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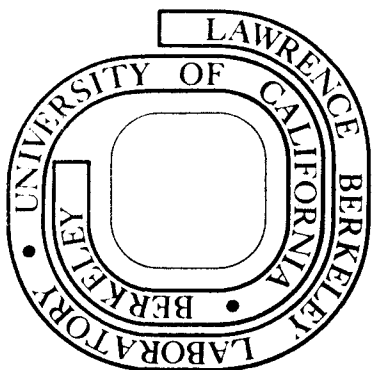
David Warshawsky, Edward Kerns, Mina J. Bissell, and
Melvin Calvin

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CHARACTERIZATION OF A PHOTOPRODUCT OF 7,12-DIMETHYLBENZ(A)ANTHRACENE
AND ITS EFFECTS ON CHICK EMBRYO CELLS IN CULTURE

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Running title: Biochemical and Morphological Effects of DMBA-endoperoxide.

SUMMARY

Warshawsky, David; Kerns, Edward; Bissell, Mina and Calvin, Melvin.

A common impurity of 7,12-dimethylbenz(a)anthracene (DMBA) is shown to be more potent than DMBA in inducing morphological alterations, and in causing an increase in glucose uptake, DNA synthesis and cell number in chick embryo fibroblasts. Gradual morphological transformation follows the increase in DNA synthesis after two days when either primary or secondary cultures are treated with 3 $\mu\text{g}/\text{ml}$ of the compound. The compound, isolated from DMBA by alumina column chromatography, was characterized by thin-layer chromatography, mass spectroscopy, carbon-hydrogen analysis, uv, nmr spectroscopy and thermal decomposition. It was found to be the photooxidation product of DMBA, 7,12-epidioxy-7,12-dimethylbenz(a)anthracene. This compound has been reported previously, but not as an impurity in DMBA preparation or in relation to possible carcinogenesis in cell culture.

INTRODUCTION

Many polycyclic aromatic hydrocarbons (PAH) have been shown to be able to induce specific alterations in cell lines in culture (1-8) after treatment for an extended period of time. Primary cell cultures by definition cannot be carried indefinitely and are not usually very sensitive to short range effects of these carcinogens. Studies with primary cell cultures (as opposed to cell lines), therefore, are not very extensive. Chick embryo cells have been reported to increase their DNA synthesis after treatment with 100 $\mu\text{g}/\text{ml}$ of DMBA (9). Benzo(a)pyrene and 3-methylcholanthrene were reported to have no appreciable effect (9). However, preliminary experiments with commercial preparations of DMBA in our laboratory indicated appreciable toxicity even at 10 $\mu\text{g}/\text{ml}$. After purification by column chromatography and recrystallization,

the toxicity and general morphological effects of DMBA were reduced. We suspected that a potent impurity in commercial preparation of DMBA must be present. We therefore isolated and characterized the compound which proved to be 7,12-epidioxy-7,12-dimethylbenz(a)anthracene (endoperoxide). The following report deals with morphological and biochemical effects of the derivative, as well as DMBA, on chick embryo cells in culture and describes the methods used for identifying the compound. An abstract of this work has appeared (10).

MATERIALS AND METHODS

Cell culture. Primary cell cultures were prepared from ten day old chick embryos essentially as described (10a,11). They were plated at 8.0×10^6 cells per 100 mm diameter Falcon plastic Petri dish in growth medium 199 supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% chicken serum. The cultures were incubated in an atmosphere of 5% CO₂ in air at 39°C. The medium was changed on day three. Cells were trypsinized and reseeded as secondary cultures on day four at 5.0×10^5 cells per 35 mm diameter dish in medium 199 supplemented as above, except the concentration of calf serum and glucose were doubled to 2% and 11 mM respectively.

Stock solutions of the endoperoxide and DMBA were prepared by dissolving the compounds in dimethylsulfoxide (DMSO); (10 mg/ml). An appropriate aliquot of the stock solution was then dissolved in 0.5 ml of culture medium and this solution was added to the cell culture medium. Cultures were treated continuously as primaries and/or secondaries with the carcinogen. All cultures also received 1.0 µg/ml fungizone at this time, which has been shown to increase cell permeability to some drugs (12,13). Control cultures received appropriate concentration of DMSO.

One set of primary cell cultures was treated with 3 µg/ml of purified

endoperoxide (0.3% DMSO by volume) and another set was treated with 0.3% DMSO as control. 10 µg/ml of purified DMBA in DMSO was used as a comparison. Chemical treatment was continued when the cells were transferred as secondary cultures. Half of the secondary control cells received 3 µg/ml of endoperoxide and the other half received only 0.3% DMSO treatment. Thus, in addition to control cultures, we had two sets of carcinogen treated culture that were treated either continuously from primary seeding, or at the secondary stage. Cultures were monitored visually by microscope and analyzed on subsequent days in terms of cell number, DNA synthesis and glucose uptake.

Incorporation of acid precipitable ^3H -thymidine (^3H -Tdr) and retention of ^3H -2-deoxy-D-glucose (^3H -2DG) were taken as measurements of DNA synthesis and glucose transport, respectively (11). Analysis of protein was by the method of Lowry *et al.* (14). Cell number was measured using a Coulter counter. ^3H -2-Deoxy-D-glucose and ^3H -thymidine were obtained from New England Nuclear Corporation.

Chemical analysis. TLC's were run on Eastman Kodak (6066) 0.1 mm silica gel plates. All melting points were recorded on a Thomas Hoover capillary melting point apparatus. The uv spectra were recorded on a Cary 118, the nmr spectra were run on a Varian HR 220 MHz spectrometer at 17°C and the mass spectra were obtained on a DuPont 491-2 with a 21-094 data system.

DMBA (Sigma) was chromatographed on a neutral alumina column surrounded with aluminum foil, and eluted with benzene. After evaporation of the solvent, the material was recrystallized from a benzene-isopropanol mixture, giving pale yellow plates; m.p. 122-123°C; $\lambda_{\text{max}}^{\text{EtOH}}$ 296 (ϵ 72,000), 287 (ϵ 64,000), 276, 264, 398 (w), 382 (w), 362 (w), 236; m^+/e , 256 (parent ion); nmr (CDCl_3) 3.05 δ (s,3P), 3.3 (s,3P), 7.6 (m,5P), 7.8 (m,1P), 8.1 (d,1P), 8.4 (m,2P), and 8.5 (m,1P).

The endoperoxide was isolated from DMBA on a neutral alumina column by elution with chloroform after the DMBA had been eluted from the column. After

evaporation of the solvent, the material was recrystallized from a benzene-isopropanol mixture, giving a colorless powder; m.p. 199-200°C, decomposition; $\lambda_{\text{max}}^{\text{EtOH}}$ 232 (ϵ 36,000), 298 (w), 288 (w), 275 (w), 267 (w); m^+/e 288 (parent ion) and m^+/e 256 highest abundance; nmr (CDCl_3) 2.25 δ (s,3p), 2.70 (s,3p), 7.3 (m,2p), 7.4 (m,5p), 7.75 (t,2p) and 8.56 (s,1p); C,H anal. for $\text{C}_{20}\text{H}_{16}\text{O}_2$ calcd: C, 83.31; H, 5.59. Found: C, 83.08; H, 5.45.

The above operations were carried out either in the dark or in the absence of direct sunlight. This was particularly necessary during chromatography (15). DMBA is readily converted to the endoperoxide in room light in a solution of benzene containing a small amount of alumina.

Thermal decomposition (16-19) of the endoperoxide was carried out as follows: 10.5 mg of the compound was refluxed for 24 hr at 140°C in 25 ml of xylene. The reaction was monitored by tlc. The reaction mixture was evaporated down and the residue chromatographed on a 0.5 mm silica gel plate (EM Reagents, Cat. 7748; silica gel PF254 + 366) with benzene as the solvent. The various bands were scraped off the plate, extracted in chloroform, filtered, and uv spectra recorded. Based on the extinction coefficients 3.7 mg (46%) of DMBA was generated and 3.2 mg of the endoperoxide was recovered.

RESULTS

Cell culture. Secondary cultures treated at 3 $\mu\text{g}/\text{ml}$ are compared to untreated cultures in terms of DNA synthesis, sugar transport, and cell density (Table I). As the control cultures reach density dependent inhibition they decrease their overall DNA synthesis. At the same time, the treated cultures show a higher rate of DNA synthesis, glucose uptake and an eventual increase in cell number. Rous sarcoma virus (RSV) transformed chick cells show similar effects in increased DNA synthesis and increased glucose uptake over control cultures.

Figure 1 shows the morphological alterations when primary cultures are exposed to 3 $\mu\text{g/ml}$ of endoperoxide for 3 days (75 hr). Many of the fibroblasts appear darkened and rounded compared to control cultures. Also the cells are more rounded up and appear darker in just 3 $\mu\text{g/ml}$ of the endoperoxide as compared to 10 $\mu\text{g/ml}$ DMBA. At 3 $\mu\text{g/ml}$ the DMBA treated cultures look very similar to the controls (not shown).

Similar effects are observed when 2° cultures are treated with these compounds (Fig. 2). In contrast to control cultures which appear as a uniform monolayer, the cells treated with 3 $\mu\text{g/ml}$ of endoperoxide appear spindle shaped and variable. While there are some morphological effects of DMBA, it should be noted that the DMBA concentration is three times that of the endoperoxide. The similarity of morphological effects of the endoperoxide to that caused by Rous sarcoma virus is shown in Fig. 3.

Characterization of the compound. The endoperoxide of DMBA is a derivative of DMBA that is produced in the workup procedure. Cook and Martin (20) showed that by passing a stream of oxygen through a solution of the hydrocarbon in CS_2 in the presence of a 200 watt lamp they could generate the endoperoxide. Sandin and Fieser (15) generated the same compound by exposing a benzene solution of DMBA to room light while in contact with a small amount of alumina. This compound, however, as demonstrated in our laboratory, can be synthesized by passing DMBA down a neutral alumina column exposed to room light and eluting with benzene and CHCl_3 (Scheme I).

The tlc and solubility of the endoperoxide indicate that it is a more polar compound than DMBA, and the fact that it is colorless is an indication of loss of some aromaticity (Table II).

The UV spectrum shows a loss of the anthracene para bands and a shift of the beta band to 232 nm (Fig. 4). This type of spectrum is characteristic of a naphthalene-type structure. The mass spec in Fig. 5 shows a m^+/e (parent

ion) of 288 and an m^+/e 256 of highest abundance for the endoperoxide as compared to m^+/e 256 (parent ion) of highest abundance for DMBA. The difference in the molecular weight is 32, which could be either CH_3OH or 2 oxygens. However, the carbon-hydrogen analysis indicates that 2 oxygens have been incorporated into DMBA. The nmr data (Fig. 6) shows an upfield shift of the 2 methyl peaks which is consistent with the loss of some aromaticity. Finally, the thermal decomposition of the compound in xylene leading to formation of DMBA and the decomposition on melting are characteristic of an endoperoxide. Therefore, we concluded that the compound has a naphthalene-type structure, a molecular weight of 288 with the incorporation of 2 oxygens, a shift of methyl protons upfield from the DMBA methyl protons and the regeneration of DMBA under thermal conditions. These characteristics correspond to those of endoperoxide, i.e., 7,12-epidioxy-7,12-dimethylbenz(a)-anthracene.

The amount of endoperoxide in the impure DMBA was determined as follows: a mass spectrum of impure DMBA gave an m^+/e of 288. Visualization of a tlc plate under a UV lamp of impure DMBA eluted in benzene revealed two spots: one fluorescent spot with an R_f of 0.62 corresponding to pure DMBA, and a dark spot with an R_f of 0.45 corresponding to pure endoperoxide. The uv spectra of pure and impure DMBA in ethanol were recorded. Using absorbance units, the peak ratios of 296 nm/232 nm of DMBA and the impure DMBA were $(1.42/.30) = 4.73$ and $(1.30/.34) = 3.82$ respectively. The fact that the ratio was smaller for impure DMBA indicated to us that the absorbance value at 232 nm is higher because of the presence of some endoperoxide. The value at 232 nm was then determined for pure DMBA based on the value of 1.30 at 296 nm and the ratio of 4.73 (the absorbance of the endoperoxide at 296 nm is negligible with respect to DMBA). This gave a value of .27 absorbance units at 232 nm. Therefore, .07 delta absorbance units were due to the endoperoxide. Using extinction coefficients for the endoperoxide at 232 nm and for DMBA at

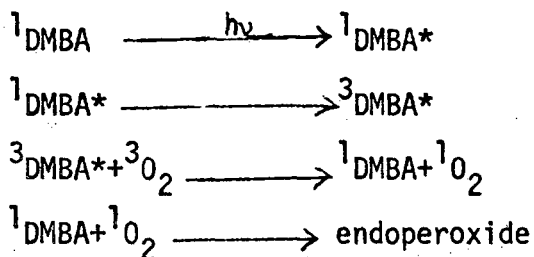
296 nm, the concentrations for the endoperoxide and DMBA were 1.9×10^{-6} M and 1.8×10^{-5} M respectively, indicating that approximately 10% of the commercial DMBA sample was endoperoxide. Tlc of a small sample of the impure DMBA resulted in only two bands which were scraped off, filtered, and weighed, yielding 11% of the endoperoxide in the DMBA sample.

The amount of endoperoxide in a DMBA sample, however, will vary depending on the preparation, storage conditions and solvent.

DISCUSSION

The endoperoxide of DMBA is readily produced in the presence of light (15,17,20). The commercial preparations of DMBA may thus contain appreciable amounts of this compound. This photo-oxidation product causes morphological and biochemical changes indicative of "transformation" in tissue culture cells. These effects, while produced by both DMBA and the endoperoxide, are more pronounced for the latter.

The formation of endoperoxides have been shown to be photosensitized autooxidation (21-24) reaction which in reality involves light and singlet oxygen.



Other workers (16-19,25) have shown that endoperoxides under mild conditions lose O_2 with the regeneration of the parent compound or undergo breakage of the oxygen-oxygen bond to form dihydroxy compounds. The loss of O_2 from this compound and other similar type PAH under mild thermal conditions is in the form of singlet oxygen (18,19). Singlet oxygen is a very reactive

species which can attack purine and pyrimidine bases (26) and protein residues (27) in the vicinity of its formation and has been postulated to be involved in the carcinogenic activity of PAH (28-30). It is, therefore, possible that some of the biological effects observed after addition of the endoperoxide to cells may be due to the formation of singlet oxygen, followed by nucleophilic attack on cell components leading to genetic or epigenetic changes. On the other hand, breaking of the oxygen-oxygen bond or carbon-oxygen bond could lead to metabolic products, i.e., 7,12-dihydroxy, or 7-hydroxy-12-hydroperoxy, capable of initiating these changes.

Boylard and Sims (31) studied the metabolism of DMBA in liver homogenates and found that the endoperoxide was only formed during the work up procedure and not as a metabolic product of DMBA. However, some of the biological effects observed after treatment of cultures with DMBA may be due in part to the presence of its endoperoxide. Primary chick cells have no measurable level of aryl hydrocarbon hydroxylase (T. Meehan and M. J. Bissell, unpublished). It has been suggested that polycyclic aromatic hydrocarbons such as DMBA require metabolic activation by this enzyme complex for their carcinogenic activity (32). It is possible that the endoperoxide impurity, after conversion to the hydroperoxide derivative (16), can act in place of the hydroxylase for conversion of DMBA to more carcinogenic products. This possibility is under investigation.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

- Figure 1. Morphological effects of DMBA and endoperoxide 75 hours after primary seeding. Procedure as described in Methods.
- Figure 2. Morphological effects of DMBA and endoperoxide 75 hours after secondary seeding.
- Figure 3. Comparison of morphological effects of endoperoxide and cells transformed with Rous sarcoma virus 75 hours after secondary seeding. (A) Normal cells. (B) Endoperoxide treated cells (these were treated with 3 $\mu\text{g}/\text{ml}$ both as primary and secondary cultures). (C) Virus-transformed cells. Cells were transformed as described in Methods.
- Figure 4. UV spectra recorded on a Cary 118; DMBA (—) 1.98×10^{-5} M, endoperoxide (----) 3.3×10^{-5} M in 95% ethanol.
- Figure 5. Mass spectra comparison of DMBA and endoperoxide. The data was obtained on a DuPont 491-2 with a 21-094 data system at a concentration of 1 $\mu\text{g}/\text{ml}$ in ethylacetate.
- Figure 6. Nmr spectra. The nmr spectra was obtained on a Varian HR 220 MHZ spectrometer at 17°C in CDCl_3 and 1% TMS at a concentration of 50 mg/ml with a sweep width of 2500 HZ and a sweep time of 500 sec.

Table 1

Cellular activity of secondary cultures after 75 hrs of treatment
with endoperoxide

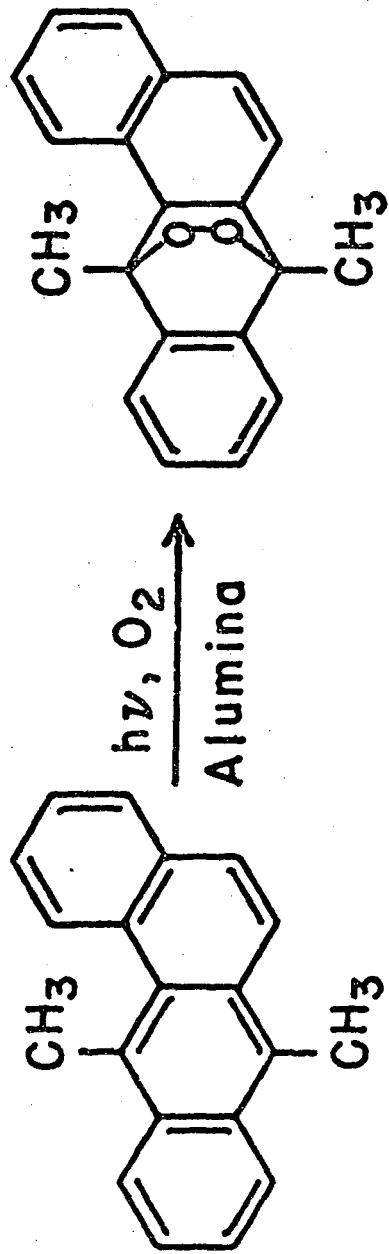
	DNA Synthesis*	Glucose Uptake**	Cell #/plate***
Control (in 0.3% DMSO)	100%	100%	100%
Endoperoxide (3 μ g/ml)	172%	165%	112%

* Measured with ^3H -Tdr incorporation

** ^3H -2-deoxyglucose uptake

*** Cell number was 1.7×10^6 /35 mm dish in control plates

Average of 3 experiments.



XBL7410-5382

Scheme I

00004304502
-15-

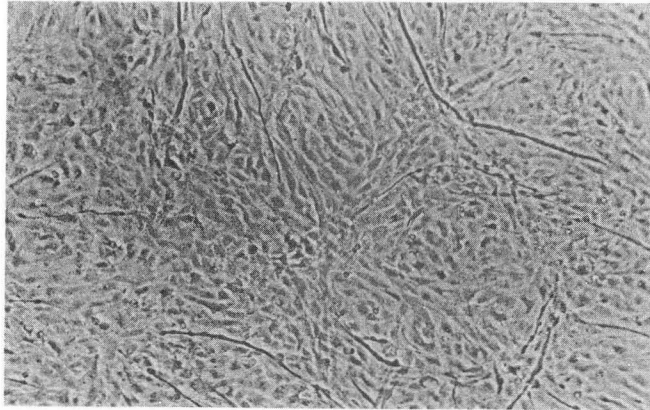
COMPARISON OF PHYSICAL DATA

	<u>DMBA</u>	<u>Endoperoxide</u>
m.p.	122-123°C	199-200°C Decomposes on melting-- turns dark yellow
	pale yellow plates	White powder
Solubility	v.s. in CHCl ₃ and hexane	s.s. in hexane v.s. in CHCl ₃
Rf*		
Benzene:EtOH (19:1)	0.62	0.62
Benzene only	0.62	0.45

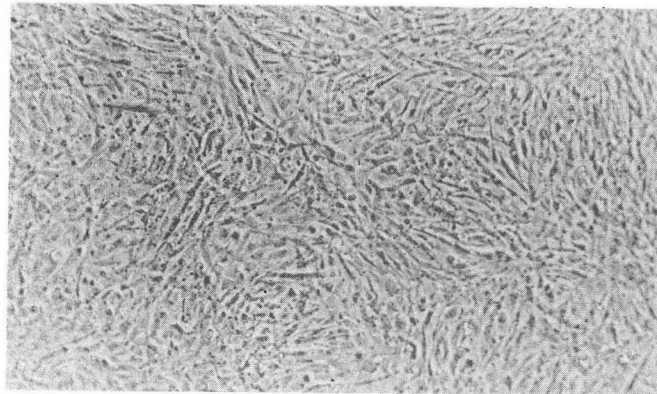
*Eastman Kodak silica gel plates 0.1 mm

XBL 7410-5385

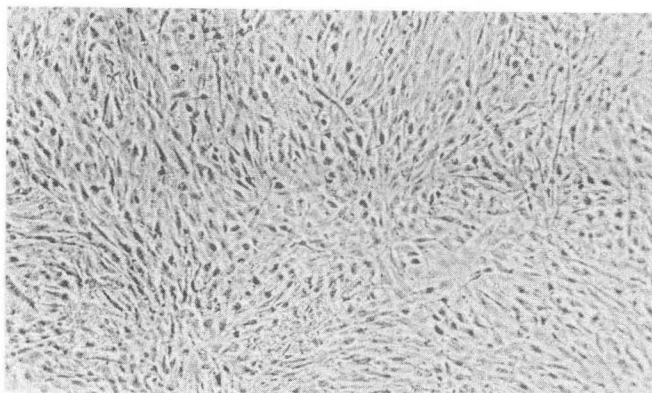
MORPHOLOGICAL EFFECTS IN PRIMARY CULTURES
75 HRS AFTER 1° PLATING



Control



DMBA
10 µg/ml



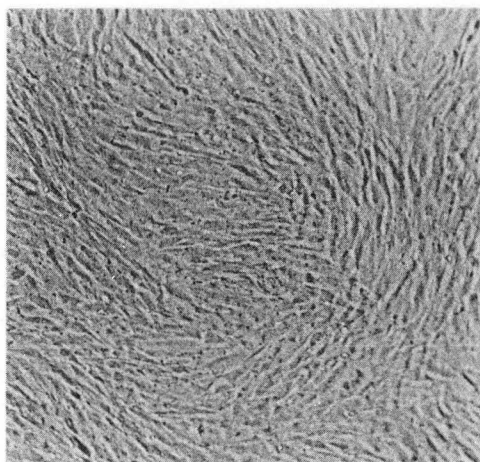
Endoperoxide
3 µg/ml

200 µ

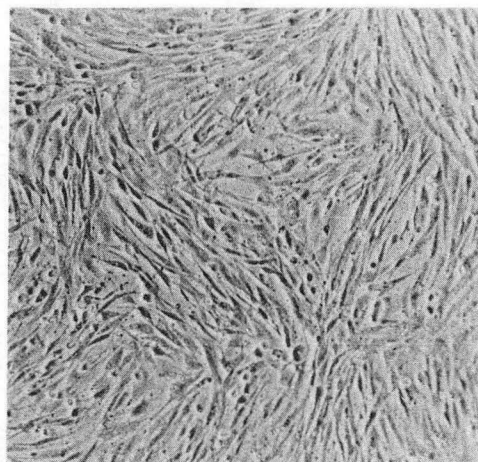
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Figure 1

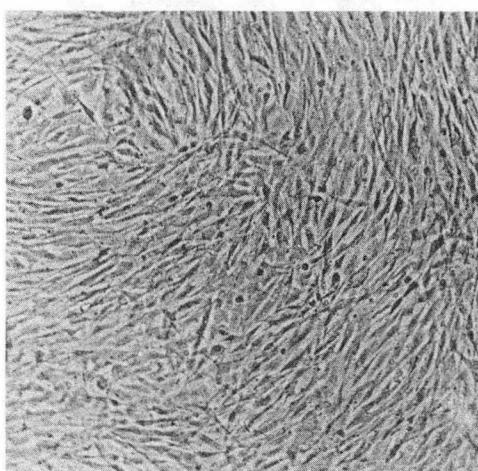
MORPHOLOGICAL EFFECTS OF DMBA AND ENDOPEROXIDE
75 HRS AFTER 2° PLATING



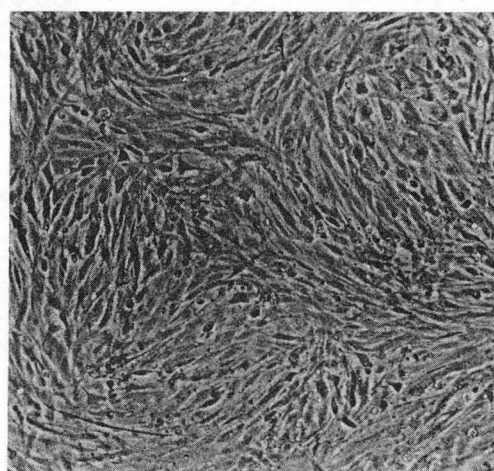
Control



Endoperoxide, 3 μ g/ml
added after 2° plating



DMBA, 10 μ g/ml, added
at both 1° and 2° plating

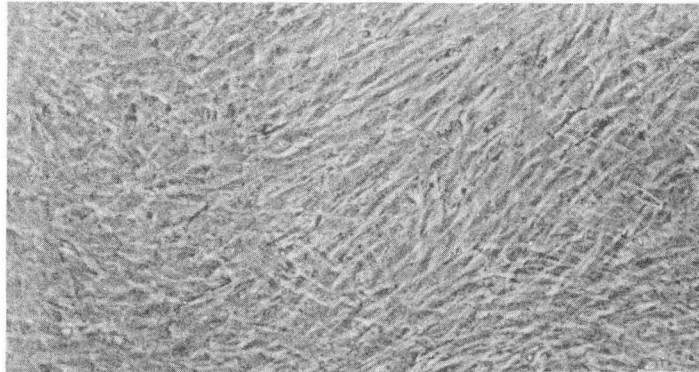


Endoperoxide, 3 μ g/ml, added at
both 1° and 2° plating

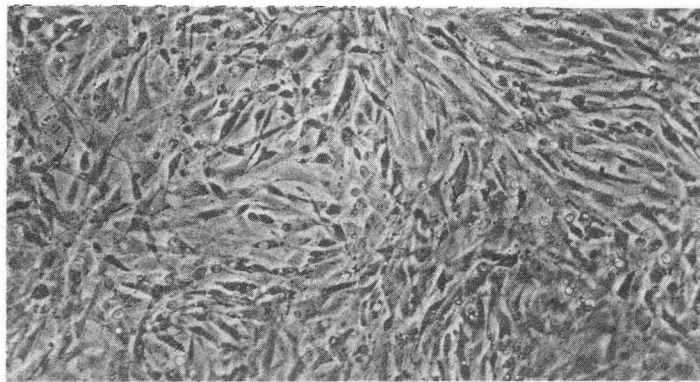
200 μ
|-----|

XBB 7410-7468

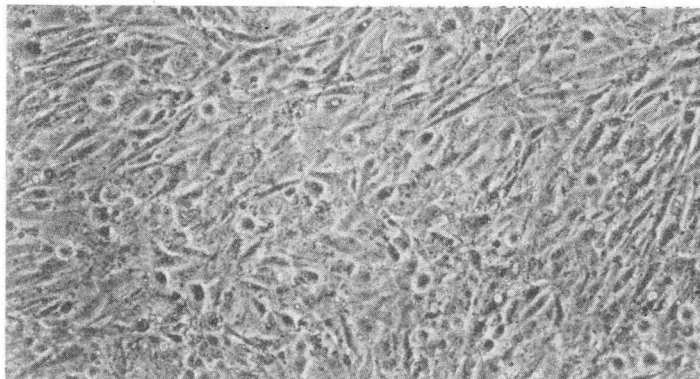
Figure 2



(A)



(B)



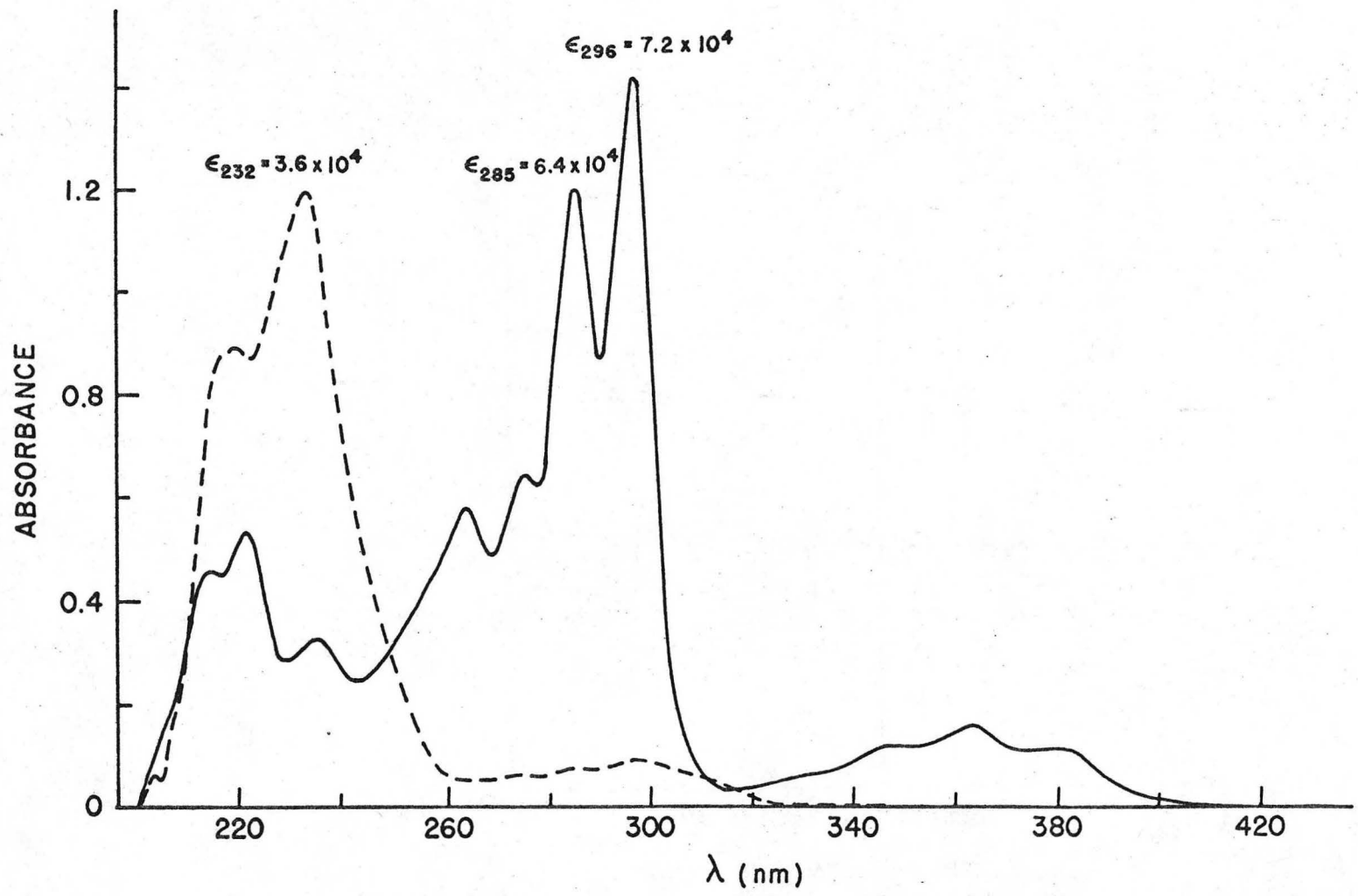
(C)

200 μ

XBB 7511-8451

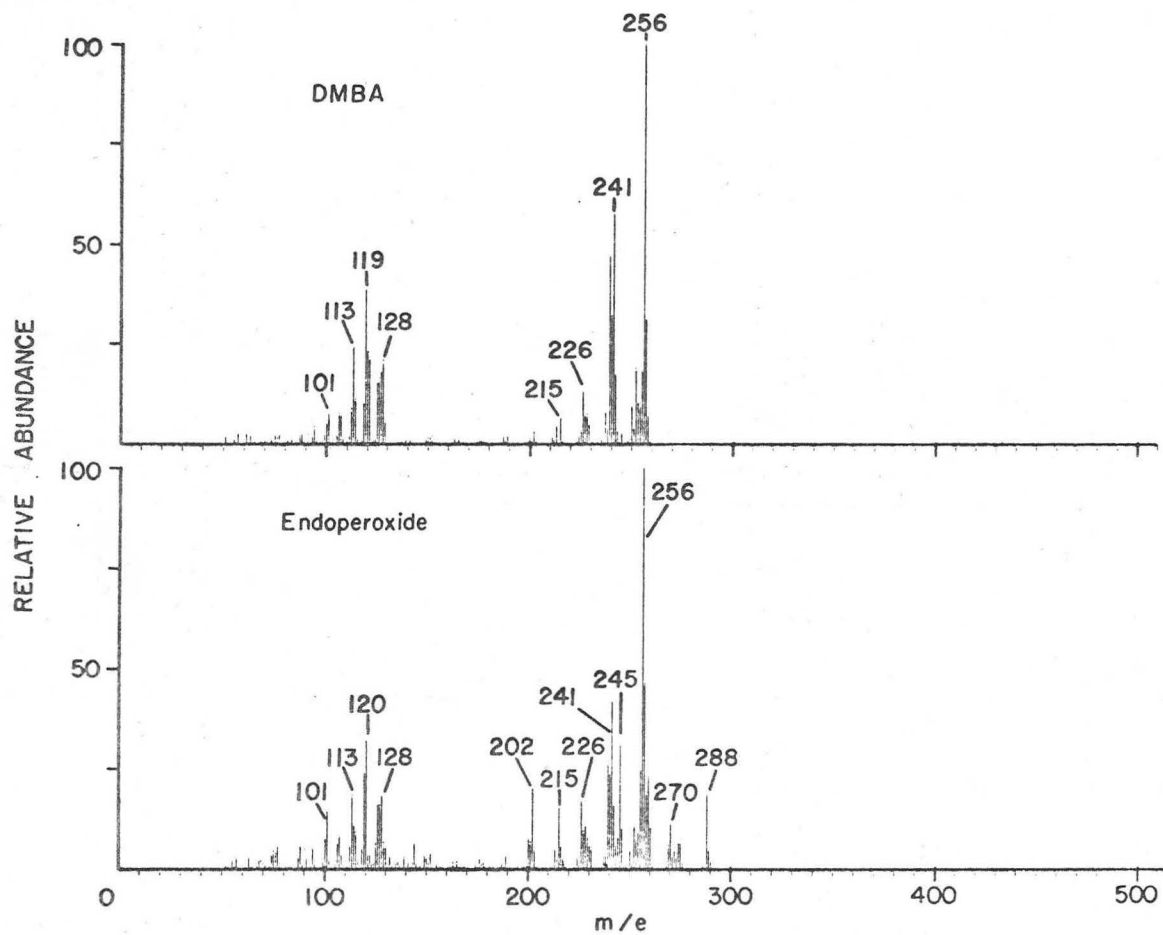
Figure 3

00004304504



XBL7410-5387

Figure 4



XBL7410-5388

Figure 5

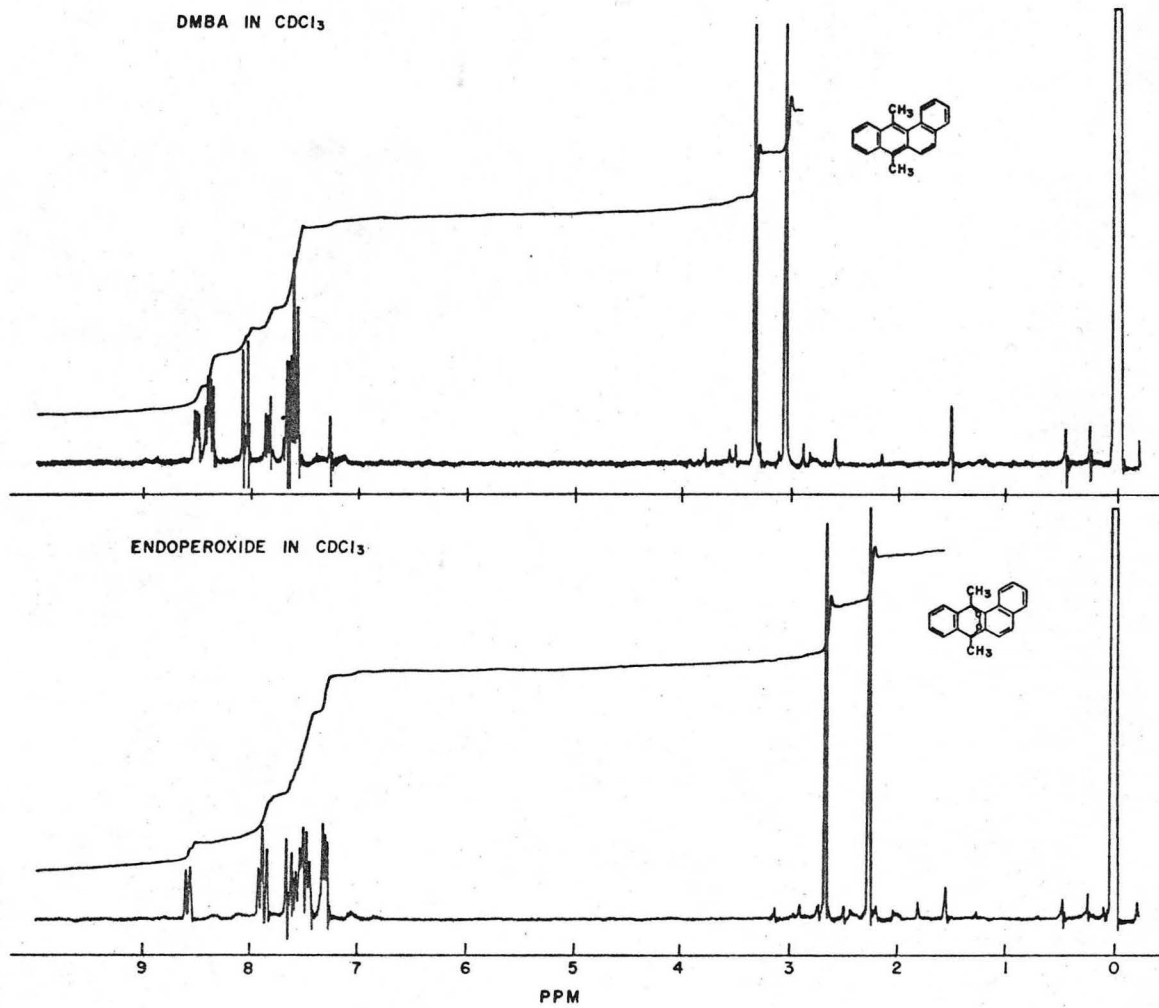


Figure 6

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