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INTERIM REPORT

API RESEARCH REPORT MC-1

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THE KETTERING LABORATORY

in the

Department of Preventive Medicine and Industrial Health

College of Medicine

UNIVERSITY OF CINCINNATI, CINCINNATI, OHIO

INTERIM REPORT

API RESEARCH PROJECT MC-1

I. Introduction

This report will summarize briefly recent developments in the investigation of the relationships between the composition of various refinery streams and the relative rates at which they are capable of producing cancer of the skin of mice under selected experimental conditions.

The earlier work on this project brought out the fact that, in general, the rate at which an oil will induce cancer of the skin under any particular set of conditions in the laboratory depends upon the concentration of a group of carcinogenic substances within the oil, and upon the extent to which two additional groups of hydrocarbons are represented in the oil, i.e., their relative concentration.

The primary carcinogens, as a group, are polycyclic aromatic compounds. To a greater or lesser extent these carcinogenic organic compounds also share the pharmacological properties of a second group, the accelerators. The second group includes representatives from all classes of hydrocarbons from normal paraffins to alkylnapthalenes.

An increase in the concentration of either or both of the first two groups tends to increase the rate at which an oil induces cancer of the skin of mice. In contrast, the third important group retards the rate. Hence, for present purposes, the compounds composing this group are termed

inhibitors. The concentration of this group appears to play an important role in governing the rate at which uncracked products such as lubricating oils and slack waxes induce tumors of the skin of mice.

The following sections designated by headings describe the current experimental program which was designed to characterize the most important class of aromatic carcinogens and the types of hydrocarbons composing the third group, i.e., the inhibitors. The importance of sulfur compounds as accelerators is also discussed. The description of methods includes efforts to apply relatively rapid methods in biological assays of the carcinogens.

Work has been started on a final report on the experimental and technologic phases of API Research Project MC-1. It is expected that this report will be ready for distribution by July 1, 1959.

II. Polycyclic aromatic carcinogens in cracked oils

In the broad screening program in which the relative carcinogenic potencies of a variety of refinery streams for the skin of mice were determined, it was found that a number of oils having end boiling points of approximately 750° F. showed surprisingly high activity. Accordingly, an intensive program of investigation of the carcinogenic components having boiling points in the range 675° to 760° F. has been carried out. It will be seen that the experimental results point to a type of phenanthrene the origin of which can probably be traced back to the plant and animal steroids.

A. Materials and methods

The carcinogenic oil from which these relatively low boiling carcinogens are being concentrated is the catalytically cracked residuum from Los Angeles basin feed stocks, API-8. Concentration of the more volatile carcinogens was carried out by fractional distillation of the aromatic components in vacuo through an 80 plate column of the Stedman type. Each distillate fraction was tested for its carcinogenic potency for the skin of mice by the usual technique of repeated application until tumors had been produced. The most volatile fractions having significant potency, those boiling from 730° to 760° F., were selected for further concentration.

The three distillates so selected were washed with aqueous alkali and acid to eliminate any acidic or basic

components. Further, since it was supposed that anthracenic derivatives in this molecular weight range would contribute little to the observed potency, they were eliminated by extraction with maleic anhydride. Biological testing of the extract confirmed its supposedly negligible potency.

The method used for the next step in the concentration of the desired materials was chromatography on activated alumina. By this technique, as demonstrated by subsequent biological testing of the fractions produced, the majority of the active carcinogens were concentrated into a fraction which amounted to approximately 20 per cent of the original distillates boiling at 730° to 760° F. The methods of ultraviolet absorption spectroscopy and mass spectrometry have been used to characterize the major components of this concentrate as methyl and dimethyl derivatives of pyrene, cyclopentenophenanthrene, and fluoranthene.

A reasonably good separation of pyrenes from the other two classes was accomplished by the partition between concentrated sulfuric acid and isooctane at approximately -10° F. (the pyrenes being recovered from the acid by dilution with ice). The raffinate from this acidic extraction was separated by chromatography on activated alumina (the fluoranthenes being, in general, more strongly adsorbed than the phenanthrene derivatives). The formation of complexes with picric acid served to remove fluoranthenes from intermediate fractions containing

the two classes of hydrocarbons.

Five fractions are being tested currently by applying them repeatedly as solutions in benzene upon the skin of mice (see Table 1). In parallel, eac of the fractions is being tested for its ability to destroy sebaceous glands. This is a relatively rapid method of biological assay developed originally by Suntzeff and Carruthers, and now being used by investigators in a number of institutions. The method consists basically of a series of six applications (2 each day for 3 days) of the material upon the skin of mice. On the eighth day the skin is removed, subcutaneous fat is scraped off, and sections of the skin are prepared for microscopic examination as whole mounts, after being stained with Nile Blue Sulfate. In these preparations, the sebaceous glands appear as pink spots around hair follicles which are stained blue. Most but not all of the known aromatic carcinogens destroy the sebaceous glands under these conditions. Certain non-carcinogenic compounds also destroy these glands to some extent. Hence, the test, although very convenient from the standpoint of time, must be used and interpreted with caution.

B. Results and discussion

In Table 1 are shown the results of standard tests of the carcinogenicity of solutions of each of the five fractions in benzene. The tests are not complete, but they point definitely to the importance of the fractions in which

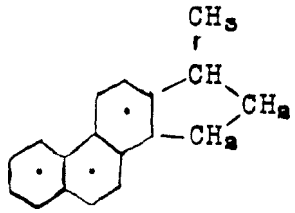
the cyclopentenophenanthrenes are the major components (it is possible that the activity of the pyrene fractions is due to their contamination with phenanthrenes).

TABLE 1

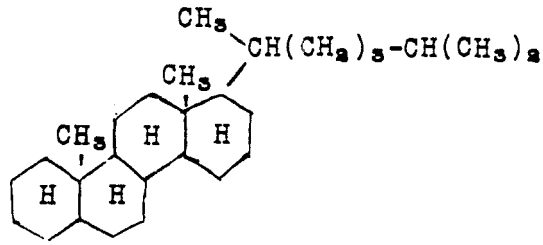
API fraction number	Major components	Per cent of original concentrate	Effective number of mice	Number of mice bearing tumors after 99 applications
8-26-8A	cyclopenteno-phenanthrenes	28	11	4
8-26-8B	80% cyclopente-nophenanthrenes 20% fluoran-thenes	6	12	7
8-26-8C	fluoranthenes	6	12	0
8-26-8D	methyl pyrenes	46	12	2
8-26-8E	di- and tri-methyl pyrenes	13	12	2

In the highest concentration tested, each of the fractions was found to destroy most of the sebaceous glands in the skin of Swiss mice on which 6 applications had been made. These short-term tests are being repeated with lower concentrations of the aromatic fractions, to determine whether a differentiation corresponding to their relative carcinogenic potency can be effected. If the use of the rapid test proves to be successful, it will be used to speed the further work on this class of carcinogens.

The structural relationships between the cyclopentenophenanthrenes and the steroids will be seen from the following examples:



methylcyclopentenophenanthrene



cholestane

This particular cyclopentenophenanthrene derivative is reportedly non-carcinogenic. No pure dimethyl derivatives of the type present in 8-26-8A have been tested.

III. The role of sulfur compounds as accelerators of carcinogenesis

An effort has been made to determine experimentally the relative contributions of carcinogens and accelerators to the effective potency of a series of different types of refinery streams. Accelerators were separated from carcinogens by chromatography and the two fractions from each oil assayed separately. From the results reported on April 10, 1957, it was apparent that the extent of the agreement between the observed potency of a cracked oil and that calculated from the contribution of its parts was modest. It was especially evident that the distillate oils from catalytic cracking and delayed coking were aberrant in this respect.

Information from another field provided a clue to this behavior. It has been shown that sulfur compounds play an important role as naturally-occurring inhibitors of oxidation in lubricating oils. In combination with certain alkylbenzenes and alkylnapthalenes acting as primary inhibitors, the sulfur compounds markedly enhance the protection of the oil against oxidation.

The particular cracked oils in question, API-89 and API-33, are relatively rich in sulfur compounds. Therefore, the possibility that these non-hydrocarbons might be important factors in their behavior was investigated. Preliminary results suggest that this may be the case.

A. Materials and methods

A distillate gas oil, API-89, from fluid catalytic cracking (containing 0.64 per cent sulfur) was used in this series of experiments. Earlier work had indicated that the relative accelerating activity of the oil, A, was equal to 3.5, whereas the accelerating activity of of the fraction containing the saturates, the alkylbenzenes, and the alkylnaphthalenes, but none of the sulfur compounds, was only 2.3.

Sulfur compounds were removed from API-89 by two methods. The first, which took out the majority of the compounds was a standard procedure involving a catalytic hydrogenation by Raney nickel in absolute alcohol solution. The second method involved an extraction with alcoholic sodium hydroxide, presumably removing only the acidic sulfur compounds.

To determine whether the effect of the sulfur compounds removed by the Raney nickel could be replaced by inclusion of a typical oxidation-inhibiting sulfur compound, benzyl disulfide, a blend of 1.36 per cent of this compound (which contains 26.0 per cent of sulfur) with the desulfurized API-89 was prepared for biological testing.

B. Results and discussion

In Table 2 are shown the experiments which have been set up to determine the contribution of certain sulfur compounds to the carcinogenic potency of the catalytically cracked oil, API-89.

TABLE 2

API sample number	Description of sample	Percentage of sulfur	Schedule of applications (number per week - dose in mg.)	Effective number of mice	Number of mice with tumors at the 8th week
89-B	API-89, catalytically cracked distillate	.64	2-50	6	3
89-D	same as 89-B	.64	2-10	6	0
89-9	API-89, desulfurized with Raney nickel	.24	2-50	8	0
89-10-B	Blend of 89-9 with 1.36% of dibenzyldisulfide	.60	2-50	6	0
89-10-D	same as 89-10-B	.60	2-10	6	0
89-11	API-89 desulfurized by alcoholic NaOH	.54	2-50	8	0

The rapid development of tumors at the higher level of dosage of the original API-89 is consistent with the results of an earlier experiment. This difference in rate with difference in dosage per application has been associated with the contribution of accelerators to the activity of the oil. It represents part of the evidence leading to the belief that the accelerators exert a systemic effect, rather than a local action on the epidermis itself.

It is too early in the experiment to claim that a significant retardation has been effected by removal of the

sulfur compounds. It will be fortunate if the extraction by alcoholic sodium hydroxide produces a significant effect, since these compounds will be readily available for identification, whereas those eliminated by the Raney nickel have been converted to hydrogen sulfide.

IV. Inhibitors of carcinogenesis occurring naturally in petroleum

The inhibiting activity of naphthenic mineral oils has been suggested by a number of results of earlier experiments. When white mineral oils, containing a predominance of cyclic saturated hydrocarbons, were blended with powerful accelerators such as dodecylbenzene or normal dodecane, a marked reduction of accelerating activity was observed. In no case has any animal developed a papilloma following an initial application of a very strong carcinogen followed by repeated applications of the white mineral oil, whereas, in parallel experiments involving only the single application of the strong carcinogen, 5 to 15 per cent of the mice have developed tumors.

Thus, it has become apparent that a relatively small amount of such inhibitors might compensate for a relatively high concentration of accelerators. To make it possible to determine when a sufficient concentration of such inhibitors is actually present in any given refinery stream, it is necessary to characterize the components of the white mineral oils having this property.

A. Materials and methods

The white mineral oil API-80 (95SSU/100°F.) was separated into two portions by the use of thiourea. The isoparaffins and part of the monocyclic naphthenes form crystalline adducts which were removed from the oil. The remainder, constituting

about 85% of the oil, was distilled under vacuum to remove components boiling below 610°F.

The residue was then subjected to liquid thermal diffusion in order to separate the remaining monocyclic paraffins from the condensed polycyclic paraffins. A 6 foot, 10 take-off column was employed with the temperature of the cold wall at 75°F., and the temperature of the hot wall at 150°F.

The composition of the fractions from the diffusion column is being investigated by mass spectrometry (at 70 volts ionization potential) and absorption spectra in the near infrared (at about 1200 mμ).

Blends of selected fractions with normal dodecane and benzopyrene are being tested by multiple applications upon the skin of mice to determine which of the fractions are responsible for the inhibiting properties of the original white oil. The experiments have not been in progress long enough to allow for appraisal of results.

V. Summary

A. The most volatile class of aromatic carcinogens present in cracked oils appears to be composed of derivatives of cyclopentenophenanthrene.

B. The three and one half ring aromatic components of catalytically cracked oil are capable of destroying sebaceous glands. As yet it is uncertain whether a differentiation between the carcinogenic and the non-carcinogenic members of this class of hydrocarbons can be made by this technique.

C. There are preliminary indications that sulfur compounds, which may be removed by alcoholic sodium hydroxide, are playing an important role as accelerators in certain catalytically cracked oils.

D. It has been found possible to concentrate the polycyclic paraffins of an inhibiting white mineral oil by liquid thermal diffusion. Determination of the structures required for inhibitory activity will be made from the results of current biological tests of these fractions.

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