation of the aromatic carcinogens.[1]

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1. The research departments of a number of the major oil companies furnished the mass spectra required in this project.

PROPOSED PROGRAM FOR 1957-58

In approaching the goal of an analytical tool for prediction of the rate at which an oil will induce tumors of the skin, two parallel and closely related paths are being followed. The first is the investigation of the identity of the polycyclic carcinogens, which must be present if the oil is to have any cancer-inducing activity whatsoever. The second path is that leading to an understanding in quantitative terms of the relationships between the composition of the medium in which the aromatic carcinogens are dissolved and the extent to which it is capable of effecting an acceleration of the rate of the development of tumors.

The three major classes of carcinogens may be described as the 5-ringed, the 4- (and 4 1/2) ringed, and the 3 1/2-ringed compounds. The development of an analytical method for the 5-ringed benzopyrenes completed the work on the first class. Considerable progress has been made during the past year towards the characterization of the 3 1/2-ringed class (those polycyclics based upon a nucleus having 16 or 17 carbon atoms). Two additional cycles of biological tests (about 9 months for each) are estimated to be sufficient to complete the identification of this class. The attention that is being devoted to the 3 1/2-ringed group of carcinogens is justified by the evidence that almost all petroleum oils that are carcinogenic contain significant quantities of this group.

A major part of the 4- and 4 1/2-ringed carcinogens may be extracted from an oil with maleic anhydride. Such extracts from a variety of oils are being tested to determine what part of the basic potency they contribute.

During the coming year the experimental work on the second important aspect of this problem (the catalytic activity of the medium) will be concerned with the development of quantitative data that define the influence of the polycycloparaffins which seems to interfere with catalytic activity. A technical white oil that was shown to have this "inhibitory" property has been fractionated to concentrate the suspected materials. These will be tested in synthetic blends

with a long-chain accelerator and a standard carcinogen. Mass spectrometric analysis will be applied in parallel to a variety of fractions already tested for their catalytic activity, in an effort to quantitate these relationships.

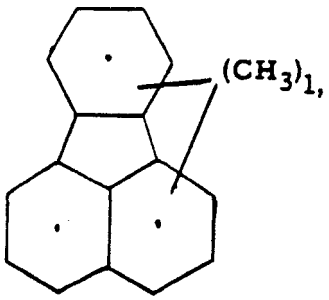
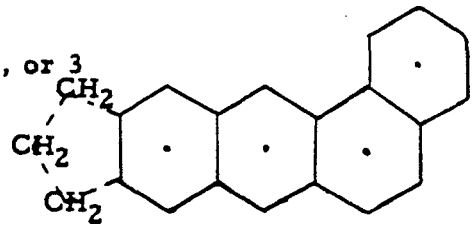
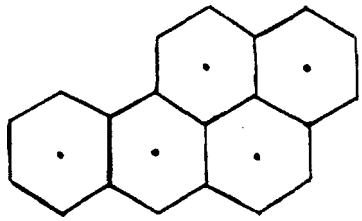
APPENDICES

APPENDIX 1

EXPERIMENTAL METHODS USED TO DETERMINE THE RELATIVE CONTRIBUTIONS MADE BY THE CARCINOGENS AND THE MEDIUM TO THE POTENCY OF A COMPLEX OIL

A. Total Carcinogens

The term carcinogen is applied to specific types of polycyclic aromatic components of petroleum oils known or suspected to have cancer-inducing properties. The following structures indicate important types in cracked oils:

3 1/2-ringed	4 and 4 1/2-ringed	5-ringed
		
Methylfluoranthenes	Benz(a)anthracenes	Benzo(a)pyrene
suspected	known	known

Such compounds are easily separated from more saturated components having less than 3 rings per molecule by chromatography on alumina (Alcoa F-20). The polycyclic fraction is then blended into a non-accelerating medium (benzene, for fastest results) at a selected level of concentration. The rate at which the blend will induce papillomas of the skin of C3H mice is determined in the usual manner.

Since the rate at which a polycyclic carcinogen will induce tumors is directly related to the dosage per unit area of the skin (see Figure 1), the difference in the area covered by equal quantities of the blend in benzene and the original oil must be taken into account. (The method used to measure the relative tendencies of different materials to spread upon the skin of C3H mice is described in section B of this appendix.) The spreading tendencies of the oils which have been fractionated in the manner described above have not been measured as yet, but reasonable estimates of the relative tendencies may be made for immediate purposes.

The rate at which the original oil would have induced papillomas if the medium had had no catalytic influence is estimated by correcting the rate at which the blend of the polycyclics in benzene acted upon the skin. The estimated rate (symbolized by e_c) is slower than the rate for the blend in benzene because the blend usually used has a higher concentration of the carcinogens than the original oil. Moreover, the blend in benzene yields a larger dose of the carcinogen per unit area of the skin because it spreads to a lesser extent than the original oil.

To obtain e_c reference is made to the dose-response curves for the standard carcinogens, benzopyrene and methylcholanthrene, in relatively non-volatile, non-accelerating solvents, such as white mineral oil (340 S. S. U. at 100°), decahydronaphthalene, or sec.-amylbenzene (Figure 1). It is assumed for the purpose of this estimate that the corresponding curves for the carcinogens of the oils have slopes intermediate between those of methylcholanthrene and benzopyrene.

The curve for such a hypothetical reference carcinogen is shown on Table 1.

The following example should illustrate the use of the relationships discussed above in arriving at the value of e_c:

The polycyclic aromatic fraction of oil API-89 is obtained in 19 percent yield by a chromatographic fractionation. A blend containing 40 percent of this fraction and 60 percent of benzene (by weight) is applied repeatedly upon the skin of mice (three 20 mg. applications each week). The average duration of tumor-free life proves to be 27.1 weeks. From the relationships in Figure 1 it is noted that this rate of response corresponds to that obtained with approximately 0.54 mg. of the reference carcinogen (applied per unit area of the skin each week, the unit area being defined as that covered by 100 mg. of the reference solutions having a relative spread equal to one).

The original oil has only one-half as much carcinogen as the blend. Further, it is found that its tendency to spread over the skin is approximately 1.6 times that of the blend. Therefore the dose, d_o, of carcinogen per unit area when the original oil is applied is equivalent to $\frac{0.54}{2 \times 1.6}$, or 0.16 mg. of the reference carcinogens per week. This rate of application of this dosage would result in the induction of papillomas in approximately 43 weeks (from Figure 1), which is the value of the estimated effect, e_c, of oil API-89 if it has no catalytic influence on the activity of its polycyclic carcinogens.

The total content of carcinogens C_{rc} (expressed as the equivalent percentage of the reference carcinogen) also may be derived from the value of d_o, using the following relationship:

$$d_o = \frac{f\, C_{rc}}{s_o} \qquad (1)$$

where f is the number of applications per week (three) and s_o the relative spread of the oil. In the case of oil API-89, s_o is estimated to be 1.3; therefore, the content of carcinogens,

$$C_{rc} = \frac{0.16 \cdot 1.3}{3},$$

is equivalent to 0.07 percent of the reference carcinogen.

Alternatively C_{rc} and e_c may be calculated directly from the equation for the dose-response curve of the reference carcinogen,

$$d = \frac{10}{e - 13} - 0.17 . \qquad (2)$$

As above, $$d_o = d_b \cdot \frac{s_b}{s_o} \cdot \frac{c_o}{c_b}, \qquad (3)$$

where d_b is the rate of dosage of carcinogen obtained when the blend of the polycyclics in benzene was applied upon the skin (0.54 mg. per unit area per week in the case of oil API-89) resulting in an average duration of tumor-free life, e_b; s_b and s_o are the estimated relative spread of the blend and the original oil, respectively; and c_b and c_o are the concentration of the polycyclic fraction in the blend and the original oil, respectively.

Combining equations (1) and (3),

$$f\, C_{rc} = d_b \cdot s_b \cdot \frac{c_o}{c_b},$$

and substituting the expression for $\underline{d}$ of equation (2) for $\underline{d}_b$, the resulting equation for the calculation of C_{rc} becomes:

$$C_{rc} = \frac{\underline{s}_b \underline{c}_o}{\underline{fc}_b} \left(\frac{10}{\underline{e}_b - 13} - 0.17 \right) \qquad (4)$$

Since $\underline{e}_c$ is the average duration of tumor-free life resulting from a rate of dosage, $\underline{d}_o$, these factors are also related by equation (2). Therefore, an expression for $\underline{e}_c$ may be derived by the combination of equations (1) and (2), as follows:

$$\underline{e}_c = \frac{10}{\frac{\underline{fC}_{rc}}{\underline{s}_o} + 0.17} + 13, \qquad (5)$$

the value of C_{rc} being obtained from the calculation of equation (4).

In the case of the oil, API-89,

$$C_{rc} = \frac{0.8 \cdot 19}{3 \cdot 40} \left(\frac{10}{27.1 - 13} - 0.17 \right) = 0.07$$

and $$\underline{e}_c = \frac{10}{\frac{3 \cdot .07}{1.3} + 0.17} + 13 = 43.$$

B. Measurement of the Relative Spreading Tendencies of Various Materials

A direct measurement of this factor is being made in selected cases by the following procedure:

A measured quantity of the material (2-10μl.) is applied upon the skin of each of 20 mice (during the resting phase of the hair cycle), which have been anesthetized with carbon dioxide to immobilize them. After intervals of 30 and 60 minutes, the animals are again anesthetized. Each is then placed under a glass slide and the apparent area of distribution of the polycyclic hydrocarbon is traced on the slide by its fluorescence under an ultraviolet lamp. Several tracings of each spot are made, measured with a planimeter, and the average area calculated. From the average for the group of mice used in a given test and the quantity of solution applied to each, the average area of skin covered per μl. of solution is calculated.

The absolute values obtained vary from one operator to another, depending in part upon the experience in observing the fluorescence of the aromatic hydrocarbon. Hence, an instrumental pick-up of this radiation would be necessary to obtain significant absolute values. However, it is the relative spreading of the different materials that is the important consideration in this application. By use of standard reference solutions, the relative values obtained by two experienced operators are in good agreement.

C. Individual Classes of Carcinogens

It has proved to be useful to consider the basic (non-accelerated) carcinogenic potency of an oil as a summation of the concentrations of three individual classes of carcinogens (those indicated in section A of this appendix) when each concentration is expressed in equivalent weights of a hypothetical reference carcinogen having a dose-response curve intermediate between that of methylcholanthrene and benzopyrene.

The content of the five-ringed benzopyrenes is determined by an analytical procedure developed in an earlier period of this investigation[1]. The equivalent value of the reference carcinogen is obtained from the dose-response curves in Figure 1 (the value is essentially equal to the content of benzopyrenes unless the latter exceeds 0.10%; for higher values, the rate of dosage per unit area must be taken into account).

The available evidence indicates that most of the 4- (and 4 1/2-) ringed carcinogens are derivatives of benz(a)anthracene and may be extracted from an oil by formation of an adduct with maleic anhydride. The carcinogens may be concentrated further by chromatographic fractionation, which eliminates most of the 3-ringed non-carcinogens (determined by absorption and mass spectra). The yield of the

1. Determination of Benzo(a)pyrene in Complex Mixtures, Tye R., Graf, M.J., and Horton, A.W., Anal. Chem., 27, 248 (1955).

4'-ring compounds is noted and the fraction is then blended with benzene at a definite level of concentration. The rate at which the blend will induce papillomas in the skin of C3H mice is then determined and expressed in equivalents of the reference carcinogen. In order to arrive at the contribution of this class of carcinogens to the potency of the original oil, this value must be corrected in the manner described previously with reference to the total carcinogens (Section A, page 2).

In attacking the classification of the 4- and 5-ringed carcinogens it has been possible to proceed with a considerable background of published knowledge on the carcinogenic potencies of pure compounds. In contrast, only a very few of the methyl- or dimethyl- derivatives of the 3 1/2-ringed class have been tested for their potency, and none of these were reported to be active. Since it is apparent that a significant part, and in some cases a major part, of the potency of petroleum oils is due to some compounds in this general class it is essential that these carcinogens be identified to permit the development of adequate analytical methods. Therefore a systematic approach to the problem is being made. By fractional distillation, chromatography, and extraction with cold, concentrated sulfuric acid, the various 3 1/2-ringed classes, fluoranthenes, cyclopentanophenanthrenes, pyrenes, and benzofluorenes, have been separated and are being tested to determine their relative contributions to the potency of the fraction distilling from 670-760°F. of the catalytically cracked oil, API-8.

D. The Catalytic Influence of the Non-carcinogenic Medium

By the "medium" is meant the combination of all of the non-carcinogenic components of an oil. The influence of the medium is determined by comparing e_c, the rate of tumor induction expected on the basis of the carcinogens present if the medium had no catalytic activity (as determined by the method discussed in section A of this appendix), with the observed rate of induction of tumors by the oil. Thus the relative catalytic activity, A, is expressed simply as the ratio between the expected and observed duration of tumor-free life:

$$A = \frac{e_c}{e_{obs.}} . \qquad (6)$$

Thus, in the example at the end of section A of this appendix the expected duration of tumor-free life for oil API-89 was 43 weeks if there was no catalytic contribution to its activity. Actually the original oil induced tumors quite rapidly ($e_{obs.} = 12.3$ weeks). The relative catalytic activity of the medium of this oil is obtained from the calculation:

$$A = \frac{43}{12.3} = 3.5 .$$

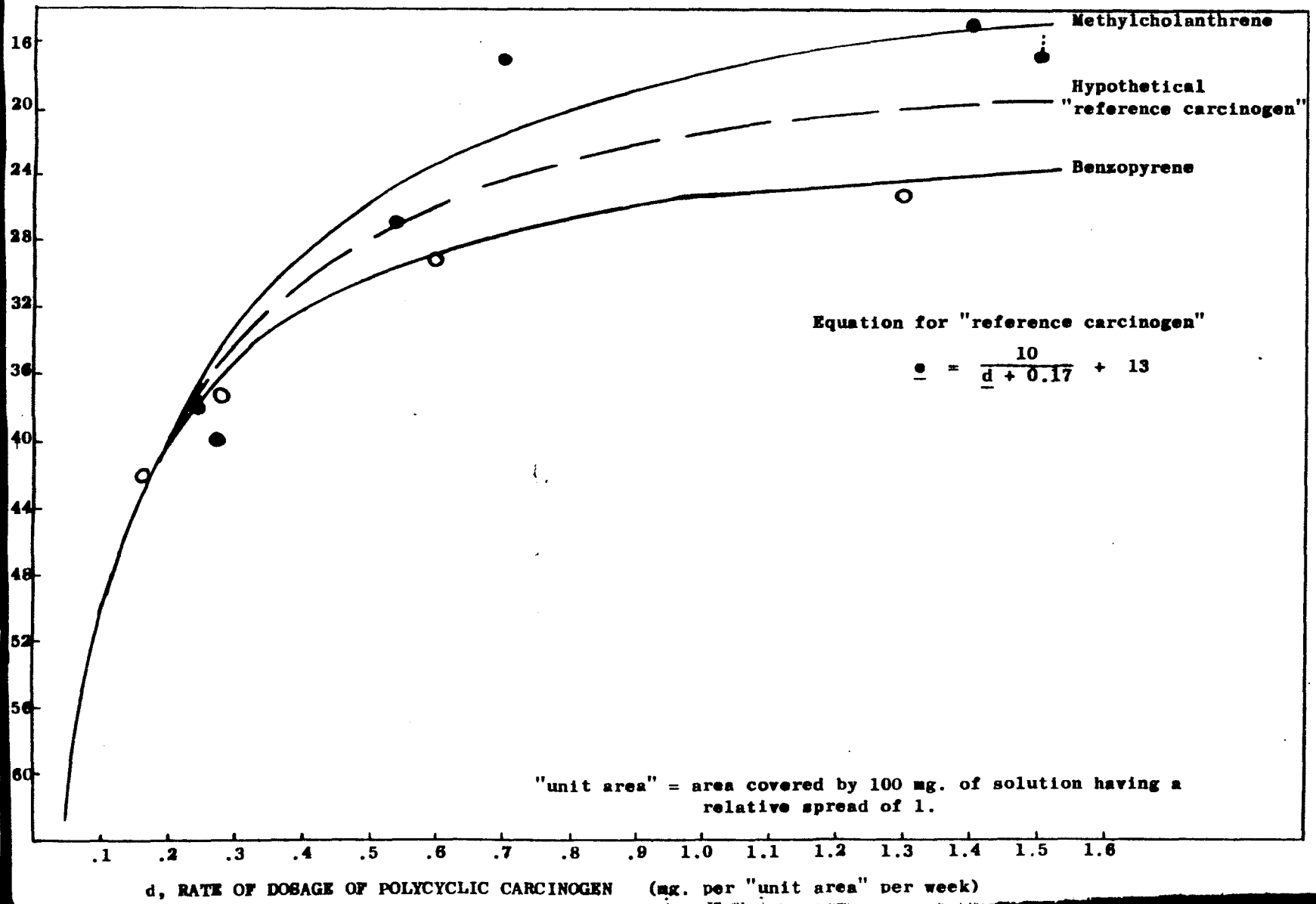
Solutions of 0.05 percent methylcholanthrene in the non-carcinogenic fraction separated chromatographically from the polycyclic aromatics are being applied to mice. From these tests is determined the extent to which this fraction, in the absence of certain more strongly absorbed non-carcinogens, is capable of accelerating the rate of carcinogenesis.

E. Materials

The methods discussed in the previous sections have been applied to a spectrum of the oils which were tested for their effective carcinogenic potency in an earlier period of this investigation. Samples have been included from Straight Run Distillation, Thermal Cracking, Catalytic Cracking, and Delayed Coking. An effort has been made to cover a sufficient variety of oils to be sure of the general applicability of analytical methods for estimating carcinogenic potency developed from this investigation.

FIGURE 1

THE RATE OF INDUCTION OF PAPILLOMAS IN C3H MICE BY THE REPEATED APPLICATION OF 3-METHYLCHOLANTHRENE AND BENZO(a)PYRENE IN NON-ACCELERATING SOLVENTS



APPENDIX 2

RESULTS

A comparison of oils from various refining operations according to the relative rates at which they induced tumors of the skin of C3H and CFW mice is shown in Table 1 (the measurements involved repeated applications without washing). The rates are expressed as their reciprocals, i.e., the fractions of the average life span of the mice (~85 weeks) required to induce papillomas.

TABLE 1

NUMBER OF OILS	REFINERY OPERATION	PRODUCT FROM FRACTIONATOR	AVERAGE DURATION OF TUMOR-FREE LIFE, $\bar{e}_{obs.}$ (weeks)	FRACTION OF LIFE SPAN OF MICE TO INDUCE TUMORS[1] ($\bar{e}_{obs.}/85$)
11	Straight Run Distillation	Lube Distillate	15-81	0.2 - 1.0
2	" " "	Gasoline	(>85)	>1.0
2	" " "	Residuum	70-(>85)	0.9 - 1.0
7	Solvent Extraction	Extract	11-78	0.13- 0.9
2	Lubricant Manufacture	Finished Lubricating oil	31-(>85)	0.4 - 1.0
3	White Oil Manufacture	White Mineral Oil	(>85)	>1.0
6	Thermal Cracking of Virgin Gas Oil	Residuum	15-53	0.2 - 0.6
6	Thermal Reforming	Residuum	22-27	0.26-0.32
12	Thermal Cracking of Catalytic Gas Oil	Residuum	9-34	0.1 - 0.4
10	Coking	Cycle Gas Oil	10-30	0.1 - 0.35
11	Catalytic Cracking	High Boiling Sidestream	10-43	0.1 - 0.5
27	" "	Bottoms	5-36	0.06- 0.4

1. A value of 1.0 indicates that a full life span was required.

The polycyclic aromatic fractions were separated from a number of these oils. In Table 2 are shown the estimated rates, e_c, at which the carcinogens in these fractions would induce tumors if applied upon the skin of mice in a non-accelerating solvent (at a concentration chosen so that the dosage applied per unit area per week was the same as that obtained when the original oil was applied). The catalytic activity of the total medium, A, is calculated as the ratio $\frac{e_c}{e_{obs.}}$ (a value of A = 2 indicates that the rate was doubled). In addition, the catalytic activity, a, of the fraction of the oil separated from the polycyclics was determined.

TABLE 2

API SAMPLE NUMBER	DESCRIPTION OF ORIGINAL OIL	OBSERVED DURATION OF TUMOR-FREE LIFE FOR TOTAL OIL,[1] $e_{obs.}$ (weeks)	ESTIMATED DURATION DUE TO CARCINOGENS ALONE,[1] e_c (weeks)	RELATIVE CATALYTIC ACTIVITY OF TOTAL MEDIUM, A	RELATIVE CATALYTIC ACTIVITY OF PART OF THE MEDIUM,[2] a
89	FCC Heavy Gas Oil	12.3±1.3	43	3.5	2.3
33	Coker Gas Oil	10.2±1.0	36	3.5	2.4
120	Lube Stock (naphthenic)	33.2±3.4	~44[3]	~1.3	<1.4
115	"	≦56X			~1.5
105	Lube Stock (paraffinic)	23.2±4.4			2.0
65	Thermal Tar	≦21X	26	≧1.2	~2.0

1. For tests involving three 50 or 100 mg. applications each week.
2. That part containing less than 3 rings per molecule (tested as the solvent in a solution of 0.05% methylcholanthrene).
3. The experiments from which the values preceded by ~ are derived are not completed.

The values derived for e_c, A, and a in Table 2 should be regarded as having relative, but not absolute significance since the correction

factors which must be applied for the spreading characteristics of the different materials were estimates. Measurements of these spreading characteristics are being made in the current program.

Information that has been obtained (by mass spectrometric analysis) on the composition of certain non-carcinogenic chromatographic fractions of the oils is shown in Table 3. Data are also included on the composition of the technical white oil API-80 (95 SSU at 100°), which previously has been shown to contain accelerating fractions, on the one hand, and components capable of counteracting acceleration, on the other.

TABLE 3

PERCENT OF CLASS IN NON-CARCINOGENIC FRACTIONS OF

	API-89			API-120			API-65			API-80
CLASS OF HYDROCARBONS	1[1]	1 2[1]	1 + 2 + 3 (estimated)[2]	1[1]	2[1]	1 + 2 + 3 (estimated)[2]	1[1]	2[1]	1 + 2 + 3 (estimated)[2]	
Paraffins (n- & iso-)	53	5	38	4	0	3	42	21	14	12
Alkylcyclopentanes	22	( )[3]	18	34	( )[3]	24	34	( )[3]	15	28
Alkylcyclohexanes	8	29	8	15	0	10		11		23
Condensed Cycloparaffins	15	( )[3]	14	43	(8)	35	20	(21)	20	33
Alkylbenzenes	2	42	5	4	84	25	3	32	15	3
Alkylnaphthalenes	-	23	12	-	8	3	1	15	25	1
3-ringed Aromatics	-	-	5	-	-	-	-	-	11	-
Percent of fraction in original oil	61	8	81	45	22	67	2.5	27	44	(100)

1. Fraction 1, most easily eluted; fraction 2, next most easily eluted.
2. Tested as a solvent for 0.05% methylcholanthrene.
3. Values for cyclopentanes and condensed cycloparaffins are uncertain in the fractions indicated due to interference from the aromatics.

As indicated by its analysis the thermal tar API-65 has a composition that is somewhat unique. In chromatography very little of the oil could

TABLE 4 - THE CATALYTIC ACTIVITY OF SOLUTIONS OF REFERENCE CARCINOGENS

API EXPERIMENT NUMBER	SOLVENT	CONCENTRATION OF CARCINOGEN % MC OR BP[1]	SCHEDULE OF APPLICATION OF SOLUTION: Applications Per Week	SCHEDULE OF APPLICATION OF SOLUTION: mg. Per Application	RELATIVE SPREAD OF SOLUTION	$e_{obs.}$ (weeks)	e_c (weeks)	A
204a	Benzene	0.17 MC	3	100	0.5	15.7±1.6	18	1.1
222	sec-Amylbenzene	0.18 MC	3	100	1.0	26.7±2.7	25	0.9
249a	t-Dodecyl benzene[2]	0.17 MC	3	100	1.4	8.8±0.9	30	3.4
249b	"	0.17 MC	3	20	1.4	9.9±0.9	30	3.0
296	White Oil II (340SSU/100°)	0.20 BP	3	50	1.0	28.0±3.4	29	1.0
299	"	0.08 BP	3	5	1.0	37.3±2.0	38	1.0
602	n-Octane	0.21 BP	3	100	0.8 est.	27.1±1.8	27	1.0
278	n-Decane	0.21 BP	3	50	1.4 est.	16.6±1.2	31	1.9
295	n-Dodecane	0.20 BP	3	50	1.6	14.6±1.1	33	2.3
666	"	0.08 BP	2	50	1.6	20.4±2.2	50	2.5
665	"	0.04 BP	2	50	1.6	23.7±1.8	60	2.5
661	20% Dodecane 80% Benzene	0.08 BP	3	100	0.8 est.	24.0±1.5	35	1.5
659	50% Dodecane 50% Decalin	0.08 BP	2	50	1.3 est.	20.0±2.6	48	2.4
660	50% Dodecane 50% b-methyl-naphthalene	0.08 BP	2	50	1.3 est.	19.1±3.1	48	2.5
297	50% Dodecane 50% White Oil II	0.20 BP	3	50	1.3 est.	20.6±2.2	31	1.5
614	75% Dodecane 25% White Oil II	0.20 BP	3	50	1.4 est.	20.4±1.3	31.5	1.5
258	API-80, White Oil I (95SSU/100°)	0.23 BP	1	50	1.3	35.1±1.9	42	1.2

1. Methylcholanthrene (MC) or benzopyrene (BP).
2. Product of alkylation of benzene with the tetramer of propylene.

be removed from alumina before compounds containing one or two aromatic rings appeared.

In Table 4 is shown the catalytic activity of various solutions of reference carcinogens. This data will facilitate the interpretation of the catalytic activity of the complex oils in Table 2 and 3 in terms of their composition.

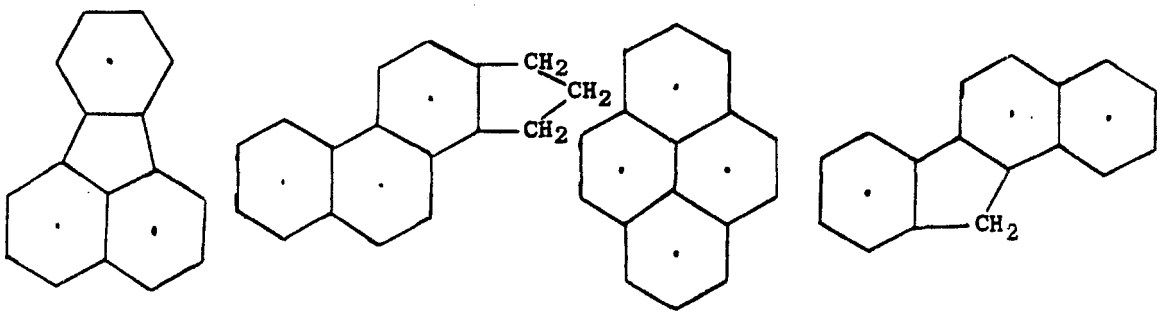
In the following pages, the results of the investigation of individual classes of polycyclic carcinogens from various oils are presented. In Table 5 is shown the proportion of the total carcinogens represented by benzopyrenes, and the 4-ringed benzanthracenes.

TABLE 5

API SAMPLE NUMBER	DESCRIPTION	TOTAL CONTENT[1] OF CARCINOGENS, C_{rc} (%)	CONTENT[1] OF 4-RINGED CARCINOGENS[2] (%)	CONTENT OF BENZO-PYRENES (%)
89	FCC Heavy Gas Oil	0.07	0.007	0.001
33	Coker Gas Oil	0.11	0.07	0.008
65	Thermal Tar	0.20	0.04	0.05
120	Lube Stock (naphthenic)	0.05	0.004	$\leq$0.003
8	FCC Clarified Oil	0.6 est.	0.10	0.42
44	FCC Clarified Oil	-[3]	incomplete	0.15
45	Tar from thermal cracking of API-44	-[3]	incomplete	0.38

1. Expressed in equivalents of the reference carcinogen of Figure 1.
2. Extracted from the oil by formation of adduct with maleic anhydride.
3. Not determined to date.

The investigation of the 3 1/2-ringed class of carcinogens (by appropriate fractionation of the FCC clarified oil, API-8) has narrowed the field to three groups of hydrocarbons, fluoranthenes, cyclopentanophenanthrenes, and pyrenes, the last being the least likely to be significantly carcinogenic. The benzofluorenes, which have been separated from the above by chromatography, apparently contribute only a small fraction of the potency due to this class as a whole.



Fluoranthene Cyclopentano-Phenanthrene Pyrene Benzo(a)fluorene

The pyrenes have been extracted on a small scale from the fraction containing the first three groups by cold concentrated (90%) sulfuric acid. Some loss due to sulfonation occurs; therefore, the use of liquid hydrogen fluoride for this purpose will be investigated.

Mass and absorption spectra have shown that the raffinate from this acid extraction contains about equal amounts of fluoranthenes and cyclopentanophenanthrenes together with a small amount of a group having molecular weights of 220 and 234. Initially it was supposed that the members of this last group were derivatives of phenanthrene (3 or 4 alkyl carbons), but their chromatographic

behaviour makes this assumption questionable. For all groups in the raffinate (from the most carcinogenic fraction of API-8 in the distillation range 727 to 759°F.) the 18-carbon atom derivatives predominate (in the case of fluoranthene these would be dimethyl or ethyl).

The separation of the fluoranthenes from the cyclopentanophenanthrenes by use of chromatography and picric acid has proved to be feasible. These groups will be tested separately on mice to determine their respective potencies.

APPENDIX 3

DISCUSSION

The evidence developed in the course of this investigation indicates that an analytical estimation of the rate at which a given oil may be expected to induce tumors in the skin of mice must take into account two different types of influences. The basic and essential one is that of the polycyclic aromatic carcinogens. As their concentration increases, so is the rate of induction of tumors expected to increase. An augmentation of this effect may, and frequently does, occur if the remaining non-carcinogenic portion of the oil, i.e., the "medium" in which the carcinogens are dissolved, is of such composition as to act as a catalyst in the mechanism of carcinogenesis. Although the nature of this mechanism is not understood, considerable progress has been made in describing the types of components which exert this accelerating effect.

It has been found to be useful, for the purpose of orienting the collection of experimental information, to express the contributions of the carcinogens and of the medium as a simple equation which expresses rate. The rate at which a given oil may be expected to induce tumors, $\underline{r}_{obs.}$,

$$\underline{r}_{obs.} = A\underline{r}_{c} , \qquad (7)$$

where A is the factor due to the catalytic influence of the medium, and the rate, $\underline{r}_{c}$, is that at which the oil would produce papillomas if its composition were such that A equals one.

The rates at which certain classes of petroleum oils induce cancer of the skin of mice is dealt with semiquantitatively in the

following sections in terms of the general relationships expressed by equation (7). In a subsequent section the progress toward a quantitative analytical tool will be discussed in detail.

A. Refined Lubricants and White Oils

A few oils of the types indicated by this heading have been tested and found to be completely non-carcinogenic. Solvent extraction of lubricating stocks appears to be particularly effective in eliminating the polycyclic aromatic carcinogens.

B. Unrefined and Partially Refined Lubricating Stocks and Slack Waxes

When materials of this class are applied repeatedly upon the skin of mice, the rate of induction of tumors varies over an extremely wide range, depending upon the composition of the sample. Among the unrefined stocks (having a given distillation range) there is as much as a three-fold variation in the content of polycyclic carcinogens. The ratio of naphthenes to paraffins also appears to be of major importance. It seems probable that the naphthenes (polycycloparaffins) of high molecular weight counteract most of the catalytic effect of any long-chain accelerators present in the oil (see Appendix 2).

The two paraffinic distillates which have been tested produced tumors fairly rapidly (in 4 to 6 months on the average). Although the content of polycyclic aromatics in these oils is relatively

low, the ratio of long-chain accelerators to polycycloparaffins is probably high. The press oil from dewaxing of one of these distillates showed the same level of activity as the original oil. The available evidence indicates that solvent extraction of such stocks produces an oil having little or no carcinogenic activity. It is unnecessary to carry the refining to the stage of a white oil to eliminate this property effectively.

C. Straight Run Residua

In all likelihood the straight run naphthenic residua which we have tested contained the same types of carcinogens, in somewhat lower concentration, as were present in the raw lubricating stocks. However, the potencies of these residua in general have been negligible. The possible presence of inhibitors has been considered in an earlier Interim Report (September 24, 1952). The recent findings indicate that the apparent "inhibition" may be due to the dilution of accelerators by polycycloparaffins.

D. Products of Thermal and Catalytic Cracking and Delayed Coking

Wide variations have been found in the potencies of the products of the cracking of heavy oils by these processes (0.06 to 0.6 of a life span required to induce papillomas in the skin of mice). With one exception, the tars produced by the thermal cracking process have failed to induce tumors in less than 20 weeks, on the average (so long as the stock charged to the unit was free of catalytically cracked components). In comparing the effect of thermal cracking with that

of catalytic cracking it is important to note the relative fate of the paraffins and the polycycloparaffins in the charge. Although both classes are decomposed much more rapidly in the catalytic than in the thermal process, the increase in rate for the naphthenes is much greater than the increase in rate for the paraffins (2000 times versus 50 times, respectively).[1] Thus, the catalytic operation tends to increase the ratio of accelerators to those compounds suspected of interfering with acceleration, whereas the thermal process does not. Catalytic cracking also builds up the concentration of polycyclic carcinogens somewhat more rapidly than does the thermal process. Hence the products of catalytic cracking generally will have higher potency than tars from thermal cracking. The one exceptionally active thermal tar mentioned above was produced from a charge stock which was relatively free of naphthenes (Pennsylvania crude).

Catalytic cracking usually provides an effective build-up of carcinogens together with an increase in the catalytic activity of the medium to the point whereby the product, in the extreme cases, may induce tumors of the skin of mice in less than 10 weeks. More severe catalytic cracking may result eventually in complete destruction of all long-chain compounds, reducing the catalytic activity of the medium to a low level. Usually the concentration of highly active carcinogens in such oils has reached very high levels (>0.5 percent). Hence such materials will still induce tumors almost as rapidly as those produced by milder cracking conditions.

1. B.S. Greensfelder, et al, Industrial and Engineering Chemistry, Vol. 37, pages 514, 983, 1083, and 1168, 1945.

The effect of thermal cracking on the potency of a catalytically cracked charge stock will vary, depending upon the relative rates of two competing phenomena. The carcinogen content (as measured by the benzopyrenes) normally increases during this operation. However, the catalytic activity, if any, usually decreases.

The composition and effective potency of recycle gas oils from delayed coking units appear to be similar to those of the heavy catalytically cracked sidestreams. Apparently, the coke building up in the drum has a catalytic influence on the direction of the decomposition of the various classes of compounds in the feed comparable to that of the silica-alumina catalysts. The one sample of a coker gas oil which has been examined in detail has shown one unusual characteristic, in that two-thirds of its basic carcinogenic activity can be ascribed to compounds extracted by maleic anhydride.

E. Progress in the Development of a Quantitative Analytical Tool

The expression of the relative influences of the carcinogens and the medium, symbolized by equation (7) (this Appendix), must be expressed in parameters which can be measured experimentally. In the typical test, an oil of a given composition is applied repeatedly upon the skin of mice until papillomas appear on the back of each mouse. The average time, calculated by standard statistical methods, is called the average duration (or expectancy) of tumor-free life, $\underline{e}_{obs.}$. Obviously the more rapid the rate of induction of tumors, the smaller the calculated value of $\underline{e}_{obs.}$. In other words, the rate is inversely

proportional to $\underline{e}_{obs.}$. Thus equation (7) may be restated in terms of the experimental measurements in the following manner:

$$\frac{1}{\underline{e}_{obs.}} = A \times \frac{1}{\underline{e}_c} \quad , \qquad \text{equivalent to (6)}$$

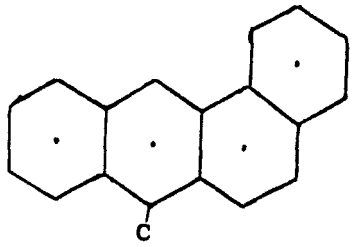
or,

$$\underline{e}_{obs.} = \frac{\underline{e}_c}{A} \quad .$$

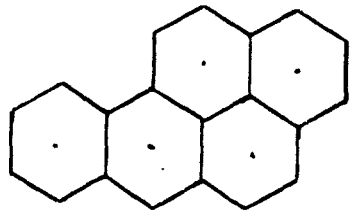
For the sake of clarity, the two important parameters, A and $\underline{e}_c$ will be discussed separately in the following paragraphs.

An understanding of the relationship between the composition of the medium and resulting value of A has been approached by two parallel paths. To furnish a background of reference, various solutions of these synthetic carcinogens, methylcholanthrene and benzopyrene, have been tested to determine the relative catalytic activities, A, of the solvents. The solvents employed have included pure compounds and blends of these compounds to simulate the variety of compositions of the non-carcinogenic portions of petroleum oils. The results of these tests, shown in Table 4 of Appendix 2, have demonstrated that the catalytic activity of a solution rises as the molecular structure of its chief component approaches that of the typical 4-ringed carcinogen in general size. The similarity in molecular size between carcinogens and accelerators will be noted from the examples on the following page. A variety of compounds of similar molecular size have been found to be capable of increasing the rate at which the polycyclic carcinogens may induce tumors. Larger compounds, e.g., di-sec.-nonylnaphthalene, do not seem to share this property.

CARCINOGENS

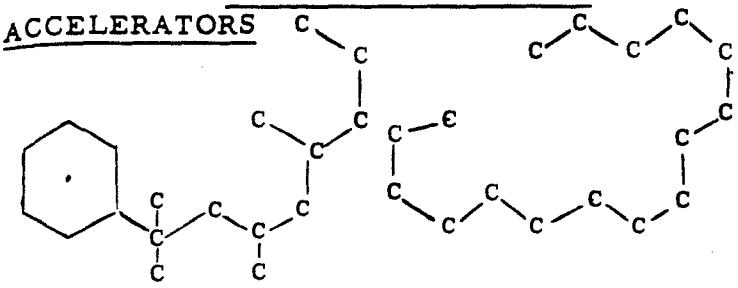


7-Methylbenz(a)anthracene



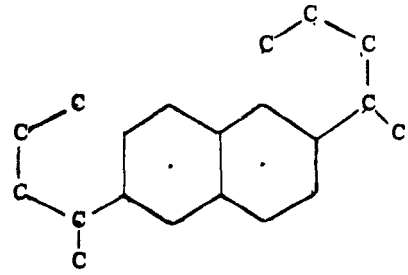
Benzo(a)pyrene

ACCELERATORS



t-Dodecylbenzene

Cetane



Di-sec-amylnaphthalene

Almost all refinery streams will include some quantities of long-chain accelerators of this type. However, the accelerating, or catalytic, activity of the total medium will vary over a wide range depending partly upon the concentration of such accelerators, which in some cases may be quite high, and also to a very important extent upon the relative concentration of various classes of those components which in themselves have neither accelerating nor carcinogenic activity.

The results of experiments in which the long-chain accelerators, t-dodecylbenzene and n-dodecane, were diluted with various solvents having little or no catalytic activity, led to a recognition of the potential importance of such diluents. In Table 4 it will be noted that, when the diluent has a molecular structure appreciably smaller than that of typical carcinogens, no significant reduction of A occurs until the concentration of the diluent exceeds 50 percent.

In contrast with the effect of diluents possessed of only one or two rings in their molecular structure, is the very marked interference with the accelerating activity of dodecane (and t-dodecylbenzene) on dilution with saturated oils containing appreciable amounts of condensed cycloparaffins comparable in molecular size to the typical carcinogens (experiments 297 and 614). It should be emphasized that this inhibitory property counteracts the catalytic influence of accelerators, without reducing the basic activity of an oil due to the carcinogens themselves.

Direct proof that it is the content of condensed polycyclo-paraffins of these white oils which is responsible for their ability to counteract the influence of long-chain accelerators has not as yet been obtained (fractionation of the white oil API-80 has been initiated, but the biological tests of the fractions are still to be carried out). However, the tentative application of this assumption to the problem of the relationship between the composition of the non-carcinogenic fractions of complex oils and their comparative catalytic activity has proved to be fruitful. Thus, as has been indicated, the testing of synthetic solutions of pure compounds has been paralleled by tests on non-carcinogenic fractions of a variety of refinery streams. The results of initial work on the composition of these fractions (by mass spectrometric analysis) has correlated well with the explanation of the inhibitory properties of the white oils in terms of their content of condensed cycloparaffins.

In Tables 2 and 3 are shown the data which have been collected on the composition and relative catalytic activities of the fraction of a series of oils containing less than three rings per molecule (separated from the polycyclic aromatic components by chromatography). The composition of the fraction from the naphthenic lube stock, API-120, is comparable to that of the white oil, API-80, each containing about 50 percent of mono-cycloparaffins (some of which may act as accelerators) and about 35 percent of condensed cycloparaffins. The catalytic activity A of this fraction of API-120 was found to be essentially the same as that of API-80, approximately 1.3. It is expected that the composition of the non-carcinogenic fraction of the other naphthenic lube stock, API-115, will have a similar pattern. In contrast, that of the waxy lube stock, API-105, will be expected to show a high ratio of long-chain paraffins and monocycloparaffins to condensed cycloparaffins. In line with the tentative hypothesis, the catalytic activity of the fraction of API-105, containing less than 3 rings per molecule, was almost as high as that of pure n-dodecane.

As indicated in section D of this Appendix, catalytic cracking tends to increase the ratio of long-chain compounds to condensed cycloparaffins. This is shown by the composition of the non-carcinogenic fraction of API-89 in Table 3. The vigorous catalytic activity predicted for such a composition is confirmed by the result of the biological test shown in Table 2. Similarly it is to be expected that the intense catalytic activity of the non-carcinogenic

fraction of API-33 is due to a composition containing a relatively small quantity of condensed cycloparaffins.

Difficulty has been experiences in obtaining satisfactory biological measurements of the potency of thermal tars because of their toxicity for the mice. However, it appears that the influence exerted by catalysts in their total carcinogenic effect is very much less than it is in oils produced by catalytic cracking or coking. The composition of the non-carcinogenic fraction of one fairly typical example, API-65, shows an unusually high ratio of alkylbenzenes and naphthalenes to saturated hydrocarbons. Although such a fraction might be expected to show catalytic activity, it is apparent that its influence, in the total composition of API-65 (of which it represents less than 50 percent), is relatively small. The one thermal tar, API-72, which has been found to be significantly more potent than the average material from this type of process, is expected to be relatively free of condensed cycloparaffins, on the basis of its crude source. A direct analysis will be made by mass spectroscopy to test this assumption.

F. Progress Toward the Analytical Estimation of the Content of Carcinogens in any Given Petroleum Oil

The application of biological methods to estimate the rate (i.e., its reciprocal, e_c) at which a given oil may be expected to produce tumors if its catalytic activity A were equal to 1, has been described in Appendix 1. The procedure involved an estimate of the

intermediate parameter, $\underline{d}_o$, the dosage of carcinogen in milligrams per "unit area" per week supplied by the oil in question. The approach to chemical methods to estimate $\underline{e}_c$ is designed similarly to evaluate $\underline{d}_o$ as an intermediate step. The parameters which must be measured by analytical methods in order to arrive at the value of $\underline{d}_o$ are the total content of carcinogens C_{rc}, and the relative spreading tendency of the oil, $\underline{s}_o$. The investigation has been designed to estimate C_{rc} by adding up the contributions of three separate classes of polycyclic aromatic carcinogens (expressed in equivalents of the reference carcinogen):

$$C_{rc} = C_5 + C_4 + C_{3\,1/2}. \qquad (8)$$

The method of estimating the value of C_5 from the measured content of benzopyrenes has been described in section C of Appendix 1. The development of methods for C_4 and $C_{3\,1/2}$ is being pursued vigorously. The preliminary results indicate the major importance of the 3 1/2-ringed carcinogens in almost all types of petroleum oils (the relative contribution of the 4-ringed benzanthracenes and the 5-ringed benzopyrenes increases with increasing severity of catalytic cracking).

The progress made in concentrating the 3 1/2-ringed carcinogens indicates that their identification will be completed satisfactorily in the course of two additional cycles of biological tests (about 9 months each). Since the investigation of the chemical and physical properties of these fractions will be carried on concurrently, the

step from the identification to the analytical determination of this class (and the value of $C_{3\ 1/2}$ in equation (3)) should be a short one.

The contribution of the 4'-ringed compounds extracted by maleic anhydride varies from almost nil in the case of raw lube stocks, such as API-120, to as much as 2/3 of the basic potency in the case of the coker gas oil, API-33 (Table 5). The lack of significant quantities of the 4- and 5-ringed carcinogens in API-89 may be explained by its low final boiling point (750°F.). Only the carcinogens of lower molecular weight (the 3 1/2-ringed class) are present.

Currently additional rests are being carried out to determine whether the content of 4-ringed carcinogens increases during the thermal cracking of a catalytically cracked charge stock. The content of 5-ringed benzopyrenes more than doubled. In addition, the tests, which are being carried out at a series of concentrations, should provide information on the slope of the dose-response curve for the 4-ringed class of carcinogens.

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