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PHOTOCHEMICAL COUPLING OF BENZO[a]PYRENE WITH 1-METHYLCYTOSINE. POSSIBLE MECHANISM OF THE LINKAGE *IN VIVO*.*

E. Cavalieri and M. Calvin

Running Title:

Photochemical Coupling of Benzo[a]pyrene with Methylcytosine

*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: Irradiation of benzo[a]pyrene 1 with 1-methylcytosine hydrochloride 2a (molar ratio 1:10) at 3500 Å in methanol-acetone produces the 6-(1-methylcytos-5-yl)-benzo[a]pyrene 3. The structure of the photoproduct shows the hydrocarbon bound through the most active 6-carbon atom to the nucleophilic 5-position of the base. The specific substitution of both moieties combined with other data allows us to understand the carcinogenic activity of the hydrocarbon (reported in the previous paper of the present issue) and thereby to propose a possible mechanism of their linkage *in vivo*. In this model, the K region does not play a role in triggering the cancer process.

Introduction

Forty years have elapsed since the first polycyclic aromatic hydrocarbon dibenzo[a,h]anthracene (Kennaway, 1930) was found to be carcinogenic. Since that time many of the polycondensed aromatic compounds have been isolated and an extensive research activity initiated with the hope of finding a relationship between carcinogenic activity and molecular structure. The resolution of this first step contained the hope of shedding light on their likely mechanism of carcinogenesis.

Unfortunately, the results have been unsatisfactory so far. The major goals offering a challenge in this field can be formulated with the following questions: What is the distinctive feature of these hydrocarbons that render them specifically carcinogenic? Are the hydrocarbons *per se* or some metabolites the ultimate carcinogens responsible for the crucial reaction with the cellular receptor? Is the reaction physical or chemical? What is the essential cellular receptor with which they interact? What is the biological specificity capable of provoking the irreversible change from the normal cell to the neoplastic cell? All these questions still await a definitive answer.

Pullman and Pullman (1955) from MO calculations on these hydrocarbons succeeded to some extent in correlating the carcinogenic activity with the relatively high electron density of the meso-phenanthrenic double bond (K region) and the relatively low electron density of the meso-anthracenic positions (L region). They have proposed further that the critical reaction in cancer induction is an addition of the K region to the essential cellular component.

Boyland (1964) postulated that the K region epoxide, which presumably might be a metabolite of the hydrocarbons, is the ultimate carcinogen.

Dipple et al. (1968) offer an alternative hypothesis. The ultimate carcinogen for unsubstituted aromatic hydrocarbons is the carbonium ion corresponding to the open form of the K region epoxide. As far as the initial triggering of the carcinogenic process is concerned, several rival theories have been suggested in the past years.

A line of thought (Boyland, 1962; Pitot and Heidelberger, 1963) argues that the chemical agents bind to the protein repressor molecules which govern the expression of the genome. The imbalance of the genetic information originated by this

modification can provoke a loss of response to the growth-regulating mechanism of the cell. On the other hand, the somatic mutation theory, for the first time formulated by Boveri (1929),suggests the nucleic acids as the essential cellular target. In such a case, the importance of the DNA, as critical cellular component, compared to the RNA, can be considered directly proportional to the extent of validity of the central dogma (Baltimore, 1970; Temin and Mizutani, 1970).

Strong support could be provided to the mutational assumption of tumor initiation, if the different structures of the ultimate chemical carcinogens are capable of modifying the nucleic acid bases and thereby of producing a common biological effect.

The mutagenic properties of the acridine dyes attributed to the intercalation model with DNA, by analogy, suggested an intercalation mechanism for the physical binding of the aromatic hydrocarbons to DNA (Boyland and Green, 1962). However, carcinogenic and non-carcinogenic hydrocarbons show an extent and an affinity of physical binding to DNA which cannot be correlated with the carcinogenic activity (Lesko _et al_., 1968).

Brookes and Lawley (1964) found a relationship among chemical complexes of DNA with aromatic hydrocarbons and carcinogenicity after painting mice with these compounds and isolating their DNA. A chemical linkage between benzo[a]pyrene and DNA by the action of ultraviolet light (Ts'o and Lu, 1964; Kodama and Nagata, 1969/1970) was also obtained.

A covalent binding of benzo[a]pyrene (Lesko et al., 1969) (or 3-methylcholanthrene, or 7,12-dimethylbenz[a]anthracene, Morreal et al., 1968) to DNA was induced by iodine, hydrogen peroxide in the presence or absence of ferrous ion and the ascorbic acid model hydroxylating system.

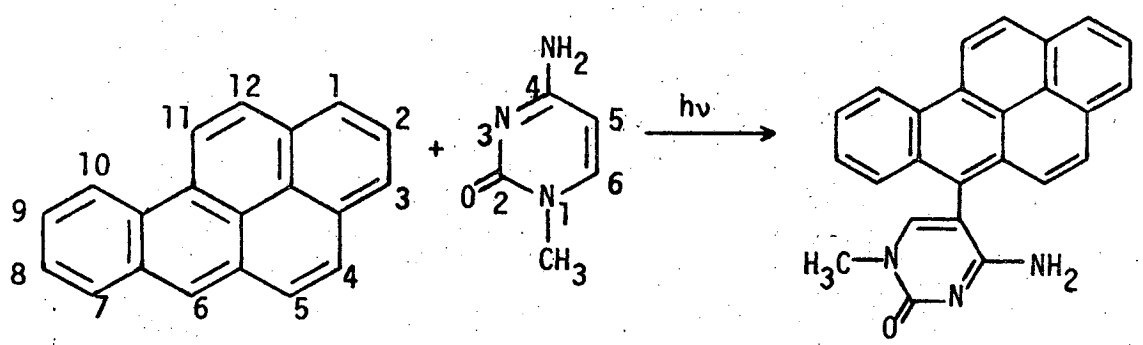
The rat liver microsomal hydroxylating enzyme systems also promoted the same reaction between DNA and carcinogenic aromatic hydrocarbons (Grover and Sims, 1968; Gelboin, 1969).

This paper describes the model photochemical reaction of benzo[a]pyrene 1 with 1-methylcytosine 2. The elucidation of the structure of the photoproduct displays the coupling positions for the compounds 1 and 2. The hydrocarbon is found to bind through the most active 6-carbon atom to the nucleophilic 5-position of the base. The specific substitution of both moieties taken together with other data permits us to understand the carcinogenic

activity of the hydrocarbon (Cavalieri and Calvin, 1970) and to propose a possible mechanism of their linkage *in vivo*.

Results

Photolysis of Benzo[a]pyrene 1 and of 1-Methylcytosine Hydrochloride 2. A mixture of benzo[a]pyrene 1 (0.400 g) and 1-methylcytosine hydrochloride[1] 2a (2.570 g) (molar ratio 1:10) is irradiated at 3500 Å using a Rayonet Photochemical Reactor in 400 ml of a solution of methanol acetone[2] (70:30) for 24 hr.



1 2a, HCl at 3-N 3

[1]The protonation of the nitrogen at the 3-position has been clearly demonstrated by Miles *et al.* (1963).

[2]In an alternate run, water replaced methanol with the same results.

Before the irradiation a stream of nitrogen was bubbled through the solution for 1 hr. Under these photochemical conditions only the aromatic hydrocarbon was excited. At the end of the photolysis a small amount of a new compound, shown to be 3, in addition to the two starting materials, was detected on thin-layer chromatography (tlc); it had an Rf value between those of the hydrocarbon 1 (less polar) and of the base 2a. After the evaporation of the solvents, the residue was treated with ether and most of 1 was dissolved. The remaining solid was then dissolved in a mixture of water-chloroform and the new compound was extracted in the organic solvent. The chloroform solution was washed with 5% sodium bicarbonate and then with water.

After purification on column chromatography and then on preparative tlc, the yellow compound 3 was recrystallized from acetone-hexane and had m.p. 331-33° (dec.). The elemental analysis is consistent with 1:1 benzo[a]pyrene-methylcytosine complex. The spot of the compound 3 on silica gel tlc using the aprotic solvent systems, benzene-acetone (17:30) or pure acetone, does not move. A solution containing only 2% of water, using acetone-benzene-water (58:40:2), is sufficient to displace the same spot. No decomposition of the compound 3 is indicated by

tlc and ultraviolet (UV) spectra after boiling for 24 hr in methanol-water. The product is equally stable after treatment with 1 N hydrochloric acid for 2 hr at 100° or with 1 N sodium hydroxide for 12 hr at room temperature.

When a mixture of 1 and 1-methylcytosine 2 rather than 2a was irradiated only trace amounts of the new product were revealed by tlc. The comparison of this product with the compound 3 showed the same Rf on tlc and the same UV spectrum. In contrast, the non-carcinogenic benzo[e]pyrene 1 and 1-methylcytosine hydrochloride 2a or 1-methylcytosine 2, when photolyzed under the same conditions, formed no new product.

A chemical attempt to get the same coupling between the compounds 1 and 2a (molar ratio 1:10) in acetone-water (66:33), using as promoter iodine or hydrogen peroxide in the presence of ferrous sulfate, was unsuccessful.

Elucidation of the Photoproduct Structure 3. The structure of compound 3 has been elucidated by the elemental analysis (vide supra and Experimental), the infrared spectrum, the ultraviolet spectrum, and the nuclear magnetic resonance (NMR) spectrum.

The infrared spectrum shows characteristic absorptions at 3520 and 3400 cm^{-1} attributable to the NH primary amine, 1655 and

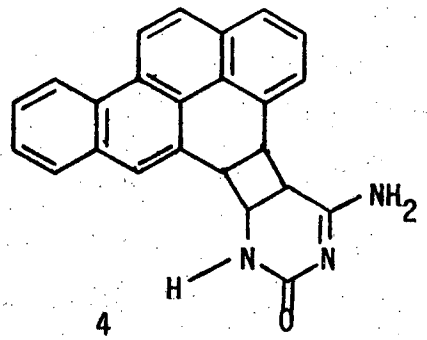
1590 cm^{-1} ascribable to the carbonyl vibration of a δ-lactam and to the bending vibration of an amine, respectively. The disappearance of the characteristic strong absorption of the benzo[a] pyrene at 870 cm^{-1}, corresponding to the out-of-plane bending band of the CH group at the 6-position (penta-substituted benzene) offers some evidence that such a position is involved in the attachment to the base.

The UV spectrum (Figure 2C) has the same absorption maximum as benzo[a]pyrene (Figure 2A) but all the bands are, as compared to the hydrocarbon, shifted to longer wavelengths by 3-10 mμ. This displacement toward the red is the same as 6-methylbenzo[a]pyrene (Figure 2B). The molar absorption coefficients for the three compounds, corresponding to the wavelengths of maximum absorptions are reported in Table I. The striking similarity of the two UV spectra of the methyl- and methylcytosyl-benzo[a]pyrene strongly suggests for the latter the attachment to the hydrocarbon through a substitution at the 6-position.

Before discussing the NMR spectrum of the photoproduct 3, which will enable us to establish the specific points of linkage of the base and of the hydrocarbon, it is useful to take into account the structures formerly proposed for these kinds of compounds.

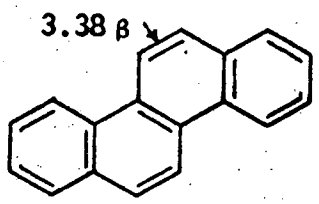
Rice (1964) obtained stable products in poor amounts when benzo[a]pyrene was irradiated in the presence of uracyl, thymine, cytosine, 5-methylcytosine, guanine and 6-azathymine. The UV spectra of these compounds presented the same absorption maximum

and were the same as in Figure 2C. He proposed for cytosine and benzo[a]pyrene the cyclobutane adduct 4, involving the 4,5-double



4

bond of the hydrocarbon and the 5,6-double bond of the base. The 4,5-double bond of the benzo[a]pyrene is the K region proposed by the Pullmans (see Introduction). The rationale of their theory largely contributed to the formulation of the adduct 4. This structure offers two major discrepancies. (i) The UV spectrum of the hydrocarbon, where the 4,5-double bond is reduced as in 4, is supposed to be similar to the UV spectrum of chrysene. The comparison of the UV spectra of chrysene 5 and 4,5-diacetoxy-4,5-dihydrobenzo[a]pyrene 6,[3] as shown in Figure 3, are truly similar



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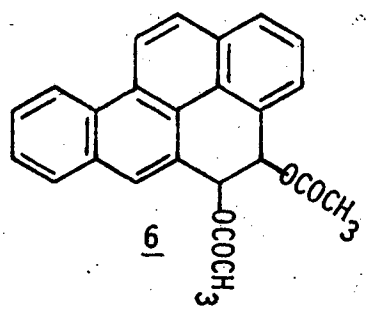

6

[3]This compound was prepared in the manner described by Cook and Schoental (1948).

and thus, the structure 4 for the photoproduct can be regarded as unlikely. (ii) The same UV spectra were obtained from the photoproducts of benzo[a]pyrene and pyrimidine bases or purine bases as, e.g., guanine. While it is easy to speculate about the possibility of a cycloadduct of the hydrocarbon with the active 5,6-double bond of the pyrimidine bases, it is arduous to imagine the same derivative with guanine.

The latter argument is also valid in causing serious doubts about the structure of the cyclobutane adducts proposed by Antonello et al. (1968) for the same photochemical products. In this case the 7,8-double bond of the hydrocarbon is suggested to form cycloaddition with the pyrimidine bases. The structure of the photoproducts is supported by the similarity of these compounds with the 7,8-dihydrobenzo[a] pyrene.

The analysis of the 220 MH_Z NMR (Figure 4) shows the characteristic methyl protons of the cytosine moiety at δ 3.51. The same protons in the NMR spectrum of 1-methylcytosine in dimethyl-d_6-sulfoxide are at δ 3.15. The two broad singlets at δ 4.52 and 6.64 are exchangeable with deuterated water and thus correspond to the amino protons. In 1-methylcytosine these two acidic protons possess a unique

chemical shift at δ 6.92. The difference of their chemical shifts in the compound 3 results from hindered rotation of the amino group, as already suggested earlier for the cytosine hydrochloride (Katritzky and Waring, 1963), and for the 1-methylcytosine hydrochloride (Miles et al., 1963).

The two doublets at δ 5.54 and 7.44 in 1-methylcytosine, corresponding respectively to the protons in 5- and 6-position have disappeared in the compound 3 (Figure 4). This might imply, at first glance, that the 5,6-double bond of 2 is involved in the reaction with the hydrocarbon. However, if this bond has been reduced, the corresponding protons must be strongly shifted to the higher field, as it can be observed from the NMR spectrum of 5,6-dihydro-1-methylcytosine in chloroform-d (see Experimental), where the protons in 5- and 6-position are found at δ 2.65 and 3.33, respectively.

The expanded spectrum of 3 in the low field region (Figure 5) permits one to visualize the aromatic protons. The integrated spectrum shows twelve protons determined relatively to the protons of the methyl group (Figure 4) and provides the relative ratio from left to right as 2:1:1:2:1:1:3:1.

The comparison of this spectrum with the spectra of benzo[a] pyrene 1 and 6-methylbenzo[a]pyrene (Cavalieri and Calvin, 1970), which have been completely interpreted, shows the absence of the characteristic singlet peak at δ 8.41, corresponding to the proton in the 6-position. A similar absence has been observed in the spectrum of the 6-methylbenzo[a]pyrene. Therefore, the hydrocarbon is substituted at the 6-carbon atom. The two angular protons H_{10} and H_{11} are clearly identified for their largest downfield shift. The H_{12} is also assigned on the basis of the same coupling constant as H_{11} and about the same chemical shift (slightly deshielded) as in benzo[a]pyrene. The two broad bands centered at δ 8.68 and 9.36 are spinning side-bands.

The other protons of the hydrocarbon moiety are only tentatively assigned. The doublet at δ 8.24 (J = 7.0 H_z) is probably the proton in 7-position, which is not deshielded compared to the benzo[a]pyrene, although the presence of the base substituted in 6-position should provide a deshielding effect. The H_4 is suggested to be the doublet at δ 7.92 for its chemical shift and coupling constant (J = 8.8 H_z). The chemical shift and the coupling constant (J = 7.5 H_z) also decide for the attribution of H_1. In the higher field the

complex multiplet centered at δ 7,80 contains three protons, which for their chemical shift are tentatively assigned as the protons at the 2-, 8-, and 9-positions. The two protons at δ 8.08 are the H_3 and H_5. This band system is a superimposition of two doublets. The singlet at δ 7.35, that was not found in the spectra of benzo[a]pyrene and 6-methylbenzo[a]pyrene must necessarily belong to the methylcytosine moiety. The chemical shift is consistent with the proton at the 6-position. The methylcytosine in dimethyl-d_6-sulfoxide (the base is insoluble in chloroform) shows this signal at δ 7.44. The slight shift difference is compatible with the solvent effect. The spectra of 5,6-dihydro-1-methylcytosine in chloroform-d and dimethyl-d_6-sulfoxide present a shift difference of the respective protons of the same order of magnitude (see Experimental). The absence of the signal corresponding to the proton in the 5-position and the resulting singlet in the 6-position clearly signify that the base is substituted at the 5-position.

The UV spectrum, previously discussed, has the same absorption maximum as the 6-methylbenzo[a]pyrene, indicating that the planar ring of the pyrimidine base is not conjugated to the aromatic hydrocarbon and thus, must be perpendicular to the same.

The hydrocarbon is substituted at the 6-carbon atom, which is known to be the most active one.

The same UV spectra for the photoproducts (Rice, 1964; Antonello et al., 1968) arising from benzo[a]pyrene and all bases of the nucleic acids suggest that the same position of the aromatic hydrocarbon is bound to the bases. Therefore, the 4,5-double bond (K region) does not play any role in this reaction with these biological substrates.

Rationalization of the Photochemical Coupling. Although no evidence is reported about the electronic distribution of the polycondensed aromatic hydrocarbons in their excited states, it is possible to infer a possible photochemical mechanism of coupling between benzo[a]pyrene and methylcytosine based on the following data: (i) The cationic radicals are the species produced by chemical (Lewis and Singer, 1965; Fried and Schumm, 1967) and electrochemical (Marcoux et al., 1967) oxidations. The one electron oxidation in these conditions gives rise to the reactive intermediates, which can be stabilized by addition of a nucleophile followed by dimerization of the resulting radical or further oxidation of the latter to yield a second cation and subsequent reaction with a second nucleophile. (ii) The methylcytosine

is substituted by the hydrocarbon at the 5-position, which has been postulated by theoretical calculations to be the most nucleophilic one, both in the free base and in the cytosine-guanine complementary pair (Pullman and Pullman, 1963). The reactive intermediate of the hydrocarbon produced by UV irradiation is then presumably a radical cation derived from the positive hole created by the excitation of the molecule. The positive charge which results is localized on the meso-anthracenic position. (iii) Further evidence concerning the above-proposed photochemical mechanism is given by the comparison with the mechanism of coupling between benzo[a]pyrene and pyridine in the presence of iodine, suggested by Rochlitz (1967) and presented in Chart 1.

The photochemical coupling is thus rationalized as in Chart 2. The excited benzo[a]pyrene, possessing the electronic distribution of a radical cation[4] undergoes the nucleophilic substitution of the base on the positive 6-position. The loss of hydride ion following the return of the excited electron to the ground state and subsequent rearrangement restores the

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[4]The radical is suggested to be stabilized on the 1-carbon atom as shown in Chart 2 or on the 3-carbon atom (Cavalieri and Calvin, 1970).

Chart 1

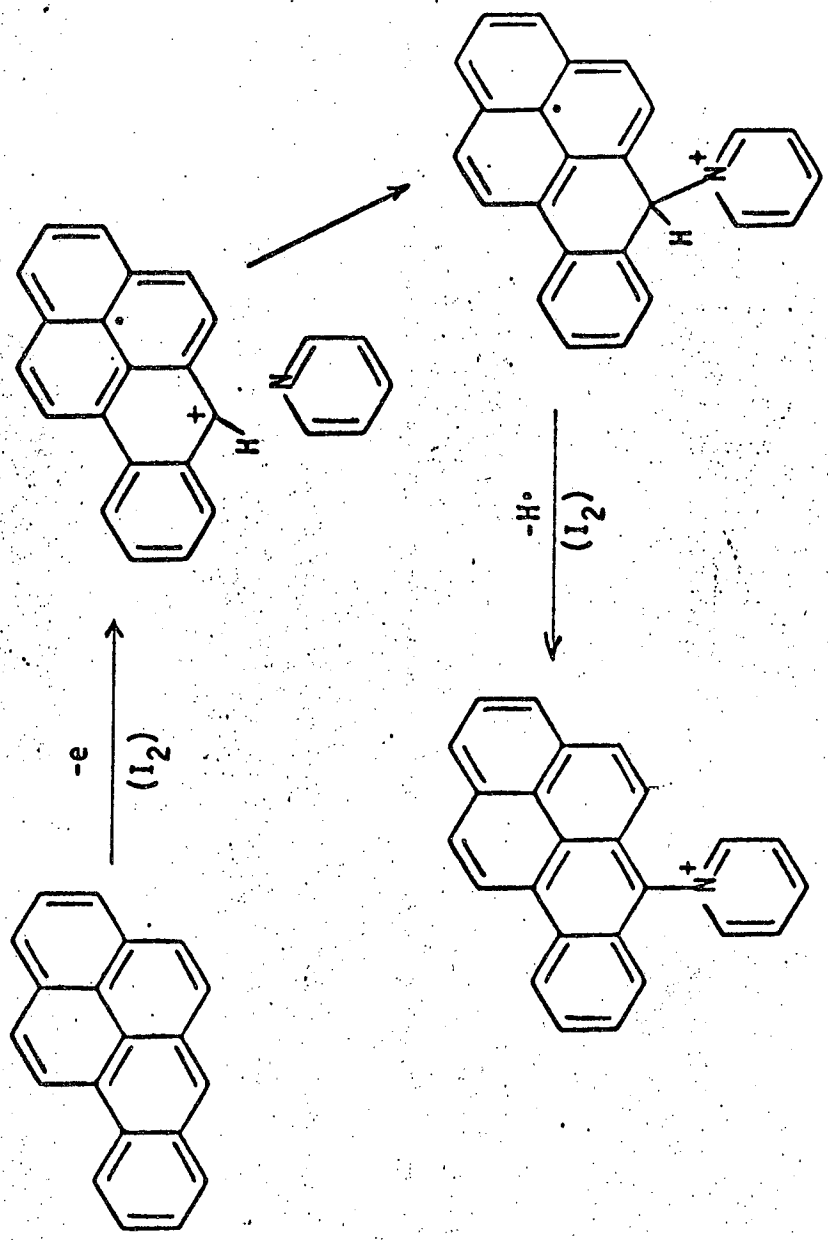
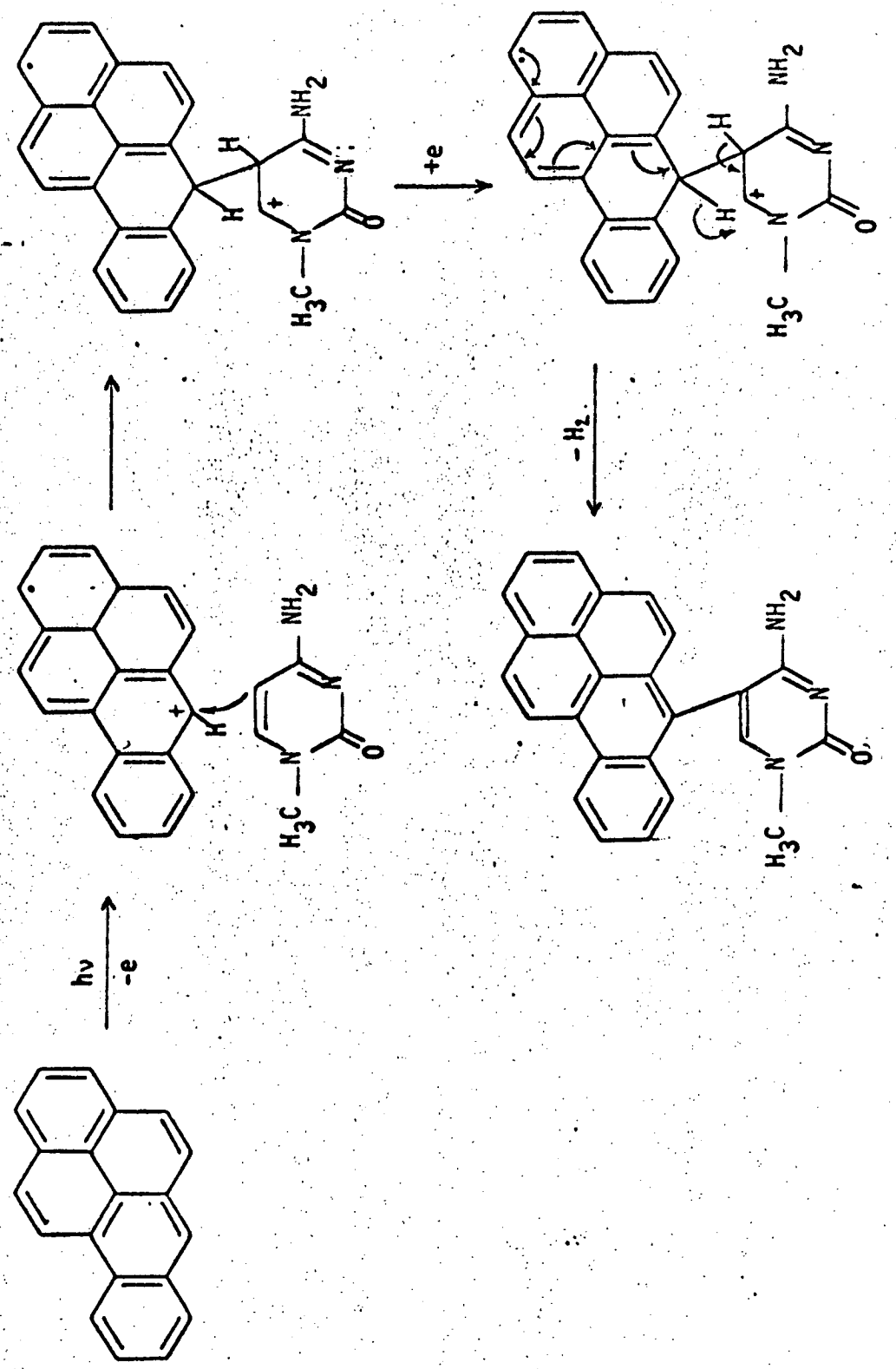


Chart 2



aromatic configuration of the hydrocarbon, whereas the loss of a proton on the 5-carbon atom of the base reestablishes the 5,6-double bond.

Negative Evidence of the K Region. It has been demonstrated that cancer induction on the mouse skin is increased by UV light (Santamaria et al., 1966) when a definite concentration of benzo[a]pyrene and intensity of radiation are utilized. The photodynamic action of the aromatic hydrocarbon might be interpreted as an excitation of the molecules which, so activated, should bind to a higher extent on the cellular receptor than the non-irradiated one.

The capacity of binding, in accordance with the Pullman theory (see Introduction) is directly related to the presence of the electronically rich K region. If the energy required to localize the two electrons is below a set value[5] the aromatic hydrocarbon forms an addition reaction with the biological receptor and thereby induces cancer.

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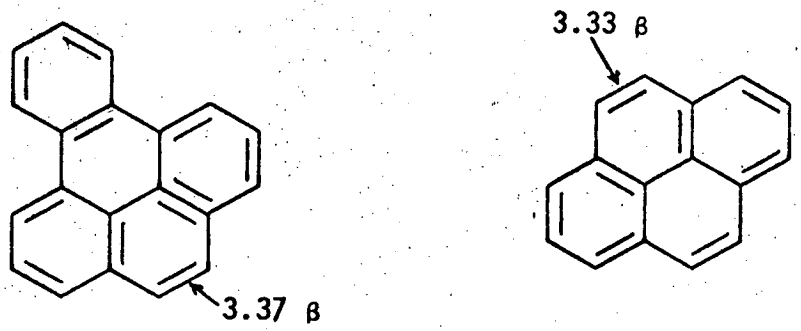
[5]The limit value of the K region set by Pullman and Pullman (1955) is a complex index equal to 3.31 β, where β for the polycondensed aromatic hydrocarbons is about 20 kcal/mole.

The possibility of getting a carcinogenic effect from non-carcinogenic hydrocarbons, under the action of the UV light, was investigated. The intention was to provide an experimental support to the Pullmans' theoretical assumptions.

The hydrocarbons benzo[e]pyrene 7 and pyrene 8 were chosen, since they are characterized by a similar structure to the benzo[a] pyrene 1 (K value, 3.23 β) and possess K values slightly higher



7 8

than the limit of carcinogenicity.[5] The latter reason was likewise responsible for the choice of chrysene 5.

The experiment was carried out on 180 mice. Swiss albino mice of both sexes, seven weeks old, were used. The mice were shaved once a week in the interscapular area and painted twice a week with 0.1 mg of benzo[a]pyrene 1 in acetone (one drop of acetone solution delivered by an automatic dispenser) or with the corresponding molar amount of the other non-carcinogenic hydrocarbon (vide supra). They were fed with Simonsen food and water

was provided ad libitum. The 180 mice were divided into nine groups treated respectively: a) benzo[a]pyrene, a') benzo[a] pyrene plus UV light, b) benzo[e]pyrene, b') benzo[e]pyrene plus UV light, c) pyrene, c') pyrene plus UV light, d) chrysene, d') chrysene plus UV light, e) only UV light. The painting was performed twice weekly by delivering a drop in the clipped interscapular area which was about 1 cm^2. The irradiations were carried out four times a week and two hours a day, using a 100 watt UV lamp with maximum output at 3660 Å and keeping each group of 20 mice in plastic containers 10 x 10 cm and 3 cm high, covered by a metallic net. The intensity of the UV light, delivered by a bulb lamp at 20 cm from the mice, was 8550 uW/cm^2. A fan system directed at the light beam regulated the temperature at 25° at the level of the mice. The experiment lasted 18 weeks. After that period no tumors were disclosed in the irradiated only group or in the groups both irradiated and non-irradiated, treated with the non-carcinogenic chrysene 5, benzo[e]pyrene 7 and pyrene 8. The group treated with the benzo[a]pyrene plus irradiation presented 63% of the tumors.

These data for the hydrocarbon 1 confirmed the previous results, namely, that there is an increase of cancer induction in the mice painted and irradiated as compared to the mice only

painted with the same compound. If the extent of binding to the biological constituents accounts for the photodynamic action of 1, the K region is not responsible for this effect since the most active position is the 6-carbon atom, as above described for the photochemical reaction between the hydrocarbon and methylcytosine. The absence of tumors in mice treated with benzo[e] pyrene, pyrene, and chrysene and irradiated is another bit of evidence against the biological importance of the K region in triggering the cancer process.

Discussion

The rationalized mechanism of the photochemical reaction enables us to correlate the excited state chemistry of the benzo[a] pyrene 1 to its ground state chemistry.[6] Therefore, the model system is adequate for understanding either the carcinogenicity of the hydrocarbon or the possible mechanism of binding *in vivo*. Microsomal hydroxylating enzyme systems are likely to induce the

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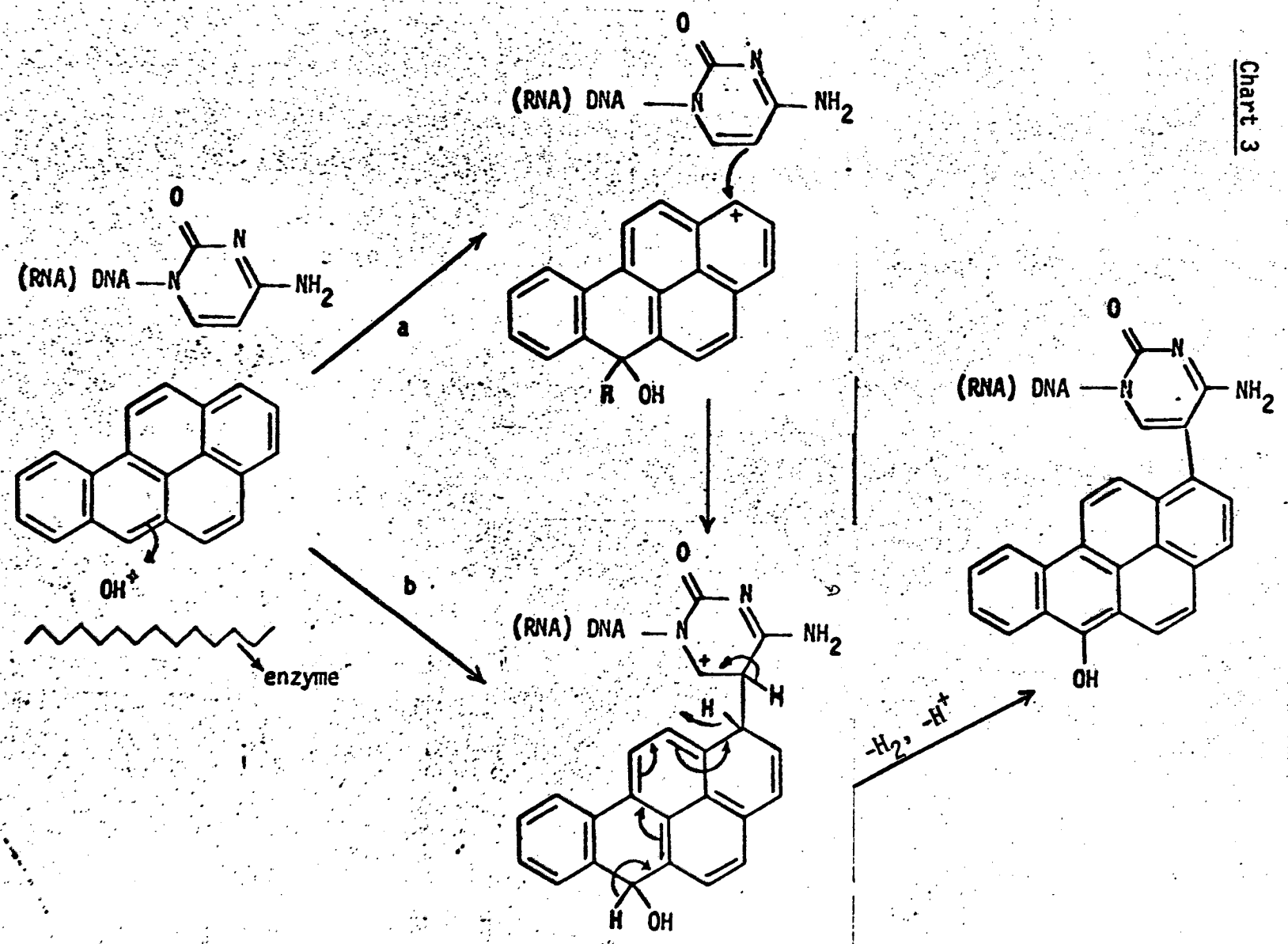
[6]An outline of the chemistry of 1 is presented in the previous paper (Cavalieri and Calvin, 1970).

covalent binding of the polycyclic aromatic hydrocarbons to the nucleic acids *in vivo* (Grover and Sims, 1968; Gelboin, 1969). In the previous paper it has been described how the electrophilic active oxygen produced by these enzyme systems engenders electrophilic active intermediate(s) for 1, which can react with the nucleophilic positions of the nucleic acid bases. The carcinogenicity of 1 has been attributed to the presence of at least two complementary, active, interrelated positions, which must necessarily be separated by an even number of carbon atoms.

Supposing that cytosine is one of the critical receptor sites of the nucleic acids, the following scheme of activation-reaction (Chart 3) can be formulated. Path a shows an initial electrophilic attack of the active oxygen on the 6-carbon atom. The positive charge produced is mainly localized in the 1-position (as depicted in Chart 3) and the 3-position. This reactive intermediate is stabilized further by addition to the nucleophile cytosine. The loss of hydride ion and two protons yields the cytosylhydroxybenzo[a]pyrene. Path b presumes a concerted electrophilic and nucleophilic attack of the active oxygen and cytosine, respectively, and the final stabilization as in path a.

Chart 3



In an alternative possibility, if the 1- or 3-positions of the hydrocarbon undergo the first electrophilic substitution of the active oxygen, the reactive intermediate exhibits the positive charge localized at the 6-position. In this case the latter position will add to the biological nucleophile following the pattern a or b, as above described.

The proposed mechanism implies a specific stereochemical arrangement of the benzo[a]pyrene with respect to the nucleic acids. In fact, one of the three active carbon atoms of the hydrocarbon must be situated in close proximity to the nucleophilic positions of the nucleic acid bases. The requirement is quite obvious in suggesting the concerted mechanism (path b). Indeed, the same requirement is also essential for path a. In this case, if the biological nucleophile is not closely available, the reactive carbon ion will be stabilized by loss of a proton to give a hydroxy derivative of 1, or by addition of water to form a diol derivative of 1. Thus, although the physical benzo[a]pyrene-nucleic acids complex does not constitute in itself the determining carcinogenic step (see Introduction), it participates in the preliminary step that leads to the formation of the critical covalent bond. The suitable physical complex might also depend on other factors like molecular shape and size of the hydrocarbon.

According to the above mechanism, the nucleic acid can be contemplated as a key site in the carcinogenic process only if it is located in the vicinity of the hydroxylase enzyme systems, which are found on the endoplasmic reticulum. The ribosomal and messenger RNA satisfy this prerequisite. The coupling of the hydrocarbon with these RNA molecules probably induces an anomalous expression of the genome. Nevertheless, it is not known how critical this disturbance can be in determining the neoplastic transformation of the cell.

The mutational hypothesis of the cancer initiation invokes preferably the chromosomal DNA as essential cellular receptor. The few data supporting the existence of the oxidative enzymes in the nuclei (Rees and Rowland, 1961; Penniall et al., 1964) have been considered doubtful since the possibility of contamination cannot be excluded. However, the recent results demonstrating that nuclei have their own unique electron transport system (Berezney et al., 1970) validate the previous findings. Therefore, the DNA also possesses the requirement of being closely associated with oxidative enzyme systems.

All bases of the nucleic acids react photochemically with benzo[a]pyrene to give traces of products (Antonello et al., 1968).

The same UV spectra for all products presumably indicate that the 6-position of the hydrocarbon is involved.

MO calculations (Pullman and Pullman, 1963) predict the 5-position of the cytosine and the 8-position of the guanine to be the most susceptible to an electrophilic attack. Recent results show that when the benzo[a]pyrene is mixed with the four homopolynucleotides in the presence of iodine, the hydrocarbon reacts more extensively with polyguanine (Hoffman et al., 1970). It is also the 8-position of the guanine that undergoes an electrophilic attack by the carcinogen 2-acetylaminofluorene N-sulfate (De Baun et al., 1970).

The similar modification of the nucleic acid bases produced by benzo[a]pyrene and fluorene derivative leads to the idea of a common primary biological effect, as a consequence of these changes.

Experimental Details

Irradiations were carried out in a quartz vessel, using a Rayonet Photochemical Reactor, Model RPR-208 (8 low pressure Hg lamps, 2500 Å). The alumina used for column chromatography was obtained from Woelm and the activity described is in accordance

with the prescriptions of the Company. Thin-layer chromatography was carried out on Eastman Chromagram sheets coated with silica gel containing a fluorescent indicator. Preparative thin-layer chromatography was done on glass plates coated with 1 mm thick silica gel containing fluorescent indicator. Melting points were determined on Büchi apparatus and are uncorrected. The infrared spectra were recorded on a Perkin-Elmer, Model 257. The NMR spectra were scanned on a Varian high resolution HR 220 MH_z spectrometer with tetramethylsilane as internal standard.

The UV spectra were recorded on a Cary, Model 14, recording spectrophotometer. The 100 watt UV lamp for irradiating mice was a Black-Ray, Model B-100A, with flood bulb (maximum outout 3660 Å) and 5-inch round filter produced by Ultraviolet Products, Inc. (San Gabriel, Calif.).

The Swiss albino mice were obtained from Bio-Sciences Laboratory (Oakland, Calif.).

<u>1-Methylcytosine 2</u>. 1-Methylcytosine was obtained from Cyclo Chemical and had m.p. 305-307° (lit., m.p. 303°; Hilbert, 1934). The NMR (dimethyl-d_6-sulfoxide) had absorptions at δ 3.15 (3H, singlet), 5.54 and 7.44 (1H each, doublet, J = 7.0 H_z) and 6.92 (2H, broad singlet).

1-Methylcytosine Hydrochloride 2a. 1-Methylcytosine (1.950 g, 1.56×10^{-2} moles) was dissolved in 150 ml of methanol. To that solution an excess of hydrochloric acid dissolved in methanol was added. By dilution with 300 ml of ether the 1-methylcytosine hydrochloride (2.260 g, 90%) precipitated and had m.p. 292-293°.

Photolysis of Benzo[a]pyrene 1 and 1-Methylcytosine Hydrochloride 2a. A solution of 1 (0.400 g, 1.59×10^{-3} moles) in 120 ml of distilled acetone was mixed with a solution of 1-methylcytosine hydrochloride (2.570 g, 1.59×10^{-2} moles) in 280 ml of absolute methanol. The resulting solution was bubbled with a stream of oxygen-free nitrogen for 1 hr and further irradiated at 3500 Å for 24 hr. The tlc on silica gel using acetone-benzene-water (70:20:10) indicated a small amount of a new compound, showing the Rf between the two starting materials, still present in large amount. After evaporation of the solvent, the residue was taken up in ether and most of 1 passed into solution. The insoluble material was dissolved in a mixture of water-chloroform. After extraction the organic layer containing the new product and some residual 1 was washed with 5% sodium carbonate and further with water saturated by ammonium sulfate. The chloroform solution was dried (Na_2SO_4) and, after evaporation, was chromatographed

on Woelm alumina activity IV. The first fractions, eluted with chloroform-benzene (1:1) contained 6-(1-methylcytos-5-yl)-benzo[a] pyrene 3 (30 mg). The new compound was then better purified on preparative tlc coated with silica gel and recrystallized from acetone-hexane. A yellow solid with m.p. 331-333° (dec.) was obtained.

Anal. Calcd. for $C_{25}H_{19}N_3O$: C, 79.56; H, 5.07; N, 11.13.
Found: C, 79.46; H, 5.22; N, 10.61.

The infrared spectrum showed bands at 3520, 3460, 3400, 1655, 1590 cm^{-1}. The UV spectrum had maximum absorptions at 258, 268, 278, 290, 302, 340, 357, 375, 395, 407.5 mμ. The NMR spectrum possessed absorptions at δ 3.51 (3H, methyl group, singlet), 4.52 (1H, NH broad band exchanged with D_2O), 6.64 (1H, NH broad band exchanged with D_2O), 7.35 (1H, H_{6MC}, singlet), 7.70-7.89 (3H, H_2, H_8, and H_9, multiplet), 7.92 (1H, H_4, doublet, J = 8.8 H_z), 7.99 (1H, H_1, doublet, J = 7.5 H_z), 8.08 (2H, H_3 and H_5, doublet), 8.24 (1H, H_7, doublet, J = 7.0 H_z), 8.34 (1H, H_{12}, doublet, J = 8.8 H_z), 9.02 (1H, H_{11}, doublet, J = 8.8 H_z, and 9.04 (1H, H_{10}, doublet).

1-Methyl-5,6-dihydrocytosine. 1-Methyl-5,6-dihydrocytosine was prepared by hydrogenation of 1-methylcytosine according to

the method of Green and Cohen (1957) with some modifications. 1-Methylcytosine (125 mg, 1 x 10^{-3} moles) was dissolved in 40 ml of absolute methanol. To that solution 5% of rhodium on alumina (200 mg) was added. When one equivalent of hydrogen was consumed, the reaction was terminated. After evaporation of the solvent, the solid obtained revealed by tlc (acetone-benzene-water, 70:20:10) the new product with traces of 1-methylcytosine. Two recrystallizations from methanol-acetone removed the starting material. The purified product had m.p. 223-225° (lit., m.p. 223-25°, Cheng and Lewis, 1964). The NMR spectrum (dimethyl-d_6-sulfoxide) showed at δ 2.79 (3H, singlet), 2.41 and 3.20 (each 2H, triplet, J = 7.0 H_z) and 7.70 (2H, broad singlet). The NMR spectrum in chloroform-d exhibited at δ 2.93 (3H, singlet), 2.65 and 3.33 (each 2H, triplet, J = 7.0 H_z).

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TABLE I: Molar Absorption Coefficients $[\lambda_{max}(\epsilon_{max})]$.

Benzo[a]pyrene	254.5(53,100) 265(56,500) 272(36,400) 284(49,000) 296(59,500) 332(4930) 347(11,900) 365(22,900) 385(24,900) 404(3150)
6-Methylbenzo[a]pyrene	256(31,000) 267(34,200) 277(18,200) 288(32,400) 300(45,800) 340(4550) 356(11,500) 374(21,600) 394(22,800) 409(5000)
6-(1-Methylcytos-5-yl-benzo[a]pyrene	258(62,700) 268(64,000) 278(43,000) 290(50,300) 302(48,700) 340(6080) 357(13,600) 375(27,800) 395(30,600) 407.5(9430)

Figure Legends

Figure 1. The infrared spectrum in chloroform of the 6-(1-methylcytos-5-yl)benzo[a]pyrene.

Figure 2. The ultraviolet spectra in 95% ethanol of benzo[a] pyrene (A), 6-methylbenzo[a]pyrene (B), 6-(-methylcytos-5-yl)-benzo[a]pyrene (C).

Figure 3. The ultraviolet absorption spectra of 4,5-diacetoxy-4,5-dihydrobenzo[a]pyrene (A) and chrysene (B).

Figure 4. The 220 MH_z NMR spectrum of 6-(1-methylcytos-5-yl)-benzo[a]pyrene in saturated solution of chloroform-d (isotopic purity 100%) at 17°.

Figure 5. The 220 MH_z NMR expanded spectrum of 6-(1-methylcytos-5-yl)-benzo[a]pyrene.

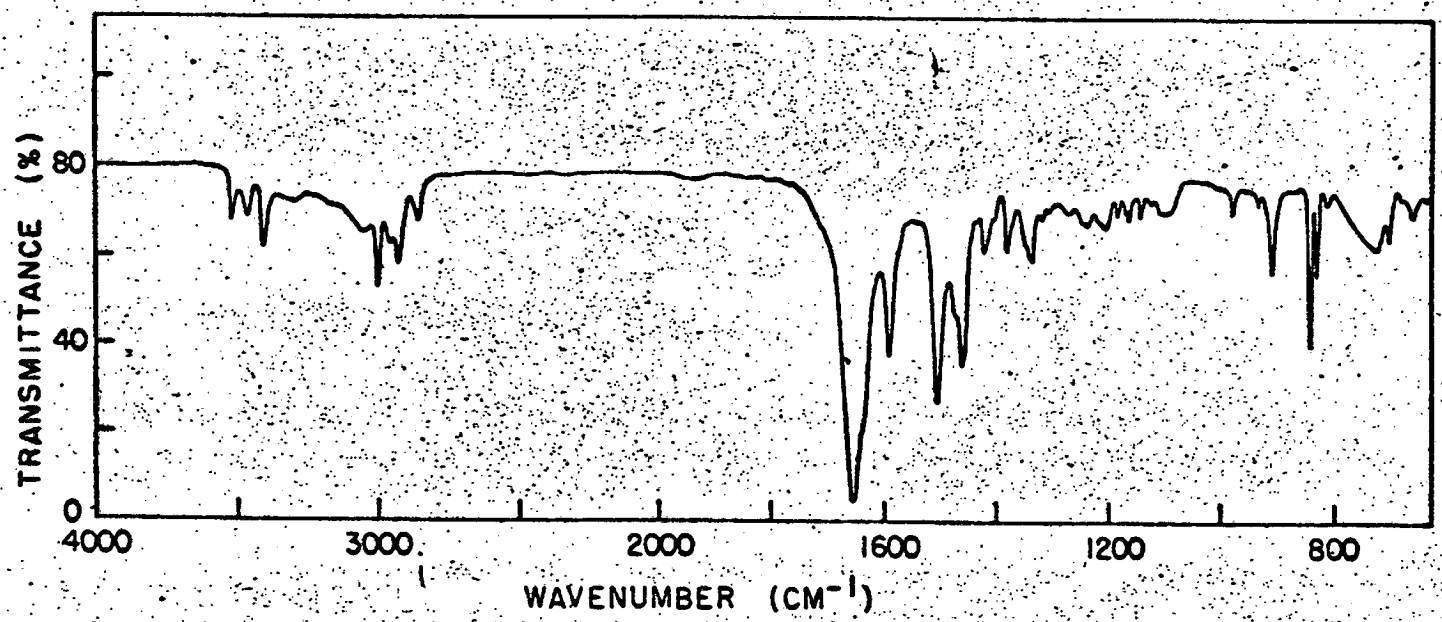

Figure 1 Cavatieri + Calvin

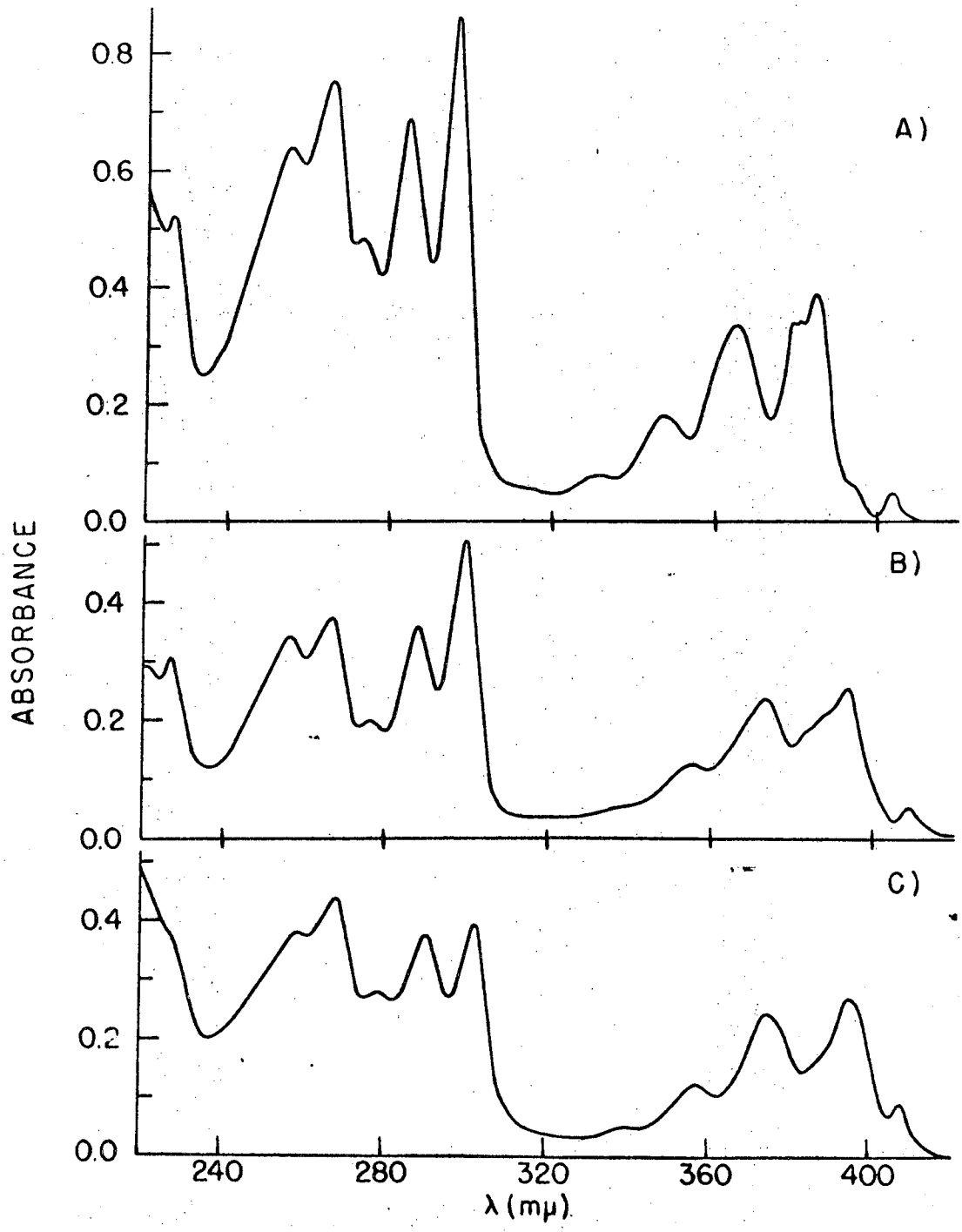

Figure 2. Cavalieri & Calvin

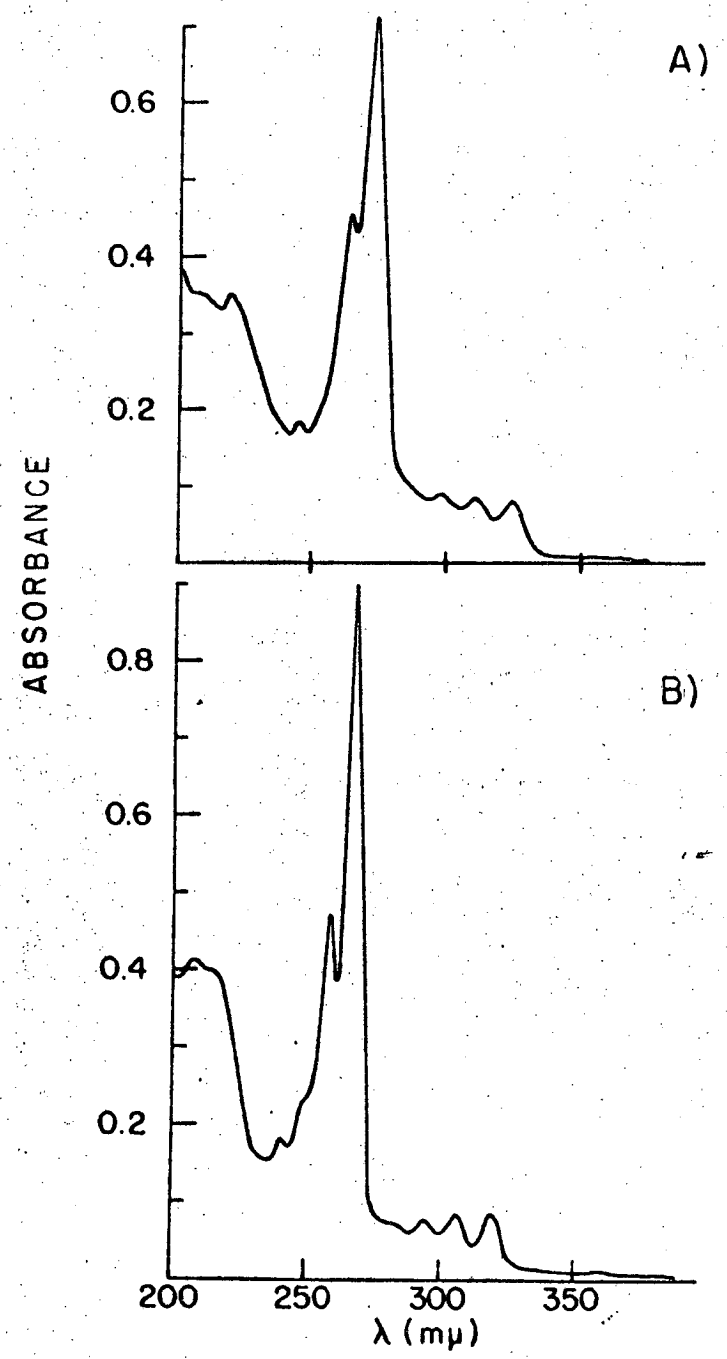

Figure 3. Cavalieri & Calvin

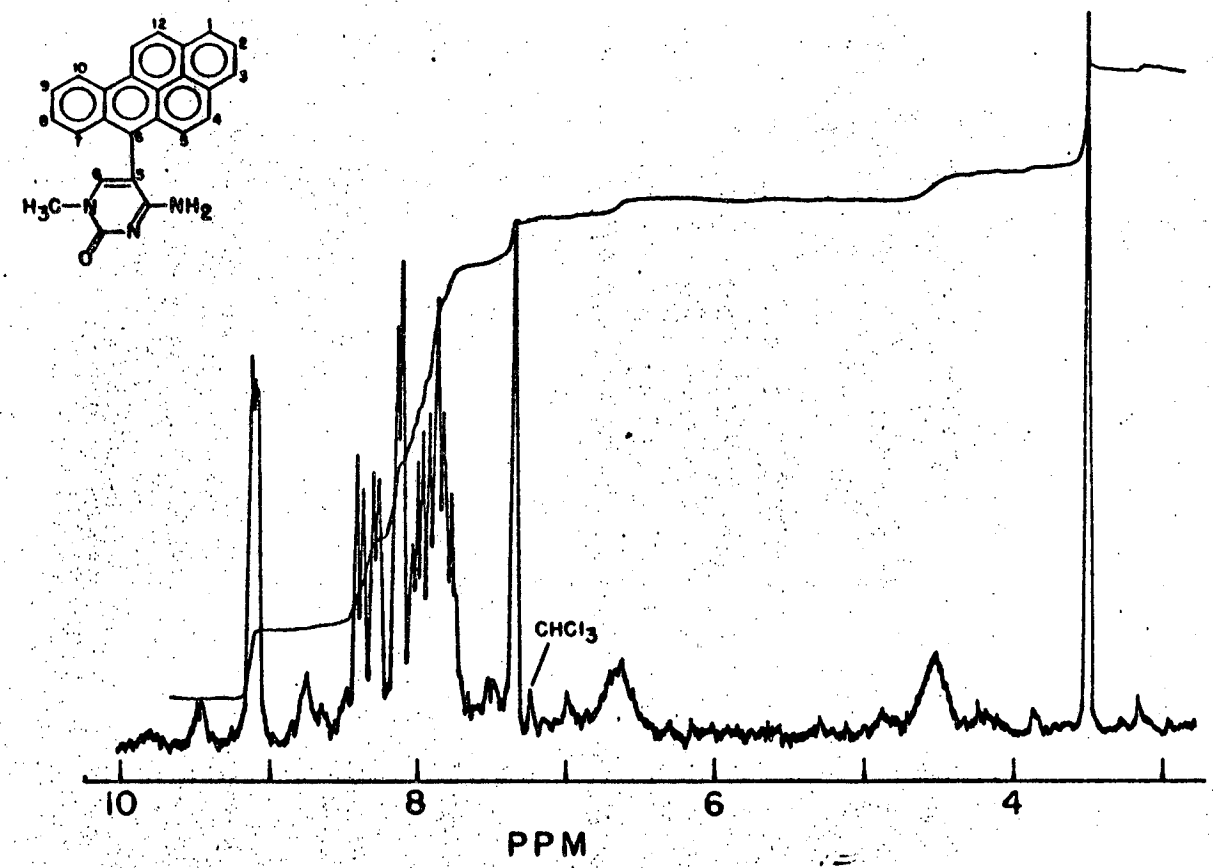

Figure 4. Cavalieri + Calvin

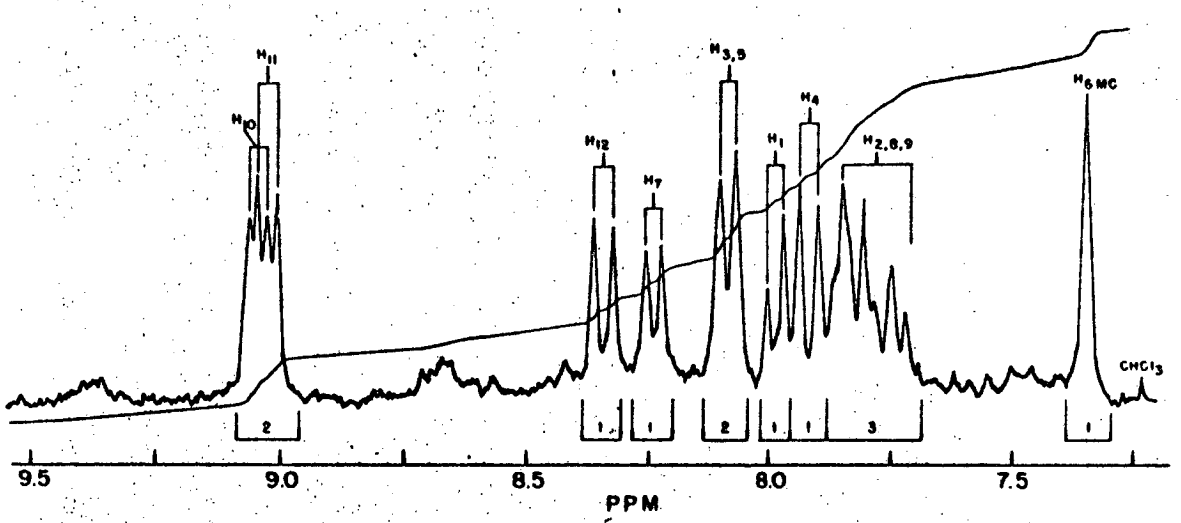

Figure 5. Cavalieri & Calvin

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