ULTRAVIOLET-MEDIATED CYTOTOXIC ACTIVITY OF PHENYLHEPTATRIYNE FROM *BIDENS PILOSA* L.

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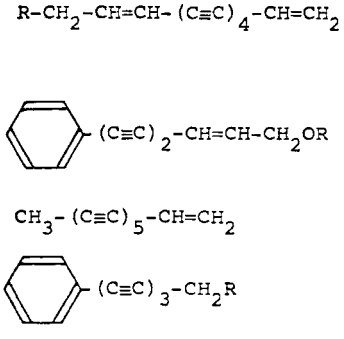
ABSTRACT.—The tropical weed *Bidens pilosa* L. (Asteraceae) contains a number of polyacetylenes which are phototoxic to bacteria, fungi, and human fibroblast cells in the presence of sunlight, artificial sources of long-wave ultraviolet light, or cool-white fluorescent light. The principle photoactive compound in the leaf, phenylheptatriyne, is present in the cuticle as well as in the underlying cells. Experiments with calf thymus DNA indicate that, unlike photoactive furanocoumarins, phenylheptatriyne does not form interstrand cross linkages with DNA in ultraviolet light.

The ecologically well adapted tropical weed *Bidens pilosa* L. (Beggar's Ticks or Spanish needles), a member of the Asteraceae, contains a number of polyacetylenic compounds (I–IV) identified by Bohlmann *et al.* (1)



In our survey of the Asteraceae for antibiotics, we found that the leaves of *B. pilosa* contain photoactive chemicals and that compounds Ia and III are lethal to *Candida albicans* and other microorganisms when the cultures are exposed to long-wave ultraviolet light (2). In a further study of this species, collected in Miami, Florida, we identified compound IVa, phenylheptatriyne (PHT), as the major polyacetylenic constituent of leaves and stems and have found that it is a phototoxic compound in the *Candida* test.

This report is concerned with details of the distribution of PHT within the plant, its biological activity and experiments in which its mode of action is compared with that of 8-methoxypsoralen (8-MOP), a well-known naturally occurring phototoxic compound which is capable of forming cross-linkages with DNA in long-wave ultraviolet light (3).

EXPERIMENTAL

ISOLATION OF PHENYLHEPTATRIYNE FROM BIDENS PILOSA.—Leaves were ground in methanol with a Waring blender and the suspension filtered. The methanolic extract was diluted with an equal volume of water and extracted with petroleum ether (bp 30–60°). The petroleum ether extract, after reduction in volume, was passed through a column of activated alumina. Petroleum ether containing increasing amounts of ether was used as the developing solvent.

The presence of phenylheptatriyne (PHT) in the eluate was monitored by uv spectroscopy. Fractions containing PHT were pooled and further purified by preparative tlc on alumina with petroleum ether as the solvent. Recrystallization from petroleum ether gave colorless prisms, mp 55–56° (8). Uv spectrum: λ max (petroleum ether) (log ε) 238, 250.2, 259, 274, 291, 310, 331.5 (4.9, 5.2, 4.0, 4.2, 4.4, 4.5, 4.3). Pmr spectrum: τ2.6 (m, 5H, aromatic-H), 7.99 (s, 3H, CH_3).

TISSUE CULTURE OF BIDENS PILOSA.—Techniques described by Gamborg were followed (13). Seeds of *Bidens* were sterilized with 70% ethanol and 20% sodium hypochlorite and germinated on moistened sterile filter papers in Petri dishes in the dark for 7–10 days at room temperature. Radicals, hypocotyls, and cotyledons were separated and transferred onto plates containing modified Gamborg's medium (2 gm sucrose replacing 20 gm) and 5 ml/liter Townsley Fe Stock (prepared with mono-sodium EDTA, 0.747 gm/100 ml and $Fe_2SO_4 \cdot 7H_2O$, 0.557 gm/liter). Plates were incubated in the dark at 26° until there was indication of callus formation and then exposed to continuous light at the same temperature.

LIGHT SOURCES.—Long-wave uv light was obtained from four Sylvania black-light blue fluorescent lamps (F20T12-BLB). Plates were placed 20 cm from the light source. The incident energy was 1.5×10^{-3}joule/sec/cm^2 as determined by ferrioxalate actinometry (14). Fluorescent light was obtained from eight Sylvania Lifeline lamps (F24T12/CW/HO) used in a Psycrotherm incubator; plates were placed 35 cm from the source.

BACTERIAL AND FUNGAL CULTURES.—Some of the microorganisms were used previously and their sources have been described (2). *Micrococcus roseus* UBC#289 and *Proteus mirabilis* UBC#5 were obtained from the Department of Microbiology, UBC. *Rhodotorula rubra* Y96, *Sporobolomyces* sp. Y138 and *Torula* sp. were obtained from Dr. B. Dill, Department of Microbiology, UBC.

CULTURE CONDITIONS.—Bacteria were cultured on nutrient agar plates at 37°. Yeasts and filamentous fungi were cultured on Sabouraud dextrose agar plates at 30°.

PHOTOTOXICITY ASSAY.—The method of Daniels was used for the general assays with bacteria and yeasts, as described previously (2). Filamentous fungi were macerated in distilled water and aliquots were spread evenly on the agar plates. Cultures were incubated overnight before the discs, containing the compounds, were placed on them.

QUANTITATIVE ASSAY WITH SACCHAROMYCES CEREVISIAE.—The yeast was cultured in a medium containing potassium hydrogen phthalate (0.06M), glucose (10 mM) and yeast extract (0.5%) and adjusted to pH 6.8 with NaOH. PHT, in petroleum ether (54 μg/100 μl), was added to 2 ml cultures in Falcon plastic Petri dishes (35×10 mm) to give a final concentration of 10 μg/ml. The cultures were incubated with shaking in the dark for 30 min. before exposure to uv light. Samples were taken at various intervals and plated to determine the number of viable cells.

IN VITRO ASSAY WITH HUMAN SKIN FIBROBLAST CELLS.—Normal diploid human skin fibroblast cells (ATCC #CRL(1221) were cultured in Dulbecco's Modified Eagle's Medium (MEM) with 10% fetal bovine serum, fungizone (25 μg/ml) and gentamycin (50 μg/ml) in Falcon tissue culture dishes (100×20 mm). The cells were split 1:5 at the tenth passage into smaller size dishes (35×100 mm) and incubated until confluent monolayers were obtained. The medium of each culture was aspirated and replaced with 2 ml Hepes buffer in MEM. PHT in petroleum ether was added to each dish to give a final concentration of 10 μg/ml. After shaking for 30 min. in the dark at 30°, the cultures were exposed to the various conditions as indicated in the results. The viability of the cells was tested by the trypan blue exclusion method. Viable cells excluded the dye, whereas non-viable cells were stained blue.

REACTION OF 8-METHOXYPSORALEN AND PHENYLHEPTATRIYNE WITH CALF THYMUS DNA.—Samples of calf thymus DNA (Sigma) were dissolved in 0.01N NaCl to give an O.D. of approx. 2 units/ml. Two ml samples were treated with 10 μg of the photoactive compound for each ml of DNA in Falcon plastic Petri dishes (35×10 mm). One set was incubated with shaking in the dark and the other maintained with shaking under uv light. Samples were removed at various times, and the following experiments were carried out. *Hyperchromicity Test* (10): Each sample was diluted 1:1 with 0.01N NaCl. After measuring the O.D. of the solution, it was heated in boiling water for 10 min., cooled rapidly in ice water, and the O.D. of the solution was read again. *Hydroxylapatite Column Chromatography* (11): One ml of denatured sample was applied to a column of DNA-grade Bio-Gel HTP (4 gm) in a 30 ml disposable plastic syringe and a gradient, prepared from 100 ml each of 0.01M and 0.3M sodium phosphate (with equimolar amount of Na_2HPO_4 and NaH_2PO_4) was used for chromatography. Fractions of 3.5 ml were collected, and the O.D. of each fraction was measured at 260 nm. *Sucrose Gradient Ultracentrifugation* (12): Samples (0.5 ml) were placed on top of a 4.4 ml sucrose gradient (5–20%) at pH 11 and centrifuged for 17 hr at 30,000 rpm in a Beckman SW-50 rotor. Fractions of 5 drops each were collected and diluted with 1 ml of water, and the O.D. of each fraction was read at 260 nm.

RESULTS AND DISCUSSION

OCCURRENCE OF PHENYLHEPTATRIYNE IN TISSUE AND CELLS OF B. PILOSA.—Petroleum ether extraction of crude aqueous-methanolic extracts of one or two leaves gives a solution with the characteristic uv absorption of the phenylheptatriyne chromophore. PHT is present to the extent of 400–600 μg/gm fresh weight. Uv spectra of crude leaf extracts show that there is hardly any detectable phenylheptadiynene in leaves, although this compound is easily discernible together with PHT in pith, stelar, and cortical extracts of stems. PHT occurs in cuticular extracts obtained by rapid immersion of intact, undamaged leaves in chloroform. After removal of the cuticular PHT, further amounts can be obtained from the epidermis, trichomes, and underlying cells. In fact, it would appear that the compound is present in all cells of the leaf. Resin canals are associated with vascular bundles in leaves and stems, but it has not been possible to tap these for their contents because of their very small diameters. The yellow exudate of the resins canals was, however, obtained by blotting 50 cut petioles on phase-separating Whatman filter paper. On elution with petroleum ether, the solution gave a uv spectrum corresponding to PHT.

PHT was also found to be present in ligules and ray florets of the flower heads. In root tissues, PHT appears to be absent. Instead, the roots contain a straight chain ene-poly-yne-ene type of compound with λ max at 257.5, 271, 288, 315, 357, 361.5 and 391 nm in petroleum ether (1).

Although we isolated PHT from mature leaves of *B. pilosa* collected in Hawaii, we could not detect it in cotyledons or hypocotyls of seedlings or from callus cultures obtained from seeds of Hawaiian or Jamaican plants. On the other hand, it was easily detectable in cotyledons and hypocotyls of seedlings and from young callus cultures obtained from seeds collected in Florida. One-month-old callus cultures prepared from Florida seedlings, however, did not contain PHT, which provides yet another example of the switching off of secondary metabolite syntheses in undifferentiated tissue cultures when these are maintained on certain media (6).

It has been suggested that chloroplasts are important in the formation of acetylenic linkages (1). Indeed, we found that, by sucrose density gradient fractionation (4) of a macerated leaf preparation, 50% of the PHT was located in chloroplast fractions. However, sucrose density gradient fractionation of a macerate of New Zealand spinach leaves, which do not contain PHT but to which PHT was added, also yielded chloroplast fractions containing more than 50% of the added PHT. Thus, PHT is readily adsorbed on chloroplasts. The intracellular location of PHT thus requires different methods of analysis.

UV-MEDIATED ANTIBIOTIC ACTIVITY OF PHT.—Table 1 shows the results of phototoxicity tests on bacteria, yeasts, and filamentous fungi with PHT using 8-MOP as a control. The antibiotic activity of PHT was elicited only when the cultures were kept in long-wave uv light or cool-white fluorescent light. All Gram-positive bacteria, yeasts and filamentous fungi tested were shown to be sensitive to 8-MOP and PHT when uv light was present. Of the Gram-negative bacteria, some resistant colonies were noted with *E. coli*, and *P. fluorescens* was found to be resistant. The resistance of the latter organism to a number of other polyacetylenes had been observed previously (2).

A culture of *S. cerevisiae* containing $22X10^6$ cells in log phase of growth was killed in a solution of PHT (10 μg/ml) after exposure to 1.33 joules/cm^2 of uv

TABLE 1. *Inhibition of bacterial and fungal growth by 8-methoxypsoralen and phenylheptatriyne in the presence of long-wave and cool-white fluorescence lights.*

Organisms	8-Methoxypsoralen 10 μg/disc			Phenylheptatriyne 5 μg/disc		
	UV	cool-white	dark	UV	cool-white	dark
Gram-positive Bacteria						
Bacillus subtilis	+	±	−	+	+	−
Micrococcus roseus	+	−	−	+	+	−
Staphylococcus albus	+	+	−	+	+	−
Streptococcus fecalis	+	−	−	+	+	−
Gram-negative Bacteria						
Escherichia coli	+*	−	−	+*	−	−
Proteus mirabilis	+	−	−	+	−	−
Pseudomonas fluorescens	−	−	−	−	−	−
Yeasts						
Candida albicans	+	+	−	+	+	−
Rhodotorula rubra	+	±	−	+	±	−
Saccharomyces cerevisiae	+	+	−	+	+	−
Sporobolomyces sp.	+	+	−	+	+	−
Torula sp.	+	±	−	+	−	−
Filamentous Fungi						
Fusarium sp.	+	−	−	+	+	−
Microsporum canis	+	+	−	+	+	−
Microsporum cookei	+	+	−	+	+	−
Microsporum gypseum	+	+	−	+	+	−
Trichophyton mentagrophytes	+	−	−	+	+	−

+ = phototoxic; − = inactive; * = some resistant colonies.

light (table 2). Similar treatment of a culture of human skin fibroblasts rendered the cells inviable within 60 min. (Table 3.)

Bondarenko *et al.* reported that PHT is a major antibiotic of *Bidens cernua*, inhibiting the growth of Gram-positive bacteria, yeasts, and dermatophytic fungi at dilutions of 5–10 ppm. They did not mention, however, whether their cultures were incubated in light or dark. We have found that cultures of yeasts and

TABLE 2. *Effect of phenylheptatriyne (PHT) on* Saccharomyces cerevisiae *in the presence and absence of uv light.*

Irradiation with uv light		Cell count of *S. cerevisiae* ($\times 10^6$)					
			Controls				
Duration (min.)	Dose Joules/cm²	PHT (10 μg/ml) +uv	+uv		Dark		
			−PHT	+pet ether	+PHT	+pet ether	−PHT
0	0.00						22.3
15	1.33	2.57			24.2		
30	2.67	0.02			35.8		
60	5.33	0.00			48.3		
180	16.00	0.00	47.7	44.7	53.5	48.0	46.3

TABLE 3. *Effect of phenylheptatriyne (PHT) on the viability of human skin fibroblast cells.*

Irradiation with uv light		Viable cells (%)					
			Controls				
Duration (min.)	Dose Joules/cm	PHT+UV	+uv		Dark		
			−PHT	+pet ether	+PHT	+pet ether	−PHT
20	1.78	95	100				
40	3.53	50	100				
60	5.33	0	100	100	100	100	100
90	8.00	0	100				
120	10.67	0	95				
150	1.333		80				
180	16.00		45				

Concentration of PHT: 10 μg/ml.
Fibroblast Cell Count: 2×10^6/ml.

bacteria are also sensitive to PHT when exposed to cool-white fluorescent light in our incubators. No inhibition of growth was obtained when a filter (Kodak Wratten gelatin filter #2A) which cuts off light below 400 nm was used. Thus light of wave-lengths less than 400 nm is essential for antibiotic activity. Sunlight is also effective. Exposure of plates with PHT absorbed on 7 mm paper discs (5 μg/disc) under the Vancouver noon-hour sun (May, 1978) for 20 min. produced zones of inhibition of 40 mm diameter with *S. cerevisiae* and 16 mm with *Candida albicans*. Similar effects on these yeasts were observed after 15 min. exposure to sunlight in the presence of crushed leaf pieces of *B. pilosa*.

The possibility that uv-induced products of PHT are the phototoxic compounds was examined in the following way: Discs, with either PHT or 8-MOP absorbed on them, were irradiated for various length of time before they were placed on cultures of *S. cerevisiae* or *C. albicans*. The plates were kept in the dark for 30 min. and then exposed to uv light in the usual way. The results, shown in table 4, indicate that PHT is relatively unstable, its activity being lost after 30 min. irradiation.

TABLE 4. *Effect of the duration of uv irradiation on 8-methoxypsoralen (8-MOP) and phenylheptatriyne (PHT) on their phototoxicity.*

Duration of Irradiation (min.)	*Candida albicans*		*Saccharomyces cerevisiae*	
	8-MOP	PHT	8-MOP	PHT
15	+(13 mm)	+(11 mm)	+(17 mm)	+(10 mm)
30	+(15 mm)	−	+(18 mm)	−
180	+(17 mm)	−	+(20 mm)	−

+=phototoxic; −=inactive.
Numbers in brackets: diameter of zone of inhibition.
Concentrations: 8-MOP: 5 μg/disc; PHT: 2.5 μg/disc.

The stability of PHT in the leaf cuticle of a plant exposed to tropical sunlight is, therefore, surprising and suggests either a special chemical environment for this compound in the cuticle or a continuous replenishment of it from the underlying cells. We would like to propose that the occurrence of PHT in the cuticle of *B. pilosa* is of biological importance and that it affords protection to the plant against certain microorganisms, and this protection is realized only in sunlight.

EXPERIMENTS WITH CALF THYMUS DNA AND PHT.—According to current hypothesis, 8-MOP and certain other linear furanocoumarins form covalent cross-linkages with thymine residues in native DNA in the presence of long-wave uv light. Oxygen is not required in this reaction (3). Because the antibiotic activity of PHT has a uv light component to it, we compared the photochemical behaviour of PHT with that of 8-MOP using calf thymus DNA.

Three methods were used. The first utilizes the property of hyperchromicity displayed by DNA when it is denatured (10). For example, when double-stranded DNA is denatured by boiling and fast cooling, the extinction coefficient at 260 nm increases. With 8-MOP, because of its cross reaction with two DNA strands, the strands are prevented from separating, and thus the hyperchromicity is decreased to zero as the photochemical reaction proceeds. With PHT it can be seen that there is no such decrease with time (table 5).

TABLE 5. *Hyperchromism of calf thymus DNA after incubation with 8-methoxypsoralen and phenylheptatriyne and subsequent boiling and cooling.*

Irradiation with uv light		O.D. differences at 260 nm before and after boil-cool			
Duration (min.)	Dose Joules/cm²	8-Methoxypsoralen		Phenylheptatriyne	
		UV	Dark	UV	Dark
0	0.00	0.18	0.17	0.18	0.17
30	2.67	0.03	0.17	0.18	0.17
60	5.33	0.02	0.18	0.18	0.17
120	10.67	0.01	0.19	0.17	0.17
180	16.00	0.00	0.18	0.18	0.17

The second method involves the separation of double- from single-stranded DNA on a hydroxylapatite column by increasing the molarity of the eluting buffer (11). The results are shown in fig. 1. With 8-MOP, the denatured DNA from a mixture incubated in uv light was eluted at a higher molarity than the DNA from a comparable mixture maintained in dark. With PHT, the DNA appeared as single-stranded in both light- and dark-treated samples.

The third method involves the separation of double- and single-stranded DNA by sucrose gradient ultracentrifugation at pH 11 (12). At this pH, hydrogen bonds between the two strands of native DNA are destroyed. With 8-MOP, in light, cross-linkage was indicated but with PHT only single strands were obtained (fig. 2).

Thus, the mode of action of PHT is different from that of 8-MOP. There is a

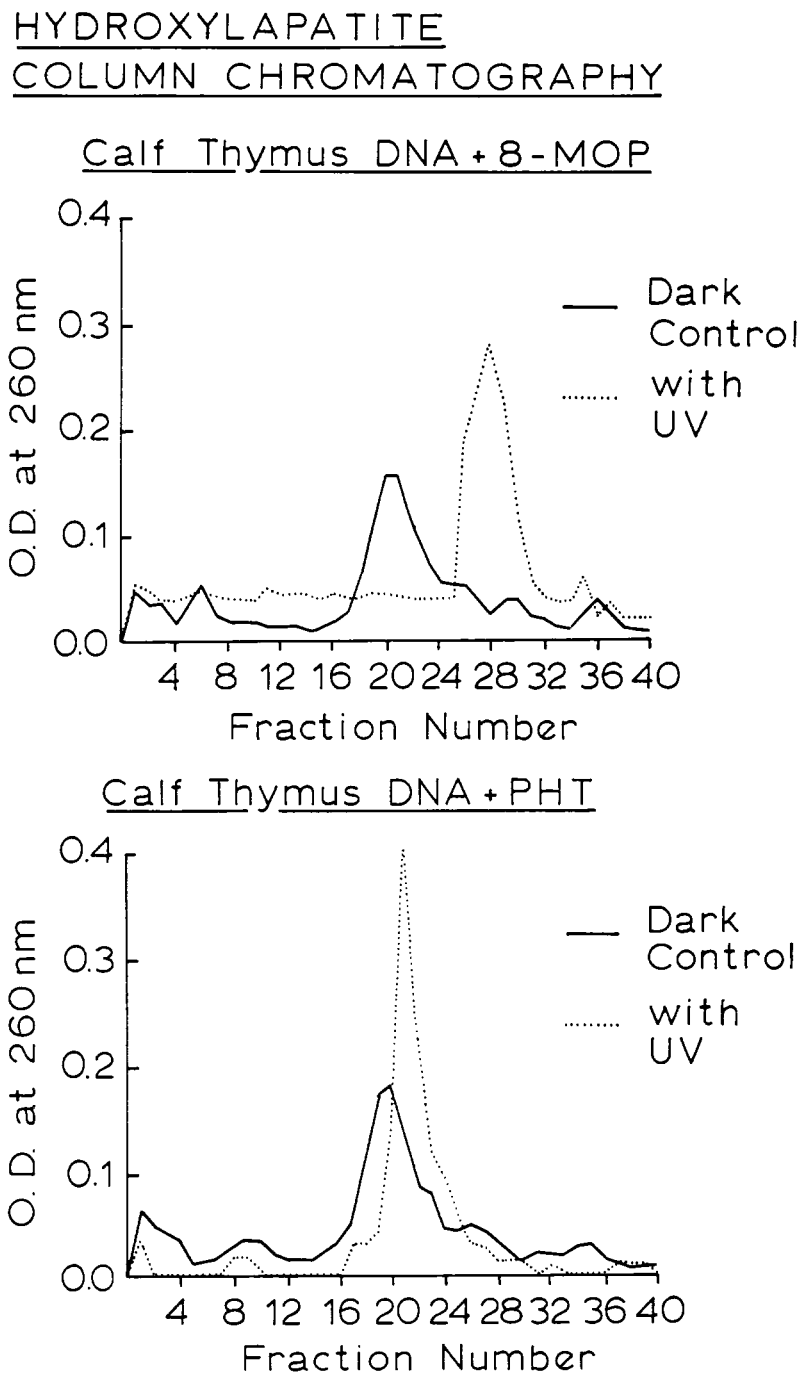


FIG. 1. *Hydroxylapatite column chromatography of calf thymus DNA after incubation with 8-MOP and PHT in long-wave uv light and in dark.*

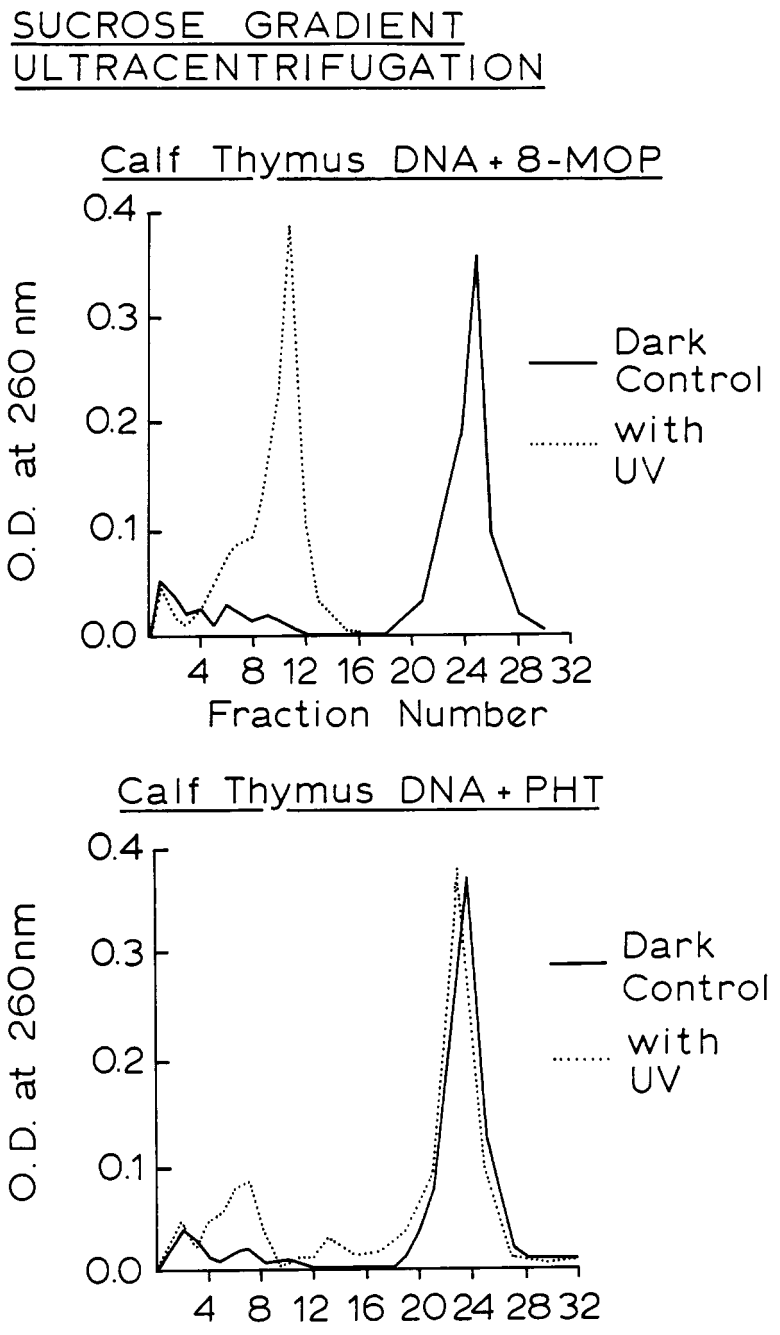


FIG. 2. *Alkaline sucrose gradient ultracentrifuagation of calf thymus DNA after incubation with 8-MOP and PHT in long-wave uv light and in dark.*

possibility that it reacts with only one strand of DNA as is the case with the angular furanocoumarins (5); this has yet to be determined.

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