Submitted to Biochemistry

UCRL-20060
Preprint

c. 2

# THE 200 $MH_Z$ NUCLEAR MAGNETIC RESONANCE ANALYSIS AND THE SELECTIVE DEUTERODEPROTONATION OF BENZO [a] PYRENE AND 6-METHYLBENZO [a] PYRENE. PROBABLE ACTIVE INTERMEDIATES IN CANCER INDUCTION

E. Cavalieri and M. Calvin

August 1970



AEC Contract No. W-7405-eng-48



LAWRENCE RADIATION LABORATORY
UNIVERSITY of CALIFORNIA BERKELEY

48

UCRL-20060

c. 2

# THE 220 $MH_z$ NUCLEAR MAGNETIC RESONANCE ANALYSIS AND THE SELECTIVE DEUTERODEPROTONATION OF BENZO[a]PYRENE AND 6-METHYLBENZO[a]PYRENE. PROBABLE ACTIVE INTERMEDIATES IN CANCER INDUCTION.*

E. Cavalieri and M. Calvin

Running title:

The 220 $MH_z$ NMR of Benzo[a]pyrene and 6-Methylbenzo[a]pyrene

*Contribution from the Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory, University of California, Berkeley, California 94720. This research was supported in part by the United States Atomic Energy Commission. One of the authors (E.C.) was a recipient of a Damon Runyon Cancer Research Fellowship, 1968-1970.

ABSTRACT: The proton magnetic resonance spectra at 220 $MH_z$ of the carcinogenic benzo[a]pyrene 1 and 6-methylbenzo[a]pyrene 2 in sulfuric acid-$d_2$ are used to determine the specific positions of electrophilic substitution.

The electrophilic attack in 1 takes place predominantly on the 6-position, followed by 1- and 3-positions, whereas in 2, where the 6-carbon atom is substituted, the active role is played by the 1-, 3- and 5-positions.

The qualitative data for 1 obtained by this reaction, taken together with its specific coupling to the nucleic acid base 1-methylcytosine (reported in the present issue), its chemical properties and its metabotism (known from the literature) suggest probable active intermediates, which by reaction with the nucleophilic positions of the nucleic acid bases could initiate the process of transformation of the normal cell into the neoplastic cell.

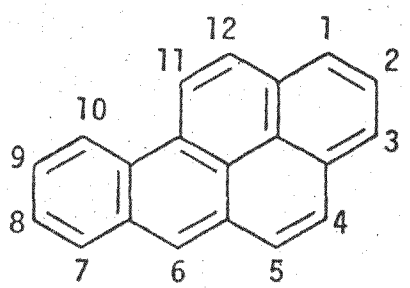
## Introduction

The proton nuclear magnetic resonance (NMR) study at 60 $MH_z$ of twenty unsubstituted polycyclic hydrocarbons (Martin et al., 1964) reveals the presence of separated band-systems.

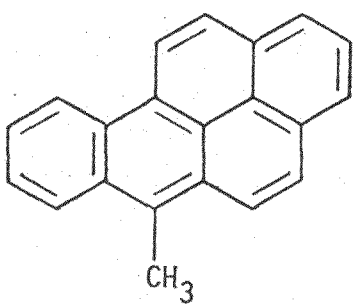
The large ring-current diamagnetic effect of these molecules generates on the peripheral protons a downfield chemical shift relatively to those of the benzene and the naphthalene series. The particular relevance of this effect on the meso-anthracenic protons leads to their separation and thus to their identification. Moreover, the angular protons exhibit an additional deshielding effect due to the non-bonding spin-spin interactions (steric compression effect) and their band systems can also be easily assigned. However, the remaining protons are normally not well separated.

A complete analysis of these hydrocarbons has been solely successful for the benzo[e]pyrene (Cobb and Memory, 1967). This has been achieved by comparison of the theoretical calculated shielding parameters, relatively less complicated for this symmetric molecule, with the experimental data.

The better resolution of the 220 $MH_z$ spectrometer taken together with the double resonance technique and the specific deuterodeprotonation with sulfuric acid-$d_2$ allow us now to report the complete interpretation of the benzo[a]pyrene 1 and 6-methylbenzo[a]pyrene 2 proton magnetic resonance spectra.




1




2

At the same time, the spectra provide qualitative information on the selective substitution positions obtained by such a deuterium ion exchange. These electrophilic substitution results for 1 are compared with other types of chemical reactions for 1 and with its enzymatic reactions in vivo. The resulting evaluation indicates with remarkable agreement the 6-, 1- and 3-positions as the most active ones. The specific coupling of the 6-carbon atom of

1 with the base 1-methylcytosine (Cavalieri and Calvin, 1970) also constitutes a matter of consideration and comparison.

Finally, the collective data obtained by these observations have shed light on the mode of activation of 1 *in vivo*, which seems to be promoted by the aryl hydrocarbon hydroxylase enzyme systems (Grover and Sims, 1968; Gelboin, 1969).

The nature of the active intermediates can now be suggested. Their attack on the nucleophilic positions of the nucleic acid bases might give rise to the crucial step responsible for initiating the carcinogenic process within the cell.

## Results

Assignment of Lines. 1. BENZO[a]PYRENE 1. The 220 $MH_z$ proton magnetic resonance of 1 is shown in Figure 1. The peaks are assigned to the corresponding protons in the following manner. The integrated spectrum provides the protons' ratio 2:1:3:1:3:2 from left to right and the empirical rules suggested by Martin (1964; Martin *et al.*, 1964) allow a first approximated interpretation.

The two angular protons $H_{10}$ and $H_{11}$ were already characterized for their largest downfield shift by Martin et al. (1964) at 60 $MH_z$. The overall superimposition of the two chemical shifts in this spectrum is a pure coincidence. At higher concentrations, or when the coupled protons are irradiated in the regions close to their resonance signal, using the double resonance technique (vide infra), the sharp doublet of $H_{11}$ becomes slightly separated by the more complex coupling of $H_{10}$. If the two protons are irradiated with a strong radiofrequency signal at their resonance frequence, the $H_{12}$, postulated on the basis of the same coupling constant, collapses to a singlet and the complex multiplet attributed to the protons $H_8$ and $H_9$ occurs at a simpler signal.

On the other hand, when the $H_{12}$ is saturated, the signal of $H_{11}$ becomes a broad singlet. The irradiation of the complex multiplet $H_8$, $H_9$ in the region of their resonance frequency creates a separation of the doublet $H_{11}$ from the $H_{10}$ which appears now as a complex signal. This complexity is reduced at the resonance frequency of $H_9$.

The multiplet peak $H_7$, partially superimposed by $H_{12}$ and $H_3$, is attributed by spin decoupling when the signal corresponding to $H_8$ is irradiated. Thus, the decoupling experiments make it

possible to assign unequivocally the protons $H_{10}$, $H_{11}$, $H_{12}$, $H_7$, $H_8$ and $H_9$.

The characteristic peak at 8.41 ppm corresponds to the meso-anthracenic hydrogen in the 6-position. Although the two protons $H_4$ and $H_5$ possess about the same chemical shift as the $H_2$, the pattern of their AB spectrum can be recognized. To ensure this point the quartet can be easily visualized when the $H_1$ and $H_3$ are selectively deuterated (vide infra and Figure 3C). In this spectrum the large left inner-band results from a superimposed singlet of the collapsed $H_2$, arising from the disappearance of the two orto-coupling constants (Figure 1) with the protons in the 1- and 3-positions.

The precise attribution of the protons $H_1$ and $H_3$ cannot be directly resolved because there does not exist a valid criterion for their differentiation. The theory (Dewar, 1952) predicts that the most active position for the substitution reactions are in decreasing order the 6-, 1- and 3-positions. Although the 6-position is by far the most active one, a bulky Friedel-Crafts reagent would manifest a steric hindrance of the meso-anthracenic type on this carbon-atom. Therefore, the

substitution is expected in this case to take place on the 1- and 3-positions. When the hydrocarbon 1 is allowed to react with the succinic anhydride and aluminum chloride the 1-acylated derivative is preferentially isolated (Buu-Hoï and Lavit, 1960).

The deuterodeprotonation of 1 in Table I indicates that the 6-position is the most basic one and the 1- and 3-positions reveal only a little difference in reactivity.

On the basis of the above described data, the slightly more reactive carbon atom is preferentially suggested to correspond to the 1-position.

2. 6-METHYLBENZO[a]PYRENE 2. At first glance, the NMR of this molecule (Figure 2) compared to the spectrum of 1 shows the two expected deshielded protons $H_5$ and $H_7$ in peri-position with respect to the methyl group (peri effect; Dudek, 1963), and an anomalous shielding effect of one of the two angular protons as it can be observed by the integrated spectrum. The ratio of the protons from left to right is 1:1:1:1:2:1:2:2. The complex multiplet of the two downfield shifted protons $H_5$ and $H_7$ cannot, to our knowledge, be easily explained. As a matter of fact, they do not seem to be coupled with the methyl group (singlet,with the line width at half height of 1.6 $H_z$) and besides, the decoupling

experiments result negative. The peculiar interaction of these protons with the methyl group does not solely affect their chemical shifts but probably also their relaxation times.

The double resonance technique by irradiation of the $H_8$ and $H_9$ multiplet suggests that the proton centered at 8.96 ppm belongs to the 7-position. On the other hand, the saturation of the latter proton affects the $H_8$ and $H_9$ signal, indicating spin-spin coupling to $H_8$ and hence, their respective assignment. Furthermore, this finding suggests the multiplet centered at 8.45 ppm to be, by exclusion, the proton in the 5-position. The two peri-protons $H_5$ and $H_7$ present about the same downfield shift relative to their signals in benzo[a]pyrene, and this adds further evidence on their attribution. A negative decoupling of the proton at 8.90 ppm by irradiation of the proton $H_9$ rules out the possibility that the doublet might be the proton in the 10-position and suggests this to be the proton in the 11-position.

The protons $H_{11}$ and $H_{12}$, though coupled, do not show exactly the same coupling constant. However, a positive reciprocal decoupling experiment does not leave any doubt about their assignment. The downfield shift of the proton $H_5$ with respect to its chemical shift in the NMR of 1 (Figure 1), leaves the proton

$H_4$ as a doublet possessing about the same chemical shift and coupling constant as in 1. The characteristic triplet with two equal coupling constants defines the proton in 2-position. One of the related protons to the $H_2$ is directly disclosed by the same value of the coupling constant. The second one, partially superimposed, is disclosed after deuterium-exchange (Figure 4). They present the same patterns, the same chemical shift and the same facility to be deuterated as the protons $H_1$ and $H_3$ in 1. On this basis, their assignment is suggested. Finally, the remaining proton, namely, the doublet at 8.20 ppm, is the proton in 10-position.

Deuterodeprotonation of Benzo[a]pyrene 1 and 6-Methylbenzo[a]pyrene 2. The carcinogenic aromatic hydrocarbon 1 is easily dissolved in the concentrated sulfuric acid-$d_2$. The solution is then quenched with a chilled mixture of deuterated water and chloroform at different reaction times.

After the separation of the compound, its NMR spectra (Figure 3), compared to the NMR spectrum of 1 (Figure 1), point out specific deuterated positions by the partial or total disappearance of some of the signals. The results are summarized in Table I. When the hydrocarbon 1 is treated for 120 sec with deuterated sulfuric acid at 5-10° (Figure 3A) only the 6-position undergoes exchange.

The same reaction at room temperature for 60 sec (Figure $3_B$) gives rise to the practically total exchange on the 6-carbon atom and to the partial exchange on the 1- and 3-positions to about the same extent. Further, when this deuterated hydrocarbon is protonated with sulfuric acid, the NMR spectrum of 1 (Figure 1) is recovered. Deuteration for longer times (i.e., 480 sec, Figure 3C) shows the complete selective electrophilic substitution of the three active positions, leaving the other ones unaltered.

The compound 2 (Figure 4 and Table I), also carcinogenic, indicates after treatment with deuterated sulfuric acid for 120 sec a complete exchange of the protons in 1- and 3-positions. The 5-position seems to be active as well. The eventual reactivity of the methyl-substituted 6-position cannot be revealed by this method.

The non-carcinogenic benzo[e]pyrene does not dissolve in sulfuric acid at room temperature, suggesting rather inactive substitution positions in comparison with the two carcinogenic benzo[a]pyrenes.

Discussion

The better resolution of the NMR spectra at 220 $MH_z$ in relation to the 60 $MH_z$ has permitted the separation of the peaks of the different protons in 1 and 2 and hence, to provide their complete analysis. In this way it has been possible to follow the selective deuterodeprotonation in both compounds. The most active 6-position for 1 is distantly followed by the 1- and 3-positions possessing about the same reactivity. These data, though not quantitative, manifest a noteworthy agreement with the theoretical M.O. calculations (Dewar, 1952; Streitwieser, 1961) (vide supra), which indicate those three positions with the lowest carbon localization energies in the same order of reactivity. Furthermore, an extensive body of experimental evidence supports the concept of this localized chemical reactivity.[1]

---

[1]Compound 1 undergoes electrophilic substitution at the 6-position to yield the 6-formyl (Fieser and Hershberg, 1938), 6-chloro (Windaus and Raickle, 1939), 6-nitro (Windaus and Renhak, 1937), 6-diazophenyl (Fieser and Campbell, 1938) and the 6-thiociano (Wood and Fieser, 1941). The same 6-isomer is also obtained by radical substitution with lead tetraacetate (Fieser and Hershberg,

1938), benzoyl peroxide (Roitt and Waters, 1952) and thioglycolic acid (Conway and Tarbell, 1956). The Friedel Crafts condensation with succinic anhydride (Buu-Hoï and Lavit, 1960) produces predominantly 1-isomer and presumably the 3-isomer is the unidentified second product.

---

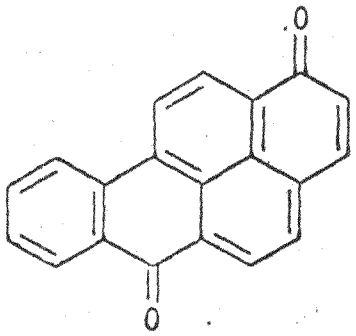
However, before discussing the σ-complex in the deutero-electrophilic substitution of 1, it is necessary to focus attention upon a special kind of reaction, namely, chromic acid oxidation (Vollmann et al., 1937; Cook and Schoental, 1950; Cook et al., 1950; Antonello and Carlassare, 1964) and ozonization (Moriconi et al., 1961).

The products in all reactions[2] were the benzo[a]pyrene-1,6-dione 3 and the benzo[a]pyrene-3,6-dione 4. These compounds suggest the existence of an interrelation between the 1- and 6-positions and the 3- and 6-positions, respectively.

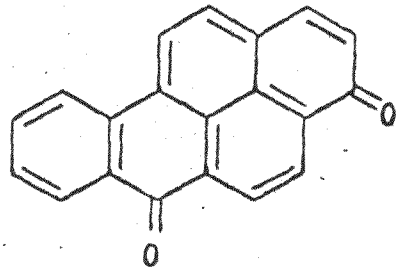
---

[2]Antonello and Carlassare (1964) indicate the presence of minor amounts of benzo[a]pyrene-6,12-dione both in chromic acid oxidation and in photooxidation.

Moriconi et al.,(1961), using one molar equivalent of ozone, obtained the diones 3 and 4 (3:1 ratio) from 1. Only trace amounts



3

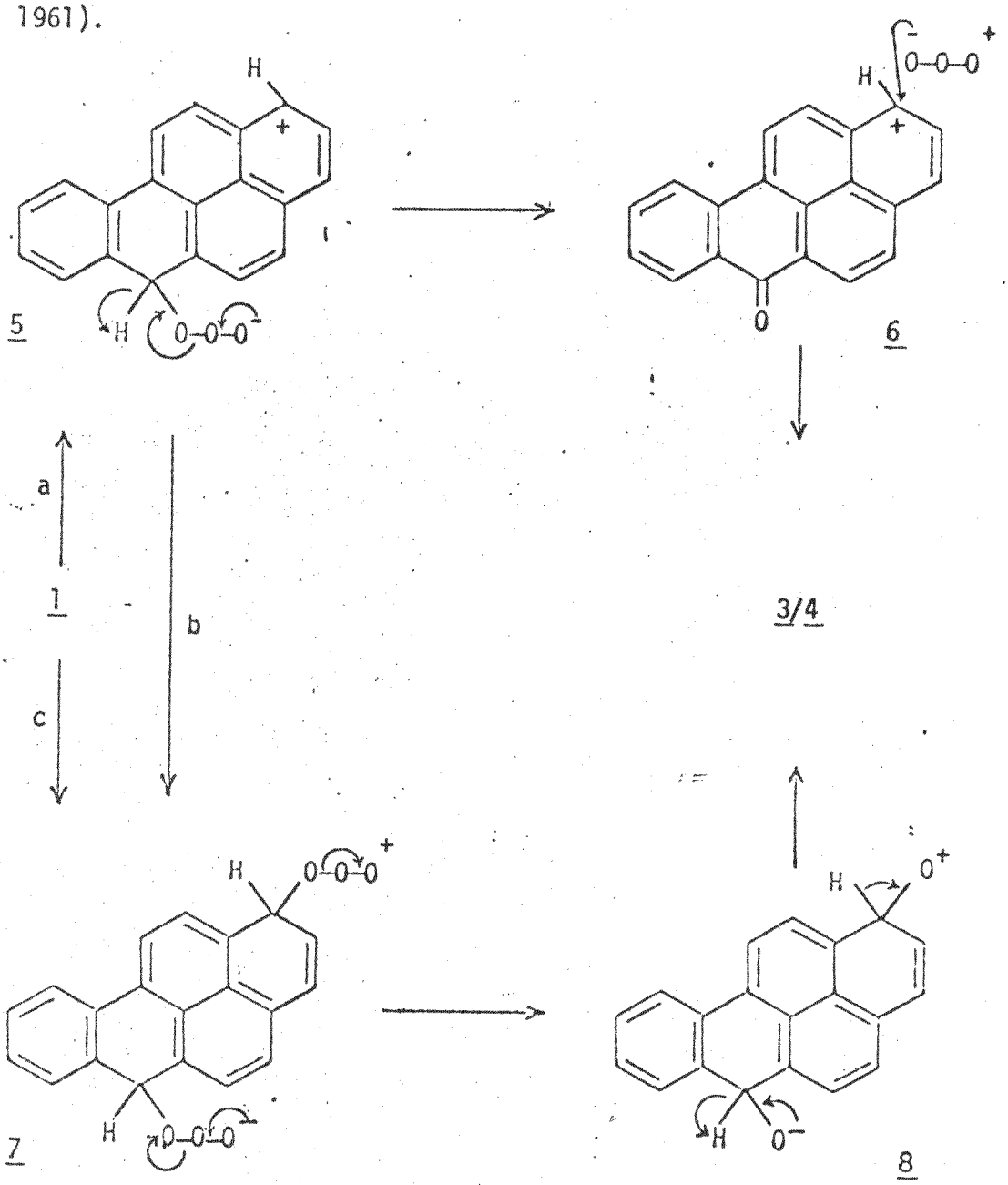


4

of benzo[a]pyrene-4,5-dione were formed . They further proposed three possible paths by which the interaction with the ozone gives rise to the two final diones.

Chart 1 points out that 1 requires two molecules of ozone to give 3 and/or 4. (a) The compound 1 undergoes an initial electrophilic attack on the positions 1, 3 or 6 (more frequently 6) to form 5, which by loss of oxygen and hydride-ion produces 6. This active intermediate by nucleophilic substitution of a second molecule of ozone and further loss of oxygen and of a proton leads ultimately to 3 and/or 4. (b) This path presumes the same first step as in (a), followed by a nucleophilic attack of a second molecule of ozone to give 7. Further, by loss of two molecules of oxygen, hydride-ion and proton, the diones are formed. (c) A

Chart 1. Three possible mechanisms in the formation of the 1,6- and 3,6-diones (the depicted one is for 1,6-dione) (Moriconi et al., 1961).



synchronous electrophilic and nucleophilic attack of two molecules of ozone engenders 7 and then, the same sequence mechanism as (b) is followed.

When the hydrocarbon 1 is treated with deuterated sulfuric acid, the electrophilic attack of the deuterium-ion takes place predominantly on the 6-position and, nearly to the same extent, on the 1- and 3-positions. Therefore, the well-accepted σ-complex (also known as Wheland intermediate) (Norman and Taylor, 1965) can be represented as in Chart 2.[3,4]

As has been suggested by the oxidation and ozonization reactions so far described, the charge of the σ-complex is located on the carbon atoms of highest free valence (or lowest localization energy). Thus, the intermediates above proposed constitute essentially the σ-complex for 1. Accordingly, the charge delocalization can be disregarded, as an important stabilizing mechanism.

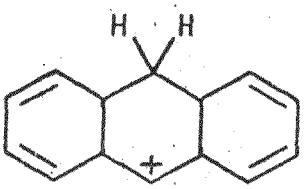
---

[3]Because of the selective deuteration on the 6-carbon at 5-10° (see Table I), 9, 10 and 11 are practically in these conditions the sole intermediates present in solution.

[4]The contribution of 11 is minor and is taken into account after the results of Antonello and Carlassare (1964).

Chart 2

In the class of the polycondensed aromatic hydrocarbons the concept of localized charge in the σ-complex has so far received experimental evidence exclusively for the anthracene. The ultraviolet spectrum of this compound in sulfuric acid (Gold and Tye, 1952) is similar to the spectra of 1,1-diphenylethylene and triphenylethylene in the same solvent, indicating a similarity of the three corresponding carbon ions. This result implies an overall localization of the positive charge in the meso-position 14, which is known to be the one of lowest localization energy.




14

The charged intermediates rationalized in Chart 2 constitute the distinctive and striking feature of the carcinogenic benzo[a] pyrene. Their possible formation *in vivo* and their attack on a nucleophilic center of the cellular target (*vide infra*) could probably give rise to the crucial step capable of inducing the carcinogenic process.

The benzo[a]pyrene 1 is converted in the whole animals (Falk *et al*, 1962) to its oxidized derivatives.

The most meaningful metabolites have been found to be the 6-hydroxy-, 3-hydroxy-, 1,6-dihydroxy- and 3,6-dihydroxy-benzo[a]pyrene, whereas the 6,12-dihydroxy- and, doubtfully, the 4,5-dihydro-4,5-dihydroxy-benzo[a]pyrene are only present in negligible amounts. Besides, compound 1 is metabolized by rat-liver homogenates mainly (Sims, 1967) to the 3-hydroxy-benzo[a]pyrene and to the benzo[a]pyrene-1,6- and 3,6-dione.[5] Therefore, the oxidation reaction *in vivo* promoted by the aryl hydroxylating enzymes takes place on the same reactive positions disclosed when 1 is treated with chemical reagents.

It is well known that the formation of these enzyme systems can also be induced by the administration of polycyclic hydrocarbons (Nebert and Gelboin, 1969). Their presence has been disclosed in mammalian cells of different species, tissues and strains. They metabolize the aromatic hydrocarbons to more polar derivatives, which can be easily eliminated, providing a suitable way of detoxification. Furthermore, the carcinogenic ones are

---

[5]The two diones are probably secondary products of oxidation of their respective diols, which are easily converted in the air.

generally transformed by hydroxylation to less or non-carcinogenic compounds.

Nevertheless, these hydrocarbons are also cytotoxic, when added to cell cultures containing the enzyme systems, whereas a lack of toxicity is noticed in the cell lines, where these enzymes are not inducible. A cogent correlation seems to exist between the presence of the enzymes and the cytotoxicity of the polycondensed hydrocarbons (Diamond, 1965) and also between toxicity and carcinogenicity. Thus, the presence of these enzyme systems can be noxious and not solely beneficial.

A chemical linkage between DNA and benzo[a]pyrene (Gelboin, 1969; Grover and Sims, 1968) (or other aromatic hydrocarbons; Grover and Sims, 1968) has been induced by the rat liver aryl hydroxylating enzyme systems.

Brookes and Lawley (1964) have indicated a chemical complex between DNA and polycyclic aromatic hydrocarbons after painting mice with these compounds and isolating their DNA. The extent of the binding correlates satisfactorily with the carcinogenicity of the hydrocarbons.

A chemical linkage has also been obtained between DNA and 1 by promotion with iodine, or hydrogen peroxide in the presence

or absence of ferrous ion, and ascorbic acid model hydroxylating system (Lesko et al., 1969). Furthermore, it is suggested that the activation of the inert aromatic hydrocarbons to react with DNA could be determined by the formation of cationic radical intermediates, and this mechanism of activation could be extended to living systems. Indeed, Rochlitz (1967) had already postulated that the coupling between pyridine and 1, promoted by iodine, proceeds by way of a cationic radical intermediate of the hydrocarbon.

The formation of the chemical complex between DNA and 1, induced either by iodine or model hydroxylating systems, render their suggestion justifiable. However, the action of the model hydroxylating systems on the aromatic substrates, as indicated by Ullrich and Staudinger (1969), is quite different from the iodine catalytic effect. In fact, the model systems produce hydroxylation on the aromatic compounds. Consequently, the benzo[a]pyrene moiety in the DNA chemical complex (vide supra) is very likely the hydroxyl-derivative.

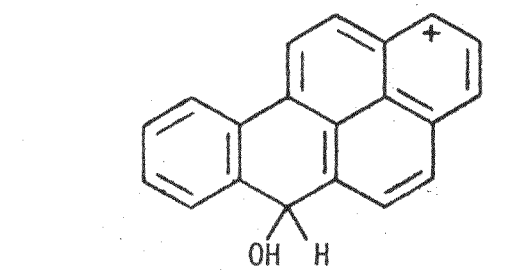
The quantitative comparison of the relative o:m:p ratio in hydroxylation of benzene-derivatives (Ullrich and Staudinger, 1969) provoked by rat-liver microsomal hydroxylase enzymes and

model systems, respectively, points to an electrophilic substitution mechanism for the enzyme systems. The electrophilic nature of the active oxygen produced by the hydroxylating enzymes is also partially substantiated by the general occurrence of the NIH shift (Udenfriend et al., 1969). Moveover, the specific coupling between the most active 6-position of 1 with the nucleophilic 5-position of the 1-methylcytosine (Cavalieri and Calvin, 1970; Pullman and Pullman, 1963) suggests that the chemical fixation of this hydrocarbon in living organisms takes place on the nucleophilic positions of the nucleic acid bases. Therefore, the active hydrocarbon intermediate of 1 should possess an electrophilic nature.

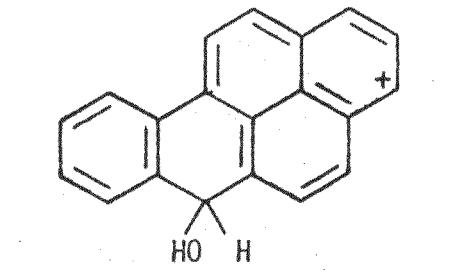
On the basis of all the previously discussed results, the active intermediates formulated in Chart 3 might represent the primary process of carcinogenesis for 1.

The hydroxyl attack induced by the aryl hydrocarbon hydroxylase enzyme should take place, for steric reasons, on the site opposite to that part of 1 that physically interacts with the nucleic acids. When, for instance, the hydroxylation takes place on the 6-carbon atom the charge is localized on the 1-carbon atom 15 and the 3-carbon atom 16. One of the two latter positions will

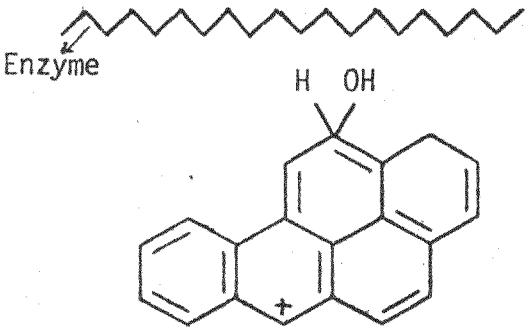
Chart 3



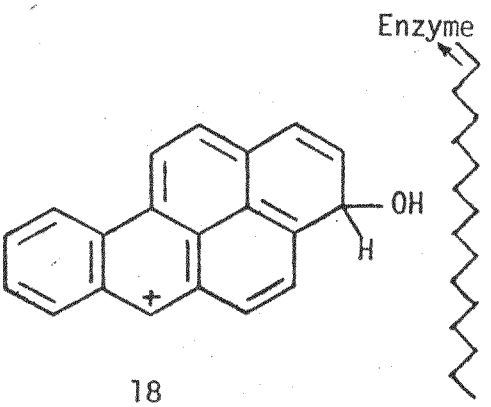

15




16




17




18

further react with the nucleic acid targets. Vice versa, when the active hydroxyls react with the 1-position 17 or the 3-position 18, the charge is completely localized on the 6-position. The criterion of carcinogenicity for 1 seems to be defined by the presence of at least two complementary, active, interrelated positions, which necessarily must be separated by an even number of carbon atoms.

The substitution of the active 6-position of 1 by a methyl group should seemingly render the hydrocarbon non-carcinogenic. However, the deuterium-exchange reaction for 2 indicates the presence of the 5-carbon atom as an active one, in addition to the 1- and 3-carbon atoms. Besides, the 6-position, though substituted, can still remain active for an electrophilic attack of the active oxygen. Further detailed study is required for assessing the carcinogenicity of 2.

## Experimental

The NMR spectra were recorded on a Varian high resolution HR 220 $MH_z$ spectrometer at the ambient temperature (17°) using deuteriochloroform with tetramethylsilane (TMS) as internal standard. The additional stationary radio-frequency field for the double resonance was provided by the oscillator 4204A Hewlett-Packard. The sulfuric acid-$d_2$ (99.5% isotopic purity) was obtained from Merck Sharp & Dohme.

Benzo[a]pyrene 1. The benzo[a]pyrene was purchased from Aldrich and further purified by filtration through a chromatography column containing neutral alumina Woelm activity I and using benzene as solvent. The compound was recrystallized from acetone-methanol and had m.p. 181-182°.

The NMR had $\delta$ 7.75 ($H_8$ and $H_9$, multiplet), 7.89 ($H_4$ and $H_5$, AB system, $J_{4,5} = 9.1\ H_z$), 7.92 ($H_2$, triplet, $J_{1,2} = 7.6\ H_z$, $J_{2,3} = 7.8\ H_z$), 8.02 ($H_1$, quadruplet, $J_{1,2} = 7.6\ H_z$, $J_{1,3} = 1.0\ H_z$), 8.16 ($H_3$, quadruplet, $J_{2,3} = 7.8\ H_z$, $J_{1,3} = 1.0\ H_z$), 8.21 ($H_7$, multiplet), 8.24 ($H_{12}$, doublet, $J_{11,12} = 9.1\ H_z$), 8.41 ($H_6$, singlet), 8.94 ($H_{10}$ and $H_{11}$, doublet).

6-Methylbenzo[a]pyrene 2. The compound was prepared by reduction of 6-formylbenzo[a]pyrene (Fieser and Hershberg, 1938) according to the method of Huang-Minlon (1946). The formylbenzo[a]pyrene (0.500 g, $1.185 \times 10^{-3}$ moles) was dissolved in the minimum amount of dioxane (10 ml). To that solution 0.397 g of potassium hydroxide was dissolved in 0.2 ml of water, 10 ml of triethylenglycol and 1 ml of hydrazine-hydrate 100%. The solution was refluxed (100°) for 1.5 h. After that period hydrazine, water and dioxane were removed by distillation and the temperature was raised to 180-200° for 5 h. The cooled solution was diluted with

water (40 ml) and was neutralized with hydrochloric acid 1 N. The colored precipitate was separated and dried ($Na_2SO_4$). It was then filtered on neutral alumina (Woelm activity I) using chloroform as solvent. The first fraction contained the yellow compound. After recrystallization from acetone-ethanol, it weighed 0.300 g (63% yield) and had m.p. 216.5-217° (lit. m.p. 216.2-216.7°; Fieser and Hershberg, 1938).

The NMR spectrum had $\delta$ 3.20 ($CH_3$ group, singlet, linewidth at half height 1.6 $H_z$), 7.76 ($H_8$ and $H_9$, multiplet), 7.84 ($H_4$, doublet, $J_{4,5}$ = 9.5 $H_z$), 7.89 ($H_2$, triplet, $J_{1,2}$ = 7.6 $H_z$, $J_{2,3}$ = 7.6 $H_z$), 8.02 ($H_1$, quadruplet, $J_{1,2}$ = 7.6 $H_z$, $J_{1,3}$ = 1.4 $H_z$), 8.11 ($H_3$, quadruplet, $J_{2,3}$ = 7.6 $H_z$, $J_{1,3}$ = 1.4 $H_z$, 8.15 ($H_{12}$, doublet, $J_{11,12}$ = 9.1 $H_z$), 8.20 ($H_{10}$, doublet, $J_{9,10}$ = 9.7 $H_z$), 8.45 ($H_5$, multiplet), 8.90 ($H_{11}$, doublet, $J_{11,12}$ = 9.3 $H_z$).

Deuterodeprotonation of Benzo[a]pyrene 1. (a) The compound 1 (25 mg) was partially dissolved under stirring in 1.5 ml of concd. sulfuric acid-$d_2$ at the temperature of 5-10° and left for 120 sec. A deep red solution appeared. The acidic solution was then poured into 10 ml of deuterated water and 5 ml of chloroform, previously chilled. Room temperature was not exceeded following the dilution. The chloroform solution after extraction was

separated and the acidic aqueous-deuterated solution was extracted again with 5 ml of chloroform. The total organic solution was washed with 5 ml of deuterated water and dried ($Na_2SO_4$). After chloroform evaporation the residue left of approximately 20 mg was dissolved in 1 ml of chloroform-d and its NMR was recorded.

(b) The compound 1 (25 mg) was dissolved in 1.5 ml of concd. sulfuric acid-$d_2$ at room temperature and stirred for 60 sec. After that time the same procedure as (a) was followed.

(c) The same conditions as (b) were used when 1 was left for 120, 180, 240, 480 sec in sulfuric acid-$d_2$.

Deuteroprotonation of 6-Methylbenzo[a]pyrene 2. The compound 2 (30 mg) was dissolved in 1.5 ml of sulfuric acid-$d_2$ and left for 120 sec at room temperature under stirring. The solution became green. The same procedure as (a) was followed.

Acknowledgements

We wish to thank Dr. Melvin P. Klein and Dr. Algis Alkaitis for helpful discussions.

References

Antonello, C., and Carlassare, F. (1964), Atti Ist. Lettere Arti, Classe Sci. Mat. Nat. 122, 9 (C.A. 64, 9139e).

Brookes, P., and Lawley, P. D. (1964), Nature 202, 781.

Brookes, P., and Lawley, P. D. (1964), J. Cell. Comp. Physiol. 64, (Suppl. 1), 111.

Bun-Hoï, N. P. and Lavit, D. (1960), Tetrahedron 8, 1.

Cavalieri, E., and Calvin, M. (1970), see the following paper in the present issue.

Cobb, T. B., and Memory, J. D. (1967), J. Chem. Phys. 47, 6, 2020.

Conway, W., and Tarbell, D. S. (1956), J. Amer. Chem. Soc. 78, 2228.

Cook, J. W., Ludwiczak, R. S., and Schoental, R. (1950), J. Chem. Soc., 1112.

Cook, J. W., and Schoental, R. (1950), J. Chem. Soc., 47.

Dewar, M. J. S. (1952), J. Amer. Chem. Soc. 74, 3357.

Diamond, L. (1965), J. Cell. Comp. Physiol. 66, 183.

Dudek, G. O. (1963), Spectrochim. Acta 19, 691.

Falk, H. L., Kotin, P., Lee, S. S., and Nathan, A. (1962), J. Natn. Cancer Inst. 28, 699.

Fieser, L. F., and Campbell, W. P. (1938), J. Amer. Chem. Soc. 60, 1142.

Fieser, L. F., and Hershberg, E. B. (1938), J. Amer. Chem. Soc. 60, 2542.

Gelboin, H. V. (1969), Cancer Res. 29, 1272.

Gold, V., and Tye, F. L. (1952), J. Chem. Soc., 2172.

Grover, P. L., and Sims, P. (1968), Biochem. J. 110, 159.

Huang-Minlon (1946), J. Amer. Chem. Soc. 68, 248.

Lesko, S. A., Ts'o, P. O. P., and Umans, R. S. (1969), Biochemistry 8, 6, 2291.

Martin, R. H. (1964), Tetrahedron 20, 897.

Martin, R. H., Defay, N., Geerts-Evrard,F., and Delavarenne, S. (1964), Tetrahedron 20, 1073.

Moriconi, E. J., Rakoczy, B., and O'Connor, W. F. (1961), J. Amer. Chem. Soc. 83, 4618.

Nebert, D. W., and Gelboin, H. V. (1969), Arch. Biochem. Biophys. 134, 76, and references therein.

Norman, R. O. C., and Taylor, R. (1965), in Electrophilic Substitution in Benzenoid Compounds, Amsterdam, Elsevier Publishing Company, Chapter 8.

Pullman, B., and Pullman, A. (1963), in Quantum Biochemistry, New York, N. Y., Interscience Publishers, Chapter 5, p. 290.

Rochlitz, J. (1967), Tetrahedron 23, 3043.

Roitt, I. M., and Waters, W. A. (1952), J. Chem. Soc., 2695.

Sims, P. (1967), Biochem. Pharmacol. 16, 613.

Streitwieser, A., Jr. (1961), in Molecular Orbital Theory for Organic Chemists, New York, N. Y., Wiley, J. and Sons, Inc., Chapter 11, p. 345.

Udenfriend, S., Daly, J. W., Guroff, G., Jerina, D. M., Zaltzman-Nirenberg, P., and Witkop, B. (1969), in Microsomes and Drug Oxidations, Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., and Mannering, G. J., Eds., New York, N. Y., Academic Press, p. 225, and references therein.

Ullrich, V., and Staudinger, H. (1969), in Microsomes and Drug Oxidations, Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., and Mannering, G. J., Eds., New York, N. Y., Academic Press, p. 199.

Vollmann, H., Becker, H., Corell, M., and Streek, H. (1937), Ann. 531, 51, 130.

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

Windaus, A., and Renhak, S. (1937), Z. Physiol. Chem. 249, 256.

Wood, J. L., and Fieser, L. F. (1941), J. Amer. Chem. Soc. 63, 2323.

TABLE I: Percent[a] of Deuterodeprotonation in Concentrated Sulfuric Acid-$d_2$ (Isotopic Purity, 99.5%).

| Hydrocarbon | Reaction Time (sec) | Positions of Substitution 6 | 1 | 3 | 5 |
|---|---|---|---|---|---|
| Benzo[a]pyrene | 120[b] | 42 | 0 | 0 | 0 |
| | 60 | 96 | 55 | 44 | 0 |
| | 120 | 99.5 | 61 | 48 | 0 |
| | 180 | 99.5 | 73 | 68 | 0 |
| | 240 | 99.5 | 85 | 80 | 0 |
| | 480 | 99.5 | 99.5 | 99.5 | 0 |
| 6-Methylbenzo[a]pyrene[c] | 120 | -- | 99.5 | 99.5 | 21 |

[a]The percent of the deuteration is calculated in the NMR spectra at 2 $H_z$ per cm by the percent ratio of the integration of the peaks corresponding to the partially substituted protons with respect to the integration of the peaks corresponding to the non-substituted protons. The signal of the proton in the 3-position (Figure 1) is half-superimposed. The integration of the visible part is considered half a proton. The substitution reactions have been carried out at ambient temperature unless otherwise specified.

[b]At 5-10°. In these conditions the hydrocarbon was not completely

(Continuation of Footnotes to Table I)

soluble in the acid. [c]Experiments with longer reaction times have produced negative results because of the almost total decomposition of the compound under these severe conditions.

Figure Legends

Figure 1. The 220 $MH_z$ NMR spectrum of benzo[a]pyrene (2% wt/v) in $CDCl_3$ at 17°. The scale is referred to TMS as internal standard. The coupling constants are in hertz ($H_z$) and have been determined by expansion at 2 $H_z$ per cm.

Figure 2. The 220 $MH_z$ NMR spectrum of 6-methylbenzo[a]pyrene (2% wt/v) in $CDCl_3$ at 17°. The scale is referred to TMS as internal standard. The coupling constants are in $H_z$ and have been determined by expansion at 2 $H_z$ per cm. The singlet signal of the methyl protons at 3.20 ppm is out of the field.

Figure 3. A) The 220 $MH_z$ NMR spectrum of benzo[a]pyrene, previously treated with sulfuric acid-$d_2$ for 120 sec at 5-10°. The compound is dissolved in $CDCl_3$ and the spectrum is recorded at 17°, using TMS as internal standard. B) The NMR spectrum of the same compound, previously treated with sulfuric acid-$d_2$ for 60 sec at room temperature, recorded in the same conditions as A). C) The NMR spectrum of the same compound, previously treated with sulfuric acid-$d_2$ for 240 sec at room temperature, recorded in the same conditions as A).

Figure Legends (continued)

Figure 4. The 220 $MH_z$ NMR spectrum of 6-methylbenzo[a]pyrene, previously treated with sulfuric acid-$d_2$ for 120 sec at room temperature. The compound is dissolved in $CDCl_3$ and the spectrum is recorded at 17° using TMS as internal standard.

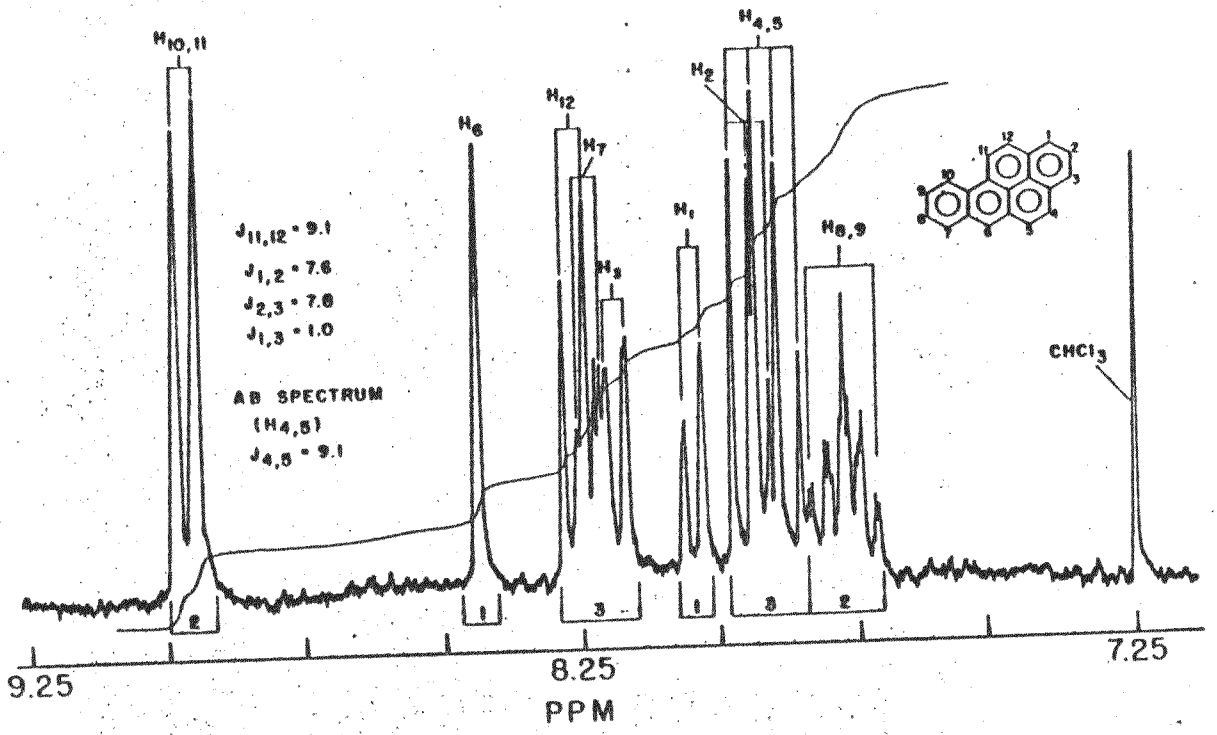



Figure 1. Cavalieri & Calvin

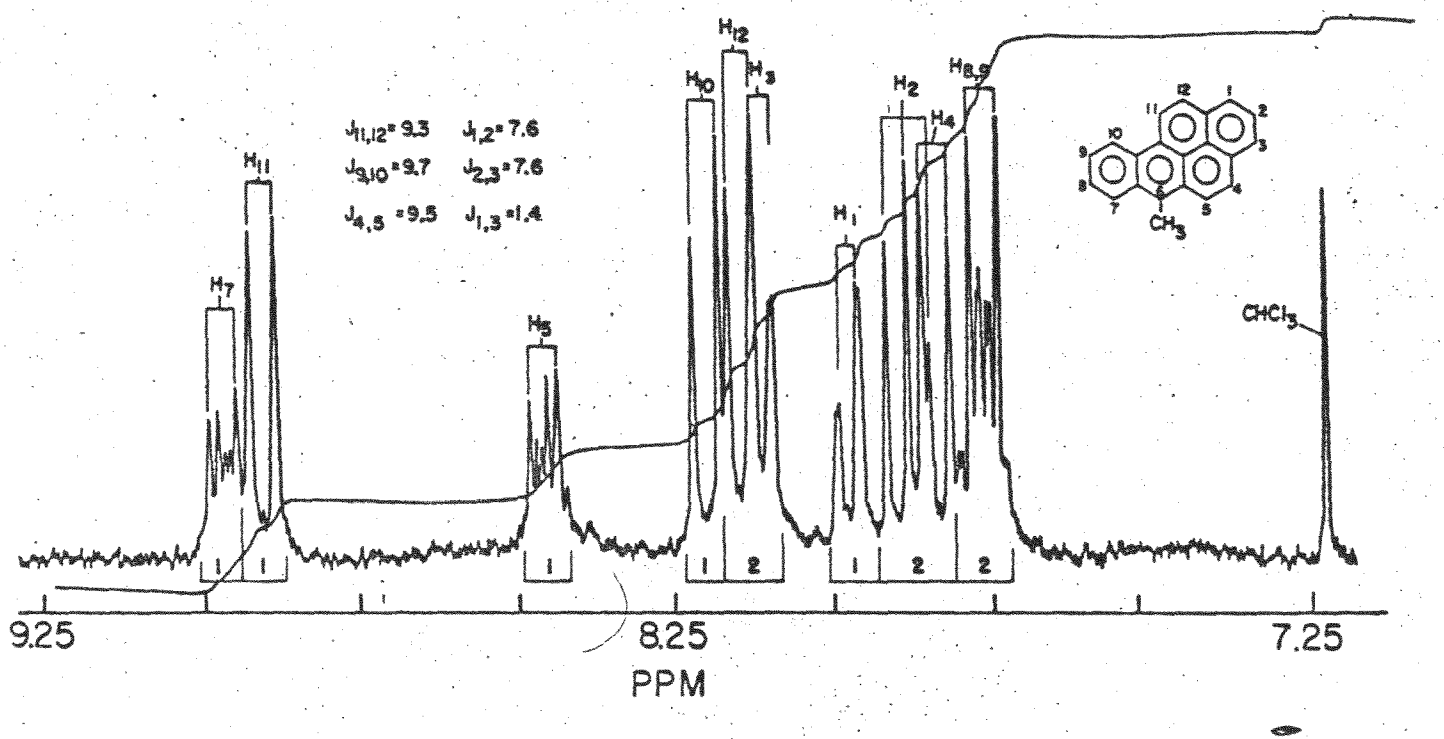



Figure 2. Cavalieri & Calvin

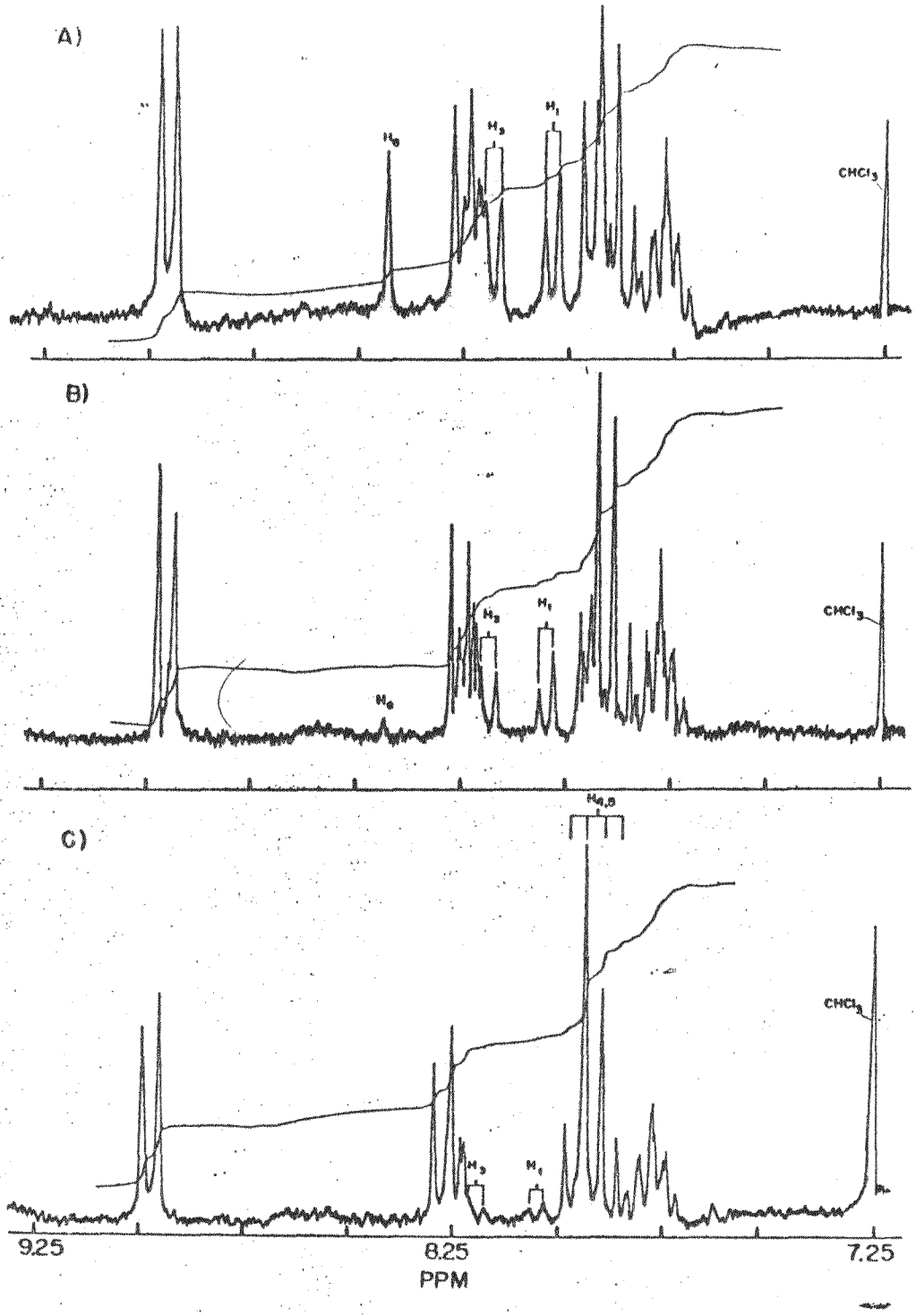



Figure 3. Cavalieri & Calvin

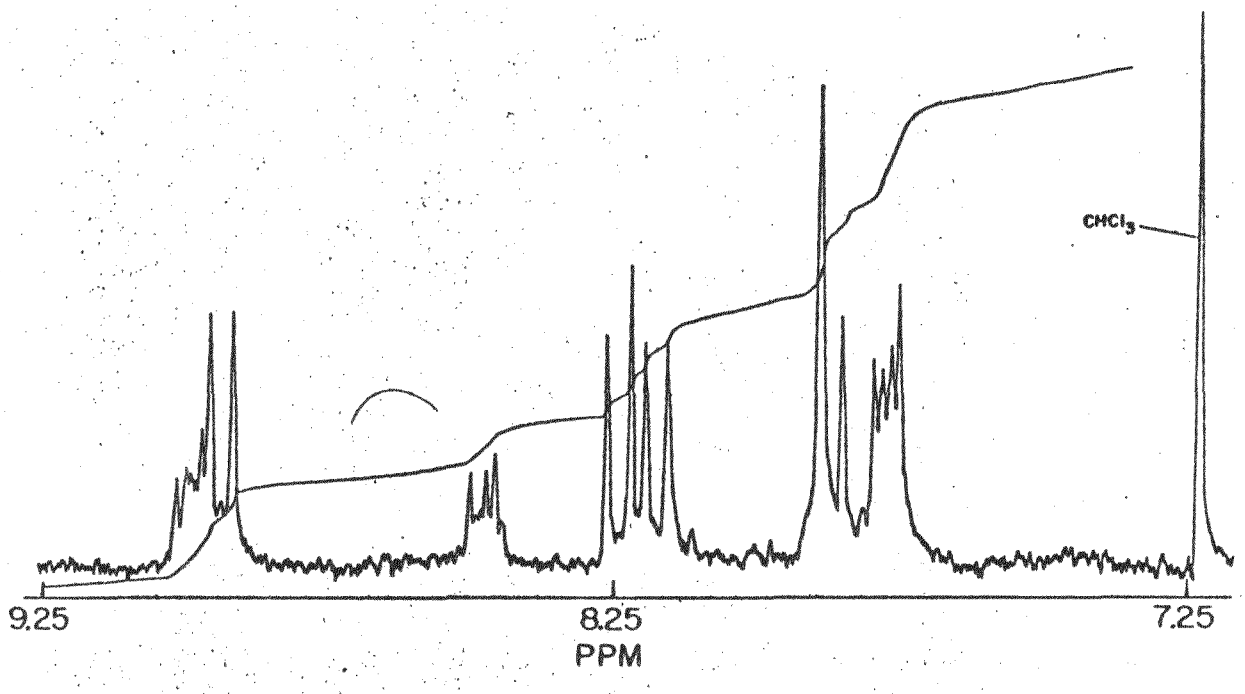



Figure 4. Cavalieri & Calvin

## LEGAL NOTICE